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GENOMICS AND PROTEOMICS

Genes differentially expressed by *Aspergillus flavus* strains after loss of aflatoxin production by serial transfers

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Abstract Aflatoxins are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species. Levels of aflatoxins in agricultural commodities are stringently regulated by many countries because of the health hazard, and thus, aflatoxins are of major concern to both producers and consumers. A cluster of genes responsible for aflatoxin biosynthesis has been identified; however, expression of these genes is a complex and poorly understood phenomenon. To better understand the molecular events that are associated with aflatoxin production, three separate nonaflatoxigenic *A. flavus* strains were produced through serial transfers of aflatoxigenic parental strains. The three independent aflatoxigenic/nonaflatoxigenic pairs were compared via transcription profiling by microarray analyses. Cross comparisons identified 22 features in common between the aflatoxigenic/nonaflatoxigenic pairs. Physical mapping of the 22 features using the *Aspergillus oryzae* genome sequence for reference identified 16 unique genes. Aflatoxin biosynthetic and regulatory gene expression levels were not significantly different between the aflatoxigenic/nonaflatoxigenic pairs, which suggests that the inability to produce aflatoxins is not due to decreased expression of known biosynthetic or regulatory genes. Of the 16 in common genes, only one gene homologous to glutathione S-transferase genes showed higher expression in the nonaflatoxigenic progeny relative to the parental strains. This gene, named *hcc*, was selected for over-expression in an aflatoxigenic *A. flavus* strain to determine if it was directly responsible for loss of aflatoxin production. Although *hcc* transformants showed six- to ninefold increase in expression, no discernible changes in colony morphology or aflatoxin production were detected. Possible roles of *hcc* and other identified genes are discussed in relation to regulation of aflatoxin biosynthesis.

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Introduction

Aflatoxins are polyketide-derived carcinogenic secondary metabolites produced primarily by two fungal species, *Aspergillus flavus* and *Aspergillus parasiticus* (Payne and Brown 1998). *A. parasiticus* isolates are typically aflatoxigenic with the percentage of naturally occurring *A. parasiticus* isolates not producing aflatoxins ranging from 3 to 6% (Barros et al. 2006; Horn et al. 1996; Vaamonde et al. 2003). In contrast, *A. flavus* isolates collected in many parts of the world vary considerably in their ability to produce aflatoxins. The percentage of aflatoxin-nonproducing *A. flavus* isolates ranges from zero to nearly 80% (Giorni et al. 2007; Horn et al. 1996; Pildain et al. 2004; Razzaghi-Abyaneh et al. 2006; Takahashi et al. 2004; Vaamonde et al. 2003). *A. flavus*

produces aflatoxin B_1 and B_2, whereas *A. parasiticus* produces aflatoxins G_1 and G_2 in addition to B_1 and B_2. Aflatoxins pose a great risk to human and animal health, and significant economic losses can result from the contamination of agricultural commodities such as corn, peanut, cotton, and tree nuts (Guzman-de-Pena and Pena-Cabriales 2005).

Biosynthesis of aflatoxins by *A. flavus* and *A. parasiticus* involves proteins encoded by genes in a 70-kb gene cluster (Ehrlich et al. 2005; Yu et al. 2004a). A defect in the *cypA* gene of *A. flavus* is responsible for the loss of G_1 and G_2 aflatoxin production (Ehrlich et al. 2004). The production of aflatoxins is influenced by environmental and nutritional factors, such as water activity (Ribeiro et al. 2006; Vaamonde et al. 2006), temperature (Giorni et al. 2007), pH (Keller et al. 1997), and nitrogen source and carbon source (Chang and Hua 2007; Ehrlich and Cotty 2002; Luchese and Harrigan 1993).

Wild-type *A. flavus* strains often degenerate after serial transfers on culture media, resulting in loss of aflatoxin production accompanied by morphological changes such as increased floccose growth, reduced sporulation, and sclerotial production, and a shift in conidial color from green to brown (Bilgrami et al. 1988; Horn and Dorner 2002; Torres et al. 1980). Strain instability associated with laboratory subculturing is common even in the absence of external mutagens. In spite of these observations, we have virtually no molecular understanding of the loss of aflatoxin production in clonal derivatives of aflatoxigenic *A. flavus* strains.

Determination of how many gene expression differences are associated with a phenotype is important for understanding the molecular basis of complex traits. In this study, we sought to identify genes differentially expressed by aflatoxigenic *A. flavus* and nonaflatoxigenic progenies using the recently available *A. flavus* cDNA microarray (Wilkinson et al. 2007a) that represents approximately 40% of the *A. flavus* transcriptome.

Materials and methods

Fungal strains and spore production

A. flavus NRRL 29459, NRRL 29474, and NRRL 29499 are aflatoxigenic strains originating from soil collection in a peanut field (Terrell, Georgia, USA) and belong to vegetative compatibility groups 6, 23, and 28, respectively (Horn and Greene 1995; Horn et al. 1996). Nonaflatoxigenic strains 29459B-20-2 (NRRL 35742), 29474A-20 (NRRL 35743), and 29499A-20 (NRRL 35744) were single spored from cultures after 20 serial transfers of the parental aflatoxigenic strains on potato dextrose agar slants (Horn and Dorner 2002). Wild-type aflatoxigenic *A. flavus* CA14 was the recipient used in the overexpression experiments. Fungal strains were grown at 30°C on V8 agar plates for 5 days for spore production. V8 medium consists of 5% V8 juice [a commercial beverage (Campbell Soup, Camden, NJ, USA) containing eight vegetable juices] and was adjusted to pH 5.2 before autoclaving. Harvested spores from plates were resuspended in 0.01% Triton X-100 solution.

RNA isolation and first-stranded cDNA synthesis

An aliquot of spore suspension from each fungal strain was added to 100 ml potato dextrose broth (PDB, Becton and Dickinson Company, Sparks, MD) to give a final concentration of 5×10^6 per milliliter. The medium containing spores was dispensed (20 ml) onto five Petri dishes (100× 15 mm). Stationary cultures were incubated at 30°C in darkness. After 48- and 72-h growth, mycelium from each of the five culture plates was harvested by filtration, pooled, and rinsed with sterilized distilled water. Mycelia were pulverized to a fine powder with a mortar and pestle in the presence of liquid nitrogen. Total RNA was prepared using TRIzol® reagent (Invitrogen, Carlsbad, CA). Three micrograms of total RNA was used to generate cDNA using Genispheres RT primers with Supercript II, Dithiotreitol, and 5× SuperScript II First Strand Buffer (Invitrogen) following the manufacturer's instructions.

Microarray design

The microarrays used in this study were constructed at The Institute for Genome Research (TIGR, Rockville, MD) with genomic DNA amplicons (approximately 530 bp) amplified using sequence-specific primers designed according to *A. flavus* expressed sequence tag (EST) sequence information (Yu et al. 2004b). A total of 5,002 genes were arrayed at least three times each for a total of 17,991 features. A gene can be represented on the array by more than one sequence, and thus, features are not a direct representation of genes. The arrays were printed by TIGR using a protocol adapted from Hegde et al. (2000) with minor modifications. Polymerase chain reaction (PCR) amplicons were purified using Millipore 96-well size exclusion vacuum filter plates. The purified PCR products were resuspended in water and diluted at a 1:1 ratio with dimethyl sulfoxide (DMSO) before printing. The PCR products were arranged in triplicate at high density on Telechem Superamine aminosilane-coated microscope slides using an Intelligent Automation Systems spotting robot.

Hybridization

Pre-hybridization was performed according to TIGR's protocols (http://pga.tigr.org/sop/M005_1a.pdf) and washed according to post-hybridization instructions for the 3DNA Array 900™ Kit (Genisphere, Hatfield, PA). Hybridizations

were performed using the 3DNA Array 900™ Kit (Genisphere) according to the manufacturer's protocol. Each experiment consisted of one aflatoxigenic parental strain and its 20th generation, nonaflatoxigenic progeny, compared after 48- or 72-h growth. Each comparison was repeated with duplicate dye-flip. cDNA hybridizations were performed overnight at 49°C in Hybridization Cassette's (ArrayIt, TeleChem International, Sunnyvale, CA) using the 2× formamide-based hybridization buffer. Unbound cDNA was removed by washing following Genisphere's instructions. Hybridization of the 3DNA Capture reagent containing the fluorescent dyes were performed using the 2× formamide-based hybridization buffer at 49°C for 4 h in Hybridization Cassette's, followed by washes to remove nonspecific background. Hybridized slides were scanned using a ScanArray5000XL (GSI Lumonics, Packard Biochip, Packard BioScience, Billerica, MA) and the independent TIFF images from each channel were analyzed using TIGR Spotfinder (http://www.tm4.org/spotfinder.html) software program and deposited with NCBI (GSE 8185).

Data analysis

To remove the nonspecific background signals, the raw data were normalized with local regression technique LOWESS (LOcally WEighted Scatterplot Smoothing) using the MIDAS software tool (http://www.tm4.org/midas.html). The LOWESS normalized dye-flips were then combined to minimize any artifacts introduced by the Cy3 or Cy5 dyes. The resulting data were averaged over duplicate gene features on each array for each replicate experiment. As some genes are represented by two separate non-overlapping fragments, a gene may be detected by each fragment. Therefore, they are defined as features so that gene counts will not be over reported. All calculated gene expression ratios were $\log_2$-transformed, and aflatoxin biosynthetic gene expressions were examined by cross comparison between experiments using TIGR MeV (http://www.tm4.org/mev.html). Differentially expressed genes at the 95% confidence level for each reference set were determined by assuming the $\log_2$ ratios for each data set from a normal distribution and by selecting genes with $\log_2$ (ratio) values >1.96 SDs from the mean. This filtration of the significantly expressed genes was conducted using MIDAS, and the resulting lists of the genes were examined further by cross comparison between experiments using TIGR MeV (http://www.tm4.org/mev.html).

Identification of the NAFDI04TV corresponding full-length gene

NAFDI04TV, a glutathione S-transferase related gene, was the only gene expressed at higher levels in the non-aflatoxigenic progenies (see Results). BLAST search of the *A. flavus* and *Aspergillus oryzae* EST and genome sequence databases and BLASTX analysis were carried out to identify the putative full-length gene (*hcc*). A 1.8-kb region containing the *hcc* gene was amplified using High Fidelity Platinum Supermix (Invitrogen) with primers ATTGGTACCTCCAGTGGTTAGT AAGTAT (the *Kpn*I site is underlined) and TATAAGCTTCACTAAACAGA CATCTAGC (the *Hin*dIII site is underlined). The PCR products were digested with *Kpn*I and *Hin*dIII and cloned into the corresponding sites of pPTRII (TaKaRa, Japan), which is an *Escherichia coli–Aspergillus* shuttle vector that replicates autonomously in *Aspergillus* cells (Kubodera et al. 2002). Clones containing the correct insert were verified by PCR and sequencing.

Overexpression of *hcc* in aflatoxigenic *A. flavus*

Vectors containing pPTRII plus the *hcc* gene (pPTRII-hcc) or pPTRII alone as a control were transformed into *A. flavus* CA14. For protoplast preparation, spores were inoculated into 100 ml of modified (2× glucose) Czapek Dox (CD) broth at a concentration of 10^7 per milliliter. The cultures were shaken at 200 rpm for 17 h at 30°C. The resulting mycelia were harvested using a 40-μm nylon cell strainer (BD Biosciences, Bedford, MA) and washed several times with a sterile 0.8 M NaCl solution. Approximately 2 g mycelium was resuspended in 40 ml enzyme digestion solution and incubated in a rotary incubator (65 rpm) at 30°C. The filter-sterilized enzyme solution consisting of 400 mg of lysing enzymes, 100 mg driselase, β-glucuronidase, 0.8 ml cell wall degrading complex (all purchased from Sigma), and 800 μl of 1 M $CaCl_2$ to 40 ml 0.8 M NaCl solution buffered with 10 mM sodium phosphate at pH 6.0. The digestion was allowed to progress for 4 h before protoplasts were harvested by filtering through a 40-μm nylon cell strainer. The protoplasts were pelleted at 2,000 rpm for 5 min, washed twice with the 0.8 M NaCl solution, and resuspended in 200 μl of solution no. 1 (0.8 M NaCl, 10 mM $CaCl_2$, 10 mM Tris–HCl, pH 8.0) followed by the addition of 40 μl of solution no. 2 [40% (*w/v*) PEG4000, 50 mM $CaCl_2$, 50 mM Tris–HCl, pH 8.0]. Approximately, 0.5 g plasmid DNA (pPTRII-hcc or pPTRII) was used in each transformation experiment. The resulting transformation mixtures were mixed gently and placed on ice for 30 min before 1 ml of solution no. 2 was added. The mixtures were then incubated at room temperature for 15 min before 8.5 ml of solution no. 1 was added. The tubes were centrifuged for 5 min at 2,000 rpm to remove PEG. A soft-agar overlay method was used for protoplast regeneration. The protoplasts were incorporated into 8 ml of warm soft CD selection media (CD in 0.8 M NaCl with 0.5% agar and pyrithiamine added at a final concentration of

0.1 μg/ml). Two milliliters of the protoplast-containing medium was loaded on top of each of four prepared CD selection (1.5%) agar plates. The solidified plates were incubated at 30°C. Pyrithiamine resistant transformants were single spored using a transfer needle and streaked onto fresh CD selection medium plates.

Quantitation of the *hcc* gene expression in *A. flavus* transformants

The expression levels of four pPTRII-hcc transformants and four pPTRII control transformants were determined by real-time reverse transcriptase (RT)-PCR in an iCycler iQ5 Multicolor Real Time PCR Detection System (Bio-Rad, Hercules, CA). Stationary cultures were grown for 48 and 72 h on Cove's liquid medium (Cove 1976) supplemented with 0.5% casamino acids and containing pyrithiamine at a final concentration of 0.1 μg/ml. Total RNA was extracted using TRIzol® reagent (Invitrogen) and treated with DNase I. First stranded cDNA was synthesized with a Super-Script™ III First Strand kit (Invitrogen). The quantitative real-time RT-PCR was performed using SYBR Green I. The sequences of the 18S ribosomal RNA primers are CA TTACCGAGTGTAGGGTTCCTAG and CCGCCGAA GCAACTAAGG. The sequences of the *hcc* gene primers are HCC-383F: TTGTGACGACTGATGGGTTT and HCC-499R: TTCTGTCAGACGGAGTTTGG. Amplification conditions were as follows: an initial denaturation step at 95°C for 3 min, followed by 40 cycles, each consisting of denaturation at 95°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 15 s. A melt curve analysis consisting of one step at 95°C for 1 min, one step at 55°C for 1 min, and one step of 81 cycles at 55°C for 30 s was carried out to confirm the specificity of the PCR products. All samples were determined in triplicate. Gene expression levels were normalized to *A. flavus* 18S rRNA gene expression levels at each time point.

Results

Transcription profile of the aflatoxin genes

A total of 5,086 features were expressed across all experiments of the aflatoxigenic parental strains and nonaflatoxigenic progenies. Included in these 5,086 are representative fragments from all aflatoxin biosynthetic and regulatory genes, except *aflA (fas1)* and *aflY (hypA)*, designed from the previously reported aflatoxin gene cluster (Ehrlich et al. 2005; Yu et al. 2004a). To determine whether loss of aflatoxin production by the progenies was related to changes in the expression levels of genes involved in aflatoxin biosynthesis, we analyzed the microarray expression data of these aflatoxin biosynthetic and regulatory genes using TIGR MeV. As seen in Table 1, the aflatoxin pathway genes *aflC* (*pksA*), *aflF* (*norB*), *aflG* (*avnA*), *aflI* (*avfA*), *aflJ* (*estA*), *aflK* (*vbsA*), *aflL* (*verB*),

Table 1 Log_2 values of aflatoxin biosynthetic and regulatory genes detected across all experiments[a]

Gene[b]	48 h D vs A[c]	48 h G vs B[c]	48 h J vs C[c]	72 h D vs A[c]	72 h G vs B[c]	72 h J vs C[c]
aflC (pksA)	−0.207	0.108	−0.033	0.197	−0.051	−0.071
aflF (norB)	−0.200	0.240	−0.024	0.080	0.063	−0.320
aflG (avnA)	0.146	0.155	0.412	0.243	0.133	0.302
aflI (avfA)	−0.328	−0.090	0.042	−0.067	0.029	−0.297
aflJ (estA)	0.230	0.116	0.090	0.199	0.070	−0.025
aflK (vbsA)	0.114	0.049	−0.038	0.079	0.066	−0.117
aflL (verB)	0.053	0.291	−0.215	0.073	0.501	−0.402
aflN (verA)	−0.001	0.221	−0.092	−0.082	0.161	0.056
aflO (omtB)	−0.072	0.124	−0.495	0.190	0.015	−0.201
aflQ (ordA)	−0.145	−0.063	0.146	−0.046	−0.001	-0.002
aflW (moxY)	−.246	0.128	−0.056	−0.071	0.220	−0.026
glcA	−0.290	−0.094	−0.106	0.228	−0.011	0.091
hxtA	0.027	−0.052	−0.016	0.030	−0.062	−0.051
nadA	0.075	−0.042	−0.716	−0.168	−0.231	0.221
aflS (aflJ)	−0.053	−0.190	0.142	0.029	−0.081	−0.109
aflT	−0.027	0.006	−0.123	0.103	0.172	0.123
laeA[d]	−0.032	−0.404	−0.102	0.334	−0.199	−0.001

[a] Values are log_2-transformed data of expression ratios after data normalization, average of dye-flip, and in-slide replications.
[b] Nomenclature from Yu et al. (2004a), named first according to location on cluster and secondary (in parentheses) by function
[c] Strain names are abbreviated A, B, and C respectively for the aflatoxigenic *Aspergillus flavus* NRRL 29459, NRRL 29474, and NRRL 29499, and D, G, and J, respectively, for the nonaflatoxigenic progeny strains *A. flavus* 29459B-20-2, 29474A-20 and 29499A-20.
[d] *laeA* is short for lack of *aflR* expression (Bok and Keller 2004).

aflN (*verA*), *aflO* (*omtB*), *aflQ* (*ordA*), *aflS* (*aflJ*), and *aflW* (*moxY*) were detected in all experiments. The immediately adjacent sugar cluster genes *glcA, hxtA*, and *nadA* (Yu et al. 2000), the transporter gene *aflT* located in the aflatoxin gene cluster (Chang et al. 2004), and the global regulator *laeA* (Bok and Keller 2004) were also detected in all experiments (Table 1). Several genes detected in most, but not all, experiments were *aflE* (*norA*), *aflH* (*adhA*), *aflU* (*cypA*), *aflV* (*cypX*), *aflX* (*ordB*), *aflB* (*hexB*), and *hypB*. The aflatoxin biosynthetic gene *aflM* (*ver1*) was not detected in any experiment. Also detected were several aflatoxin biosynthetic and regulatory gene EST sequences that are assigned function based upon the *A. oryzae* genome. These sequences include three representations of *aflD* (*nor1*) NAFAD40TV (TC8486), NAGAV36TV (TC8486), and NAGBP59TV (TC8487); two *aflJ*-like sequences NAFDK34TV (TC10671) and NAFFA10TV; two tentative norsolorinic acid reductase genes NAFCL59TV (TC9797) and NAFER13TV (TC8710) that do not correspond to *nor1*, *norB*, or *norA,*; a *hypB*-like sequence NAGDO61TV; and an *aflR*-like sequence NAFCO59TV (TC9969) (data not shown).

Genes differentially expressed in aflatoxigenic parents and their nonaflatoxigenic progenies

The distribution of the overlapping ESTs among the three aflatoxigenic parental/nonaflatoxigenic progeny sets is shown in Fig. 1. After filtration of the ESTs and examination by cross comparison using TIGR MeV, 22 significantly expressed features were identified as being in common for the three parental/progeny combinations (Table 2). Of the 22 features, several are located on the same TC (tentative consensus) sequence. For example, NAFBK48TV and NAGAT11TV are on TC8344, NAFDM52TV and NAFBX71TV are on TC8370, and NAFAG10TV and NAGAH49TV are on TC10579. The chromosomal map of *A. flavus* is not yet available; however, due to the high similarity between *A. oryzae* and *A. flavus*, it is possible to map these genes using the *A. oryzae* chromosomal map (Payne et al. 2006). Using the *A. oryzae* RIB40 genome sequence (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao), we confirmed that some of the TCs reside on the same physical spots or are located in the same vicinity on certain chromosomes (Table 3). The three TCs (8341, 8344, and 8352) and the two TCs (8370 and 11819) are at identical locations on chromosomes 2 and 1, respectively. TC9625 and the singleton NAFDK60TV are less than 3 kb apart. Others on chromosomes 1 and 6 are scattered within 300–400 kb regions (Table 3). After consolidation by physical mapping, the number of unique genes was reduced to 16.

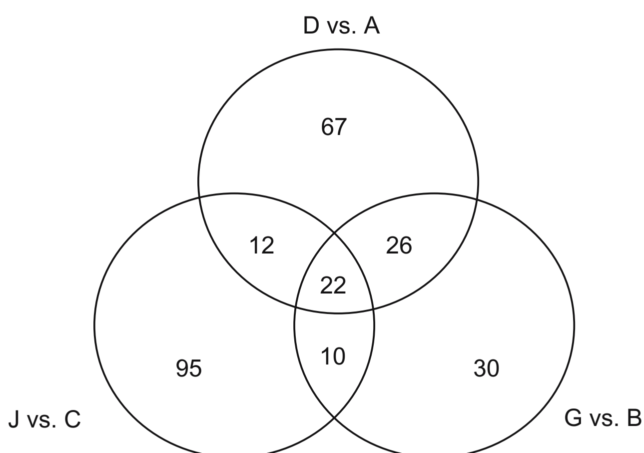

Fig. 1 Venn diagram representing genes showing differential expression from microarray experiments comparing aflatoxigenic parental strains *A. flavus* NRRL 29459, NRRL 29474, and NRRL 29499 (*A*, *B*, and *C*, respectively) and nonaflatoxigenic progenies *A. flavus* 29459B-20-2, 29474A-20, and 29499A-20 (*D*, *G*, and *J*, respectively). Genes are expression ratios of log_2-transformed data differentially expressed at the 95% confidence level. Cross comparison using TIGR MeV corresponds to the 22 significantly expressed features identified as being in common for the three combinations (all shown in Table 2)

Overexpression of a glutathione S-transferase relative gene in aflatoxigenic *A. flavus*

Of the 16 unique genes, the functions of the majority are not clear. Only the singleton NAFDI04TV showed increased expression levels in the nonaflatoxigenic progenies relative to the aflatoxigenic parental strains. To determine if NAFDI04TV plays a direct role in regulation of aflatoxin biosynthesis, the full-length gene was isolated and sequenced (GenBank Accession No. EF512548).

The full length gene corresponding to NAFDI04TV contains three introns and encodes a polypeptide of 217 amino acids. This predicted protein is homologous to members in the glutathione (GSH) S-transferase kappa (GSTK) subfamily and is closely related to the bacterial enzyme, 2-hydroxychromene-2-carboxylate isomerase. Due to this similarity, the gene NAFDI04TV was tentatively named *hcc*. To characterize the role of *hcc* in relation to morphological changes and/or aflatoxin production, we overexpressed *hcc* in aflatoxigenic *A. flavus* CA14. Compared to the averages of the four pPTRII control strains, the average *hcc* expression levels from the four pPTRII-hcc transformants were 6.0- and 8.7-fold at 48 and 72 h, respectively. No discernible changes in culture morphology or aflatoxin production were observed between the control and the *hcc* overexpression transformants when grown on agar plates of CD, CD supplemented with 0.5% casamino acids, or Cove's medium containing ammonium as the sole nitrogen source (data not shown).

Table 2 Log_2 values of the 22 significantly expressed features identified by TIGR MeV[a]

EST number[b]	TC number[b]	Putative function	48 h			72 h		
			D vs A[c]	G vs B[c]	J vs C[c]	D vs A[c]	G vs B[c]	J vs C[c]
NAFCB55TV		Unknown	−1.46	−1.18	−1.07	−1.58	−1.06	−0.89
NAFDI04TV		Related to glutathione S-transferase subunit	0.81	1.72	2.39	0.85	1.31	2.04
NAFDK60TV		Methylenetetrahydrofolate dehydrogenase	−2.32	−2.10	−2.25	−2.49	−2.50	−2.09
NAGAU64TV	TC8341	Unknown	−2.68	−2.79	−3.18	−1.03	−2.37	−3.48
NAFBK48TV	TC8344	Unknown	−2.26	−3.04	−3.79	−2.53	−2.44	−3.61
NAGAT11TV	TC8344	Unknown	−2.12	−1.73	−1.78	−2.04	−1.46	−1.95
NAFDN77TV	TC8352	Unknown	−2.80	−2.65	−3.04	−2.59	−2.19	−3.55
NAFDM52TV	TC8370	Unknown	−2.14	−2.46	−3.07	−1.38	−1.77	−2.47
NAFBX71TV	TC8370	Unknown	−1.63	−2.06	−2.89	−1.79	−1.49	−2.42
NAFCD80TV	TC8646	Unknown	−1.19	−1.00	−0.65	−1.08	−0.65	−1.42
NAFCC04TV	TC9110	Isocitrate lyase	−2.74	−1.26	−1.29	−2.18	−1.32	−1.98
NAGDL82TV	TC9112	Unknown	−2.48	−1.52	−1.49	−2.19	−1.41	−1.10
NAFAH32TV	TC9364	Integrin-like repeats	−3.14	−3.37	−2.26	−2.37	−2.01	−2.16
NAFDK57TV	TC9625	Unknown	−2.54	−1.34	−1.53	−2.59	−1.74	−1.17
NAGDF31TV	TC9632	Unknown	−1.75	−1.06	−2.22	−1.87	−1.65	−2.13
NAFAG10TV	TC10579	Unknown	−1.10	−2.25	−2.44	−1.21	−2.34	−1.73
NAGAH49TV	TC10579	Unknown	−0.86	−1.92	−1.66	−1.07	−2.16	−1.98
NAGCY65TV	TC10658	Unknown	−1.90	−1.69	−2.12	−1.83	−2.12	−1.96
NAGEF45TV	TC10748	Kinesin light chain	−1.00	−1.16	−0.85	−0.50	−1.17	−1.58
NAFDR19TV	TC11018	Cytochrome *P*450	−0.85	−0.79	−1.46	−0.51	−0.58	−1.64
NAFDY45TV	TC11819	Unknown	−1.67	−2.17	−1.99	−1.48	−1.44	−1.64
NAGDM14TV	TC11938	EsdC (cell development)	−2.67	−1.84	−1.66	−2.49	−2.06	−1.33

[a] Genes are expression ratios of log_2-transformed data and are average of dye-flip replications differentially expressed at the 95% confidence level.
[b] DNA sequence labels correspond to expressed sequence tag (EST) and tentative consensus sequences (TC) as per the DFCI *A. flavus* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=a_flavus).
[c] Strain designations are same as Table 1.

Discussion

Gene expression difference among individuals within a population can vary significantly (Oleksiak et al. 2002; Townsend et al. 2003). These individual variations when considered with the multiple environmental factors that can modulate aflatoxin biosynthesis make the task of identifying and isolating regulatory factors a difficult prospect. In our efforts to understand the important phenomena of aflatoxin regulation, we have investigated three independent sets of the *A. flavus* parent/progeny combination in which the progenies had lost their ability to produce aflatoxins after serial transfers. By examining three independently derived nonaflatoxigenic strains, we avoided biases that result from single-strain comparisons.

By comparing multiple strains and minimizing variation due to environmental factors, we have reduced the number of common features (ESTs) between parental and progeny strains (Fig. 1, Table 2). This is clearly seen when numbers of significant common features are compared to other recent microarray studies using the same platforms. Numbers of common features reported in other studies are 77 (Wilkinson et al. 2007a), 56 (Wilkinson et al. 2007b), 144 (O'Brian et al. 2007), 136 (Cary et al. 2007), and over 80 (Wilkinson et al. unpublished data). Only the studies on the *aflR* deletion strain of *A. parasiticus* by Price et al. (2006) was a small, but significant group of 23 genes detected as differentially expressed, with 18 identified within the aflatoxin cluster itself.

These microarray analyses (Price et al. 2006) comparing wild-type and *aflR*-deleted *A. parasiticus* strains showed that expression of aflatoxin biosynthetic genes were at least fourfold higher in the wild type. Our comparisons of the *A. flavus* parental strains to the nonaflatoxigenic progenies showed multiple changes in expression of both biosynthetic and regulatory genes (Table 1). Examinations of maximal changes of expression between experiments at the log2 scale showed that *omtB* expression at 48 h for J vs C to be −0.495 and *verb* at 72 h for G vs B to be 0.501, and changes in the majority of other expressed aflatoxin genes are far below this level (Table 1). However, none of these aflatoxin biosynthetic genes were found to be significantly different by our defined parameters. These results suggest that loss of aflatoxin production by the progenies is not caused directly by altered expression levels of the aflatoxin biosynthesis genes. Serial transfers of non-sporulating

Table 3 *A. oryzae* chromosomal location of significantly expressed microarray features[a]

A. flavus[b]	*A. oryzae* chromosome/ SC number[c]	Position in SC[c]	Other studies[d]
TC11819	1/009	245,866–245,206	
TC8370	1/009	245,888–245,206	Trp, *sec−*
NAFDK60TV	1/009	374,971–374,192	
TC9625	1/009	377,760–378,200	
TC10658	1/009	405,702–405,206	Trp, *veA*
TC9110	1/009	595,140–595,831	
NAFCB55TV	1/009	794,785–794,182	
TC8341	2/001	187,831–188,740	Trp, *sec−*
TC8344	2/001	187,878–188,571	Trp, *sec−*
TC8352	2/001	187,885–188,571	Trp, *sec−*
TC9364	2/003	2,540,207–2,539,082	Trp, *sec−*
TC9632	4/012	109,631–108,930	
TC10579	4/102	412,057–412,784	Temp
TC10748	5/111	2,261,532–2,260,699	
TC9112	6/020	1,121,773–1,122,740	*sec−*
NAFDI04TV	6/038	1,232,903–1,233,318	
TC8646	6/038	1,383,256–1,383,890	*veA*
TC11938	6/038	1,547,898–1,548,640	*sec−*
TC11018	8/103	1,242,950–1,241,741	*veA*

[a] Location determined using *A. oryzae* RIB40 genome sequence (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao)

[b] DNA sequence labels correspond to expressed sequence tag (EST) and tentative consensus sequences (TC) as per the DFCI *A. flavus* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=a_flavus).

[c] Chromosome number, supercontig (SC) number and the nucleotide positions in *A. oryzae* SC corresponding to the indicated *A. flavus* TC or singleton are shown.

[d] In previous and ongoing microarray studies, genes have been detected as significant, Trp (Wilkinson et al. 2007a), *sec−* (Wilkinson et al. unpublished), *veA* (Cary et al. 2007), and Temp (O'Brian et al. 2007).

mycelial macerates of *A. parasiticus sec+* (for secondary metabolism plus) strains also yielded *sec−* variants that exhibit altered phenotypes and an inability to produce aflatoxin intermediates (Kale et al. 1996). As in the results of the present study, these *sec−* variants still produce transcripts of aflatoxin genes, such as *aflR*, *aflD*, and *aflP* (Kale et al. 2003).

Although only a few microarray studies have been conducted on *A. flavus* and *A. parasiticus*, it is important to note that more than half of the differentially expressed genes identified in this study are also described as significant in several other studies (Table 3) (Wilkinson et al. 2007a, b; Cary et al. 2007; O'Brian et al. 2007). Of these, only three, TC9364, TC11018, and TC11938, have been assigned tentative functions. In addition, of the 15 genes showing reduced expression in the progenies, many are apparently located in very close physical proximity.

As seen in Table 3, the TC8341/8344/8352 gene and TC9364 are in close proximity on chromosome 2, while the TC8370/11819 forms a cluster on chromosome 1 with NAFDK60TV, TC9625, TC10658, TC9110, and NAFCB55TV. A second cluster of TC9112, NAFDI04TV (*hcc*), TC8646, and TC11938 are found on chromosome 6. Although most of the functions of these genes are not yet known, some, if not all, genes in relatively close proximity may be subject to similar levels of regulation or selection.

Of the genes with assigned functions, TC9364 encodes an integrin-like protein. The expression of TC9364 in *A. flavus* was significantly decreased when grown in YES medium supplemented with tryptophan which decreases aflatoxin production (Wilkinson et al. 2007a). It also was differentially expressed by *A. parasiticus sec−* strains that have lost the ability to produce aflatoxins (Wilkinson, unpublished). Integrin is an integral membrane protein in the plasma membrane of cells. A function of integrin proteins is the signal transduction from the extracellular matrix to the cell (Giancotti and Ruoslahti 1999). Nutritional and physiological cues may trigger changes in primary metabolism, cell cycle, and differentiation, and may indirectly affect aflatoxin production. TC11938 is homologous to *esdC*, a gene required for sexual development in *Aspergillus nidulans*. Homologs of *esdC* also have been found in asexual *Aspergillus fumigatus, A. oryzae*, *Aspergillus niger*, and *A. terreus*. The findings of decreased expression of *esdC* by the *A. parasiticus sec−* strains and the nonaflatoxigenic *A. flavus* progenies suggest that *esdC* is involved in other processes such as conidiation or growth.

Nonaflatoxigenic progenies *A. flavus* 29459B-20-2, 29474A-20, and 29499A-20 all exhibited a decrease in conidiation on potato dextrose agar (PDA) plates (data not shown). In addition, *A. flavus* 29474A-20 and 29499A-20 had white fluffy sectoring and reduced radial growth, respectively. Loss of aflatoxigenicity has been correlated with alterations in conidiation in *A. parasiticus* (Guzman-de-Pena and Ruiz-Herrera 1997). In *A. nidulans*, conidiation increases with each progressive step along the biosynthetic pathway to sterigmatocystin, the penultimate precursor in aflatoxin formation (Wilkinson et al. 2004). However, a decrease in aflatoxin production by either *A. flavus* or *A. parasiticus* did not cause changes in conidiation (Wilkinson et al. 2007a). No known conidiation-specific genes or genes of the G-protein signaling pathway that controls aflatoxin production and conidiation (Hicks et al. 1997) were differentially expressed in the nonaflatoxigenic *A. flavus* progenies. One possibility that cannot be excluded is that some of these genes might be overlooked because the *A. flavus* cDNA microarray represents only about 40% of the total genes transcribed.

The singleton NAFDI04TV (*hcc*) corresponds to a gene encoding a predicted protein belonging to glutathione

(GSH) S-transferase kappa (GSTK) subfamily (Nebert and Vasiliou 2004). GSTK is a member of the GST family but has little sequence similarity to the other members of the family. It catalyzes the transfer of the thiol group of GSH to electrophilic substrates and is specifically located in the mitochondria and peroxisomes. GSTK is presumed to have a protective role during respiration when large amounts of reactive oxygen species (ROS) are generated.

A series of oxidative steps are involved in the formation of dihydrobisfuran that leads to the formation of highly oxygenated aflatoxins by *Aspergillus* species. A lack of exposure to competitive and stressed conditions in nature may contribute to *A. flavus* losing aflatoxin production and wild-type morphological characters in the laboratory (Bilgrami et al. 1988). Horn and Dorner (2002) showed in a series of laboratory experiments that adverse environmental conditions (high temperature, low pH, and nutrient deprivation), but not competition with yeast and filamentous fungi, help maintain aflatoxigenicity over successive generations during serial transfers. The adaptive value of aflatoxin production is not fully understood, but synthesis of aflatoxins may act as a defense mechanism against oxidative stress. Studies have demonstrated that antioxidants reduce aflatoxin production (Kim et al. 2006) and that a positive correlation exists between ROS accumulation and aflatoxin production by *A. flavus* and *A. parasiticus* (Mahoney et al. 2006; Narasaiah et al. 2006; Reverberi et al. 2005). Thus, an elevated level of GSTK activity may help to combat the oxidative stress caused by ROS accumulation when the progenies have lost the ability to synthesize aflatoxins after serial transfers on nutrient-rich PDA medium.

Genome-wide comparison studies have shown that the majority of differentially expressed genes identified are apparently not correlated with a particular phenotype and that a phenotype may be affected by interactions among multiple genes (Fay et al. 2004; Lehner 2007; Yoshimoto et al. 2002). Similarly, we showed that the single gene (NAFDI04TV termed *hcc*), identified as exhibiting significantly increased expression in three separately derived nonaflatoxigenic *A. flavus* progenies, does not directly control aflatoxin biosynthesis, but instead, may play a role in combating oxidative stress. However, confirmation of this specific gene's involvement in oxidative stress or other physical adaptation must await further functional analyses. In addition, we have identified a subset of genes that appear significant through the various *A. flavus* and *A. parasiticus* microarray experiments. These include multiple unknowns, *esdC* (TC11938) and an integrin-like gene (TC9364) that await future characterization to help resolve the complex expression networks associated with *A. flavus* aflatoxin biosynthesis.

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