

# The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster

Michael S. Price<sup>1</sup>, Jiujiang Yu<sup>2</sup>, William C. Nierman<sup>3</sup>, H. Stanley Kim<sup>3</sup>, Bethan Pritchard<sup>1</sup>, Carrie A. Jacobus<sup>1</sup>, Deepak Bhatnagar<sup>2</sup>, Thomas E. Cleveland<sup>2</sup> & Gary A. Payne<sup>1</sup>

<sup>1</sup>North Carolina State University, Center for Integrated Fungal Research and Department of Plant Pathology, Raleigh, NC, USA; <sup>2</sup>Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture, New Orleans, LA, USA; and <sup>3</sup>The Institute for Genomic Research, Rockville, MD USA

**Correspondence:** Gary A. Payne, North Carolina State University, Center for Integrated Fungal Research and Department of Plant Pathology, Box 7567, Raleigh, NC 27695-7567, USA. Tel.: +1919 515 6994; fax: +1919 513 0024; e-mail: gary\_payne@ncsu.edu

**Present address:** Michael S. Price, Duke University Medical Center, Department of Molecular Genetics and Microbiology, Durham, NC 27710, USA.

Received 12 September 2005; revised 9 November 2005; accepted 22 November 2005. First published online 10 January 2006.

doi:10.1111/j.1574-6968.2005.00084.x

Editor: Derek Jamieson

## Keywords

transcription profiling; microarray analysis; aflatoxin; secondary metabolism; *Aspergillus flavus*; *Aspergillus parasiticus*.

## Introduction

The biosynthesis of the potent carcinogen, aflatoxin (AF), has been studied extensively and molecular analysis has identified a biosynthetic gene cluster (Yu *et al.*, 2004a) regulated by the transcription factor AflR (Yu *et al.*, 1996; Flaherty & Payne, 1997). AflR is transcriptionally and post-transcriptionally regulated (Shimizu *et al.*, 2003), and is believed to regulate itself (Ehrlich *et al.*, 1999). AflR is absolutely required for AF biosynthesis. It is hypothesized that AflR regulates only genes in the biosynthetic cluster, but until now it has not been possible to test this hypothesis.

In this study we sought to identify genes regulated by AflR via gene expression comparisons between *Aspergillus parasiticus* SU1 and a  $\Delta$ aflR mutant created from the same *A. parasiticus* strain. A cDNA microarray representing *c.* 40% of the *A. flavus* transcriptome was hybridized with cDNA

## Abstract

Aflatoxin contamination of food and feed is a major concern due to the carcinogenic properties of this mycotoxin. Previous studies using classical approaches have identified a cluster of genes responsible for aflatoxin production under the control of the pathway-specific transcriptional regulator *aflR*, but it is unknown whether *aflR* controls expression of other genes within the genome. Transcription profiling comparing wild type and  $\Delta$ aflR strains of *Aspergillus parasiticus* grown under conditions conducive for aflatoxin production identified only 23 upregulated genes in the wild type. These included 20 genes in the aflatoxin biosynthetic cluster, and three additional genes outside the aflatoxin biosynthetic cluster (*nadA*, *hlyC*, and *niiA*), all with AflR binding sites. This report is the first to demonstrate genes outside the biosynthetic cluster as being associated with *aflR* expression.

made from RNA of both *A. parasiticus* strains grown under conditions conducive for AF production. This comparison is possible because *A. flavus* and *A. parasiticus* share nearly identical sequence and conserved gene order in the AF biosynthetic cluster.

## Materials and methods

### Strains and culture conditions

*Aspergillus parasiticus* (Speare) strain SU1 (ATCC 56775) and a  $\Delta$ aflR derivative of SU1 (courtesy of Dr Jeff Cary, USDA-ARS, SRRC) were grown for biomass production in media conducive for AF production as described previously (Price *et al.*, 2005). Cultures to be harvested for RNA extraction were incubated at 28 °C, 200 r.p.m. for 8, 16, or 24 h. Five cultures were inoculated per strain for the 8 h

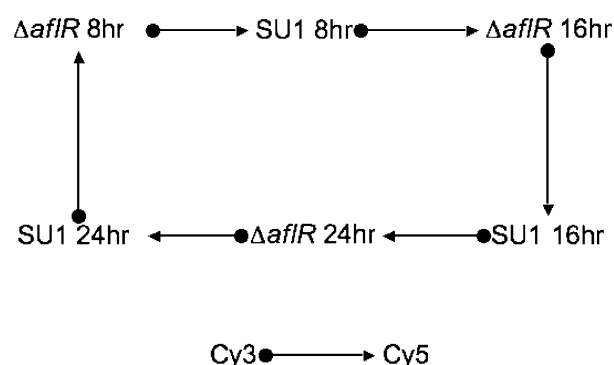
timepoint, two cultures per strain for the 16 h timepoint, and one culture per strain for the 24 h timepoint in order to procure sufficient tissue for RNA extraction. Two biological replications of the experiment were performed, with the resulting data combined and analyzed.

### RNA preparation and microarray analysis

Tissue handling and RNA preparation were performed as previously described (Price *et al.*, 2005), with the following modifications: after isolation of total RNA with Trizol (Invitrogen Life Technologies, Carlsbad, CA), the RNA was precipitated overnight in 2 M LiCl. After centrifugation, the RNA was washed once with 75% ethanol, centrifuged, and allowed to air dry for 20 min. The RNA pellet was resuspended in 50  $\mu$ L DEPC-dH<sub>2</sub>O with 40 units RNasin<sup>TM</sup> RNase inhibitor (Promega Corporation, Madison, WI) and quantified by spectrophotometry. RNA preparations were visualized by gel electrophoresis to ensure quality.

The microarrays used in this study were printed at The Institute for Genome Research (TIGR) with amplicons (c. 530 bp) generated from genomic DNA using primers selected from EST sequences (Yu *et al.*, 2004c). A total of 5002 genes, including 31 known AF and sugar utilization cluster genes, were arrayed in three duplications (or more) for a total of 17 991 spots. Total *A. parasiticus* RNA from each treatment studied was converted to cDNA and labeled as previously described (Price *et al.*, 2005).

Comparisons between treatments were made using a loop design (Fig. 1). This design allowed *in silico* comparisons between each node in the loop (Churchill, 2002). Furthermore, each treatment was labeled with each dye, removing effects on measurements caused by the individual dyes. The



**Fig. 1.** Loop design for microarray analysis of the  $\Delta aflR$  strain vs. wild type. Comparisons were made between strains at each timepoint. RNA samples from each strain/timepoint combination were isolated and cDNAs were labeled with Cy3 (solid dot) or Cy5 (arrowhead) and hybridized to the microarray. In this design, each sample is labeled once with each dye to correct for bias in dye incorporation. The construction of this comparison loop allows for *in silico* comparisons between any two treatments.

hybridized slides were scanned using a Perkin Elmer ScanArray Express Lite scanner (Perkin Elmer Life and Analytical Sciences Inc., Boston, MA). Spot intensity data were extracted from the images using UCSF-Spot (Jain *et al.*, 2002). The resulting spot-intensity data were then analyzed statistically using the mixed procedure in SAS (SAS v8, SAS Institute, Cary, NC) as described previously (Price *et al.*, 2005). Briefly, least squares estimates of gene-specific treatment effects between pairs of treatments were obtained for each gene under each treatment condition. Differences between treatment effects (least-squares estimates) for pairs of conducive and nonconductive conditions can be considered as  $\log_2$ -transformed fold changes (Wolfinger *et al.*, 2001). Genes were considered significantly up- or down-regulated in the individual microarray experiments if they possessed *P*-values less than (0.001). The value  $P < 0.001$  was chosen based on the maximum *P*-value exhibited by the AF pathway genes included on the array.

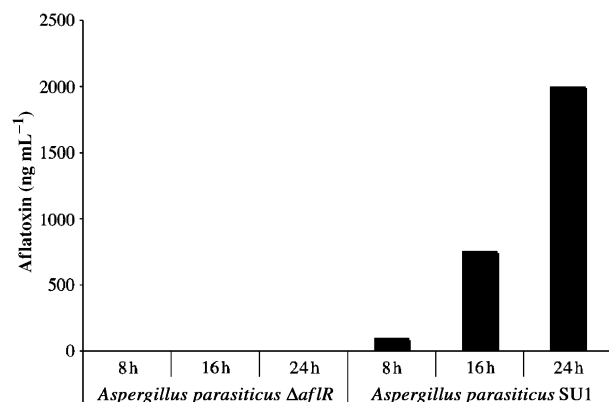
### Quantitative PCR confirmation of microarray results

Real time quantitative PCR analysis was used to support the gene expression data obtained from the microarray experiments. Primers were designed to amplify 65–72 bp amplicons of genes that were differentially expressed at 24 h: *aflD* (5'-GCTGCAGCAGTCCAAGCAA-3' and 5'-CATGTTGGTGATGGTGCTGATC-3'), *aflM* (5'-GTGGGCCTCCCTGTGGAT-3' and 5'-CACTTACCCATTCGGCTGTGT-3'), *hypB* (5'-CGACAAGTTGACCCGACTGA-3' and 5'-GAGCGTC-TATGGGCTTGCA-3'), *aflO* (5'-GCTGGGATGATCTGCTTCAAG-3' and 5'-ATTTGCGTCATGTCTTCCATGA-3'), *aflP* (5'-TGAAGCGCTGCCAATCCT-3' and 5'-AGCAAGTCGCGCATCCTT-3'), *nadA* (5'-CTCCCAGGACGCGGTAGAT-3' and 5'-ACGGTCAATCGCCTTTCG-3'), *niia* (5'-GCGTAATTCGAGCTCAATG-3' and 5'-AAGTTGCGA-TATTCATAGCCTCATC-3'), and *hlyC* (5'-TGACTGTTTGC TCGGAGAATGA-3' and 5'-GCTAGCCATCTGTTCCACGATAGC-3'). The  $2^{-\Delta\Delta C_T}$  method was used to analyze the real time quantitative PCR data (Livak & Schmittgen, 2001). For each strain, 18S control values were subtracted from raw  $C_T$  values to normalize the data. Normalized  $C_T$  values for each gene in the *aflR* deletion strain were subtracted from the corresponding wild type normalized  $C_T$  values to obtain a  $\log_2$  value. The fold change was calculated by raising 2 to the power represented by the  $\log_2$  value for each gene.

## Results and discussion

### AF production in $\Delta aflR$ strain and SU1

Aflatoxin was detected in SU1 cultures at all three timepoints, reaching a concentration of 2010 ng mL<sup>-1</sup> at 24 h.



**Fig. 2.** Aflatoxin production in cultures of *Aspergillus parasiticus* SU1 and  $\Delta$ *aflR*. Cultures of *A. parasiticus* SU1 and  $\Delta$ *aflR* mutant strains were grown for 16 h at 28 °C for mycelia production. In all, 20 mL aliquots of these cultures were then inoculated into daughter cultures and incubated for 8, 16 and 24 h at 28 °C, 200 r.p.m. for the production of tissue and aflatoxin. RNA samples were prepared from these tissues for microarray analysis.

(Fig. 2). The time course for AF production was similar to that observed in previous studies (Skory *et al.*, 1993). As predicted, no AF was detected in cultures of the  $\Delta$ *aflR* mutant at any of the three time points examined.

### Transcription profiling of SU1 and $\Delta$ *aflR* strains

An analysis of gene transcription at each of the three time points using the microarrays identified only 23 genes more highly expressed in SU1 than the  $\Delta$ *aflR* mutant at every timepoint examined (Table 1, Fig. 3). The trends in expression for eight of these genes were confirmed by quantitative PCR (Table 2). Eighteen of the genes differentially expressed on the microarrays are reported AF biosynthetic genes, three of these genes (*hypB*, *aflY*, and *nadA*) are in or adjacent to the established AF cluster (Yu *et al.*, 2004a, 2004b), and the last two genes (*hlyC* and *niiA*) are located outside the AF biosynthetic cluster. All of the AF biosynthetic genes with a putative consensus AflR binding site (5'-TCGSWNNSCGR-3') in their promoters (Ehrlich *et al.*, 1999), except for *aflR*, were upregulated in SU1 (Table 1). In addition, *aflY*, which was listed in the cluster by (Yu *et al.*, 2004b) but not assigned a function, was shown to be upregulated in SU1 and to have a consensus AflR binding site. Recently, Ehrlich and colleagues reported that *aflY* is involved in the conversion of versicolorin A to sterigmatocystin (Ehrlich *et al.*, 2005). Statistical evidence supports the functionality of the consensus AflR binding sites in these genes. In all, 3647 putative AflR binding sites are identified in the *A. flavus* genome

**Table 1.** ESTs upregulated in *Aspergillus parasiticus* SU1 at all timepoints

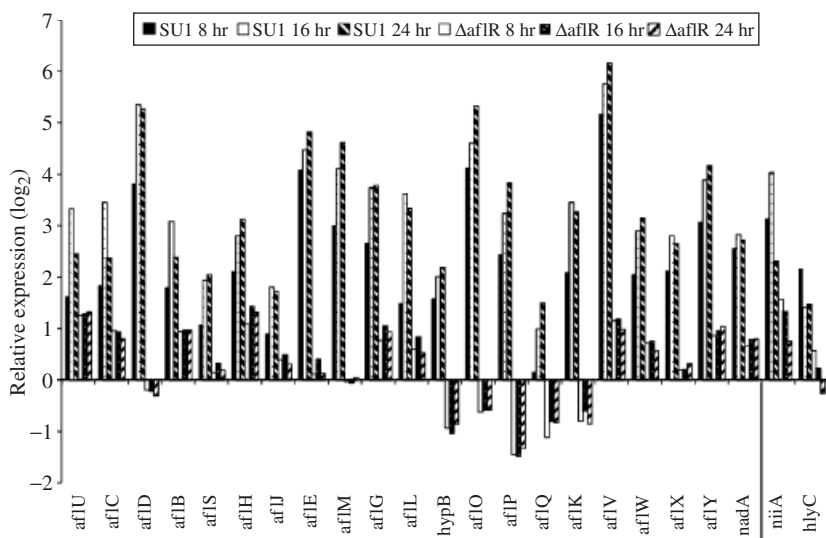
Gene name (original name)*	Annotation	AflR binding site (reference) <sup>†</sup>	Clone ID <sup>‡</sup>
<i>aflU</i> ( <i>cypA</i> )	Cytochrome P450 monooxygenase	Y (Yu <i>et al.</i> , 2004b)	<i>cypA</i>
<i>aflC</i> ( <i>pksA</i> )	Polyketide synthase	Y (Ehrlich <i>et al.</i> , 1999)	<i>pksA</i>
<i>aflD</i> ( <i>nor1</i> )	NOR reductase	Y (Ehrlich <i>et al.</i> , 1999)	TC8486
<i>aflB</i> ( <i>fas2</i> )	Fatty acid synthase	Y (Yu <i>et al.</i> , 2004b)	<i>hexB</i>
<i>aflS</i> ( <i>aflJ</i> )		Y (Ehrlich <i>et al.</i> , 1999)	TC10671
<i>aflH</i> ( <i>adhA</i> )	Alcohol dehydrogenase	Y (Ehrlich <i>et al.</i> , 1999)	<i>adhA</i>
<i>aflJ</i> ( <i>estA</i> )	Esterase	Y (Yu <i>et al.</i> , 2004b)	<i>estA</i>
<i>aflE</i> ( <i>norA</i> )	NOR reductase	Y (Ehrlich <i>et al.</i> , 1999)	<i>norA</i>
<i>aflM</i> ( <i>ver1</i> )	Dehydrogenase	Y (Ehrlich <i>et al.</i> , 1999)	TC10529
<i>aflG</i> ( <i>avnA</i> )	Cytochrome P450 monooxygenase	Y (Ehrlich <i>et al.</i> (1999)	<i>avnA</i>
<i>aflL</i> ( <i>verB</i> )	Desaturase	Y (Yu <i>et al.</i> , 2004b)	<i>verB</i>
<i>hypB</i>	Hypothetical protein	Y (this study)	TC10462
<i>aflO</i> ( <i>omtB</i> )	O-methyltransferase B	Y (Yu <i>et al.</i> , 2004b)	<i>omtB</i>
<i>aflP</i> ( <i>omtA</i> )	O-methyltransferase A	Y (Ehrlich <i>et al.</i> , 1999)	TC8384
<i>aflQ</i> ( <i>ordA</i> )	Oxidoreductase	Y (Ehrlich <i>et al.</i> , (1999)	TC10424
<i>aflK</i> ( <i>vbs</i> )	VERB synthase	Y Ehrlich <i>et al.</i> 1999)	<i>vbs</i>
<i>aflV</i> ( <i>cypX</i> )	Cytochrome P450 monooxygenase	Y (Yu <i>et al.</i> , 2004b)	<i>cypX</i>
<i>aflW</i> ( <i>moxY</i> )	Monooxygenase	Y (Yu <i>et al.</i> , 2004b)	TC10309
<i>aflX</i> ( <i>ordB</i> )	Oxidoreductase	Y (Yu <i>et al.</i> , 2004b)	NAGDE85TV
<i>aflY</i> ( <i>hypA</i> )	Hypothetical protein	Y (Yu <i>et al.</i> , 2004b)	<i>hypA</i>
<i>nadA</i>	NADH oxidase	Y (this study)	<i>nadA</i>
<i>niiA</i>	Nitrite reductase	Y (this study)	TC8843
<i>hlyC</i>	$\alpha$ -hemolysin	Y (this study)	TC11297

\*Genes are presented in the order of relative physical location in the AF gene cluster. The dotted line separates those genes within the AF cluster from those residing outside of the cluster. *nadA* was previously reported to be in the adjacent sugar utilization cluster Yu *et al.* (2000).

<sup>†</sup>These genes were previously shown or currently determined to possess an AflR binding site in their promoter regions.

<sup>‡</sup>Clone IDs are either known AF gene names (i.e. *pksA*) or EST IDs from the TIGR gene index (i.e. TC8486).

AF, aflatoxin; EST, expressed sequence tag; TIGR, The Institute for Genome Research.



**Fig. 3.** Relative expression levels for genes upregulated in *Aspergillus parasiticus* SU1. Least-squares means estimates of expression obtained from the mixed-model analysis of the microarray data (see Materials and methods for explanation and for relationship to fold changes) were plotted for genes upregulated in *A. parasiticus* SU1.

**Table 2.** qPCR confirmation of microarray results

Gene	Fold induction in <i>Aspergillus parasiticus</i> SU1
<i>aflD</i>	4598
<i>aflM</i>	28 592
<i>hypB</i>	29 875
<i>aflO</i>	102 363
<i>aflP</i>	13 247
<i>nadA</i>	4650
<i>niiA</i>	10
<i>hlyC</i>	28

sequence (unpublished data). This number is similar to the 3398 putative NirA binding sites (the nitrogen assimilation pathway-specific regulator), but more than the number of AflR binding sites that would be expected by chance in the *A. flavus* genome (2211,  $P = 0.00006$ ).

Four of the genes that have not been previously shown to be involved in AF biosynthesis were upregulated in SU1 (*hypB*, *nadA*, *niiA*, and *hlyC*) and possessed consensus AflR binding sites upstream of their putative start codons (Table 1). One of these genes (*nadA*) was previously identified (Yu *et al.*, 2000) as part of the sugar utilization cluster neighboring the AF biosynthetic cluster. Yu *et al.* (2000) stated that the genes of the sugar utilization cluster in *A. parasiticus* did not possess consensus AflR binding sites near the translational start sites (100–300 bp), but were likely related to AF production due to their location neighboring the AF biosynthetic cluster. The AflR binding site in the intergenic region of the divergently transcribed *nadA* and *aflY* genes

(752 bp to *nadA* and 124 bp to *aflY* start codons) may be shared by both genes. The reduced expression of *nadA* in the  $\Delta$ *aflR* strain and the similarity of its expression to other pathway genes in SU1 in these experiments (Fig. 3) suggest that *nadA* may in fact belong to the AF biosynthetic cluster. A role for *nadA* in AF biosynthesis is not known. It is possible that *nadA*, encoding a putative NADH oxidase, increases reducing power in the cell, and thus increases the available NADH required energy conservation in the cell. A previously undiscovered gene, *hypB* (for second hypothetical protein in AF cluster), was shown to be expressed and upregulated. *hypB* was located in the AF biosynthetic cluster between *aflI* and *aflL* (data not shown). This gene has two putative AflR binding sites located 100 bp and 1.3 kb upstream of the putative coding region.

Two genes were not located near the AF biosynthetic cluster. One of these genes, nitrite reductase (*niiA*), is located in the nitrate assimilation cluster, and is divergently transcribed from the same promoter as *niaD*, the gene encoding nitrate reductase. No putative AflR binding sites were observed in the *niiA* 5'-untranslated region. Most of the functional AflR binding sites reported reside within 100–300 bp of the transcriptional start site (Ehrlich *et al.*, 1999), although functional AflR binding sites beyond 300 bp have been observed (Fernandes *et al.*, 1998). Interestingly, *niiA* possessed a consensus AflR binding site approximately 2.3 kb upstream, within the coding region of *niaD* (data not shown). The prospects for *aflR* control of nitrite reductase expression, in light of nitrate repression of AF production, compel further investigation of this putative interaction. The *hlyC* gene, encoding a homolog of  $\alpha$ -hemolysin from

*Aeromonas hydrophila*, was located approximately 1.5 Mb from the AF cluster according to *A. flavus* genome data (data not shown) and has a putative AflR binding site approximately 1.8 kb upstream of the putative coding region. *hlyC* has no apparent role in AF production but may play a role in animal pathogenesis by *Aspergilli*.

In conclusion, our data support previous data showing that *aflR* is required for expression of the AF biosynthetic genes under conditions conducive for AF production (Price *et al.*, 2005). They also support the inclusion of *nadA* into the AF biosynthetic cluster, but confirmation of its role in AF biosynthesis must await functional analysis of this gene. Furthermore, these data argue that AflR regulates genes outside the cluster. The number of such genes is likely small, as only two were identified using an array that contains approximately 40% of the transcriptome. It will be interesting to learn if these genes are in some way involved in AF production. It is tempting to speculate that both *nadA* and *niiA* may be involved in metabolic regulation of the pathway by influencing reducing power and nitrogen metabolism, respectively.

Because *nadA* and *niiA* contained the consensus AflR binding site upstream of their coding regions, it may be fruitful to examine the genome (once it is available) for additional genes with the binding site. Clearly the AflR binding site is not required for the activation of all AF biosynthetic genes as several without the consensus site were upregulated in the wildtype strain (Ehrlich *et al.*, 1999). Finally, this study shows that DNA microarrays can be a powerful tool to identify genes regulated by transcriptional factors and points to the need for whole genome arrays for *Aspergillus flavus*.

## Acknowledgements

The authors wish to thank Greg O'Brian, Rob Holmes, Sheri Denslow, and Ryan Georgianna for their critique of this manuscript. This research was supported by Grant 2002-35201-12562 from the USDA/NRI Competitive Grants Program.

## References

Churchill GA (2002) Fundamentals of experimental design for cDNA microarrays. *Nat Genet* **32**: (Suppl): 490–495.  
 Ehrlich KC, Montalbano B, Boue S & Bhatnagar D (2005) XIII Fungal Genetics Conference. *Abstract* 521.

Ehrlich KC, Montalbano BG & Cary JW (1999) Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene* **230**: 249–257.  
 Fernandes M, Keller NP & Adams TH (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol Microbiol* **28**: 1355–1365.  
 Flaherty JE & Payne GA (1997) Overexpression of *aflR* leads to upregulation of the pathway gene transcription and increased aflatoxin production in *Aspergillus flavus*. *Appl Environ Microbiol* **63**: 3995–4000.  
 Jain AN, Tokuyasu TA, Snijders AM, Segraves R, Albertson DG & Pinkel D (2002) Fully automatic quantification of microarray image data. *Genome Res* **12**: 325–332.  
 Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>( $\Delta\Delta C_T$ ) method. *Methods* **25**: 402–408.  
 Price MS, Connors SB, Tachdjian S, Kelly RM & Payne GA (2005) Aflatoxin conducive and non-conductive growth conditions reveal new gene associations with aflatoxin production. *Fungal Genet Biol* **42**: 506–518.  
 Shimizu K, Hicks JK, Huang TP & Keller NP (2003) Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)<sub>2</sub>Cys<sub>6</sub> transcription factor in *Aspergillus nidulans*. *Genetics* **165**: 1095–1104.  
 Skory CD, Chang PK & Linz JE (1993) Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis. *Appl Environ Microbiol* **59**: 1642–1646.  
 Wolfinger RD, Gibson G, Wolfinger ED, *et al.* (2001) Assessing gene significance from cDNA microarray expression data via mixed models. *J Comp Biol* **8**: 625–637.  
 Yu J-H, Butchko RAE, Fernandes M, Keller NP, Leonard TJ & Adams TH (1996) Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr Genet* **29**: 549–555.  
 Yu J, Chang P, Bhatnagar D & Cleveland TE (2000) Cloning of a sugar utilization gene cluster in *Aspergillus parasiticus*. *Biochim Biophys Acta* **1493**: 211–214.  
 Yu J, Bhatnagar D & Cleveland TE (2004a) Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *FEBS Lett* **564**: 126–130.  
 Yu J, Chang PK, Ehrlich KC, *et al.* (2004b) Clustered pathway genes in aflatoxin biosynthesis. *Appl Environ Microbiol* **70**: 1253–1262.  
 Yu J, Whitelaw CA, Nierman WC, Bhatnagar D & Cleveland TE (2004c) *Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. *FEMS Microbiol Lett* **237**: 333–340.