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Differential Expression Pattern of Heat Shock Protein Genes in Toxigenic and Atoxigenic Isolate of *Aspergillus flavus*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RT, ST and JS conceived and designed the experiments. Author RT performed the experiments. Authors RT and JS analyzed the data. Author JS contributed reagents and materials. Authors RT, ST and JS wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aflatoxin biosynthesis in *Aspergillus flavus* requires coordinated expression of regulatory and structural genes. Aflatoxin production is optimum at 24-30℃ and inhibition occurs at temperature higher than 35℃. Chaperones or heat-sock proteins are involved in processing of cellular protein and heat-stress induced protein, hence, we studied the genes encoding for heat-shock proteins under the influence of temperature (30℃ vs. 37℃). *A. flavus* isolates, aflatoxigenic (MTCC9367) and atoxigenic (MTCC11580) were grown in glucose minimal salt broth for 24 hours for expression profile of selected genes using quantitative real-time PCR. We monitored the expression profile of genes encoding for heat-shock proteins (*hsp98, hsp90*, *hsp70* and *hsp60*) and regulatory gene of aflatoxin biosynthesis pathway *aflR*. We found the similar trend for heat-shock proteins gene expression except *hsp70* in aflatoxigenic and atoxigenic isolates of *A. flavus*. Expression for *hsp70* was found to be upregulated at 30℃ (vs 37℃)in atox igenic isolate (P<0.001) of *A. flavus* in

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comparison of toxigenic (P<0.05) isolate. Since, heat-shock proteins are involved in protein folding and conformational stability of cellular proteins to maintain the biological activity, our data on transcripts encoding for heat-shock proteins suggested it may influence the aflatoxin biosynthesis process in *A. flavus*.

Keywords: Aflatoxin; Aspergillus flavus; heat-shock protein; hsp90; hsp70; Aspergillus parasiticus.

1. INTRODUCTION

Aflatoxin is highly carcinogenic secondary metabolite produced by *Aspergillus* genus predominantly by *Aspergillus flavus* and *A. parasiticus* [1]. *A. flavus* is well known aflatoxin contaminants in economically important crops [2]. Exposures of aflatoxin to human beings cause liver cancer and various toxic syndromes in consumed persons or feeding animals [1]. There are 4-30 parts-per-billion limit of aflatoxin in food and feed in many countries including India [3]. Environmental conditions like temperature, pH, moisture and type of nutrients on which *A. flavus* grows, may influence the aflatoxin biosynthesis in vitro conditions [3,4]. Also, there were no detectable aflatoxin using ELISA in *A. flavus* infected mice model studies [5] and growth of filamentous and dimorphic fungi is influenced by hormone/temperature has been reported in the host [6]. So far, temperature has been reported to be critical for the aflatoxin biosynthesis in field conditions along with water activity and elevated CO_2 [7]. Temperature is not only involved in regulation of aflatoxin biosynthesis in *A. flavus* but also involved in formation of conidia in *Aspergillus* [8,9] and fungal dimorphism including *Paracoccidioides* [10]. Tarun et al. recently showed the effect of temperature on aflatoxin biosynthesis in *A. flavus* using triple quadrapole mass spectrometer and reported enhanced aflatoxin B1 synthesis at 24℃ to 27℃ and significantly low or undetectable aflatoxin production at 37℃ [3]. Simultaneously Schindler et al observed that aflatoxin biosynthesis is optimum at 24℃ and not above 35℃. Also there is complete inhibition of aflatoxin synthesis at 37℃ temperature, which is optimum for the growth of *Aspergillus* [11]. The early studies of aflatoxin in corn grown under high or moderate heat stress demonstrated that heat stress play an important role in aflatoxin production [12]. Aflatoxin biosynthesis in *A. flavus* requires co-ordinated expression of series of genes [13]. Regulatory genes *aflR* and *aflS* control the aflatoxin biosynthesis along with post-transcription regulation mechanisms [14]. *aflR* is the positive regulator gene and required for transcription activation of most of the aflatoxin pathway genes along with *aflS gene, a* co-activator of *aflP* and *aflD* genes. Chang and Meyers et al. showed *aflR* mRNA and AFLR regulatory protein were present at 29℃, but not at 37℃. It has been suggested that AFLS protein interacts with AFLR regulatory protein to modulate the transcription of aflatoxin biosynthesis pathway [15]. However, additional governing factors regulating the aflatoxin biosynthesis is still unclear.

The role of chaperones (heat-shock proteins) has been studied during stress conditions and that favor native folding of protein to maintain biological activity [16,17]. Evolutionary conserved heat-shock proteins protect other cellular proteins from heat by binding to them when they are denatured or incorrectly folded. They also help in the assembly and disassembly of macromolecular complex [18,19]. Thus to understand the role heat shock proteins in aflatoxin biosynthetic process, we measured mRNA of heat shock proteins (Hsp98, Hsp90, Hsp70 and Hsp60) mRNA by quantitative real-time PCR at different temperature (30℃ vs. 37℃) in toxigenic and atoxigenic isolates of *A. flavus*. Our data with heat-shock proteins mRNA at temperatures (30℃ vs. 37℃) from different isolates of *A. flavus* suggested that heat-shock proteins may influence aflatoxin biosynthesis process.

2. MATERIALS AND METHODS

2.1 *Aspergillus flavus* Inoculum Preparation and Growth Conditions

Atoxigenic (MTCC11580) and aflatoxigenic (MTCC9367) isolates of *A. flavus* were used in this study [3,20]. Freshly grown cultures of *A. flavus* isolates were used for inoculum preparation. Conidial suspension was prepared from *A. flavus* grown on Sabouraud's dextrose agar (SDA) medium (HiMedia, India) slants using phosphate buffer saline (PBS) with 0.05% (v/v) sterile polyoxyethylene sorbitan monooleate (Tween 80, Merck, India) solution (PBST). The conidial suspension was transferred to a sterile vial for further use. Conidial count was performed

using hemocytometer (Neaubaure, Germany) at 400× magnification using compound microscope. Working suspension of conidia was prepared by diluting the conidial suspension with PBST to a final count of 1×10^6 conidia/ml. colony forming units were counted from the diluted stock to confirm viability of conidia. Glucose minimal salt broth medium (5 mg/ml glucose) was used for the growth of *A. flavus* strains at different temperature conditions, which is known for aflatoxin production [15,21]. GMS medium was inoculated with 1×10^6 conidia/ml and cultures were grown at 30℃ and 37℃, respectively, for 24 hours on a shaker (Thermo Scientific, USA) at 150 rpm.

2.2 Total RNA Extraction

Total RNA was extracted from biological replicates of *A. flavus* isolates grown at 30℃ and 37℃, respectively, for 24 hrs at 150 rpm. *A. flavus* culture was harvested by centrifugation at 12000 rpm and snap chilled in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, USA) as per manufacturer's instruction and by method previously used by Gautam P, et al. [22]. The quality and quantity of extracted RNA were assessed at A_{260}nm/A_{280}nm by using Nanodrop spectrophotometer (Thermo Scientific, USA). The quality was also checked by electrophoresis using 1.2% agarose gel stained with ethidium bromide (EtBr) for the presence of intact ribosomal RNA bands (18S and 28S), visualized by UV transillumination at 302 nm.

2.3 cDNA Synthesis

The cDNA synthesis was carried out from total RNA (1µg) pooled from two biological replicates. cDNA template was synthesis using verso-cDNA synthesis kit (Thermo scientific, USA) according to manufacturer's instructions. cDNA synthesis was performed at 42℃ for 30 min for reverse transcription followed by inactivation at 95℃ for 2 min. The integrity of cDNA template was checked through housekeeping gene β-tubulin [10].

2.4 DNA Extraction

The genomic DNA (gDNA) was isolated from *A. flavus* using phenol: chloroform method. The quality and quantity of gDNA was assessed by using Nanodrop spectrophotometer (Thermo Scientific, USA) at A_{260}nm/A_{280}nm. The quality of genomic DNA was also checked through electrophoresis on 0.8% agrose gel stained with EtBr and observed under UV transillumination at wavelength of 302 nm [23].

2.5 Quantitative Real-time PCR

Quantitative Real-time PCR reactions were performed in BIORAD machine CFX96 (BIORAD, USA). Total RNA from two biological replicates were pooled, each reaction were performed two times in duplicate from pooled total RNA in 96 well format using SYBER-Green master-mix (Thermo Scientific, USA) according to manufacturer's instructions. Each quantitative PCR reaction was performed in 20 µl total volume using 100 ng of cDNA as template. The thermo-cycling conditions for Real-time PCR were comprised of initial step at 95℃ for 3 min, and 35 cycle of 95℃ for 10 sec (denaturation), 56.3℃ for 30 sec, 72℃ (extension) for 20 sec. A dissociation curve was performed to check the primer specificity. Transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed significant difference in expression at both temperatures targeted, compare to β-tubulin. Thus, β-tubulin gene was considered as internal control. The result obtained for target genes were normalized using β-tubulin threshold cycles (C_T) obtained from cDNA amplification run on the same plate by using absolute quantification method. Also β-tubulin gene was used to determine the copy number by generating standard curve from gDNA dilution ranging from 3 to 300,000 genome copies [10]. A linear relationship was obtained by plotting (C_T value) threshold cycle against the logarithm of known amount of initial template. The primers used in this study were designed from exon-exon region using Primer-Blast is described in Table 1. Expression levels of each gene were measured four times normalized by absolute quantification method with β-tubulin as endogenous reference [10].

2.6 Statistical Analysis

The statistical analysis of data was done by use of graph pad software (GraphPad prism v 5.0, GraphPad software) [10]. The significant difference of the mean value of the data at different temperature 30℃ and 37℃ after 24 hours in toxigenic and atoxigenic strains of *A. flavus* was done by two-sample unpaired t-test using GraphPad software [10]. A *P*-value <0.05 was considered significant in our data analysis.

Table 1. List of primers used in quantitative Real-time PCR

Gene	Sequence 5' to 3'	GI. NO	Product size (bp)
β-tubulin	F:GGAATGGATCTGACGGCAAG R: GGTCAGGAGTTGCAAAGCG	480538393	122
hsp90	F:CGTCAAGTCCATCACTCAGC R:GCTTGTGGATGCGCTCGGC	238503320	98
hsp60	F: GGTTTGACAGCTCCAAGG R:GCTTGTGGATGCGCTCGGC	238493600	121
hsp70	F:CCTACTCCCTCAAGAACACC R:GAGACTCGTACTCCTCCTTG	238490040	141
hsp98	F:GAGAGATGAGGCAGAACG R:TCCACCTCGAGTCTTTCG	238484242	102

3. RESULTS AND DISCUSSION

Different biotic and abiotic factors induce the stress response in fungi that affect fungal growth [19] and metabolite production such as aflatoxin biosynthesis [7]. Abiotic stress includes heat-stress/temperature, water and CO_2, and in response, heat-stress/temperature significantly affects aflatoxin biosynthesis process. Thus, it is imperative that heat shock proteins may play crucial role during aflatoxin synthesis process. Further, elevated CO_2 along with drought stress conditions (37℃) favors aflatoxin biosynthesis. So the interactions of abiotic factors become critical for the biosynthesis of aflatoxin [7]. Furthermore, biotic and abiotic factors (drought and heat stress) lead to stress on plants that affect their growth and fungal infection or aflatoxin contamination [24]. Aflatoxin biosynthesis process in *A. flavus* is complex and requires coordinated expression of regulatory and structural genes. Twenty five genes have been identified and are clustered within 70Kb region of *A. flavus* genome along with aflatoxin pathway specific genes *aflR* and *aflS* [13,15]. Regulatory genes, *aflR* and *aflS*, of aflatoxin biosynthesis pathway have been identified in *A. flavus*, *A. parasiticus* and *A. oryzae* [25]. Regulatory genes control the expression of structural genes under the influence of temperature [26]. *aflR* regulatory gene produces an AFLR protein, which contains a zinc-finger motif and interacts with promoter region of structural genes to modulate their expression.

Recently, Tarun et al. showed the influence of temperature on aflatoxin biosynthesis in *A. flavus* isolates MTCC11866 and MTCC9367 using tandem mass spectrometry with ESI interface. High amount of aflatoxin (AFB1) production in MTCC11866 at 24℃ (2.3×10^4 cps, 19.27 µg/mL) and at 27℃ (5.1×10^4 cps, 43.63 µg/mL) was observed. However at 37℃ (5967 cps, 5.07 µg/mL), 8.6 folds decreased level of aflatoxin (AFB1) was found as compared to 27℃. Whereas in case of MTCC9367, we observed the enhanced production of aflatoxin (AFB1) at 24℃ (1487cps, 1.26 µg/mL) and 27℃ (2022 cps, 1.72 µg/mL). However, we found highly reduced level of aflatoxin (AFB1) at 37℃ (21 cps, 0.018 µg/mL) [3]. We also monitored the expression of *aflR* gene at 30℃ and 37℃ in toxigenic and atoxigenic isolate of *A. flavus*. We observed the high expression of *aflR*, at 30℃ as compared to 37℃ after 24 hours in toxigenic isolate of *A. flavus* MTCC9367. Whereas the expression levels of *aflR* gene at both temperatures in atoxigenic isolate of *A. flavus* MTCC11580 was downregulated (unpublished). Thus, inhibition of AFLR protein in atoxigenic isolate possibly leads to inhibition of aflatoxin biosynthesis. Data showed that temperature significantly affect the production of aflatoxin in *A. flavus.* Since, temperature is one of the key factors of aflatoxin biosynthesis process, our study on genes encoding heat shock protein showed differentially expressed.

3.1 Heat Shock Protein Genes Respond to Temperature

Chaperones/heat-shock proteins are the key proteins involved in folding/conformational stability of the cellular protein to perform necessary biological function [16] and these heat shock proteins are highly conserved, which are either highly expressed or constitutively expressed during various stress conditions such as at high temperature condition [27]. Increased temperature destabilizes cellular protein and further lead to loss of essential protein function. So heat shock proteins protect these thermally damaged proteins from aggregation and unfolding [28]. Among heat shock proteins, Hsp90 (HtpG in prokaryotes) is an evolutionary conserved chaperone, which involves in proper

folding of proteins and assembly of complex macromolecular structure and also interacts with client proteins such as kinases and transcription factors in fungi [16,28,29]. Whereas Hsp70 (DnaK in prokaryotes) do function together with Hsp90 and interacts with partially unfolded proteins [16,28]. Hsp90 chaperone coordinates temperature with various signaling pathways, which regulate morphogenesis, virulence and development in fungi [30].

Quantitative real-time PCR data showed up-regulation of *hsp98* (P<0.01, P<0.001), *hsp90* and *hsp60* (P<0.001) transcripts at 30℃ in comparison to 37℃ in both toxigenic and atoxigenic isolates of *A. flavus* (Fig. 1). Since, Hsp90 is an essential protein involved in proper functioning of diverse set of proteins including those involved in metabolic pathways [31]. In *C. albicans* at 37℃, interaction of Hsp90 protein with repressor protein help filamentation and cellular morphogenesis, thus Hsp90 protein tunes cellular output to environmental effect [32]. Hsp90 has also been involved in cell wall remodelling of a pathogenic yeast using MAPK signalling during thermal adaptation [32]. Our data with Hsp90 mRNA suggest the appropriate folding of regulatory proteins at 30℃ to induce the expression of structural genes of aflatoxin biosynthetic pathway. However, to evaluate the role of chaperones in maintaining biological function of the proteins (AFLR and AFLS) involved in secondary metabolite production, inhibitors (geldanamycin and radicicol) for Hsp90 protein could be further explored [8,25].

Other stress induced heat-shock protein include downregulation of *hsp70* mRNA expression at 30℃ in comparison to 37℃ (P<0.05) in toxigenic isolate as compared to atoxigenic isolate of *A. flavus*, which showed up-regulation of *hsp70* mRNA at 30℃ (P<0.001) in comparison to 37℃. Previously, under drought stress conditions a heat shock protein (Hsp179) has been found up regulated in aflatoxin resistance maize [33,34]. Thus, our study suggests Hsp70 is involved in thermal adaptation to the environment and may influence the regulatory gene products (AFLR & AFLS) of aflatoxin biosynthesis pathway at 30℃. Lamoth *et al* 2015 showed that mutation in StiA-interacting domain of Hsp70 impaired thermal adaptation of *A. fumigatus* [35]. Further, previous studies on *Paracoccidioides* has shown that *hsp90* or *hsp70* are induced during Mycelia-to-Yeast transition and are important for its adaption at 37℃ temperature [10]. Several transcripts encoding for heat shock proteins (Hsp9, Hsp30, and Hsp104) including Hsp70 have also been observed in *A. niger* in dormant conidia and in transient stage of germinating conidia indicating the role of these heat-shock proteins in cell maintenance and growth [36]. Further, our data showed downregulation of transcripts of *hsp60* at 37℃ in comparison to 30℃ in both strains of *A. flavus*, where as Raggam RB *et al* showed high expression level of *hsp60* in *A. fumigatus* and *A. terreus* at 37℃ [27]. Hsp60 is an essential mitochondrial chaperone involved in the proper folding protein under oxidative stress condition [37]. We also observed downregulation *hsp98* mRNA at 37℃ in comparison to 30℃ in atoxigenic and toxigenic isolates of *A. flavus*. Whereas in a limited study available for Hsp98, Zeuthen *et al* showed synthesis of Hsp98 chaperon at high temperature (45℃) in *Candida albicans* [38]. However, the role of Hsp98 in *A. flavus* needs further study.

It is clear from various studies that heat shock proteins are involved in protein folding and their up-regulation or expression depends at temperature [28,32]. Since, AFLR protein binds to a consensus sequence upstream of structural genes of aflatoxin pathway [14], the proper folding of AFLR protein at 30℃ seems essential for the transcription of structural genes. Our data with genes encoding HSPs showed that expression of *hsp70* is significantly regulated in toxigenic and atoxigenic isolates of *A. flavus*. We found expression of *hsp70* is extremely low at 30℃ in toxigenic isolates of *A. flavus* in comparison to 37℃, suggested that low transcripts of *hsp70* may favor the aflatoxin biosynthesis process. Conversely, we observed high expression of Hsp70 mRNA at 30℃ in atoxigenic isolates of *A. flavus* correlating inhibition of aflatoxin biosynthesis. AFLR protein encoded by *aflR* gene is the positive regulator of aflatoxin pathway genes [15]. It may be possible that Hsp70 may work in association of *aflR* gene product and affect its function or there may be different regulators molecules that prevent the binding of AFLR to promoter sites in aflatoxin pathway genes. However, intense work is required on regulators of aflatoxin biosynthesis process in *A. flavus*. We have concluded that at 30℃ aflatoxin biosynthesis is inhibited due to increased expression of *hsp70* mRNA in atoxigenic isolate and their possible role on functioning of regulatory proteins of aflatoxin pathway.

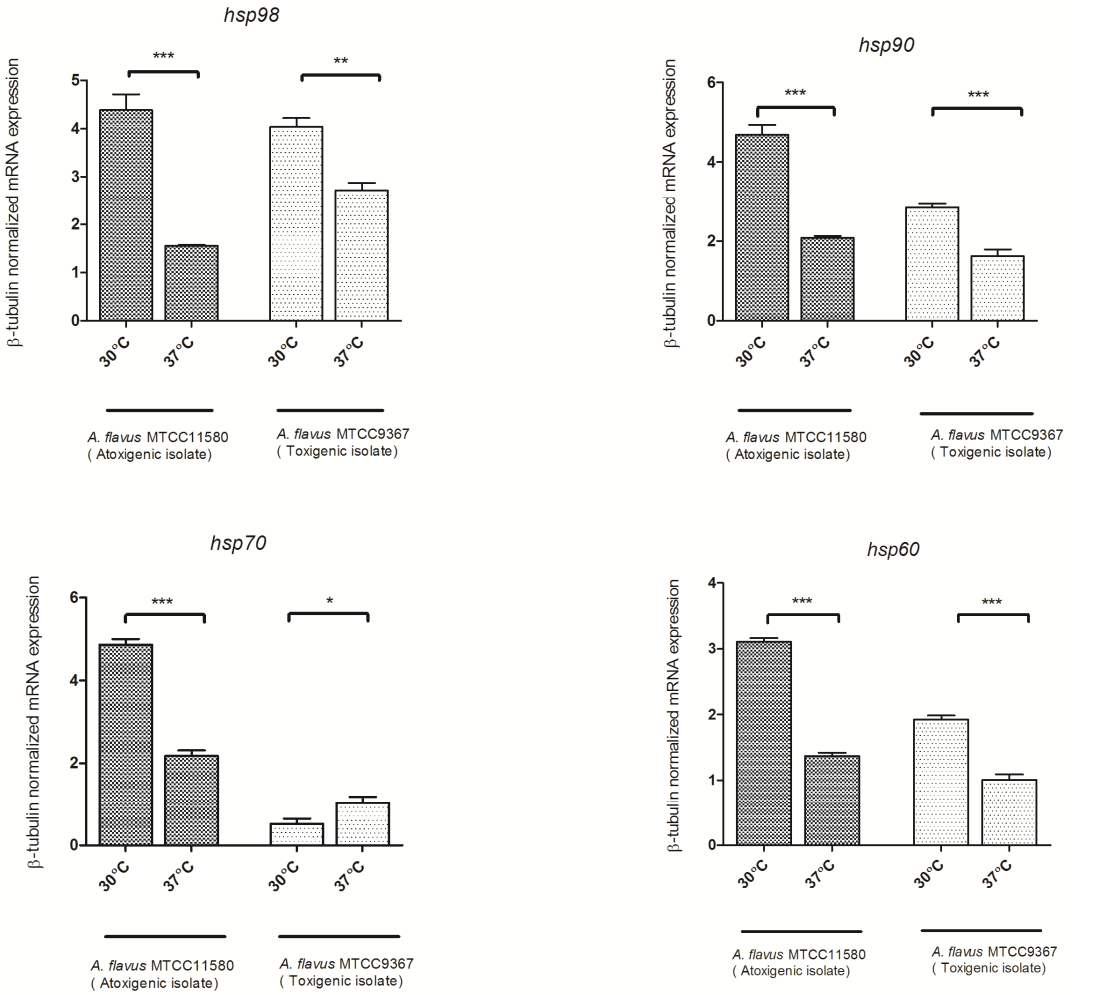

Fig. 1. Quantification of transcripts of genes encoding heat shock proteins

This figure shows genes expression level at 30℃ and 37℃ after 24hours in toxigenic and atoxigenic isolates of A. flavus. The expression value of each gene derived from β-tubulin-normalized qRT-PCR. Each gene plotted against 30℃ vs. 37℃ to find out the relative quantification at both temperatures. The expression quantity of each gene at 30℃ and 37℃ was normalized by using C_T value obtained from the β-tubulin Real-Time PCR on the same plate. The relative quantification of each gene and β-tubulin gene expression was determined by a standard curve. The C_T value plotted against logarithm of DNA copy number. The values represent number of copies of the cDNA of each gene divided by the number of copies of the cDNA of β-tubulin gene. The data represent the mean (±SEM) of duplicate of Real–time PCR run from RNA samples of two independent biological samples. Statistical comparison between 30℃ and 37℃ samples from toxigenic and atoxigenic isolates of A. flavus was done and significant difference marked on figure above the bars by asterisk, which indicates the p values (P<0.05, **P<0.01, *** P<0.001)*

4. CONCLUSION

Our analysis of quantitative real-time PCR data showed up-regulation of *hsp98* (P<0.01, P<0.001), *hsp90* and *hsp60* (P<0.001) and down-regulation of *hsp70* transcripts at 30℃ in comparison to 37℃ in toxigenic isolates of *A. flavus*. Present studies suggested the proper folding and function of gene products involved in the biosynthesis of aflatoxin at 30℃ in toxigenic isolate whereas upregulation of *hsp70* at 30℃ in atoxigenic isolate of *A. flavus* possibly does not in favor of aflatoxin biosynthesis and needs further investigation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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