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Genome Mining for Aflatoxin Biosynthesis

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Abstract

Occurrence of aflatoxins in crops has vast impacts on human health. Therefore, the study of aflatoxin biosynthesis has become important. Previous studies have shown that the aflatoxin biosynthesis gene cluster and several genes, enzymes, and regulatory elements are involved in aflatoxin biosynthesis; however, the intricate metabolic regulatory networks of aflatoxin synthesis remain unclear. Rapid development of fungal genomics and other "omics" empower us for genome mining, gene expression profiling, and gene regulation studies that could provide more comprehensive insights into the aflatoxin biosynthesis regulatory network and other secondary metabolisms in fungi. Here we review the recent advances in the field of genome sequencing of aflatoxin-producing fungi, application of genomics, transcriptomics, proteomics, metabolomics, and epigenetics in aflatoxin biosynthesis. In addition, mitochondrial genomes in *Aspergillus* spp and their function in aflatoxin biosynthesis are discussed. In summary, the availability of whole-genome sequence of aflatoxigenic fungi will undoubtedly expand our knowledge of aflatoxin biosynthesis and will provide more insights on the biosynthesis of aflatoxin and other secondary metabolites in fungi.

Keywords: Biosynthesis; Genes; Enzymes; Aspergillus flavus; Aspergillus parasiticus; Signal transduction; Transcriptional regulation

Introduction

Aflatoxin (AF) is a notorious mycotoxin with potent carcinogenicity, and consumption of AF-contaminated foods and feeds poses a serious threat to human and animal health. AF biosynthesis has become an excellent model for exploring the genetic regulation of secondary metabolism in fungi. Studying AF biosynthesis will help us to eliminate or reduce its impact on human and animal health. However, the mechanism of AF biosynthesis and its regulatory network are still beyond our understanding and the control of AF contamination remains limited.

Aspergillus flavus and *Aspergillus parasiticus* are the main producers of AF; some other *Aspergillus* species such as *Aspergillus nomius*, *Aspergillus pseudocaelatus* sp. nov. and *Aspergillus pseudonomius* sp. nov. also produce AF. In addition, several non-*Aspergillus* fungi are also known to produce AF [1].

Regulatory Factor Studies on Aflatoxin Production in Fungi

Previous studies have shown that approximately 30 identified genes required for AF biosynthesis are clustered in a 75-kb DNA region of *A. flavus* and *A. parasiticus* [2], which is an exemplary way of regulating secondary metabolisms in fungi. To date, a variety of genes, enzymes, and regulatory factors have been described in detail to be involved in AF biosynthesis. Genes in the AF gene cluster are co-regulated, and their expression level directly affects AF production. A recent study confirmed the functions of hypothetical genes such as *hypC* and *hypB* in this cluster [3]. Several genes outside the AF gene cluster such as *vrdA*, whose expression is not regulated by *aflR* [4], and an *EST* (CA747446), whose deletion in *A. flavus* genome results in the inhibition of AF production, are known to be involved in AF biosynthesis [5]. In addition, various environmental factors are known to affect AF biosynthesis, including nitrogen, carbon, microelement, lipid, ethylene, temperature, light, pH, and oxidative stress [6]. Moreover, it is generally recognized that fungal development and AF biosynthesis are co-regulated [7,8]. For instance, deletion of genes encoding transcription factors NsdC and NsdD, which are required for asexual development, results in abnormal phenotypes of conidiophore, reduced expression of some genes in the AF gene cluster, and thus no production of AF [9].

aflR and *aflS* within the AF gene cluster, which are the AF pathway-specific regulatory genes, have been extensively investigated. A change in their expression can alter the expression level of AF biosynthesis pathway genes in the AF gene cluster [10]; moreover, their expression is influenced by environment, nutritional conditions, and other factors [11]. A global regulator of secondary metabolism, LaeA, which is a part of the velvet complex (VelB/VeA/LaeA) described in *Aspergillus* spp [12], regulates gene expression in the AF gene cluster. Deletion of either *laeA* or *veA* in *A. flavus* [13,14] or deletion of both in *Aspergillus nidulans* [15] results in the failure of AF or sterigmatocystin production and down-regulation of the expression of genes in the secondary metabolism cluster. Recent studies have identified more transcription factors such as AtfB, ApyapA, Yap1, RsmA, AP-1, MsnA, and SrrA involved in AF biosynthesis [1]. However, the intricate metabolic regulatory networks about how AF-producing fungi respond and transmit environmental stimuli and how regulatory elements control AF biosynthesis remain unclear.

In the past 20 years, especially since the genomic sequence of *Saccharomyces cerevisiae*, the first eukaryotic organism to be sequenced, was completed in 1996 [16], numerous significant progress has been made in the field of fungal genomics. Fungal genomics and other "omics" have empowered us for genome mining, gene expression profiling, and gene regulation network studies that could provide more comprehensive insights into the AF biosynthesis regulatory network and other secondary metabolisms in fungi.

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Through genome analysis, we can anticipate that some fungi such as *Dothistroma septosporum* and *D. pini* cannot produce AF even though they produce intermediates of the AF biosynthetic pathway [1]. Comparative genomic studies of AF-producing fungal strains or other related *Aspergillus* strains could be leveraged for pathogenicity, secondary metabolism network, and phylogenetic researches [17,18].

Studies on mechanisms of aflatoxin biosynthesis using "omics" technologies

Genomic sequencing of 10 *Aspergillus* species including *A. flavus* has already been completed: *Aspergillus oryzae*, a close genetic cousin to *A. flavus* that is safely used in East Asian cuisines; *A. nidulans*, an important model organism for studying genetics and cell biology, which harbors the complete AF biosynthesis pathway except the final step that converts sterigmatocystin to AF; *Aspergillus fumigatus*, a human pathogen; and other species such as *Aspergillus fischeri*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus clavatus*, *Aspergillus sojae*, and *Aspergillus kawachii* [19]. The genome size of *A. flavus*, which is the focus of this review, is 37 Mb, with 14,510 genes encoding proteins larger than 50 amino acids [8,20].

Genomic sequencing has revealed that many fungi contain numerous genes and gene clusters involved in secondary metabolism. For example, genes involved or potentially involved in AF biosynthesis and other secondary metabolisms, signal transduction, transcriptional regulation, pathogenicity, and stress response have been identified [21,22]; fifty-five secondary metabolite clusters were discovered from the *A. flavus* genome sequence [23].

Global gene expression studies using microarray or RNA sequencing can facilitate the discovery of regulatory genes and shed light on environmental impact on AF biosynthesis. Research has revealed that transcription profiles of genes in the AF cluster are similar to those of cyclopiazonic acid (CPA) gene clusters [24]. Based on the complete genome sequence, the transcriptomes of *A. flavus* and *A. parasiticus* were profiled to determine the effect of temperature [25] and other environmental parameters, including carbon, nitrogen, pH, and water activity [26,27] on AF biosynthesis. In addition, the complex regulation under 4 different culture conditions in *A. oryzae* was also elucidated [28]. In our laboratory, the transcriptome of *A. flavus* indicated that the "fluffy" phenotype and inhibition of AF production by 5-azacytidine were due to the inhibition of *veA* gene transcription and up-regulation of *brlA* gene transcription [8,29].

A range of "omics" technologies toward functional and comparative genomics such as proteomics and metabolomics technologies are playing increasingly important roles in understanding the mechanism of AF synthesis and biological process in aflatoxingenic strains. Several proteomics studies have been conducted in *Aspergillus* spp, although proteomics research in filamentous fungi is relatively few. For example, protein changes in response to environmental stimuli regulating AF biosynthesis in *A. flavus* have been quantified [30], comparison of proteome profiles between *hapX* deletion mutant and wild-type strain in *A. nidulans* has been made [31], and the first proteome profile and 2-D proteome map of whole cell mycelial extract of *A. flavus* has been obtained recently [32]. Application of metabolomic analysis and a novel low cell density-dependent metabolic switch to AF production was performed in a study on the effect of peptone on growth and AF biosynthesis in *A. flavus* [33].

A new ChIP-seq method can be used to detect diverse transcription factors involved in secondary metabolism, conidiophore production, and response to environmental stimuli and to map the binding sites based on genome sequences in fungi. For instance, stress-related transcription factor AtfB was reported to bind to AF gene promoters and integrate secondary metabolism and cellular response to oxidative stress in *Aspergillus* spp [34].

Genome-wide epigenetic regulation such as histone modification, chromatin modification, and repeat-induced and repeat-associated silencing mechanisms should be linked with the production of secondary metabolites; however, current evidence from the results of bisulfite sequencing indicated that DNA methylation level is negligible in *A. flavus* [35]. Activation of secondary metabolism clusters is associated with increased acetylation of histones H3 and H4 and affects the production of secondary metabolites [36]. Direct evidence of the involvement of histone acetylation in the regulation of the AF cluster in *A. parasiticus* [37] and ST cluster in *A. nidulans* [38] has been obtained. Keller's laboratory found that deletion of *had*, which encodes histone deacetylase in *A. nidulans*, results in the transcriptional activation of sterigmatocystin biosynthesis cluster [39]. Recent findings suggest that activation process in secondary metabolism clusters are silenced by heterochromatic histone marks and that closed heterochromatic structures are reversed when secondary metabolism activation is mediated by LaeA [36]. However, it is unclear how LaeA mediates the low level of heterochromatic marks inside different clusters and how epigenetic modification affects secondary metabolism.

Future Directions

Mitochondria is the main source of cellular reactive oxygen species (ROS), indicating that mitochondrial genome is involved in cellular development and AF synthesis. Thus, research on the mitochondrial genome, which may partly encode the enzymes involved in AF biosynthesis, will be promising. In recent years, mitochondrial genomes of some *Aspergillus* species have been completely sequenced and annotated, including that of *A. flavus*, which has a mitochondrial genome size of 29.2 Mb, and eight other species such as *A. fumigatus*, *A. oryzae*, *A. niger*, *A. nidulans*, *Aspergillus tubingensis*, *A. clavatus*, *A. terreus*, and *Aspergillus fischerianus* (*Neosartorya fischeri*) [40]. These mitochondrial genome sequences can be used as a supplement of the nuclear genome to obtain insights into species identification and AF biosynthesis. In several *Aspergillus* species, the mitochondrial enzyme alternative oxidase (AOX), which is considered to play a key role in stress alleviation, has been characterized and induced by oxidative stress [41,42]. Some cytochrome P450 enzymes, generally associated with the mitochondrial or endoplasmic reticulum membrane, have been found to be involved in sterigmatocystin and AF biosynthesis [43]. However, up to now the role of mitochondria in AF biosynthesis has not been systematically studied.

The availability of whole genome sequence of AF-producing fungi undoubtedly deepens our knowledge of the AF biosynthesis network, for example, revealing the missing enzymatic steps in AF biosynthesis and identifying more regulatory networks that link AF biosynthesis to oxidative stress and other internal and external factors. Much more exploitation of genomics and other omics may unveil more mysteries of the mechanisms involved in the biosynthesis of AF and other secondary metabolites in fungi and may contribute to the development of strategies for controlling AF contamination.

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