ASSESSMENT OF GENES INVOLVED IN THE TRANSITION OF CONIDIA TO MYCELIA IN *ASPERGILLUS FLAVUS*

Thesis submitted in partial fulfillment of the degree of

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SELF- DECLARATION

I, **Anuja Thakur**, student of M.TECH. Biotechnology, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh do hereby declare that the project entitled **"Assessment of genes involved in the transition of conidia to mycelia in *Aspergillus flavus*"** submitted towards partial fulfillment for the award of the degree of Master of Technology in Biotechnology is an authentic record of the original research conducted by me under the guidance of Dr**. Jata Shankar**. I confirm that this work has not been submitted elsewhere for any other academic degree or diploma.

I recognize the significance of academic integrity and the consequences of academic dishonesty. Therefore, I assure you that all the research work presented in this thesis is genuine and original, and I have followed all the ethical guidelines prescribed by the university.

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CERTIFICATE

This is to certify that the work reported in the M. TECH thesis entitled "**Assessment of genes involved in the transition of Conidia to Mycelia in *Aspergillus flavus***" submitted by Ms. Anuja Thakur at the Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Anuja Thakur

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List of Abbreviations

A.flavus	*Aspergillus flavus*
AF	Aflatoxin
RNA	Ribonucleic Acid
DNA	Deoxy nucleic acid
PDA	Potato Dextrose agar
PDB	Potato Dextrose Broth
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered saline with tween 20
Hsp90	Heat shock protein 90
Hsp70	Heat Shock Protein 70
CRZ	Calcineurin

Abstract

Aspergillus flavus is a widely known species of *Aspergillus*, known to produce carcinogenic Aflatoxin. It is the major cause of aflatoxicosis in humans and animals. It is mostly present in Environment due to small asexual bodies known as Conidia. The transition of Conidia to Mycelium/hyphae is the key event in the *A. flavus* life-cycle, which needs further exploration to identify stage-specific genes, Candidate genes, or genes involved in the production of toxins (Aflatoxin, mycotoxin, etc.). Prodigious studies have been conducted on *A. flavus* transition from 0 hours- 72 hours, which includes both transcriptomics and genomics approaches. However, there is a huge research gap for transcripts involved in morphogenesis at the genomics level to identify major gene clusters involved in isotropic and polarized growth. Thus, in our current study objective analysis of the transcriptome of *A. flavus* at 0h,5h,10h,24h, and 72h shows dormant conidia, active conidia, swollen conidia, germ tube stage, hyphae, and mycelia. Our data suggested that a large number of genes expression changed from isotropic growth to polarized growth, with 9065 total differentially expressed genes out of which 4426 genes are upregulated and 4639 genes are down-regulated. Go annotation and KEGG pathway enrichment revealed that the top pathways which are involved in morphogenesis are signaling, cAMP, glycolysis, secondary metabolism, and amino acid metabolism played important roles. Our mapman results showed that Hsp90, Hsp70, rho-gdp, Pkc, Calcineurin, Hsp98, rho-gdp, calmodulin, Crz1, phospholipase C, showed a constitutively higher expression. This result will further be validated by quantitative real-time RT-PCR.

Chapter-1
Introduction

Aspergillus is a prevalent mold that thrives on dead-decaying organic matter and can be found in diverse ecosystems and climatic conditions. Inhaling mold spores can result in adverse health effects, including allergic reactions in susceptible individuals, fibrosis in the lungs, and hypersensitivity pneumonia [1]. In terms of causing invasive infections, *Aspergillus fumigatus* is a primary cause, with a nearly 80% incidence rate. Additionally, the second most prevalent contaminant is *Aspergillus flavus* [2]. It has gained increased significance as a human invader because they are capable of infecting individuals with weekend respite. *Aspergillus flavus* species are connected with highly regulated mycotoxin, aflatoxin B1 [3].AFB1 has been scientifically established as a causative agent of cancer in humans and classified as a Category 1 carcinogen. It is deemed to be the most hazardous natural carcinogen and the most toxic unit of the mycotoxin group [4]. Approximately 25% of the global population is altered by aflatoxin (AF) contamination, which has served long-lasting implications for human health. These health issues include hindered immunity, slow development, amplified cancer risk, and even death. When crops are contaminated with a significant amount of AF, they become substantial to regulations that can create financial hardship and constrain trade in the market [5]. It has been reported that the annual decline of crops caused by *Aspergillus flavus* in just the US results in an annualized reduction of USD 686.6 billion per year. The impact of AF on maize is massive, with some evaluations putting the yearly financial cost as high as USD 1.68 billion [6]. Aflatoxin contamination is believed to be widespread in the North Indian Environment, presumably due to changes in climatic conditions. It is responsible for causing fungal sinusitis and endophthalmitis in the population of Northern India [7].

It has been proven that AF leads to mutations in the p53 gene responsible for restraining tumors and the K-ras and H-ras protooncogenes. Moreover to AF, certain strains of *A.flavus* also generate other toxic substances comprising cyclopiazonic acid and aflatrem.CPA has an impact on the calcium-dependent ATPase found in the sarcoplasmic reticulum, which enhances modifications in calcium levels and heightened muscle contractions [8].In depth genetic research of *A.flavus* and *A.parasiticus* have manifest a 70 kb DNA region that contain a cluster of 25 genes responsible for the enzymatic reactions involved in the biosynthesis of aflatoxin [9].

Chapter -2
Review of Literature

Aspergillus flavus is serious cause of health in immunocompromised human beings. It causes aspergillosis in humans and animals. Aspergillus serves as model organism to understand the cytology of eukaryotic organisms. Conidia, mycelia, hyphae are important morphotypes of Aspergillus due to which it can survive in favorable and unfavorable conditions. Conidia size ranging from 2 to 5 µm because of small size it reached the alveoli after being inhaled by humans. *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus oryzae*, present in air but most common fungus is *Aspergillus flavus*. It is second most opportunistic fungus that causes invasive aspergillosis. It is main species that infecting insects, cause diseases in economically important crops such as peanuts, maize, and groundnuts, etc.The predominant species which induce aflatoxin (AF) contamination of crops before harvest or during storage are A. flavus and A. parasiticus. There have been at least 16 structurally related toxins, four are the aflatoxins G1, B1, B2 and G2 majorly produced by *Aspergillus flavus*. It is the most hazardous and strongly hepatocarcinogenic natural compound. In recent years there are increase cases of Aspergillus flavus induced contamination/diseases in immunocompromised patients that have developed curiosity to understand the life cycle events of Aspergillus flavus germination, expression of genes in AF biosynthesis during the transition of conidia to mycelia.

2.1 Emergence of *Aspergillus flavus* as an opportunistic Fungal Pathogen

Aspergillus is a fungus that comprises 180 species.20 species out of 180 causing infections in humans and animals. It is the second most known pathogenic species after *Aspergillus fumigatus* 180 species with different types of pathogenicity. Some of the species of Aspergillus flavus are harmless, but some cause very serious problems that can lead to death also. *Aspergillus flavus* is mostly concerned because it produces secondary metabolites as toxins such as aflatoxins, and mycotoxins. Ingestion of these pathogens into the human body can cause serious health risks it can also lead to the death of the individual. It causes other serious diseases in animals also like mycotoxicosis, and aspergillosis. Effective treatment is needed for this otherwise it will lead to economic loss and also health loss. Antifungal medications such as voriconazole and amphotericin B are often used to treat these infections, while surgical intervention may be necessary in some cases. Proper handling of food and food storage can reduce the risk of contamination by *Aspergillus flavus* and its harmful toxins [10].Aspergillosis is a condition or name given to the disease that causes infection in humans and animals by *Aspergillus flavus*. It is not

just a pathogenic species; it is also known for causing serious health problems and huge economic losses due to its ability to contaminate crops such as maize, groundnuts, peanuts, and cotton with aflatoxins. It affects maize, cotton, and peanuts, where it can produce a large number of toxins and can lead to a higher amount of contamination. Due to the presence of aflatoxins in crops, people will not be able to utilize the crops or either sell them into another market or do their work on a large scale, which will lead to loss to the agricultural industry. Toxin contamination can occur at any stage of crop harvest it can occur in pre-harvest or post-harvest or even after when the crop is fully cultivated [11].During the storage of crops, two types of aflatoxins came in to picture, B1 and B2. Aflatoxins (B1, B2) are majorly produced by *Aspergillus flavus*, not by another species in very large amounts. They can cause liver damage in humans and animals and also lead to cancer. It is very important to check the supply of food in the market [12]. It can cause diseases such as kernel rot in corn and peanut pod rot. Aspergillosis infection in animals is untreatable in the last stage that can cause the death of animals. Due to contamination of food and other eatable items export of food cannot do that can automatically lead to a deficiency of food and economic losses. Preventing economic loss, loss of trade, and human loss needs preventive measures otherwise it will create a global problem [13].It can also give rise to the loss of the textile industry due to disordered texture and shape. Yellow spot disease is the most common or most famous disease of cotton balls that is also caused due to *Aspergillus flavus* toxins. Due to this quality of fibers gets reduced and trade loss increased [14]. Aspergillus flavus is mostly present in those regions which are hot and temperature is more as compared to thermopiles. They thrive in hot areas and the condition for growing cotton, groundnuts, and peanuts is also the same, so due to this reason, they get mostly affected as compared to other crops. Aspergillus flavus is known worldwide due to its ability to produce aflatoxins, which can lead to human loss, environmental loss, and economical loss [15]. This problem is not a concern of developed countries this is a matter of both developing and underdeveloped countries. Food safety and food integrity are important otherwise it can lead to disruption of human survival, impacting up to 25% of the world's food crops [16]. In India, Allergic bronchopulmonary aspergillosis (ABPA) cases are notably more significant. Highlighting the urgent need for effective measures to manage and prevent fungal infections. Public awareness regarding this issue is not much, so firstly we have to tell people to take precautions like sanitation, and cleanliness. Doctors or health caretakers spread awareness through various campaigns, and various regular health checkups so that infection can be detected in the

first stage of infection. It is important of taking action to address this significant public health issue [17]. Apart of causes diseases in humans and animals it can also produce carcinogens in crops that are more dangerous compared to other infections. Researchers need to take considerable precautions while dealing with this most dangerous organism. Precautions are important because they can decrease the little amount of effect.

Table1.1 Scientific Classification of *Aspergillus flavus*

Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergilaceae	Aspergillus	*Aspergillus flavus*

2.2 Morphology

Aspergillus is an interesting genus with 250 different species, all of them having different biological functions. Apart from biological functions, they are also important in an ecosystem like soil, air, and water. Aspergillus is a saprophytic organism. It depends upon dead and decaying matter for its nutrients. It maintains a mutualistic relationship with plants also. It is also having industrial and biotechnology importance, as an important source of organic acids, and enzymes [18]. Due to the presence of conidia, fungi can never be dead it can spread through various mechanisms by the source of conidia like wind, water, and soil. Aspergillus flavus life cycle major component is conidia which can spread from one place to another and it can also divide its cell rapidly which can spread infections in a very short period. This information can lead to the development of those fungicides which can directly be linked to or directly act on the life cycle of the fungus [19].Conidia can also survive in any environmental conditions if there is organic stress, change in pH, or environmental stress. It can survive or can follow its life cycle of germination. These factors cannot be able to stop its life cycle. Many species of filamentous fungi need sugars, amino acids, or other nutrients for their activity. Activation of the germination cycle needs nutrients in some fungi [20]. Sclerotia are an asexual spore that is produced by some fungus, but only in special conditions. Their structure is black, hard, and small important for the survival of fungi otherwise without

this structure survival is not possible. Sclerotia can remain dormant for long periods and can germinate when conditions become favorable.

Table 1.2 Morphology of *Aspergillus flavus*

Characteristic	**Description**
Hyphae	Septate, with a diameter of 2-6 µm, and branching at 45◦ angles
Colony appearance	Yellow-green colonies with a velvety texture
Vesicles	Round or oval-shaped structures that contain Conidiogenous cells
Conidiogenous cells	Phialides that are flask-shaped with a small opening at the tip
Conidiophores	Long, unbranched, and terminating in a vesicle
Conidia	Spherical to oval-shaped, smooth, and greenish-yellow in color

2.3 Ecological and Morphological Dispersal

Aspergillus flavus is known as the most diverse fungus, a cosmopolitan fungus, well known for the production of Aflatoxins (AF). It is majorly present in peanuts, groundnuts, and cotton. India faced a very serious outbreak of aflatoxicosis, which is caused due to ingestion of AF in food. The country faced a major outbreak, with 317 cases reported with 125 fatalities. This outbreak confirms that there is exposure to aflatoxins in the environment, food, and feed that causes problems in humans and animals [21]. Spores are the main cause of spreading disease in the environment because it was stress-tolerant and present in the environment for long periods under adverse conditions. Spores can contribute to the dispersal and survival of the fungus in varied environments [22]. Spore spreads infections in crops that are ingested by humans, goes into the human gut and stay there for a long period, grows and complete their life cycle, and then cause infections. Using crop rotation, organic farming, or other advanced technology in agricultural fields

can reduce the chances of crop infection. Many countries introduced a regulatory level of aflatoxin in food due to the security reason of humans. Due to fewer resources, it is difficult for developing countries to overcome these problems related to aflatoxin. This is an important point for consideration, and innovative and scientific methods should be developed so that health problems, and food and feed security issues should be resolved globally [23].*Aspergillus flavus* grows majorly in warm climates where the temperature is high between 16◦C to 37◦C, and maximum crops grow in a warm climate so it can lead to the destruction of crops grown in warm climates. Therefore, it is important to monitor and control aflatoxin contamination in these regions. Crop management and crop monitoring can lead to less amount of toxins production in these regions [24].

2.4 Exploration of *Aspergillus flavus* Genomic Diversity

Aspergillus species is a widely known species and it also shows similarity with another genome according to recent studies. For example, Aspergillus species, like *Aspergillus terrus* and *Aspergillus fumigaticus* are of medical importance and cause diseases in immunocompromised patients. *A. flavus* and *A. parasiticus* are responsible for food spoilage. It can produce carcinogenic aflatoxins. *A. niger,* A. *aculeatus*, and *A.oryzae* have industrial applications in the production of various enzymes, and organic acids. Aspergillus species have been extensively studied, due to their industrial applications, and ecological importance. In a fungal kingdom, Aspergillus species are better understood as compared to other genera because a lot of research was conducted because of their diverse functions in ecology [25]. The genome of *Aspergillus flavus* is composed of 13,071 genes. It includes a 70Kb gene cluster that comprises 24 structural genes; those genes are involved in the production of aflatoxins. *A. flavus* has a relatively large genome size of 37.96 Mbp. It is approximately 20% larger than the average genome size of the genus (31.7 Mbp). Genome size is larger because it is involved in complex genetic modification and diverse ecological niches that directly contribute to the production of Aflatoxins. Complex genetic modification creates a knowledge gap to know how this modification is related to aflatoxin production, or involved in the Biosynthesis pathway of toxin [26]. Genomic information can lead to the development of drug targets, and it can identify those genes which are exactly involved in aflatoxin production, their expression, and their gene regulation, pathogenicity, and mycotoxin production. With a better understanding of the genetic basis of *A. flavus,* researchers can develop more efficient and

sustainable strategies to reduce its impact on human health and agriculture. Genomic Information can also give information regarding those genes which are common in humans and fungi, so that similarity between both organisms can be known, important for the drug target perspective. Genomic information regarding all known species of *Aspergillus flavus* is tabulated.

Table 1.3 Aspergillus species Genomic Information

Genome	Genome Size	Genes	Protein Coding Genes	Importance
Aspergillus oryzae	37.9 Mb	12347	98%	Industrial or Food Strong secretion of amylases, Carboxypeptidase, Low Tyrosinase, Aesthetics, Coloured substances production
Aspergillus fumigatus	29.4 Mb	9915	97.1%	Medical Recycling Environmental Carbon and Nitrogen Pathogenic, Cause Infections in Humans
Aspergillus terreus	29.3 Mb	10551	98.6%	Medical Produce Secondary Metabolites- Lovastatin Lowering Blood Cholesterol Levels
Aspergillus Niger	34 Mb	10828	97.3%	Industrial or Food Used in production of Citric acid, gluconic acid etc. Enzymes Producers- Glucoamylase, Glucose Oxidase etc.
Aspergillus flavus	37 Mb	12323	97.5%	Industrial or food Human or Animal Pathogen Produce Aflatoxins as a secondary metabolite

2.5 Characterization of *Aspergillus flavus* Transcriptomes

Due to the advancement of high throughput techniques, transcriptome study is now easy and also less expensive. RNA-Seq is a technique that overcomes the microarray due to its high expression read count in very less time. It reduces manpower also and wastage of time. It allows for the sequencing of cDNA and has become a maximum tool with accurate results for studying eukaryotic transcripts. Researchers identify single nucleotide changes with low false positive results. It increased productivity when compared to microarray analysis. RNA-Seq has several advantages over microarray analysis. It is having maximum sensitivity, a broader dynamic range, and the ability to detect novel transcripts, isoforms, and splice variants [27]. *A. flavus* DNA indicated that mapping data

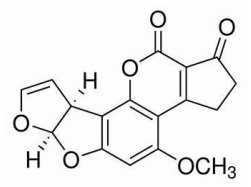
of an overlapped cosmid clone of a group of genes related to the biosynthesis of aflatoxin was organized in a cluster spanning approximately 70 kilobases. This cluster contained 25 genes that were significant for the biosynthesis process. Various studies explored the genome of species like *Aspergillus flavus*, *Aspergillus fumigaticus*, and *Aspergillus niger* under different environmental conditions studies have identified hundreds of differentially expressed genes. DEGs show involvement in secondary metabolism, stress response, and virulence. ITS region has importance in Aspergillus species; the region shows a difference between different species. But it remains the same in similar species. ITS sequencing provides a valuable resource for researchers to conduct phylogenetic analysis, species identification, and taxonomic studies. Due to the ITS region, we can find out the similarity between species and the identification of similar morphological characteristics [28]. 55 gene clusters are recognized in *Aspergillus flavus* which is responsible for AF production and also involved in the biosynthesis pathway. The specificity of genes leads to the identification of gene clusters. Polyketide syntheses, non-ribosomal peptide synthetases, and prenyltransferases genes are involved in the production of complex secondary metabolites. They are important for various biological functions, such as defense against predators and competitors, as well as facilitating the organism's growth and survival in its environment [29].Complex gene clusters in Aspergillus flavus are responsible for the formation of secondary metabolites, and toxins. Secondary metabolites are involved in various functions including defense against other organisms or signaling. Chromosome 3 contains the cluster of genes that produce aflatoxin.55 genes clusters are not responsible for aflatoxin production, only 30 genes are majorly involved in the formation of secondary metabolites. The gene cluster responsible for aflatoxin biosynthesis is located in Chromosome 3. Multiple enzymatic reactions are involved in the biosynthesis of aflatoxins. The biosynthesis of aflatoxin is also affected by various environmental conditions like temperature, pH, gene regulation, stresses, and water activity. Gene regulation of these involved in toxin production is important, otherwise, it can lead to the overproduction of toxins, and risk of crop damage also increases most. It is important to understand the biology of toxins, biosynthesis, biosynthesis pathways, genes, and gene regulation so that identification of novel, innovative ideas for controlling problems caused by aflatoxins. The study of the Aspergillus transcriptome can provide insights into the molecular mechanisms underlying the production of other secondary metabolites, which may have important applications in biotechnology and drug discovery [30].

2.6 Secondary Metabolism: Aflatoxins Production

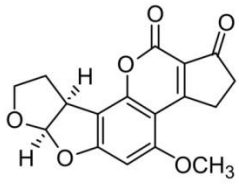
Aflatoxins are highly toxic, teratogenic, and carcinogenic. It is among the most dangerous mycotoxin found in food and agri-food systems. Toxins can cause a significant threat to human health and substantial economic losses. Aflatoxins are known as secondary metabolites produced mainly by the fungus *Aspergillus flavus* [31]. AFB1 is a highly toxic mycotoxin found in grains. It has been identified as a human carcinogen by the World Health Organization. Early detection and control of fungi and toxins are necessary to prevent contaminated substances from entering the production chain [32]. It is a prevalent aerobic disease that may infect crops and TCM materials, producing highly toxic aflatoxins that damage human health and lead to economic losses. In humans, AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2 are Group I carcinogens. *A. flavus* with aflatoxin contamination exposes approximately 5 billion people worldwide [33]. The fungal species responsible for AFB1 secretion and the primary pollutants of agricultural products and feed are *Aspergillus parasiticus* and *Aspergillus flavus*. Some chemical fungicides are successful at reducing pathogenic and toxigenic fungi. Their use can leave residues or parts that can be harmful. [34]. AFB1 is an extremely poisonous and cancer-causing compound made by fungi. It is mainly present in foods that have been improperly stored, including rice, corn, oil seeds, peanuts, and dried fruits. It can contaminate animal feed and excrete into the milk and eggs of those animals, exposing people. AFB1 is poisonous, immunosuppressive, mutagenic, and carcinogenic, among other health problems. CYP P450 isoenzymes metabolize AFB1 mostly in the liver [35]. *Aspergillus flavus*, which has two primary morphotypes: L and S, causes aflatoxin contamination. S-morphotypes fungi that produce both B and G aflatoxins are abundant in West African soils and are connected with crops such as maize, peanut, sesame, and chili. According to recent molecular research, A. aflatoxiformans and A. minisclerotigenes are more abundant in the West African sub-region [36].

Groundnut is a commonly grown crop that is subject to contamination by harmful fungi. It generates illnesses and causes an impact on the farming industry. There's no genetic resistance or chemical intervention. It is unsuccessful and potentially dangerous. Biological control utilizing non-toxic Aspergillus strains or participating microorganisms has shown the potential in decreasing contamination [37]. Milk is a prominent source of aflatoxin M1 (AFM1) contamination among food products, with pasteurized and UHT

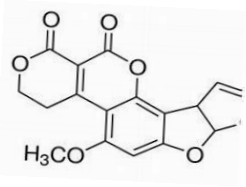
milk having the highest contamination rate and mean concentration. Aflatoxin M2 (AFM2), a metabolite of AFM1. It has similar toxicity but is detected in lower concentrations in milk. It is critical to limit aflatoxin exposure, particularly in children, by ingesting safe and well-regulated food products [38]. These metabolites may be found in milk products obtained from livestock-fed contaminated feed. The International Agency for Research on Cancer (IARC) has classified AFM1 as probably carcinogenic to humans, as well as highly toxic to the liver and mutagenic. AFM1 is relatively stable in pasteurized, sterilized, processed, and kept dairy products, data on its stability in yogurt is scarce and conflicting. It is critical to monitor and regulate the levels of AFM1 and AFM2 in milk and dairy products to reduce the risk of exposure to these harmful substances [39]. Aflatoxin biosynthesis is a complex process involving the usage of several genes, including those encoding enzymes that catalyze oxidative processes. One of these enzymes, OrdA, transforms O-methyl sterigmatocystin, a biosynthetic precursor, into aflatoxins B1 and B2. The creation of G aflatoxins, such as G1 and G2, necessitates the involvement of additional P450 monooxygenases, notably cypA, which is expressed by a gene located in the aflatoxin cluster upstream of the polyketide synthase gene [40]. The European Union has specified maximum amounts of four forms of hazardous aflatoxins in processed foods to reduce the possible harm caused by aflatoxins. Thin-layer chromatography, capillary electrophoresis, gas chromatography coupled with mass spectrometry, and high-performance liquid chromatography coupled with fluorescence or mass spectrometry detection can all be used to detect these compounds [41]. AFG1 and AFG2 are fewer hazardous variations of aflatoxins made by certain types of fungi than AFB1 and AFB2. Consumption in high quantities or over a long period can still cause health hazards. For the protection of public health, regulatory organizations set maximum amounts of certain poisons in food and animal feed products [42].



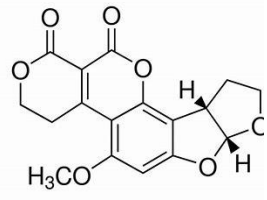
Aflatoxin B1



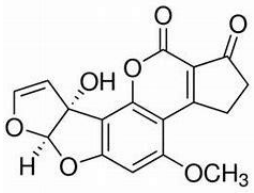
Aflatoxin B2



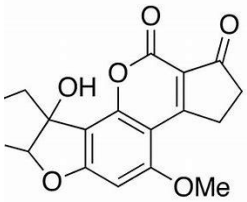
Aflatoxin G1



Aflatoxin G2



Aflatoxin M1



Aflatoxin M2

Figure 1.1 Chemical Structures of Aflatoxins B1, B2, G1, G2, M1, and M2

2.7 Genetic Regulation of Aflatoxins Biosynthesis: Biosynthesis Pathways

More than 20 genes are involved in the molecular mechanism of aflatoxin production, which is grouped in a 65-70 kb DNA area. Aflatoxin synthesis is primarily connected with *Aspergillus flavus*, which is common in warm areas. Gene flow within *A. flavus* is constrained by a vegetative compatibility system that defines several genetic groups known as vegetative compatibility groups [43]. Multiple mechanisms, including aflR, FadA, PKA, Ca2+/calmodulin, AflJ, and a repressor, regulate aflatoxin production. AflR is a pathway-specific regulatory gene that regulates the process. PKA inhibits aflR; whereas Ca2+/calmodulin-mediated protein phosphorylation affects aflatoxin synthesis. AflR transcription is self-regulated, and its function is modified post-transcriptionally by AflJ and a repressor [44]. The detection of toxic structures is followed by a succession of biochemical stages and genetic components in the aflatoxin manufacturing pathway. *A. parasiticus* and *A. flavus* have largely similar genes in the same arrangement inside a 70-kb gene cluster. Aflatoxins are produced from malonyl CoA in two phases, with at least 18 enzyme steps required for the transfer of acetyl CoA to its end products. Each gene's transcription direction is distinct, and the architecture of the gene clusters in *A. flavus* and *A. parasiticus* are mostly the same [45]. The first stable step in the biosynthesis of aflatoxins, norsolorinic acid (NA) is created. According to previous studies, NA is created

when a polyketide synthase (PKS) catalyzes the condensation of an acetyl CoA starter unit with nine malonyl-CoA extender units through ten condensations and two reduction cycles. Since the PKS implicated lacks the essential domains for reducing the six-carbon chain of NA. Recent evidence shows that a six-carbon fatty acid, hexanoyl-CoA, maybe the real beginning unit and that fatty acid synthase (FAS) may also be involved in the mechanism [46]. The transcriptional regulator genes aflR and aflS are presented adjacent to one another in the AF cluster. The majority of the genes involved in the production of the AF toxin are activated by a protein called AflR. AflR affects the expression of some genes in the AF cluster, but not of others, according to a study. This might be the case because various genes have distinct AflR binding sites. The expression of those genes is controlled by other genes that are responsible for the production of toxins [47].

2.8 Determinants of Aflatoxin Formation

The growth of the *Aspergillus flavus* fungus and the manufacturing of the deadly chemical aflatoxin depend on the availability of water. Research has shown that when the fungus is grown on cowpeas with a water activity range and temperature within particular ranges, the fungus produces the maximum quantities of aflatoxin. Higher salt concentrations result in lower levels of both aflatoxin and growth rates. It is inversely correlated with salt content. In a different investigation, *Aspergillus flavus* produced the most aflatoxin at the highest level of water activity. Researchers concluded that *Aspergillus flavus* produces aflatoxin and grows best when there is access to water [48]. The amount of aflatoxin, a dangerous toxin, produced depends on the medium in which the mold is grown. Pure substances, such as clear water and pure yeast extract, produce more of the toxin than inferior ingredients do. On the tenth day of growth, when the mold is producing the most spores, the most poisons are produced [40]. Warm and dry weather in India can promote the growth of the Aspergillus fungus. It can release toxic byproducts that contaminate maize harvests. This is a serious issue, especially in the country's tropical areas. This is a serious issue, especially in the country's tropical areas. For the protection of both human and animal health, it is crucial to make sure that the levels of these toxins in maize-based products, such as dairy and animal feed, are within safe ranges [49]. Temperature plays a crucial role in the formation of aflatoxin. Researchers have found that the ideal temperature for aflatoxin production is 30°C, while the optimal

temperature for fungal growth is 37°C, but not as conducive to mycotoxin production [50].

2.9 Health Risks and Agricultural Impacts of Aflatoxin Contamination

Aflatoxin contamination of agricultural products, including corn, peanuts, cottonseed, millet, and sorghum is possible. The Food and Agriculture Organization estimates that this contamination might result in annual worldwide food losses of about 1000 million metric tonnes. Farmers are also affected financially by mycotoxin contamination of animal feed since it reduces productivity and causes organ damage in animals [51]. According to research conducted in India, a sizable portion of food crops including groundnut and maize are infected with mycotoxins at levels that are unsafe. 26% of the analyzed samples of maize and 21% of the analyzed samples of groundnuts both surpassed tolerance levels. These results demonstrate the requirement for enhanced handling and storage procedures to avoid mycotoxin contamination of food crops in India. [52]. Aflatoxin B1 is harmful or more dangerous as compared to other aflatoxins. It can cause cancer and damage organs like the liver and kidneys. The immune system may be weakened as a result, which increases a person's susceptibility to illnesses like HIV/AIDS. These chemicals can also cause birth abnormalities and reduced growth in children [53].

2.9.1 Aspergillosis

A fungal infection that is caused by the *Aspergillus flavus* is known as Aspergillosis. It affects the various parts of the body like the lung, sinuses, and brain. It majorly affects that person who undergoes therapy like chemotherapy, or already suffered from other diseases and is more prone to aspergillosis. Studies found that when construction work is going on in hospitals during that time spores spreads more as compared to normal conditions. According to the report, 73% of spores are found in an air sample in hospitals during construction work, and in normal conditions, it is only 31%. This data concluded that during construction work there are more chances of spreading aspergillosis as compared to normal situations when there is no construction work [54]. Invasive aspergillosis mostly affects those persons who are suffering from liver diseases like chronic obstructive pulmonary diseases and who are very ill. People with mild immune deficiency or with chronic lung conditions are suffered from other types of Aspergillus

lung diseases which are known as chronic necrotizing aspergillosis (CNA). It is a dangerous form of aspergillosis in which lung tissue is filled with cavities and there fungus grows in a ball or thick, yellowish, and greenish color discharge. Weight loss, cough, fever, shortness of breath, fatigue, and restlessness are symptoms of CNA. Persons who are suffering from tuberculosis and chronic lung diseases are more susceptible to these diseases [55]. Acute distress syndrome is common in those patients who are suffering from COVID-19 and this complication leads to other types of fungal infection that are known as invasive pulmonary aspergillosis (IPA). The person who suffered from COVID-19 also suffered from IPA that is still unknown how the fungus develops in that case [48]. North America *Aspergillus flavus* is very common due to the warm climate and causes Aspergillosis in children mostly. It causes mycotitic keratitis (the cornea of the eye gets infected) [49]. Rabbits, ducks, chickens, turkeys, and geese can also get sick from *A. flavus*. Stonebrood disease is also caused by *Aspergillus flavus* in honeybees [56].

2.9.2 Aflatoxicosis

Aflatoxin produces by the *Aspergillus flavus* causes health issues in humans and animals which is known as aflatoxicosis. It affects majorly liver and disrupts the system of the liver. Due to the action of this fungus neurotransmission of liver cells is also affected majorly. Brain function is very important for proper communication of body parts, but it gets disrupted and it majorly affects by three major amino acids like leucine, valine, and isoleucine, and aromatic amino acids tyrosine, phenylalanine, and tryptophan. Increased levels of aflatoxin will create problems in the human body like mental illness, bleeding, jaundice, and mental disturbance [57]. Implementation of rules and regulations by FDA governments to control aflatoxin consumption and control aflatoxin-related health hazards. A developed country is well equipped and well-infrastructured so maintaining there is easy as compared to a developing nation because in developing nations there are no facilities that can maintain the infection level [58]. Based on the exposure it is divided into two types-acute aflatoxicosis and chronic aflatoxicosis. When the consumption of aflatoxin by animals or humans is directly in large amounts in one period of time then it is known as acute aflatoxicosis. On the other hand in the case of chronic aflatoxicosis when the exposure of aflatoxins for an extended time in a small amount is referred to as chronic aflatoxicosis [59].

2.9.3 Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is particularly lung disease which is caused by *Aspergillus flavus* in humans and animals, it majorly affects the lungs. ABPA was first reported in UK and USA in 1952. It is particularly reported in those persons who are suffered from asthma on regular basis, which mostly act on the lung. People with cystic fibrosis, allergic fungal sinusitis, hyper-IgE syndrome, and chronic granulomatous disease, are mostly get affected by fungus. ABPA can be diagnosed even if there is no bronchiectasis [60]. High range of symptoms, including coughing, wheezing, shortness of breath, chest tightness, and fever seen in ABPA. Symptoms can vary in severity and frequency. It can also cause long-term lung damage when the infection is in the last stage and the patient is unable to breathe properly. Cystic fibrosis suffered patients develop ABPA infection then there are chances of a false lung transplant. Health professionals spread awareness about ABPA so that early diagnosis of a disease can be possible [55]. India has some regions with warm climates their exposure to aflatoxin is high, it is not uncommon in India but it is misguided by tuberculosis [61].

2.10 Prevention and Control of Aflatoxin Contamination

Biological control of aflatoxin contamination involved the use of a non-toxic strain that can grow together with an aflatoxin strain and inhibit the growth of the aflatoxigenic strain. The toxigenic strain grows naturally in the environment but still, there is less circulation of strain in the air as compared to the non-toxigenic strain. The introduction of non-toxigenic strain in the environment reduces the effect of aflatoxigenic strain [62]. Recent studies concluded that the use of non-toxigenic strain reduces the contamination in peanuts, cotton, and groundnuts. Biocontrol agents are not a permanent solution because their activity decreases concerning time. Selection of non-toxigenic strain is very important according to the crop otherwise it can also lead to a decrease in the productivity of main crops, often leading to economic losses [63]. Many organisms were also selected for this procedure but they cannot be active in the field due to warm climatic conditions. Bacteria and yeasts show results in the lab but they cannot show any results in the fields. The non-toxigenic strain of *Aspergillus flavus* and *Aspergillus parasiticus* majorly neglect the effect of the toxigenic strain. A non-toxic strain is combined with grain and then cultivate in the field. Studies show up to a 90% of reduction in toxigenic strain in the field. Aspergillus well-known strain AF36 is used in the field with grains and it showed

very good results [64]. A substance that can prevent cancer is known as Chlorophyllin. It is an important substance that binds with aflatoxin and decreases activity. Chances of liver cancer in China are more due to the presence of aflatoxin in their food. Chlorophyllin can be used for treatment because it shows less amount of aflatoxin in urine after the use of Chlorophyllin [65].

Chapter-3

Objectives of Study

There is a rapid increase in the incidence of Aspergillus flavus-related infections in immunocompromised patients. Elucidation of life cycle events of *Aspergillus flavus* is important to know the expression of genes involved in the production of aflatoxin and involved in the transition of conidia to mycelium. Gene-encoding proteins that are involved in the transition from dormant conidia to mycelia are limited or less explored. Identification of critical genes could pave the way to inhibit the morphogenesis of *Aspergillus flavus* conidia; in addition, it also sheds light on genes expressed during the production of AF (Mycotoxins). Based on study Objectives of the study is:

Objective 1- To understand stage-specific genes such as conidia, germination, and regulatory genes, involved in the formation of mycelia.

Objective 2- To understand critical genes involved in the formation of Mycelia.

Chapter-4

Material and Method of Study

4.1 List of laboratory equipment used in the study

Table 1.4 Laboratory types of equipment

S.No.	Material	Quantity	Source
1.	Incubator	1	Fisher Scientific
2.	Centrifuge	1	Eppendrof 5415R
3.	Spectrophotometer	1	Thermo Scientific USA
4.	Haemocytometer	1	Fisher Scientific
5.	Gel Doc	1	Bio-Rad
6.	qRT-PCR	1	CFX96 Bio-Rad
7.	Microscope	1	Olympus
8.	PCR	1	Thermo Scientific

4.2 List of Chemicals and reagents used in the Study

Table 1.5 Composition of PBS (pH is adjusted 7.4)

S.NO.	Component	Amount per liter
1.	Sodium Chloride (NaCl)	8.0g
2.	Potassium Chloride (KCL)	0.2g
3.	Disodium Hydrogen phosphate (Na2HPO4)	1.44g
4.	Potassium dihydrogen phosphate (KH2PO4)	0.24g

Table 1.6 Composition of PBST (pH is adjusted 7.4)

S.No.	Components	Concentration
1.	Sodium Chloride (NaCl)	8g/L
2.	Potassium Chloride (KCL)	0.2g/L
3.	Disodium Hydrogen phosphate (Na2HPO4)	1.44g/L
4.	Potassium dihydrogen phosphate (KH2PO4)	0.24g/L
5.	Tween20(Polyethylene glycol sorbitan monolaurate)	0.05%

Table 1.7 Composition of TAE

S.No.	Component	Concentration
1.	Tris Base	0.4M
2.	Glacial Acetic Acid	0.2M
3.	EDTA	0.01M

4.3 Culture Medium Used in the Study

Table 1.8 Fungal Growth Media

S.No.	Components	PDB(per litre)	PDA(per litre)	Purpose
1.	Potato infusion	200g	200g	Growth and nutrients factors for fungi
2.	Dextrose	20g	20g	Carbon Source
3.	Agar	-	15g	Solidifying agent for fungus growth
4.	Distilled Water	Up to 1L	Up to 1L	Dilutes Components

4.4 Components used in qRT- PCR

Table 1.9 Chemicals Used in RNA Extraction

S.NO.	Chemical	Function
1.	Guanidine isothiocynate	Inactive RNases and disrupt cells
2.	Phenol	Denatures proteins
3.	Chloroform	Separates the aqueous and organic phases
4.	Isopropanol	Precipitates RNA
5.	Ethanol	Dries and Washes RNA pellet
6.	RNase-free Water	Resuspends RNA

Table 2.1 cDNA Synthesis Kit (Convert RNA to cDNA)

S.No.	Components	Volume
1.	5xcDNA synthesis Buffer	4µl
2.	dNTP Mix	2µl
3.	RNA primer	0.6 (oligo dT) 0.5 (Hexamer)
4.	RT enhancer	1µl
5.	Verso Enzyme Mix	1µl
6.	Template(RNA)	2µl
7.	Nuclease free Water	10µl

Table 2.2 Primers used in the study

S.No.	GENE ID	NAME	SEQUENCE
1.	480538393	Tubulin	5'-GGAATGGATCTGACGGGCAAG-3' 5'-GGTCAGGAGTTGCAAAGCG-3'
3.	238496080	Rho-gdp	5'-CGAGCTATAAATCCCGAGG-3' 5'-GTCGTTAAGAGGAAGGGTG-3'
4.	238501501	Crz1	5'-CCACCATCCATTAACGTGG-3' 5'-CGGATCAGATTTGGTACGC-3'
5.	238490040	Hsp70	5'-CCTACTCCCTCAAGAACACC-3' 5'-CCTACTCCTCAAGAACACC-3'
6.	238503320	Hsp90	5'-CGTCAAGTCCATCACTCAGC-3' 5'-GCTTGTGGATGCGCTCGGC-3'

4.5 Tools and pipelines used in the Study

Table 2.3 RNA Seq data analysis tools and pipeline

S.No.	Step	Tool	Description	Link
1.	Data Retrieval	SRA toolkit	Conversion of SRA files to FASTQ files	https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=toolkit_doc
2.	Quality Check	FastQC	Quality check of FASTQ files	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
3.	Trimming	Trimmomatic	Remove low-quality reads and adaptors	http://www.usadellab.org/cms/?page=trimmomatic
4.	Expression Quantification	Cufflinks	Calculation of FKPM values and assembling transcripts	http://cole-trapnell-lab.github.io/cufflinks/
5.	Differential expression	EdgeR	Identification of DEGs in two different conditions	https://bioconductor.org/packages/release/bioc/html/edgeR.html

4.6 Organism and Culture Conditions

Aspergillus flavus 9367 was used in the study. PDA (Himedia, Mumbai, India) was used as culture media at 28-30 °C.

Aspergillus flavus isolate used in the study

Isolate Name	Personal Reference Code	MTCC No.	Aflatoxin Producer
Aspergillus flavus	Af67	9367	YES

4.7 Spore Harvesting

A. flavus culture was grown on Potato Dextrose Agar slants. After 72 spores were harvested using a solution of PBST (Phosphate Buffered Saline and 0.05% Tween20). Spores were purified by centrifugation and washing with PBS. Haemocytometer was used for calculating CFUs (colony-forming units). 2×10^6 cells/ml was used as a working conidial culture in studies. The spores were then resuspended in fresh PDB and grown for specific time points at 28°C -30°C.

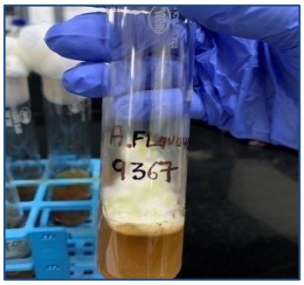
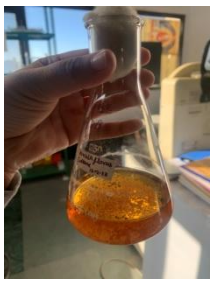


Figure1.2

PDA Slants

Figures1.3

PDA media for maintaining *A. flavus* Cultures



Figures1.4

PDB Liquid Media

Figure1.5

Temperature-controlled Incubator

Figure1.6

PDB flask in an incubator for *A.flavus* Growth

Figure1.7

Conidial Cultures

4.8 RNA Extraction and qRT- PCR

RNA extraction was done by the TRIzol reagent method. The RNA concentration was determined by using Spectrophotometer (Thermo Scientific) and gel electrophoresis. CDNA was synthesized using the Verso cDNA kit (Thermo Scientific) using 1.5 micrograms RNA. CFX96 PCR machine (BIO-RAD, USA) was used to perform qPCR analysis.

RNA Quality Assessments- Spectrophotometer was used for assessing the quality of RNA and contaminants. The absorbance ratios at 260/280 nm were measured. 260/280 nm ratios range from 1.8-2.0; It showed the high quality of RNA.

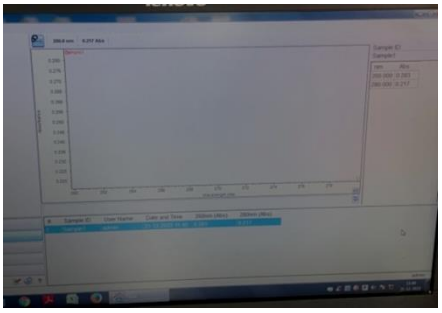


Figure 1.8 OD at 260/280 nm (RNA 0hour)

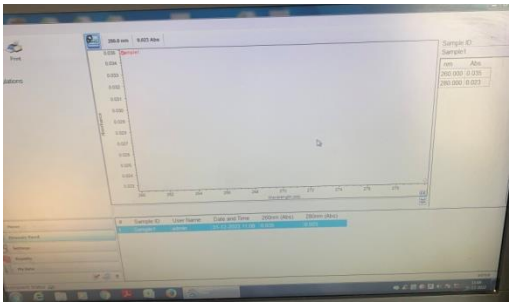


Figure 1.9 OD at 260/280 nm (RNA 24hr)

Figure 2.1

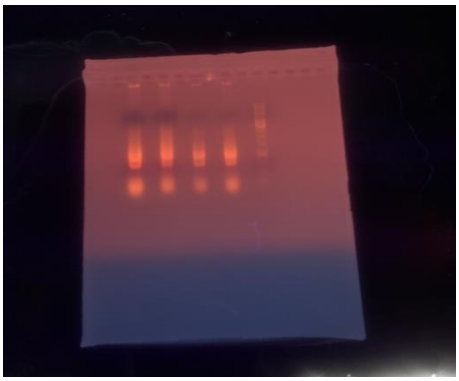


Figure 2.2

Figure 2.1 Figure 2.2 Image of Agarose gel electrophoresis of RNA extracted from *Aspergillus flavus* strain 9367 after 24 hours of growth

qRT-PCR technique was used for the identification of gene expression. Comparison of the amount of RNA for the gene of interest to a reference gene. Delta Delta Ct method was used for the analysis of gene expression in different methods.

Chapter-5
Results

The biosynthesis of aflatoxin is a complex and conserved process in *Aspergillus flavus*. There is coordinated expression of regulatory or structural genes in aflatoxin biosynthesis. To better understand the regulation of aflatoxin biosynthesis analysis of several genes including Aflr, Hsp90, Hsp70, CRZ, and Rho-GDP was done, by using qRT-PCR and RNA-seq data analysis at different time points. Aflr and Afls majorly involved in aflatoxin biosynthesis, the positive regulator gene is Aflr. It is required for transcriptional activation of most of the aflatoxin pathway. Co-activators such as Aflp and AflS are also involved in AF biosynthesis. The zinc-finger motif is present in Aflr and modulates the gene expression by doing interaction with the promoter region of structural genes. Heat shock proteins are majorly involved in the folding of proteins and conformational stability of cellular proteins. Data shows that these genes are also may influence aflatoxin biosynthesis. The regulatory biosynthesis pathway is also controlled by Hsp90 and Hsp70, which also play an important role in toxin formation and also in morphogenesis of fungi. Hsp90 proper folding plays an important role in AF production. Regulatory genes such as aflR and aflS production are also affected by Hsp70.

Calcium signaling machinery is highly conserved and linked with signaling pathways also.CRZ-1 is a transcription factor that activates nuclear genes responsible for cell remodeling, polarity, and conidiophores development in response to various stress signals. Deletion of CRZ-1 leads to a reduction in the level of AF production. Modulation of expression of key regulatory genes involved in AF biosynthesis done by CRZ-1.It is also involved in calcium signaling pathways and other signaling pathways. Vesicle trafficking, cell polarity, organization, and cellular processes are controlled by a family of small G-proteins known as RHO-GTPases. It is divided into two forms based on activity, one form is inactive known as the GDP-Bound state and the other is inactive state that is known as the GTP-Bound state. Studies show expression of genes leads to a significant reduction in AF production. Overexpression leads to increased levels of AF.

5.1 Cq values of Hsp90 gene expression at different time points

Table 2.4 Cq values of Hsp90

Time Point	Replicate 1	Replicate2	Replicate3	Cq Mean
0h	26.74	28.59	28.27	27.87
10h	19.91	19.6	19.55	19.687
24h	19.34	19.35	20.21	19.633

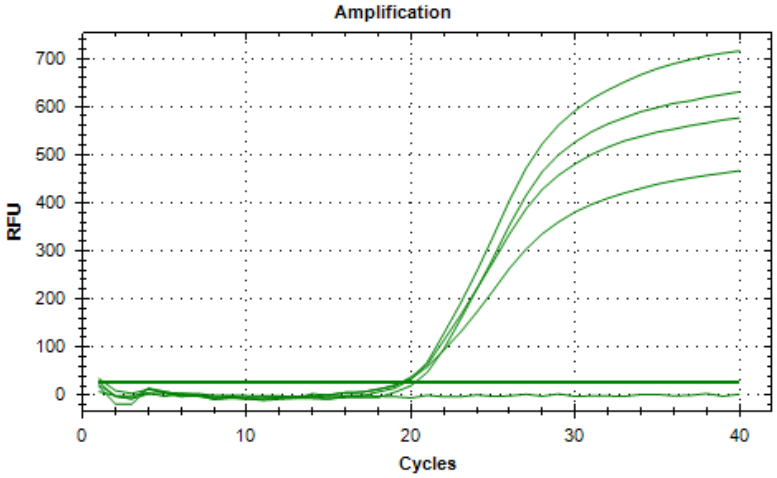


Figure 2.3 Amplification Plot of Hsp90 at 10h time point

qRT-PCR analysis showed that the expression of Hsp90 changes significantly over time. The highest expression is observed at 24 hr time points.Hsp90 is well known for playing an essential role in protein folding and conformational change. It is important for the production of structural genes which are majorly involved in the Biosynthesis of AF. Therefore, the upregulation of Hsp90 may impact the production of aflatoxins. However, at 0hour, Expression levels of Hsp90 decreased significantly. Data indicates a reduction in the level of AF at 0 hours.

5.2 Cq values of CRZ gene expression at different time points

Table 2.5 Cq values of CRZ

Time points	Replicate1	Replicate2	Replicate3	Cq Mean
0h	29.58	29.48	32.82	30.6267
10h	27.34	27.07	27.22	27.21
24h	19.65	20.34	20.56	20.1833

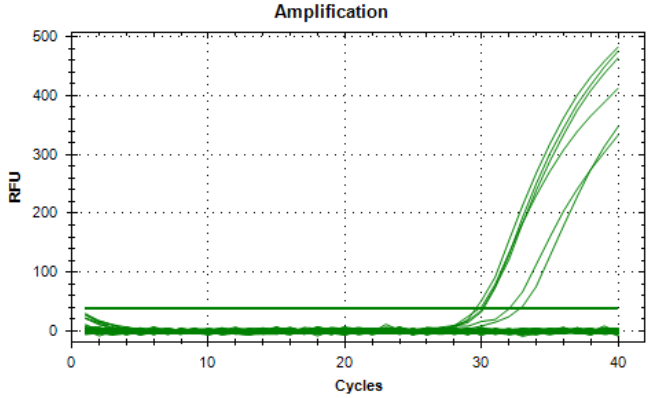


Figure 2.4 Amplification Plot of CRZ at 0h time point

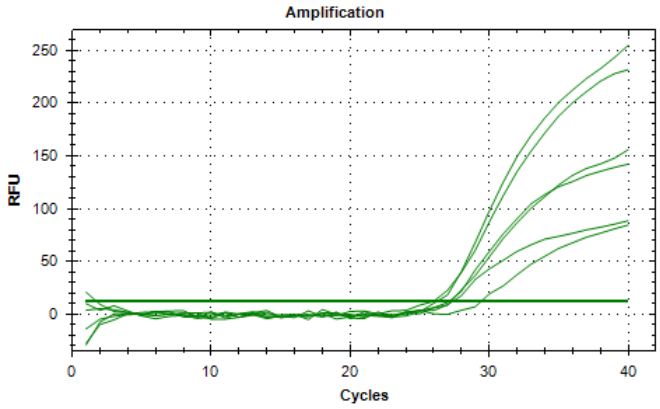


Figure 2.5 Amplification Plot of CRZ at 10h time point

Based on results obtained from qRT-PCR analysis Cq value of CRZ at 0h is 30.6267 means it takes 30 cycles to cross the threshold value so it suggests that expression of CRZ at 0h is low. At 10h higher expression of the CRZ gene as compared to 0h.This may indicate that there is an increase in transcription of the CRZ gene. At 24 hours expression

is more as compared to 0h and 10h. This may show that CRZ is responsible for aflatoxin production at 24h because the expression is more as compared to 0h and 10h.

5.3 Cq values of Hsp70 gene expression at different time points

Table 2.6 Cq values of Hsp70

Time	Replicate1	Replicate2	Replicate3	Cq Mean
0h	24.02	23.29	23.6	23.637
10h	19	19.22	18.74	18.987
24h	25.57	25.66	25.19	25.4733333

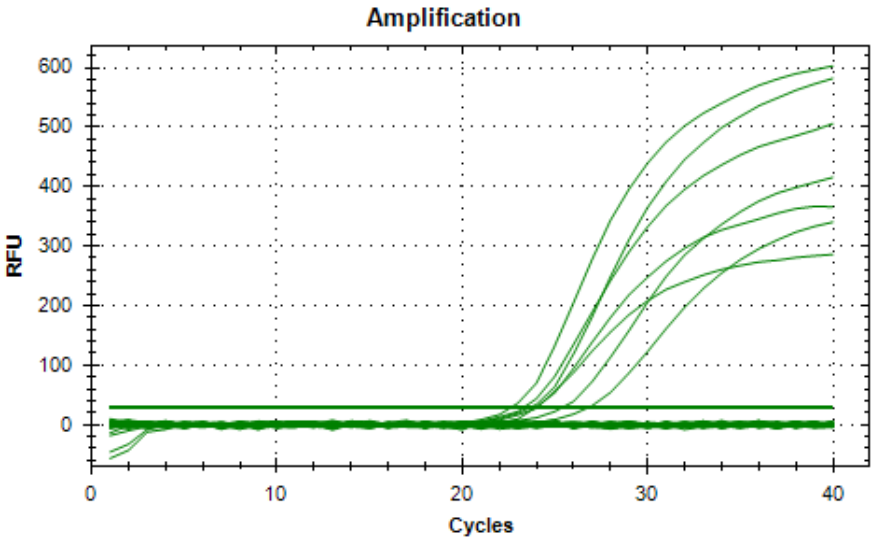


Figure 2.6 Amplification plot of Hsp70 at 0h

As per qRT-PCR analysis expression of Hsp70 changes significantly during morphogenesis of conidia to mycelia. The highest expression is observed at a 10h time point as compared to 0h and 24h. It shows that the expression of Hsp70 is more at 0h as compared to 24h, it takes 25 cycles to achieve the threshold. This data indicates that Hsp70 is responsible for aflatoxin biosynthesis at 10h where expression is more as compared to other time points.

5.4 Cq values of Rho-GDP gene expression at different time points

Table 2.7 Cq values of Rho-GDP

Time	Replicate1	Replicate2	Replicate3	Cq Mean
0h	29.66	30.07	29.74	30.033
10h	19.41	20.19	21.19	20.263
24h	24.78	24.28	19.45	22.84

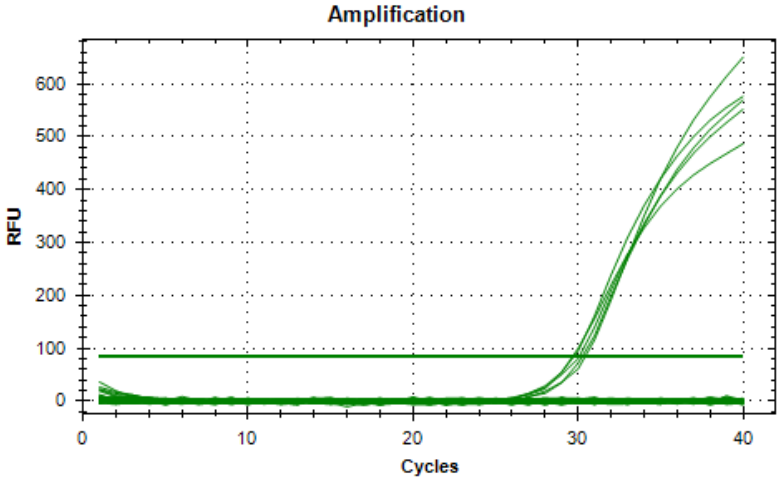


Figure 2.7 Amplification plot of RHO-gdp at 0h

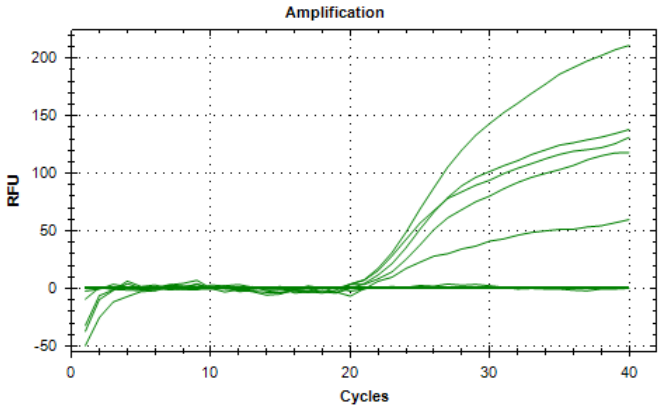


Figure 2.8 Amplification plot of RHO-gdp at 10h

qRT-PCR analysis showed that the expression of RHO-GDP changes significantly over time. Higher expression of RHO-GDP at 10h as compared to 0h and 24h. At 0h expression of RHO-GDP is low as compared to 24h. The main role of RHO-GDP is to activate the regulatory genes involved in the aflatoxin biosynthesis pathway. Previous

studies also show expression of genes leads to a significant reduction in AF production. This study indicates that an increase in expression of RHO-GDP leads to aflatoxin production at a 10h time point.

5.5 Cq Values of Aflr gene expression at different time points

Table 2.8 Cq values of Aflr

Time	Replicate1	Replicate2	Replicate3	Cq Mean
oh	N/A	N/A	N/A	-
10h	N/A	N/A	N/A	-
24h	18.61	21.38	20.66	20.2167

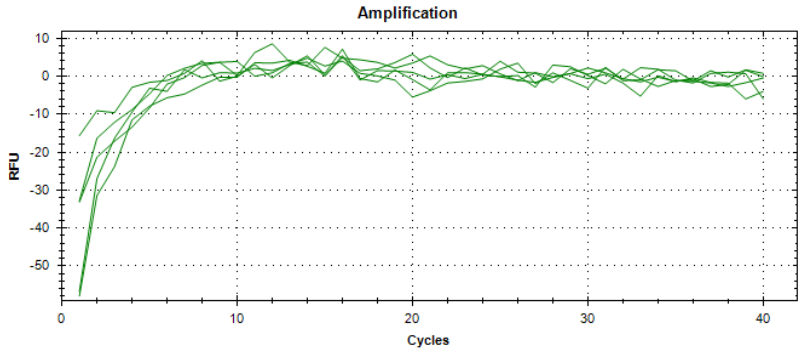


Figure 2.9 Amplification plot of Aflr at 0h

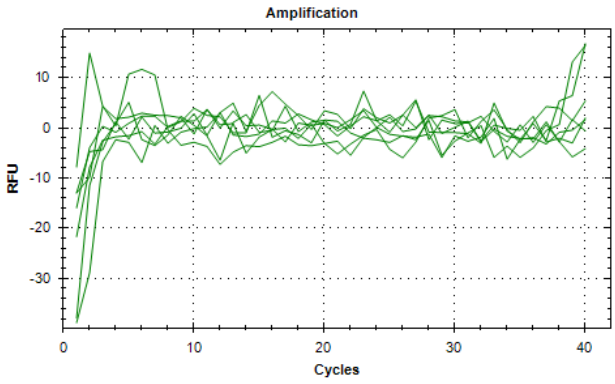


Figure 2.10 Amplification plot of Aflr at 10h

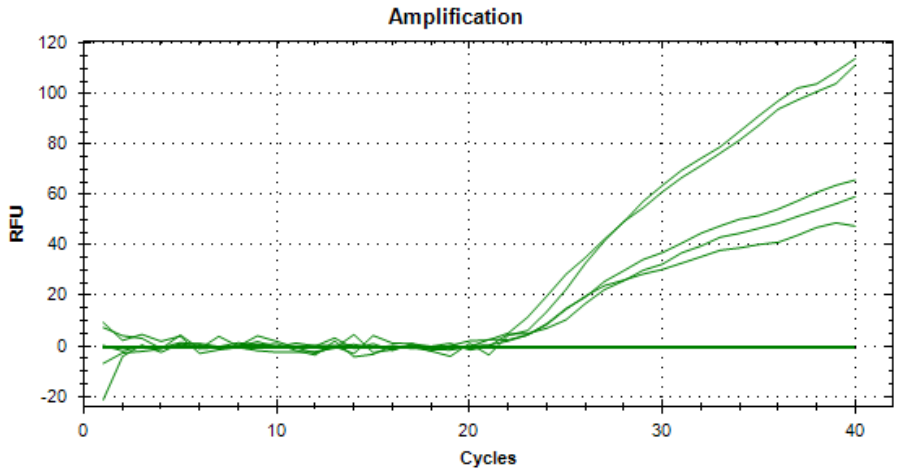


Figure 3.1 Amplification plot of Aflr at 24h

qRT-PCR data revealed that there was no detectable expression of the Aflr gene, which is the key structural and regulatory gene for aflatoxin biosynthesis. Gene Expression is detected at 24h and it revealed that aflatoxins genes are upregulated at this time point. It could potentially also lead to an increase in the production of aflatoxin.

5.6 Expression Profile

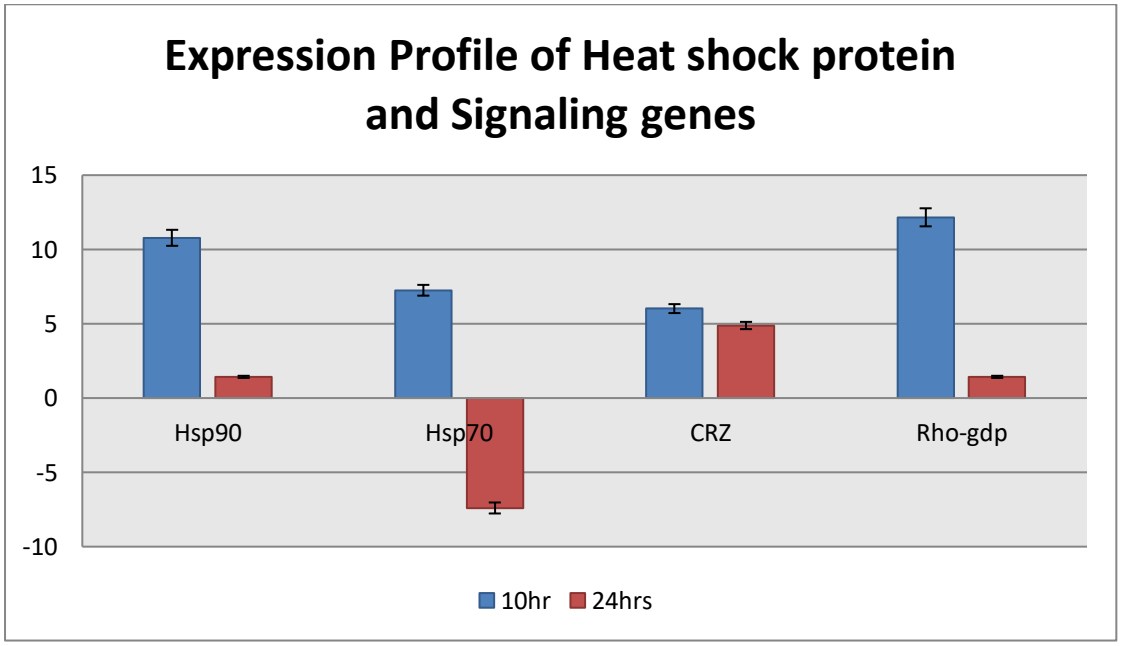


Figure 3.2 Expression profiles of Heat shock protein and signaling genes

5.7 RNA-seq Data Analysis

Differential Genes profiling: 9065 genes differentially expressed between the conidial and mycelial stage according to the transcriptome analysis. The gene showing upregulation is 4426 and the gene showing downregulation is 4639.

Table 2.9 List of Differentially Expressed Genes

Samples	Total DEGs	Significant DEGs	Significant up DEGs	Significant Down DEGs
0h_5h	9596	1959	749	1210
0h_10h	9844	2585	962	1623
0h_24	9890	3978	1899	2079
0h_72h	9288	3297	1665	1632
5h_10h	9784	550	58	492
5h_24h	10071	2842	1497	1345
10h_24h	10116	2479	1379	1100
10h_72h	9844	2447	1569	878
24h_72h	9732	3222	1828	1394

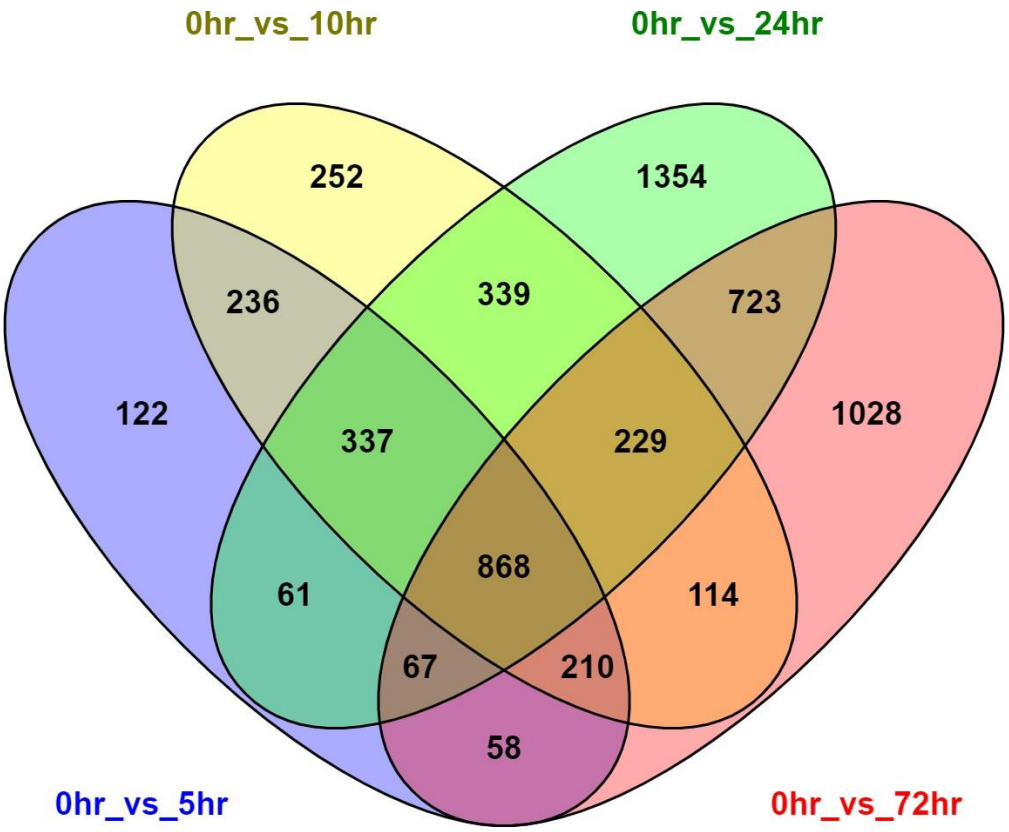


Figure 3.3 Venn Diagrams show common genes at different time points

Chapter-6

Conclusions

A. flavus is a fungal pathogen of oil crops, known for developing AF in pre-post-harvest maize, peanuts, cotton, and tree nuts. This fungus potentially causes aspergillosis and liver cancer. Although the aflatoxin gene clusters have been studied extensively, the regulation of aflatoxin biosynthesis remains unknown. Aflatoxin biosynthesis is a challenging process according to studies [66]. In previous studies, we observed that *A.flavus* had production capability, suggesting that aflatoxin generation might very well play a part in stress tolerance [67]. Regardless, the fact that the entire genome sequence of *A. flavus* has been released but mRNA that is involved in the biosynthetic pathway, morphogenesis has not been reported completely yet. RNAi can especially work down two genes associated with aflatoxin biosynthesis. Previous study indicates that *A. flavus* must have functional RNAi machinery that generates toxins [68]. Due to advancements in technology, a variety of studies on *A. flavus* have been conducted, providing useful information that fungus fungi belong to Aspergillus section Flavi. A. flavus has been studied mostly for its toxin production and pathogenicity, but understanding all of this requires knowledge of asexual development, the primary reproductive mode of Aspergillus species. Therefore, conclusively, the data generated by transcriptome analysis provided the annotated collection of critical genes, regulatory genes, significant genes, significantly upregulated genes or significantly downregulated genes transition from conidia to mycelium.

Due to limited data available on the transcriptome of A. flavus transition from conidia to mycelia stage-specific genes or aflatoxin genes are less explored. Thus, our transcriptome data further can be explored for understanding transcripts involved in aflatoxin production at what germination stage. Mechanisms of conidial germination are important. Conidium plays an important role in growth, food spoilage, and pathogenic infections of animals and plants. It breaks dormancy when the circumstances are favorable [69].Therefore, an improved understanding of Conidia germination is important to know key pathways, critical genes, and stage-specific genes. It can provide significant contributions to studies and is also helpful for the safety of food and feed. There are three different transitions of conidia germination: Dormant stage, Isotropic stage, and polarized stage [70]. The goal is to understand transcripts of the transition from germinating conidia of the filamentous fungi to the mycelial stage. Presumably, our study is the first report to analyze critical genes, transcripts that are involved in the transition from conidia to mycelia by using bioinformatics and molecular biology methods. Our study is also important to understand genes that are involved in aflatoxin production. The goal is to

understand transcripts of the transition from germinating conidia of the filamentous fungi to the mycelial stage. Presumably, our study is the first report to analyze critical genes, transcripts that are involved in the transition from conidia to mycelia by using bioinformatics and molecular biology methods. Our study is also important to understand genes that are involved in aflatoxin production. For future prospects, development of new therapy or new drug for the contamination and pathogenicity of *A. flavus.*

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