The Transcriptional Regulator Hbx1 Affects the Expression of Thousands of Genes in the Aflatoxin-Producing Fungus Aspergillus flavus

Jeffrey W. Cary,*1 Sarah Entwistle,* Timothy Satterlee,* Brian M. Mack,* Matthew K. Gilbert,* Perng K. Chang,* Leslie Scharfenstein,* Yanbin Yin,* and Ana M. Calvo†1

*Food and Feed Safety Research Unit, USDA/ARS, Southern Regional Research Center, New Orleans, Louisiana and †Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois

ABSTRACT In filamentous fungi, homeobox proteins are conserved transcriptional regulators described to control conidiogenesis and fruiting body formation. Eight homeobox (hbx) genes are found in the genome of the aflatoxin-producing ascomycete, Aspergillus flavus. While loss-of-function of seven of the eight genes had little to no effect on fungal growth and development, disruption of hbx1, resulted in aconidial colonies and lack of sclerotial production. Furthermore, the hbx1 mutant was unable to produce aflatoxins B1 and B2, cyclopiazonic acid and afattoxin. In the present study, hbx1 transcriptome analysis revealed that hbx1 has a broad effect on A. flavus gene expression, and the effect of hbx1 increases overtime, impacting more than five thousand protein-coding genes. Among the affected genes, those in the category of secondary metabolism (SM), followed by that of cellular transport, were the most affected. Specifically, regarding the effect of hbx1 on SM, we found that genes in 44 SM gene clusters where upregulated while 49 were downregulated in the absence of hbx1, including genes in the SM clusters responsible for the synthesis of asparasone, piperazine and aflavarin, all known to be associated with sclerotia. In addition, our study revealed that hbx1 affects the expression of other transcription factor genes involved in development, including the conidiation central regulatory pathway and fib genes.

The opportunistic phytopathogen, Aspergillus flavus, is often found colonizing oil seed crops such as peanut, corn, sorghum, tree nuts and cotton (Robens and Cardwell 2003). Dispersal of this fungus proceeds rapidly in the field through production of asexual spores termed conidia present on specialized structures denominated conidiophores. Once the fungus has colonized the crop it can survive in the field under harsh conditions for several years by forming resistant structures termed sclerotia (Horn et al., 2014). Upon colonization of the plant, A. flavus produces a number of mycotoxins, including the highly carcinogenic family of toxins known as aflatoxins (Bhatnagar et al., 2018). Contaminated crops are often destroyed or significantly reduced in value leading to substantial economic losses in the range of one billion US dollars annually during years of severe aflatoxin outbreaks (Robens & Cardwell 2003, & Wu et al. 2008). In developing nations where legislation is often not in place to regulate the allowable levels of aflatoxins in susceptible crops, consumption of aflatoxin-contaminated food can lead to immunosuppression, liver cancer, and in some cases death (Yard et al. 2013).

Successful control of aflatoxin contamination in crops will depend in part on research efforts directed toward understanding the regulatory mechanisms controlling A. flavus dissemination and survival, aseexual and sexual development as well as production multiple secondary metabolites in many fungal genera (Calvo et al., 2016), including Aspergillus (Kato et al., 2003, Duran et al. 2007, Dhingra et al. 2013, Lind et al. 2015).
In *A. flavus*, loss of VeA results in increased conidiation, absence of sclerotia, and suppression of secondary metabolite production including aflatoxin, aflatem, and cyclopiazonic acid (Duran et al. 2007). In addition to VeA, the arginine methyltransferase RmtA has been shown to be a positive regulator of both aflatoxin production and assexual development (Satterlee et al. 2016). Another example is RfA, a homolog of a putative member of the *Saccharomyces cerevisiae paf1* complex (Warner et al., 2007), that is also required for normal aflatoxin biosynthesis, sclerotial development and conidiation (Lohmar et al. 2016). Other examples are the genes encoding transcription factors *MtfA* (Zhuang et al. 2016) *nsdC*, and *nsdD* (Cary et al. 2012). The global regulator *MtfA* is a negative regulator of conidiation, required for normal maturation of sclerotia, and a positive regulator of aflatoxin production (Zhuang et al. 2016). Both *nsdC* and *nsdD* also demonstrated a role in the regulation of conidiophore development, and are essential for sclerotial formation, as well as influencing production of aflatoxin (Cary et al. 2012).

Recently homeobox domain transcription factor genes were identified in *A. flavus*, and disruption of the homeobox 1 (*hbx1*) gene abolished production of conidia and sclerotia as well as production of several mycotoxins (Cary et al. 2017). The *hbx1* gene was also shown to regulate expression of several development regulators such as *brlA* (Cary et al., 2012), a keystone in the induction of conidiation (Adams et al. 1998). Alongside the effect on developmental regulators, expression of the aflatoxin specific transcription factor *aflA* and the global regulator *veA* were altered in the absence of *hbx1* possibly contributing to the observed decrease in the production of several mycotoxins such as aflatem, cyclopiazonic acid, and aflatoxin (Cary et al., 2017).

Based on the profound effect that *hbx1* has on development and secondary metabolism in *A. flavus*, this gene represents a potential target for new strategies to control aflatoxin contamination of food and feed crops by *A. flavus*. To gain further insight into the regulatory scope of *hbx1* we performed a transcriptome analysis. The impact of *hbx1* on the gene expression profile of *A. flavus* was assessed over three-time points. Several thousand genes were under *hbx1* control indicating that *hbx1* is a global regulator, and its influence increased with time. An elevated number of transcription factors and developmental regulators were shown to be *hbx1*-dependent. Furthermore, a large numbers of secondary metabolite gene clusters are also affected by *hbx1*, among them seven are associated with known metabolites.

**MATERIALS & METHODS**

**Strains used and growth conditions**

*Aspergillus flavus* strains used in this study were the AF70 control, AF70 *Δhbx1* and a genetically complemented *Δhbx1* mutant (designated AF70 *Δhbx1*-COM) as described in Cary et al. (2017). Strains were point inoculated onto double strength 5/2 agar (50 ml V8 juice, 40 g agar, pH 5.2 per liter of medium (Chang et al. 1993) supplemented with 3.0 g ammonium sulfate and 1 mg/ml uracil (termid 2X V8 ASU) and incubated in the light at 30° for 6 days to promote conidiation. Conidia were collected from plates in 0.01% Triton X-100 and stored at 4°. Due to the inability of the *Δhbx1* mutant to conidiate, cultures were maintained at -80° as glycerol stocks containing agar plugs of fungal mycelia.

**Sequence analysis of plant homologs**

Using the amino acid sequence for Hbx1 as query (XP_002380469.1) a BLASTp search was performed to identify possible homologs of Hbx1 in selected plant species. Species and sequences used were *Arachis hypogaea* (AKN10291.1), *Zea mays* (NP_001140916.1), *Gossypium arboretum* (XP_017643272.1) and *Arabidopsis thaliana* (AAA56907.1). A MAFFT multiple sequences alignment (https://mafft.cbrc.jp/alignment/software/) was performed to align the sequences and visualized using BoxShade (https://embnet.vital-it.ch/software/BOX_form.html).

**RNA sequencing study**

**RNA preparation and sequencing** Inoculated approximately 5 × 10⁵ conidia/ml of the AF70 control and the *Δhbx1*-COM mutant into 500 ml peptone minimal salts (PMS; not conducive to aflatoxin production) (Buchanan and Lewis 1984) broth supplemented with 1 mg/ml uracil (PMSU) in 1 liter Ehrlenmeyer baffle flasks. Cultures were incubated at 30° in the dark with shaking at 250 rpm for 24 h. Mycelia of the AF70 *Δhbx1* mutant were scraped from the surface of four 2X V8 ASU top agarose (0.5% agarose, I. Amresco, Solon, OH) plates and placed in 25 ml 2X V8 ASU broth in a 50 ml Sarstedt tube. Equal amounts of mycelia were macerated for 10 sec using a tissue grinder (Tissumizer SDT1810, Tekmar, Cincinnati, OH) then transferred into 500 ml PMSU broth in 1 liter Ehrlenmeyer baffle flask. Incubated at 30° in the dark with shaking at 250 rpm for 24 h. Collected mycelia from cultures of each of the three strains by filtering through sterile miracloth, transferred 0.5g wet weight into 25 ml of PDBU broth in 250 ml Ehrlenmeyer flasks (4 replicates) and incubated statically in the dark for 6 h (point time for initiation of aflatoxin gene expression), 24 and 48 h (approximate time points for initiation of conidia and sclerotia production, respectively). Cultures were filtered through sterile miracloth and the fungal tissue collected, frozen in liquid nitrogen and stored at -80°. The frozen mycelial samples were ground under liquid nitrogen with mortar and pestle until powdered and transferred to a 50ml Sarstedt tube and stored frozen at -80 until ready for RNA extraction. RNA was isolated from 100-200 mg of the frozen ground mycelial samples using the TRI Reagent (Sigma T9424-100ML) and following the standard Direct-zol RNA MiniPrep kit (ZYMO Research, Irvine, CA) protocol using the double washes modification. RNA quality and quantity were determined using the Experion Automated Electrophoresis Station (Bio-Rad). Frozen RNA samples were shipped overnight on dry ice to North Carolina State University’s Genomics Sciences Laboratory for RNA sequencing. RNA libraries were prepared using the Ultra Directional RNA library prep kit from NEB using the manufacturer’s protocol for NEBNext PolyA mRNA magnetic isolation module. Sequencing was carried out by Illumina HiSeq 2500 at 125 bp single end reads.

**RNA data analysis**

**Read mapping** The single-end reads of three strains (Control, *Δhbx1*, *Δhbx1*-COM) each with three replicates at three time points (6 h, 24 h, and 48 h) were separately aligned to the reference genome (Nierman et al., 2015) using HISAT2 (Kim et al., 2015) version 2.0.5. The command used was hisat2 -x reference_genome_index -U fastq_file -s output_file.sam. HISAT2 utilizes Bowtie2 (Langmead & Salzberg 2012) and was run using software version 2.3.

**Read counts** The mapped reads in SAM format were then analyzed using the feature Counts tool from the Subread package (Liao et al., 2013) version 1.6.0. This tool was employed to return a table of read counts for each gene. The command used was featureCounts -a reference_genome. gtf -p -s 2 -o output_file.sam. A bash script was used to combine all the separate read count files into one table.

**Differentially expressed coding genes (DEGs)** The table of read counts was used as input for the R limma package (Ritchie et al., 2015). This package was used to determine DEGs by comparing read counts between two strains: Control vs. *Δhbx1* and Control vs. *Δhbx1*-COM. These comparisons were made at all three-time points: 6 h, 24 h, 48 h.
The replicates of each condition at each time point were combined during this step of the analysis. The RPKM function in the R edgeR package (Robinson et al., 2009) determined the reads per kilobase per million (RPKM) values for all the genes.

Bash and Perl scripts were developed to parse the DEGs and RPKM data. An Excel file was created with the RPKM values for all genes across all conditions. FungiFun2 (Priebe et al., 2014) was used for FunCat term annotation of DEGs from Control vs. \( \Delta hbx1 \) and Control vs. \( \Delta hbx1\text{-COM} \). FungiDB (Basenko et al., 2018) was used for GO term annotation.

Functional annotation and generation of gene lists The metabolic gene clusters (SMGs) were extracted from Ehrlich and Mack (2014). In addition, a list of transcription factors (TFs) were derived from the Fungal Transcription Factor Database (http://ftfd.snu.ac.kr/intro.php) (Park et al. 2008) for \( A.\ flavus \) and mapped to differentially expressed genes in \( A.\ flavus \). Functional annotations of these transcription factors were obtained from NCBI. R (R Core Team 2017) version 3.4.1, specifically the ggplot2 package (Wickham 2009), was used to make statistical figures. The Venn diagrams were made using the R package VennDiagram (Chen & Boutots 2011). Fungal development-related genes from \( A.\ flavus \) species were reviewed in Table S2 from Kriegsheld et al. (2013). FASTA sequences of these genes were used to search against the \( A.\ flavus \) genome to identify developmental genes.

The list of DEGs from the study performed by Dolezal and collaborators (Dolezal et al. 2013) was compared to the \( hbx1\) DEGs to search for potentially \( hbx1\)-dependent virulence genes. Furthermore, we specifically looked for virulence-related secretory genes. The list of possible virulence-related \( hbx1\) genes was compared to the \( A.\ flavus \) secretome-related genes in the FunSecKB2 database (Meinken et al., 2014). For higher confidence in results only the list of “curated secreted” and “highly likely secreted” genes in FunSecKB2 were used.

Weighted gene network co-expression analysis The gene co-expression network was made using WGCNA (Weighted Gene Network Co-expression Analysis) with a signed network, the biweight mid-correlation method, and a soft-thresholding power of 9. Variance stabilized counts from DESeq2 were used as input to WGCNA (Langfelder and Horvath 2008). Genes with missing values or zero variance were filtered out using the goodSamplesGenes function within the WGCNA package. Visualization of gene networks using wild-type \( A.\ flavus \) data were shown using Cytoscape v3.6.0 with the Edge-weighted Spring Embedded layout with minor manual adjustment. Relative edge weight values were calculated for the entire module containing \( hbx\) and First Neighbor nodes were selected for additional analysis.

Data availability Table S1 contains calculated expression values of sequenced RNA samples along with corresponding p-values. Table S2 contains a selected list of fungal developmental regulators that are shown to be \( hbx1\)-dependent. Table S3 is a subset of Table S1 that shows all known transcription factors in \( A.\ flavus \) and their corresponding expression pattern in regard to presence or absence of \( hbx1\). The data are publicly available at NCBI’s SRA repository with the SRA Accession #: PRJNA494425. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7304252.

RESULTS

\( hbx1 \) is not conserved in plants species

To determine if the \( hbx1 \) product is conserved among plants and thus a possible viable target for control of \( A.\ flavus \), a BLASTp search was performed to identify potential homologs. The best BLASTp hit for \( Hbx1 \) from \( Arachis hypogaea \), \( Zea mays \), \( Gossypium arboreum \), and \( Arabidopsis thaliana \) were chosen for comparison to the \( A.\ flavus \) protein. Among these hits the query coverage of the results was very low (9–18%) localizing only around the homeobox domain located in \( Hbx1 \). Amino acid sequences of all species were then run through a MAFFT multiple sequence alignment. The results of the alignment are visualized in Figure 1. This result, together with the low percentage values, indicates that \( Hbx1 \) from \( Aspergillus \) is not conserved in these common plant hosts.

\( hbx1 \) is a global genetic regulator in \( A.\ flavus \)

Across the time points assessed, absence of \( hbx1 \) caused a significant change in expression levels in more than 5000 genes in the \( A.\ flavus \) genome. Nearly 2000 genes were downregulated at each time point. In addition, while at the 6 h time point only 980 genes presented an increase in their expression, that number approximately doubled at the later time points (Figure 2, Table 1). Although a similar total amount of genes showed altered expression at each time point, there were not always the same DEGs at all three-time points. Only 350 genes of the entire genome were consistently upregulated by the loss of \( hbx1 \), while 507 genes experienced a significant decrease in expression at all three time points (Figure 3).

\( hbx1 \) is indispensable for normal secondary metabolism in \( A.\ flavus \)

To elucidate the regulatory scope of \( hbx1 \) in \( A.\ flavus \), a series of functional enrichment analyses were performed with the transcriptome data. Using the FungiFun2 platform we performed a Gene Ontology search using FunCat terms (Figure 4) (Priebe et al. 2014). The analysis revealed multiple enriched categories, with the largest one being related to metabolism, followed by cellular transport and cell rescue (the former particularly at 48 h) (Figure 4). Within this division of categories, metabolic involved in secondary metabolism were the largest group affected by loss of \( hbx1 \). At all-time points, most of the DEGs associated with secondary metabolism in the absence of \( hbx1 \). GO terms were also used for functional analysis from FungiDB and are represented in Figure S2. This data also supports the pattern that secondary metabolism is the largest \( hbx1\)-affected category.

In a previous study we discovered that \( hbx1 \) is a positive regulator of aflatoxin, aflatrem, and cyclopiazonic acid biosynthesis (Cary et al. 2017). The current transcriptome analysis provides further insight into \( hbx1 \) regulation of biosynthetic gene clusters of those mycotoxins (Figure 5). In the aflatoxin cluster the majority of the genes are suppressed in the mutant strain. Previously mRNA transcripts from the aflatrem clusters were detected at approximately 48 h in the wild-type (Nicholson et al. 2009), coinciding with our observations (Figure 5), however such an increase was not observed in the \( hbx1 \) deletion mutant. In addition, all the genes in the cyclopiazonic acid genes cluster were downregulated in the absence of \( hbx1 \) (Figure 5).

Other gene clusters involved in the production of secondary metabolites known to be associated with sclerotial development were also affected, particularly at the last time point, such as the asparasone, piperazine, and allavarin gene clusters (Figure 6). In addition, other secondary metabolite gene clusters in \( A.\ flavus \) without a described associated product (orphan clusters) were shown to be regulated by \( hbx1 \) (Figure S3). Other clusters were also affected but to a lesser extent (Table S1).

\( hbx1 \) is a master regulator of developmental regulatory genes and other transcription factors in \( A.\ flavus \)

The \( hbx1 \) gene is necessary for normal conidiation and sclerotial development in \( A.\ flavus \) (Cary et al., 2017), and it was shown to affect...
### Figure 1 - Multiple sequence alignment of *Aspergillus flavus* Hbx1 and possible closest predicted proteins in selected plant species.

BLASTp search was carried out to find possible homologs in selected plant species and with the best hits. A MAFFT Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/mafft/) was performed to show homology of amino acid sequences.

| Protein   | Amino Acid Sequence                                                                 |
|-----------|-------------------------------------------------------------------------------------|
| *A. flavus* | AYPAGAVRIVYTHYDEWNPVTQIMDYEE--YAENL--SRPLTK                                         |
| *A. hypogaee* | LPVQFIDQGQSGQGQPENSNPDVEETDKFQKQSSQDTPMSDDRTKMPHNSR                              |
| *A. thaliana* | TKOOQATIANSKDSTLGZQVLRQNL--LDKQFQVFFFFQNNRTRKLKOTVE                              |
| *G. arboreum* | 67 DEYVEKELSFRERKLETRKNKHALAEKG---LDFKQFAVQWQOFDRKHKKLKLEE                      |
| *Z. mays*    | 122 --FALKQAHDAAIHLKCCHLE--                                                   |
| *A. flavus* | 216 SKHHKTDASEARETAFSLQRALHAAVAREHYSDEGQQPATIHEGSQPPTTYSORMNN                  |
| *A. hypogaee* | 190 --IQEL--                                                                |
| *A. thaliana* | 196 --IQEL--                                                                |
| *G. arboreum* | 185 --IQEL--                                                                |
| *Z. mays*    | 143 --IQEL--                                                                |
| *A. flavus* | 276 HGDSRAQQSSSTTFSEWNEAKETAMSWASQSPHEGYSARESALTVELEDGSQHNVQH                  |
| *A. hypogaee* | 180 --IQEL--                                                                |
| *A. thaliana* | 190 --IQEL--                                                                |
| *G. arboreum* | 189 --IQEL--                                                                |
| *Z. mays*    | 147 --IQEL--                                                                |
| *A. flavus* | 336 SDTLQFHSQEESNVPQGQTPKFGYHSSDHAEYSAQAETLQHPLCSSRLGSQSSDDLA                 |
| *A. hypogaee* | 190 --IQEL--                                                                |
| *A. thaliana* | 200 --IQEL--                                                                |
| *G. arboreum* | 189 --IQEL--                                                                |
| *Z. mays*    | 147 --IQEL--                                                                |
| *A. flavus* | 396 DSLEGICHIAGLPIRTDSSWEAKELDARKKRPAAAIGTSSSSMLASASMHS                       |
| *A. hypogaee* | 199 --IQEL--                                                                |
| *A. thaliana* | 219 --IQEL--                                                                |
| *G. arboreum* | 189 --IQEL--                                                                |
| *Z. mays*    | 147 --IQEL--                                                                |
| *A. flavus* | 456 PTTTLPSYADGRVQKSAQCLNSYAVGKASSQASPFLNLSSAEAGALGTSPERM                    |
| *A. hypogaee* | 199 --IQEL--                                                                |
| *A. thaliana* | 219 --IQEL--                                                                |
| *G. arboreum* | 189 --IQEL--                                                                |
| *Z. mays*    | 147 --IQEL--                                                                |
the expression of key developmental regulators, such as brlA, the master regulator of the asexual development (conidia) in Aspergillus. In addition, hbx1 also affected the expression of veA, which regulates multiple aspects of fungal development as well as influencing production of secondary metabolites (Calvo et al., 2016). In our current study, a list of selected A. flavus developmental regulatory genes (experimentally characterized in A. flavus and/or in other fungi), was applied to our data analysis to better understand the mechanism of action of hbx1 in A. flavus regulatory networks (Table S2). Our results revealed that several additional developmental transcription factors were also hbx1-dependent, such as the terminal gene in the central conidiation pathway, wetA (Wu et al., 2017) the fluffy genes flbA, flbC, flbD, and fluG (Purschwitz et al., 2008, Garzia et al., 2010, & Kwon et al., 2010, Chang et al. 2012), as well as the mating gene MAT1-1 (Ramirez-Prado et al., 2008).

Our hbx1 data analysis also extended to other A. flavus transcription factors. A list of A. flavus transcription factors were obtained from the Fungal Transcription Factor Database and used to search for DEGs. Annotations of the transcription factors were derived from NCBI. From these results, of the over six hundred transcription factors identified in this fungus, almost four hundred of them are influenced by hbx1 (Table S3). A subset of well-characterized genes from that list are also represented in Table 2.

Prediction of hbx1-dependent genes possibly involved in virulence

Previously Dolezal et al. (2013) analyzed the transcriptome of A. flavus during the infection of maize kernels. This study compared the gene expression profile of A. flavus during infection of viable kernels to that of non-viable. The data from this study was assessed into two groups, genes that were upregulated during infection of viable kernels and those downregulated. We compared these groups of genes to the hbx1-dependent transcriptome from our study. In total, 1125, 1451, and 1672 genes were differentially expressed in both studies at the 6, 24, and 48 h, respectively (Table S4). Table S4 also shows genes that exhibited the same trend over three-time points as well as genes that presented an expression pattern opposite that depicted in the Dolezal et al. virulence study compared to hbx1-dependent DEGs. Among them, 75 genes were found upregulated during infection of viable seeds but downregulated in the hbx1 mutant. Conversely 20 genes were found to present lower expression levels during infection while having increased expression in the hbx1 mutant.

In the aforementioned study, genes encoding transcription factors, involved in secondary metabolism, as well as the fungal secretome were upregulated and predicted to be potential virulence factors (Dolezal et al., 2013). We further compared differentially expressed secretory genes from that study to the hbx1 transcriptome study. From this selected data set, 164, 196, and 235 secretory genes where differentially expressed in the absence of hbx1 at 6, 24, and 48 h respectively (Table S5).

Identification and visualization of gene regulatory networks correlated with hbx1 expression and knockout

Expression values (variant stabilized read counts) from the RNA-seq data were subjected to WGCNA to identify networks of genes that are

---

**Table 1** - Percentage of A. flavus hbx1-dependent DEGs at each time point

|                      | 6 h   | 24 h   | 48 h   | All 3 time points |
|----------------------|-------|--------|--------|-------------------|
| **Up regulated**     |       |        |        |                   |
| Percent of total DEGs| 16.17%| 28.76% | 36.42% | 5.78%             |
| Percent of total genome| 7.27%| 12.93% | 16.37% | 2.60%             |
| **Down regulated**   |       |        |        |                   |
| Percent of total DEGs| 30.45%| 32.90% | 31.14% | 8.37%             |
| Percent of total genome| 13.68%| 14.79% | 13.99% | 3.76%             |
co-expressed with wild-type hbx1, and therefore potentially impacted by the hbx1 deletion. The homeobox transcription factor showed highest co-expression correlation with a hypothetical protein (AFLA_061410), (Figure 7), which was significantly upregulated in the hbx1 mutant (~4 fold), and shared a high sequence similarity to a putative DNA methyltransferase. The putative hypothetical proteins down-regulated in the hbx1 mutant (AFLA_013180 and AFLA_066950) share a sequence similarity with an S-adenosyl-methionine dependent methyltransferase and a flavin adenine dinucleotide-binding proteins, respectively. These putative methyltransferases highly associated with hbx1 expression (and affected in the hbx1 mutant) are excellent candidate regulatory genes for near-downstream regulation by hbx1.

DISCUSSION

Previous studies showed that development is genetically associated with secondary metabolism, (Calvo et al. 2002; Calvo and Cary 2015). Recent
published work also demonstrated that the \( hbx1 \) gene is one of those genetic links that is required for normal conidiation, sclerotial formation as well as secondary metabolism in the aflatoxin-producer and agriculturally important fungus \( A.\ flavus \) (Cary et al., 2017). In the current study we showed that while homeobox domains similar to that present in the \( Hbx1 \) protein are found throughout various phyla, the rest of the \( A.\ flavus \) \( Hbx1 \) amino acid sequence is not conserved \( in\ planta \). This suggests that \( hbx1 \) could be a good target for strategies to control \( A.\ flavus \) infection that would not result in any off-target effects in agriculturally important crops susceptible to this opportunistic pathogen.

To gain insight into \( A.\ flavus \ hbx1 \) mechanism of action we investigated the extent of its regulatory scope performing a transcriptome study using RNA sequencing. Our analyses revealed a broad effect of \( hbx1 \) on the genome; in the absence of \( hbx1 \) more than 20% of the \( A.\ flavus \) genome presents changes in expression. In addition, we observed that the number of genes governed by \( hbx1 \) increases with time in the fungal culture. Based on our results, \( Hbx1 \) is a dynamic transcriptional regulator that, while it controls the expression of 857 genes at all time points assessed, a greater number of \( hbx1 \)-dependent genes were affected at specific time points analyzed.

Our functional enrichment analysis indicated that while categories involved in cell rescue and defense, development and cellular transport were shown to be under the control of \( hbx1 \), the largest group of \( hbx1 \)-dependent genes corresponds to the category of metabolism. Twelve secondary metabolite gene clusters, out of 56 clusters identified in \( A.\ flavus \), including the kojic acid cluster (Ehrlich and Mack, 2014 & Ammar et al. 2017), were under \( hbx1 \) control. Of these 12 clusters, five of them were not associated with a known metabolic product, however the remaining clusters have already been characterized. Four of these clusters have been shown to be involved in the synthesis of potent mycotoxins, aflatoxin, aflatrem (split into two clusters), and cyclopiazonic acid (Yu et al., 2004, Chang et al., 2009, Nicholson et al., 2009). In addition, genes in the clusters involved in the production of asparasone, piperazine, and aflavarin were also shown to be suppressed in the absence of \( hbx1 \). These three metabolites are associated with sclerotial development (Calvo and Cary 2015). Both asparasone and aflavarin are specifically found within these structures (Cary et al. 2014, & Cary et al. 2015), and genes located in the piperazine cluster have been shown to

**Figure 5** Heat map of RPKM values of genes on a log scale found in secondary metabolite gene clusters of aflatrem, aflatoxin, and cyclopiazonic acid (CPA). The RPKM value of each gene was calculated by averaging all the RPKM values of all replicates corresponding to that treatment at three different time points: 6 h, 24 h, and 48 h.

**Figure 6** Heat map of RPKM values of genes on a log scale found in sclerotia related secondary metabolite gene clusters of asparasone, piperazine, and aflavarin. The RPKM value of each gene was calculated by averaging all the RPKM values of all replicates corresponding to that treatment at three different time points: 6 h, 24 h, and 48 h.
Table 2 Annotated hbx1-dependent transcription factors. A list of A. flavus transcription factors was obtained from the Fungal Transcription Factor Database and compared to the list of hbx1 dependent DEGs. Annotations were retrieved from NCBI (full list is shown in Table S3). Expression values are those between the wild type (WT) and Δhbx1 at all time points assayed.

| Gene     | AFLA ID   | Description                        | 6 h   | 24 h    | 48 h    |
|----------|-----------|------------------------------------|-------|---------|---------|
| abp2     | AFLA_081210 | ARS binding protein Abp2, putative | -0.02695 | -1.05237 | -0.83165 |
| aRO      | AFLA_139220 | aRO/ omA/ dmtA/ O-methyltransferase B | -0.81939 | -0.97277 | 2.94881 |
| aRI      | AFLA_139210 | aRI/ omA/ omA/ O-methyltransferase A | -9.18684 | -11.8992 | -2.50201 |
| amdA     | AFLA_048870 | C2H2 transcription factor (AmdA), putative | -0.27305 | 0.369593 | -0.62522 |
| amdR     | AFLA_028560 | C6 transcription factor (AmdR), putative | -1.0392 | -0.75555 | -0.63691 |
| amdx     | AFLA_002290 | C2H2 transcription factor (Amdx), putative | -1.02402 | -1.62432 | -0.79849 |
| areA     | AFLA_049780 | GATA transcriptional activator AreA | 0.718895 | 1.399027 | 1.196918 |
| areB     | AFLA_136100 | GATA transcription factor (AreB), putative | 0.136046 | -1.10235 | 0.431972 |
| azf1     | AFLA_054800 | C2H2 transcription factor (Azf1), putative | -0.59027 | -3.74734 | -0.30994 |
| brlA     | AFLA_082850 | C2H2 transcription factor (BrlA), putative | 0.391684 | -2.19702 | -2.37438 |
| cnbB     | AFLA_051900 | zinc knuckle transcription factor (CnbB), putative | 3.893041 | 0.290236 | 2.115113 |
| creA     | AFLA_134680 | C2H2 transcription factor (CreA), putative | -0.50862 | -0.56638 | -1.0953 |
| ctf1B    | AFLA_012010 | C6 transcription factor (Ctf1B), putative | -0.04607 | 0.620485 | 1.55645 |
| erg2     | AFLA_069460 | C2H2 transcription factor (Erg2), putative | -1.55121 | -0.57147 | -1.05583 |
| fibC     | AFLA_137320 | C2H2 transcription factor (FibC), putative | -0.72449 | -2.36931 | -0.71241 |
| fibD     | AFLA_080170 | MYB family conidiophore development protein FibD, putative | -1.18991 | -2.3671 | -3.69073 |
| hpa3     | AFLA_131640 | HLH transcription factor (Hpa3), putative | -0.97532 | -1.5551 | -2.37946 |
| MATα-1   | AFLA_103210 | mating-type protein MAT alpha 1 | 0.301373 | 0.231333 | 3.258805 |
| mbf1     | AFLA_086430 | coactivator bridging factor 1 (Mbf1), putative | 0.637772 | 1.803474 | 0.237383 |
| nirA     | AFLA_093040 | C6 transcription factor (NirA), putative | -0.36009 | -1.05781 | 0.124633 |
| nosA     | AFLA_025720 | C6 transcription factor NosA | -0.57861 | -6.05653 | 3.24851 |
| nsdD     | AFLA_020210 | sexual development transcription factor NsdD | -0.29566 | -1.18101 | -0.97544 |
| pcaG     | AFLA_012100 | NDT80_PhoG domain protein PcaG | -1.3881 | -1.5446 | -0.20405 |
| regA     | AFLA_073870 | C6 transcription factor RegA, putative | -0.25045 | 0.20309 | 1.643673 |
| rfcC     | AFLA_044060 | C2H2 transcription factor (RfcC), putative | -0.42645 | -1.10049 | 0.597632 |
| rpn4     | AFLA_017640 | C2H2 transcription factor (Rpn4), putative | 0.491655 | 0.477596 | 3.176475 |
| seb1     | AFLA_110650 | C2H2 transcription factor (Seb1), putative | -0.18746 | -0.02585 | 1.021753 |
| sep1     | AFLA_048110 | forkhead transcription factor (Sep1), putative | -0.65362 | -1.43862 | -0.5192 |
| smt2     | AFLA_029990 | PHD finger and BAH domain protein (Smt2), putative | 0.07165 | -0.12486 | 1.163666 |
| srrA/Skn7| AFLA_034540 | stress response transcription factor SrrA/Skn7, putative | -0.77849 | -1.28158 | 0.119597 |
| ssb3     | AFLA_093820 | ssDNA binding protein Ssb3, putative | -0.00201 | 0.813483 | -1.02252 |
| steA     | AFLA_048650 | sexual development transcription factor SteA | -0.53006 | -1.08547 | -0.48395 |
| stuA     | AFLA_046990 | APSES transcription factor StuA | -1.62823 | -2.5412 | -1.79524 |
| swi5     | AFLA_031400 | C2H2 transcription factor Swi5 | -0.54244 | -1.11596 | -0.0182 |

Fungi concentrate secondary metabolites in reproductive structures for defense against herbivores and insects (Wicklow 1988, Gloer 1995, Gloer 1997, & Gloer 2007). Horn et al. (2009, 2014) reported ascospore-bearing ascocarps embedded within sclerotia of A. flavus and A. parasiticus. In these aflatoxin producers, sclerotia play an important role as resting structures capable of surviving environmental extremes remaining viable after several years in the crop fields (Coley-Smith and Cooke 1971), and the hbx1-dependent secondary metabolites present in them contribute to their survival against biotic stress and possibly abiotic stresses. Since deletion of hbx1 results in abolishment of sclerotia in the fungus, it is possible that the effect of hbx1 on the expression of some of these secondary metabolite gene clusters specifically associated with a particular morphological structure could be indirect, by affecting developmental regulators that are repressed in the absence of hbx1. Whether the effect on these clusters is direct or indirect, these studies indicate an important role of hbx1 in A. flavus survival, promoting the formation of resistant structures and a chemical arsenal critical for defense against microbes, predators and other environmental insults.

The hbx1 gene is also necessary for conidiation. Our transcriptome analysis also indicated that the brlA central regulatory pathway is under hbx1 control, not only affecting brlA, but also wetA, a developmental regulator conserved in Aspergillus species (Wu et al., 2018). Furthermore, the aconidial phenotype of Δhbx1 resemble that of the fluffy mutants described in A. nidulans that revealed the flb regulatory pathway (reviewed by Ruge-Herreros et al. 2011; Krijgsheld et al. 2013). Indeed, A. flavus flbA, flbC, flbD, and flbE homologs (Chang et al., 2012) are down regulated in the absence of hbx1 while in the same strain at the early time point fluG had a significant increase of expression. This indicates that hbx1 is a regulator of these conidiophore biogenesis genes, and expression of some of these genes over time is significantly different from that observed in the control strain.

It is possible that Hbx1 might not bind directly to the promoters of the central regulatory pathway genes but affects their expression by controlling expression of genes upstream in the regulatory hierarchy. Examples of these might be genes like ppoC and stuA. Both of these genes have been shown to affect conidiophore development via brlA (Dutton et al., 1997, Tsitsigiannis et al., 2004, & Sheppard et al., 2005). In addition, other developmental genes were also under the influence of hbx1, for instance, the spore hydrophin gene rodA (Carrión et al., 2013) and also the nosA gene, encoding a putative Zn(II)2Cys6 transcription factor previously described in several Aspergillus species (Vienken and Fischer 2006, Soukop et al., 2012, Zhao et al., 2017). In A. nidulans, nosA is necessary for cleistothecial
primordium maturation (Vienken and Fischer 2006), and its homolog in A. flavus has been reported to be required for sclerotial production (Zhao et al., 2017). It is likely that the reduction in the expression of nosA in the A. flavus hbx1 mutant could contribute to prevent sclerotial formation in this strain.

Since hbx1 has a broad effect on A. flavus development and metabolism, we also investigated possible connections between hbx1 and virulence during corn infection based on the previous report by Dolezal et al. (2013). In our study, the DEGs identified from the hbx1-dependent transcriptome were compared to those identified by Dolezal et al. (2013) in viable and non-viable infected corn kernels. This allowed us to predict genes possibly involved in virulence that are controlled by hbx1. Genes identified as upregulated in the corn infection study but suppressed in the hbx1 transcriptome study could potentially be involved in virulence. Approximately 300 DEGs were identified at all time points that fit this description. Among them is the pes1 gene (AFLA_069330), that in Aspergillus fumigatus was found indispensable for virulence in the Galleria mellonella model (Reeves et al., 2006). Other genes in this group were shown to affect spore germination and secondary metabolism, such as sfk1. In Penicillium roqueforti, silencing of sfk1 alters conidial germination and prevents production of roquefortine C, andrastin A, and mycophenolic acid (Torrent et al., 2017). Out of the mentioned group of 300 DEGs, 75 were consistently suppressed in the hbx1 mutant (Table S4). In this subgroup, beyond genes located in the aflatoxin gene cluster, most of these genes have not been investigated, and could be potential genes of interest in future studies to identify A. flavus virulence factors.

Secreted proteins such as hydrolytic enzymes are essential for successful infection of the host by the fungus (Lo Presti et al., 2015; de Jonge et al., 2011 & Kale and Tyler 2011). With this in mind, we focused on analyzing components of the secretome regulated by hbx1, specifically those genes that may play a role in virulence. FunSeqKB2 analysis revealed that among the genes in the Dolezal et al. (2013) study, approximately 50 secretome-related genes were upregulated during infection of viable seeds, while those same genes were downregulated in our transcriptome study of the hbx1 deletion mutant, at least at one time point, suggesting that this set of hbx1-dependent genes could be potentially involved in virulence, for example genes encoding proteases (i.e. AFLA_057670), amylases (i.e., AFLA_123170) and other hydrolases (i.e., AFLA_065010, AFLA_088610, and AFLA_125970).

Weighted gene co-expression network analysis has been used to identify novel gene interactions by determining patterns of co-expression among several biological samples, which infers a functional relationship between genes. This process has been used to analyze RNA-seq data from Aspergillus species (Baltussen et al. 2018, Korani et al. 2018), and functional studies have demonstrated the validity of WGCNA (Calabrese et al., 2017, Wang et al. 2017). Here we identify the network of co-expressed genes using the isogenic control strain and identified several genes that are both significantly co-expressed with hbx1 and show altered expression patterns in the hbx1 knockout mutant. Three genes of particular interest that demonstrated altered regulation and relatively high correlation values are annotated as hypothetical proteins (AFLA_013180, AFLA_061410, and AFLA_066950). The impact of these hypothetical genes on A. flavus biology will be the focus of future studies of hbx1-dependent gene regulation.

We demonstrated that the expression of thousands of genes is affected by hbx1. In addition, we showed that hbx1-dependent regulation in A. flavus is dynamic in a time-dependent manner. The hbx1 gene is required for the production of structures needed for dissemination and survival of A. flavus and the production of detrimental secondary metabolites. This, together with the fact that Hx1 is not conserved in other phyla suggest that this global regulator could be a target to develop novel methodologies to control the adverse health and economic impacts due to infection and aflatoxin contamination of many important crops by A. flavus.

ACKNOWLEDGEMENTS
This work was supported by USDA grant 58-6435-4-015 and the Department of Biological Sciences at Northern Illinois University. SE and YY are supported by NSF (DBI-1652164) and NIH (1R15GM114706) grants.

LITERATURE CITED
Adams T., M. Boylan, and W. Timberlake, 1998 BrlA is necessary and sufficient to direct conidiophore development in Aspergillus nidulans. Cell. 54: 353–362. pmid:3293800
Ammar, H. A., A. Y. Srour, S. M. Ezzat, and A. M. Hoseny, 2017 Identification and characterization of genes involved in kojic
acid biosynthesis in *Aspergillus flavus*. Ann. Microbiol. 67: 691–702. https://doi.org/10.1007/s13213-017-1297-8

Baltussen, T. J. H., J. P. M. Coolen, J. Zoll, P. E. Verweij, and W. J. G. Melchers, 2018 Gene co-expression analysis identifies gene clusters associated with isotropic and polarized growth in *Aspergillus fumigatus* conidia. Fungal Genet. Biol. 116: 62–72. https://doi.org/10.1016/j.fgb.2018.04.013

Basenko, E., J. Pulman, A. Shanmuga, O. Harb, K. Crouch et al., 2018 FungiDB: an integrated bioinformatic resource for fungi and oomycetes. J. Fungi (Basel) 4: 39. https://doi.org/10.3390/jof4010039

Bhatnagar, D., K. Rajasekaran, M. Gilbert, J. W. Cary, and N. Magan, 2012 Deletion of the *flaG* cluster in *Aspergillus nidulans* reduces conidiation and promotes production of sclerotia but does not abolish aflatoxin biosynthesis. Appl. Environ. Microbiol. 78: 7557–7563. https://doi.org/10.1128/AEM.01241-12

Chen, H., and P. C. Bouttros, 2011 VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics 12: 35. https://doi.org/10.1186/1471-2105-12-35

Coley-Smith, J. R., and R. C. Cooke, 1971 Survival and germination of fungal sclerotia. Annu. Rev. Phytopathol. 9: 65–92. https://doi.org/10.1146/annurev.py.09.090171.000433

Dhingra, S., A. L. Lind, H. Lin, Y. Tang, A. Rokas et al., 2013 The fumagillin gene cluster, an example of hundreds of genes under veA control in *Aspergillus fumigatus*. PLoS One 8: e77147. https://doi.org/10.1371/journal.pone.0077147

Dolezel, A. L., G. R. Obrian, D. M. Nielsen, C. P. Woloshuk, S. R. Boston et al., 2013 Localization, morphology and transcriptional profile of *Aspergillus flavus* during seed colonization. Mol. Plant Pathol. 14: 898–909. https://doi.org/10.1111/mpp.12056

Duran, R. M., J. W. Cary, and A. M. Calvo, 2007 Production of cyclopiazonic acid, aflattrem, and aflatoxin by *Aspergillus flavus* is regulated by veA, a gene necessary for sclerotial formation. Appl. Microbiol. Biotechnol. 73: 1158–1168. https://doi.org/10.1007/s00253-006-0581-5

Dutton, J. R., S. Johns, and B. L. Miller, 1997 StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J. 16: 5710–5721. https://doi.org/10.1093/emboj/16.18.5710

Ehrlich, K., and B. Mack, 2014 Comparison of expression of secondary metabolism biosynthesis cluster genes in *Aspergillus flavus*, *A. parasiticus*, and *A. oryzae*. Toxins (Basel) 6: 1916–1928. https://doi.org/10.3390/toxins6061916

Forseth, R. S., A. Amaiké, D. Schwenk, K. J. Aafedt, D. Hoffmeister et al., 2012 Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. Angew. Chem. Int. Ed. 52: 1590–1594. https://doi.org/10.1002/anie.201207456

Garzia, A., O. Extebeste, E. Herrero-García, U. Ugale, and E. A. Espeso, 2010 The concerted action of bZip and cMyb transcription factors FbB and FbD induces brlA expression and assexual development in *Aspergillus nidulans*. Mol. Microbiol. 75: 1314–1324. https://doi.org/10.1111/j.1365-2958.2010.07063.x

Gloer, J. B., 1995 Antisense natural products from fungal sclerotia. Acc. Chem. Res. 28: 343–350. https://doi.org/10.1021/ar00056a004

Gloer, J. B., 1997, pp. 249–268 in “Applications of fungal ecology in the search for new bioactive natural products,” in The Mycota, Ed. 1st, edited by Wicklow, D. T., and B. Soderstrom. Springer, Berlin.

Gloer, J. B., 2007 “Applications of fungal ecology in the search for new bioactive natural products,” in The Mycota, Vol. IV, Ed. 2nd, edited by Kubicek, C. P., and I. S. Druzhinina. Springer-Verlag, New York, NY.

Horn, B. W., G. Geromy, M. Carbone, and I. Carbone, 2009 Sexual reproduction in *Aspergillus flavus*. Mycologia 101: 423–429. https://doi.org/10.3852/09-011

Horn, B. W., R. B. Sorensen, M. C. Lamb, V. S. Sobolev, R. A. Olarte et al., 2014 Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. Phytopathology 104: 75–85. https://doi.org/10.1094/PHYTO-05-13-0129-R

de Jonge, R. M. D. Bolton, and B. P. Thomma, 2011 How filamentous pathogens co-opt plants: The ins and outs of fungal effectors. Curr. Opin. Plant Biol. 14: 400–406. https://doi.org/10.1016/j.pbi.2011.03.005

Kale, S. D., and B. M. Tyler, 2011 Entry of oomycete and fungal effectors into plant and animal host cells. Cell. Microbiol. 13: 1839–1848. https://doi.org/10.1111/j.1462-5822.2011.01659.x

Kato, N., W. Brooks, and A. M. Calvo, 2003 The expression of sterigmatoctysin and penicillin genes in *Aspergillus nidulans* is controlled by veA, a gene required for sexual development. Eukaryot. Cell 2: 1178–1186. https://doi.org/10.1128/EC.2.6.1178-1186.2003

Kim, D., B. Langmead, and S. L. Salzberg, 2015 HISAT: A fast spliced aligner with low memory requirements. Nat. Methods 12: 357–360. https://doi.org/10.1038/nmeth.3317
Yard, E. E., J. H. Daniel, L. S. Lewis, M. E. Rybak, E. M. Paliakov et al., 2013 Human aflatoxin exposure in Kenya, 2007: A cross-sectional study. Food Additives & Contaminants: Part A 30: 1322–1331. https://doi.org/10.1080/19440049.2013.789558

Yu, J., P. Chang, K. C. Ehrlich, J. W. Cary, D. Bhatnagar et al., 2004 Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70: 1253–1262. https://doi.org/10.1128/AEM.70.3.1253-1262.2004

Zhao, X., J.E. Spraker, J.W. Bok, T. Velk, Z.M. He, and N.P. Keller. 2017 A cellular fusion cascade regulated by LaeA is required for sclerotial development in Aspergillus flavus. Front Microbiol. Oct 5:1925. eCollection 2017. https://doi.org/10.3389/fmicb.2017.01925

Zhuang, Z., J. Lohmar, T. Satterlee, J. W. Cary, and A. M. Calvo, 2016 The master transcription factor mtaA governs aflatoxin production, morphological development and pathogenicity in the fungus Aspergillus flavus. Toxins (Basel) 8: 29. https://doi.org/10.3390/toxins8010029

Communicating editor: A. Rokas