Genomic analysis of aflatoxin biosynthesis

Gary A. Payne*1, Ahmad M. Fakhoury*2, Kimberly A. Scheidegger*1, Michael S. Price*1 and Gregory R. O'Brien*1

*1 Department of Plant Pathology, North Carolina State University (Raleigh, NC 27695-7567, USA)
*2 Current address: Southern Illinois University (Carbondale, IL, USA)

Summary

Aflatoxins are toxic and carcinogenic secondary metabolites produced only by a few species of Aspergillus. Growth of these fungi on a number of plant host species can result in aflatoxin contamination and an unmarketable product. No plant genotype with adequate resistance to aflatoxin contamination is commercially available. The application of genomic sciences to better understand the factors that regulate aflatoxin biosynthesis and describe the interaction of A. flavus with its hosts holds promise for identifying new target sites for control of aflatoxin contamination. Gene expression analysis, along with gene disruption and protein-protein interaction studies, is being used in our laboratory to identify new regulatory circuits in aflatoxin biosynthesis. A targeted DNA microarray composed of 753 elements is being used to identify genes expressed during aflatoxin biosynthesis under a number of physiological conditions. A comparison of conducive and non-conducive conditions for aflatoxin production based on nitrogen source, time, and genetic regulation in A. flavus has identified several genes differentially expressed during aflatoxin biosynthesis. Many of the differently expressed genes have no known function. Two genes are currently being examined for a role in aflatoxin biosynthesis, an alkyl hydroperoxide reductase (afahpl) and a 14-3-3 gene homolog (mafl). Disruption of (mafl) resulted in loss of aflatoxin production. Yeast two-hybrid analysis identified several genes (including some members of our EST library) that potentially interact with mafl. The function of these genes in aflatoxin biosynthesis is being evaluated.

Key words : mycotoxins, microarrays, 14-3-3

Introduction

Aflatoxin contamination of crops in the field remains an economic concern for
growers and producers in the United States, and a health concern for many parts of the world. Epidemiological data show a strong correlation between incidence of primary liver cancer in humans and consumption of contaminated maize\(^1\). More than 40 years have lapsed since the discovery of aflatoxin in the feed that resulted in the deaths of thousands of turkeys\(^2\). Research during this period has greatly increased our understanding of the biosynthesis of aflatoxin and its toxic and carcinogenic effects on animals. Effective and practical control practices, however, are still lacking. In US alone it is estimated that aflatoxin contamination of corn and peanuts results in losses that exceed $272 million yearly\(^3\).

Management strategies that reduce losses are available, but these practices are not adequate in years conducive for aflatoxin contamination. Biocontrol procedures using a non-aflatoxigenic strain of *A. flavus* have proven to be effective in reducing aflatoxin levels in cotton seed\(^4\). No such strategy has been commercialized for any other crops affected, including maize, peanuts, and tree nuts. Traditionally, host plant resistance has proven to be the most successful method of control of plant diseases. While progress has been made in identifying genotypes of maize, peanuts, and tree nuts with some resistance to aflatoxin contamination, resistant commercial genotypes are not available. Difficulty in identifying resistance genes and the movement of these genes into desirable genotypes has limited the development of resistant plants. Certainly, the identification of genetic targets could help in screening for resistance genes.

The aflatoxin pathway represents one of the best-studied pathways of fungal secondary metabolism. Aflatoxins are synthesized by only five Aspergillus species: *A. flavus*, *A. parasiticus*, *A. pseudotamarii*, *A. bombycis*, and *A. nomius*\(^5-7\). However, a number of fungi produce sterigmatocystin (ST), the penultimate compound in the aflatoxin (AF) pathway. Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*, and sterigmatocystin biosynthesis in *A. nidulans*, led to the cloning of 17 genes responsible for 12 enzymatic conversions in the aflatoxin/sterigmatocystin pathways. All of the known genes for both aflatoxin and sterigmatocystin biosynthesis are clustered within 70kb of DNA\(^6-11\); this information has been well described in several review articles\(^6,10,12\). Transcriptional control of the pathway genes is regulated by the activity of *aflR*, a zinc binucleate cluster DNA binding protein\(^6\). The AF and ST pathways appear to follow a similar biosynthetic process up to the formation of ST, and thus information gained from both pathways has been used to construct a common biosynthetic scheme.

Although it has long been noted that biosynthesis of natural products is usually associated with cell differentiation or development, the mechanism of this connection was not clear. A critical advance in this regard was the recent finding that the regulation of sporulation and ST production is by a shared G-protein-mediated growth pathway in *A. nidulans*\(^13\). Mutations in *A. nidulans* *flbA* and *fadA* genes, early acting members of a
G-protein signal transduction pathway, resulted in failure to produce ST, ST gene transcripts, and spores \(^1\). Recent results have shown that this regulation is partially mediated through protein kinase A \(^2\). Furthermore, the effect of fadA mutations on *A. parasiticus* and *A. flavus* sporulation and aflatoxin production \(^3, 4\) provides evidence for the maintenance of conserved genetic mechanisms governing fungal differentiation processes. Recent evidence suggests that the signaling through FadA is mediated in part by a cAMP-dependent protein kinase, PKA. PKA has been reported to negatively regulate aflR both transcriptionally and post-transcriptionally \(^5\). \(Ca^{2+}\) calmodulin-mediated protein phosphorylation also has been reported to influence aflatoxin production \(^6\). In addition to these controls, aflR is autoregulated \(^7\). AflR also appears to interact with the product of another pathway gene, aflJ, to enhance the transcription of genes in the early steps of the pathway \(^8\).

Less is known about the effect of host factors on aflatoxin production. Burrow et al.\(^9\) proposed that lipoxygenases affect the ability of species of *Aspergillus* to produce aflatoxin. Their data show that the products of lipoxygenases, mainly the 13S-hydroperoxy fatty acids, interfere with aflatoxin and sterigmatocystin biosynthesis by mimicking psi factors (precocious sexual inducers) in Aspergilli. Psi factors are believed to be necessary for the fungus to reach a state of competence precluding conidiation or toxin production \(^9\). Recently, a protein was identified from mature maize seed that inhibits aflatoxin formation but not growth \(^10, 11\). These data indicate that host factors directly regulate both growth and aflatoxin production by the fungus.

Aflatoxin biosynthesis is also regulated by a number of physiological factors including pH, carbon and nitrogen source, redox potential, adenylate concentration, and energy charge \(^3\). pH regulation appears to be mediated through PacC \(^2\), although the details of this regulation are not yet known. Less understood are how the other physiological factors regulate aflatoxin and ST biosynthesis. It is interesting that while the overall regulation of ST production in *A. nidulans* and aflatoxin production in *A. flavus* appear similar, the two pathways respond differently to carbon, nitrogen, and temperature. High sugar, low temperature, and ammonium nitrogen sources favor aflatoxin biosynthesis but repress ST production \(^3\). These differences likely result from an adaptation of the two strains to different ecological niches. For this reason, understanding the regulation of aflatoxin by these factors may be important in designing control strategies for this fungus.

A goal of our research program is to better understand the regulation of aflatoxin biosynthesis by physiological and plant host factors. Because the regulation of aflatoxin is complex, we have chosen to employ a genomics approach to address this problem. Our approach is to use DNA microarrays to first identify new genes involved in aflatoxin biosynthesis and, second, to study their expression during aflatoxin biosynthesis. Because the genome sequence of *A. flavus* is yet to be completed, we chose to do our
studies with a targeted array of 753 elements.

**Gene expression analysis**

*Construction of the EST library and DNA microarray*  cDNA clones obtained from a library enriched in genes expressed during aflatoxin biosynthesis were sequenced to construct an EST library of 753 elements. The protocol used has been described previously. Briefly, *A. flavus* grown in stationary culture on peptone mineral salts and glucose was harvested during the peak of aflatoxin biosynthesis. RNA was extracted and used to construct a cDNA library. Over 90,000 clones from this library were arrayed on nylon filters. Hybridization of duplicate filters with either mRNA from a toxin producing or non-producing culture identified clones for sequencing. The PCR products from the sequenced ESTs were spotted on glass slides and hybridized with mRNA from cultures grown under different conditions.

At the onset of this study we anticipated that many genes would be induced during aflatoxin production. To enrich for candidate genes with a direct role in aflatoxin production we have chosen to select those genes that are expressed during aflatoxin production under more than one physiological condition. We realize that we may overlook some genes that are uniquely expressed during a certain condition and would be of extreme interest to us, but because gene disruption remains a bottleneck in functional genomics we wanted to start with a few candidate genes. Our initial studies have focused on three conditions in which a comparison can be made between cultures that either produce or do not produce aflatoxin. The comparisons are: nitrate vs. ammonium; 8 hrs after resuspension vs. 24 hrs after resuspension; and a wild type strain vs. a dominant activating FadA mutant strain.

*Developmentally expressed genes*  Cultures of *Aspergillus parasiticus* were grown for 36 hours on yeast extract with sucrose (YES) and resuspended into fresh YES. Cultures harvested at 8 hours (no aflatoxin production) and at 24 hrs (aflatoxin producing) were evaluated for gene expression. Eighteen genes were more highly expressed prior to aflatoxin production and 24 genes were more highly expressed during aflatoxin production. Of these 24 genes, no predicted function could be ascribed to 18 of the genes.

*Differentially expressed genes on nitrate and ammonium nitrogen sources*  To study the role of nitrogen source in the regulation of aflatoxin biosynthesis, we decided to employ cDNA microarray technology to explore the differences in gene expression between NO₃ and NH₄ grown cultures. In order to determine what transcriptional variation exists between nitrogen sources, we harvested RNA from tissue grown with either NH₄ or NO₃ as the nitrogen source. These RNA samples were labeled and probed with our glass slide microarrays.
Differences in transcriptional profiles were seen between the treatments. All the aflatoxin pathway genes represented on the array were differentially expressed between the treatments, with the exception of aflR. The lack of differential expression of aflR could be due to the overall low level of pixel intensities observed. Thirty-five of the differentially expressed genes on the array had no significant homology to anything in either the nucleotide or protein databases. Twenty-seven of these genes were up regulated in response to NO₃, and eight were up regulated in response to NH₄.

**Differentially expressed genes in comparison between a FadA mutant and a wild type strain**  
Comparative gene expression analysis of an *Aspergillus flavus* FadA mutant (obtained from Nancy Keller, University of Wisconsin) and wild type strain provided results consistent with previous experimental findings. The dominant activating mutation in fadA blocked conidiation and aflatoxin production in the mutant strain. DNA microarrays were used to compare gene expression profiles between the FadA mutant strain and the wild type strain. Probes were constructed from mRNA isolated from liquid cultures grown under conditions conducive for aflatoxin production. Two microarrays printed on a single slide were probed with cDNA labeled with cy3 and cy5 in a Latin Square Design experiment. Based on paired T-test analysis, 331 genes were differentially expressed between the FadA mutant and the wild type (df = 5; p < 0.01). 186 genes showed higher levels of expression in wild type than in the FadA mutant. These genes included the aflatoxin biosynthetic genes nor-1, ver-1, and omtA as well as the pathway specific regulator aflR. Of the 186 genes, 89 have no known function. In contrast, 145 genes were shown to be more highly expressed in the FadA mutant than in the wild type strain. 137 genes showing differential expression under either condition have no known function.

**A common set of genes is expressed during more than one condition**  
A goal of our research is to compile a list of genes that are more highly expressed in all conditions conducive to aflatoxin production. To date, we have found three genes that are not in the aflatoxin biosynthetic cluster that are more highly expressed in each of the three conducive conditions tested. These genes show no homology to sequences in the public databases. The function of these genes is not yet known. We have also found a number of genes that are more highly expressed in more than one aflatoxin conducive condition. These genes may also be involved in aflatoxin production. Subsequent studies are planned to more carefully examine each condition for genes expressed specifically in one physiological condition.

**Functional analysis of candidate genes**

**afahp1, a functional homolog of yeast AHPI**  
One gene positively associated with aflatoxin production is *afahp1*. This gene was identified from a subset of genes that were
more highly expressed during aflatoxin-producing conditions in both the developmental study and the FadA study. The deduced amino acid sequence of *afahp1* (168aa) has 86% identity with the *Aspergillus fumigatus* allergen Asp f 3 and 36% identity with an alkyl hydroperoxide reductase from *Saccharomyces cerevisiae* (Ahp1p). A peroxisomal localization has been suggested for Asp f 3 on the basis of sequence and functional homology to peroxisomal membrane proteins from *Candida boidinii*25, and Ahp1p contains a putative peroxisomal signal sequence at the C-terminus26. Peroxisomes hold some interest for researchers in the area of secondary metabolism. The actual site of aflatoxin biosynthesis within the cell remains unknown, and peroxisomes have been proposed as a potential site for the activity of this metabolic pathway27. However, visualization of a GFP-Ahp1p fusion protein and immunoblot analysis point to a cytoplasmic localization28, and sequence analysis of Afahp1 indicates that it is also likely to be present in the cytosol.

In an effort to assign putative functions to Afahp1 and to Anaahp1, a predicted homolog of Ahp1p in *A. nidulans*, a *S. cerevisiae* AHPI mutant strain29 was transformed with overexpression constructs containing coding sequences for *afahp1*, *anaahp1*, or the native AHPI gene or with a plasmid with no insert. In yeast, Ahp1p has been described as a hydroperoxide reductase that functions to reduce alkyl hydroperoxides using the thioredoxin system. The antioxidant activity of Ahp1p serves to protect the cell against metals that can generate reactive oxygen species and induce lipid peroxidation30. The specificity of Ahp1p for organic peroxides is visualized by the increased sensitivity of the AHPI mutant strain to tert-butyl hydroperoxide and cumyl hydroperoxide26, 28, 30. Each of the yeast transformants described above were tested against a range of concentrations of tert-butyl hydroperoxide to determine whether the genes from *A. flavus* and *A. nidulans* could rescue the sensitivity observed in the mutant strain. Preliminary results indicate that *afahp1* and *anaahp1* are able to protect the yeast cells against toxicity induced by exposure to tert-butyl hydroperoxide.

**maf1, a 14-3-3 homolog** Sequence comparisons between sequences in our EST library and sequence databases revealed the presence of a 14-3-3 homolog (*maf1*). Disruption of this gene in *A. flavus* by site-directed mutagenesis abolished aflatoxin production. Aflatoxin production was restored in the mutant transformed with a wild type copy of the gene. The maf1 mutant was morphologically similar to the wild type strain, but had reduced conidiation and growth on some media.

14-3-3 proteins are widespread in nature and are involved in many different aspects of cell biology. Their role in plants has been well studied, and members of this protein family have been implicated in the regulation of nitrogen, carbohydrate, amino acid, and lipid metabolism in plants. Holtman *et al*31 showed that a 14-3-3 protein interacts with a 13-S-lipoxygenase from barley in a phosphorylation-dependent manner. Lipoxygenases are instrumental in the dioxygenation of polyunsaturated fatty acids, an important step in
lipid metabolism. As discussed earlier, lipoxygenases have been shown to influence aflatoxin formation.

The maf1 mutation also adversely affected conidial germination. A role of a 14-3-3 proteins in the regulation of conidial germination has recently been proposed in A. nidulans where the over-expression of ArtA, the 14-3-3 homologue in A. nidulans, caused a delay in the polarization of conidiospores\textsuperscript{32}. Our preliminary data showed that upon disruption of ArtA, the generated mutant strain was unable to produce sterigmatocystin, an intermediate in aflatoxin biosynthesis.

We are currently in the process of identifying potential partners interacting with MAF1 using a yeast 2-hybrid approach. We have also investigated a possible interaction between MAF1 and AflR, the aflatoxin pathway specific regulator, since it contains (RXSX(S/T)XP), a 14-3-3 putative binding domain\textsuperscript{33}. However, our preliminary data does not provide evidence for a direct interaction between the two proteins.

**Conclusion**

Gene expression analysis is a powerful tool for studying complex gene interactions, allowing for the simultaneous measurement of expression levels of thousands of genes. Such an approach is ideal for studies on aflatoxin biosynthesis because its regulation involves overlapping regulatory pathways. Our current studies have initially focused on the use of a DNA microarray for the identification of genes. Using this approach, we have identified a number of candidate genes for further characterization. An alkyl hydroperoxide reductase has been identified from expression profiles linking the gene to aflatoxin in two independent experiments. Functional analysis in yeast has shown that afahpl from A. flavus and anahpl, the respective gene in A. nidulans, may have antioxidant activity similar to the AHP1 gene in S. cerevisiae. Gene disruption experiments in the two Aspergillus species are planned in order to further characterize the functions of these genes. Also, we have shown that another candidate gene, maf1, has a role in aflatoxin biosynthesis. Disruption of this gene results in loss of aflatoxin production. 14-3-3 proteins often participate in protein-protein interactions. Yeast 2-hybrid analysis has identified several putative partners for maf1. Two of these genes are represented in our EST library.

Our approach has been to use a targeted DNA microarray containing elements from a cDNA library enriched in transcripts for genes expressed during aflatoxin biosynthesis. This approach has proved successful. Such an array contains sufficient elements to identify novel genes and at the same time keeps the number of candidate genes to a small number that is manageable for functional genomics. As we begin gene expression profiling, it will be desirable to have arrays with a larger number of elements.

The ability to evaluate a complete array of A. flavus genes soon will be possible.
Funding has been obtained from the Microbial Genome Sequence Project, USDA/National Research Initiative to complete a whole genome sequence and assembly of *A. flavus*. The sequencing will be done at The Institute for Genomics Research (TIGR). The USDA/ARS is providing EST sequencing information to aid in the annotation. Manual annotation and data release will be done at North Carolina State University under the supervision of Gary Payne and Ralph Dean. Information on the progress of the sequencing project can be found at http://aspergillusflavus.org.

**References**

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