

Role of *aflR* gene expression from *A. flavus* to cause disease in human

Abstract

The *aflR* gene is a regulatory gene for aflatoxin biosynthesis, encodes a protein containing a zinc-finger DNA-binding motif. There is a positive regulatory gene, *aflR* which required for transcriptional activation of most, if not all, of the structural genes by binding to the palindromic sequence 5'-TCGN5CGA-3' in the promoter region of the structural genes in *A. parasiticus*, *A. flavus* and in *A. nidulans*. Adjacent to the *aflR* gene, a gene, *aflS aflI*, is also involved in the regulation of transcription. Finally, the *laeA* gene, for loss of *aflR* expression, was shown to be involved in the global regulation of secondary metabolites, aflatoxins, sterigmatocystin (ST), penicillin and gliotoxin, in several fungal species. Aflatoxins are polyketide-derived secondary metabolites produced by *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus nomius* and a few other species. The toxic effects of aflatoxins have opposing consequences for the health of human and agricultural economics. This study will examine to understand the mechanisms of *aflR* gene regulation, documentation of transcriptional activation.

Keywords: sterigmatocystin, *aspergillus parasiticus*, *aspergillus nomius*, aflatoxins, penicillin

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Introduction

Aspergillus flavus is an opportunistic pathogen.¹ The genus *Aspergillus* is a member of the phylum Ascomycota include over 185 known species. There are 20 species of them have been reported to cause harmful infections in humans and animals² causing invasive and non-invasive aspergillosis in humans, animals and insects. It also causes allergic reactions in humans.³ *A. flavus* infects crops, stored grains and produces the most toxic and potent carcinogenic metabolites such as aflatoxins and other mycotoxins.⁴ Aflatoxins are a family of toxins, polyketide-derived metabolites from *Aspergillus flavus* and *Aspergillus parasiticus*.⁵ Many aflatoxin pathway genes revealed that are clustered within a 60-kb DNA region in *A. parasiticus* and *A. flavus*.⁶ This finding has renewed interest in the study of the regulation of aflatoxin biosynthesis.⁵ Former studies have suggested that one of these genes i. e. *aflR*, is involved in some aspect of the regulation of aflatoxin biosynthesis.⁷ The *aflR* gene product, *AFLR*, contains a GAL4-type binuclear zinc finger cluster Cys-(Xaa)2-Cys-(Xaa)6-Cys-(Xaa)6-Cys-(Xaa)2-Cys-(Xaa)6-Cys.⁸ The(Cys)6-type structure is a distinct feature of some of the pathway-specific regulatory proteins in fungi and yeasts. Also, *aflR* contains a highly acidic domain adjacent to the carboxyl terminus,⁹ similar to that found in GAL4 and GCN4. In *Saccharomyces cerevisiae*, this region is necessary for the DNA-binding protein's ability to activate transcription of other pathway related genes.¹⁰ The transformation with *aflR* of a mutant of *A. flavus*, which inhibit the production of all aflatoxin pathway precursors and *aflR* restored it to aflatoxin proficiency.¹¹ Wild-type and blocked *A. parasiticus* strains, on transformation with *aflR*-containing vectors,⁵ over produced aflatoxin precursors in aflatoxin permissive media, such as potato dextrose broth and Adye and Mateles medium.¹² Aflatoxins or precursors were also produced by these transformants when cells were grown in nitrate medium, which normally inhibits aflatoxin production.¹³ Interestingly, sclerotial morphogenesis was also affected

by these *aflR* transformations grown in the nitrate medium.

Nitrogen regulates the biosynthesis of secondary metabolites in a variety of microorganisms.⁵ Nitrate represses the production of polyketide-derived mycotoxins in *A. parasiticus* and *Alternaria alternata*. Niehaus suggested that repression by nitrate is due to an increased cytoplasmic NADPH/NADP ratio, which favors a biosynthetic reduction, promoting utilization of malonyl coenzyme A and NADPH for fatty acid synthesis, rather than for polyketide synthesis.⁷ Other researchers suggested that nitrate represses formation of enzymes involved in polyketide synthesis.⁵ In this report, we show that nitrate inhibits transcription of aflatoxin pathway genes in *A. parasiticus*, including *aflR*, *nor-1*, *ver-1*, and *omt-1* but the transcription of these genes is restored in transformants containing an additional copy of *aflR*.¹⁴

Fungal Strains and Isolation of Total RNA

A. flavus, a wild-type aflatoxigenic strain, and SU1-N3(pHSP), a derivative of SU-1 that transformed with the *aflR*-containing vector pHSP, were maintained on Potato Dextrose Agar(PDA).¹⁵ *A. flavus* mycelia grows in parallel for 48, 72, 96 and 120h in Cove's minimal salt medium (CMSM) supplemented with ten mM nitrate (nitrate medium),⁵ collected on Miracloth, blotted dry, quickly frozen in liquid nitrogen, and stored at 28°C until use.¹¹ Mycelia is grind to a fine powder in a mortar and pestle in the presence of liquid nitrogen.¹⁶

Northern (RNA) hybridization analysis

Twenty micrograms of total RNA per sample fractionated in a 0.4M formaldehyde-1.2% agarose gel and transferred to a Gene Screen Plus membrane (Dupont NEN Research Products, Boston, Mass.),¹⁷ for probing with [a-32P]dCTP-radiolabeled DNA probes prepared by using the Random Primed DNA Labeling Kit.¹⁸

Cloning and sequencing of *aflR* cDNA

A cDNA library in lambda phage vector constructed with a directional ZAP-cDNA synthesis kit.⁵ Screening of the cDNA library performed with [α -³²P] dCTP-labeled probes generated from a 1.9-kb BamHI-BamHI genomic DNA fragment. In vivo excision of pBluescript SK2 phagemids from positive Uni-ZAP XR lambda clones conducted with the ExAssist/SOLR system (Stratagene) by a helper phage coinfection protocol. Several positive cDNA clones isolated and analyzed by restriction enzyme digestion.¹⁹ The clone containing the largest cDNA insert sequenced by the dideoxy chain termination method with Sequenase version.⁵ The *aflR* cDNA sequence including the 59 upstream sequences and the deduced amino acid sequence.²⁰ The copy number of the *aflR* transformants determined by a quantitative slot hybridization technique with a GeneScreen Plus nylon membrane.²¹

Determination of *aflR* copy number in transformants

The copy number of the *aflR* transformants determined by a



Figure 1 *AflR* and *aflJ* are regulatory genes. The positively acting transcriptional factor is *aflR* requisite for the expression of the *stc* genes.¹⁵

Temperature and nitrogen effect on aflatoxin biosynthesis

There are several biotic and abiotic environmental factors influence aflatoxin biosynthesis.²² According to the temperature and nitrogen effect on aflatoxin biosynthesis *aflR* play key role. Nitrate play a suppressive effect on aflatoxin production, also over expression of the *aflR* gene by extra copies of *aflR* overcomes and negative regulatory outcome on gene transcription of aflatoxin pathway. Aflatoxin formation is exactly affected by temperature.²³ RT-PCR analyzed enough quantity of transcripts of commonly regulatory genes *aflR* and *aflS*.²³

Conclusion

The *aflR* sequence identified in these studies *aflR* gene cloning, expression and its pathogenicity of aflatoxin in human. We learn focus on the mechanisms of aflatoxin biosynthesis, we need to examine its regulatory mechanisms. The regulation of aflatoxin gene expression occurs at numerous levels and by several regulatory components. The nutritional and environmental factors effect on aflatoxin formation through the presence of *AflR*. The regulated expression of *aflR* also occurs in *A. oryzae*, which is generally used in the food industry. We expect that examination will lead to a finding of genes and molecules involved in pathogenicity and fungal continued existence that may give efforts to control diseases.

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quantitative slot hybridization technique with a GeneScreen Plus nylon membrane. The SU1-N3 and SU1-N3(pHSP) genomic DNAs denatured in 1ml of 0.25M NaOH–0.5M NaCl for 10min at room temperature. Four 200-ml portions of denatured DNA is loaded into individual sample wells of the apparatus. PCR performed by using Ampli Taq DNA polymerase and a DNA thermal cycler.^{5,11}

Expression and purification of recombinant *aflR*

The SmaI-XhoI digest of *aflR* cDNA ligated to SmaI-XhoI-digested pET29c (Novagen, Madison, Wis.) to give the plasmid *pAFLR1*. This construct has in-frame codons for six histidines at the C-terminal side of the *aflR* insert. The plasmid transformed into Escherichia coli BL21 (DE3) pLysS, and expression is induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM to log-phase E. coli cells grown in Luria broth (LB) medium containing kanamycin and chloramphenicol. Expression of the fusion protein is monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in aliquots taken at hourly intervals.⁵

Conflict of interest

The author declares no conflict of interest.

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