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Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters

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SUMMARY

Species of *Aspergillus* produce a diverse array of secondary metabolites, and recent genomic analysis has predicted that these species have the capacity to synthesize many more compounds. It has been possible to infer the presence of 55 gene clusters associated with secondary metabolism in *Aspergillus flavus*; however, only three metabolic pathways—aflatoxin, cyclopiazonic acid (CPA) and aflatrem—have been assigned to these clusters. To gain an insight into the regulation of and to infer the ecological significance of the 55 secondary metabolite gene clusters predicted in *A. flavus*, we examined their expression over 28 diverse conditions. Variables included culture medium and temperature, fungal development, colonization of developing maize seeds and misexpression of *laeA*, a global regulator of secondary metabolism. Hierarchical clustering analysis of expression profiles allowed us to categorize the gene clusters into four distinct clades. Gene clusters for the production of aflatoxins, CPA and seven other unknown compound(s) were identified as belonging to one clade. To further explore the relationships found by gene expression analysis, aflatoxin and CPA production were quantified under five different cell culture environments known to be conducive or nonconducive for aflatoxin biosynthesis and during the colonization of developing maize seeds. Results from these studies showed that secondary metabolism gene clusters have distinctive gene expression profiles. Aflatoxin and CPA were found to have unique regulation, but are sufficiently similar that they would be expected to co-occur in substrates colonized with *A. flavus*.

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INTRODUCTION

Aspergillus flavus first came to notoriety for its production of the highly carcinogenic secondary metabolite aflatoxin. Aflatoxins represent a family of mycotoxins that occur worldwide in several important food and feed crops, including maize, peanuts and seeds of nut trees, colonized by *A. flavus* or *Aspergillus parasiticus*. Aflatoxin B1 (AF) is the most carcinogenic member of the family and that most carefully studied. The presence of aflatoxins in food and feed is regulated by the Food and Drug Administration in the USA, and by other agencies in most countries. Because of health concerns and the worldwide distribution of AF contamination, environmental and genetic regulation of the AF biosynthetic pathway has been studied extensively since its discovery in the early 1960s (Bhatnagar *et al*., 2006; Georgianna and Payne, 2008; Yu and Keller, 2005).

Aflatoxins are not the only mycotoxins produced by *A. flavus*. Over 14 described mycotoxins are known to be produced by *A. flavus* (see http://www.aspergillus.org.uk), although 10 of these are derived from the AF biosynthetic pathway. All characterized mycotoxins in *A. flavus* are secondary metabolites produced by genes organized in physical clusters throughout the genome. Genomic analysis of *A. flavus* has led to the prediction of 55 secondary metabolism gene clusters by the Secondary Metabolite Unknown Regions Finder (SMURF), available at http://www.jcvi.org/smurf (N. Khaldi *et al*., Smurfit Institute of Genetics, Trinity College, Dublin, Ireland and Department of Infectious Disease, The J. Craig Venter Institute, Rockville, MD, USA). Cluster prediction was performed using the SMURF program which searches for genes encoding multifunctional enzymes (called backbone genes by SMURF) associated with four classes of secondary metabolites. These included nonribosomal peptide synthetases (NRPSs) for nonribosomal peptides, polyketide synthases (PKSs) for polyketides, hybrid NRPS–PKS enzymes for hybrids and prenyltransferases (PTRs) for terpenoids

(Hoffmeister and Keller, 2007; Keller *et al*., 2005). The structures for aflatoxin, a polyketide, and cyclopiazonic acid (CPA), a hybrid NRPS–PKS, are shown in Fig. 1. Once these putative multifunctional enzymes are identified, SMURF explores neighbouring genes for domains commonly found in enzymes associated with secondary metabolism. This process defines the extent of the physical genomic regions for each identified cluster.

The metabolites produced by most of the 55 predicted secondary metabolism clusters in *A. flavus* are unknown. The fact that one secondary metabolism cluster can produce numerous products suggests that *A. flavus* has the potential to produce a very diverse repertoire of secondary metabolites. Although secondary metabolites are, by definition, not essential, they play many important roles. As an example they can be important in host–pathogen relationships, with some providing protection against environmental stresses as well as fungivory (Rohlfs *et al*., 2007). Many secondary metabolites also include beneficial products for human health, such as the antibiotic penicillin and the cholesterol-lowering drug lovastatin (Endo *et al*., 1976; Hoffmeister and Keller, 2007; Keller *et al*., 2005). Thus, understanding the conditions under which the genes of these clusters are expressed may allow us to predict when these compounds may be found in food and feed, as well as give potential clues for ecological roles.

We recently began a series of studies examining gene expression in *A. flavus* with a whole-genome Affymetrix GeneChip. Expression datasets were collected from a variety of experimental conditions. These conditions included field inoculation of maize kernels, inoculation of kernels in different stages of development, inoculation of autoclaved mature kernels and kernel tissues, incubation of infected kernels at temperatures conducive and nonconducive for AF biosynthesis, and field inoculation of maize with *Aspergillus oryzae*, a species thought to be domesticated from *A. flavus*, which is commonly used for food fermentations (Payne *et al*., 2006). Other experiments have examined the effect of AF conducive and nonconducive culture media and temperature, and the effect of *laeA*, a global secondary metabolism regulator of gene expression (Kale *et al*., 2008). LaeA misexpression was also examined during conditions favourable for sclerotia development (S. Horowitz-Brown *et al*., unpublished work). The overall goal of these studies was to better understand the factors governing the morphological development, secondary metabolism biosynthesis and pathogenicity of *A. flavus*.

The goals of our study were to establish the expression patterns of the putative secondary metabolism clusters under a variety of experimental conditions and to associate gene clusters with known functions to others having no currently accepted roles. We hypothesized that the variety of conditions examined would result in the identification of groups of secondary metabolites that are likely to be produced under similar conditions or environments. We show that the genes expressed in the cluster responsible for the secondary metabolite CPA share a similar transcription profile with genes in the AF cluster.

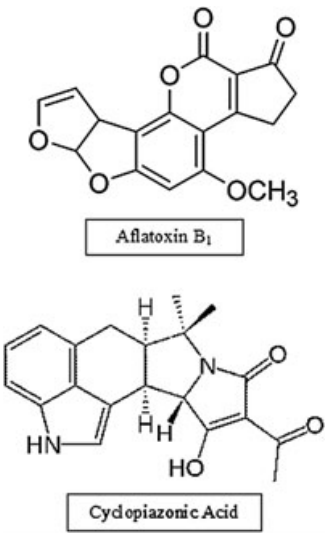

Fig. 1 Structures of aflatoxin B_1 (top) and cyclopiazonic acid (bottom).

RESULTS

Transcriptional analysis of 55 predicted gene clusters in *A. flavus*

Expression data obtained from 28 experiments (Table 1) were used to identify patterns among the genes in the 55 different secondary metabolism clusters predicted by SMURF. A hierarchical clustering analysis was used to determine which clusters showed the most similar patterns of transcription over the 28 conditions. Attempts to use all of the predicted genes contained within each of the 55 secondary metabolism clusters yielded results that were not easily interpreted. Therefore, the genes encoding the 'backbone' enzymes predicted with SMURF for each cluster were chosen (Table 2). These enzymes are likely to be essential for metabolite biosynthesis, and our assumption is that the expression of the corresponding gene should be representative of gene cluster biosynthetic activity. In 13 of the 55 clusters, more than one backbone gene was found. Another advantage of the method used was that it was not affected by errors in predicted cluster boundaries. Our method is not biased by the expression of nonenzymatic genes within the cluster, such as transcription factors, which may follow a different expression pattern from other genes within a secondary metabolism gene cluster (O'Brian *et al*., 2007).

Distance relationships derived from the hierarchical clustering analysis revealed four discernible expression patterns, designated clades A–D (Fig. 2). Notably, a few backbone genes from

Table 1 Experimental conditions used for the analysis of secondary metabolism gene clusters.

Number	Name	Description
1	Blister	Inbred maize line B73 kernels identified at the blister, milk, dough and dent stages of development were used for each treatment. Pin-bar needles were dipped into a 1E6 conidia/mL conidial suspension of NRRL3357. The needles were then inserted into a row of kernels, introducing approximately 11–13 conidia into each kernel. Five rows were inoculated per ear and harvested after 4 days
2	Milk	
3	Dough	
4	Dent	
5	Autoclaved endosperm	Kernels from maize line B73 were autoclaved and dissected to include the endosperm, germ or whole kernel. Tissue sections were placed in vials, inoculated with 1E5 conidia NRRL3357 and incubated at 29 °C for 5 days
6	Autoclaved germ	
7	Autoclaved whole kernel	
8	B73 48 h	At the late milk–early dough stage of development, maize line B73 kernels were inoculated as described above for the blister, milk, dough and dent experiments. Harvesting took place 48, 60, 72 and 96 h after inoculation
9	B73 60 h	
10	B73 72 h	
11	B73 96 h	
12	28C maize	Kernels from maize line B73 were placed in vials, inoculated with 1E6 conidia NRRL3357 and incubated at 28 °C or 37 °C for 5 days
13	37C maize	
14	28C A&M	100 mL of modified A&M medium in a 500-mL flask were inoculated with conidia from NRRL3357 at 1E6 conidia/mL. Shake cultures (200 rpm) were grown for 24 h at either 28 or 37 °C
15	37C A&M	
16	RIB40 maize	Inbred maize line B73 ears in the milk stage were inoculated with a pin-bar dipped in 1E6 conidia/mL of strain NRRL3357 or RIB40 and harvested after 4 days
17	NRRL3357 maize	
18	RIB40 wheat bran	A mix of 5 g wheat bran and 5 mL dH_2O was autoclaved and mixed with 1E8 conidia of either strain NRRL3357 or RIB40 and analysed after 2 days of growth
19	NRRL3357 wheat bran	
20	WT 6 h	50 mL liquid YEP medium (6% peptone, 2% yeast extract) was inoculated with 10E6 conidia/mL of *A. flavus* NRRL 3357, an *laeA* deletion strain or an *laeA* overexpression strain in 50-mL flasks, incubated with shaking at 250 rpm at 29 °C. After 24 h, the mycelium was collected and incubated in the aflatoxin-stimulating YEP medium for 6 and 24 h (220 rpm, 29 °C)
21	WT 24 h	
22	*laeA* deletion 6 h	
23	*laeA* deletion 24 h	
24	*laeA* OE 6 h	
25	*laeA* OE 24 h	
26	WT sclerotia	10E5 conidia were inoculated into liquid glucose minimal media (GMM) + 2% sorbitol in a 60 × 15-mm Petri plate. The plates were incubated at 29 °C under continuous darkness for 6 days
27	*laeA* deletion sclerotia	
28	*laeA* OE sclerotia	

the same cluster failed to group together. For example, the three backbone genes of cluster 26 exhibited expression patterns associated with clades A, C and D, respectively. It is possible that cluster 26 is more than one cluster merged together, or that it contains nonfunctional backbone genes.

Clade A contains the aflatoxin cluster (cluster 54) and eight other predicted gene clusters (1, 16, 19, 23, 24, 26, 36 and 55). Cluster 55 was later found to be necessary for the biosynthesis of CPA. Moderate to high levels of gene expression are characteristic of this clade in most of the 28 experimental conditions examined. On the basis of the predicted multifunctional synthases (Table 2) in these clusters, we inferred that their products would contain diverse classes of secondary metabolites, including polyketides, nonribosomal peptides, pigments and siderophores.

Clade B contains 11 gene clusters (4, 5, 9, 10, 11, 20, 23, 31, 37, 48 and 50). The characteristic of this group is low gene expression in liquid shake culture medium [A&M medium (Mateles and Adye, 1965) experiments and *laeA* experiments]. The expression values in other experiments were also not as high as those associated with genes in clade A.

Clade C contains genes from 25 of the 55 predicted clusters. Gene expression in these clusters appeared to be low across almost all experimental conditions. These expression results suggest that products from these clusters are not being actively synthesized under the conditions tested. For example, these metabolites may be involved in competition with other fungi, bacteria and other soil inhabitants, and only synthesized during co-cultivation with these microorganisms.

Clade D contains 18 different gene clusters. Most of the backbone genes monitored for these clusters appeared to be only slightly more expressed than those in clade C. A group of eight clusters within clade D were highly expressed during only two conditions. Included in this small group is the aflatrem biosynthesis cluster (cluster 15). The biosynthesis of aflatrem is thought to require two clusters located on separate chromosomes (Saikia *et al*., 2008; Zhang *et al*., 2004).

Characterization of the CPA cluster

Among the nine clusters in clade A, cluster 55 is located nearest the AF cluster in the genome. Cluster 55 spans a 20-kb region of DNA near the telomere of chromosome 3, immediately distal to the AF cluster (Fig. 3). Until recently, the genes in cluster 55 had remained uncharacterized. Tokuoka *et al*. (2008) reported that the PKS–NRPS gene encoded within this cluster is necessary for CPA production in *A. oryzae*. On the basis of its proximity to PKS, we disrupted a gene (designated *dmaA*) encoding a PTR with homology to dimethylallyl tryptophan synthases, and studied its function in CPA biosynthesis. By homologous recombination, we obtained a transformant, number 39, with a deleted *dmaA*.

Table 2 Secondary metabolism gene clusters and accession numbers for backbone enzymes predicted by SMURF.

Cluster	Gene name	Accession
1	Polyketide synthase, putative	AFLA_002900
2	Dimethylallyl tryptophan synthase, putative	AFLA_004300
4	Nonribosomal peptide synthetase, putative	AFLA_005440
5	Polyketide synthase PksP	AFLA_006170
6	Nonribosomal peptide synthetase, putative	AFLA_008770
7	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_009120
7	Polyketide synthase-like enzyme, putative	AFLA_009140
8	Polyketide synthase, putative	AFLA_010000
8	Nonribosomal peptide synthetase, putative	AFLA_010010
8	Nonribosomal peptide synthetase, putative	AFLA_010020
9	Nonribosomal peptide synthetase, putative	AFLA_010580
9	Nonribosomal siderophore peptide synthase Sid2	AFLA_010620
10	Conidial pigment biosynthesis scytalone dehydratase Arp1	AFLA_016140
11	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_023020
12	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_028720
13	Nonribosomal peptide synthetase, putative	AFLA_038600
14	Enterobactin esterase IroE-like, putative	AFLA_041050
15	Dimethylallyl tryptophan synthase, putative	AFLA_045490
16	L-Ornithine *N*5-oxygenase SidA	AFLA_047190
16	Palmitoyltransferase SidR	AFLA_047200
17	Polyketide synthase-like enzyme, putative	AFLA_053770
17	Polyketide synthase-like enzyme, putative	AFLA_053780
17	Polyketide synthase, putative	AFLA_053870
18	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_054270
19	Dimethylallyl tryptophan synthase, putative	AFLA_060680
20	Polyketide synthase, putative	AFLA_062820
20	Polyketide synthase, putative	AFLA_062860
21	Nonribosomal peptide synthetase, putative	AFLA_064560
22	Nonribosomal peptide synthetase, putative	AFLA_066720
23	Hybrid nonribosomal peptide synthetase–polyketide synthase enzyme, putative	AFLA_066840
23	Polyketide synthase, putative	AFLA_066980
24	Nonribosomal peptide synthetase Pes1	AFLA_069330
25	ACV synthetase PcbAB	AFLA_070860
25	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_070920
26	Polyketide synthase-like enzyme, putative	AFLA_079360
26	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_079380
26	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_079400
27	Polyketide synthase, putative	AFLA_082150
28	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_082480
29	Dimethylallyl tryptophan synthase, putative	AFLA_084080
30	Dimethylallyl tryptophan synthase, putative	AFLA_090190
30	Nonribosomal peptide synthetase, putative	AFLA_090200
31	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_095040
31	Dimethylaniline monooxygenase, putative	AFLA_095050
33	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_096700
33	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_096710
33	Polyketide synthase, putative	AFLA_096770
35	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_101700
36	Polyketide synthase-like enzyme, putative	AFLA_104210
36	Polyketide synthase-like enzyme, putative	AFLA_104240
36	Polyketide synthase-like enzyme, putative	AFLA_104250
37	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_105190
38	Polyketide synthase, putative	AFLA_105450
39	Polyketide synthase, putative	AFLA_108550
40	Polyketide synthase, putative	AFLA_112840
41	Polyketide synthase, putative	AFLA_114820
42	Polyketide synthase, putative	AFLA_116220
43	Polyketide synthase-like enzyme, putative	AFLA_116500
44	Polyketide synthase, putative	AFLA_116890
45	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_118440
46	Polyketide synthase, putative	AFLA_118940
46	Polyketide synthase, putative	AFLA_118960
47	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_119110
48	Nonribosomal peptide synthetase-like enzyme, putative	AFLA_121520
49	Polyketide synthase-like enzyme, putative	AFLA_125630
49	Polyketide synthase-like enzyme, putative	AFLA_125640
50	Polyketide synthase, putative	AFLA_126710
51	Polyketide synthase, putative	AFLA_127090
52	Polyketide synthase, putative	AFLA_128060
53	Nonribosomal peptide synthetase, putative	AFLA_135490
54	aflC/pksA/pksL1/polyketide synthase	AFLA_139410
55	Dimethylallyl tryptophan synthase, putative	AFLA_139480
55	Hybrid polyketide synthase–nonribosomal peptide synthetase enzyme, putative	AFLA_139490

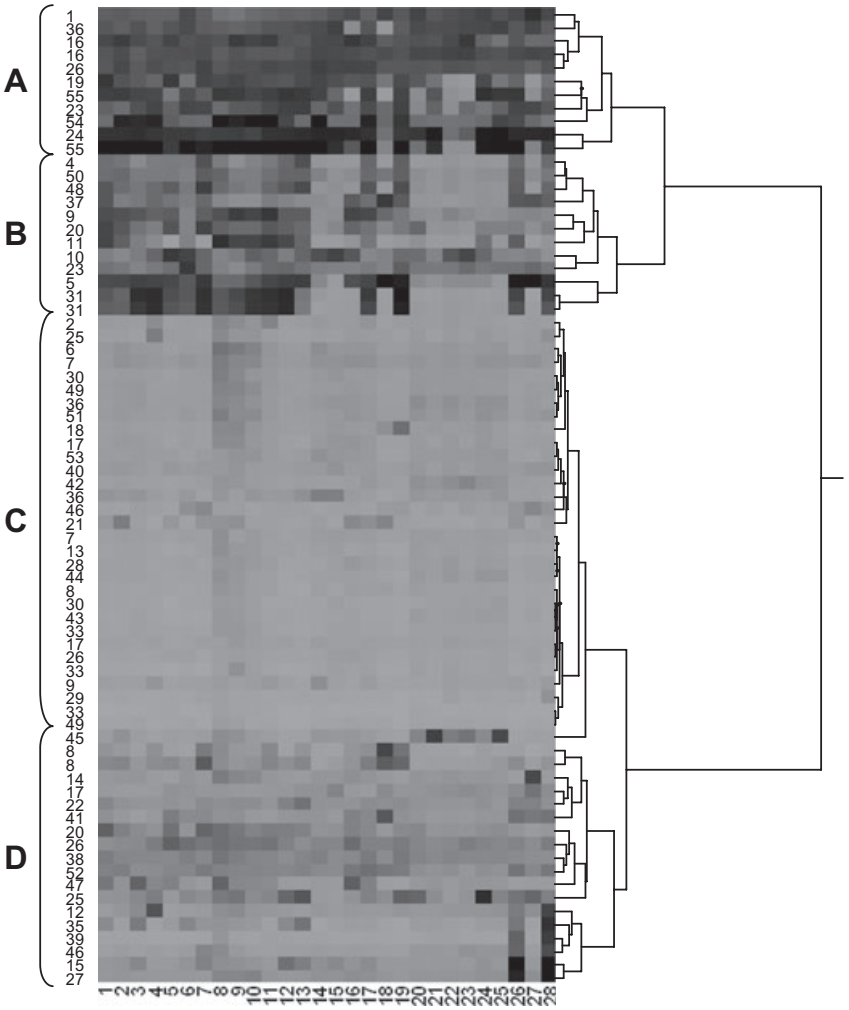

Fig. 2 Hierarchical clustering of backbone enzymes. The cluster numbers of each backbone enzyme are given on the left. Experiments 1–28 (Table 1) are listed along the bottom. Shading represents the intensity of gene expression, with darker shading indicating higher expression. Letters A–D represent the four clades discussed in more detail in the text.

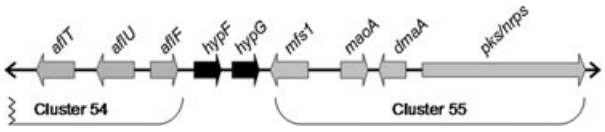

Fig. 3 Location of cluster 55 in the genome of *Aspergillus flavus*. Cluster 55 is located immediately distal to the aflatoxin cluster (cluster 54, entire 70-kb cluster not shown) near the telomere of chromosome 3. Cluster 55 spans approximately 20 kb and contains four genes: a major facilitator superfamily protein (*mfs1*), a flavin adenine dinucelotide oxidoreductase (*maoA*), a predicted dimethylallyl tryptophan synthase (*dmaA*) and a hybrid polyketide–nonribosomal peptide synthase. AFLA_139450 (*hypF*) and CO1467294 (*hypG*) have also been characterized in our results.

Integration of the disruption vector at the *dmaA* locus was verified by amplification of a 5-kb DNA fragment from the mutant with PCR primers 7 and 8 (Fig. 4A,B). To determine the impact of *dmaA* deletion on CPA biosynthesis, transformant 39 and the wild-type (WT) strain were grown in potato dextrose broth medium. After 24 h, the medium was analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the presence of CPA. As can be seen in Fig. 5, a distinct peak with a mass of 337.2 Da at a retention time of 5.45 min, representing CPA, was observed in the analysis of culture medium from the WT strain, but not in culture medium from transformant 39. Two additional transformants with predicted gene deletions for *dmaA* were also selected, and these transformants also tested negative for CPA (data not shown).

We also examined the potential role of two putative genes, *hypF* and *hypG*, located between the AF cluster and the CPA cluster in AF and CPA production. *hypF* encodes the hypothetical protein AFLA_139450, and *hypG* (CO146294.10) (Fig. 3) was identified in an expressed sequence tag (EST) library. The *hypG* gene was not predicted during annotation of the *A. flavus* genome. The predicted gene product from *hypG* was found to have weak homology to transcription factors in *Aspergillus niger* and *Penicillium chrysogenum*. We hypothesized that they may be necessary for CPA cluster gene transcription. Both genes were deleted in strain CA14Δku70ΔpyrG. The deletion strains Δ*hypF*

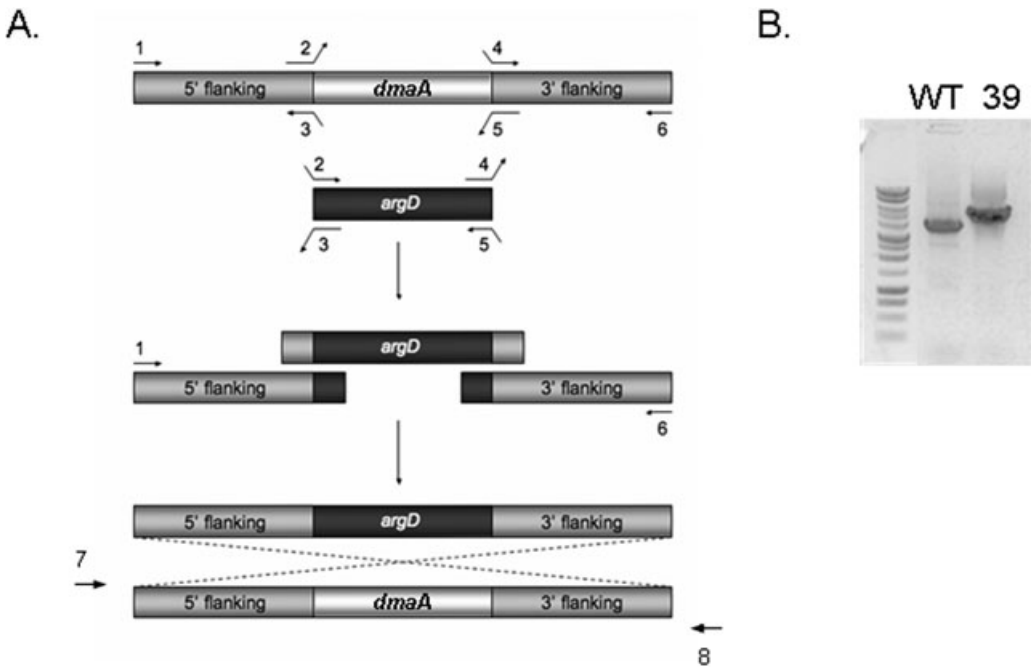

Fig. 4 Overlap polymerase chain reaction (PCR) strategy for *dmaA* deletion construct and predicted integration into the genome. (A) Using overlap PCR with primers numbered 1–6, a DNA deletion construct was prepared with the upstream and downstream flanking regions of *dmaA* flanking the *argD* marker. After transformation, the presence of this construct at the *dmaA* locus was determined by screening DNA from the transformants with external PCR primers 7 and 8. (B) Gel showing DNA bands amplified from wild-type (WT) and transformant 39 (39) when screened with primers 7 and 8. The PCR band of 5 kb is predicted for replacement of the WT gene, *dmaA*, with the deletion construct.

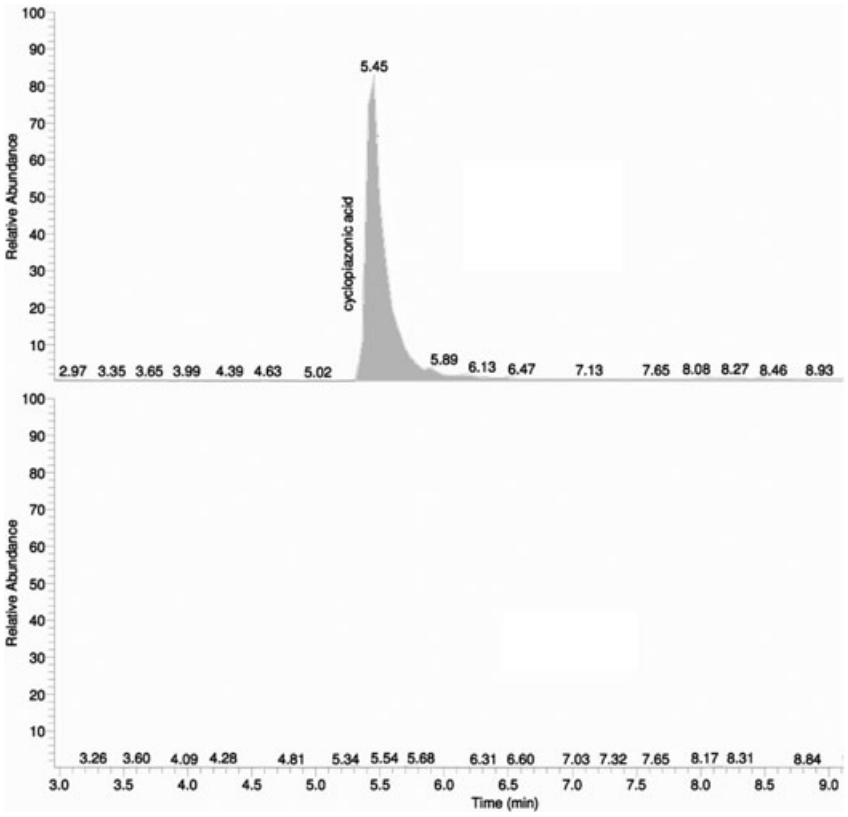

Fig. 5 Selected ion chromatogram for cyclopiazonic acid (CPA). Full-scan liquid chromatography-mass spectrometry (LC-MS) chromatogram displayed for the detection of mass 337 Da, corresponding to CPA.

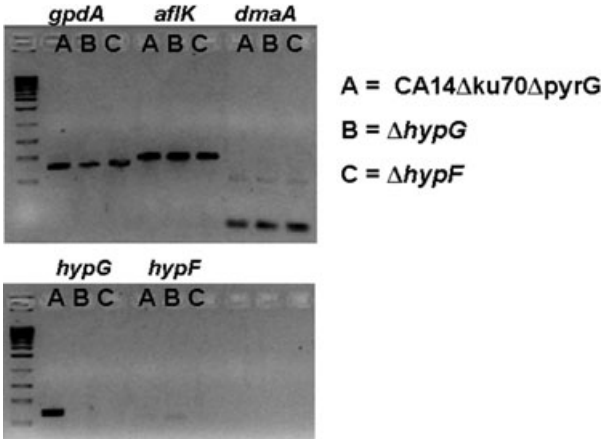

Fig. 6 Reverse transcriptase-polymerase chain reaction (RT-PCR) for gene products in Δ*hypG*, Δ*hypF* and parent strain CA14Δku70ΔpyrG. Each strain was assayed for the expression of the aflatoxin cluster gene *aflK*, cyclopiazonic acid (CPA) cluster gene *dmaA*, *hypG* and *hypF*. The *gpdA* gene was used as a control.

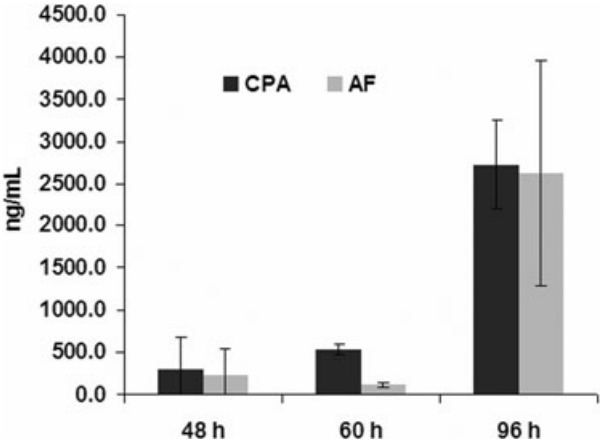

Fig. 7 Cyclopiazonic acid (CPA) and aflatoxin (AF) concentrations in maize kernels after 48, 60 and 96 h of infection.

and Δ*hypG* were grown in potato dextrose broth medium for 48 h at 28 °C. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Fig. 6) showed that neither deletion of *hypF* nor *hypG* had an effect on the transcription of genes within the AF (*aflK*) or CPA (*dmaA*) clusters. In addition, mycotoxin analysis showed no effect on the accumulation of CPA or AF in either gene deletion mutant (data not shown). The *hypG* gene was clearly expressed, whereas *hypF* showed very weak expression (Fig. 6). Neither of the predicted genes was expressed in their respective deletion strain; however, it appears that *hypF* may either be needed for the expression of *hypG* or the promoter for *hypG* may be contained within *hypF*, as the expression of *hypG* was not detected in Δ*hypF*. Regardless, the data indicate that these two genes do not play an essential role in AF or CPA biosynthesis.

Accumulation of CPA and AF

Studies have shown that many strains of *A. flavus* commonly produce both AF and CPA (Chang *et al*., 2005; Gallagher *et al*., 1978; Martins and Martins, 1999; Widiastuti *et al*., 1988). We further examined the production of these mycotoxins more closely by testing the effects of specific culture conditions that are known to affect AF biosynthesis. Concentrations of both AF and CPA increased over time (48, 60 and 96 h) in maize kernels infected by strain NRRL3357, indicating that this substrate is favourable for the production of the two mycotoxins (Fig. 7). Growth of the fungus on defined media, however, showed AF and CPA biosynthesis to respond differently to the carbon and nitrogen sources of the media and to culture temperature.

The medium most conducive for AF production contained sucrose as the carbon source, ammonium salt as the nitrogen

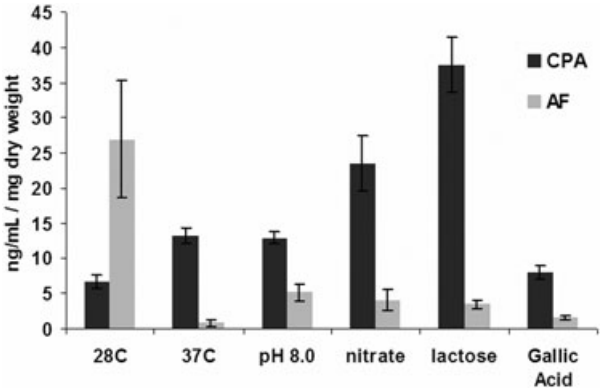

Fig. 8 Response of cyclopiazonic acid (CPA) and aflatoxin (AF) concentrations to changes in temperature, pH, nitrogen source, carbon source and addition of the anti-oxidant gallic acid. Each medium used for the growth of *Aspergillus flavus* was identical to the most AF-conducive condition, listed as 28C, except for the modification of pH, nitrogen source, carbon source, temperature or addition of anti-oxidant.

source and had a pH value of 4.5 (shown as the 28 °C treatment in Fig. 8). Although CPA was produced on all media examined, the most favourable carbon and nitrogen sources for CPA production differed from those for aflatoxin production. Lactose, for example, supported the greatest amount of CPA production. Increasing the temperature from 28 to 37 °C, or the pH from pH 4.0 to pH 8.0, supported more CPA production but inhibited AF production. Addition of the anti-oxidant gallic acid, a compound known to inhibit AF (Mahoney and Molyneux, 2004), to the medium reduced AF production but had no measurable affect on CPA production.

DISCUSSION

The results presented in Fig. 2 allow further insights into the regulation and ecological roles of secondary metabolites in *A.*

flavus. The functions for most secondary metabolites, including CPA and AF, are not known. They are assumed to be important for niche adaptation, and thus confer a fitness advantage in particular environments. From a functional perspective, multiple secondary metabolites may have overlapping or synergistic effects to optimize fitness (Challis and Hopwood, 2003). The characterization of fungi lacking the ability to produce multiple mycotoxins may provide further insight into the potential ecological roles for these compounds.

The four different gene clusters (1, 16, 26, 36) represented in the upper grouping of clade A of Fig. 2 appear to be constitutively expressed at moderate to high levels. It is plausible that their products may have important roles in basal functions, such as the growth and development of the fungus. As an example, secondary metabolites can be important effectors of differentiation, often acting as signalling molecules known to influence sporulation and germination (Demain and Fang, 2000). Siderophores are known to influence germination through their ability to solubilize iron (Horowitz *et al*., 1976). Consistent with our hypothesis of these secondary metabolites being involved in basal functioning, the *sidA* gene, an orthologue of the *A. flavus* siderophore biosynthesis gene from cluster 16, has been shown to be essential for the growth of *Aspergillus nidulans* (Eisendle *et al*., 2003).

A possible ecological role for the products from the three better characterized gene clusters within the remainder of clade A [clusters 24, 54 (AF) and 55 (CPA)] could be linked to oxidative stress. CPA, produced by cluster 55, was found to prevent lipid peroxidation cause by patulin (Riley and Showker, 1991). Mutants for *pes1*, the NRPS in cluster 24, show increased sensitivity to oxidative stress (Reeves *et al*., 2006). Finally, AF, produced by cluster 54, has been proposed to be involved in the quenching of reactive oxygen (Campbell, 2005). Researchers have shown that aflatoxin is induced by oxidative stress (Jayashree and Subramanyam, 2000). In *A. parasticus*, deletion of the anti-oxidant enzyme gene *yapA* resulted in altered timing of AF biosynthesis in culture media and maize seeds (Reverberi *et al*., 2007, 2008). A possible functional relatedness of the products from three of the gene clusters in clade A as anti-oxidants may be one possible explanation for their close grouping. We predict that the other secondary metabolite gene clusters in this clade may also produce metabolites that are related to oxidative stress responses as well as basal functioning.

In plants, exposure to a fungal pathogen results in many defence responses, including the production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl free radicals (Ferreira *et al*., 2006). The potential shared functions of the three members of clade A may explain the strong expression of each of these gene clusters in living maize kernels (Fig. 2). The deletion of *laeA* in *Aspergillus* species has been shown to result in reduced pathogenicity (Bok *et al*., 2005; Rohlfs *et al*., 2007; Sugui *et al*., 2007), including *A. flavus* (Amaike and Keller, 2009; Kale *et al*., 2008). Although the regulation of gene clusters by LaeA, as observed in our study, extends beyond just those in clade A, it is conceivable that the lack of one of the metabolites or an antagonistic affect from a lack of all of these metabolites in the previous LaeA studies could have resulted in the decreased potential for pathogenicity/survival of *A. flavus*.

We observed that the metabolites in clade B of Fig. 2 were more highly expressed on solid rather than liquid medium. This led us to hypothesize that some of these clusters are involved in conidiogenesis. Within clade B, the gene *pksP* (*alb1*) from cluster 5 has been implicated in *Aspergillus fumigatus* for the production of dihydroxynaphthalene (DHN)-like melanin (Tsai *et al*., 1998). Consistent with our hypothesis, conidia from *A. fumigatus* strains lacking the *pksP* gene appear white instead of the typical bluish-green and smooth rather than echinulate (Tsai *et al*., 1998). Strains lacking *pksP* were also less virulent than WT strains (Langfelder *et al*., 1998; Tsai *et al*., 1998). The DHN cluster contains six different genes in a 19-kb region in *A. fumigatus* (Tsai *et al*., 1999). The genes from this cluster do not appear to be contained within a single intact cluster in *A. flavus*; *arp1*, another gene in the *A. fumigatus* DHN cluster, is found in *A. flavus* cluster 10 (clade A). The gene *arp2* does not appear to be present in *A. flavus*, and *abr1*, *abr2* and *ayg1* have genes sharing significant homology located elsewhere in the *A. flavus* genome. Among the clusters in clade B, cluster 31 appears to follow the most similar expression pattern to that of cluster 5. The only conditions that appear to contrast are expression in *A. oryzae* and expression in the *ΔlaeA* strain under sclerotia-favouring conditions. Interestingly, *ΔlaeA* strains have been found to show slightly reduced conidiation (Kale *et al*., 2008).

For clades A and B, we observed that expression often appeared to be greater in *A. flavus* strain NRRL3357 relative to *A. oryzae* strain RIB40. The different expression patterns of these identified clusters between the two species could provide clues for their function. Expression in *A. flavus* and not *A. oryzae*, a domesticated fungus important for food fermentations, may indicate a role for these compounds unique to the ecology of *A. flavus*. Alternatively, but not exclusive of the role postulated above, these compounds may be toxic. During the domestication of *A. oryzae*, strains would have been selected for the lack of toxic compounds.

Many of the biosynthetic pathways present in *A. flavus* may be silent. One possible reason for low/no expression for the 25 clusters from clade C is that they may have been silenced by epigenetic factors. Researchers have observed enhanced chemical diversity when treating *A. flavus* and other fungal species with DNA methyltransferase inhibitors and histone deacetylase inhibitors (Shwab *et al*., 2007; Williams *et al*., 2008). It is also conceivable that secondary metabolism pathways represented in

clade C could produce their respective metabolites in very low quantities, or simply be expressed in response to conditions not encountered within these experiments.

Based on our results, clade D appears to contain some metabolites that are only expressed in the dark during conditions favourable for sclerotia production. Although aflatrem has no known role, deletion of *veA*, a gene necessary for sclerotia formation, was found to inhibit the biosynthesis of aflatrem (Duran *et al*., 2007). Interestingly, several anti-insectan products thought to prevent fungivory have been found in sclerotia (Whyte *et al*., 1996; Wicklow *et al*., 1996). We hypothesize that the six clusters within clade D (12, 15, 27, 35, 39, 46) from the subgroup that shows expression under conditions 26 and 28 will be involved in the development or survival of sclerotia. These six clusters were not expressed in the Δ*laeA* strain from condition 27, which was grown under the same conditions as 26 and 28. Fittingly, Δ*laeA* strains do not produce sclerotia (Kale *et al*., 2008).

We have more closely characterized cluster 55, which has now been shown to be necessary for CPA production (Chang *et al*., 2009; Tokuoka *et al*., 2008). Although the function of the major facilitator superfamily (MFS), predicted to be part of the CPA cluster, has not yet been verified, Chang *et al*. (2009) recently characterized *dmaA*, which they named *dmaT*. They also described an essential role in CPA biosynthesis for the predicted flavin adenine dinucleotide oxidoreductase in cluster 55, and named its gene *maoA*. The shared regulation we have observed for the four genes from cluster 55 shown in Fig. 3 provides strong evidence that they are part of a gene cluster necessary for CPA biosynthesis. Whether there are additional structural genes required for the biosynthesis of CPA, located elsewhere in the genome, is not yet known. No pathway-specific transcription factor has been described for CPA biosynthesis and no gene with predicted sequence to a transcription factor resides in cluster 55. Chang *et al*. (2009) deleted a predicted zinc-finger transcription factor found distal to PKS–NRPS, but observed no effect on CPA. This gene is on the *A. flavus* Affymetrix GeneChip and was not significantly transcribed under any of the 25 experimental conditions examined in this study. In addition, we have shown that the two predicted genes located between the CPA and AF clusters do not appear to be essential for the biosynthesis of either metabolite.

As the AF and CPA gene clusters are located next to each other, we also investigated whether the AF pathway-specific transcription factor AflR could control genes in the CPA cluster. Price *et al*. (2006) studied the effect of AflR on gene expression by deleting the AF pathway transcription factor *aflR*. A closer look at the microarray data revealed no significant differences for any of the three CPA biosynthetic genes contained on the 5002-element arrays used for this study. There is one caveat to the *aflR* deletion experiment regarding CPA. These studies were performed using *A. parasiticus*, which is not known to produce CPA (Dorner *et al*., 1984); however, a closer examination of the data shows a strong expression signal in both the WT and deletion mutant for two of the three genes needed for CPA production present on the 5002-element array. This result indicates that the lack of CPA production in *A. parasiticus* is probably not a result of a deficiency in gene expression from these two genes.

With our newfound knowledge of the location of the CPA cluster, we were able to further explore some previous results examining *A. flavus* strain 649. The *afl-1* mutation in *A. flavus* strain 649 is the only known dominant mutation inhibiting AF biosynthesis, and thus AF biosynthesis cannot be restored in parasexual diploids resulting from the pairing of *afl-1* mutants with WT AF-producing strains (Papa, 1979). This dominant mutation is thought to inhibit AF biosynthesis through an unknown silencing mechanism which can be overcome through the addition of ectopic copies of the pathway regulatory gene *aflR* (Smith *et al*., 2007). Strain 649 does not contain the AF cluster or the CPA cluster as a result of a large chromosomal loss. Unknown to Smith *et al*. (2007), when they assayed for the effects of *afl-1* on genes outside the aflatoxin cluster, one of the genes measured was the predicted dimethylallyl tryptophan synthase (*dmaA*) of the CPA cluster [denoted 16TV in the study by Smith *et al*. (2007)]. This gene was found to be expressed at WT levels in the diploid between the *afl-1* mutant strain 649 and AF-producing strain 86. We examined the same 86×649 diploid and found it to produce CPA (data not shown), indicating that all of the genes necessary for the biosynthesis of CPA in the WT strain are expressed in the diploid. On the basis of these results, we hypothesize that, if there is a regulatory gene specific to the CPA cluster, it is at a locus outside the deleted region on chromosome 3 of the *afl-1* mutation.

Until now, very little was known about the regulation of CPA in *A. flavus*. CPA appears to respond more similarly to sterigmatocystin biosynthesis in *A. nidulans* with regard to nitrogen source. Nitrate, like temperature, affects the biosynthesis of sterigmatocystin differently from that of AF, with more sterigmatocystin being produced on nitrate rather than the more AF-conducive nitrogen source, ammonium (Feng and Leonard, 1998). Nitrogen source, pH and carbon source are thought to directly affect the expression of genes through *cis*-regulatory elements related to the environmental sensing of nitrogen, pH and carbon. The proteins that interact with these regulatory elements include global transcription factors, such as AreA for nitrogen (Caddick *et al*., 1994), PacC for pH (Penalva *et al*., 2008) and the various regulatory DNA-binding proteins associated with carbon catabolite repression (Ebbole, 1998). Many aflatoxin genes possess these regulatory elements, but appear to respond differently from sterigmatocystin and CPA; however, proteins such as PacC can act as both activators and repressors of gene

expression (Cary *et al*., 2006; Georgianna and Payne, 2008; Penalva *et al*., 2008). From these data, it appears that, although CPA does not respond identically to AF for the culture conditions tested, it responds very similarly to how we would expect sterigmatocystin to accumulate in *A. nidulans*. Despite the differential accumulation of CPA and AF in response to the culture medium environment, Fig. 7 shows that these two metabolites accumulate almost identically in a natural environment such as maize.

The goal of our study was to establish expression patterns and to associate gene clusters with known functions to others having no currently accepted roles. In addition, we were able to determine the identity of the metabolite produced by predicted gene cluster 55 as CPA. CPA has the potential to become an overlooked mycotoxin that could have significant health risks (Nishie *et al*., 1985). Our data show that, under conditions favourable for the production of AF, CPA will also probably be produced, assuming that the strain has the capacity to produce CPA. As not all strains of *A. flavus* produce aflatoxin, some do not produce CPA because of mutations or a loss of the gene cluster (Chang *et al*., 2005; Geiser *et al*., 2000). As both metabolites can be detected simultaneously (see Experimental procedures), it is not difficult to include CPA testing when checking for AF. In addition, CPA may be more commonly encountered than AF because of its biosynthesis by both *Aspergillus* and *Penicillium* species (Frisvad and Thrane, 2000; Hermansen *et al*., 1984; Le Bars, 1979; Pitt *et al*., 1986), and its production over a wide range of conditions.

EXPERIMENTAL PROCEDURES

Fungal strains

Aspergillus flavus strains NRRL3357, AFC-1 and CA14Δku70ΔpyrG were used in these studies. Strain 3357-5, a uracil auxotroph of NRRL3357 (He *et al*., 2007), was used to create a new strain with both an arginine and uracil requirement, called AFC-1. This strain was created through the use of *pyr4*-blaster, a plasmid containing complementary kanamycin cassette sequences flanking the *pyr4* gene from *Neurospora crassa*. The construct is designed such that the marker gene can be forced to loop out under selection on 5-fluoro-orotic acid (5-FOA) (5-FOA is metabolized to the toxic compound fluorodeoxyuridine in strains containing an active 5′-orotidine decarboxylase gene such as *pyr4*), similar to the method used by d'Enfert (1996) with *pyrG* in *A. fumigatus* (d'Enfert, 1996). The loss of this marker on growth on medium containing 5-FOA and uracil restored the requirement for uracil in the fungus.

We used *pyr4*-blaster to delete the *argD* gene in 3357-5, with subsequent mutagenesis with 5-nitroquinoline 1-oxide (5-NQO), as described by He *et al*. (2007), and selection on 5-FOA (100 μg/mL) (Zymo Research, Orange, CA, USA)-containing MLS medium (Czapeck-Dox Broth (Difco) plus 0.4 M $(NH_4)_2SO_4$ and 1% agar) with nucleic acid supplements uracil (7.5 mM) and uridine (7.5 mM) plus the amino acid arginine (1.5 mM) at pH 3 to create the AFC-1 double mutant (-*pyrG*, -*argD*) (note: agar was autoclaved separately and added to a filter-sterilized pH 3 solution of the 5-FOA-containing medium). The 5-NQO step was added because the background in strains simply selected on 5-FOA was not sufficient for genetic transformations. Strain AFC-1 shows no background when selecting against either marker, and produces AF at similar levels to the parent strains 3357 and 3357-5.

The *A. flavus* strain CA14Δku70ΔpyrG has a *ku70* deletion background which results in high rates of homologous recombination for more efficient gene targeting. CA14Δku70ΔpyrG was kindly provided by Perng-Kuang Chang (Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture, New Orleans, LA, USA).

Gene deletion, fungal transformation and DNA isolation

An overlap PCR method (Davidson *et al*., 2002) was used to create gene deletion constructs with *argD* as the selectable marker (Fig. 6A). Primers were designed from the DNA sequence downloaded from the genome browser (Stein *et al*., 2002) for *A. flavus* (http://www.aspergillusflavus.org). Genomic DNA from strain NRRL3357 was used as a PCR template for both the 5′ and 3′ homologous flanking regions of the genes targeted for deletion in this study. Amplification of *argD* included sufficient upstream sequence to include the native promoter. For fungal transformation, we used the methods suggested by He *et al*. (2007) and currently available at www.aspergillusflavus.org. The transformation selection medium, MLS, was supplemented with 1.12 g/L of uracil in order to use the *argD*-containing deletion construct with strain AFC-1. DNA was isolated from transformants for PCR-based screening using the cetyltrimethylammonium bromide (CTAB) method described by He *et al*. (2007). Screening primers were designed to amplify outside of the *dmaA* deletion construct to screen for its integration in the genome (Fig. 6A, primers 7 and 8). Other genes were deleted in strain CA14Δku70ΔpyrG using the same method, but with *pyr4* as the selectable marker, with the appropriate ~1-kb flanking regions and MLS as the selective medium.

Culture media for CPA and AF production

A total of five different cell culture conditions known to influence AF biosynthesis (Mahoney and Molyneux, 2004; Price *et al*., 2005) were tested for their effect on the accumulation of CPA and AF. NRRL3357 was grown in either A&M medium (Mateles and Adye, 1965) or A&M medium modified for carbon source

(50 g/L), nitrogen source (3 g/L), pH or the presence of anti-oxidants. All media were buffered at pH 4.5 with 100 mM citric acid, except for the pH 8.0 treatment, which was buffered with 100 mM tris(hydroxymethyl)aminomethane (Tris). The following five conditions were compared: (i) the AF-conducive carbon source, sucrose, was compared with lactose; (ii) the AF-conducive nitrogen source, ammonium sulphate, was compared with sodium nitrate; (iii) the AF-conducive temperature, 28 °C, was compared with 37 °C; (iv) the AF-conducive pH 4.5 was compared with pH 8.0; and (v) A&M medium containing 2.5 mM gallic acid (*n*-propyl gallate; MP Biomedicals, Inc., Solon, OH, USA), an anti-oxidant that inhibits AF (Mahoney and Molyneux, 2004), was compared with standard A&M medium. A 2-mL aliquot of the medium was placed in 24-well plates (Corning Life Sciences Inc., Lowell, MA, USA) and inoculated with *A. flavus* to a final concentration of 1×10^6 condia/mL. The plates were wrapped with parafilm and incubated at 28 °C, except for the 37 °C treatment. Each treatment was replicated three times.

RNA isolation and RT-PCR

Mycelia were filtered from 48 h potato dextrose broth cultures grown at 28 °C with shaking at 200 rpm using miracloth filters together with vacuum flasks. This tissue was lyophilized and used for RNA isolation with the Qiagen Plant RNeasy kit (Qiagen Inc., Valencia, CA, USA). After RNA isolation, the concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

A total of 3 μg of RNA was used for DNase treatment with Promega RQ1 DNase according to the manufacturer's suggestion (Promega, Madison, WI, USA). After DNase treatment, 1.5 μg of RNA was used for reverse transcription employing the first-strand cDNA sythesis protocol for SuperScript™ II RT (Invitrogen Corp., Carlsbad, CA, USA). PCR was performed on the resulting cDNA with the appropriate gene-specific primers with Takara ExTaq (Takara Bio, Otsu, Shiga, Japan), following the manufacturer's suggestion, with a total of 30 1-min extension cycles. Primers were purchased from Sigma-Aldrich (St Louis, MO, USA). PCR products were analysed on a 0.8% Tris-acetate-ethylenediaminetetracetic acid (TAE) agarose gel.

Microarray analysis

Data from experiments using the *A. flavus* Affymetrix GeneChip microarrays were imported into JMP genomics (SAS, Cary, NC, USA). All array data was deposited in the National Center for Biotechnology Information (NCBI) GEO database as experiment GSE15435. The arrays included data from the following experiments: (i) a 28 °C vs. 37 °C comparison in A&M culture medium from Georgianna *et al*. (2008); (ii) a 28 °C vs. 37 °C experiment on detached field-grown corn kernels from Smith *et al*. (2008); (iii) a time course study in which developing seeds were inoculated in the field with *A. flavus* and the seeds were assayed every 24 h from 48 to 96 h; (iv) a study in which developing seeds were inoculated at four different corn development stages and assayed 4–5 days later (A.L. Dolezal *et al*., unpublished work); (v) *A. flavus* grown on either the endosperm or embryo of autoclaved corn kernels (C.P. Woloshuk *et al*., unpublished work); (vi) a comparison of WT *A. flavus* with an *laeA* deletion strain and *laeA* overexpression strain at 6 and 24 h (J. Bok and N.P. Keller, unpublished work); (vii) a comparison of *A. flavus* (NRRL3357) with *A. oryzae* (RIB40) grown on field-inoculated corn and wheat bran koji (D.R. Georgianna *et al*., unpublished work); and (viii) a comparison of WT *A. flavus* with an *laeA* deletion strain and *laeA* overexpression strain under sclerotia-forming conditions (S. B. Sigal *et al*., Department of Medical Microbiology and Immunology, Department of Plant Pathology, University of Wisconsin, Madison, WI 53706, USA). These experiments are described in further detail in Table 1. Data from each experiment were corrected for background, normalized and summarized in JMP genomics using the robust multichip average (RMA) normalization procedure (Irizarry *et al*., 2003a, b). The mean value for all replicates of each individual experiment was taken. We analysed the subset of values for all backbone genes associated with secondary metabolism clusters predicted in SMURF. Hierarchical clustering using the Fast Ward procedure was performed in JMP genomics for the backbone gene expression values across all microarray experiments.

Detection and quantification of CPA and AF

All experiments were performed with the Thermo LTQ ion trap instrument (Thermo Scientific, Bremen, Germany) at the North Carolina State University Genome Sciences Laboratory (GSL; http://gsl.cals.ncsu.edu/). For the quantification of CPA and AF, standards were purchased (Sigma-Aldrich). The solvent system used consisted of 25 mM morpholine and methanol (MeOH) at 50 μL/min on a Thermo Hypersil Gold C18 column (3 μm particle size, 150 × 1 mm). A 15-min linear gradient was used, starting at 20% MeOH, increasing to 80% MeOH, followed by a hold at 80% MeOH for 9 min, and column equilibration at 20% MeOH for 12 min. The mass spectrometer was set to run in electrospray ionization (ESI) positive mode with six different scans: one full scan from 200 to 2000 *m/z*, followed by five selected ion MS/MS mode scans for 331 Da (AFG_2), 329 Da (AFG_1), 315 Da (AFB_2), 313 Da (AFB_1) and 337 Da (CPA). Metabolites were identified from MS/MS scans using the National Institute of Standards and Technology Mass Spectral Search Program. All samples used for quantification in A&M medium were diluted by 1/10th with 1 : 4 MeOH : 25 mM morpholine. The dilution of the sample and the use of the highly basic (>pH 10) morpholine limited signal-suppressing matrix effects that were observed for CPA using

undiluted sample or other carrier ions. A standard curve was created in a similar dilution of A&M medium and, for each different condition examined, a single standard addition was used to verify the accuracy and comparability of quantitative measurements. Values for the accumulation of CPA and AF in A&M medium were adjusted for dry weights by determining the mass of lyophilized tissue for each replicate. Maize samples were analysed similarly to the A&M experiments, except that the concentrations were not normalized for biomass.

Qualitative analysis, employed to determine the absence or presence of mycotoxins, was performed using a similar method with direct injection of the sample medium onto the column and no reference standards. This method was used for the detection of CPA in the deletion mutants created in this study, where the deletion was hypothesized to contain no detectable CPA.

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