Characterization of Natural Antisense Transcript, Sclerotia Development and Secondary Metabolism by Strand-Specific RNA Sequencing of Aspergillus flavus

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Abstract

Aspergillus flavus has received much attention owing to its severe impact on agriculture and fermented products induced by aflatoxin. Sclerotia morphogenesis is an important process related to A. flavus reproduction and aflatoxin biosynthesis. In order to obtain an extensive transcriptome profile of A. flavus and provide a comprehensive understanding of these physiological processes, the isolated mRNA of A. flavus CA43 cultures was subjected to high-throughput strand-specific RNA sequencing (ssRNA-seq). Our ssRNA-seq data profiled widespread transcription across the A. flavus genome, quantified vast transcripts (73% of total genes) and annotated precise transcript structures, including untranslated regions, upstream open reading frames (ORFs), alternative splicing variants and novel transcripts. We propose natural antisense transcripts in A. flavus might regulate gene expression mainly on the post-transcriptional level. This regulation might be relevant to tune biological processes such as aflatoxin biosynthesis and sclerotia development. Gene Ontology annotation of differentially expressed genes between the mycelia and sclerotia cultures indicated sclerotia development was related closely to A. flavus reproduction. Additionally, we have established the transcriptional profile of aflatoxin biosynthesis and its regulation model. We identified potential genes linking sclerotia development and aflatoxin biosynthesis. These genes could be used as targets for controlled regulation of aflatoxigenic strains of A. flavus.

Citation: Wu X, Zhou B, Yin C, Guo Y, Lin Y, et al. (2014) Characterization of Natural Antisense Transcript, Sclerotia Development and Secondary Metabolism by Strand-Specific RNA Sequencing of Aspergillus flavus. PLoS ONE 9(5): e97814. doi:10.1371/journal.pone.0097814

Editor: Juan Mata, University of Cambridge, United Kingdom

Received February 5, 2014; Accepted April 23, 2014; Published May 21, 2014

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Funding: The authors wish to acknowledge the financial support by National Natural Science Foundation of China (Project 31100026), the 863 (Hi-tech research and development program of China) program (contract NO. 2012AA022108), Guangdong Provincial Department of Science and Technology Research Project (NO. 2012B010900028 and 2012A080800013). Part of this work was supported by Guangdong Provincial Natural Science Foundation of China (No. S2011040003020) and the Fundamental Research Funds for the Central Universities (NO. 2012ZM0080). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Aspergillus flavus is a ubiquitous pathogenic fungus that infects plants and animals. Recently, studies of A. flavus gained tremendous attention owing to its health impact on agricultural commodities and related fermented products induced by aflatoxin contamination. Initially, studies using A. flavus as a model organism have been focused mainly on the aflatoxin biosynthesis pathway and on mechanisms of regulation of aflatoxin formation [1–5]. The highly negative impact of infection of agriculturally relevant plants by A. flavus caused gradual expansion of studies into the related fundamental areas of biology of this fungus, including the A. flavus secondary metabolism, sclerotia morphogenesis and propagation [2,4,6–9]. The recently revealed genomic sequence of A. flavus NRRLI3557 provided a powerful tool for detailed analysis of biology of this fungus [10].

Sclerotia morphogenesis is an physiological process important for A. flavus propagation and involves various secondary metabolism pathways, including aflatoxin biosynthesis. Sclerotia are pigmented, specialized structures composed of compact A. flavus mycelia, which makes A. flavus resistant to harsh environmental conditions. Sclerotia likely derive from cleistothecia and might represent a vestige of sexual ascospore production [6]. According to the size of sclerota, A. flavus could be divided into two groups: the L strain has sclerotia that are >400 μm in diameter and the S strain has sclerotia that are <400 μm in diameter [11,12]. The S strains produce greater amounts of sclerotia and aflatoxin compared to the L strains under the same conditions of medium and cultivation [13], suggesting sclerotia morphogenesis and aflatoxin biosynthesis are closely related. To interpret this correlation, Chang and colleagues proposed the “substrate (acetate) competition” hypothesis. In their proposal, the increased production of aflatoxin results in a progressive decrease in sclerotial size, alterations in sclerotial shape and causes weakening of the sclerotial structure [6]. Recent efforts provided insights into the regulation of sclerotia morphogenesis. Ammonium, light, oxidative stress, temperature, organic acids and endogenous levels of cAMP might influence sclerotia formation and maintenance [14–19]. It was reported that A. flavus laeA and veA mutants are not able to form sclerotia [20,21]. Cary and colleagues used DNA array to analyze sclerotia-related genes by comparing the A. flavus wild type strain and a veA mutant strain [22]; however, a detailed comprehensive description of sclerotia morphogenesis is lacking.
Natural antisense transcripts (NATs), a subset of non-coding RNAs (ncRNAs), are endogenous RNA molecules transcribed from the opposite DNA strand and can be complementary to the sense RNA through base pairing [23–25]. Expressed sequence tag (EST) sequencing, tiling microarrays, SAGE libraries, asymmetric strand-specific analysis of gene expression and global run-on sequencing (GRO-seq) could be used to identify NATs [26]. NATs are involved in transcriptional and post-transcriptional gene regulation by RNA interference (RNAi) [27,28], chromatin-level gene silencing [23,26,29–31], chromatin remodeling [28,32] and local chromatin modifications [33–35]. Global analysis of NATs has been done for mammals [23], insects [36], worms [37] and plants [38,39]. It was reported that a large portion of the mammalian genome could produce transcripts from both strands [23,40,41]. For instance, >70% of mouse transcripts contain NATs and, owing to their prevalence, NATs are treated as pervasive features of mammalian genomes [26]. *Aspergillus* spp. have been used as model organisms to investigate many molecular processes that govern the life of eukaryotic cells. Moreover, *Aspergillus* spp. have been used extensively to study fungal-specific pathways. NATs have been found in a divergent group of fungi, including the ascomycetes *Saccaromyces cerevisiae*, *Candida albicans*, *A. flavus*, *Magnaporthe oryzae*, *Tuber melanosporum* and *Schizosaccharomyces pombe*, and the basidiomycetes *Cryptococcus neoformans*, *Ustilago maydis* and *Schizophyllum commune* [28]. There are two distinct drawbacks in the study of *A. flavus* NATs, however; first, there were only 352 NATs (2.8% of the total open reading frames (ORFs)) found in *A. flavus* by analyzing ~23,000 *A. flavus* cDNAs from cells grown under different nutritional conditions [25], while this number soars to 16.7–85.2% in comprehensively analyzed transcriptomes such as *A. flavus* (23,000) [23,40,41]. For instance, 20% of mouse transcripts contain NATs and, owing to their prevalence, NATs are treated as pervasive features of mammalian genomes [26]. *Aspergillus* spp. have been used extensively to study fungal-specific pathways. NATs have been found in a divergent group of fungi, including the ascomycetes *Saccaromyces cerevisiae*, *Candida albicans*, *A. flavus*, *Magnaporthe oryzae*, *Tuber melanosporum* and *Schizosaccharomyces pombe*, and the basidiomycetes *Cryptococcus neoformans*, *Ustilago maydis* and *Schizophyllum commune* [28]. There are two distinct drawbacks in the study of *A. flavus* NATs, however; first, there were only 352 NATs (2.8% of the total open reading frames (ORFs)) found in *A. flavus* by analyzing ~23,000 *A. flavus* cDNAs from cells grown under different nutritional conditions [25], while this number soars to 16.7–85.2% in comprehensively analyzed transcriptomes such as *S. cerevisiae* [16.7%], *C. albicans* (40.0%) and *S. pombe* (85.2%) [29]. Second, the biological function of NATs in *A. flavus* remained elusive due to the limited amount of NATs detected [42].

Owing to the importance of transcriptional regulation in the development of fungi, transcriptome profiling could be a valuable tool for establishing an in-depth understanding of *A. flavus* biology. The transcriptome of *A. flavus* has been studied by several groups, but these studies were not focused on sclerotia development or secondary metabolism. ESTs and microarrays have been used to identify genes involved in aflatoxin production using 7218 unique ESTs and microarrays containing more than 5000 unique *A. flavus* gene amplicons [1–3]. However, only 263 genes were expressed differentially and only 20 of the 29 aflatoxin pathway genes were identified owing to the limitation of microarrays in detecting genes with low levels of expression. Even an important transcriptional factor in the aflatoxin biosynthesis pathway, *gffR*, has not been detected [1]. Chang et al identified 22 features common to the aflatoxigenic/non-aflatoxigenic pairs by cross comparison. Possible roles of these identified genes have been discussed in relation to the regulation of aflatoxin biosynthesis [3]. Yu et al profiled the transcriptome of *A. flavus* under various temperature conditions in order to understand the effect of temperature on mycotoxin biosynthesis using RNA-seq technology. Only 23–29% of the total reads were mapped to genes, however, and >50% of the reads were mapped to rRNA genes and mitochondria and, therefore, were regarded as useless data [4].

We used strand-specific RNA sequencing (ssRNA-seq) to obtain an extensive transcriptome profile of *A. flavus* that might facilitate comprehensive understanding of the physiological processes of *A. flavus*. ssRNA-seq technology has all of the advantages of conventional RNA-seq technology, including low detection background, high dynamic detection range, high reproducibility and precise definition of transcript structure [43]. Furthermore, in contrast to conventional RNA-seq technology, ssRNA-seq data contain RNA polarity information and could decode a complex eukaryote transcriptome, including genome annotation, de novo transcriptome assembly and accurate digital gene expression analysis [44,45]. ssRNA-seq technology has been used to parse the transcriptome of many organisms, including *S. cerevisiae* [46], *Mycoplasma pneumoniae* [47], *Mus musculus* [47] and *Oryza sativa* [48]. By using ssRNA-seq data of the *A. flavus* mycelia and sclerotia cultures, we profiled genome-scale transcription, annotated precise transcript structure and provided in-depth depiction of *A. flavus* NATs. Analysis of differentially expressed genes might contribute to explicating sclerotia development and aflatoxin biosynthesis. In summary, our ssRNA-seq-based annotation provided a more extensive depiction of the *A. flavus* transcriptome and might contribute to the reduction of detrimental effects of *A. flavus* infection on agriculture.

### Materials and Methods

#### Strain and Culture Conditions

*A. flavus* CA43 was kindly provided by Professor Perng-Kuang Chang (Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Washington, DC, USA). *A. flavus* CA43 belongs to the S strain isolates, which can produce numerous sclerotia and high amounts of aflatoxin [13].

Potato dextrose agar (PDA) medium (20 g of dextrose, 15 g of agar and the infusion from 200 g of potatoes per 1 L of medium, pH = 6.0) was used for *A. flavus* cultivation. For harvesting the *A. flavus* culture, a layer of cellophane was placed over the PDA medium plate (PDA-cellophane plate). *A. flavus* sclerotia (1.2 x 10^6) were inoculated onto each PDA-cellophane plate and cultivated at 30°C in darkness. For RNA sequencing, the *A. flavus* mycelia were harvested after 48 h of cultivation (*Aflavus_CA43_M*) and sclerotia were collected after 7 days of cultivation (*Aflavus_CA43_S*).

#### Strand-specific RNA-seq Library and Sequencing

The total RNA of each sample was extracted using RNAiso™ Plus (TaKaRa, Japan), treated with RNase-free DNase I (TaKaRa, Japan) and purified with a NucleoSpin® RNA Clean-up Kit (Macherey-Nagel, Germany). RNA integrity was analyzed by an Agilent Technologies 2100 Bioanalyzer. All samples had an RNA integrity number (RIN) >7. The strand-specific RNA-seq library was prepared essentially as described but with some modifications [49]. Sera-mag magnetic oligo(dT) beads were used to isolate poly(A) mRNA from 20 µg of purified total RNA. mRNA was eluted by 10 mmol/L Tris buffer and fragmented into small pieces in the range of 100–500 bp by treatment with divalent cations. Taking these short fragments as templates, random hexamer primers were used to synthesize the first-strand cDNA using SuperScript II, RNaseH and DNA polymerase I. The first-strand cDNA was purified using a QIAquick PCR Purification Kit and used as the template for synthesizing dUTP-containing second-strand cDNA by adding buffer, dNTPs with dTTP replaced by dUTP, RNaseH and DNA polymerase I. Double-stranded cDNA fragments were purified with a QIAquick PCR Purification Kit and eluted with elution buffer for end-repair, phosphorylation and 3’-adenylation. After that, cDNA fragments were purified with a Qiagen MinElute PCR Purification Kit. Illumina sequencing adapters were ligated to the 5’-adenylated cDNA fragments followed by purification using a MinElute PCR Kit. Fragments of 100–500 bp were purified using a QIAquick Gel Extraction Kit from the modified double-stranded cDNA fragments described above separated by TAE-agarose gel electrophoresis (2% (w/v) agarose; Certified...
Low-Range Ultra Agarose, Biorad). Uracil-DNA glycosylase (UNG) was added to digest dUTP-containing second-strand cDNA in an alkaline solution at high temperature. The remaining first-strand cDNA was purified using a MinElute PCR Purification Kit and enriched by 15 rounds of PCR amplification using primers P7 and P5, which were homologous to the Illumina sequencing adapters. After purification using a QIAQuick Gel Extraction Kit, fragments of 100–500 bp (average 200 bp) were selected as a strand-specific RNA-seq cDNA library. Finally, after quantification on an Agilent Technologies 2100 Bioanalyzer, the cDNA library was sequenced on the Illumina HiSeq2000 platform by a 90 bp paired-end sequencing strategy (using primers P7 and P5). The raw Illumina sequencing data for the A. flavus CA43 transcriptome was deposited in the SRA (http://www.ncbi.nlm.nih.gov/sra/) with ID no.SRP018670.

Primary Splitting of Raw Strand-specific Sequencing Data

In the course of strand-specific paired-end (PE) sequencing, reads consistent with the reverse strand of a transcript were first obtained by primer P5-triggered sequencing (read1), and reads consistent with the forward strand of the same transcript were then obtained by primer P7-triggered sequencing (read2). Read1 and read2 are PE reads.

According to strand-specific sequencing features, read1 mapped to the forward strand of a transcript and read2 reversely mapped to the reverse strand of the transcript could not reflect the real transcription and was discarded. The remaining mapping reads were used to analyze gene expression and genes expressed differentially.

As the forward strand of a gene might be located in either the forward or the reverse strand of the genome, we classified read1 reversely mapped to the forward strand of the genome and read2 mapped to the forward strand of the genome into one group representing transcription from the forward strand of the genome. We classified read1 mapped to the forward strand of the genome and read2 reversely mapped to the forward strand of the genome into a group representing transcription from the reverse strand of the genome. The mapping result with genome sequence was used for the following analysis, including novel transcript prediction, untranslated region (UTR) and alternative splicing (AS) events. This splitting strategy was designed according to the BGI analysis pipeline (http://www.genomics.cn/index).

Read Mapping and Normalization of Gene Expression

The reference genome (A. flavus NRRL3357) was downloaded from GenBank (accession no. AAIIH00000000.2) and the gene annotation data were downloaded from the AspGD website (http://www.aspergillusgenome.org/download/sequence/A_flavus_NRRL_3357/current/). After removing the reads containing sequencing adapters and low-quality reads (reads containing Ns ≥ 9), the remaining 90 bp clean reads were aligned to the A. flavus reference genome and annotated genes using SOAP2 software [50], allowing up to five base mismatches for genotype alignment and three base mismatches for gene alignment. Reads that failed to be mapped were first trimmed off 1 base from the 5′-end. If there was still no match, these reads were trimmed off progressively two bases each time from the 3′-end and mapped to the genome until a match was found (unless the read was trimmed <48 bases for genome alignment or <28 bases for gene alignment). The insert between PE reads was set to 1 bp-10 kb, allowing them to span introns or the intergenic regions of various size in the genome. When PE reads were mapped to non-redundant genes, the insert was set to 1 bp-1 kb. The coverage per base in the genome and the reads count covering each transcript could be calculated by reads mapping.

We used the popular RPKM method to normalize the transcript level, which was expressed as the number of reads per kilobase of exon region per million mapped reads (RPKM) [51, 52]. The cutoﬀ value for gene transcriptional activity was determined on the basis of the 95% conﬁdence interval (CI) for all RPKM values. GO annotation of the A. flavus genes was done by Blast2GO (version 2.6.3) software [53] and visualized by WEGO software [54].

Non-redundant EST Sequence

Non-redundant ESTs were obtained by mapping all A. flavus EST sequences [http://comphio.dfci.harvard.edu/cgi-bin/cgi/est_ann.pl?gudb = a_flavus] to A. flavus annotated genes using BLAT software [55, 56]. ESTs that could not be mapped to annotated genes [http://www.aspergillusgenome.org/download/sequence/A_flavus_NRRL_3357/current/] were selected as non-redundant ESTs.

UTR and Upstream Open Reading Frame Analysis

UTRs were defined as regions flanking a gene coding sequence, with contiguous expression of each base supported by at least two uniquely mapped reads. Transcripts whose ends overlapped with each other were discarded. The length of 5′- or 3′-UTRs was limited to a maximum length of 1000 bp. The upstream ORFs (uORFs) were searched for in the 5′-UTRs of genes. The length of uORFs was set to 9–150 bp, and the distance between a uORF and a gene start codon should be <500 bp.

Detection of Novel Transcripts

Novel transcriptionally active regions (TARs) were determined in the intergenic regions by contiguous expression of each base supported by at least two uniquely mapped reads and length > 35 bp [43]. A novel TAR should be separated by at least 200 bp from the upstream or downstream region of the transcript. A novel transcript is composed of novel TARs connected by at least one paired read. Novel transcripts of length >150 bp were selected for further analysis.

Alternative Splicing Events in A. flavus

Potential junction sites were detected using the TopHat program [57]. Reads used to identify junction sites could not map to the genome but were able to map to the genome after several terminal bases were trimmed off. An acknowledged junction site should be supported by at least two mapped reads with different mapping positions within the junction site region and with a minimum of 5 bp mapping on each side of the junction site and a tolerance of 2 bp mismatch [43]. A novel junction site should be supported by at least four such reads. Using the method described by Wang et al [58], seven types of AS events were analyzed in A. flavus CA43, including skipped exons (SE), retained introns (RI), alternative 5′-splice sites (A5SS), alternative 3′-splice sites (A3SS), mutually exclusive exons (MxEx), alternative first exons (AfEx) and alternative last exons (AlEx).

Natural Antisense Transcript

Putative antisense transcripts were detected for A. flavus annotated genes with RPKM ≥3. For a certain annotated transcript, the corresponding antisense transcript was denoted as a complementary overlapping region on the opposite strand with contiguous expression of each base supported by at least one uniquely mapped read and length >200 bp. In order to obtain
reliable results, the detected antisense transcripts should have an average coverage depth ≥ 2. To eliminate the influence of an intronic region, antisense transcripts were analyzed in the transcriptional exons and UTRs.

Analysis of Differentially Expressed Genes between A. flavus Mycelia and Sclerotia States

The DEGseq R package was used to analyze differentially expressed genes (DEGs) by reads count covering each gene on the basis of the Random Sampling model [59]. Functional enrichment analysis of certain DEGs was performed with Blast2GO software using Fisher’s exact test with robust false discovery rate (FDR) correction of 0.05.

Results and Discussion

Sequencing Summary

To obtain an elaborate transcriptome profile of A. flavus, the isolated mRNA of A. flavus CA43 mycelia (Aflavus_CA43_M) and sclerotia (Aflavus_CA43_S) were subjected to high-throughput sequencing by the strand-specific paired-end sequencing strategy. In total, 26.9 and 27.7 million of 90 bp Illumina reads were obtained for Aflavus_CA43_M and Aflavus_CA43_S, reaching an average genome coverage depth of 60-fold and 62-fold, respectively. About 85% of all reads were mapped uniquely to the A. flavus genome with a tolerance of 5 bp mismatch (84.48% for Aflavus_CA43_M and 83.91% for Aflavus_CA43_S; Fig. 1A and Table S1). Approximately 53% of all reads were mapped uniquely to annotated A. flavus genes (Table S1), which represents much greater accuracy compared to earlier A. flavus transcriptome data (23–29%) [4]. The 30% of reads mapped uniquely that could be mapped to the genome but could not be mapped to the annotated genes might represent novel transcripts hidden in the intergenic regions. Massive non-redundant ESTs in the A. flavus genome (Table S2) suggest the presence of a large number of novel transcripts. For all A. flavus transcripts, 38.34 million reads were mapped to their sense strand but only 0.63 million reads (1.7%) were mapped to their opposite strand, suggesting our RNA-seq library is strand specific. The reads mapped to the opposite strand might represent natural antisense transcripts that occur with a length of 848 bp and an average sequencing depth of 11.61.

The transcribed genome sequence assembled by our ssRNA-seq reads was 39.91 Mb that can be mapped to the genome (39.91 Mb) was expressed as ssRNA-seq reads, with a sequencing coverage of 90% (Fig. S1) and the 3′-UTRs of the gene, which have important roles in post-transcriptional regulation of gene expression. One of the identified novel transcripts was >500 bp with a median length of 167 bp for 5′-UTRs and 206 bp for 3′-UTRs (Fig. 3B). The median length of UTRs in A. flavus CA43 was longer compared to A. oryzae RIB40 (107 bp for 5′-UTRs and 156 bp for 3′-UTRs) [43] and Schizosaccharomyces pombe (152 bp for 5′-UTRs and 169 bp for 3′-UTRs) [63]. According to the study reported by Lackner et al suggesting the most stable transcripts have short 5′-UTRs and the least stable transcripts have long 5′-UTRs [64], A. flavus CA43 has less stable transcripts and a much higher RNA turnover rate compared to A. oryzae and S. pombe. It was reported that ORFs in 5′-UTRs upstream of annotated start codons (uORFs) might constitute important regulatory factors for gene expression [65,66]. We have predicted 2600 uORFs for A. flavus annotated genes (19.28%; Table S5),

We analyzed the transcriptional activity of A. flavus transcriptional factors (TFs) to address extensive transcription of the A. flavus CA43 genome. Fungal TF information was downloaded from the web (http://flytd.smu.ac.kr/tf.php?act=summary&data = 0). The A. flavus genome contains 667 TFs and 617 of them were expressed as RNA-seq reads, while A. oryzae contains 603 TFs and 571 of them were transcribed (Table S3; the data for A. oryzae TFs were obtained from our earlier work [43]). GO analysis showed A. flavus CA43 TFs were enriched mainly in the GO categories of binding, catalytic, biological regulation, nucleotide binding, cellular process and metabolic process (Fig. 2). Particularly, A. flavus CA43 contains transcribed TFs involved in secondary metabolic process and sporulation.

The non-homologous end joining (NHEJ) pathway is important for DNA repair and gene recombination. NHEJ-deficient strains could be used efficiently for gene targeting and homologous gene recombination [60], ku70, ku80 and LigD are the essential genes in the NHEJ pathway of Aspergillus spp. [61]. For A. oryzae RIB40, the transcriptional level of ku80 (AO0900260000842) reached RPKM 82.62 in the solid-state culture, and ku70 (AO090701000906) and LigD (AO090113000208) were not expressed [43]. For A. flavus CA43, ku70 (AFL2G_06276), ku80 (AFL2G_06694) and LigD (AFL2G_08329) were all transcribed under both mycelia and sclerota cultures (RPKM 23.57, 18.84 and 14.28 for mycelia culture, and 150.82, 31.34 and 231.45 for sclerota culture) (Table S2), suggesting A. flavus CA43 possessed highly efficient non-homologous gene targeting.

Improved Annotation of A. flavus Gene Models

Besides the reads mapped to the 13,487 annotated A. flavus genes, 30% of reads mapped uniquely were located in the intergenic regions. Extensive mapping and clustering of these intergenic reads revealed 939 and 1196 previously unrecognized novel transcripts for mycelia and sclerota samples, respectively (Table S4). The levels of expression of these novel transcripts were almost as high compared to exons (Fig. 1B) and 62.62% of the identified novel transcripts were longer than 500 bp (Table S4). This ratio is much higher compared to A. oryzae RIB40 [43]. Most of the identified novel transcripts were non-coding RNAs (ncRNAs) and only 25 (1.17%) novel transcripts were predicted to be potential protein-coding genes with an ORF length of ≥ 150 bp. The vast majority of transcribed ncRNAs might act as NATs to regulate gene expression. One of the identified novel transcripts (TU134) in mycelia culture is illustrated by Fig. 3A, with a length of 848 bp and an average sequencing depth of 11.61.

The transcribed genome sequence assembled by our ssRNA-seq reads could be used to define or extend untranslated regions (UTRs) of the gene, which have important roles in post-transcriptional regulation [62]. The 5′-UTRs for 5994 transcripts and the 3′-UTRs for 6407 transcripts were determined in this study (Table S5). Most (88.87%) of these identified UTRs were < 500 bp with a median length of 167 bp for 5′-UTRs and 206 bp for 3′-UTRs (Fig. 3B). The median length of UTRs in A. flavus CA43 was longer compared to A. oryzae RIB40 (107 bp for 5′-UTRs and 156 bp for 3′-UTRs) [43] and Schizosaccharomyces pombe (152 bp for 5′-UTRs and 169 bp for 3′-UTRs) [63]. According to the study reported by Lackner et al suggesting the most stable transcripts have short 5′-UTRs and the least stable transcripts have long 5′-UTRs [64], A. flavus CA43 has less stable transcripts and a much higher RNA turnover rate compared to A. oryzae and S. pombe. It was reported that ORFs in 5′-UTRs upstream of annotated start codons (uORFs) might constitute important regulatory factors for gene expression [65,66]. We have predicted 2600 uORFs for A. flavus annotated genes (19.28%; Table S5),
which is much higher compared to *A. oryzae* RIB40 (11.14%) [43] and *S. cerevisiae* (6%) [67]. An example of a uORF and the corresponding 5′-UTR is shown in Fig. 3C. Genes containing uORFs are enriched specifically for GO terms of cellular protein modification process, protein serine/threonine kinase activity, Ras GTPase activator activity, positive regulation of Ras GTPase

Figure 1. Summary of RNA sequencing of *A. flavus* CA43. (A) Matching summary of ssRNA-seq reads to the *A. flavus* genome. (B) Box and whisker plots of log2-transformed RPKM for exons, novel transcripts, introns and intergenic regions. Horizontal lines in boxes, the first, median and third quartile. Other horizontal lines, the inner boundaries. Diamonds, data outside the inner boundaries. (C) Mapping coverage of the transcribed *A. flavus* genes.

doi:10.1371/journal.pone.0097814.g001

Figure 2. WEGO comparison of *A. flavus* and *A. oryzae* transcribed transcriptional factors. In total, 525 of the 617 *A. flavus* transcribed TFs and 461 of the 571 *A. oryzae* transcribed TFs were assigned to GO categories.

doi:10.1371/journal.pone.0097814.g002
activity, phosphorylation, autophagy, phospholipid binding and protein transport (FDR-adjusted \( p < 0.05 \); Fig. S2 and Table S5).

AS events contribute to producing multiple proteins from genes with two or more exons in fungi. This could enrich the proteomic diversity of fungi and provide the ability to survive in a hazardous environment. AS events were analyzed in 9545 \( A.\text{flavus} \) multi-exon genes (70.77\% of all \( A.\text{flavus} \) genes) using the method described by Wang et al [58]. A total of 1220 AS events took place in 941 \( A.\text{flavus} \) genes (Table S6 and Fig. 4A), including retained introns (RIs), skipped exons (SEs), alternative 5’-splice sites (A5SSs) and alternative 3’-splice sites (A3SSs). About 12.78\% of the \( A.\text{flavus} \) multi-exon genes produced AS isoforms, similar to \( A.\text{oryzae} \) RIB40 (11.10\%) [43] and many more than \( P.\text{pastoris} \) (4.78\%) [68]. This is in agreement with the conclusion that the frequency of AS events is proportional to the ratio of multi-exon genes in a genome (76.98\% for \( A.\text{oryzae} \) and 11.91\% for \( P.\text{pastoris} \)) [69]. AS events might alter the amino acid composition and the structure of the target protein. For example, AFL2G_07666 (Fig. 4A), encodes sphingosine kinase (\( \text{SphK} \)), which participates in the sphingosine 1-phosphate (S1P) metabolism. Together with S1P phosphatase (\( \text{S1PP} \)) and S1P lyase (\( \text{SPL} \)), AFL2G_07666 controls the intracellular S1P level and has important roles in the regulation of cell migration, survival, differentiation, angiogenesis and development through an extracellular signaling pathway mediated by a family of specific G protein-coupled receptors. We performed homologous modeling using SWISS-MODEL Workspace [70,71] and constructed a 3D model of the AFL2G_07666 transcript and its AS variant (Fig. 4B). The skipped exon alters the 3D structure of AFL2G_07666 and might influence its biological function. To investigate the mechanism of AS events, we calculated the ratio of the amount of RIs to cassette exons (CEs, including SE, AFE, ALE and MXE). The high RI/CE ratio (34.75) indicates \( A.\text{flavus} \) might recognize splicing sites and produce AS events mainly by the intron definition (ID) mechanism, according to the study reported by McGuire et al [69].

**Natural Antisense Transcript**

The “G-value paradox”, that the amount of protein-coding genes does not correlate with the complexity of an organism, suggests that so-called junk DNA in a genome contains rich regulatory information exerting via transcribed natural antisense transcripts (NATs) [23,28]. So far, little is known about the role of NATs in \( Aspergillus \) spp. It was suggested that NATs might have an important role in differential expression of genes involved in secondary metabolism in \( A.\text{oryzae} \) [42]. According to an earlier study, the most prominent NAT type is the non-protein-coding antisense RNA partner of a protein-coding gene [26]. We searched for NATs in our data for all \( A.\text{flavus} \) protein-coding genes with the RPKM > 33 using stringent criteria. In all, 1124 and 839 NATs were identified in the AflavusCA43_M and AflavusCA43_S samples, respectively (Table S7). This is many more compared to \( A.\text{flavus} \) NATs on the basis of EST data (352, 2.8\%) and \( Cryptococcus\text{neoformans} \) NATs (53, 0.8\%) [25]. The number of RNA-seq-based \( A.\text{flavus} \) NATs is in the same range as \( S.\text{cerevisiae} \) (1103, 16.7\%) [46]. However, this frequency is much lower compared to \( \text{Candida\text{albicans}} \) (2458, 40\%) and \( \text{M.\text{oryzae}} \) (4215, 32.8\%) [28].

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**Figure 3. Detailed annotation of the \( A.\text{flavus} \) gene model.** (A) A novel transcript (TU134) identified in contig gi|156128545|gb|AAIH02000077.1| in the forward orientation. Red bar, the novel transcript TU134 identified in the mycelia sample. Red curve, expression level (log2-transformed reads count) of the transcripts located in the forward strand. Green curve, the expression level of the transcripts located in the reverse strand. (B) Scatterplot and histograms showing the length distribution of the 5’- and 3’-UTRs of \( A.\text{flavus} \) CA43 genes. (C) UTR and uORF illustration for an annotated \( A.\text{flavus} \) gene (AFLG2_08360). doi:10.1371/journal.pone.0097814.g003
There are more NATs located inside coding regions (768, 39.14%) compared to UTR regions (466, 23.76%, Fig. 5A). This means NATs in *A. flavus* might regulate gene expression mainly at the post-transcriptional level, which is consistent with the conclusion reported by Donaldson et al [28]. The inside-biased NAT distribution in *A. flavus*, however, was different from the 3′-
biased antisense transcription in *A. nidulans* [72] and the mammalian NAT distribution, where NATs are usually enriched in the region of the 250 bp upstream sequence and the 1.5 kb downstream sequence [26]. The transcriptional level of *A. flavus* genes with NATs (average RPKM 253.59) was much higher compared to genes without NATs (average RPKM 54.50; Fig. 5B). This is consistent with the earlier study by Katayama et al [23], strongly denying the simple hypothesis that NATs are just negative regulatory elements and artifacts of leaky bidirectional transcription [26,28,73].

Among the 352 EST-based NATs in *A. flavus*, only 19 were verified by our RNA-seq data (Table S7). One of the EST-based NATs is that of the aflatoxin biosynthetic regulator gene (*aflR*, AFL2G_07224), which was identified in our study when the RPKM cutoff for NAT analysis was set at 5 (Table S7). The existence of a NAT in the transcriptional factor *aflR* suggests the interaction between a NAT and its sense transcript can occur in the nucleus, consistent with the conclusion from studies in mammals [23,26]. Another example is *SdeA* (AFL2G_00446), which is involved in the regulation of morphology under temperature change and in the production of multicellular developmental structures (conidiophores, cleistothecia and sclerotia) [25]. Changes in the expression of *SdeA* and its NAT in the mycelia and sclerotia states suggests NAT participates in the regulation of *SdeA* and thus biological processes controlled by this gene (Fig. 5C).

GO enrichment analysis demonstrated genes with NATs were enriched specifically in GO terms of protein complex, protein binding, RNA binding, translation, ribosome, intracellular protein transport, cellular amino acid metabolic process, vesicle-mediated transport, hexose catabolic process and biological regulation (FDR-adjusted *p*<0.05; Fig. S3 and Table S7). Thus, these NAT-containing genes are closely related to protein expression, secretion and energy production in *A. flavus*. For example, the *A. flavus* *Set1* gene (AFL2G_02936) has a NAT located inside its coding sequence (Table S7). According to the report that the corresponding NAT could prevent *Set1*-mediated transcription initiation in *S. cerevisiae* [28], NAT might influence the *Set1*-mediated transcription initiation in *A. flavus*. The discussion about NAT function in fungi was focused mainly on the NAT-mediated alteration of physiological processes in response to environmental nutrient starvation [28] and nitrogen metabolism [72]. Our analysis is the first global investigation of NAT function in *A. flavus*.

**Sclerotia Development and Reproduction**

Sclerotia are considered to derive from cleistothecia, which is the sexual reproductive organ in *Aspergillus* spp. [6]. Genes involved in sexual reproduction and the balance of sexuality and asexuality of *Aspergillus* spp. are given in Table S8 [74–76]. To identify genes involved in *A. flavus* sclerotia development, differentially expressed genes (DEGs) between the *A. flavus* mycelia and sclerotia cultures were detected. For the 13,487 *A. flavus* genes, 9871 (73.19%) were transcribed under both conditions. A total of 661 genes were expressed specifically in the mycelia state and 343 genes were specific for the sclerotia state. These genes might represent factors critical for the physiological development of *A. flavus*. There were 7609 DEGs (56.42%) between the *A. flavus* mycelia and sclerotia states, with 1821 up-regulated genes and 5788 down-regulated genes in the sclerotia state (*p*<0.001; Table S9). DEGs between the *A. flavus* mycelia and sclerotia states are much more abundant compared to *A. flavus* cultivated at different

Figure 5. NAT analysis in *A. flavus* CA43. (A) The relationship between the location of NAT and its corresponding genes. Downstream, Upstream and Inside, NATs located in the downstream, upstream or inside of their corresponding genes, respectively. Intersected, NAT located across one gene and its 5’- or 3’- flanking region. (B) Box and whisker plots of log2-transformed RPKM for *A. flavus* annotated genes with or without NATs. (C) Occurrence of NAT in the *SdeA* gene (AFL2G_00446). Orange curves, the NAT in the *SdeA* gene. doi:10.1371/journal.pone.0097814.g005
Strand-Specific RNA-Seq Transcriptome of A. flavus

A

Percent of genes

Number of genes

0.01
2 37

Mycelia state  Sclerotia state

2 37

432

Cellular Component

Molecular Function

Biological Process

B

Signal Transduction - Asexual Reproduction

Conidiophore Development

Mating Processes

Signal Transduction - Sexual Reproduction

Ascomata (Fruit Body) Development

B

C

Mycelia state

Sclerotia state

D

Mycelia state

Sclerotia state

Expression

Expression

Expression

Expression

AFL2G_07468

AFL2G_00999

AFL2G_00999

AFL2G_07468
temperatures (2709) [23], indicating different developmental stages are accompanied by a high level of diversity in gene expression.

To identify genes closely related to the developmental stages, DEGs with change >2-fold were selected for further analysis: 1149 up-regulated genes and 4492 down-regulated genes were detected for the sclerotia state. WEGO illustration showed GO terms of the reproductive cellular process, the reproductive process, sexual reproduction and sporulation contained more up-regulated genes in the sclerotia state compared to the mycelia state (Fig. 6A), indicating sclerotia is closely related to A. flavus reproduction instead of being only a sexual vestige. Additionally, the abundance of residual mating process genes in A. flavus suggests it might be capable of sexual development.

DEGs related to reproduction are shown in Fig. 6B, with 14 up-regulated genes and 37 down-regulated genes in the sclerotia state. The zinc finger protein-encoding gene brlA (AFL2G_00999) is the primary activator of asexual conidiation reproduction [77,78]. Down-regulated brlA in the sclerotia state (Fig. 6D), together with a series of conidiation-related genes (abaA, wetA, ffbB, ffbC and ffbE; Fig. 6B), suggests conidial development is repressed during sclerotia development. These findings might contribute to understanding the sexual and asexual balance of A. flavus.

Secondary Metabolism

The secondary metabolic (SM) pathways in fungi consist of many genes encoding polyketide synthetases (PKS), fatty acid synthases, dehydrogenases, reductases, oxidoreductases, epoxide hydrolases, cytochrome P450 monooxygenases and methyltransferases [1]. However, it is difficult to determine whether such genes are involved in SM pathways, because vast numbers of genes are categorized as belonging to the gene clusters mentioned above. On the basis of the web tool SMURF [9], 55 putative SM pathways were identified in A. flavus, including 22 PKS and 27 non-ribosomal peptide synthetase (NRPS) pathways. It is important to study secondary metabolism in A. flavus because it was reported that secondary metabolism was often related to sporulation and sclerotia development [2,76]. In our RNA-seq data, backbones of 38 SM pathways were transcribed in both the mycelia and sclerotia cultures (Table S10), including the aflatoxin (AF) biosynthetic pathway (cluster #54).

The AF biosynthesis pathway is the best studied SM pathway in A. flavus, containing at least 23 enzymatic reactions and 29 genes in a 75 kb cluster on chromosome III (Table S10) [79]. Only eight transcribed genes in the AF pathway were scored by microarray technology [1]. Our RNA-seq data provided precise information about AF biosynthesis. The transcriptional factor aflR (AFL2G_07224) was transcribed under the mycelia and sclerotia conditions and the NAT of aflR was transcribed under the sclerotia condition, suggesting AF biosynthesis might be down-regulated by NAT-mediated RNAi during sclerotia development (Table S10). This is consistent with the fact that the transcripts of all AF pathway genes were detected in the mycelia state but three genes (aflD, aflCa and aflP) were not detected in the sclerotia state (Table S10). Additionally, changes in expression of the global SM transcriptional factor veA (AFL2G_07468) during sclerotia development suggest it has roles in linking sclerotia development with secondary metabolism (Fig. 6C and Table S8) [80]. These findings are in agreement with the transcriptional profile of AF biosynthesis (Fig. 7A) and the down-regulation of most AF biosynthetic structural genes in the sclerotia state (Fig. 7B).

Genes participating in the AF metabolic pathway contain biosynthesis genes, signal transduction genes, regulatory genes and genes involved in the stress response [2]. Although most of the AF structural genes are in a single cluster, this cluster might be regulated by genes spread throughout the A. flavus genome [81].
Among the AF-related genes given in Table S10, 11 were downregulated in the sclerotia state and two were up-regulated (Fig. 7B). According to our analysis of sclerotia development (Table S8 and Fig. 6B), these AF genes were also related to sclerotia development, suggesting they link sclerotia development and aflatoxin biosynthesis. Therefore, high throughput RNA-seq data brought new insights into A. flavus mycotoxin production and other secondary metabolism pathways in this fungus. AF contamination of crops is a heavy economic burden on farmers and it is important, therefore, that the target genes identified by RNA-seq data can be used in the biocontrol of aflatoxigenic strains by genetic manipulation.

Conclusions

The A. flavus transcriptome has been studied intensively by several research groups in recent years. Despite these efforts, however, the mechanism underlying the regulation of A. flavus physiology is unknown. Our data profiled the transcriptional activity of A. flavus transcriptome on the genomic scale and annotated transcript structures precisely. UTR annotation revealed A. flavus might have a much higher RNA turnover rate compared to A. oryzae or S. pombe. A. flavus might recognize the splicing sites and produce AS events mainly by the intron definition (ID) mechanism owing to the high R1/CE ratio (34.75). Among the novel transcripts we were able to identify, the vast majority of transcribed ncRNAs might act as NATs to regulate gene expression at the post-transcriptional level. The transcriptional activity of A. flavus genes with NATs was much higher compared to genes without NATs, suggesting NATs are true transcripts rather than artifacts of leaky bidirectional transcription. Our analysis is the first global investigation of NAT function in A. flavus. As for DEGs, it is quite likely that the 14 up-regulated and 37 down-regulated reproduction-related genes in the sclerotia state link A. flavus reproduction and sclerotia development. In our ssRNA-seq data, the backbones of 38 SM pathways were transcribed in both mycelia and sclerotia states, and we identified genes linking sclerotia development and aflatoxin biosynthesis. Our data could be used to develop strategies to control aflatoxin synthesis by aflatoxigenic strains. Therefore, ssRNA-seq data provided in this study could expand our understanding of A. flavus.

Supporting Information

Figure S1 Global transcriptional profile of A. flavus CA43 in the mycelia state, denoted by log-transformed reads count. Coverage, the percentage of the genomic region covered by ssRNA-seq reads with a window size of 5 kb. Gene, the number of A. flavus genes within a window size of 5 kb. The chromosome number is shown at the left. (TIF)

Figure S2 GO functional enrichment analysis of A. flavus uORF-containing genes. The abscissa is the percentage of genes in each GO term. The ordinate is in GO terms. (TIF)

Figure S3 GO functional enrichment analysis of A. flavus genes with NATs. The abscissa is the percentage of genes in each GO term. The ordinate is in GO terms. (TIF)

Table S1 Mapping summary of ssRNA-seq reads. (XLS)

Table S2 Expression level of A. flavus genes and non-redundant ESTs at different development stages revealed by ssRNA-seq. (XLS)

Table S3 The transcription status of transcriptional factors in A. flavus CA43 and A. oryzae RIB40. (XLS)

Table S4 Novel transcripts in A. flavus CA43. (XLS)

Table S5 UTRs and uORFs identified for A. flavus annotated genes. (XLS)

Table S6 Alternative splicing events in A. flavus CA43 mycelia state. (XLS)

Table S7 Antisense transcripts of A. flavus in the mycelia and sclerotia states. (XLS)

Table S8 Genes related to asexual and sexual reproduction in A. flavus. (XLS)

Table S9 Genes expressed differentially between A. flavus mycelia and sclerotia states. (XLS)

Table S10 Transcriptional status of A. flavus secondary metabolism pathways. (XLS)

Acknowledgments

We are grateful to Dr Peng-Kuang Chang (Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Washington, DC) for providing A. flavus strains and for helpful suggestions concerning experimental design.

Author Contributions

Conceived and designed the experiments: YG YL LP BW. Performed the experiments: XW BZ. Analyzed the data: XW CY BW. Wrote the paper: LP BW.

References

1. Yu J, Cleveland TE, Wilkinson JR, Campbell BC, Kim JH, et al. (2006) Aspergillus flavus expressed sequence tags for identification of genes with putative roles in aflatoxin biosynthesis. MycoKeys Res 22: 16–21.
2. Yu J, Whitelegge CA, Nierman WC, Bhatnagar D, Cleveland TE (2004) Aspergillus flavus expressed sequence tags for identification of genes with putative roles in aflatoxin biosynthesis of crops. FEBS Microbiol Lett 237: 333–340.
3. Chang PK, Wilkinson JR, Horn BW, Yu J, Bhatnagar D, et al. (2007) Genes differentially expressed by Aspergillus flavus strains after loss of aflatoxin production by serial transfers. Appl Microbiol Biotechnol 77: 917–925.
4. Yu J, Fedorova ND, Montalbano BG, Bhatnagar D, Cleveland TE, et al. (2011) Tight control of mycotoxin biosynthesis gene expression in Aspergillus flavus by temperature as revealed by RNA-Seq. FEBS Microbiol Lett 322: 145–149.

5. Wilkinson JR, Yu J, Bland JM, Nierman WC, Bhatnagar D, et al. (2007) Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in Aspergillus flavus NRRL 3357 and Aspergillus parasiticus SRRC 143. Appl Microbiol Biotechnol 74: 1308–1319.
6. Chang PK, Bennett JW, Coty PJ (2002) Association of aflatoxin biosynthesis and sclerotial development in Aspergillus parasiticus. Mycopathologia 153: 41–48.
7. Coty PJ (1988) Aflatoxin and sclerotial production by Aspergillus flavus: influence of pH. Phytopathology 78: 1250–1253.
8. Greiner DM, Timberlake WE, Arnold ML (1996) Loss of meiosis in Aspergillus. Mol Biol Evol 13: 809–817.
9. Khalidi, N, Seifeddin, FT, Turner, G, Haft, D, Nierman, WC, et al. (2010) SMURF: Genomic mapping of fungal secondary metabolite clusters. Fungal Genet Biol 47: 736–741.
10. Payne GA, Nierman WC, Wortman JR, Pritchard BL, Brown D, et al. (2006) Whole genome comparison of Aspergillus flavus and A. sojae. Med Mycol 44: 9–11.
11. Horn BW, Dorner JV (1999) Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of Aspergillus flavus along a transect within the United States. Appl Environ Microbiol 65: 1445–1449.
12. Cotty P (1989) Virulence and cultural characteristics of two Aspergillus flavus strains pathogenic on cotton. Phytopathology 79: 808–814.
13. Bayman P, Cotty PJ (1993) Genetic diversity in Aspergillus flavus: association with aflatoxin production and morphology. Can J Bot 71: 29–31.
14. Bennett J, Ferrelle E, Lee L (1976) Effect of light on aflatoxins, arachidonic acids, and sclerotia in Aspergillus flavus and A. parasiticus. Mycologia 70: 104–116.
15. Littley E, Rahe J (1992) Sclerotial morphogenesis in Sclerotium crepivorum in vitro. Can J Bot 70: 772–778.
16. Kawasaki H, Taira K (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature 431: 211–217.
17. Murrell A, Heeson S, Reik W (2004) Interaction between differentially methylated regions and DNA methylation. Nat Rev Genet 5: 433–440.
18. Chang P-K, Hua S (2007) Molasses supplementation promotes conidiation but suppresses aflatoxin production by small sclerotial Aspergillus flavus. Lett Appl Microbiol 46: 241–246.
19. Calvo AM, Bok J, Brooks W, Keller NP (2004) veA is required for toxin and sclerotial development in Aspergillus nidulans. Mol Microbiol 54: 141–157.
20. Malamud-Borloz M, Pfeffer M, Pfeffer S (1995) Molasses supplementation promotes conidiation but suppresses aflatoxin production by small sclerotial Aspergillus flavus. Lett Appl Microbiol 21: 131–137.
21. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, et al. (2005) The transcriptional landscape of the mammalian genome. Science 309: 1559–1563.
22. Parkhomchuk D, Borodina T, Amstislavskiy V, Sirota M, Iwamoto K, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
23. Smith CA, Robertson D, Yates B, Nielsen DM, Brown D, et al. (2008) The effect of 5′-methylation of conserved DNA via enhanced-BLAST and EvoPrinterHD analysis. Genome Biol 9: RESEARCH0083.
24. Wang XJ, Gaasterland T, Chua NH (2005) Genome-wide prediction and identification of cis-natural antisense transcripts in Arabidopsis thaliana. Genome Res 15: 145–155.
25. Parkhomchuk D, Borodina T, Amstislavskiy V, Sirota M, Iwamoto K, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
26. Dillies MA, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, et al. (2012) A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief Bioinform. doi: 10.1093/bib/bbs046.
27. Latchman DS (2001) The role of the glucocorticoid receptors in gene expression. Adv Drug Deliv Rev 46: 131–149.
28. Geisler S, Yang X, Wang D, McKeon C, Wilson B, et al. (2005) Identification of conserved DNA via enhanced-BLAST and EvoPrinterHD analysis. Genome Biol 9: RESEARCH0083.
29. Hauser G, Houthoofd K, Van Der Veken J, Vermeire P (2006) Identification of conserved DNA via enhanced-BLAST and EvoPrinterHD analysis. Genome Biol 9: RESEARCH0083.
30. Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Nat Methods 5: 621–628.
31. Parkhomchuk D, Borodina T, Amstislavskiy V, Sirota M, Iwamoto K, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
32. Li R, Yu C, Li, Yam LW, Yin SM, et al. (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25: 1966–1967.
33. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptional units by RNA-Seq. Nat Methods 5: 621–628.
34. Zeng X, Liu L, Wang D, Su M, McKeon C, et al. (2005) Identification of conserved DNA via enhanced-BLAST and EvoPrinterHD analysis. Genome Biol 9: RESEARCH0083.
35. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, et al. (2005) The transcriptional landscape of the mammalian genome. Science 309: 1559–1563.
36. Beck A, Pedersen AT, Pedersen C, Mikkelsen TS, Bernstein BE, et al. (2005) Dicer and RNAi are required for the initiation of transcriptional gene silencing in human cells. Science 305: 1289–1292.
37. Wang S, Wang X, Zhang Y, Chen J, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
38. Wang X, Zhang Y, Chen J, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
39. Wang X, Zhang Y, Chen J, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
40. Wang X, Zhang Y, Chen J, et al. (2006) WEGO: a web tool for annotation, visualization and analysis in functional genomics studies. Bioinformatics 22: 1625–1627.
41. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, et al. (2005) The transcriptional landscape of the mammalian genome. Science 309: 1559–1563.
42. Wu Y, Jansen WC, Fedorova ND, Bhatnagar D, Cleveland TE, et al. (2011) What can the Aspergillus flavus genome offer to mycotoxin research. Mycologia 2: 210–216.
43. Wang B, Guo G, Wang C, Lin Y, Wang X, et al. (2010) Survey of the transcriptome of Aspergillus flavus via massively parallel mRNA sequencing. Nucleic Acids Res 38: 5075–5087.
44. Latchman DS (2001) The role of the glucocorticoid receptors in gene expression. Adv Drug Deliv Rev 46: 131–149.
45. Li R, Yu C, Li, Yam LW, Yin SM, et al. (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25: 1966–1967.
46. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptional units by RNA-Seq. Nat Methods 5: 621–628.
47. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, et al. (2005) The transcriptional landscape of the mammalian genome. Science 309: 1559–1563.
48. Zeng X, Liu L, Wang D, Su M, McKeon C, et al. (2005) Identification of conserved DNA via enhanced-BLAST and EvoPrinterHD analysis. Genome Biol 9: RESEARCH0083.
49. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, et al. (2005) The transcriptional landscape of the mammalian genome. Science 309: 1559–1563.
72. Sibthorp C, Wu H, Cowley G, Wong PW, Palaima P, et al. (2013) Transcriptome analysis of the filamentous fungus Aspergillus nidulans directed to the global identification of promoters. BMC Genomics 14: 847.
73. Neil H, Malabat C, d’Aubenton-Carafa Y, Xu Z, Steinmetz LM, et al. (2009) Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature 457: 1038–1042.
74. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, et al. (2007) Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat Biotechnol 25: 221–231.
75. Galagan J, Calvo SE, Cuomo C, Ma LJ, Wortman JR, et al. (2005) Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature 438: 1105–1115.
76. Jorgensen TR, Nielsen KF, Arendshorst M, Park J, van den Hondel CA, et al. (2011) Submerged conidiation and product formation by Aspergillus niger at low specific growth rates are affected in aerial developmental mutants. Appl Environ Microbiol 77: 5270–5277.
77. Yamada O, Lee BR, Gomi K, Imura Y (1999) Cloning and functional analysis of the Aspergillus oryzae conidiation regulator gene brlA by its disruption and misscheduled expression. J Biosci Bioeng 87: 424–429.
78. Wieser J, Adams TH (1995) flbD encodes a Myb-like DNA-binding protein that coordinates initiation of Aspergillus nidulans conidiophore development. Genes Dev 9: 491–502.
79. Yu JJ, Cleveland TE, Nierman WC, Bennett JW (2005) Aspergillus flavus genomics: gateway to human and animal health, food safety, and crop resistance to diseases. Rev Iberoam Micol 22: 194–202.
80. Calvo AM (2008) The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet Biol 45: 1053–1061.
81. Georgianna DR, Payne GA (2009) Genetic regulation of aflatoxin biosynthesis: from gene to genome. Fungal Genet Biol 46: 113–125.