



## Mini Review

# Advances in research of the structural gene characteristics of the aflatoxin biosynthetic gene cluster

Qi-Zhang Li and Zhu-Mei He\*

Guangdong Provincial Key Laboratory for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China

**\*Address for Correspondence:** Prof. Zhu-Mei He, School of Life Sciences, Sun Yat-Sen University, Guangzhou, People's Republic of China, People's Republic of China, Tel.: +86 20 84113065; Fax: +86 20 84036551; E-mail: lsshezm@mail.sysu.edu.cn

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## Abstract

Aflatoxins, produced by *Aspergillus* spp., are strongly toxic and carcinogenic fungal secondary metabolites. Aflatoxin biosynthesis is a complex process and involves at least 30 genes clustered within an approximately 75 kB gene cluster. In this paper, we reviewed current status of the researches on the characterized structural genes involved in aflatoxin biosynthesis and their roles in aflatoxin-producing fungi, especially in *A. flavus* and *A. parasiticus*, which will improve our understanding of the mechanism of aflatoxin biosynthesis and regulation and provide reference for further study.

## Introduction

As early as 1960, animal poisoning incidents happened in England due to a toxin produced by *Aspergillus flavus*, and this toxin is called aflatoxins (AFs) later. Aflatoxins produced by *Aspergillus* spp. are secondary metabolites which have strongly toxicity and carcinogenicity, and an obviously harmful effect on liver [1]. The agricultural contamination caused by aflatoxins is a worldwide problem and a potential threat to animal and human health [2,3]. *A. parasiticus* and *A. flavus* are two major aflatoxin producers, and their aflatoxin biosynthesis pathway genes are highly homologous and orders are similar [4]. The aflatoxin production involves a series of complex enzymatic reactions. Every enzyme is encoded by a corresponding structural gene. Therefore, a research on these structural genes directly reflects the mechanism of aflatoxin biosynthesis. This is an important and necessary process to further study the mechanism of synthesis and regulation of aflatoxin and its prevention and control. In this paper, on the basis of previous reviews and research papers [5-9], we mainly focus on the characterizations and roles of structural genes involved in aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* and provide reference for more in-depth studies.

## Overview of aflatoxin biosynthesis

Four different types of aflatoxins are biosynthesized by *Aspergilli*. Based on different fluorescence under ultraviolet light, these aflatoxins are divided into two groups: one showing blue fluorescence is called B-group aflatoxins, containing AFB<sub>1</sub> and AFB<sub>2</sub>; and the other exhibiting green is called G-group aflatoxins, including AFG<sub>1</sub> and AFG<sub>2</sub>. Moreover, the four aflatoxins are all different in structure (Figure 1). G-group aflatoxin (AFG<sub>1</sub> and AFG<sub>2</sub>) differs from B-group (AFB<sub>1</sub> and AFB<sub>2</sub>) by having an extra oxygen atom in the A-ring; while, 1-group aflatoxin (AFB<sub>1</sub> and AFG<sub>1</sub>) contains a dihydrobisfuran ring and 2-group aflatoxin (AFB<sub>2</sub> and AFG<sub>2</sub>) contains a tetrahydrobisfuran ring [10]. Different *Aspergilli* produces different kinds of aflatoxins. For example, *A. flavus* mainly

produces B-group aflatoxins, while *A. parasiticus* produces both B- and G-group aflatoxins. It's important to note that AFB<sub>1</sub> is the most toxic mycotoxin among the four aflatoxins [1]. In addition to aflatoxins, these fungi produce other secondary metabolites as well, such as cyclopiazonic acid, kojic acid, etc. [11,12].

The aflatoxin biosynthesis is complex and involves more than 20 enzyme-catalyzed reactions (Figure 2) [5,8,13]. At least 30 aflatoxin pathway genes are clustered within a 75 kB region of the genome on chromosome III (Figure 3) [6,7]. In the aflatoxin cluster, *aflF* is at the proximal end of the cluster and *nadA* is at the distal end. In the 5' end of the cluster sequence, there is no identifiable open reading frame (ORF) in an approximately 2 kB DNA region and a sugar utilization gene cluster is present in the 3' end [7]. There are two regulator genes involved in aflatoxin gene cluster and regulating aflatoxin biosynthesis [5].

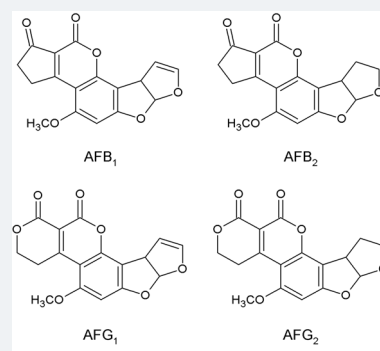


Figure 1: Structures of aflatoxins.

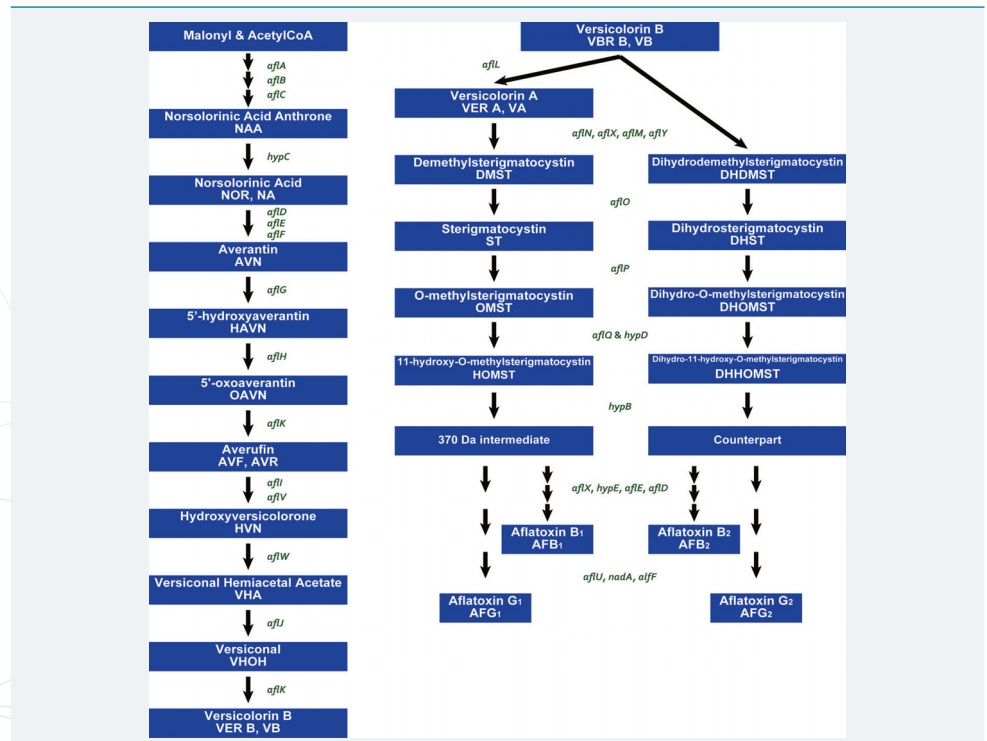


Figure 2: Schematic diagram of aflatoxin biosynthetic pathway.



Figure 3: Schematic presentation of aflatoxin biosynthetic pathway gene cluster..

## Structural genes in aflatoxin biosynthesis

Aflatoxin biosynthesis involves a set of enzymatic reactions which need a variety of enzymes encoded by at least 30 genes. These genes encode two fatty acid synthases, a polyketide synthase, eleven oxidoreductases, seven cytochrome P450 monooxygenases, one cyclase, one esterase, two O-methyltransferases and other enzyme.

**Fatty acid synthase (FAS)-encoding genes:** *aflA* and *aflB*, two fatty acid synthase encoding genes, are involved in the production of norsolorinic acid anthrone (NAA) from hexanoate produced by acetyl-CoA at the beginning of aflatoxin biosynthesis [14-17]. *aflB* cloned from *A. parasiticus* by genetic complementation contains four exons and three introns, and encodes a 7.5 kb transcript of FAS  $\beta$  subunit [6,18]. Besides *aflB*, the other FAS gene, *aflA*, which encodes FAS  $\alpha$  subunit and contains three exons and two introns, is also found by sequence analysis [6]. The 701 bp of intergenic region between *aflA* and *aflB* possesses several elements, such as an atypical AflR-like binding motif or probably two overlapping motifs, three cAMP response element (CRE)-like motifs, two stress-response element (STRE) motifs, a SrrA-binding motif, a CCAAT box and multiple AP-1 motifs [6,19-21] (Figure 4A). On the aspect of protein structure, both AflB and AflA contain some domains. For example, AflB contains acyl transferase (AT) domain, enoyl reductase (ER) domain, dehydrase (DH) domain and malonyl-CoA:acyl-carrier protein (ACP) transacylase (MAT) domain; while, ACP domain,  $\beta$ -ketoacyl synthase (KS) domain, ketoreductase (KR) domain and 4'-phosphopantetheinyltransferase (PPT) domain are found in AflA [8,22].

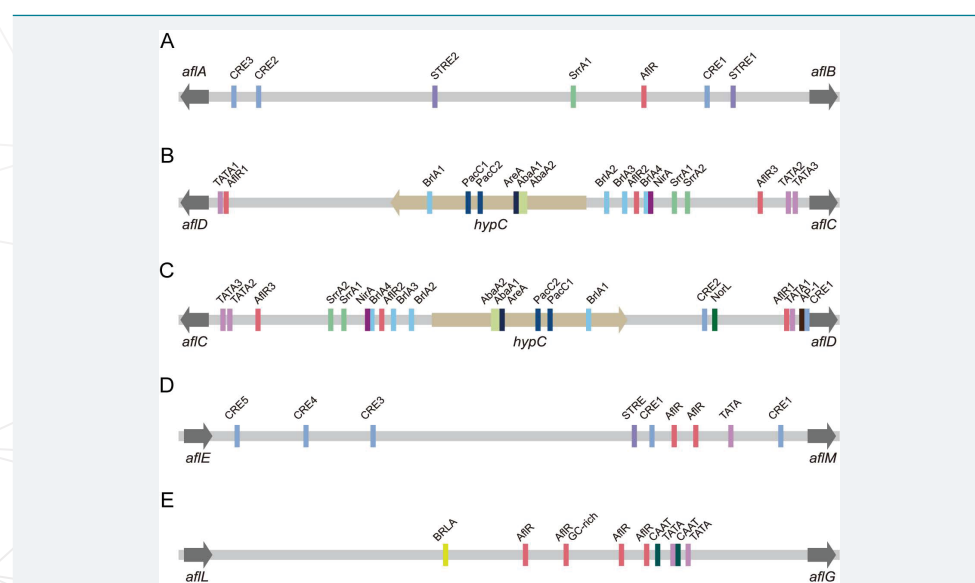
**Polyketide synthase (PKS)-encoding gene:** PKS is involved in synthesis of AFB<sub>1</sub> polyketide backbone [22,23]. *aflC* encodes polyketide synthase and is cloned from *A. parasiticus* [24-26]. This gene has 6.8 kb of genomic DNA, and contains six exons collectively coding for a protein of 2,109 amino acid residues and five introns of 65 bp, 66 bp, 52 bp, 59 bp and 54 bp. In the 1,687 bp of intergenic region between *aflC* and *aflD*, the sequence contains many cis-acting elements [19-21,26-28] (Figure 4B). *AflR1* have no effect on *aflC* activity because of the position probably. Both *AflR2* and *AflR3* are required for expression of *aflC*, although not showing the binding for lacking a C or G at position 8, but having the strongly and moderately competing oligonucleotides 5'-TCG(C/G)(A/T). *BrlA3* and *BrlA4* help recruit AflR, and *BrlA3* is necessary for *aflC* activity. *PacC1* appears to have a positive effect on *aflC* expression at acid pH and a negative effect at basic pH. Additionally, two AbaA sites, one AreA site, one NirA site, 15 CRE-like motifs, two SrrA-binding motifs, three potential TATA boxes and two inverted CCAAT boxes exist in the promoter sequence. The polyadenylation site of *aflC* exhibits heterogeneity [26]. Besides the domains recognized by comparing amino acid sequence, more domains are found in AflC: starter unit: ACP transacylase (SAT) domain, KS, MAT, product template (PT), ACP and thioesterase/Claisen cyclase (TE/CLC) [22,29-31].

**Oxidoreductase-encoding genes:** Eleven oxidoreductases are involved in aflatoxin biosynthesis and encoded by corresponding genes. Some genes only participate in one reaction, such as *hypC*, *aflH*, etc. While, some involve in more reactions, such as *aflD*, *aflE*, etc.

*hypC* Norsolorinic acid (NA or NOR) is the first stable precursor in aflatoxin biosynthesis [32]. HypC, an anthrone oxidase, catalyzes the introduction of oxygen into activated aryl moieties, which results in the formation of NA (NOR) from NAA [13]. A putative gene *hypC* encoding HypC is discovered in the region between *aflC* and *aflD* in *A. parasiticus* [6]. It has the length of 542 bp, contains an intron, and encodes a protein of 139 amino acid residues. Because *hypC* is located between *aflC* and *aflD*, some of the elements mentioned above which located between *aflC* and *hypC* may influence the transcription of *hypC* (Figure 4B). *hypC* is predicted to encode approximately 17 kDa proteins and has a catalytic active site Q-L-X-X-Q-W-S-R-I-F-Y [13].

*aflD* NADPH-dependent NA (NOR) ketoreductase (alcohol dehydrogenase) encoded by *aflD* catalyzes the conversion of 1' keto group of NA (NOR) to 1' hydroxyl group of averantin (AVN) and may be involved in the formation of AFB<sub>1</sub> [13,33,34]. *aflD* transcript is 1.25 kb in length, and contains four exons collectively coding for a 29 kD protein of 294 amino acid residues and three introns of 52 bp, 58 bp and 64 bp [35]. Three transcriptional start sites, two of which are major and one is minor, are located at upstream from the ATG. The polyadenylation site locates approximately 215 bp downstream of translation termination codon. The promoter contains the same elements mentioned in the intergenic region between *aflC* and *aflD* (Figure 4C). Multiple transcription factors are required for maximum activity of *aflD* promoter. *AflR1*, which has no effect on *aflC* activity, dose not bind with AflR in EMSA because the A/T at position 5 is replaced by a C, but is necessary, not sufficient for maximum transcriptional activation; while *AflR2* is required for expression of both *aflC* and *aflD* [20,27,28,36]. *NorL* appears only in *aflD* promoter and is necessary for maximum transcriptional activation *in vivo* [36]. Two *CREs* are identified between *aflD* and *hypC*, and only *CRE1* is functional although they have only one nucleotide difference [20,36]. An *AP-1*-like site is located at 12 nucleotides upstream from *CRE1*, and both of them form composite regulatory element [21]. A TATA box is required for maximum transcriptional activation *in vivo* [20,36]. *PacC* and *BrlA* sites do not affect *aflD* transcriptional regulation, and may not be required for transcriptional activation [36]. A negative regulator presents in the region from position -298 to position -332 by deletion analysis. AflD contains some conserved sequences, such as Y-G-V-S-K-L-A-A-N-Y-M, G-X-G-X-X-L and Y-L-V-T [35]. Additionally, it has high identity and similarity to these of *Flavobacterium* sp. *N*-acyl-D-mannosamine dehydrogenase and *Streptomyces violaceoruber* PKS.

*aflE* and *aflF* *aflE* and its homologous gene *aflF* encode aryl alcohol dehydrogenases [37]. AflE participates in the formation of AVN and oxidization of AFOH which is yielded in *norA* disruptant cultures back to AFB<sub>1</sub> [13]. *aflE* is cloned from *A. parasiticus* using a monoclonal antibody raised against NA (NOR) reductase [38]. An additional copy of *aflE*, *aflE2*, is identified by Southern blot analysis, and this may explain higher-level stable aflatoxin produced in *A. parasiticus* than that in *A. flavus* which has only one copy. A single ORF of 1,167 bp encodes a 43.7 kDa protein of 388 amino acids. Its promoter region has an AflR-binding motif, a TATA box and a CAAT box [27,38]. The polyadenylation site of *aflE* transcript is located at nucleotide 1500, and a



**Figure 4:** Schematic diagram of cis-acting elements of gene promoter. A, cis-acting elements in the intergenic region between *aflA* and *aflB*. B and C, cis-acting elements in the intergenic region between *aflC* and *aflD*. D, cis-acting elements in the intergenic region between *aflE* and *aflM*. E, cis-acting elements in the intergenic region between *aflL* and *aflG*.



polyadenylation signal sequence is located 25 bp upstream. *aflE* and its gene product from *A. parasiticus* share high degree of identity with its homolog from *A. flavus* at both the nucleotide and amino acid levels [6,38]. However, they show a low identity to *aflD* and AflD, respectively [8]. Moreover, there are high homology between *aflE* and an aryl-alcohol dehydrogenase (*aad*) gene from *Phanerochaete chrysosporium* or their products [38]. The amino acid sequence of AflE has an adenine nucleotide binding motif G-X-G-X-X-G and one N-glycosylation site N-X-T.

Besides participation of AVN formation, AflF is responsible for the final oxidation step in AFGs formation [33]. AflF catalyzes an intermediate given from the rearrangement and decarboxylation of the NadA reduced 386 Da intermediate to produce an ester which could be de-esterified by esterases, then this product upon dehydration would yield AFGs. *aflF* at 5' end of the gene cluster is cloned by chromosome walking from *A. parasiticus* [39]. The ORF of *aflF* consists of 1,146 nucleotides encoding a protein of 382 amino acid residues and no intron is found. However, a portion of *aflF* is deleted in *A. flavus* [40]. In the promoter, there is an AflR-binding motif [6,39]. *aflF* does not show significant homology with *aflE* at nucleotide level, but their products share 68% similarity at amino acid level [7].

*aflH* AflH, a typical short-chain alcohol dehydrogenase, is involved in dehydrogenation of 5'-hydroxyaverantin (HAVN) to 5'-oxoaverantin (OAVN) [41,42]. AflH is a homodimer composed of identical 28 kDa subunits and the native molecular mass is estimated to be 60 kDa [43]. *aflH* is cloned by chromosome walking and its transcript has approximately 1 kb with no intron [41]. There are two copies of *aflH* gene, *aflH* and *aflH1*, in *A. parasiticus* SU-1, but only one in *A. parasiticus* SRRC 2043. Eight nucleotide substitutions presenting in *aflH1* results in five amino acid mutations, in which the replacement of G by C in the glycine-rich loop may affect the binding of NAD<sup>+</sup>-NADP<sup>+</sup>. *aflH* promoter region contains an AflR-binding motif [27]. AflH contains two conserved functional motifs, a glycine-rich loop motif G-X-X-G-X-G and a motif Y-X-X-X-K [41]. *aflH* genes or their products in *A. flavus* and *A. parasiticus* share no significant homology at either nucleotide level or amino acid level [5].

*aflI* AflI may catalyze the oxidation of the hydrated intermediate catalyzed by *aflV*-encoded product, in which *aflI*-encoded product catalyzes alcohol oxidation of hydroxyversicolorone (HVN) precursor and the ring closure is catalyzed to form the hydroxyfuran [13]. It is reasonable to hypothesize that AflI may form a complex with AflV. *aflI* is cloned by complementation, and there is only one copy in the *A. parasiticus* genome [4,44]. *aflI* consists of 858 bp with no intron and encodes a 30.9 kD protein of 285 amino acid residues. An intergenic region between transcripts of *aflO* and *aflI* has only 34 bp, and no TATA motif or AflR-binding site is found [6,44,45]. However, the region at positions -55 and -150 to the translation start contains two TATA boxes. The sequences of *aflI* and its product from *A. parasiticus* share very high identity to these from *A. flavus* and *A. sojae* either at nucleotide level or at amino acid level. Sequence comparison suggests that the key amino acid at 179 may be responsible for the enzymatic activity.

*aflW* In the conversion of HVN to versiconal hemiacetal acetate (VHA), *aflW*-encoded HVN monooxygenase catalyzes Baeyer-Villiger reactions [46]. An oxygen atom is inserted into a C-C bond adjacent to the carbonyl group of an aliphatic or alicyclic ketone on the side chain. *aflW* contains a coding sequence of 1,446 bp encoding a 55 kDa protein of 481 amino acid residues and no intron [47]. *aflW* is adjacent to *aflV* and they share the promoter, so *aflW* has the same promoter elements as these of *aflV*. Although high homology is shown between AflW and monooxygenases in the GenBank database, no characteristic motif is found.

*aflX* *aflX*-encoded oxidoreductase catalyzes the reaction of epoxide ring-opening step during the process of versicolorin A (VER A or VA) to demethylsterigmatocystin (DMST) [48], and may also catalyze an oxidative decarboxylation/dehydration to

yield a 326 Da metabolite followed by demethylation to giving AFB<sub>1</sub> [13]. *aflX*, located between *aflW* and *aflY*, is cloned from *A. parasiticus* with no intron and encodes a protein of 266 amino acid residues [6,39]. There is a canonical AflR-binding motif in its promoter [39]. The amino acid sequence of AflX shows 27% identity with that of AflI catalyzed one of the steps in the conversion of averufin (AVR or AVF) to HVN [48], and AflX, like AflI, may be a helper protein for AflV [5,13]. AflX possesses a NADH-flavin reductase motif and several conserved sequences, such as A-X-X-G-A-T-G-X-T-G, R-X-X-X-K-L, L-X-S-A and Y-X-D [39,48].

*aflM* Deoxygenation of *aflM*-encoded NADH-dependent deoxygenase/ketoreductase is responsible for formation of DMST and dihydrodemethylsterigmatocystin (DHD MST), in which AflM catalyzes the dienone intermediate given by AflX [49]. *aflM*, the first gene found involved in aflatoxin biosynthesis, is cloned from *A. parasiticus* by genetic complementation [50,51]. Two copies are found but only one is functional. *aflM* is located between *aflE* and *hypE* with an intergenic region of 811 bp from *aflE* [38]. The genomic DNA sequence contains three exons collectively coding for a protein of 262 amino acid residues and two introns of 50 bp and 61 bp [51]. The transcription initiation site is at -82 bp from the translation start codon. In the intergenic region between *aflE* and *aflM*, there are two AflR-binding motifs, five CRE-like sites, a SrrA-binding motif, a STRE motif, a TATA box and several AP-1 motifs, but no CAAT box motif is found [19-21,27,51] (Figure 4D). No binding of recombinant AflR with the corresponding motif at -182 bp is observed because the A/T at position 5 in the motif is replaced by a C [27]. The polyadenylation motif is located 98 bp downstream from the translation termination codon and polyadenylation site is located 131 bp [51]. The amino acid sequence of AflM shows significant identity with those of some reductases and dehydrogenases [51-54], and contains an adenine nucleotide binding motif [51,55,56].

*aflY* *aflY*-encoded Baeyer-Villiger monooxygenase catalyzes the Baeyer-Villiger oxidation of a dienone intermediate formed by epoxidation of the anthraquinone ring of VER A (VA) [49]. The genomic DNA sequence of *aflY* contains three exons collectively coding for a protein of 495 amino acid residues and two introns [39]. Its promoter region contains a canonical AflR-binding motif. AflY possesses a highly conserved nicotinate phosphoribosyltransferase (NAPRTase) family motif and several highly conserved regions, such as G-F-H-(N/D)-H-X-X-H-(H/Q), G-X-(L/V)-H-P-(L/I/V)-I-(H/N/Q)-(L/I)-X-X-X-X-E, D-F-X-X-X-H, D-D-G-H-X-X-K-X-X-R-A and W-V-R-W-C-G-(E/D)-X-A-W [49].

*HypB* HypB can introduce an oxygen into the keto-tautomer of 11-hydroxy-O-methylsterigmatocystin (HOMST), followed by rearrangement to the 370 Da intermediate. While some researchers suggest that this oxidation is catalyzed by *aflQ* encoded protein [13,33,57]. *hypB* is located between *aflL* and *aflI* and possesses an intron and encodes a 17 kDa protein of 163 amino acid residues [6,13]. In the promoter, there are two AflR-binding motifs [58].

**Cytochrome P450 monooxygenase-encoding genes:** Although these gene-encoded enzymes participate in redox reaction, they all belong to cytochrome P450 monooxygenase.

*aflG* AflG catalyzes the hydroxylation of AVN to form HAVN [37]. Yu et al. clones *aflG*, located between *hypD* and *aflL*, from *A. parasiticus* by sequencing [4,59]. *aflG* contains three exons collectively assembling a 1,485 bp of coding region encoding a 56.3 kDa protein of 495 amino acid residues and two introns of 46 bp and 67 bp. Transcription start point of *aflG* is presented 32 bp upstream of the ATG by primer extension analysis [60]. The 367 bp of intergenic region between *aflL* and *aflG* contains four AflR-like binding motifs, an BrIA binding site, an GC-rich dyad symmetric inverted repeat sequence, two functional TATA-like elements and two CAAT boxes [27,59,60] (Figure 4E). Besides, negative regulatory elements may be in the region from -196 to -118 since expression is enhanced by truncation [60]. While, the region from -367 to

-196 is not required for *aflG* gene activity and SrrA-binding site is not found [19,21,60]. The amino acid sequence of AflG contains some conserved motifs, such as F-X-X-G-X-X-X-C-X-G, E-X-X-R and A-G-X-X-T [59].

*aflV* Disruption of *StcB* encoding a P450 monooxygenase in the sterigmatocystin (ST) gene cluster of *A. nidulans* leads to the lack of ST and the accumulation of AVR (AVF), suggesting that this enzyme is involved in the conversion of AVR (AVF) [61]. *aflV* is homologous to *StcB*, which is required for the similar reaction in aflatoxin biosynthesis. *aflV* contains three exons and two introns of 67 and 55 bp, and a coding sequence of 1,473 bp encodes a 56.3 kDa protein of 508 amino acid residues [6,47]. A 620 bp of intergenic region between *aflV* and *aflW* contains a canonical AflR-binding motif and four possible TATA boxes. AflV contains three highly conserved motifs, F-X-X-G-X-X-X-C-X-G, E-X-X-R and A-X-X-X-T, which are believed to be the active sites

*aflL* Versicolorin B (VER B or VB) is desaturated to VER A (VA) by *aflL* encoded cytochrome P450 monooxygenase/desaturase, and is also a branch point between biosynthesis of 1-group aflatoxins and that of 2-group aflatoxins [62-64]. Disruption of *stcL* in *A. nidulans* abolishes the synthesis of ST and results in the accumulation of VER B (VB) [65]. *aflL* in *A. flavus* and *A. parasiticus* is the homologous gene to *stcL* [GenBank: AF106958] and is presumed to be involved in this reaction [9]. It is located between *aflG* and *hypB*, and is transcribed in a direction same as them. The genomic DNA sequence of *aflL* contains two exons and an intron [6]. Its promoter region possesses an AflR-binding site but SrrA-binding site is not found [19,21].

*aflN* *stcS* is found to be involved in the conversion of VER A (VA) to DMST and its homolog in *A. parasiticus* is *aflN*, presuming that *aflN* is involved in the same conversion in aflatoxin biosynthesis [7,66]. AflN catalyzes epoxidation of the B ring of VER A (VA) [49]. *aflN* is cloned from *A. parasiticus* by genomic DNA sequencing [39]. The genomic DNA sequence of *aflN* contains two exons and an intron, whose ORF encodes a protein of 492 amino acid residues. In the promoter, a canonical AflR-binding motif and three SrrA-binding motifs exist [21,39].

*aflQ* AflQ catalyzes the epoxidation of the A-ring of *O*-methylsterigmatocystin (OMST) or dihydro-*O*-methylsterigmatocystin (DHOMST), then NIH shift to HOMST or dihydro-11-hydroxy-*O*-methylsterigmatocystin (DHHOMST) [33]. HypD may assist AflQ in oxidation of OMST [13,67-69]. *aflQ* belongs to a new P-450 gene family named CYP64, and they show more than 90% identity either at nucleotide level or at amino acid level in *A. flavus* and *A. parasiticus* [70,71]. Both of them contain seven exons and six introns, although the number of nucleotides in the last two introns are different [71,72]. *aflQ* codes for a 60.2 kDa protein of 528 amino acid residues. In the promoter region, there are an AflR-like binding site, a TATA box and some elements located in the intergenic region between *aflQ* and *aflP* [72]. By site-directed mutagenesis and sequence analysis, H at position 400 is critical for the enzyme activity and A at 143 plays a significant role [71]. The difference in key amino acid residues may influence the ability of aflatoxin production between *A. parasiticus* and *A. flavus*. AflQ contains three highly conserved regions, heme-binding motif F-X-X-G-X-X-X-C-X-G, hydrogen bond region E-X-X-R and a highly hydrophobic region [71,72].

*aflU* AflU can catalyze the 370 Da intermediate to give a 386 Da AFG<sub>1</sub> precursor [33]. *aflU* contains five exons collectively coding for a protein of 498 amino acid residues and four introns of 63 bp, 50 bp, 70 bp and 76 bp [7,39]. Its promoter possesses a canonical AflR-binding motif. Like *aflF*, a deletion of portion of *aflU* is present in *A. flavus*, which may lead to fail to the transcription and loss the ability to produce G-group aflatoxin [40]. AflU possesses some conserved regions of P450 enzymes, such as a heme-binding motif, an E-X-X-R motif and a protein transfer groove A/G-G-X-D/E-T-T/S, and a membrane-binding motif P-X-P [39,40].



*NadA* *NadA*-encoded product may catalyze the last conversion from NADA and probably the hypothesized DHNADA (dihydroderivative of NADA) to AFG<sub>1</sub> and AFG<sub>2</sub> through demethylation in the aflatoxin biosynthesis, in which the NADA produced from AflU catalyzed intermediate has a molecular mass of 360, and is converted to produce AFG<sub>1</sub> [73]. Ehrlich et al. [33] shows that *NadA* is unlikely to catalyze the oxidation of the 360 Da intermediate to AFG<sub>1</sub>, but *NadA* catalyzes the 386 Da intermediate given from AflU oxidation to produce a 362 Da intermediate, then converted to 360 Da AFG<sub>1</sub> precursor probably corresponding to NADA by NorB. *nadA* is cloned by gene profiling study using microarray, which is firstly believed to be a sular utilization gene encoding a NADH oxidase [74]. It is the end of the aflatoxin pathway gene cluster at 3' end. The intron assignment for *nadA* gene and their protein size are different considerably in different strains. For example, this gene from *A. parasiticus* has two exons collectively encoding a protein of 444 amino acid residues with 48.5 kD and one intron of 61 bp [33,74]. However, the one from S-strain *A. flavus* AF70 (GenBank accession number AY510453) contains three exons, encoding a protein of 407 amino acid residues, and two introns; while other from L-strain *A. flavus* AF13 (GenBank accession number AY510451) contains six exons, encoding a protein of 355 amino acid residues, and five introns [33]. In the promoter, there is an AflR-binding site [58]. The *NadA* from *A. parasiticus* possesses a conserved old yellow enzyme (OYE)-related FMN binding domain reductases family domain, and some critical amino acids, such as H at 195, N at 200, and Y at 197. [33,75,76]. In addition to the gene structure and protein size, amino acid composition of *NadA* is difference, although these diversities are not in the other aflatoxin biosynthesis genes [33,74]. Some amino acids are missing or replaced [33,75]. These sequence differences will result in the inactivation of *NadA* [33,73,74].

**Cyclase-encoding gene:** *aflK*-encoded OAVN cyclase is involved in the formation of AVR (AVF) from OAVN, and is also named versicolorin (VHOH or VAL) cyclase or versicolorin B synthase which catalyzes the conversion of VHOH (VAL) to VER B (VB) [43,77,78]. *aflK* gene product catalyzes intramolecular acetal formation among the given 5'-ketone and two hydroxyl groups (3-OH and 1'-OH) from HAVN [43], and also catalyzes the dehydrative cyclization of racemic VHOH (VAL) to optically active VER B (VB) [62,78,79]. This gene is located between *aflQ* and *aflV*, and transcribes in a direction same to *aflQ* with an intergenic region of 511 bp and opposite to *aflV*. Its 1,985 bp of genomic DNA sequence contains two exons assembling a continuous ORF of 1,932 bp and one intron of 53 bp, and the coding sequence encodes a protein of 643 amino acid residues with a molecular mass of 70,271 Da and an isoelectric point of 5.06 [80]. The molecular mass and isoelectric point estimated by calculation differ from those observed in experiments. The monomeric molecular mass of native AflK which is a dimer is approximately 78 kDa and the isoelectric point is 4.7 ± 0.1 [78,80]. These differences can be attributed to the post-translational modification. The promoter region contains an AflR-binding site, a pyrimidine-rich motif, a TATA box and a CAAT box [20,27,80]. Besides, this region lacks CRE-like motif and does not bind AftB as well [19-21]. A polyadenylation site at 161 bp after the termination codon does not correspond to the canonical one [80]. In comparison of amino acid sequences, AflK shows high homology to many flavin-dependent oxidases and dehydrogenases. Its amino acid sequence contains three N-linked glycosylation sites N-X-S/T, a G-X-G-X-X-G motif and a calmodulin binding domain (CaMBD) [80-82].

**Esterase-encoding gene:** A esterase encoded by *aflJ* involved in the conversion of VHA to VHOH (VAL) has been purified from *A. parasiticus* [83,84]. An additional copy of *aflJ*, *estA2*, whose gene product has 9 amino acid substitutions, is outside the aflatoxin pathway gene cluster but is not expressed for position effect likely [85]. The 1,000 bp of *aflJ* genomic DNA contains two exons collectively assembling a coding sequence of 945 bp encoding a 34 kDa protein of 314 amino acid residues and one intron of 55 bp. AflJ is an about 60 kDa of isomeric dimer protein consisting of two 32 kDa of monomer [83,84]. The promoter of *aflJ* contains consecutive two canonical AflR-binding motifs, a SrrA-binding motif, five TATA boxes and one CAAT box [21,85].



***O*-methyltransferase-encoding genes:** There are two *O*-methyltransferases involved in aflatoxin biosynthesis [86]. The primary structures of the two *O*-methyltransferase genes and their products exhibit many differences, so that their biological functions are different [45,86]. *O*-methyltransferase I encoded by *aflO* catalyzes methylation of the 6-hydroxyl group of DMST or DHDMST in order to produce ST or dihydrosterigmatocystin (DHST), then *O*-methyltransferase II, the other *O*-methyltransferase encoded by *aflP*, further methylates 7-OH of ST or DHST to form OMST or DHOMST [86].

*aflO* The cDNA sequence of *aflO* has the length of 1,373 bp with three introns of 63 bp, 50 bp and 62 bp, and encodes a 43 kDa protein of 386 amino acid residues [6,45,87]. The transcription start site is at -140 bp upstream before the translation start codon. The promoter region possesses an AflR-binding site and three TATA boxes, but no SrrA-binding site is found [19,21,45]. The polyadenylation signal sequence is at 26 bp downstream of the translation termination codon and the polyadenylation site is at 44 bp. AflO contains a SAM binding consensus sequence and a CaMBD [45,82].

*aflP* The genomic DNA sequence of *aflP* contains a 1,484 bp ORF composed of five exons collectively coding for a 46 kD protein of 418 amino acid residues and four introns of 6 bp, 59 bp, 52 bp and 59 bp [88,89]. In the promoter region, there are two AflR-binding sites, nine CRE-like motifs, a STRE motif, two or three TATA boxes and four CAAT boxes but no SrrA-binding site [19-21,27,88]. The nucleotide sequences of *aflP* from *A. parasiticus* and *A. flavus* share more than 97% identity and the same as at the amino acid level [88]. The native AflP has a leader peptide of 41 amino acid residues, and the mature one consists of 377 amino acid residues which has a molecular weight of 42 kD [89]. AflP possesses a SAM-binding site V-L(E/D)-X-G-X-G-X-G, a CaMBD and a sequence Y-L-V-T near the N terminus [35,82,88,89].

**Other enzyme-encoding gene:** *hypE* encodes a product which can catalyze the 328 Da intermediate from the 326 Da intermediate with an unknown cytochrome P450 enzyme to lose the methyl residue for the production of AFB<sub>1</sub> [13]. HypE has a catalytic ethD domain required for ethyl-t-butyl ether hydrolysis [90].

### Prospects

Researches of aflatoxins, the highly toxic and carcinogenic fungal secondary metabolites produced by *A. flavus* and *A. parasiticus*, have been extensively carried out more than 50 years since the 1960s. Complete nucleotide sequences of aflatoxin biosynthesis clusters contained in several aflatoxin-producing varieties including *A. flavus* and *A. parasiticus* are already known [6]. Genes involved in aflatoxin biosynthesis, including structural and regulatory genes, have been reported [5]. With the identification of functions of the genes in the cluster, the mechanism of aflatoxin biosynthesis seems to have been unveiled.

Actually, in addition to structural genes in the cluster, aflatoxin biosynthesis is regulated by other factors, such as regulatory genes, modification, environmental elements, etc. DNA methylation, an epigenetic modification, plays a vital role in the regulation of gene expression in eukaryotes. DNA methyltransferase inhibitor 5-azacytidine (5-AC) can induce a fluffy phenotype and inhibit aflatoxin biosynthesis in *A. flavus* [91]. Unexpectedly, our results reveal that the DNA methylation level of *A. flavus* is negligible [92]. In such situations, we believe that DNA methylation does not act to regulate the expressions of the genes involved in aflatoxin pathways and is not a target of 5-AC. Moreover, we find that DmtA, a DNA methyltransferase homologue, is important in regulation of aflatoxin biosynthesis and for *A. flavus* to adapt to stressful environments and for survival [93]. This increases the complexity in the regulation of aflatoxin biosynthesis. The canonical regulatory genes within the AF gene cluster, aflR and aflS, have been extensively investigated [94]. Beyond them, some other regulatory factors, such as LaeA, Ham, NosA, FarB, CreA, etc., can also influence aflatoxin formation in *A. flavus* [91,95,96].

In this review, we pay more attention to structural genes. Some details in the whole process of aflatoxin biosynthesis are not very clear, especially in the last steps which may need additional enzyme(s) but has not been identified yet. In order to do that, many more studies have been made in biochemical and molecular mechanisms of aflatoxin biosynthesis. In the process of structural gene cloning, multiple methods are carried out, such as genetic complementation (*aflB*, *aflD*, *aflI*, etc.), reverse genetics (*aflE*, *aflP*, etc.), chromosome walking (*aflF*, *aflH*, *aflQ*, etc.), sequence analysis (*hypC*, *aflV*, *aflN*, etc.), etc., in which genetic complementation is considered to be an effective strategy [97]. The functions of the most of these genes have been confirmed by gene knockout, gene complementation studies, or enzymatic studies [7]. Every structural gene can catalyze a specific reaction or multiple reactions in aflatoxins formation. Regulatory genes, such as *aflR*, and other biotic and abiotic elements, such as carbon source, nitrogen source and bacteria, also affect aflatoxin biosynthesis [5,98]. With the report of genome sequence of *A. flavus* [99], more information will be obtained to research whether-it exists other structural gene(s) and regulatory gene(s) needed in the aflatoxin biosynthesis in or beyond the aflatoxin biosynthetic gene cluster. Therefore, identification of all the structural and regulatory genes involved in the aflatoxin biosynthesis becomes imperative. Using some new strategies including the Next Generation Sequencing technology will gain more knowledge about genetic regulation, signal transduction and interaction with the environment [91]. With more information being summarized, we will have a better understanding of mechanisms of aflatoxin biosynthesis and regulation and its prevention and control.

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