Study of Genetic Regulators that Control Development and Secondary Metabolism in the Genus aspergillus

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ABSTRACT

STUDY OF GENETIC REGULATORS THAT CONTROL DEVELOPMENT AND SECONDARY METABOLISM IN THE GENUS ASPERGILLUS.

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Department of Biological Sciences
Northern Illinois University, 2019
Ana M. Calvo, Director

In this study, I investigated the role of two different regulatory genes in two different species of pathogenic fungi from the genus Aspergillus. The first study involves a transcriptome analysis of the epigenetic regulator rmtA in the plant pathogen Aspergillus flavus. A. flavus colonizes numerous oil seed crops such as corn, peanuts, treenuts and cotton worldwide, contaminating them with aflatoxins and other harmful potent toxins. Previously our lab characterized the gene rmtA, which encodes an arginine methyltransferase in A. flavus, and demonstrated its role as regulator of the expression of the aflatoxin gene cluster and concomitant synthesis of toxin. Furthermore, our studies revealed that rmtA also controls conidial and sclerotial development. Due to this role as an epigenetic regulator in A. flavus, we performed a transcriptome analysis to further ascertain the role rmtA may have on A. flavus. In this analysis we identified over 2000 genes that were rmtA dependent. Of those genes, we identified those that were involved in production of secondary metabolites, response to environmental stress, and genes active during plant virulence.

The second project that was undertaken involves the characterization of the homeobox transcriptional regulator HbxA in the opportunistic human pathogen Aspergillus fumigatus. A. fumigatus is the leading cause of invasive aspergillosis, which in immunocompromised patients has a mortality rate as high as 90%. Earlier studies showed that HbxA is a global regulator in A. flavus regulating morphological development and secondary metabolism. Here we determined its
role in *A. fumigatus* examining whether *hbxA* influences regulation of asexual development, secondary metabolism, and virulence of this fungus. Our analysis demonstrated removal of *hbxA* caused a near complete loss of conidial production in the mutant strain as well as a slight defect in colony growth. Other aspects of asexual development are affected as well such as size and germination of the conidial spores. Furthermore, we showed that in *A. fumigatus* loss of *hbxA* decreased the expression of the *brlA* central regulatory pathway involved in asexual development, as well as the expression of the “fluffy” genes *flbB*, *flbD*, and *fluG*. HbxA was also found to be a regulator of secondary metabolism affecting production of multiple secondary metabolites. Using a neutropenic mouse model for infection, the role of *hbxA* in pathogenicity of *A. fumigatus* was assayed where *hbxA* was found to have a negative impact on the virulence of this pathogen.
STUDY OF GENETIC REGULATORS THAT CONTROL DEVELOPMENT AND SECONDARY METABOLISM IN THE GENUS *ASPERSGILLUS*.

TIMOTHY SATTERLEE
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A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

Doctoral Director:
Ana M. Calvo
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PREFACE

Since the ancestral use of fungi for traditional fermentation of food and beverages, scientific research has expanded our knowledge of the mechanism of fungal development and metabolism. This new knowledge allows an improved manipulation of these organisms and their traits. For instance, new insight has been relevant in industrial fermentation using yeast or antibiotic production using *Penicillium* species. Research on the characterization of fungal genes has been particularly useful to improve their beneficial abilities, manipulating them using molecular biology techniques. This research has been particularly fruitful in studying filamentous fungal species of the genus *Aspergillus*.

An example of such a research effort in the genus *Aspergillus* is the utilization of *Aspergillus niger* in industrial settings, primarily for the production of citric acid. Before optimization is achieved, fungal metabolism and development as well as the genetic regulatory mechanisms that control them need to be elucidated. Another example is *Aspergillus terreus* production of lovastatin, used for cholesterol treatment. The identification and characterization of the biosynthetic gene cluster and its genetic regulators has the potential to enhance production.

While the fungi mentioned above possess beneficial traits, others fungal species cause severe detrimental effects. In the latter case, genetic studies can provide the bases to design methodology to control/restrict infection of pathogenic fungi and/or colonization of mycotoxigenic fungi that jeopardize our food supply. This is the realm in which my research is focused, studying two *Aspergillus* species: one is the plant pathogen *Aspergillus flavus*, a fungus that causes a major agricultural impact by primarily infecting important oil seed crops and
contaminating them with potent carcinogenic toxins; the other is the opportunistic human pathogen *Aspergillus fumigatus*. This fungus is the leading cause of invasive aspergillosis. While *A. fumigatus* infections do not normally occur in individuals with a healthy immune system, they do occur frequently in immunocompromised patients. Once the host is infected, this disease has a mortality rate as high as 90%.

In the Calvo Lab at NIU we search for genetic strategies to control the negative and positive impacts of *Aspergillus* spp. Several genetic regulators have been previously studied. One classic example is the C$_2$H$_2$ transcription factor BrlA. Originally studied in the model fungus *Aspergillus nidulans*, this transcription factor was found to be one of the central regulatory genes needed for asexual development in this fungus (Adams et al., 1998). Removal of this gene resulted in a mutant unable to produce conidia (asexual spores). Studies in other fungi within the genus *Aspergillus* showed that *brlA* has a similar role in other species (Yamada et al., 1999; Maj & Yu, 2006). This constitutes an important potential target to decrease dispersal of spores, which are the main method of dissemination for these organisms.

While the transcription factor *brlA* is an example of a regulatory gene that controls one aspect of fungal development, asexual development, other regulators with a broader regulatory scope influence multiple areas of fungal development and/or metabolism. Interestingly, in fungi there have been numerous studies demonstrating that fungal development and secondary metabolism are genetically linked, as reviewed in Calvo and Cary (2015). While there are many examples of such conserved global regulators, such as *laeA* (Bayram et al., 2010), *nsdD* (Cary et al., 2012), and *rtfA* (Myers et al., 2017), the best-characterized might be the *velvet* gene, *veA*. The
The *veA* gene product, VeA, is a fungal-specific global regulator that is part of a protein complex affecting the expression of hundreds of genes, influencing multiple aspects of fungal development and secondary metabolism. In *Aspergilli*, veA regulates production of conidia as well as the formation of sexual structures, such as cleistothecia and sclerotia. In addition, veA has shown often to be a key regulator of secondary metabolism. Several studies of the veA-dependent transcriptome have revealed previously unidentified veA-dependent secondary metabolite gene clusters in *Aspergillus* species. Examples of this are the identification of the aflavarin gene cluster in *A. flavus* and the fumagillin gene cluster in *A. fumigatus* (Dhingra et al., 2013; Cary et al., 2015).

Another example of a global regulator that was studied in the Calvo lab is the master transcription factor MtfA, originally identified in *A. nidulans* via a mutagenesis screening (Ramamoorthy et al., 2013). Mutant strains without *mtfA* were shown to be unable to produce multiple secondary metabolites, including penicillin. This study also showed that *mtfA* was required for normal asexual development by regulating *brlA* (Ramamoorthy et al., 2013). The corresponding homologs in *A. flavus* and *A. fumigatus* also control development and secondary metabolism (Smith & Calvo, 2014; Zhuang et al., 2016).

My Dissertation work involves the investigation of two different regulatory genes. The first project involves the study of the epigenetic regulator *rmtA* in *A. flavus*. RmtA is an arginine methyltransferase that methylates histones. RmtA was previously shown to regulate asexual development and secondary metabolism in *A. flavus* (Satterlee et al., 2016). In the current work, a transcriptome analysis was performed to further define the role of *rmtA* in *A. flavus*. The
second project that will be presented here involves the characterization of the homeobox
transcriptional regulator HbxA in *A. fumigatus*. Earlier studies showed that HbxA is a global
regulator in *A. flavus* (Cary et al., 2017; Cary et al., 2019). Here we determined its role in this
important opportunistic human pathogen, examining *hbxA* effects on asexual development,
secondary metabolism, and virulence in *A. fumigatus*.

REFERENCES


TRANSCRIPTOME ANALYSIS OF \textit{rmtA} AND ITS IMPLICATIONS IN SECONDARY METABOLISM, ENVIRONMENTAL STRESS, AND VIRULENCE IN \textit{ASPERGILLUS FLAVUS}

Introduction

\textit{Aspergillus flavus} is an opportunistic plant pathogen of great economic importance that infects oil seed crops such as corn, peanuts, cotton and soybeans, producing potent mycotoxins (Hedayati et al., 2007), including the highly carcinogenic mycotoxins called aflatoxins (Sarma et al., 2017). Ingestion of contaminated crops causes a plethora of disorders, depending on the doses and length of exposure. Short-term exposure to large amounts of aflatoxin can result in jaundice, edema of the limbs, pain, vomiting, necrosis, and potentially acute liver failure or death (Lancaster et al., 1961; CDC, 2004; Fung & Clark, 2004; Lewis et al., 2005). Long term exposure can lead to suppression of the immune system and several types of cancers, such as those affecting the liver, lungs and gastrointestinal tract (CDC, 2004; Lewis et al., 2005; Marchese et al., 2018). In developed nations, legislation has been implemented to prevent these crops from entering the market; however, in developing nations, lacking such guidelines or restrictions, exposure becomes more prevalent.

In the United States and other developed nations, the major impact caused by \textit{A. flavus} is the economic loss associated with aflatoxin contamination. Government agencies such as the U.S. Food and Drug Administration and the European Union have set limits on the amount of
aflatoxin allowed in food (Ojiambo et al., 2018). If crops are found to contain more than the limit, they are destroyed before reaching market. It has been estimated that associated economic loss of corn infected with aflatoxin can reach up to a billion dollars annually in the United States alone (Mitchell et al., 2016).

Due to the detrimental impact of *A. flavus*, it is paramount to gain insight into its dispersal and survival mechanisms as well as the regulatory pathways controlling the production of mycotoxins. This knowledge could reveal novel genetic elements that could be used as possible targets to reduce the negative effects of this opportunistic pathogen and mycotoxigenic fungus.

Morphological development and secondary metabolism (SM) are genetically linked (i.e. Calvo et al., 2002; Calvo & Cary, 2015). Previously studied in *A. flavus*, one of those genetic links, *rmtA*, encodes for an arginine methyltransferase and regulates aflatoxin biosynthesis as well as development (Satterlee et al., 2016). Specifically, *rmtA* is a repressor of the production of conidia, air-borne asexual spores that constitute an efficient form of fungal dissemination, and a positive regulator in the formation of sclerotia, stroma that act as resistant structures (Horn et al., 2014; Satterlee et al., 2016). Homologs of RmtA have been shown to be involved in transcriptional regulation, signal transduction, RNA processing and transport (Bedford and Clark, 2009). RmtA is known to have a role in methylation of histones, which in turn affects the transcription/expression of multiple genes. RmtA methylates histones by adding methyl groups of S-adenosylmethionine (SAM) to the guanidine nitrogen atoms of arginine residues on the histone’s tails (Trojer et al., 2004). This methylation results in conformational changes of histone
structure and affects which parts of the genome are wound around the nucleosome, which in turn affects the gene’s accessibility for transcription and expression (Tessarz & Kouzarides 2014).

Due to the function of \textit{rmtA} in epigenetic regulation and the important effect of this gene on morphological development and secondary metabolism, in our current study we performed a transcriptome analysis to further assess its role in \textit{A. flavus}. In this analysis we identified over 2000 genes that are \textit{rmtA} dependent; some of those genes are associated with SM, response to environmental stress and virulence of this agriculturally important fungus.

\textbf{Materials and Methods}

\textbf{Strains Used and Growth Conditions}

All strains used in this work are listed in Table 1. Strains were grown on potato dextrose agar (PDA) at pH 5.6 in the dark at 30 °C, unless otherwise stated. Stocks of each strain were maintained at -80 °C in 30% glycerol stocks.

\begin{table} [h]
\centering
\caption{List of strains used in transcriptome analysis of \textit{rmtA} in \textit{Aspergillus flavus}}
\begin{tabular}{|c|c|c|}
\hline
Strain & Pertinent Genotype & Source \\
\hline
CA14-WT & Δ\textit{ku70} & USDA \\
\hline
CA14-Δ\textit{rmtA} & Δ\textit{rmtA::pyrG}\textsuperscript{A.fumigatus}, niaD\textsuperscript{A.fumigatus}, Δ\textit{ku70} & Satterlee et al., 2016 \\
\hline
CA14-com-\textit{rmtA} & Δ\textit{rmtA::pyrG}\textsuperscript{A.fumigatus}, \textit{rmtA::niaD}\textsuperscript{A.fumigatus}, Δ\textit{ku70} & Satterlee et al., 2016 \\
\hline
CA14-\textit{OErmtA} & \textit{gpdA(p)::rmtA::trpC(t)::pyrG}\textsuperscript{A.fumigatus}, niaD\textsuperscript{A.fumigatus}, Δ\textit{ku70} & Satterlee et al., 2016 \\
\hline
\end{tabular}
\end{table}
The wild-type, deletion \( rmtA (\Delta rmtA) \), and overexpression \( rmtA \) (\( OE_{rmtA} \)) strains were grown on potato dextrose top agar. Spores \( (5 \times 10^6) \) were inoculated into 5 ml of melted PDA top agar, which was then placed onto 25 ml solid PDA medium. After 72 h of incubation, mycelia was collected, dried and frozen in liquid nitrogen. Total RNA was extracted from lyophilized mycelia using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol. RNA was further purified using Dynabeads® mRNA Purification Kit. RNA quality was assessed using an Agilent Bioanalyzer. Sequencing was performed as a HiSeq 2000 single-read 1x100bp lane. The experiment was carried out with two biological replicates.

**Analysis of RNA Sequencing Data to Identify Differentially Expressed Genes**

*Read mapping:* The single-end reads of three conditions (WT, DEL, OE) were separately aligned to the *A. flavus* NRRL 3375 reference genome using TopHat version 2.1.1. The command used was `tophat --o output_dir_name fastq_file`. TopHat utilizing Bowtie2 version 2.3.1 was used. SAMtools version 1.3.1 was implemented to convert the SAM output file from TopHat into a BAM file for the next step.

*Read counts:* The mapped reads in BAM format were then analyzed using the HTSeq.scripts.count command from the HTSeq Python package version 0.9.1. This tool was employed to return a table of read counts for each gene. The command used was `python --m HTSeq.scripts.count --i Parent gff_file`. The GFF file downloaded from NCBI contains the pre-annotated gene models as well as their genomic locations.

*Differentially expressed genes* (DEGs): The table of read counts was used as input for the R limma package. This package was used to determine DEGs by comparing read counts between
two conditions: WT versus DEL and WT versus OE. The two replicates of each condition were combined during this step of the analysis. The RPKM() function in the R edgeR package determined the RPKM (reads per kilobase per million) values for all the genes. Bash and Perl scripts were developed to parse the DEGs data and RPKM data. An Excel file was created with the RPKM values for all genes across all conditions. FungiDB (Basenko et al., 2018) was used for functional enrichment of the datasets using GO Term (GO) annotations.

Selected groups of genes: To gain more biological significance from the datasets, the differentially expressed genes were mapped to other databases. The list of SM gene clusters (SMGCs) information was extracted from Ehrlich and Mack (2014). A full list of transcription factors (TFs) in *A. flavus* were derived from the Fungal Transcription Factor Database (http://ftfd.snu.ac.kr/intro.php; Park et al., 2008). Functional annotations of these transcription factors were obtained from NCBI. Genes related to environmental stress response were extracted from the database established by Miskei et al. (2009). The list of DEGs from the study performed by Dolezal and collaborators (2013) was compared to this dataset to search for potentially *rmtA*-dependent virulence genes. R version 3.4.1 (R Core Team 2017), specifically the ggplot2 package (Wickham 2009), was used to make statistical figures.

Environmental stress assay

To assess *rmtA* involvement in survival when exposed to environmental stresses in *A. flavus*, we exposed the strain to a range of osmotic stress inducers and to temperatures above and below optimum growth conditions. For all assays, wild-type, *ΔrmtA*, com-*rmtA*, and OE*rmtA* strains were point-inoculated on PDA plates and grown for 48 h. For testing of osmotic stress,
1M sucrose, 1.2M sorbitol, 0.6M KCl, or 0.7NaCl were added to the medium. For the temperatures assay, cultures were exposed to 25 °C, 28 °C, 30 °C, 37 °C, 40 °C and 42 °C.

**Results**

**rmtA-Dependent Transcriptome in *A. flavus***

Transcriptome analysis of *rmtA* revealed that both deletion and overexpression of *rmtA* results in similar ratios of up or down DEGs with greater than two-fold difference in expression compared to the wild type, constituting more than 2,000 *rmtA*-dependent DEGs, as shown in Figure 1. Absence of *rmtA* affected the expression of more genes than when *rmtA* was overexpressed. Only 27 DEGs showed an opposite expression pattern, DEGs that are downregulated in the absence of *rmtA* while they are upregulated when *rmtA* is overexpressed and vice versa. Most of the DEGs require wild-type levels of *rmtA* expression to function properly, as both deletion and overexpression of *rmtA* cause alterations in their transcription, either decreasing it or increasing it. There are 719 genes with reduced expression when *rmtA* is not expressed at wild-type levels, and 632 genes with increased transcription when this occurs. Enrichment analysis of the dependent transcriptome does not indicate any particular areas of regulation that *rmtA* governs either by its absence or forced expression (Figure 2).
Figure 1 - Number of upregulated and downregulated genes when the expression of *rmtA* is altered by *rmtA* deletion or overexpression. Number of up-regulated (red) and down-regulated (green) DEGs in DEL/WT and OE/WT comparisons.
**Figure 2- GO term enrichment of differentially expressed genes.** GO terms associated with DEGs in DEL/WT (left) and in OE/WT (right) comparisons. The count of DEGs in each term is proportional to the heights of the bars. GO terms associated with biological processes are in green, cellular components are in blue, and molecular functions are in red. Downregulated genes are to the left of the origin, upregulated to the right. The top 10 terms for each broad category are presented in this figure for each condition.
<table>
<thead>
<tr>
<th>Term</th>
<th>WT/smtA</th>
<th>WT/OE</th>
</tr>
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<tbody>
<tr>
<td>Amino sugar metabolic process</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular component disassembly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate derivative binding</td>
<td>4.8x10^-2</td>
<td></td>
</tr>
<tr>
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<td>1.7x10^-2</td>
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</tr>
<tr>
<td>Cell adhesion</td>
<td>4.8x10^-2</td>
<td>4.8x10^-2</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>1.7x10^-2</td>
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<tr>
<td>Mitochondrial electron transport</td>
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<td>1.7x10^-2</td>
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<tr>
<td>Nitric oxide synthesis</td>
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<td>RNA splicing, via endonucleolytic cleavage and ligation</td>
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**rmtA-Dependent Expression of SMGCs**

Our results revealed that several genes within two SMGCs (defined as in Georgianna et al., 2010) were *rmtA* dependent (Figure 3). One cluster, initially denominated cluster #54, corresponds to the already characterized aflatoxin gene cluster. As shown previous, *rmtA* regulates production of aflatoxin (Satterlee et al., 2016). The other SMGC is the novel cluster #21. At the moment this cluster is uncharacterized in *A. flavus*, but several of its genes present homologous in the gliotoxin gene cluster in *A. fumigatus*. 
Figure 3 - Heat maps of aflatoxin and gliotoxin-like fene cluster in the rmtA transcriptome.

Heat map of RPKM values of genes found in secondary metabolite gene cluster #54 aflatoxin (left) and cluster #21 gliotoxin-like cluster (right).
**rmtA-Dependent Transcription Factors**

Based on our analysis, 251 out of the over 600 putative transcription factor genes in *A. flavus* are regulated by *rmtA* (Figure 4). Some of the transcription factors shown to be governed by *rmtA* by this study are known to be involved in the regulation of development and metabolism, such as the aflatoxin transcription factor AflR (Woloshuk et al., 1994), MetR (Gai et al., 2019), a regulator of sulfur metabolism, and Rum1, jain which in *A. flavus* regulates both asexual development and metabolism (Hu et al., 2018). Wild-type levels of *rmtA* expression were required for proper expression of these genes. In addition, multiple transcription factors have previously associated with pathogenicity (Bultman et al., 2016; Issi et al., 2016). In other species genes such as *con7, ctf1, metR* and *sreA* were found to be connected with virulence, and their homologs are dependent on *rmtA* in *A. flavus* (Schrettl et al., 2008; Ramierz & Lorenz, 2009; Ruiz-Roldán et al., 2015; Gai et al., 2019). Additionally, transcription factors attributed to different types of environmental stress response in *Aspergillus* were also found to be regulated by *rmtA*, such as SrrA (oxidative and osmotic; Hagiwara et al., 2011), HacA (thermal; Zhou et al., 2016), AtfA (oxidative and osmotic; Balazs et al., 2010), and Seb1(osmotic, oxidative, and thermal; Seidl et al., 2004).
**Figure 4 – rmtA-dependent transcription factor genes.** Graphical representation of *rmtA*-dependent transcription factor genes. This graph represents DEGs transcription factors whose expression is affected by either loss of or forced expression of *rmtA*. Red color indicates upregulated genes while green color indicates genes that are downregulated.
**rmtA Acts as a Repressor of Conidiation Even Under Environmental Stress**

Based on the finding that stress response transcription factors are regulated by *rmtA*, we further investigated whether other genes involved in fungal stress response are also under *rmtA* control. A list of genes associated with stress response from Miskei et al., (2009) was used to parse our transcriptome data. In both cases, lack of *rmtA* and overexpression of this gene, expression of approximately 100 genes deviated from that of the wild type and were related to stress response (Figure 5A). Our data analysis indicates that *rmtA* does not regulate genes responding to a single type of stress but affect the expression of genes in response to multiple types of stresses.
Figure 5 – Impact of \textit{rmtA} on the expression of stress-related genes in \textit{A. flavus}. Graphical representation of the number of \textit{rmtA}-regulated stress response genes based on the Miskei et al., (2009) database.
Stress-Related Genes

Number of DEGs

WT/ΔrmtA

WT/OE

Regulation

Up
Down

61
53
51
56
Whether \textit{rmtA} plays a role in resistance to oxidative stress in \textit{A. flavus} was previously assessed and it was found that when exposed to increasing concentrations of menadione, alterations in \textit{rmtA} improved resistance of \textit{A. flavus} under this condition (Satterlee et al., 2016). However, the possible implications of \textit{rmtA} on the effect of other environmental stresses have not been studied. Based on the fact that the expression of several genes involved in osmotic and thermal stress response is influenced by \textit{rmtA}, we examined whether \textit{rmtA} is involved in the resistance to those environmental stresses. Unlike oxidative stress, vegetative growth was slightly reduced in the absence of \textit{rmtA} when cultures were exposed to high concentrations of NaCl (Figure 6A). However, colony growth was not affected when the strains were grown on high concentrations of sucrose, sorbitol of KCl. The hyperconidation phenotype of the deletion mutant was still detected even in the presence of high osmotic stress, although it was greatly attenuated.
Figure 6 – Evaluation of the effect of rmtA on the resistance to osmotic stress in A. flavus.

To induce osmotic stress, 1 M sucrose, 1.2 M sorbitol, 0.6 M KCl, and 0.7 M NaCl were added to PDA in each case. Wild-type (WT), ΔrmtA, complementation (com), and overexpression rtmA (OE) were point-inoculated and grown at 30 °C in the dark. A) Photographs of the cultures after 48 h of incubation. B) Diameter of fungal colonies growing on PDA plus 0.7 M NaCl.
Unlike when exposed to oxidative or osmotic stress, changes in expression of \textit{rmtA} did not cause any changes in colony growth when exposed to an array of temperatures. While no change in growth was observed, the deletion mutant phenotype pertaining to hyperconidiation persisted at all temperatures tested, except 42 °C (Figure 7).
Figure 7 – Assessment of the effect of *rmtA* on the resistance to different temperatures in *A. flavus*. Wild-type (WT), Δ*rmtA*, complementation (com), and overexpression *rmtA* (OE) were point-inoculated on PDA and grown at 25 °C, 28 °C, 30 °C, 37 °C, 40 °C and 42 °C in the dark. Photographs of the cultures after 48 h of incubation are shown.
**rmtA regulates genes that are active during virulence**

During *A. flavus* infection of maize kernels, Dolezal and collaborators (2013) performed a transcriptome analysis and found differentially expressed genes during active infection versus saprotrophic growth of this fungus using viable and nonviable maize kernels. Some of these genes were upregulated and downregulated during infection. We used this list of genes and applied it to our RNAseq data analysis. As shown in Figure 8, multiple genes that are differentially expressed during infection are also *rmtA* dependent. Any modification of *rmtA* expression caused a decrease in the expression of 96 genes that were upregulated during infection. Conversely, we found 118 upregulated genes by changes in the *rmtA* locus that were suppressed during infection.
Figure 8 - Effect of *rmtA* on genes active during plant infection. Graphical representation of *rmtA*-dependent genes that are differentially expressed during corn infection. This graph represents DEGs transcription factors whose expression is affected by either loss of or forced expression of *rmtA*. Red color indicates upregulated genes while green color indicates genes that are downregulated. The two left columns represent genes with increased expression during infection of plant tissue; the two columns on the right indicate genes with decreased expression under that condition.
**Discussion**

This study provides further insight into the role of *rmtA* in *A. flavus* by performing a transcriptome analysis. Previously, it was shown that *rmtA* was a regulator of conidial and sclerotial production as well as aflatoxin biosynthesis (Satterlee et al., 2016). Specifically, we found that *rmtA* is a negative regulator of conidiation by suppressing expression of members in the *brlA* pathway. In terms of sclerotial production removal, of *rmtA* halted production of these structures where increased levels of *rmtA* increased production in respect to the WT. RmtA was identified as a positive regulator of aflatoxin production by directly affecting expression of the aflatoxin gene cluster (Satterlee et al., 2016). Our present study revealed a broad regulatory scope for *rmtA*, where a significant portion of the *A. flavus* genome is under its control.

Hundreds of genes presented altered expression when *rmtA* expression levels were different than those in the wild type, by deleting *rmtA* or overexpressing this gene; 719 genes in the *A. flavus* genome showed a reduction of their expression with this criterion, while 632 genes experienced an increase. In the model fungus *A. nidulans*, the *rmtA* homolog presented strong specificity for the methylation of H4 histone (Trojer et al., 2004). Epigenetic modifications of histone cores, such as histone methylation, affect nucleosome structures, leading to changes in the transcription of numerous genes (Tessarz & Kouzarides 2014). This agrees with the extensive effect of *rmtA* on the *A. flavus* transcriptome. In addition, based on our results, a balanced stoichiometry of RmtA with other partners seems required for its proper function.

As mentioned above, *rmtA* was found to be necessary for production of aflatoxin (Satterlee et al., 2016). Our transcriptome analysis revealed that out of 24 genes present in the
aflatoxin SMGC (cluster #45 as in Georgianna et al., 2010), 11 genes were found to be rmtA dependent. Outside of the aflatoxin cluster, only one other cluster, cluster #21, presented a large number of rmtA-dependent DEGs. In A. flavus cluster#21 has yet to be characterized. However, some genes in this cluster have homology to genes in the gliotoxin cluster in A. fumigatus (Dolan et al., 2015). Although there are similarities between these two clusters, the A. flavus cluster has nearly double the number of genes compared to that in the A. fumigatus genome. Gliotoxin belongs to a class of metabolites known as epidithiodiketopiperazine. In Aspergillus oryzae, a cluster akin to the one described in A. flavus was characterized and found to produce another compound in this same family known as aspirochlorine (Chankhamjon et al., 2014). Production of this compound has been previously shown in A. flavus and documented to possess antifungal properties (Klausmeyer et al., 2005). Whether SMGC#21 is responsible for the synthesis of aspirochlorine or possibly another epidithiodiketopiperazine compound in A. flavus is an ongoing question.

While methylation of histones by rmtA may directly regulate the expression of certain genes in the genome, it would also affect the transcription of others indirectly, including transcription factor genes. In our study we identified over 200 transcription factor genes with altered expression patterns caused by modifications in the rmtA locus, by deletion or overexpression means. While they are not all characterized, some of these transcription factors are known to play a crucial role in fungal development, metabolism and response to environmental stresses and others have been associated with virulence. A few examples of these genes that have been investigated in A. flavus are aswA, a regulator of sclerotial production and
related metabolism (Chang et al., 2017), and aflR, which is the primary regulator of aflatoxin production in *A. flavus* (Masanga et al., 2015). Another example is *rum1*, which is a transcription factor that has a wide range of regulatory effects, controlling aflatoxin biosynthesis and development of conidia and sclerotia in *A. flavus* (Hu et al., 2018). However, the majority are still uncharacterized or have been studied in other fungi such as the medusa transcription factor MedA, which is shown to regulate conidiation in multiple fungi (Clutterbuck, 1969; Gravelat et al., 2010; Chacko & Gold, 2012).

Unexpectedly, *A. flavus ΔrmtA* strain is more resistant to sources of oxidative stress than the wild type (Satterlee et al., 2016). This was in contrast to with the phenotype of the *rmtA* mutant in *A. nidulans* (Trojer et al., 2004), suggesting a specialization in the regulatory output of *rmtA* in both fungi with respect to environmental stress resistance. In our transcriptome analysis several genes such as *atfB* (Sakamoto et al., 2008), *fhda* (Malavazi et al., 2006), *alb1* (Tsai et al., 1998), and *pes1* (Reeves et al., 2006) were found to be upregulated by absence *rmtA*. Expression of these genes has been shown to be indispensable for resistance to oxidative stress. It is possible that regulation by these genes and others is responsible for the phenotype that was previously described as they are *rmtA*-dependent.

Furthermore, we identified additional *rmtA*-regulated elements involved in the response to other environmental insults, such as those involved in osmotic and thermal stress. Examples of these genes include members of the HOG pathway, *nikA* and *shoA*, a well-studied network that regulates osmotic stress in fungi (Furukawa et al., 2005; Hagiwara et al., 2013). Also, *hacA* and *cypB* are *rmtA*-dependent DEGs, where HacA is a heat shock protein and *cypB* is expressed at
high levels during heat shock conditions (Joseph et al., 1999; Zhou et al., 2016). Based on these transcriptome results, we also examined whether \textit{rmtA} influences the growth of \textit{A. flavus} colonies when challenged by osmotic or thermal stresses. However, in most cases no changes in vegetative growth were detected. Only high concentrations of NaCl resulted in a slight growth reduction compared to the wild type under the same experimental conditions, suggesting an effect of \textit{rmtA} on sodium metabolism. The fact that, although some stress response genes were affected by alteration in \textit{rmtA} modifications, the fungal colony growth was not notably changed suggests possible redundancies of a robust genetic system in \textit{A. flavus} protecting this fungus from environmental stresses. Interestingly, the hyperconidiation phenotype of \textit{\Delta rmtA} persisted in the presence of the stressors assayed, and it was only partially attenuated under osmotic stress, suggesting that even under exposure to environmental stress \textit{rmtA} is still a required regulator of asexual development in \textit{A. flavus}.

Li et al. (2017) reported that \textit{rmtA} affects development and aflatoxin production during infection of peanuts seeds and maize kernels. While this study did not examine whether removal of \textit{rmtA} influenced fungal burden during infection, \textit{rmtA} was found to regulate lipase and protease activity (Li et al., 2017). In our transcriptome analysis we investigated connections related to virulence in genes regulated by \textit{rmtA} based on a study of Dolezal et al. (2013) that shows DEGs during \textit{A. flavus} infection of maize. Our study revealed that several DEGs corresponding to classes of secretory enzymes such as lipases (PlaA & PLD), proteases (Pim1 & MEP1) and several putative hydrolases (AFLA_025360, AFLA_004540, & AFLA_062930) (Hong et al., 2005; Brown et al., 2007; Zhang et al., 2014; Ciesielski et al., 2016) were \textit{rmtA}
dependent. In addition, other genes possibly involved in animal infections such as those involved in iron (sreA; Schrettl et al., 2008) and sulfur (metR; Amich et al., 2013; Gai et al., 2019) metabolism; changed their expression when rmtA was absent, suggesting that rmtA could have an effect on *A. flavus* virulence also in animals. Furthermore, other rmtA-dependent DEGs involved in pathogenicity include pes1, which in *A. fumigatus* was shown to be necessary for full virulence in *Galleria mellonella* (Reeves et al., 2006). As mentioned earlier MedA, is regulated by rmtA and regulates conidiation in *Aspergillus* (Clutterbuck, 1969), but it is also important in virulence, since it is required for biofilm formation and normal adhesion as shown in *A. fumigatus* studies (Gravelat et al., 2010). Additionally, its homolog in *Ustilago maydis* is also necessary for full virulence in maize (Chacko & Gold, 2012).

In conclusion, we have shown that the epigenetic regulator *rmtA* governs the expression of over 2,000 genes, affecting multiple aspects of the *A. flavus* biology, including development, metabolism, virulence and some aspects of environmental stress response. It is interesting that although some of these genes have been previously characterized, the function of most of the *rmtA*-dependent genes remains unknown, constituting a new avenue to be further explored in future research. Importantly, although RmtA is well conserved in eukaryotes (Satterlee et al., 2016), outside the domain the similarity is low at the N-terminal and C-terminal regions of this protein. These regions could be potentially used as a target to develop a strategy to reduce the detrimental effects of this agriculturally important fungus.
References


THE TRANSCRIPTIONAL REGULATOR HBXA GOVERNS DEVELOPMENT, SECONDARY METABOLISM, AND VIRULENCE IN *ASPERGILLUS FUMIGATUS*

**Introduction**

The fungal opportunistic human pathogen *Aspergillus fumigatus* is a known cause of a wide range of illnesses, including invasive aspergillosis (IA). Immunodeficient individuals are particularly susceptible to these diseases (Knutsen & Slavin, 2011; Osherov, 2012), including organ transplant patients, individuals with genetic immunodeficiencies or receiving chemotherapy, and HIV patients (Denning, 1998; Pagano et al., 2001; Marr et al., 2002; Wiederhold et al., 2003; Kliasova et al., 2005; Post et al., 2007). The main site of entry leading to *A. fumigatus* infections is the respiratory tract. Healthy individuals are able to eliminate the inhaled fungal conidia (asexual spores) through mucociliary clearance. The remaining fungal spores encounter epithelial cells or alveolar macrophages, responsible for phagocytosis and killing of spores and for the initiation of a proinflammatory response that recruits neutrophils. These neutrophils destroy hyphae from germinated conidia that evaded macrophages. However, patients who are neutropenic are at high risk of developing IA, with a mortality rate of up to 90% (Schmitt et al., 1990; Latge, 1999; Kontoyiannis & Bodey, 2002; Oren & Goldstein, 2002; Sherif & Segal, 2010).
The small size of the *A. fumigatus* conidia contributes to its pathogenicity, reaching the lung alveoli and establishing an infection that can eventually become systemic (Osherov, 2012). In addition, *A. fumigatus* produces numerous secondary metabolites (Frisvad and Samson, 1990; Botterel et al., 2002; Panaccione & Coyle 2005; Coyle et al., 2007; Khoufache et al., 2007; Dagenais & Keller, 2009; Gauthier et al., 2012; Lim et al., 2014;), that are considered part of the fungal chemical arsenal required for niche specialization (Calvo et al., 2002) including host-fungus interactions. Some of these metabolites act as immunosuppressants, which may be in association with pathogenic processes. For most IA infections, early diagnosis is critical, followed by treatment with antifungal drugs such as azoles, typically voriconazole (Patterson et al., 2016; Lestrade et al., 2018). Recently, strains of *A. fumigatus* have been shown to be gaining drug resistance, thus it is necessary to identify new targets to control or prevent the potentially lethal infections caused by *A. fumigatus* (Lestrade et al., 2018).

Fungal regulatory genes could constitute some of these novel genetic targets to design antifungal therapies. Among them is the homeobox transcriptional regulator gene *hbxA*. Homeobox (Hbx) proteins are a class of transcriptional regulators that govern development in many eukaryotes; including animals, plants and other fungi (reviewed in Holland, 2012). A homolog of this gene in *Aspergillus flavus* has been shown to regulate aspects of morphological differentiation, including asexual development (Cary et al., 2017). A recent *hbxl*-dependent transcriptome analysis revealed that this gene controls the *brlA* pathway, as well as multiple “fluffy” genes (Cary et al., 2018). These genes have been studied in several *Aspergillus* spp, including *A. fumigatus*, where they were shown to be required for normal conidiation, as
reviewed by Park and Yu (2016). Additionally, *hbxl* was found to regulate secondary metabolism in *A. flavus*, positively controlling the expression of multiple secondary metabolite gene clusters (Cary et al., 2017; Cary et al., 2018). We hypothesize that the homolog of *hbxl* in *A. fumigatus*, *hbxA*, may play a similar role in the regulation of development and secondary metabolism in this important opportunistic human pathogen.

Our study revealed that in *A. fumigatus* lack of *hbxA* leads to a slight reduction in colony growth and a near complete loss of conidial production. Furthermore, we showed that loss of *hbxA* decreased the expression of genes in the *brlA* central developmental pathway as well as the expression of the “fluffy” genes *flbB*, *flbD*, and *fluG*. The conidial defect is partially restored in the presence of high concentration of sucrose or fructose, but not when exposed to other carbon sources or high concentrations of other solutes causing osmotic stress. Other aspects of asexual development were affected in the absence of *hbxA*, such as the size and germination rate of conidia. In addition, metabolomics analysis of the *hbxA* mutant indicated that this gene is a master regulator that governs the production of numerous secondary metabolites. With respect to the possible *hbxA* effect on virulence, a pathogenicity test using a neutropenic mouse infection model indicated an increase in virulence in the absence of *hbxA* compared to the *A. fumigatus* wild-type strain.

**Materials and Methods**

**Culture Conditions**

All strains used in this work are listed in Table 2. Strains were grown on glucose minimal
media (GMM) at pH 6.5 in the dark at 37 °C, unless otherwise indicated. Agar at a concentration of 1% was used for solid cultures. Stocks of each strain were maintained at -80 °C in 30% glycerol. In order to obtain abundant spore inoculum, the strains were grown on GMM supplemented with 1M sucrose. Spores were washed with water to remove residual medium components.

Table 2

List of Strains Used in Characterization of \( \text{hbxA} \) in \( \text{Aspergillus fumigatus} \)

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<th>Genotype</th>
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<td>Wild type</td>
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</tr>
<tr>
<td>CEA17</td>
<td>( \text{pyrG1} )</td>
<td>Gift from Robert Cramer</td>
</tr>
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<td>TTRS8</td>
<td>( \text{pyrG1, } \Delta \text{hbxA::pyrG}^{A,\text{parasiticus}} )</td>
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<td>TTRS12</td>
<td>( \text{pyrG1, } \Delta \text{hbxA::pyrG}^{A,\text{parasiticus}}, \text{hbxA::ptrA}^{A,\text{oryzae}} )</td>
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**Strain Constructions**

**Generation of the Deletion \( \text{hbxA} \) Strain (\( \Delta \text{hbxA} \))**

To obtain the \( \text{hbxA} \) deletion strain, an \( \text{hbxA} \) deletion cassette was first generated by fusion PCR as described by Szewczyk et al., (2006). Primers Afum_hbxA_P1 and Afum_hbxA_P2 were used to PCR amplify the 5’ UTR of the \( \text{hbxA} \) locus in the \( \text{A. fumigatus} \) genome, while Afum_hbxA_P3 and Afum_hbxA_P4 primers were used to amplify the 3’ UTR fragment. The middle fragment containing the selection marker was PCR amplified from plasmid pPG28 (Takashi et al., 2002) using primers Afum_hbxA_P5 and Afum_hbxA_P6. The marker used was
pyrG from *Aspergillus parasiticus*. The three fragments were then fused by PCR using primers Afum_hbxA_P7 and Afum_hbxA_P8. All primers used in this study are listed in Table 3. The fused PCR product was transformed into *A. fumigatus* CEA10 (pyrG-, ptrA-) by a polyethylene-glycol-mediated transformation as previously described (Cary et al., 2017). Transformants were selected on half-strength PDA without uracil. Potassium chloride (0.6 M) was used as an osmotic stabilizer in the regeneration medium. Transformants were confirmed by diagnostic PCR with primers Afum_hbxA_P0 and Apar_pyrG_R. A selected hbxA deletion transformant, TTRS8, was used in this study.
### Table 3
Primers Used in Characterization of *hbxA* in *Aspergillus fumigatus*

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</table>


**Generation of the complementation hbx1 strain (Com)**

To generate the complementation strain, a two-fragment fusion PCR method was utilized joining the *hbxA* locus and the selection marker gene *ptrA* from *Aspergillus oryzae*. First, the *hbxA* locus was amplified from the *A. fumigatus* genomic DNA, using primers Afum_hbxA_Com_P1 and Afum_hbxA_Com_P2, while the *ptrA* marker was amplified from the plasmid pPTRI (TakaraBIO, Mountain View, CA, USA) with primers Afum_hbxA_Com_P3 and Afum_hbxA_Com_P4. The two PCR products were then fused in a similar manner as described above using primers Afum_hbxA_Com_P1 and Afum_hbxA_Com_P4. The fusion cassette was transformed into the Δ*hbxA* strain TTRS8. Transformants were selected on Czapek Dox (CZ, Difco, Franklin Lakes, New Jersey, USA) medium containing 1 µg/ml of pyrithiamine. Confirmation of the reinsertion of *hbxA* in the transformants was carried out by diagnostic PCR. Transformants were confirmed by diagnostic PCR with primers Afum_hbxA_qPCR_F and R_ptrA_Check. A selected *hbxA* complementation strain, TTRS12, was used in this study. Pertinent genotypes of all strains are listed in Table 2.

**Morphological Analysis**

*Colony Growth*

Wild-type, Δ*hbxA*, and complementation strains were point-inoculated on GMM. Colony diameter was measured after five days of incubation at 37 °C. The experiment was carried out with three replicates.

*Conidial Production*

To assess whether *hbxA* regulates conidiation in *A. fumigatus*, 10⁶ spores/ml of each (wild-type, Δ*hbxA*, and complementation) strains, were inoculated into 25 ml of liquid GMM, allowing an air interphase. Cultures were grown under stationary conditions, allowing an air interphase to promote development. Cores (7 mm diameter) were collected from the mycelial mats to quantify conidia after 48 h.
and 72 h of incubation at 37 °C. The cores were homogenized in water and spores were counted under a Nikon Eclipse E-400 bright-field microscope (Nikon Inc., Melville, NY, USA) using a hemocytometer (Hausser Scientific, Horsham, PA). Experiments were performed in triplicate.

**Effect of Osmotic Stress on hbxA-Dependent Growth and Conidiation**

To examine the possible role of *hbxA* on osmotic stress resistance, the wild-type, Δ*hbxA*, and complementation strains were point-inoculated on solid GMM and GMM plus either 0.6 M KCl, 1 M sucrose, 0.7 M NaCl, or 1.2 M sorbitol. Cultures were incubated in the dark at 37 °C for 7 days. Colony growth was assessed as colony diameter. Under these conditions conidia were also quantified. Cores were collected from the colonies and spores were counted as described above.

**Effect of Carbon Sources on hbxA-Dependent Growth**

Different carbon sources were added to minimum medium. The 1% glucose concentrations in GMM was replaced with 1% concentrations of either fructose, sucrose, potassium acetate, citrate, glycerol or ethanol. GMM cultures were used as control. Strains were point-inoculated and allowed to grow for 72 h. In addition, to assess the possible effect of carbon starvation on the *hbxA* function, all strains were grown on GMM with different glucose concentrations (1%, 0.5%, 0.2%, .15%, 0.1% and 0.05%)

In a separate experiment, the strains were grown on minimum medium replacing the standard 1% glucose with 1M concentrations of different sugars (glucose, fructose, and sucrose). Plates were grown under the same conditions as above for seven days.

**Germination Assay**

Flasks with 50 ml of liquid GMM were inoculated with conidia (10⁶ spores/ml) of wild type, Δ*hbxA*, and complementation strains. Every 2 hours post-inoculation, 500 microliters of culture was
collected from each flask for spore quantification under the microscope using a hemocytometer. This experiment was performed in triplicate.

**Gene Expression Analysis**

Petri dishes containing 25 ml of liquid GMM were inoculated with conidia (10⁶ spores/ml) of *A. fumigatus* CEA10 WT, ΔhbxA, and complementation strains. Cultures were incubated in stationary conditions at 37 °C in the dark. Total RNA was extracted from lyophilized mycelial samples using TRIsure (Bioline, Taunton, MA, USA) reagent according to the manufacturer’s instructions. cDNA was synthesized with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with the Applied Biosystems 7000 Real-Time PCR System using SYBR green dye for fluorescence detection. cDNA was normalized to *A. fumigatus* 18S ribosomal gene expression, and the relative expression levels were calculated using the 2^{−ΔΔCT} method (Livak & Schmittgen, 2001). Primer pairs used are indicated in Table 3.

**Liquid Chromatography and Mass Spectrometry Analysis**

Sample analysis was performed using HPLC coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Les Ulis, France). Extracts were resuspended in 400 μl methanol and 10 μL of this suspension were injected into a reversed-phase (150 mm × 2.0 mm) 5 μm Luna C18 column (Phenomenex, Torrance, CA, U.S.A.) operated at a flow rate of 0.2 mL/min. A gradient program was performed with 0.1% formic acid (phase A) and 100% acetonitrile (phase B) with the following elution gradient: 0 min 20% B, 30 min 50% B, from 35 to 45 min 90% B, from 50 to 60 20% B. HRMS acquisitions were achieved with electrospray ionization (ESI) in the positive and negative modes as follows: spray voltage +4.5
kV, capillary temperature 350°C, sheath gas (N2) flow rate 40 au (arbitrary units), auxiliary gas (N2) flow rate 6 au in the positive mode, and spray voltage −3.7 kV, capillary temperature 350°C, sheath gas (N2) flow rate 30 au, auxiliary gas (N2) flow rate 10 au in the negative mode. Full MS spectra were acquired at a resolution of 60,000 with a range of mass-to-charge ratio (m/z) set to 50–800.

Pathogenicity analysis

Insect model

Spores from WT, ΔhbxA, and Com strains were collected using a solution of 1x PBS with 0.1% tween, after which spores were washed 5 times with additional volumes of 1x PBS. Spores were then diluted with 1x PBS to a concentration of either 1x 10^5 in 10 µl⁻¹. The infection procedure was carried out as previously described by Fuchs et al. (2010). Briefly, Galleria mellonella larvae (The Bug Company, Ham Lake, Minnesota) with a weight range between 275–300 mg and lacking grey markings were selected for the experiment. For the experiment groups of 30 larvae were infected with the different fungal spores. An additional two group of 30 larvae each were used as controls. One group received injections of 10 µl of 1x PBS while the other groups received no injections. Larvae were then placed in glass petri plates (90 mm x 15 mm) and wrapped in aluminum foil. Plates were placed in 37 °C in the dark. Larvae were checked every 2 h after 16 h of incubation until one group of larvae experienced complete mortality. No ethical approval was required for this species because they are unregulated animals.

Mouse model

Pathogenicity studies using mouse model were carried out as previously described by
Myers et al. (2017), with minor modifications. Briefly, six-week old female, outbred ICR Swiss mice, weighing approximately 25 g, were used for this experiment. Fifty mice divided into five separate groups were used, each group contained 10 mice. Animals were rendered neutropenic by intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on days -4, -1 and 3 days post-infection and Kenalog (40 mg/kg) on the day of infection. The immunosuppressed mice were infected with fungal spores of *A. fumigatus* CEA10 wild-type, ΔhbxA, and complementation strains. Sedated mice (10 mice per strain) were infected by nasal instillation of 2x10^6 spores/40 µl of PBS. Post infection mice were observed three times daily. Mice that survived to Day 8 were euthanized.

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. The protocol was approved by the Institutional Animal Care and Use Committee of Northern Illinois University (Permit #12–0006). All efforts were made to minimize suffering. Humane euthanasia by CO₂ inhalation was performed when mice met criteria indicating a moribund state; these endpoints include behaviors of unresponsiveness to tactile stimuli, inactivity, lethargy, staggering, anorexia and/or clinical signs of bleeding from the nose or mouth, labored breathing, agonal respirations, purulent exudate from eyes or nose, abnormally ruffled fur, or greater than 20% weight loss. The method of euthanasia by CO₂ inhalation is consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

**Statistical Analysis**

Statistical analysis was applied to analyze all of the quantitative data in this study
utilizing ANOVA (analysis of variance) in conjunction with a Tukey's multiple comparison test using a p-value of $p < 0.05$ for samples that are determined to be significantly different. The exception was for pathogenicity assays, in which a Kaplan-Meyer Survival test was applied.

**Results**

*hbxA is required for normal fungal growth and asexual development in *A. fumigatus*

To determine the role of *hbxA* in *A. fumigatus* two strains, an *hbxA* deletion and complementation strains, were generated (Figure 9A and 9B). Deletion of *hbxA* was confirmed by PCR, yielding the expected 2.8 kb PCR product. The complementation strain was also verified by PCR and produced a 2.7 kb DNA fragment. In addition, the strains were confirmed by assessing *hbxA* expression levels by qRT-PCR (Figure 9C). *hbxA* transcripts were absent in the Δ*hbxA* strain, while the complementation strain showed a restoration of *hbxA* expression similar to that of the wild type. Next, the growth of all the strains was evaluated in point-inoculated cultures growing on solid GMM for seven days. Our results indicated an approximately 1.4-fold reduction in the growth of Δ*hbxA* compared to the controls (Figure 10).
**Figure 9 – Construction of *hbxA* deletion and complementation strains.** (A) Representation of the strategy used to create an *hbxA* knockout strain via homologous recombination, replacing *hbxA* with the *pyrG* selection marker. Primers Afum_hbxA_P0 and Apara_pyrG_R, represented as F and R, were used to amplify a 2.8 kb band in the Δ*hbxA* strain, indicating successful gene replacement. (B) Schematic of the fusion cassette used to transform Δ*hbxA* to generate the complementation (Com) strain. Primers F and R (corresponding to Afum_hbxA_Com_P1 and R_ptrA respectively) were used for diagnostic PCR, resulting in the expected 2.7 kb PCR product. (C) Expression analysis of *hbxA* by qRT-PCR. Strains were inoculated in liquid GMM (10⁶ spores/ml), and cultures were grown statically for 72 h at 37 °C. The error bars represents standard errors. Values were normalized to the expression levels in the wild type, considered as 1. Different letters on the columns indicate values that are statistically different (p < 0.05).
Figure 10- *hbxA* is required for normal colony development. CEA10 wild type (WT), Δ*hbxA*, and complementation (Com) strains were point-inoculated on solid GMM and allowed to grow in the dark at 37 °C. (A) Images of the colonies after five days of growth. (B) Quantification of colony growth measured as colony diameter. Different letters on the columns indicate values that are statistically different (p < 0.05).
Absence of *hbxA* resulted in an almost aconidial strain. Specifically, a significant reduction of approximately 100-fold in conidia with respect to the wild type was observed (Figure 11A). Adding back the *hbxA* wild-type locus restored normal conidiation. To gain insight into the *hbxA* mechanism that controls sporulation in *A. fumigatus*, we examined the expression of the genes in the conidiation central regulatory pathway, *brlA*, *abaA*, and *wetA* (Park & Yu, 2016). Deletion of *hbxA* resulted in a significant reduction in the expression of all three genes compared to the wild type (Figure 11B, 11C and 11D).
Figure 11 – Asexual development is strongly regulated by *hbxA* in *A. fumigatus*. Wild-type (WT), Δ*hbxA*, and complementation (Com) strains were grown in liquid GMM stationary cultures for 72 h. Cores from the mycelial mats were collected at 48 h and 72 h, and conidia were quantified (A). RNA was also extracted from mycelia to perform qRT-PCR. Relative gene expression of *brlA* (B), *abaA* (C), *wetA* (D), *flbB* (E), *flbD* (F), and *fluG* (G) is shown. All values were normalized to the wild-type 48 h samples. Different letters on the columns indicate values that are statistically different (p < 0.05).
Additionally, expression of the fluffy genes *flbB*, *flbD*, and *fluG* was also downregulated in the absence of *hbxA* (Figure 11E, 11F and 11G). Interestingly, microscopic observations indicated that conidial size in Δ*hbxA* was approximately 50% larger than those formed by the wild type (Figure 12).
Figure 12 – Size of conidia is influenced by \( hbxA \). Wild-type (WT), \( \Delta hbxA \), and complementation (Com) strains were grown on GMM and spores were collected after 72 h. Samples were observed under the microscope, and spore diameter was measured. (A) Micrographs of conidia. (B) Measurements of spore diameter from 40 spores of each strain (WT, \( \Delta hbxA \), and com). Different letters on the columns indicate values that are statistically different (\( p < 0.05 \)).
Absence of \textit{hbxA} affects germination rates of \textit{A. fumigatus} conidia

To further examine the role of \textit{hbxA} on conidia in \textit{A. fumigatus}, an assay was performed to evaluate germination rates. As early as 2 hours post-inoculation, spores from the \textit{hbxA} mutant started to produce germ tubes, while spores from wild-type or complementation strain remained ungerminated (Figure 13). Only after 6 hours did the wild-type spores begin to germinate, but at that point more than 70\% of the \textit{ΔhbxA} spores were already germinated (Figure 13).
Figure 13 - Absence of *hbxA* results in earlier spore germination in *A. fumigatus*. Liquid GMM cultures of wild-type (WT), Δ*hbxA*, and complementation (Com) strains were inoculated with 10⁶ spores/ml. Every 2 h post-inoculation an aliquot of 500 µl of culture was collected to observe conidial germination under a microscope. Different letters on the columns indicate values that are statistically different (p < 0.05).
Exposure to high concentration of sucrose partially rescues the *hbxA* conidiation defect

Osmotic stress has been shown to affect development of filamentous fungi, for example, increasing conidiation (Han et al., 2003; Baidya et al., 2014). We examined whether *hbxA* still influences conidiation when exposed to osmotic stress. For most of osmotic stressors tested, no significant difference was noticed in any of the strains except when Δ*hbxA* was grown in presence of 1M sucrose (Figure 14). Under this condition, the Δ*hbxA* the strain produced more conidia than when grown on GMM alone. To further investigate the possible interaction between *hbxA* and carbon sources, we tested a variety of saccharides at a concentration of 1%. However, at this concentration none of the carbon sources tests altered the mutant phenotype (Figure 15).
Figure 14 – Evaluation of the role of *hbxA* on morphogenesis in *A. fumigatus* when exposed to osmotic stress. Wild-type (WT), Δ*hbxA*, and complementation (Com) strains were point-inoculated on GMM supplemented with different osmotic stressors (0.6 M KCl, 0.7 M NaCl, 1.2 M sorbitol, and 1 M sucrose). Cultures growing on GMM alone were used as controls. (A) Photographs were acquired after seven days of incubation. (B) Quantification of conidiation in the presence of high concentration of sucrose in GMM. Different letters on the columns indicate values that are statistically different (p < 0.05).
A

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B

- WT: 6 ± 1 × 10⁴ spores/mm²
- ΔhbxA: 2 ± 1 × 10⁴ spores/mm²
- Com: 2 ± 1 × 10⁴ spores/mm²

Significance levels:
- a: Significant difference from WT
- b: Significant difference from ΔhbxA
Figure 15 – Assessment of ΔhbxA morphological phenotype in cultures with different carbon sources. Minimum medium was supplemented with various carbon sources (1% concentration) instead of glucose. GMM was used as control. The strains were incubated for 72 h.
To further understand the effect of high sucrose concentration on ΔhapeA conidiation pattern, we also cultured the strain set in the presence of high concentrations of glucose and fructose. The result in Figure 16 reveals that high amounts of fructose, and to a lesser degree glucose, also cause an increase in conidiation in ΔhapeA. However, high concentration of sucrose still promoted the greatest amounts of spores in this A. fumigatus mutant. Starvation, however, did not affect the almost aconidial ΔhapeA phenotype, as shown in Figure 17.
Figure 16 – High concentrations of sucrose, fructose and glucose partially rescue Δhbx4 conidiation defect. Wild-type (WT), Δhbx4, and complementation (Com) strains were grown on solid GMM, with 1% glucose concentrations being replaced with 1M glucose, sucrose, or fructose. Cultures of GMM alone were used as control. Strains were grown in the dark for seven days at 37 °C.
Figure 17 – ΔhbxA phenotype does not change under carbon starvation. Wild-type (WT), ΔhbxA, and complementation (Com) strains were grown on GMM plates containing a range of glucose from 1% to 0.05%. Strains were grown for 72 h at 37 °C.
Secondary metabolism in *A. fumigatus* is regulated by *hbxA*

In *A. flavus*, *hbx1* is a regulator of the production of numerous secondary metabolites, including aflatoxin, aflatrem, and cyclopiazonic acid (Cary et al., 2017). Similarly, we observed a broad regulatory scope of *hbxA* on secondary metabolism in *A. fumigatus* (Figure 18). Our results reveal that production of fumigaclavines A, B, and C was absent in the deletion mutant while these compounds were present in the wild-type cultures grown under the same experimental conditions. Fumiquinazolines C/D, chaetomine, and fumagillin were detected in the Δ*hbxA*, but at significantly lower levels than either the wild-type or complementation strain.
**Figure 18** – HbxA regulates the production of multiple secondary metabolites in *A. fumigatus*. Spores of *A. fumigatus* wild-type (WT), deletion (ΔrtfA) and complementation (com) strain were inoculated in liquid YES medium. Cultures were incubated at 37°C, and supernatant was collected after seven days for secondary metabolite extraction and analysis. (A, B, & C) Analysis of ergot alkaloids fumigaclavine A, B and C. (D) Analysis of fumiquinazoline C/D. (E) Analysis of chaetominine. (F) Analysis of fumagillin. Different letters above the bars indicate significantly different values (p ≤ 0.05). Error bars represent standard error.
Deletion of \textit{hbxA} increases virulence of \textit{A. fumigatus} in both insect and murine models

Due to the fact that \textit{hbxA} plays an important role in regulating \textit{A. fumigatus} development as well as its metabolome, we hypothesize that \textit{hbxA} could also be relevant in virulence. To investigate this possibility, we first used the \textit{Galleria mellonella} infection model. As shown in Figure 19, the $\Delta$\textit{hbxA} strain was more virulent than the controls when tested in this insect model. Based on this result, \textit{hbxA} association with virulence was also examined in a mammalian model, specifically in mouse. In this case, removal of \textit{hbxA} also increased \textit{A. fumigatus} virulence (Figure 20).
**Figure 19 – HbxA is a negative regulator of virulence in the *G. mellonella* model.** *Galleria mellonella* larvae were infected with *A. fumigatus* wild type (WT), deletion (ΔhbxA) and complementation (com) strains as described in Material and Methods section. Two control groups were present in this experiment; one received an injection of 1x PBS instead of fungal spores and another received no injection. Statistical analysis of survival was carried out by a Kaplan-Meyer pairwise comparison using a long rank test.
**Figure 20 – HbxA is a negative regulator of virulence in mouse model.** Six-week old mice were rendered neutropenic by administration of cyclophosphamide and Kenalog-10 treatments. Neutropenic mice were infected with $2 \times 10^6$ conidia/mouse of *A. fumigatus* wild-type (WT), deletion ($\Delta hbxA$) and complementation (com) strains and monitored daily for a total of seven days. Two controls that did not receive fungal spores were included in this analysis: a group that was rendered neutropenic and another group not treated with cyclophosphamide or Kenalog-10. Statistical analysis of survival was carried out by a Kaplan-Meyer pairwise comparison using a long rank test.
Discussion

In humans the primary route of *A. fumigatus* infections is through the inhalation of airborne conidia that can eventually germinate in the lungs of a host. Investigation of genes that influence fungal development, as well as other aspects of *A. fumigatus* biology, could provide interesting targets to develop treatments against this opportunistic human pathogen. In the phylogenetically close and agriculturally important fungus *A. flavus*, the transcription regulatory gene *hbx1*, a homolog of *hbxA*, was found to regulate several aspects of morphological differentiation, including asexual development, as well as the synthesis of several secondary metabolites (Cary et al., 2017). Homologs of *hbxA* have also been identified in other fungi beyond the *Aspergillus* genus, for example, in *Magnaporthe oryzae* and in species of the genus *Fusarium*. In these species, while no connection of the possible role of *hbx1/hbxA* homologs with virulence or secondary metabolism was reported, the studies indicated a role in asexual development (Kim et al., 2009; Liu et al., 2010; Zheng et al., 2012). Our analysis of *A. fumigatus* *hbxA* demonstrated that this gene has indeed a conserved role in the regulation of conidial production. Additionally, other aspects of conidial formation and function were influenced by *hbxA* in this fungus. In the absence of *hbxA*, conidia appear enlarged and present a fast germination rate.

The mechanism of action of *hbxA* on conidiation includes a connection with the central regulatory pathway, *brlA, abaA*, and *wetA* genes (Park & Yu, 2016). The expression of the three genes in this signaling pathway is significantly reduced in the absence of *hbxA*. This decrease in expression could lead to the observed reduction in conidial production in the *hbxA* mutant. In
addition, examination of the effect of *hbx*A on the expression of other regulatory elements upstream of the *brl*A-central regulatory pathway showed that *flu*G, a well-known developmental regulator (Mah & Yu, 2006), is also *hbx*A dependent. In addition, expression of *flb*B and *flb*D was also found to be positively regulated by *hbx*A. FlbB has been previously shown to promote asexual development in *A. fumigatus* (Xiao et al., 2010). FlbD has not been characterized in *A. fumigatus* (Park & Yu, 2016); however, in *A. nidulans*, *flb*D has been shown to also promote conidiation (Garzia et al., 2010). It is possible that HbxA could regulate the expression of these “fluffy” developmental genes directly, which could consequently affect the *brl*A pathway. In addition, since *flb*D expression is dependent on the FlbB/FlbE protein complex (Garzia et al., 2010; Xiao et al., 2010), it is possible that *hbx*A could affect expression of *flb*D indirectly by controlling *flb*B transcription. Additionally, the presence of high concentrations of sugars, particularly sucrose and fructose, resulted in a significant increase in conidial production in the *hbx*A deletion mutant. Currently the mechanism that triggers conidiation under these conditions is unknown.

In *A. fumigatus* and other fungi, development and secondary metabolism are genetically linked (Calvo et al., 2002; Bayram & Braus, 2012; Calvo & Cary, 2015). As in *A. flavus* (Cary et al., 2017), in our study we show that *hbx*A not only regulates development in *A. fumigatus* but also the production of multiple metabolites. Analysis of the *hbx*A-dependent metabolome revealed four different classes of secondary metabolites under the influence of this regulator, among them ergot alkaloids (fumigaclavine) and fumiquinazoline. These compounds present bioactive properties; fumigaclavine has been shown to affect the nervous systems of the host and
induce apoptosis, while fumiquinazoline present cytotoxicity that inhibits neutrophils and aids *A. fumigatus* during infection (Coyle et al., 2007; Gauthier et al., 2012; Li et al., 2013).

Fumigaclavines and fumiquinazolines accumulate in asexual structures and their production is linked to *brlA* expression (Panaccione & Coyle, 2005; Coyle et al., 2007; Lim et al., 2014). It is possible that *hbxA* regulation of the expression of genes involved in the synthesis of these compounds is indirect and mediated, at least in part, by its effect on *brlA*. Fumagillin production was also found to be governed by *hbxA*. Fumagillin prevents blood cell formation and has been found to damage lung tissue during infections (Sin et al., 1997; Guruceaga et al., 2018). In addition, the synthesis of chaetominine, a metabolite that is being tested to combat leukemia cells (Yao et al., 2016), is also controlled by *hbxA*. At the moment, knowledge on how this compound is produced or regulated in *A. fumigatus* is limited. To our knowledge, this is the first report of a gene in *A. fumigatus* that controls productions of chaetominine.

Due to the fact that *hbxA* is important in *A. fumigatus* for morphogenesis and secondary metabolism, we investigated whether this gene is relevant in virulence. To test this possibility, we used a neutropenic mouse infection model. Surprisingly, lack of *hbxA* did not attenuate virulence of this fungus, but enhanced it, resulting in higher mortality rates in the group of mice infected with the deletion strain compared to the group inoculated with the wild-type strain. While production of secondary metabolites was hampered in the mutant and conidial size was larger, these traits did not reduce virulence in the mutant strain. It is possible that the premature germination observed in the *hbxA* mutant could have accelerated and enhanced fungal infection in neutropenic mice compared to those infected with the wild type, resulting in higher mortality
Due to the fact that \textit{hbxA} is important in \textit{A. fumigatus} for morphogenesis and secondary metabolism, we investigated whether this gene is relevant in virulence. To test this possibility, we used two models, the insect \textit{Galleria mellonella} model and also the neutropenic mouse infection model. Surprisingly, lack of \textit{hbxA} did not attenuate virulence of this fungus, but enhanced it in both models, resulting in higher mortality rates in the group of animals infected with the deletion strain compared to the group inoculated with the wild-type strain. While production of secondary metabolites was hampered in the mutant and conidial size was larger, these traits did not reduce virulence in the mutant strain. It is possible that the premature germination observed in the \textit{hbxA} mutant could have accelerated and enhanced fungal infection in insects and neutropenic mice compared to those infected with the wild type, resulting in higher mortality rates.

In conclusion we have established that the homeobox gene \textit{hbxA} is a global regulator of development, secondary metabolism, and virulence in \textit{A. fumigatus}. Specifically, we have shown that \textit{hbxA} is necessary for normal asexual development, governing the expression the fluffy genes \textit{fluG}, \textit{flbB} and \textit{flbD} as well as expression of those genes in the central developmental signaling pathway, \textit{brlA}, \textit{abaA} and \textit{wetA}. The effect of \textit{hbxA} on conidiation appears to be influenced by high concentration of sugars, such as sucrose and fructose, in the environment. With respect to the role of \textit{hbxA} on \textit{A. fumigatus} metabolome, our study revealed that production of several fungal alkaloids, as well as fumagillin and chaetominine, are under its control. We also established that \textit{hbxA} negatively affects pathogenicity, as removal of \textit{hbxA} increases virulence. Future research on \textit{hbxA} may provide further insight into the identification of additional \textit{hbxA}-
dependent genetic elements involved in the regulation of conidiation and synthesis of fungal natural products.

References


