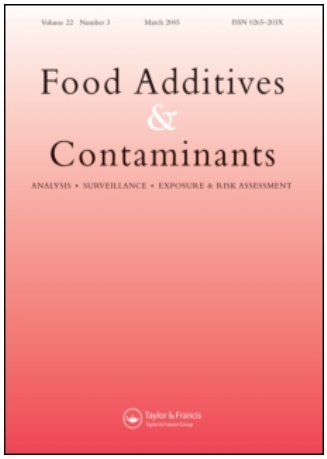


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# Gene profiling for studying the mechanism of aflatoxin biosynthesis in **Aspergillus flavus** and **A. parasiticus**

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# Gene profiling for studying the mechanism of aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus*

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**Abstract**

Aflatoxins are toxic and carcinogenic polyketide metabolites produced by certain fungal species, including *Aspergillus flavus* and *A. parasiticus*. Many internal and external factors, such as nutrition and environment affect aflatoxin biosynthesis; therefore, we analyzed the transcriptome of *A. flavus* using expressed sequence tags (ESTs) from a normalized cDNA expression library constructed from mycelia harvested under several conditions. A total of 7218 unique ESTs were identified from 26 110 sequenced cDNA clones. Functional classifications were assigned to these ESTs and genes, potentially involved in the aflatoxin contamination process, were identified. Based on this EST sequence information, a genomic DNA amplicon microarray was constructed at The Institute for Genomic Research (TIGR). To identify potential regulatory networks controlling aflatoxin contamination in food and feeds, gene expression profiles in aflatoxin-supportive media versus non-aflatoxin-supportive media were evaluated in *A. flavus* and *A. parasiticus*. Genes consistently expressed in several aflatoxin-supportive media are reported.

**Keywords:** Aspergillus flavus, *mycotoxin, aflatoxin, expressed sequence tags (EST), microarray, gene profiling, aflatoxin biosynthesis, genetic regulation, pathogenesis*

## Introduction

*Aspergillus flavus* and *A. parasiticus* are the two major species that produce aflatoxins (Cotty et al. 1994; Cotty 1997; Bennett and Klich 2003), the toxic and most carcinogenic natural secondary metabolites known. *A. flavus* is the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage (Cotty et al. 1994; Bennett and Klich 2003). The acute toxicity and carcinogenic properties of aflatoxins have been recognized for over 40 years (Lancaster et al. 1961). Due to the significant health and economic impacts of aflatoxin contamination, the chemistry, enzymology and genetics of the aflatoxin biosynthetic pathway in *A. flavus* and *A. parasiticus* have been actively studied (Cleveland and Bhatnagar 1990; Bhatnagar et al. 1991; Bennett et al. 1997; Yabe and Nakajima 2004; Yu 2004; Yu et al. 2004b, 2005) to decipher the biological mechanism of toxin production. Studies on aflatoxin biosynthesis and its genetic regulation in *A. flavus* and *A. parasiticus* led to the cloning of 25 clustered genes within a 75-kb DNA region responsible for enzymatic conversions in the aflatoxin biosynthetic pathway (Yu et al. 2004a, 2004b). Regulatory genes within the gene cluster, such as

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*aflR* and *aflS* (*aflJ*), have also been cloned (Chang et al. 1993; Payne et al. 1993; Meyers et al. 1998) and their mode of action on activating aflatoxin pathway gene expression reported (Chang et al. 1995; Chang 2003). Fungal nutritional and environmental factors (Payne and Brown 1998; Yu et al. 2003; OBrian et al. 2007), as well as stage of fungal development (Calvo et al. 2002; Calvo et al. 2004), were also found to affect aflatoxin formation. However, the global regulatory control of aflatoxin formation beyond its biosynthetic gene cluster and the process of signal transduction in turning aflatoxin production on or off remains unknown. The *A. flavus* EST project identified 7218 unique ESTs (Yu et al. 2004c). Genes that are potentially involved in aflatoxin formation have been categorized (Yu et al. 2004c) and include those in the aflatoxin biosynthetic pathway, signal transduction/global regulation, virulence/pathogenicity of the fungus and stress response/antioxidation (Yu et al. 2004c). We report here additional categories of genes that are potentially involved in secondary metabolism, transport or drug resistance, and fungal development or sporulation. These genes may also be important in aflatoxin formation. Microarray technology is a powerful tool in profiling genes or regulatory components that are differentially expressed under specific conditions. Previous works using microarray for profiling aflatoxin biosynthetic genes have been very successful (OBrian et al. 2003; Price et al. 2005; Wilkinson et al. 2007; OBrain et al. 2007). Price et al. (2005) using targeted 763 gene-element microarray studied the gene expression under conducive and non-conducive conditions of carbon source, nitrogen source, pH and temperature in *A. parasiticus*. In a more focused investigation on carbon source effects, we studied the gene expression in both *A. flavus* and *A. parasiticus*, as well as *A. oryzae*, under two sets of carbon source media, YES vs. YE and GMS vs. PMS. In this report, we present, in addition to the results of EST data (unpublished due to page limitation in a previous publication), the genes consistently expressed in seven experiments in several medium shift experiments in *A. flavus* and *A. parasiticus*, as well as *A. oryzae*.

## Materials and methods

### *Fungal strains and culture conditions*

*A. flavus* NRRL 3357 (ATCC# 20026; *A. flavus* SRRC 167), the wild type strain widely used in laboratory and field studies, was selected for making the EST library. To make the library as representative as possible for gene expression, fungal mycelia were grown in eight separate media and collected at five time points (Table I), as reported earlier (Yu et al. 2004c). The mycelia were harvested by filtration through miracloth at five different time points (18, 24, 36, 48, 72, 96 h) following inoculation with a conidial suspension. The harvested mycelial samples were mixed and frozen in liquid nitrogen for RNA purification.

For microarray experiments, *A. flavus* NRRL 3357, *A. flavus* #13, *A. parasiticus* SRRC 143 (ATCC # 56775 or SU-1) and *A. oryzae* RIB 40 were chosen. *A. flavus* NRRL 3357 is the same strain that was used for the EST (Yu et al. 2004c) and for the whole genome sequencing project (Payne et al. 2006). *A. flavus* #13 belongs to S strain that produces small sclerotia and large amount of aflatoxins (Cotty, personal communication). *A. parasiticus* SRRC 143 is a wild-type strain used widely in laboratories for molecular genetic studies on aflatoxin biosynthesis. *A. oryzae* RIB 40 is a food grade organism that is widely used in fermentation industry (Machida et al. 2005).

### *Normalized cDNA library construction, sequencing and annotation*

A normalized cDNA expression library was constructed by Incyte Genomics Inc. (Palo Alto, CA, USA) with the mixed mycelia described in Table I as starting material. Single pass, unidirectional (5′ end) sequencing was completed at TIGR on ABI 3700 sequencing machines. The sequence assembly was performed using the CAP3 program (Huang and Madan 1999) and Paracel Transcript Assembler (version 2.6.2, http://www.paracel.com) with modifications by the TIGR bioinformatics team (Yu et al. 2004c).

Table I. Fungal mycelia collected from cultures and at various time-points (in g).

| Culture media | 18 h | 24 h | 48 h | 72 h | 96 h | Aflatoxin production |
|---|---|---|---|---|---|---|
| Wheat bran solid | 20 | 20 | 20 | 20 | 20 | Supportive |
| GMS liquid (Glucose minimal salt) | 10 | 10 | 10 | 10 | 10 | Supportive |
| PMS liquid (Peptone minimal salt) | 10 | 10 | 10 | 10 | 10 | Non-supportive |
| PDB (Potato dextrose broth) | 10 | 10 | 10 | 10 | 10 | Supportive |
| Rice solid | – | – | – | – | 10 | Supportive |
| YES liquid | – | – | 10 | 10 | – | Supportive |
| GMS + oil | – | – | 10 | – | – | Supportive |
| PMS + oil | – | – | 10 | – | – | Supportive |

*Culture conditions and RNA isolation for microarray experiments*

Erlenmeyer flasks containing 100 ml of the appropriate media were inoculated with 100 µl of $10^8$ spores to obtain a final concentration of $10^5$ spores $ml^{-1}$. The cultures were maintained at 30°C with constant shaking at 150 rpm for 48 or 96 h. The four types of culture media used were yeast extract (YE), yeast extract sucrose (YES), peptone minimal salt (PMS) and glucose minimal salt (GMS). The fungal mycelia were harvested by filtration through miracloth and frozen in liquid nitrogen, then ground to a fine powder in liquid nitrogen using mortar and pestle. Total RNA was extracted from approximately 100 mg of ground mycelium in 1 ml of TRIZOL® (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and visualized by gel electrophoresis to ensure quality.

*Microarray design and experiments*

The genomic DNA amplicon microarray used in this study was constructed at The Institute for Genome Research (TIGR, Rockville, MD, USA) using sequence-specific primers designed according to *A. flavus* EST annotation (Yu et al. 2004c). A total of 5031 genes were arrayed at least three times, each for a total of 18 078 features or spots. A gene can be represented on the array by more than one sequence, thus, features are not a direct representation of genes. The microarrays were printed at TIGR. PCR amplicons were purified using Millipore 96-well size exclusion vacuum filter plates. The purified PCR products were resuspended in water and diluted 1 : 1 with DMSO before printing. Using an Intelligent Automation Systems spotting robot, the PCR products were arranged in triplicate at high density on Telechem Superamine aminosilane-coated microscope slides. The outline of the microarray experiments are shown in Table II. The microarray hybridization was performed by aflatoxin-producing medium vs. non-producing medium. Probe labeling by dye-flips was made to balance dye bias during labeling. No 96-h time-point *A. flavus* and *A. oryzae* samples were used in array hybridization due to degradation of RNA samples at the later growth stage. Data from the 48-h time-point from *A. parasiticus* SRRC 143 was obtained. Consistent comparative results were demonstrated only by the 48-h in *A. flavus* (*A. oryzae*) with 96-h *A. parasiticus* under different medium conditions

*cDNA synthesis, labeling and hybridization*

Three similar protocols were used in cDNA synthesis, labeling and hybridization for comparison, including The Institute for Genomic Research Aminoallyl RNA labeling protocol and microarray hybridization protocol (http://pfgrc.tigr.org/protocols.shtml); 3DNA Array 900 protocol (Genisphere Inc., Hatfield, PA, USA); and Array 900 MPX™ protocol (Genisphere Inc., Hatfield, PA, USA). Pre-hybridization was performed according to TIGR's protocols. When using the 3DNA Array 900™ and Array 900 MPX™ kit, the manufacturer's post-hybridization instructions were followed. Each experiment consisted of one sample grown in an aflatoxin-productive condition coupled to a sample from a non-productive condition. Each hybridization was repeated with duplicate dye-flip.

*Data acquisition and analysis*

Labeled and washed slides were scanned using either a ScanArray5000XL (GSI Lumonics, Packard Biochip, Packard BioScience Company, Billerica, MA, USA) or a GenePix 4000B (Axon Instruments) and the independent TIFF images from each channel were analyzed using TIGR Spotfinder (http://pfgrc.tigr.org/tools.shtml). To remove non-specific background signals, the raw data were normalized using a local regression technique, LOWESS (LOcally WEighted Scatterplot Smoothing) with the MIDAS software tool (http://pfgrc.tigr.org/tools.shtml). The LOWESS normalized dye-flips were then combined to minimize any artifacts introduced by the Cy3 or Cy5 dyes;

Table II. Microarray experimental design.

| Experiment | Strain | Hybridization protocol | Time-point (h) | Array pair |
|---|---|---|---|---|
| 1 | *A. flavus* NRRL 3357 | TIGR | 48 | GMS vs. PMS |
| 2 | *A. oryzae* RIB40 | TIGR | 48 | GMS vs. PMS |
| 3 | *A. flavus* #13 | Array 900 | 48 | YES vs. YE |
| 4 | *A. parasiticus* SRRC 143 | Array 900 | 96 | YES vs. YE |
| 5 | *A. parasiticus* SRRC 143 | Array 900 | 96 | GMS vs. PMS |
| 6 | *A. parasiticus* SRRC 143 | Array 900 MPX | 96 | YES vs. YE |
| 7 | *A. parasiticus* SRRC 143 | Array 900 MPX | 96 | GMS vs. PMS |

the resulting data were averaged over duplicate gene features on each array for each replicate experiment. Some genes are represented by two separate non-overlapping fragments, therefore, since a gene may be detected by each fragment, they are defined as features so that gene count 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 (Saeed et al. 2003) (http://pfgrc.tigr.org/tools.shtml). To determine genes that were significantly differentially expressed across all experiments, only those genes with $\log_2$-transformed expression levels equal to or higher than 1.96 standard deviations from the mean (95% confidence) were selected. This filtration of 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 (Saeed et al. 2003) (http://www.tm4.org/mev.html).

## Results and discussion

### *EST sequences obtained*

Of more than 26 000 sequenced *A. flavus* cDNA clones, 19 618 high-quality EST sequences were obtained and submitted to the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). The functional classification of these ESTs has been presented in Gene Index by The Institute for Genomic Research (TIGR, http://www.tigr.org; currently is managed by The Dana Farber Cancer Institute at the web site http://compbio.dfci.harvard.edu/tgi). The high-quality sequences were assembled into 7218 unique ESTs consisting of 3749 tentative consensus sequences (TCs) and 3469 singletons (Yu et al. 2004c). These unique ESTs represent putative expressed genes and account for an estimated 60% of the predicted 12 000 functional genes in the *A. flavus* genome (Payne et al. 2006).

### *Genes coregulated with aflatoxin biosynthesis*

Several categories of genes that are presumably involved in aflatoxin biosynthesis, either directly or indirectly, have been published previously (Yu et al. 2004c), such as genes associated with the aflatoxin biosynthetic pathway, global regulation and signal transduction, virulence/pathogenicity, or stress response and antioxidation. The additional categories of ESTs, having homology to sequences in the existing GenBank databases and not published in a previous paper due to page limitation, are presented in Tables III–V below.

Fungal developmental processes, such as sporulation and sclerotia formation, are related to secondary metabolite formation (Calvo et al. 2002; Chang et al. 2002), and mutants deficient in sporulation are unable to produce aflatoxins (Calvo et al. 2002). The genes involved in fungal development and conidiation were identified in the *A. flavus* EST library (Table III). *A. flavus* is known to be resistant to almost all of the antibiotics available in the market, making it difficult for targeted mutagenesis using an antibiotic-resistant marker system. This feature also presents a significant obstacle for developing effective fungicides to protect crops, animals and even human beings from fungal infections. The aflatoxin pathway cluster gene *aflT* may be involved in aflatoxin efflux by pumping toxins out of fungal cells, has been cloned in both *A. flavus* and *A. parasiticus* (Yu et al. 2004a). However, our study indicates that its function may be insignificant to aflatoxin production or secretion (Chang et al. 2004). Many ESTs with homologies to genes that confer drug resistance have been identified (Table IV). The identification of these transporters could help explain why *A. flavus* is resistant to most

Table III. Genes involved in fungal development/sporulation.

| EST ID | Hit accession # | Putative function | Organism | % ID | % Sim | *E*-value |
|---|---|---|---|---|---|---|
| NAGAZ57TV | PIR\|S29903 | Cell wall proline-rich protein | *Hedera helix* | 38.89 | 52.78 | 0.00012 |
| NAGBG48TV | GB\|BAA35140.1 | Chitinase | *Emericella nidulans* | 68.55 | 84.68 | 1.30E-44 |
| TC10379 | GB\|AAO50865.1 | Similar to *Leishmania major*. Ppg3 | *Dictyostelium discoideum* | 19.67 | 41.84 | 0.045 |
| NAGBA10TV | GB\|AAD34461.1 | Ascus development protein 3 | *Neurospora crassa* | 36.19 | 54.29 | 2.70E-10 |
| NAGBD49TV | SP\|P40552 | Cell wall protein TIR3 precursor | *Saccharomyces cerevisiae* | 27.97 | 45.45 | 4.20E-06 |
| NAGBU14TV | SP\|P10169 | Conidiation-specific protein 8 | *Neurospora crassa* | 41.11 | 51.11 | 5.90E-08 |
| TC10246 | SP\|P10169 | Conidiation-specific protein 8 | *Neurospora crassa* | 41.11 | 51.11 | 5.80E-08 |
| TC11956 | SP\|P10713 | Conidiation-specific protein 10 | *Neurospora crassa* | 71.6 | 80.25 | 2.40E-25 |
| TC12083 | SP\|P10713 | Conidiation-specific protein 10 | *Neurospora crassa* | 67.86 | 73.21 | 7.00E-12 |
| NAFEA74TV | GI\|585141 | Developmental regulator *flbA* | *Emericella nidulans* | 93 | 96 | 1.00E-126 |
| NAGBI96TV | SP\|O60032 | Conidiophore development protein *hymA* | *Emericella nidulans* | 89.66 | 95.4 | 4.50E-39 |
| TC8878 | SP\|O60032 | Conidiophore development protein *hymA* | *Emericella nidulans* | 90.83 | 95.83 | 3.30E-53 |

Table IV. Genes involved in transport and drug resistance.

| EST ID | Hit accession # | Putative function | Organism | % ID | % Sim | *E*-value |
|---|---|---|---|---|---|---|
| NAFCX67TV | GB\|CAB46647.1 | Putative multiple drug resistance protein | *Penicillium olsonii* | 60.42 | 77.60 | 5.50E-60 |
| NAGCB21TV | GB\|CAC28822.2 | Multidrug resistance-associated protein | *Neurospora crassa* | 52.56 | 67.31 | 6.70E-30 |
| NAGDC32TV | SP\|P32767 | Pleiotropic drug resistance regulatory protein 6 | *Saccharomyces cerevisiae* | 36.43 | 57.36 | 1.20E-08 |
| NAFCV74TV | GB\|CAD21494.1 | Conserved hypothetical protein | *Neurospora crassa* | 48.61 | 70.83 | 4.80E-11 |
| NAGCB47TV | GB\|CAD71016.1 | Probable mportin-alpha export receptor | *Neurospora crassa* | 51.20 | 72.80 | 1.20E-27 |
| TC11203 | GB\|CAD70375.1 | Probable fluconazole resistance protein | *Neurospora crassa* | 55.62 | 69.10 | 9.00E-51 |
| TC11795 | PIR\|T30882 | Multidrug resistance protein 1 | *Aspergillus flavus* | 100 | 100 | 0 |
| TC9584 | GB\|AAA50353.1 | Metal resistance protein | *Saccharomyces cerevisiae* | 49.70 | 72.26 | 4.30E-94 |
| TC9769 | SP\|P32386 | ATP-dependent bile acid permease | *Saccharomyces cerevisiae* | 56.28 | 74.49 | 9.80E-75 |
| TC10317 | GB\|CAD56485.1 | Fructose facilitator | *Zygosaccharomyces bailii* | 70.18 | 81.58 | 1.00E-81 |
| TC8832 | PIR\|G83503 | Probable MFS transporter PA1131 | *Pseudomonas aeruginosa* | 25.62 | 43.84 | 0.043 |

Table V. Genes potentially involved in secondary metabolism.

| EST ID | Hit accession # | Putative function | Organism | % ID | % Sim | *E*-value |
|---|---|---|---|---|---|---|
| NAGCA18TV | SP\|P32637 | Glyceraldehyde 3-phosphate dehydrogenase | *Podospora anserina* | 79.67 | 89.43 | 3/E-48 |
| NAGCA40TV | GB\|BAB49569.1 | NADH-dependent dyhydrogenase | *Mesorhizobium loti* | 29.63 | 50.62 | 0.038 |
| NAGCB08TV | GB\|AAF26281.1 | Monooxygenase | *Aspergillus parasiticus* | 100 | 100 | 2.30E-26 |
| NAGCB61TV | SP\|O47950 | NADH-ubiquinone oxidoreductase 19.3 kDa subunit | *Neurospora crassa* | 92.42 | 96.21 | 1.60E-66 |
| NAGCB73TV | GB\|CAC87049.1 | Mitochondrial NADH : ubiquinone oxidoreductase | *Bos taurus* | 38.79 | 61.21 | 1/E-14 |
| NAGCO63TV | GB\|AAF26274.1 | NADH oxidase | *Aspergillus parasiticus* | 87.32 | 92.25 | 1.30E-60 |
| NAGCQ74TV | PIR\|T38538 | Probable oxidoreductase | *Schizosaccharomyces pombe* | 43.07 | 64.23 | 4.40E-24 |
| NAGDC76TV | GB\|AAD53257.1 | Dioxygenase alpha subunit | Australian soil clone OD16 | 28.81 | 49.15 | 0.92 |
| TC10309 | GB\|BAC20334.1 | Monooxygenase | *Aspergillus oryzae* | 98.69 | 99.22 | 1.20E-213 |
| TC10899 | GB\|AAF26280.1 | Cytochrome *P*450 monooxygenase | *Aspergillus parasiticus* | 98.35 | 100 | 4.60E-63 |
| TC8634 | PIR\|S55328 | Serine-type carboxypeptidase precursor | *Aspergillus phoenicis* | 80 | 85.79 | 1.40E-79 |
| TC8800 | SP\|P36060 | NADH-cytochrome b5 reductase precursor | *Saccharomyces cerevisiae* | 42.72 | 61.17 | 3.70E-49 |
| TC10233 | SP\|P41747 | Alcohol dehydrogenase I | *Aspergillus flavus* | 99.71 | 99.71 | 2.10E-184 |
| NAGBA01TV | GB\|AAD13655.1 | T-2 toxin biosynthesis protein | *Fusarium sporotrichioides* | 45.11 | 63.59 | 6.50E-32 |
| TC8750 | PIR\|AF3007 | Epoxide hydrolase | *Agrobacterium tumefaciens* | 33.33 | 46.53 | 3.80E-07 |

of the antibiotics available and the difficulty in developing antifungal drugs effective in the filamentous fungi, especially *A. fumigatus* (Moore et al. 2000). Preliminary assembly of the *A. flavus* whole genome sequence revealed at least 65 genes encoding for ABC-2 type transporter family proteins (Payne et al. 2006); these proteins could play important roles in multi-drug resistance.

*Aspergillus flavus* produces many types of secondary metabolites, of which aflatoxins are the most notorious. In addition to the genes that are directly involved in aflatoxin biosynthesis, we have identified a category of genes that are putatively involved in the biosynthesis of secondary metabolites, such as those encoding polyketide synthases, fatty acid synthases, carboxylases, dehydrogenases, reductases, oxidases, oxidoreductases, epoxide hydrolases, mono- or di-oxigenases, cytochrome *P*450 monooxygenases and methyltransferases (Yu et al. 2004b). In addition to aflatoxin pathway genes, we have identified many ESTs that could be involved in the biosynthesis of additional secondary metabolites (Table V). Preliminary annotation of the *A. flavus* genome indicated that there are a number of gene clusters or genes that potentially encode for enzymes involved in secondary metabolite production (Payne et al. 2006), including 35 putative polyketide synthases, 24 putative non-ribosomal peptide synthases and over 130 cytochrome *P*450 enzymes (Payne et al. 2006).

Table VI. Genes expressed in common under several aflatoxin-producing carbon source media in *A. flavus* and *A. parasiticus* as detected by microarray experiments.

| Experiment | | 1<br>TIGR<br>*Af* 3357<br>48 h | 2<br>TIGR<br>*Ao* RIB 40<br>48 h | 3<br>Array 900<br>*Af* #13<br>48 h | 4<br>Array 900<br>*Ap* 143<br>96 h | 5<br>Array 900<br>*Ap* 143<br>96 h | 6<br>Array 900MPX<br>*Ap* 143<br>96 h | 7<br>Array 900MPX<br>*Ap* 143<br>96 h |
|---|---|---|---|---|---|---|---|---|
| TIGR EST# | Putative function | GMS vs. PMS | GMS vs. PMS | YES vs. YE | YES vs. YE | GMS vs. PMS | YES vs. YE | GMS vs. PMS |
| NAFAY22TV | Indolepyruvate decarboxylase | 3.8079698 | 5.1009502 | 3.6314514 | 5.8113933 | 3.7582371 | 4.3477554 | 2.0790656 |
| NAFCO95TV | Peptide transporter ptr2-a | −3.3024704 | −4.6637373 | −5.3831525 | −5.291969 | −5.321002 | −2.8490677 | −3.7928588 |
| NAFDC06TV | Indolepyruvate decarboxylase | 3.7094684 | 5.2063975 | 3.7822225 | 5.8895564 | 3.9719558 | 3.5608275 | 3.0449924 |
| NAFDH31TV | Antigenic cell wall protein MP1 | −2.991371 | −4.1788764 | −3.7679496 | −2.872515 | −4.855476 | −2.4336514 | −3.0006616 |
| NAGAC91TV | RNA export mediator GLE1 | 1.9787863 | 2.296317 | 3.9615142 | 3.5454264 | 2.7678428 | 2.688219 | 1.4570416 |
| NAGAG12TV | Unknown | 4.1175394 | 4.962635 | 3.6760864 | 4.8225274 | 4.4131126 | 3.859379 | 2.7965271 |
| NAGAS67TV | Unknown | 4.1640916 | 5.1858807 | 3.8899755 | 4.9367576 | 4.2946796 | 3.6684997 | 3.2086217 |
| NAGAT37TV | Aspartate aminotransferase | −4.972739 | −5.4448504 | −4.886115 | −3.8674343 | −4.5452228 | −2.6529703 | −2.184085 |

Notes: The numbers are log2 ratios showing gene expression up-regulation (positive values) and down-regulation (negative values). Log2 = 2 represents a 4-fold increase in gene expression; log2 = 3 represents an 8-fold increase in gene expression; log2 = 4 represents a 16-fold increase in gene expression, and so on.

*Genes profiled through microarray analysis under aflatoxin-supportive carbon source media*

Gene profiling experiments using microarrays were performed under aflatoxin-supportive in contrast to non-supportive conditions. A total of 2340 scorable expressed features were detected across all experiments. All of the aflatoxin pathway genes and associated sugar cluster genes were detected in this comparison in most, but not all experiments (data not shown). However, the aflatoxin pathway regulatory genes *aflR* and *aflS (aflJ)* (Chang et al. 1993; Payne et al. 1993; Meyers et al. 1998) were not detected in any of the experiments, possibly as levels of their gene transcripts are not high enough to be detected by microarray. However, the expression levels of the regulatory genes *aflR* and *aflS* were high enough to be detected by RT-PCR and real-time PCR methods in previous experiments performed in this laboratory (data not shown). Even under non-aflatoxin-supportive medium condition (such as PMS), a very low level of *aflR* transcript was detected by the sensitive RT-PCR procedure in *A. flavus* (unpublished data and Chang, personal communication). The *aflR* transcript level and, hence, the AflR regulator protein under the non-supportive condition is too low to activate the transcription of aflatoxin biosynthetic pathway structural genes for producing aflatoxins. After filtration of expressed genes at the 95% confidence level and examination by cross comparison using TIGR MeV program, five genes that were consistently up-regulated in response to the carbon source and three genes down-regulated, including two genes with unknown function, were identified as being in common for all of the culture conditions (Table VI). It is apparent that these genes are most likely involved in primary metabolism rather than directly involved in secondary metabolism. These genes could be co-regulated and co-expressed with aflatoxin pathway genes under aflatoxin-producing conditions. It could also represent shift from one metabolic pathway to another, more favourable for secondary metabolite formation, such as aflatoxins. More importantly, these genes could be the links between primary and secondary metabolism. The exact nature of biological involvements of these up-and down-regulated genes can only be deciphered by gene knock-out experiments. It is also interesting to find that these genes are consistently expressed in *A. oryzae* RIB 40, the non-aflatoxin producer used in food fermentation, indicating that the three species, *A. flavus*, *A. parasiticus* and *A. oryzae* share a common biological pathway response to carbon-rich medium. The non-toxigenicity of *A. oryzae* is most likely due to loss of ability to produce aflatoxins at a later stage of its regulatory hierarchy.

## Conclusion

EST and microarray analyses are robust tools for profiling genes involved in specific biological processes. However, for certain low level expressed genes such as regulatory genes, more sensitive protocols are needed for future research. The genes identified in this study by EST and microarray techniques are putative candidates for further investigation. Without additional biological evidence, it is very difficult to predict their exact biological functions. Traditional targeted mutagenesis and high throughput gene knock-out systems are necessary to elucidate the functions of a large number of genes in the post-genome era.

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