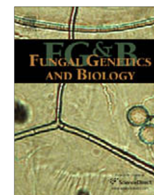
Fungal Genetics and Biology 46 (2009) 113–125

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi



Review

Genetic regulation of aflatoxin biosynthesis: From gene to genome

D. Ryan Georgianna, Gary A. Payne *

Department of Plant Pathology, North Carolina State University, 851 Main Campus, Dr. Partners III Suite 267, Raleigh, NC 27606, Campus Box 7244, USA

ARTICLE INFO

Article history:
Received 13 May 2008
Accepted 10 October 2008
Available online 5 November 2008

Keywords:
Aflatoxin
Aspergillus
Genomics
Secondary metabolism

ABSTRACT

Aflatoxins are notorious toxic secondary metabolites known for their impacts on human and animal health, and their effects on the marketability of key grain and nut crops. Understanding aflatoxin biosynthesis is the focus of a large and diverse research community. Concerted efforts by this community have led not only to a well-characterized biosynthetic pathway, but also to the discovery of novel regulatory mechanisms. Common to secondary metabolism is the clustering of biosynthetic genes and their regulation by pathway specific as well as global regulators. Recent data show that arrangement of secondary metabolite genes in clusters may allow for an important global regulation of secondary metabolism based on physical location along the chromosome. Available genomic and proteomic tools are now allowing us to examine aflatoxin biosynthesis more broadly and to put its regulation in context with fungal development and fungal ecology. This review covers our current understanding of the biosynthesis and regulation of aflatoxin and highlights new and emerging information garnered from structural and functional genomics. The focus of this review will be on studies in *Aspergillus flavus* and *Aspergillus parasiticus*, the two agronomically important species that produce aflatoxin. Also covered will be the important contributions gained by studies on production of the aflatoxin precursor sterigmatocystin in *Aspergillus nidulans*.

© 2009 Published by Elsevier Inc.

1. Introduction

Secondary metabolism is a hallmark of filamentous fungi. The diversity and complexity of secondary metabolites is astounding, and species of *Aspergillus* are rich in genes for secondary metabolism (Kobayashi et al., 2007; Nierman et al., 2005; Rokas et al., 2007). In *Aspergillus oryzae* there are 48 predicted NRPS and PKS proteins and in *Aspergillus fumigatus* there are at least 26 different secondary metabolism clusters (Nierman et al., 2005). As noted by Keller et al. (2005), it is often difficult to define secondary metabolites, and even more difficult to ascribe biological roles for these compounds. A common working definition is that secondary metabolites are not required for growth of the organism in culture, but do contribute to the fitness of the organism in its natural environment.

Understanding and appreciation of fungal secondary metabolites remained relatively obscure until the discovery of penicillin in 1929 by Alexander Fleming. Since that time they have been extensively studied due to their bioactive nature and economic importance. Secondary metabolites impact our daily lives either as toxins or as beneficial compounds. Beneficial secondary metabolites made by species of *Aspergillus* include food additives such as kojic acid, antibiotics such as penicillin, and cholesterol reducing drugs such as lovastatin (Adrio and Demain, 2003; Endo et al., 1976).

In contrast, the repertoire of fungal secondary metabolites also includes harmful products known as mycotoxins. Aflatoxin (AF), originally discovered as the cause of Turkey-X disease, is a classic mycotoxin that has been studied for nearly 50 years (Nesbitt et al., 1962). It is a stable, small molecule that contaminates major grain and nut crops colonized by species of *Aspergillus*. It is both toxic and carcinogenic and exposure to aflatoxins has been associated with many veterinary toxic syndromes (Bressac et al., 1991; Hsu et al., 1991; Richard and Payne, 2003). Comprehensive studies have shown that AF is a risk factor for human hepatocellular carcinoma, especially in Asia and sub-Saharan Africa (Groopman et al., 2005). While death is an uncommon outcome of aflatoxicosis in humans, several deaths were attributed to aflatoxicosis as recently as 2004 (Nyikal et al., 2004). Because of its toxicity, over 100 countries restrict the content of AF in the food and feed supplies (van Egmond et al., 2007). A guideline of 20 parts AF per billion parts of food or feed substrate is the maximum allowable limit imposed by the US Food and Drug Administration for interstate shipment. In the United States, agricultural economic losses due to AF contamination of food and feed amount to $270 M annually (Richard and Payne, 2003).

Secondary metabolites owe their diverse structures to the many pathways involved in their biosynthesis, and the versatility of large multifunctional proteins, such as polyketide synthases (PKSs) and non ribosomal peptide synthases (NRPSs). There are several path-

* Corresponding author. Fax: +1 919 513 0024.
E-mail address: Gary_Payne@ncsu.edu (G.A. Payne).

1087-1845/$ - see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.fgb.2008.10.011

ways leading to the production of secondary metabolites, with polyketides being the most abundant class (Keller et al., 2005). The mycotoxins AF and aflatrem represent a classic polyketide and non ribosomal peptide, respectively, produced by species of *Aspergillus* (Duran et al., 2007; Keller et al., 2005).

AF remains one of the best-characterized fungal secondary metabolites. The genes for AF biosynthesis along with the pathway specific regulator *aflR*, reside in a 70 kb DNA cluster near the telomere of chromosome 3 (Chang et al., 1993; Payne et al., 2006; Woloshuk et al., 1994; Yu et al., 2004). The seminal research on AF biosynthesis was conducted with *Aspergillus flavus* and *Aspergillus parasiticus*, the two species most commonly associated with AF contamination of agricultural commodities. AF is produced by several species of *Aspergilli* including *A. flavus*, *A. parasiticus*, *Aspergillus nomius*, *Aspergillus pseudotamarii*, and *Aspergillus bombycis* (Cary et al., 2005; Frisvad et al., 2005; Varga et al., 2003). Elegant research on production of the AF precursor sterigmatocystin (ST) with the genetic model *Aspergillus nidulans*, has contributed greatly to our understanding of the aflatoxin pathway, and has been essential for our understanding of the global regulatory mechanisms discussed later.

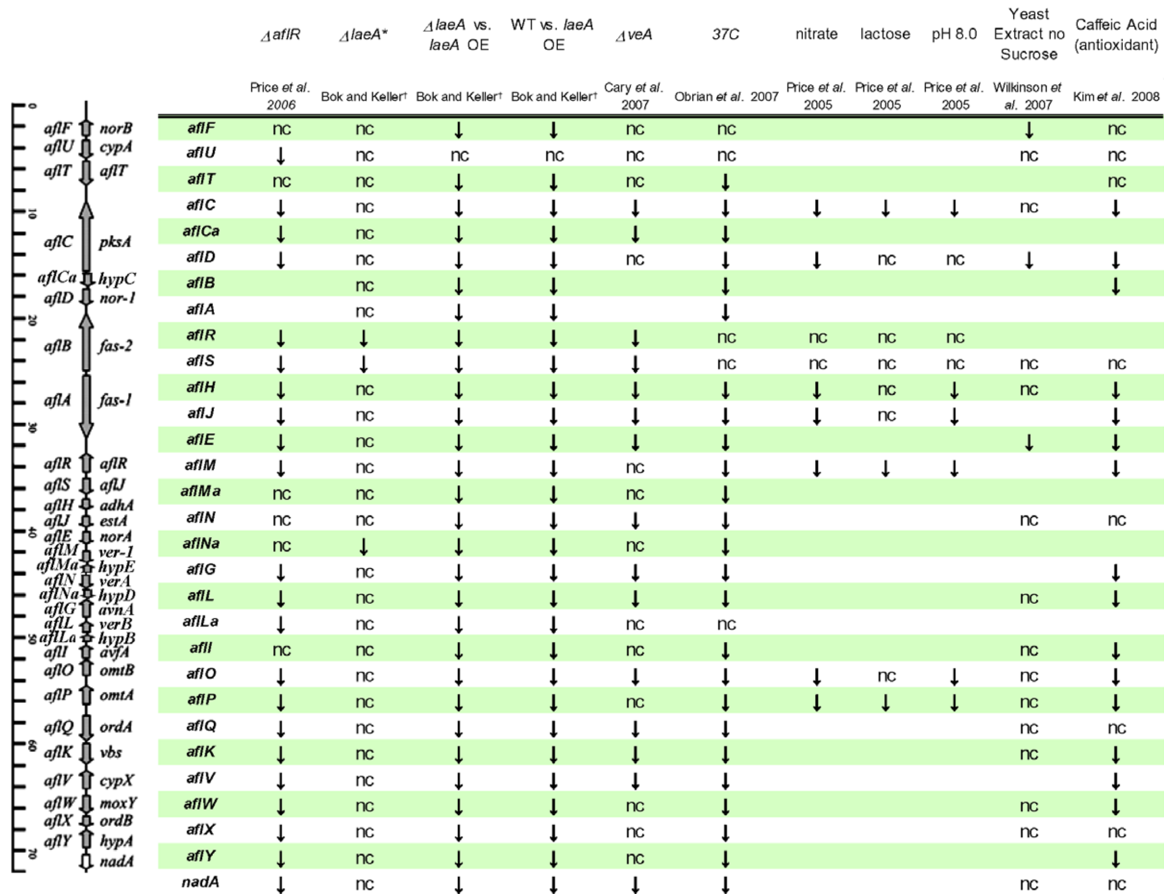
There are excellent reviews describing the chemistry and enzymology of AF biosynthesis as well as its genetic regulation (Bennett and Klich, 2003; Bhatnagar et al., 2003, 2008; Hicks et al., 2002; Keller et al., 2005; Yu et al., 2004). A goal of this review is to cover recent research on structural and functional genomics and put this information in context with current knowledge about aflatoxin biosynthesis.

1.1. Regulation of aflatoxin biosynthesis

The biosynthesis of AF has many layers of regulation, some of which are almost entirely specific to the pathway while others display a more global regulation of secondary metabolism. The use of genetics and tools from genomic sciences over the past decade (Bhatnagar et al., 2008) has expanded our knowledge of regulatory machinery beyond the AF pathway specific regulator AflR to show that the AF pathway is regulated by many mechanisms (Bok and Keller, 2004; Perrin et al., 2007). Many environmental factors control AF biosynthesis, including development, light (Calvo et al., 2002), carbon source, temperature, and pH (Fig. 1) (O'Brian et al., 2007; Price et al., 2005). We anticipate that many of these higher order processes regulating the biosynthesis of AF will also regulate the synthesis of other secondary metabolites as has been shown for those linked with the proteins VeA and LaeA discussed later in this review (Bayram et al., 2008; Duran et al., 2007; Keller et al., 2005).

2. Regulation within the aflatoxin biosynthetic cluster

The regulation of AF biosynthesis is complex and involves several interconnecting networks. At the risk of being somewhat arbi-



	Δ*aflR*	Δ*laeA**	Δ*laeA* vs. *laeA* OE	WT vs. *laeA* OE	Δ*veA*	37C	nitrate	lactose	pH 8.0	Yeast Extract no Sucrose	Caffeic Acid (antioxidant)
	Price *et al.* 2006	Bok and Keller†	Bok and Keller†	Bok and Keller†	Cary *et al.* 2007	Obrian *et al.* 2007	Price *et al.* 2005	Price *et al.* 2005	Price *et al.* 2005	Wilkinson *et al.* 2007	Kim *et al.* 2008
aflF	nc	nc	↓	↓	nc	nc				↓	nc
aflU	↓	nc	nc	nc	nc	nc				nc	nc
aflT	nc	nc	↓	↓	nc	↓					nc
aflC	↓	nc	↓	↓	↓	↓	↓	↓	↓	nc	↓
aflCa	↓	nc	↓	↓	↓	↓					
aflD	↓	nc	↓	↓	nc	↓	↓	nc	nc	↓	↓
aflB		nc	↓	↓		↓					↓
aflA		nc	↓	↓		↓					
aflR	↓	↓	↓	↓	↓	nc	nc	nc	nc		
aflS	↓	↓	↓	↓	↓	nc	nc	nc	nc	nc	nc
aflH	↓	nc	↓	↓	↓	↓	↓	nc	↓	nc	↓
aflJ	↓	nc	↓	↓	↓	↓	↓	nc	↓		↓
aflE	↓	nc	↓	↓	↓	↓				↓	↓
aflM	↓	nc	↓	↓	nc	↓	↓	↓	↓		↓
aflMa	nc	nc	↓	↓	nc	↓					
aflN	nc	nc	↓	↓	↓	↓				nc	nc
aflNa	nc	↓	↓	↓	nc	↓					
aflG	↓	nc	↓	↓	↓	↓					↓
aflL	↓	nc	↓	↓	↓	↓				nc	↓
aflLa	↓	nc	↓	↓	nc	nc					
aflI	nc	nc	↓	↓	nc	↓				nc	↓
aflO	↓	nc	↓	↓	↓	↓	↓	nc	↓	nc	↓
aflP	↓	nc	↓	↓	nc	↓	↓	↓	↓	nc	↓
aflQ	↓	nc	↓	↓	↓	↓				nc	nc
aflK	↓	nc	↓	↓	↓	↓				nc	↓
aflV	↓	nc	↓	↓	↓	↓					↓
aflW	↓	nc	↓	↓	nc	↓				nc	↓
aflX	↓	nc	↓	↓	nc	↓				nc	nc
aflY	↓	nc	↓	↓	nc	↓					↓
nadA	↓	nc	↓	↓	↓	↓				nc	nc

Fig. 1. Changes in the expression of genes within the AF cluster in response to conditions favorable or not favorable for AF biosynthesis. *All conditions are compared to a WT culture in media strongly supporting AF production except for the Δ*laeA* comparison in which the WT had low expression values for AF genes. Genes are ordered in position along the chromosome with the most left being nearer to the telomere, a schematic of the cluster genes and their orientation is represented on the left. A down arrow indicates decreased and an up arrow indicates increased gene expression in response to the non-conducive condition compared to the controls used. Blank spaces represent genes for which no data were obtained or were presented in the study. WT vs. *laeA* OE represents wild-type cultures of *A. flavus* compared to strains overexpressing the *laeA* gene, a down arrow in this case only means the gene was more lowly expressed in the WT compared to *laeA* OE. For caffeic acid experiments (Kim et al., 2008) statistics were not available in the article; medium and high level changes are indicated with arrows and low level changes as nc. †Array data provided by Bok and Keller (personal communication).

trary as to how we describe this complex regulation, we have divided the regulation into three parts, starting with regulation within the biosynthetic cluster. AF was one of the first fungal secondary metabolites shown to have all its biosynthetic genes organized within a DNA cluster. These genes, along with the pathway specific regulatory genes *aflR* and *aflS*, reside within a 70 kb DNA cluster. We now know that clustering of genes for secondary metabolism is a common feature (Keller and Hohn, 1996), although in some cases biosynthetic and pathway regulatory genes reside outside the cluster (Desjardins and Proctor, 2007). As will be discussed later, the position of genes within a DNA cluster has implications for its regulation.

Research on *A. flavus*, *A. parasiticus* and *A. nidulans* has led to our current understanding of the enzymatic steps in the AF biosynthetic pathway, as well as the genetic organization of the biosynthetic cluster. *A. nidulans* does not produce AF but has all of the genes and enzymatic steps preceding the production of sterigmatocystin (ST). The AF and ST pathways appear to have a common biosynthetic scheme up to the formation of ST, and thus information gained from both pathways has been used to study AF regulation (Hicks et al., 2002; Yu et al., 2004). Interestingly, the order of the genes in *A. nidulans* differs from that of *A. flavus*. This shuffling of genes reflects the relative age of the cluster, based on estimates of protein similarities between *A. nidulans*, *A. fumigatus* and *A. oryzae* the cluster could be as old as 450 million years (Galagan et al., 2005). However, within section Flavi evidence exists suggesting that for at least 25 million years gene order, location of AflR binding sites, and intergenic distances have been conserved (Ehrlich et al., 2005).

2.1. Transcriptional regulators (AflR and AflS)

Two genes, *aflR* and *aflS*, located divergently adjacent to each other within the AF cluster are involved in the regulation of AF/ST gene expression (Fig. 1). The gene *aflR* encodes a sequence-specific DNA-binding binuclear zinc cluster ($Zn(II)_2Cys_6$) protein, required for transcriptional activation of most, if not all, of the structural genes (Chang et al., 1993, 1995; Chang et al., 1999a,b; Ehrlich et al., 1998; Flaherty and Payne, 1997; Payne et al., 1993; Price et al., 2006; Woloshuk et al., 1994; Yu et al., 1996b). The *aflR* locus has been compared among strains within section *Flavi*, especially among isolates of AF producers such as *A. parasiticus* and *A. flavus* (Chang et al., 1995; Lee et al., 2006). These comparisons revealed differences in many promoter regulatory elements such as PacC and AreA binding sites (Ehrlich and Cotty, 2002; Lee et al., 2006). The *aflR* gene is also found in *A. nidulans* and *A. fumigatus* (Carbone et al., 2007). Despite clear differences in the sequence of AflR between *A. nidulans* and *A. flavus*, function is conserved. AflR from *A. flavus* is able to drive expression of the ST cluster in an *A. nidulans aflR* deletion strain (Yu et al., 1996a).

To more carefully examine gene regulation by AflR, Price et al., (2006) examined gene expression in a wild-type and *aflR* deletion strain of *A. parasiticus* using a 5002 element microarray. They found some genes in the AF biosynthetic cluster that showed essentially no expression in the Δ*aflR* strain, suggesting that AflR is absolutely required for their transcription. Other genes in the AF pathway showed some transcription, albeit lower than in the wild-type. The first column in the microarray expression summary of Fig. 1 shows six genes within the AF cluster whose expression appears to be more independent of AflR regulation. These results argue that the expression of these genes is either not tightly regulated, i.e., they have a moderate level of basal expression, or that their expression is modulated by regulatory factors other than AflR.

One explanation for these changes in expression for some genes but not others may be differences in AflR consensus binding sites. AflR binds to the palindromic sequence 5′-TCGN$_5$CGA-3′ (also called AflR binding motif) in the promoter region of many of the structural genes in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Fernandes et al., 1998). While a good predictor of AflR binding, the AflR binding motif is complex. Electrophoretic mobility shift assays (EMSA) have been used to thoroughly examine promoters for AflR binding in 11 different genes from the AF cluster, with three of these genes having sites that deviate from the predicted AflR binding motif, and an additional three AF genes for which AflR binding sites could not be demonstrated (Ehrlich et al., 1999b). Among these genes, *aflE*, *aflC*, *aflJ*, *aflM*, *aflK*, *aflQ*, *aflP*, *aflR*, and *aflG* all have predicted sites and demonstrate some degree of AflR binding in EMSA assays. All of these genes were differentially expressed between WT and the Δ*aflR* mutant (Fig. 1), suggesting that AflR is required to activate their expression (Price et al., 2006). When differences in gene expression were examined across several transcriptional profiling studies these differences do not always correspond with the differences seen in the comparison between WT and the Δ*aflR* mutant (Fig. 1). This suggests that there are other regulatory factors modulating AF gene cluster expression.

AF biosynthesis is also regulated by *aflS* (formerly *aflJ*), a gene that resides next to *aflR*. The genes *aflS* and *aflR* are divergently transcribed, but have independent promoters. The intergenic region between them, however, is short and it is possible they share binding sites for transcription factors or other regulatory elements (Ehrlich and Cotty, 2002). The precise role of AflS in AF biosynthesis remains unclear. Meyers et al., (1998) initially characterized AflS through use of an *aflS* disruption strain and observed that mRNA levels for the aflatoxin biosynthetic pathway genes, *aflC*, *aflD*, *aflM*, and *aflP*, remained unchanged but despite this pathway intermediates could not be converted to aflatoxin (Meyers et al., 1998). Chang (2004) showed that AflS binds AflR and that AflS is required for AflR activation in *A. parasiticus* (Chang, 2004). However, Du et al. (2007) were unable to show an absolute requirement for AflS in AF biosynthesis or activation of AflR. The roles of AflR and AflS were examined by studying the expression of pathway genes in transformants of *A. flavus* strain 649 that received the respective genes individually and in concert (Du et al., 2007). Strain 649 lacks the AF biosynthetic cluster but has the necessary upstream regulatory elements to drive the transcription of *aflR* (Prieto et al., 1996; Woloshuk et al., 1995). These studies showed that AflR is sufficient to initiate gene transcription of early, mid, and late genes in the pathway, and that AflS enhances the transcription of early and mid aflatoxin pathway genes. Roles for AflS have been suggested to be as diverse as aiding in transport of pathway intermediates to the interaction of AflS with AflR for altered AF pathway transcription. The observation that AflS binds to AflR argues that AflS modulates aflatoxin expression through its interaction with AflR (Chang, 2003).

Consistent with earlier data, microarray experiments have shown that aflatoxin biosynthesis is always associated with the expression of *aflR*. However, in experiments comparing conditions conducive and non-conducive for the biosynthesis of aflatoxin, the expression of *aflR* and *aflS* is not always significantly different (Fig. 1). This could be the result of inherently low levels of *aflR* and *aflS* transcripts, making their quantification difficult. Another possible explanation is that regulation of AF cluster gene expression is complex, and factors other than transcript levels of *aflR* and *aflS* are important in its regulation. *AflS* transcript is thought to be dependent on *aflR* (Du et al., 2007; Ehrlich et al., 1999b; Price et al., 2006). This presents the argument that many of the non-conducive conditions described in Fig. 1, where *aflS* and *aflR* do not change but most of the pathway is strongly down-regulated, are independent of regulation by AflR or AflS.

2.2. Other possible cluster regulatory mechanisms

There is no direct evidence for the involvement of regulatory RNAs in AF biosynthesis, however a Naturally occurring Antisense Transcript (NAT), *aflRas*, was identified at the same time as *aflR* (Woloshuk et al., 1994). Recently, a global analysis of NAT expression in *A. flavus* during its colonization of maize seeds incubated at 28 or 37 °C identified 32 of 352 putative cis NATs whose expression was directly or inversely correlated with expression of their respective sense gene (Smith et al., 2008). The expression of one of these NATs was inversely correlated with the expression of *aflD*. Thus, a higher expression of the NAT for *aflD* was associated with less aflatoxin production. While this does not prove a role for a NAT in the regulation of aflatoxin biosynthesis, it is tempting to speculate that they may have some role in modulating gene expression as has been shown for the NAT for *frq*, a gene involved in circadian rhythm in *Neurospora crassa* (Kramer et al., 2003). In strains that did not accumulate the NAT for *frq*, the internal clock was delayed and unable to be reset by light. Kramer et al. (2003) observed that maximal accumulation of the NAT for *frq* occurred directly opposite of the sense transcript for *frq* which reaches highest levels in the dark.

In addition to the binding sites for AflR, there are binding sites within the cluster for other transcriptional factors that may play important roles in transcriptional regulation of the AF cluster. A novel cAMP-response element, CRE1, site has been studied specifically in the *aflD* (*nor-1*) promoter of *A. parasiticus*. The CRE1 site and the regulation of aflatoxin by cAMP and glucose concentrations are discussed in further detail in Sections 3.2 and 4.2. In addition AreA binding sites (Section 4.4) and PacC binding sites (Section 4.5) are found in promoter regions throughout the AF biosynthetic cluster and may impart a role in the regulation of AF biosynthesis by nitrogen and pH respectively.

3. External control of the aflatoxin cluster and global regulation of secondary metabolism

Although there is no known role for AF in the ecology of the fungus, AF biosynthesis is tightly regulated by environmental and development cues. The entire signaling network for processes regulating AF biosynthesis is unclear, but components of these networks have been characterized (Yu and Keller, 2005). Fig. 2 summarizes our current understanding of the regulatory networks that transmit the environmental and development signals acting on AF biosynthesis. One regulatory element shown in Fig. 2, LaeA, appears to be involved in a novel type of global regulation and will be discussed in Section 3.4.

3.1. Heterotimeric G-protein signaling and PKA

Early research on AF biosynthesis uncovered an association between development and AF production (Kale et al., 1996). This was not surprising as a tight link between secondary metabolism and development in bacteria had been established with the *bldA* gene controlling aerial mycelia, conidia, and secondary metabolites (Demain, 1992). In *Aspergillus*, several observations linked a fluffy phenotype to loss of AF/ST production. The available well-characterized fluffy mutants in *A. nidulans* were instrumental in the discovery of a signal transduction pathway regulating both

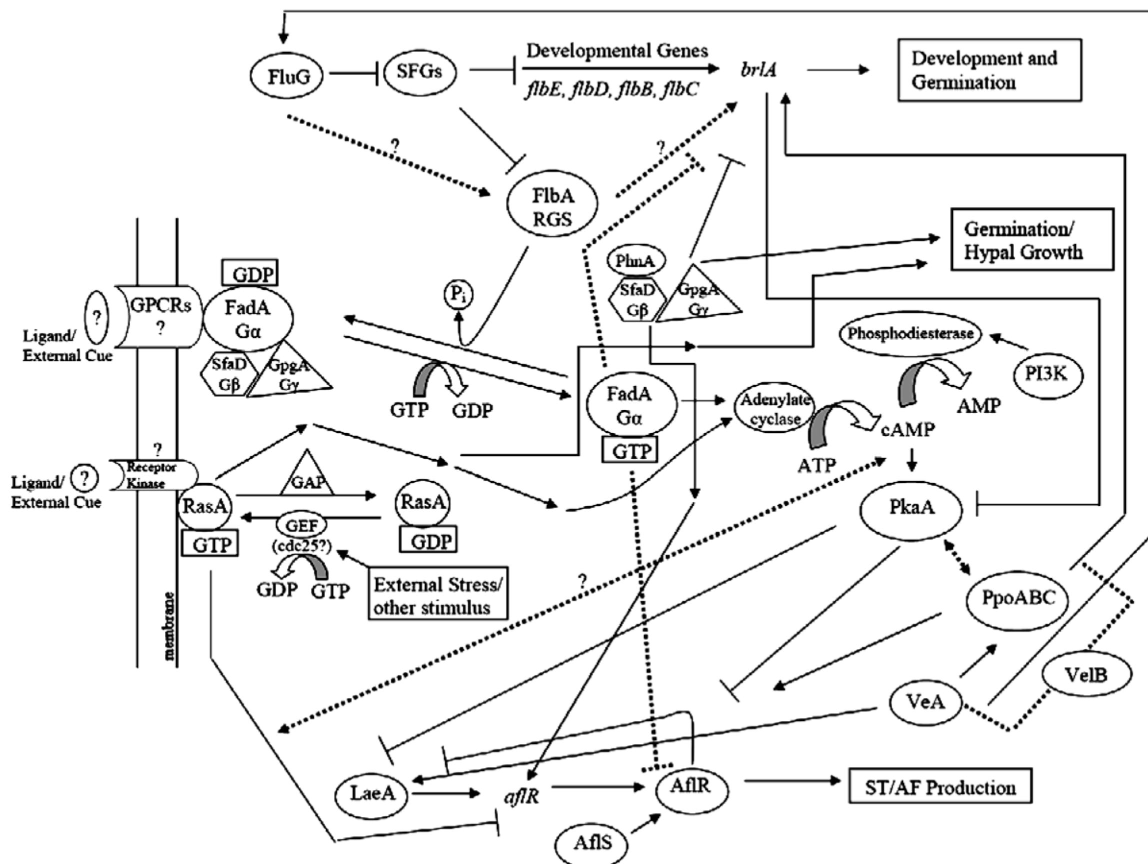


Fig. 2. Signaling pathways regulating AF biosynthesis and their ties to growth and development. Dashed lines indicate hypothesized pathways. Adapted from Calvo (2008), Seo and Yu (2006), Keller et al. (2005), Yu and Keller (2005), and Shimizu et al. (2003).

conidiation and ST/AF biosynthesis. Proteins identified as belonging to this signal transduction pathway include FlbA, an RGS (*R*egulator of *G*-protein *S*ignaling) protein, FluG, an early acting development regulator (Dohlman and Thorner, 1997; Hicks et al., 1997), FadA, the alpha subunit of a heterotrimeric G-protein (Yu et al., 1996c) and PkaA, encoding the catalytic subunit of protein kinase A (Shimizu et al., 2003; Shimizu and Keller, 2001) (Fig. 2). Activation of both FadA and PkaA inhibit conidiation and ST biosynthesis in *A. nidulans*, whereas FlbA suppresses FadA activation (Calvo et al., 2002). FlbA activation of gene expression is dependent on FluG (Hicks et al., 1997). FadA is thought to activate adenylate cyclase, resulting in an upregulation of PkaA which is able to inhibit AflR activity by preventing nuclear localization through phosphorylation of AflR, as well as cause down-regulation of a host of conidiation transcription factors (Shimizu and Keller, 2001). Later experiments have also implicated the Gβ (SfaD) and Gγ (GpgA) subunits of this heterotrimeric G-protein and PhnA, a phosducin-like protein that is an activator of Gβγ-mediated signaling, as positive regulators of *aflR* expression needed for ST biosynthesis (Fig. 2) (Seo and Yu, 2006).

Although the initial characterization of the heterotrimeric G-protein signaling pathway that regulates ST production and conidiation was established in *A. nidulans*, evidence shows that a similar signaling pathway exists in both *A. parasiticus* and *A. flavus* to regulate AflR (Hicks et al., 1997; McDonald et al., 2005; Roze et al., 2004a; Tag et al., 2000; Yu and Keller, 2005). Rather than responding to a specific external stimulus, this G-protein mediated pathway appears to play a role in regulation of AF as a function of vegetative growth and cellular development (Seo and Yu, 2006; Yu et al., 1996c).

Recent genomic evidence has revealed G-protein coupled receptors (GPCRs) to be highly conserved among *A. oryzae*, *A. fumigatus*, and *A. nidulans*, but G-proteins themselves to be more divergent (Lafon et al., 2006). *A. oryzae* and *A. flavus* are nearly identical at the genomic level, with no detected differences in G-proteins and their receptors. These receptors enable the fungus to respond to various environmental and developmental stimuli through heterotrimeric G-protein signals. The GPCR tied to the FadA-mediated signaling to control AF and ST still remains to be discovered. A total of nine GPCRs (GprA-I) in *A. nidulans* have been grouped into five different classes, class I and II (GprA and GprB) analogous to yeast pheromone receptors, class III (GprC-E) possibly involved in carbon source sensing, class IV (GprF and GprG) which are putatively linked to nitrogen sensing, and class V (GprH and GrpI) which are proposed to play a role in cAMP sensing (Han et al., 2004; Lafon et al., 2006). Six of the nine identified GPCRs have been disrupted in *A. nidulans*, with GprD proposed to control sexual development and PKA, but not through FadA (Han et al., 2004). No reports have been made on whether any of these receptor knockout mutants affected ST production.

3.2. cAMP signaling

Cyclic-AMP is an important signaling molecule. The synthesis, by adenylate cyclase, and degradation, by phosphodiesterase, of cAMP is regulated by various G-protein signaling cascades. The addition of cAMP to *Aspergillus* growth media causes an increase in AF biosynthesis (Tice and Buchanan 1982). Roze et al. (2004a,b) measured PKA activity as a function of exogenously applied cAMP and found total PKA activity to increase *in vitro* with added cAMP; however when cultures were grown in the presence of 5mM cAMP, basal and total PKA activity levels did not increase, but instead appeared to decrease (Roze et al., 2004a). It appears that high physiologically relevant cAMP levels are able to inhibit AflR through an increase in phosphorylation by cAMP dependent PKA activity. However, greatly exceeding these levels inhibits cAMP dependent PKA to cause the observed induction in aflatoxin biosynthesis (Roze et al., 2004a). Accordingly, Roze et al., (2004b) closely examined a CRE1 (cAMP-response element) site necessary for *aflD* induction by cAMP in the *aflD* promoter that was dependent on AflR (Roze et al., 2004c). This site controls *aflD* expression in response to an unidentified 32-kDa CRE1 binding protein (p32) that is hypothesized to physically interact with and assist binding of AflR to the single AflR binding site in the *aflD* promoter (Roze et al., 2004c). In order to produce increased levels of AF in response to cAMP, it seems likely that all genes within the AF cluster would have enhanced expression upon cAMP treatment. At least 10 genes examined so far from the aflatoxin cluster appear to have CRE1-like sites in their promoters (Roze et al., 2004c). However, only *aflD* and *aflM* have been reported to show increased expression in response to exogenous cAMP and it is not reported how close the CRE1 site must be to an AflR binding site for p32 to interact with and stimulate AflR binding.

It has been shown that PKA and cAMP levels are regulated through a negative feedback loop affected by a phosphodiesterase in *Cryptococcus neoformans* (Hicks et al., 2005). Phosphodiesterases have also been implicated to control cAMP levels in *Saccharomyces cerevisiae* with this regulation postulated to be controlled by stress responses triggered by the Ras-cAMP pathway (Park et al., 2005). Recent evidence has shown that a potential phosphatidyl inositol (PI) 3-kinase pathway regulates aflatoxin through control of cAMP levels and PKA activity (Lee et al., 2007). Lee et al. (2007) came to these conclusions after observing that wortmannin, a secondary metabolite commonly made by *Penicillium* and *Fusarium* species, inhibited aflatoxin biosynthesis and promoter activity for *aflD* and *aflM* (Lee et al., 2007). No effect on the expression of other genes was reported specifically but it would be interesting to know whether wortmannin affects the activity or expression of AflR. Presumably, treatment with wortmannin would activate PKA causing phosphorylation of AflR and inhibition of aflatoxin biosynthesis. This research has led to a proposed regulatory pathway where PI 3-kinase is able to activate phosphodiesterase, causing a decrease in cAMP levels and correspondingly a decrease in PKA activity (Fig. 2).

3.3. Ras family GTPase signaling

The Ras family is a highly conserved group of GTPases composed of six sub-families: Ras, Rho, Ran, Rab, Arf, and Kir/Rem/Rad (Ehrhardt et al., 2002; Reuther and Der, 2000). This family of GTPases composes a key intracellular signaling network having the ability to transmit extracellular signals to the nucleus and control transcription in response to certain stimuli (Roze et al., 1999). GTPases are in their active signaling state when associated with GTP and inactive state when bound to GDP. The Ras-cAMP pathway mediates global responses connected to stress tolerance (temperature, osmolarity, oxidative, and others) and nutrient sensing (Park et al., 2005).

In comparison to heterotrimeric G-protein signaling in *Aspergilli*, relatively little work has been reported on the possibilities of Ras-like GTPase involvement in AF biosynthesis. Examination of the *A. flavus* genome using HMM analysis for Ras superfamily proteins has predicted a total of 31 potential Ras family GTPases. Ras members and their regulators are known to be highly similar within and across species (Cetkovic et al., 2007; Colicelli, 2004; Field, 2005). Amino acid similarities between Ras family members within a species are usually between 40% and 95%, yet most if not all have their own unique set of biological functions (Ehrhardt et al., 2002; Reuther and Der, 2000).

The *A. nidulans rasA* gene encodes a homolog of the human *ras* proto-oncogene (Som and Kolaparthi, 1994). Examination of *A. nidulans* mutants overproducing a dominant active form of RasA

(RasA^{G17V}) supports a signaling role for this protein in spore germination and carbon sensing (Osherov and May, 2000; Som and Kolaparthi, 1994). Further, RasA has been shown to regulate ST biosynthesis by both transcriptional and post-transcriptional control of *aflR* (Shimizu et al., 2003). Interestingly, RasA transcriptional control of *aflR* is PkaA independent, but the post-transcriptional control is at least partially regulated by PkaA. How this partial regulation works is unclear, but RasA post-transcriptional control is not affected by the AflR phosphorylation state at PKA sites. This has been shown by use of an *A. nidulans* phosphorylation mutant AflR^{S323,381,382A} overexpressed in a dominant active (permanent GTP bound state) RasA^{G17V} background (Shimizu et al., 2003). Despite many hypotheses, the pathway for transcriptional control of *aflR* by RasA is not known. Other non-PKA mediated systems of AflR phosphorylation or other post-translational modifications also remain unknown.

Regulation of GTPase activity and its relation to PKA is a complicated multi-component process and could explain the partial mediation of Ras signaling related to phosphorylation of AflR. Crosstalk between Ras and cAMP could prove to be very important in tying together many of the described signals regulating AF and has potential involvement with results such as the increased levels of AF resulting from exogenously applied cAMP in culture. Interestingly, PD98059, an inhibitor of a mitogen-activated protein kinase kinase, inhibited the stimulatory affect of DcAMP on conidiation, but not AF (Roze et al., 2004a).

Ras proteins are not limited to activation of PKA but can activate numerous other kinases often tied to stress-sensing pathways needed for survival (Thevelein and de Winde, 1999). While the specific kinase responsible is not known, it is clear that another factor must be involved in Ras control of AflR, as shown by the aforementioned deletion of PKA phosphorylation sites in AflR which cannot restore AF in dominant active Ras mutants (Shimizu et al., 2003).

3.4. Chromosomal remodeling and silencing

LaeA, for "loss of *aflR* expression", controls the expression of genes involved in ST production in *A. nidulans* (Bok and Keller, 2004; Butchko et al., 1999). Deletion of *laeA* results in loss of *aflR* gene expression and ST/AF synthesis in *A. nidulans* and *A. flavus*. Furthermore, LaeA was hypothesized to be a global regulator of secondary metabolism when it was shown to regulate penicillin and lovastatin biosynthesis in *A. nidulans* (Bok and Keller, 2004). Using microarray data from *laeA* deletion and overexpression mutants, Bok et al. (2006) mapped expression patterns to genomic regions to identify new secondary metabolite gene clusters regulated by LaeA. These data supported the role of LaeA as a global regulator of secondary metabolism (Bok et al., 2006). The mechanism of this control is not currently known; because of its homology to arginine and histone methyltransferases, it has been suggested that LaeA may function through chromatin remodeling of metabolic gene clusters (Bok and Keller, 2004; Yu and Keller, 2005; Zhang et al., 2004). In *A. flavus* LaeA is clearly a strong regulator of *aflR* and *aflS* and has inhibitory effects for every gene in or around the AF cluster as shown by comparison of the *laeA* deletion mutant to the *laeA* overexpression strain (Fig. 1) (Bok and Keller, personal communication). So far there is no common motif or other physical signature that has been found among all genomic clusters regulated by this protein. Mechanisms of inhibiting LaeA could prove important in applied settings for the control of mycotoxins and other metabolites in fungi. Orthologs to LaeA have been found in all filamentous and dimorphic fungi that have been studied (Yu and Keller, 2005).

Although it has been difficult to prove that LaeA has a direct role in chromosomal remodeling, recent work by Roze et al. (2007a,b) has specifically addressed the role of histone H4 acetylation in activation of the aflatoxin gene cluster (Fig. 3). They hypothesized that p32, the CRE1 binding protein mentioned in Section 3.2, was responsible for recruitment of histone acetyltransferase (HAT) and AflR for activation of aflatoxin pathway promoters. Using chromatin immunoprecipitation analysis it was determined that histone acetylation along aflatoxin gene promoters was positively correlated with AflR binding (Roze et al., 2007a). Histone acetylation was shown to be independent of AflR activity through use of an *A. parasiticus aflR* deletion strain, AFS10. As noted by the authors, this conclusion may be complicated by the presence of a

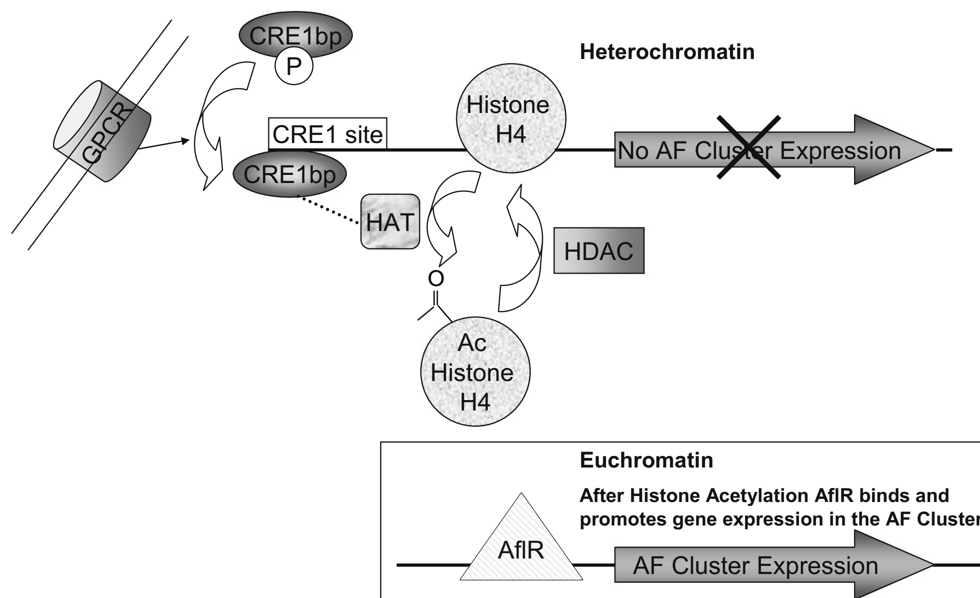


Fig. 3. Epigenetic control of the aflatoxin cluster. Histone H4 associated with heterochromatin prevents activation of aflatoxin cluster gene expression. Histone H4 is acetylated by acetyltransferase (HAT) to relax DNA and form euchromatin upon activation of CRE1bp (cAMP-response element binding protein) by dephosphorylation through a GPCR mediated pathway during declining periods of glucose/sucrose. CRE1bp recruits HAT, causing acetylation of Histone H4 with subsequent formation of euchromatin (inset box) allowing access for AflR binding and transcription of aflatoxin cluster genes. Histone deacetylase (HDAC) is able to remove acetyl groups from histones causing reformation of heterochromatin and inhibition of gene expression. LaeA, a described global regulator of secondary metabolism, is also thought to function through chromosomal remodeling, however, it remains unclear how regulation by LaeA occurs.

non-functional copy of *aflR* in the genome of AFS10, called *aflR-2*, predicted to lack the ability to activate RNA Pol II but still encode a protein (Cary et al., 2002). AflR-2 was observed bound to promoters in the *aflR* deletion strain, indicating that it has a functional binding activity. This observation reveals that transcriptionally active AflR is not necessary for histone acetylation but does not rule out that histone acetylation is dependent on AflR binding since other domains of AflR-2 are active. Results from Roze et al. (2007a,b) also displayed a dependence on physical gene order for activation that results in a temporal pattern of gene expression corresponding with histone H4 acetylation, where the "early", "middle", and "late" steps of the biosynthetic pathway are expressed in an order corresponding to their stage in the biosynthetic pathway. Interestingly, the gene order of the ST cluster in *A. nidulans* is not identical to *A. flavus* or *A. parasiticus* and a predicted sequence for the CRE1 binding protein, p32, is not present in *A. nidulans* (Roze et al., 2007a). This difference between species could prove to be important in explaining regulatory processes not observed in *A. nidulans*, such as the regulation of aflatoxin biosynthesis by temperature, which is discussed in Section 4.7.

Complementary to the work on histone acetyltransferase, a histone deacetylase (HDAC) gene (Fig. 3), *hdaA*, was deleted in *A. nidulans*. Deletion mutants of *hdaA* showed increased accumulation of secondary metabolites and gene expression for both the ST and penicillin gene clusters (Shwab et al., 2007). The *hdaA* deletion was also able to restore ST as well as penicillin in a *laeA* deletion background, however not to the same levels as the *hdaA* deletion alone. Additionally, the terraquinone A gene cluster was not affected by deletion of *hdaA* but is inhibited by deletion of *laeA*. These data suggest that *hdaA* and *laeA* function differently to control gene expression of metabolite clusters. Shwab et al. (2007) suggested that the lack of an effect of the hdaA deletion on terraquinone A biosynthesis may be due to its lack of proximity to the telomere and repetitive DNA sequence.

Not surprisingly, many of the secondary metabolites in fungi appear to be under epigenetic control. In a recent study in which *A. flavus* and other fungi were grown in the presence of several known inhibitors to histone deacetylases and methyltransferases, a wide variety of new compounds were observed to accumulate (Williams et al., 2008). While many of these new compounds are from what the author's called "silent biosynthetic pathways" which never appear to be expressed, it is clear from previously described work that epigenetics is capable of playing a major role in regulation of actively expressed secondary metabolites such as aflatoxin.

Chromosomal silencing/remodeling may be involved in the silencing of aflatoxin pathway genes in diploids formed between *A. flavus* strain 649, which contains the *afl-1* mutation, and wild-type strains (Prieto et al., 1996; Woloshuk et al., 1995). Papa (1979) described the *afl-1* mutation in 649 and it remains the only known dominant mutation for AF production. Further characterization of this strain showed that it is missing 317 kb of chromosome III, including the known genes for aflatoxin biosynthesis (Prieto et al., 1996; Woloshuk et al., 1995). In addition, 939 kb of chromosome II is present as a duplication on chromosome III in the region that originally contained the aflatoxin gene cluster (Smith et al., 2007). Inhibition of AF biosynthesis in diploids formed with 649 and aflatoxin producing strains is not due to the activation of a repressor of *aflR* or the result of currently described silencing mechanisms in fungi such as repeat-induced point mutation (RIP), quelling (RNAi), or transvection (Smith et al., 2007). The silencing appears to be restricted to the aflatoxin cluster; genes on either side of the cluster are expressed in the *afl-1* diploids. Interestingly, diploids formed between a wild-type strain and a transformant of 649 containing an ectopic copy of *aflR* produce AF. Thus the presence of an additional copy of *aflR* not in the biosynthetic cluster prevents silencing of the genes in the cluster. This mechanism may not be related to that of Δ*laeA* mutants, but it is interesting that an ectopic copy of *aflR* restores AF both in silenced diploids and in Δ*laeA* mutants.

4. Environmental conditions regulating aflatoxin biosynthesis

Several environmental and cultural conditions modulate AF biosynthesis including light, temperature, pH, nitrogen, carbon source and metals (Calvo et al., 2004; Luchese and Harrigan, 1993; Price et al., 2005). Table 1 summarizes the effect of known environmental and nutritional conditions affecting AF biosynthesis. For a more detailed discussion refer to the following reviews: (Bhatnagar et al., 2006; Cotty and Jaime-Garcia, 2007; Luchese and Harrigan, 1993; Payne and Brown, 1998). An understanding of how these factors impact AF biosynthesis is critical as it will be important in determining the role of AF in the ecology of the producing organism, and it may identify target sites for control of AF formation. Unfortunately, the regulatory networks involved in sensing and transmitting environmental and nutritional stimuli are not well understood. Price et al. (2005) examined the effect of four cultural and environmental conditions on gene transcription specific to the AF pathway. They found temperature to have the most profound effect followed by pH, nitrogen source, and then carbon source (Price et al., 2005). Schmidt-Heydt et al. (2008) examined temperature and water activity in relation to growth on secondary metabolism genes in several fungal species, including the AF cluster in *A. parasiticus*. They observed that conditions of intermediate stress to the organism were most favorable for production of mycotoxins (Schmidt-Heydt et al., 2008). Calvo et al. (2004) showed light to affect the transcription of several genes, including genes in the AF gene cluster and genes putatively involved in the development of sclerotia in *A. flavus* (Calvo et al., 2004). The effect of nutrition and environmental conditions on the transcription of the AF cluster is summarized in Fig. 1.

4.1. Light mediated regulation of aflatoxin biosynthesis

Of the many environmental conditions that modulate AF biosynthesis, the effect of light is becoming among the best-characterized. Production of cyclopiazonic acid, aflatrem, and AF by *A. flavus* is regulated by *veA*, a gene necessary for sclerotial formation (Kato et al., 2003). Velvet A protein (VeA) has been studied in many *Aspergillus* species including *A. flavus*, *A. parasiticus*, and *A. nidulans* and has been shown by these researchers to regulate, in response to light, *aflR* expression as well as formation of the environmentally resilient structures, cleistothecia in *A. nidulans* and sclerotia in *A. flavus* and *A. parasiticus*. Recent work has begun to uncover how VeA may function at the protein level. VeA is included in Fig. 2 to show that it is needed for *aflR* expression and linked to development and conidiation, these two are likely not independent processes. Purschwitz et al. (2008) has shown that FphA, a phytochrome in *A. nidulans* that responds to blue and red light, interacts with VeA. They found that red light and white light inhibited mycotoxin biosynthesis whereas blue light had a stimulatory affect

Table 1
Nutritional and environmental effects on AF biosynthesis.

Conditions	Conducive (+)	Non-conducive (−)
Carbon source	Simple sugars	Complex sugars
Nitrogen source	Reduced	Oxidized
Oxidative stress	Oxidants	Anti-oxidants
Temperature	<35 °C	>36 °C
pH	Acidic (pH 4.5)	Basic (pH 8)

(Purschwitz et al., 2008). For a detailed review of VeA see the recent review by Calvo (2008). Recently, Bayram et al. (2008) discovered through tandem affinity purification that VeA functions in a complex consisting of another velvet-like protein, VelB, and a global regulator of secondary metabolism, LaeA. These interactions allow for the coordinated control of both development and secondary metabolism by light (Bayram et al., 2008).

Microarray analysis of an *A. flavus veA* deletion strain has confirmed that VeA regulates the transcription of several genes in the fungus. Cary et al. (2007) used a 5002 element DNA microarray to assess transcriptional regulation by VeA by comparing the deletion strain to a wild-type. A total of 136 genes were differentially expressed between the two strains (Cary et al., 2007). Included in these differentially expressed genes were gene products that were regulated in other array experiments by O'Brian et al. (2007) and Wilkinson et al. (2007) such as the AAA family ATPase (clone NAFBE02TV), two different conidiation-specific proteins (clones NAGDC44TV and NAFEY43TV), and a Rieske 2Fe-2S family-like protein (clone NAFCL17TV). Many of the genes in the AF cluster were similarly affected when compared to prior array experiments (Fig. 1). The gene for the transcriptional regulator AflR was not found to be differentially expressed in the array study, however quantitative PCR results indicated a significant decrease in *aflR* expression in the VeA mutant compared to the wild-type strain; this would be expected if VeA is controlling AF biosynthesis through activation of LaeA (Bayram et al., 2008; Cary et al., 2007). An interesting experiment to further reiterate that VeA functions through LaeA would be to see if the *hdaA* deletion, described in Section 3.4, could partially relieve repression of the ST cluster in a *veA* deletion background as done with previously with *laeA*.

4.2. Regulation of aflatoxin by carbon source

While many investigators have examined the effect of carbon sources on AF biosynthesis (Abdollahi and Buchanan, 1981a, 1981b; Buchanan, 1984; Davis and Diener, 1968; Mateles and Adye, 1965; Wiseman and Buchanan, 1987), it is unclear if carbon source directly regulates AF biosynthesis or modulates its synthesis through general metabolism. Unlike the production of most secondary metabolites that are repressed by simple sugars, production of AF biosynthesis is stimulated by simple glucose containing sugars, such as sucrose, or derivatives of glucose, such as fructose (Davis and Diener, 1968). A high degree of glucose utilization has been correlated with production of AF (Applebaum and Buchanan, 1979; Shih and Marth, 1974).

In an attempt to better understand the source of carbon for AF biosynthesis, researchers have studied rates of labeled acetate incorporation into AFs relative to glucose (Hsieh and Mateles, 1970). Interestingly, it was found that acetate preferentially incorporated into AF even during conditions where the concentration of glucose was 4-fold higher than acetate. This result suggests that the aflatoxins are synthesized extramitochondrially from glucose-derived acetyl coenzyme A (Hsieh and Mateles, 1970; Shantha and Murthy, 1981). This conclusion is supported by additional studies measuring the flux of the tricarboxylic acid cycle (TCA) intermediates. TCA intermediates were found to inhibit AF production and an active functioning of the TCA cycle was linked to lack of AF production. These results suggest that the reduction of intracellular acetyl-CoA concentrations through complete oxidation of Acetyl-CoA via the TCA cycle limits AF production (Buchanan and Ayres, 1977; Gupta et al., 1977; Shantha and Murthy, 1981).

Interestingly, carbon sources non-conducive for AF biosynthesis appear to result in less repression of the cluster genes as compared to non-conducive temperature, nitrogen source, and pH (Price et al., 2005). Similar results were obtained when AF production was examined in *A. flavus* grown with and without sucrose in yeast extract media (Wilkinson et al., 2007). While carbon source clearly has many effects on gene/protein expression, a lack of strong gene down-regulation within the AF cluster could suggest that carbon source is not having a specific regulatory effect on the AF pathway (Fig. 1). One possibility is that instead of a direct regulatory affect there is an alteration in the metabolic pool of precursor products needed for unique secondary metabolites such as AF. One possible role for glucose in AF biosynthesis is its influence on cAMP signaling/catabolite repression. Glucose and cAMP levels are typically inversely related; however in *Aspergillus* it was shown that cAMP levels were high during exponential growth, a period of high glucose utilization, and then lowered later during stationary phase, with the majority of AF accumulating during a period where cAMP levels are shifting (Applebaum and Buchanan, 1979).

Within the AF cluster several genes have CreA sites near promoter regions despite the apparent induction of aflatoxin by glucose. It appears that these sites are not active or function differently in the aflatoxin cluster. It is interesting to note that *aflD* (*nor-1*) mentioned in conjunction with cAMP in Section 3.2 was among the most strongly upregulated in response to sucrose (Fig. 1) (Wilkinson et al., 2007).

4.3. Lipids-key precursors and important signaling molecules

The AF polyketide synthase uses hexanoate, formed by a fatty acid synthase encoded by two genes within the AF biosynthetic cluster, as the starter unit, unlike most PKSs which use acetyl-CoA (Yabe et al., 2003). Early studies revealed that lipid synthesis was highly correlated with AF production (Shih and Marth, 1974). The first step in fatty acid biosynthesis is the conversion of Acetyl-CoA to Malonyl-CoA by Acetyl-CoA carboxylase. Not surprisingly, evidence has shown that Malonyl-CoA is needed for biosynthesis of AF (Dutton, 1988). In corn, *A. flavus* is preferentially associated with the germ region of infected kernels (Fennell et al., 1973; Lillehoj et al., 1974); this lipid rich germ tissue also appears more amenable to AF production than the whole kernel (Mellon et al., 2005).

Oxylipins (also called psi factors) are signaling molecules derived from fatty acids that have been shown to regulate developmental and pathogenic processes as well as secondary metabolism in *A. nidulans* (Fig. 1) (Tsitsigiannis and Keller, 2006, 2007). Three genes (*ppoA*, *ppoB*, and *ppoC*) encoding fatty acid oxygenases were found in the *A. nidulans* genome and predicted to be responsible for production of these psi factors. Deletion mutants were made for each of these three genes (Tsitsigiannis et al., 2005). Tsitsigiannis et al. (2005) presented evidence that the asexual conidiation regulatory gene *brlA* was clearly regulated by these genes and additionally showed evidence that *veA* was possibly displaying a different temporal pattern of gene expression in a triple deletion mutant background of *ppoA*, *ppoB*, and *ppoC*. VeA has also been shown to alter *brlA* transcripts however it is not clear whether VeA must function through oxylipins to exhibit this regulation (Fig. 2) (Calvo, 2008). Interestingly, certain *ppo* mutants were found to have increased expression of *aflR* and *stcU* (*aflM equivalent*) while other *ppo* deletion strains showed a decrease in expression of these genes in the ST pathway. It was shown that this regulation of *aflR* linked to psi factors was not by LaeA (Tsitsigiannis and Keller, 2006). Tsitsigiannis and Keller (2006) proposed that *aflR* inhibition by *ppoA* and *ppoC* products may function in a PKA mediated manner since PKA has been shown to inhibit ST and increase penicillin accumulation. They also propose that this is regulated by a G-protein coupled receptor (GPCR) signaling cascade, possibly involving the heterotrimeric Gα-subunit FadA (Tsitsigiannis and Keller, 2006). Clearly use of previously described AflR PKA phosphorylation mutants and FadA mutants in a delta-*ppoA*, *ppoC*

background would provide excellent evidence as to whether there is a link between oxylipins and PKA or G-Protein mediated regulation of the ST/AF cluster.

4.4. Regulation of aflatoxin by nitrogen source

Nitrogen source has been widely reviewed as important in the biosynthesis of AF (Luchese and Harrigan, 1993; Payne and Brown, 1998). Various sources of nitrogen have been tested and it has been observed that organic nitrogen sources are superior for AF biosynthesis (Davis et al., 1967) and that nitrate inhibits AF production (Kachholz and Demain, 1983). However, ST production in *A. nidulans* is not regulated similar to AF in response to nitrogen source, with nitrate inducing ST and ammonia causing a reduction(Feng and Leonard, 1998). Among various amino acids, proline has been reported as supporting the most AF. Asparagine, casein, and ammonium sulfate also support robust AF production (Payne and Hagler, 1983). Nitrate has been shown to result in decreased expression of genes involved in AF biosynthesis (Fig. 1) (Feng and Leonard, 1998; Price et al., 2005). Worth noting is the presence of AreA binding sites in the intergenic region between *aflR* and *aflS*. Results have shown that the AreA protein from *A. parasiticus* is able to bind to this region (Chang et al., 2000b). Nitrate resulted in active AreA binding to this region, and actually increased expression of *aflS*.

In a comparison between divergent populations of *A. flavus* from North America and West Africa nitrate regulation of aflatoxin biosynthesis was found to differ. North American strains were found to be less inhibited by nitrate and it was proposed that this was due to the presence of additional AreA binding sites in these strains in the intergenic region between *aflR* and *aflS* when compared to West African strains (8 sites compared to 6 sites) (Ehrlich and Cotty, 2002). Expression of the regulatory gene *aflS* was found to be 2.6-fold higher in nitrate media for North American strains examined (Ehrlich and Cotty, 2002). Interestingly, researchers have tried to overcome nitrate repression of AF by increasing expression of *aflR*, but not *aflS*. Initially, Chang et al. (1995) found that insertion of an additional copy of *aflR* into *A. parasticus* was able to restore expression of cluster genes when grown in nitrate; however, AF levels were not reported. Flaherty and Payne (1997) used a constitutively-expressed *aflR* which resulted in a similar restoration of transcripts for *aflD, aflM* and *aflC*. However, they found that AF was still not made when nitrate was the sole nitrogen resource (Flaherty and Payne, 1997). These results show that while gene expression is controlled by nitrate, the actual biosynthesis of AF is inhibited by another control outside of the pathway. One hypothesis was that a change in redox potential caused by nitrate increasing the activity of mannitol dehydrogenases (Niehaus and Jiang, 1989) would play a role in the shunting of precursor metabolites such as Malonyl-CoA to fatty acid synthesis instead of polyketide synthesis due to a lack of reducing power. However, recent experiments have suggested that changing redox by supplementing media with 100 μM NADPH has not resulted in a reversal of nitrate repression on AF biosynthesis (Price, 2005).

4.5. Regulation of aflatoxin by pH

Acidic conditions are more favorable for the biosynthesis of AF than alkaline conditions. Keller et al. (1997) showed that transcripts for cluster genes were down-regulated in basic conditions relative to acidic (Keller et al, 1997). The regulation of AF by pH stems from the alkali transcription factor PacC and appears to function inversely to the role of pH regulation on penicillin production. PacC is a zinc finger transcription factor that regulates gene expression depending on ambient pH (Tilburn et al., 1995). PacC is synthesized as an inactive precursor requiring a two-step proteolytic activation for its activity under alkaline conditions. At acidic pH it adopts an inactive conformation through intramolecular interactions in the C-terminus of the protein (Diez et al., 2002; Penas et al., 2007). The intracellular cytoplasmic pH does not appear to fluctuate in fungi so regulation must come from ambient pH (Parton et al., 1997).

It is not clear whether PacC sites have a specific role in the control of aflatoxin biosynthesis by pH. PacC binding sites have been identified in the promoter of *aflR* so it would have been expected that alkaline pH would enhance AF cluster gene expression (Ehrlich et al., 1999a). In addition to a down-regulation of expression for AF cluster genes at alkaline pH (Fig. 1), a regulation of 19 genes induced by alkaline pH was found in microarray data comparing pH 4.5 and pH 8; included in these genes was *pkaA* (Price et al., 2005). PkaA has been shown to negatively regulate AflR through phosphorylation (Shimizu et al., 2003; Shimizu and Keller, 2001) and is discussed in Section 3.1.

4.6. Regulation of aflatoxin by metals/trace elements

It has been known since the 1960's that metals are important factors needed for high levels of AF accumulation in synthetic media, especially zinc (Davis et al., 1967; Mateles and Adye, 1965; Nesbitt et al., 1962). Early on it was observed that corn steep liquor resulted in a significant increase in AF production for fungi grown in synthetic liquid media (Schroeder, 1966). This increase was thought to come from a nutrient, possibly a metal, in the corn liquor that was needed for efficient synthesis of AFs. Several studies were done on important commodities to analyze metal content in relation to *Aspergillus* infection and AF production. Lillehoj et al. (1974) found that levels of metals stayed the same or increased when corn seeds were infected with *A. flavus*. They demonstrated that biologically available trace elements were limited in corn seed because of phytate levels, and found that the addition of the metals zinc or lead caused significant increases in AF production in corn germ (Lillehoj et al., 1974). Metal availability appears critical; addition of EDTA to A&M media metal mix inhibits AF biosynthesis, presumably due to the chelation of available metal ions (unpublished data). Researchers also noted that copper has an inhibitory effect on the production of AF (Lillehoj et al., 1974; Marsh et al., 1975).

Cuero et al. (2003) explored the molecular significance of metals and AF. They found that a mixture of copper, iron, and zinc caused a significant increase in total RNA, biomass, *aflP* (*omtA*) transcription, and AF (Cuero et al., 2003). A subtractive cDNA expression library was used in *Fusarium graminearium* to show that metals cause an increase in transcription among many genes, several of which were involved in cell growth and division. The expression of one gene encoding an alcohol dehydrogenase was measured by RT-PCR and found to increase in metal treated cultures of both *F. graminearium* and *A. flavus* (Cuero and Ouellet, 2005). Alcohol dehydrogenase, *aflH* (*adhA*) is a gene located in the AF biosynthetic gene cluster needed for the conversion of 5′-hydroxyaverantin to averufin (Chang et al., 2000a). Cuero and Ouellet (2005) measured a gene they called *adh1*, but it is not clear whether this is the cluster gene or another alcohol dehydrogenase within the fungus; if it is the cluster gene then the transcription of at least two genes in the cluster *aflH* and *aflP* are stimulated by the addition of metals.

4.7. Regulation of aflatoxin by temperature

Aflatoxins are produced optimally between 28 and 30 °C, and production decreases as temperatures approach 37 °C, the optimum temperature for fungal growth. AF is known to be a thermally stable compound (Mann et al., 1967; Yazdanpanah et al., 2005).

Research into the effects of temperature on AF production is ongoing. Early studies showed that AF was produced maximally at 24 °C, but was not produced at temperatures lower than 18 °C or higher than 35 °C (Schindler et al., 1967). Diener and Davis (1967) reported AF production in peanuts at 40 °C; however, (Schroeder and Hein, 1967) showed AF was not made in significant amounts at temperatures any higher than 35 °C on cottonseed, peanuts, and rice. The general response of AF biosynthesis to temperature therefore appears to be independent of substrate. Studies with both solid and liquid defined A&M media have shown that AF production decreases linearly as the temperature is increased or decreased from its optimum temperature for biosynthesis (28–30 °C) (O'Brian et al., 2007).

At least one effect of temperature occurs at the level of gene transcription. Feng and Leonard (1995) showed by Northern analysis that the AF polyketide synthase gene of *A. parasiticus* was expressed at 27° C but not at 37 °C (Feng and Leonard, 1995). Another AF pathway gene, *aflP*, was similarly transcribed in *A. parasiticus* at 29 °C but not 37 °C (Liu and Chu, 1998). Temperature shift studies in which cultures are moved from a conducive temperature to a non-conductive temperature, or vice versa, appear to have no lasting effect on AF biosynthesis, indicating that the inhibition of AF is caused by temperature and not the lack of a needed precursor molecule nor presence of an inhibitory molecule (Georgianna and Payne, unpublished).

Microarray data from a 5002 element array revealed that among 144 differentially expressed genes between 28 and 37 °C; a majority of the AF biosynthetic cluster genes are repressed at the higher temperature, despite transcript levels of *aflR* and *aflS* remaining constant (Fig. 1) (O'Brian et al., 2007). Liu and Chu (1998) showed that both *aflR* transcripts and AflR protein are present at 37 °C but at a 4-fold lower concentration (Liu and Chu, 1998), suggesting that the transcriptional inhibition among cluster genes could be from an inactivating modification of the protein at higher temperatures. Interestingly, *A. nidulans* is able to produce ST and the requisite transcripts at 37 °C, thus temperature affects this pathway differently from the AF pathway. Although the reason for these differences is not known, it could be related to differences in the sequence of AflR between the two species.

Current data argue that inhibition of AF biosynthesis at 37 °C is not due to an inactivation of AflR by phosphorylation. The localization of AflR using a strain expressing a GFP:AflR fusion protein was recently examined and AflR was found to be localized in the nucleus at both 28 and 37 °C (Georgianna and Payne, unpublished data). These data effectively rule out phosphorylation by PKA since the localization does not change and AflR is unable to enter the nucleus when phosphorylated (Shimizu et al., 2003). It seems likely that temperature regulation of AF biosynthesis is more complex than AflR simply being inactive at 37 °C especially since *A. flavus* AflR has been shown to function in *A. nidulans* at 37 °C suggesting that protein stability/folding at higher temperatures is also not effected (Yu et al., 1996b). Temperature inhibition of AF appears to represent a regulatory mechanism not present in *A. nidulans*.

4.8. Regulation of aflatoxin by oxidative stress

Research over the past decade has made clear that oxidative stress stimulates AF production (Jayashree and Subramanyam, 2000; Reverberi et al., 2008); and anti-oxidants, such as gallic acid in walnut, have an inhibitory effect on AF production (Mahoney and Molyneux, 2004). It has been reported that gallic acid causes an inhibition of several AF pathway genes, including *aflM* (*ver-1*) and *aflD* (*nor-1*) (Mahoney and Molyneux, 2004). More recently, Kim et al. (2008) analyzed the effects of the anti-oxidant caffeic acid on gene expression using microarrays. They found that most genes in the AF biosynthetic pathway appeared to be down-regulated by caffeic acid treatment (Fig. 1). Reverberi et al. (2005) also reported that the induction of anti-oxidant enzymes by β-glucans from *Lentinula edodes* inhibited AF biosynthesis and delayed transcription of *aflR* as well as AF cluster genes. *A. flavus* has also been grown under anaerobic conditions and still shown to make AF, albeit at much lesser quantities (Clevstrom et al., 1983).

Kim et al. (2005) identified several genes needed to cope with oxidative stress using *Saccharomyces cerevisiae* as a model for fungal stress. Identified genes included many oxidative stress genes (glutaredoxins, catalases, superoxide dismutases) and others such as a MAPK gene like the yeast *hog1* (Kim et al., 2005). Interestingly, differential expression for several MAPK genes was also observed in the aforementioned temperature comparison arrays. One pitfall of the yeast studies is that for many of the genes identified only some have actually been shown to have an effect on AF production. A knockout of a superoxide dismutase gene in *A. flavus* has been shown to decrease AF production (He et al., 2007). One of the most important recent discoveries stemming from the work by Kim et al. (2005) was that the anti-oxidant response transcription factor Yap1 has an apparent role in regulation of AF biosynthesis (Reverberi et al., 2008). Reverberi and coworkers (2008) found that deletion mutants of *yap1* in *A. parasiticus* had an increased accumulation of AF. It is not clear whether the increase in AF can be directly related to deletion of Yap1 or a result of increased reactive oxygen, however, it was proposed that there may be Yap1 binding sites in the *aflR* promoter. *aflR* was induced earlier in *yap1* deletion strains compared to wild-type and a known binding site for the human ortholog of Yap1, AP-1, is found in the promoter region of *aflR* (Reverberi et al., 2008).

5. Outlook for genomics

Functional genomics promises to provide important information on metabolites with no known function. For example, gene expression experiments have been valuable in characterizing regulatory mechanisms controlling the AF cluster (Fig. 1). Further analyses of these types of array experiments may lead to identification of secondary metabolism gene clusters under similar regulatory controls. The newly created *A. flavus* database (Smith and Payne, unpublished) should help facilitate complex genomic mining studies to discover important functional traits and further our understanding of the many complex genetic relationships described throughout this review.

Perhaps the most immediate impact of functional genomics will be a better understanding of the regulatory networks controlled by global regulators like VeA and LaeA. These two genes, and perhaps additional genes, appear to be important in the regulation of several pathways of secondary metabolism. Genome sequences and whole genome DNA microarrays will undoubtedly provide new insight into how these genes modulate development and metabolism. It is logical to speculate that other global regulators that control secondary metabolism also will be uncovered. The striking control of AF biosynthesis, but not conidiation, by temperatures within the cardinal ranges for growth provides strong evidence that the fungus uses temperature as a cue to regulate secondary metabolites in addition to AF, and in the case of AF overriding AflR. Environmentally relevant volatiles have emerged as a promising area of research for regulation of AF biosynthesis with ethylene and crotyl alcohol both exhibiting some inhibition of accumulation of AF cluster gene transcripts, potentially independent of AflR (Roze et al., 2007b, 2004b).

The genomes of several *Aspergillus* species have recently been sequenced; comparative genomics between these species are revealing many new insights about the genetic diversity within the genus (Rokas et al., 2007). The *Aspergillus* community is just starting to reap the benefit of many new functional genomics tools.

In the *A. flavus* community, several different array platforms (cDNA and Affymetrix) are currently available. Additionally, high throughput quantitative proteomics methods have been applied to *A. flavus* (Georgianna et al., 2008). With the push for more information-intensive methods of systems analysis, it will be important for consistent methodology to be applied between different platforms and to make data easily accessible. To this end, in addition to the newly created *A. flavus* database, the *A. flavus* community has adopted the Purdue Genomics Laboratory as the center for all Affymetrix array hybridization and scanning. Systems databases, for incorporation of genomic, transcriptomic, metabolomic, proteomic, and phenomic data will prove to be a very powerful tool for functional analysis of the complex processes being studied in *A. flavus*.

References

Abdollahi, A., Buchanan, R.L., 1981a. Regulation of aflatoxin biosynthesis: characterization of glucose as an apparent inducer of aflatoxin production. J. Food Sci. 46, 143–146.

Abdollahi, A., Buchanan, R.L., 1981b. Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. J. Food Sci. 46, 633–635.

Adrio, J.L., Demain, A.L., 2003. Fungal biotechnology. Int. Microbiol. 6, 191–199.

Applebaum, R.S., Buchanan, R.L., 1979. Intracellular concentration of cAMP and cGMP in an aflatoxigenic strain of *Aspergillus parastiticus*. J. Food Sci. 44, 116–117.

Bayram, O., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., et al., 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320, 1504–1506.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clin. Microbiol. Rev. 16, 497–516.

Bhatnagar, D., Cary, J.W., Ehrlich, K., Yu, J., Cleveland, T.E., 2006. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. Mycopathologia 162, 155–166.

Bhatnagar, D., Ehrlich, K.C., Cleveland, T.E., 2003. Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61, 83–93.

Bhatnagar, D., Rajasekaran, K., Payne, G.A., Brown, R.L., Yu, J., et al., 2008. The 'omics' tools: genomics, proteomics, metabolomics and their potential for solving the aflatoxin contamination problem. World Mycotoxin J. 1, 3–12.

Bok, J.W., Hoffmeister, D., Maggio-Hall, L.A., Murillo, R., Glasner, J.D., et al., 2006. Genomic mining for *Aspergillus* natural products. Chem. Biol. 13, 31–37.

Bok, J.W., Keller, N.P., 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp.. Eukaryot. Cell 3, 527–535.

Bressac, B., Kew, M., Wands, J., Ozturk, M., 1991. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 350, 429–431.

Buchanan, R.L., Ayres, J.C., 1977. Effect of various glycolytic and tca intermediates on aflatoxin production. J. Food Saf. 1, 19–28.

Buchanan, R.L.a.S.H.G., 1984. Ability of various carbon sources to induce and support aflatoxin biosynthesis in *Aspergillus parasiticus*. J. Food Saf. 6, 271–279.

Butchko, R.A., Adams, T.H., Keller, N.P., 1999. *Aspergillus nidulans* mutants defective in stc gene cluster regulation. Genetics 153, 715–720.

Calvo, A.M., 2008. The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet. Biol. 31, 31.

Calvo, A.M., Bok, J., Brooks, W., Keller, N.P., 2004. *VeA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 70, 4733–4739.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. 66, 447–459.

Carbone, I., Ramirez-Prado, J.H., Jakobek, J.L., Horn, B.W., 2007. Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. BMC Evol. Biol. 7, 111.

Cary, J.W., Dyer, J.M., Ehrlich, K.C., Wright, M.S., Liang, S.H., et al., 2002. Molecular and functional characterization of a second copy of the aflatoxin regulatory gene, aflR-2, from *Aspergillus parasiticus*. Biochim. Biophys. Acta 1576, 316–323.

Cary, J.W., Klich, M.A., Beltz, S.B., 2005. Characterization of aflatoxin-producing fungi outside of *Aspergillus* section *Flavi*. Mycologia 97, 425–432.

Cary, J.W., O'brian, G.R., Nielsen, D.M., Nierman, W., Harris-Coward, P., et al., 2007. Elucidation of *veA*-dependent genes associated with aflatoxin and sclerotial production in *Aspergillus flavus* by functional genomics. Appl. Microbiol. Biotechnol. 76, 1107–1118.

Cetkovic, H., Mikoc, A., Muller, W.E., Gamulin, V., 2007. Ras-like small GTPases form a large family of proteins in the marine sponge *Suberites domuncula*. J. Mol. Evol. 64, 332–341.

Chang, P.K., 2003. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. Mol. Genet. Genomics 268, 711–719.

Chang, P.K., 2004. Lack of interaction between AflR and AflJ contributes to nonaflatoxigenicity of *Aspergillus sojae*. J. Biotechnol. 107, 245–253.

Chang, P.K., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Bennett, J.W., et al., 1993. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. 59, 3273–3279.

Chang, P.K., Ehrlich, K.C., Yu, J., Bhatnagar, D., Cleveland, T.E., 1995. Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl. Environ. Microbiol. 61, 2372–2377.

Chang, P.K., Yu, J., Bhatnagar, D., Cleveland, T.E., 1999a. Repressor-AflR interaction modulates aflatoxin biosynthesis in *Aspergillus parasiticus*. Mycopathologia 147, 105–112.

Chang, P.K., Yu, J., Ehrlich, K.C., Boue, S.M., Montalbano, B.G., et al., 2000a. *AdhA* in *Aspergillus parasiticus* is involved in conversion of 5′-hydroxyaverantin to averufin. Appl. Environ. Microbiol. 66, 4715–4719.

Chang, P.K., Yu, J.J., Bhatnagar, D., Cleveland, T.E., 1999b. The carboxy-terminal portion of the aflatoxin pathway regulatory protein AflR of *Aspergillus parasiticus* activates GAL1::lacZ gene expression in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 65, 2508–2512.

Chang, P.K., Yu, J.J., Bhatnagar, D., Cleveland, T.E., 2000b. Characterization of the *Aspergillus parasiticus* major nitrogen regulatory gene, *areA*. Biochim. Biophys. Acta Gene Struct. Expr. 1491, 263–266.

Clevstrom, G., Ljunggren, H., Tegelstrom, S., Tideman, K., 1983. Production of aflatoxin by an *Aspergillus flavus* isolate cultured under a limited oxygen supply. Appl. Environ. Microbiol. 46, 400–405.

Colicelli, J., 2004. Human RAS superfamily proteins and related GTPases. Sci STKE 2004, RE13.

Cotty, P.J., Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int. J. Food Microbiol. 119, 109–115.

Cuero, R., Ouellet, T., 2005. Metal ions modulate gene expression and accumulation of the mycotoxins aflatoxin and zearalenone. J. Appl. Microbiol. 98, 598–605.

Cuero, R., Ouellet, T., Yu, J., Mogongwa, N., 2003. Metal ion enhancement of fungal growth, gene expression and aflatoxin synthesis in *Aspergillus flavus*: RT-PCR characterization. J. Appl. Microbiol. 94, 953–961.

Davis, N.D., Diener, U.L., 1968. Growth and aflatoxin production by *Aspergillus parasiticus* from various carbon sources. Appl. Microbiol. 16, 158–159.

Davis, N.D., Diener, U.L., Agnihotri, V.P., 1967. Production of aflatoxins B1 and G1 in chemically defined medium. Mycopathol. Mycol. Appl. 31, 251–256.

Demain, A.L., 1992. Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry. In: Chadwick, D.J., Whelan, J. (Eds.), Secondary Metabolites: Their Function and Evolution. John Wiley and Sons, Chichester West Sussex, England.

Desjardins, A.E., Proctor, R.H., 2007. Molecular biology of *Fusarium* mycotoxins. Int. J. Food Microbiol. 119, 47–50.

Diener, U.L., Davis, N.D., 1967. Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by *Aspergillus flavus* in sterile peanuts. J. Am. Oil Chem. Soc. 44, 259–263.

Diez, E., Alvaro, J., Espeso, E.A., Rainbow, L., Suarez, T., et al., 2002. Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolytic steps. EMBO J. 21, 1350–1359.

Dohlman, H.G., Thorner, J., 1997. RGS proteins and signaling by heterotrimeric G proteins. J. Biol. Chem. 272, 3871–3874.

Du, W., O'brian, G.R., Payne, G.A., 2007. Function and regulation of *aflJ* in the accumulation of aflatoxin early pathway intermediate in *Aspergillus flavus*. Food Addit. Contam. 24, 1043–1050.

Duran, R.M., Cary, J.W., Calvo, A.M., 2007. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. Appl. Microbiol. Biotechnol. 73, 1158–1168.

Dutton, M.F., 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. 52, 274–295.

Ehrhardt, A., Ehrhardt, G.R., Guo, X., Schrader, J.W., 2002. Ras and relatives—job sharing and networking keep an old family together. Exp. Hematol. 30, 1089–1106.

Ehrlich, K.C., Cary, J.W., Montalbano, B.G., 1999a. Characterization of the promoter for the gene encoding the aflatoxin biosynthetic pathway regulatory protein AflR. Biochim. Biophys. Acta Gene Struct. Expr. 1444, 412–417.

Ehrlich, K.C., Cotty, P.J., 2002. Variability in nitrogen regulation of aflatoxin production by *Aspergillus flavus* strains. Appl. Microbiol. Biotechnol. 60, 174–178.

Ehrlich, K.C., Montalbano, B.G., Bhatnagar, D., Cleveland, T.E., 1998. Alteration of different domains in AflR affects aflatoxin pathway metabolism in *Aspergillus parasiticus* transformants. Fungal Genet. Biol. 23, 279–287.

Ehrlich, K.C., Montalbano, B.G., Cary, J.W., 1999b. Binding of the C6-zinc cluster protein, AflR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. Gene 230, 249–257.

Ehrlich, K.C., Yu, J., Cotty, P.J., 2005. Aflatoxin biosynthesis gene clusters and flanking regions. J. Appl. Microbiol. 99, 518–527.

Endo, A., Kuroda, M., Tanzawa, K., 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. FEBS Lett. 72, 323–326.

Feng, G.H., Leonard, T.J., 1995. Characterization of the polyketide synthase gene (*pksL1*) required for aflatoxin biosynthesis in *Aspergillus parasiticus*. J. Bacteriol. 177, 6246–6254.

Feng, G.H., Leonard, T.J., 1998. Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. Nidulans*. Appl. Environ. Microbiol. 64, 2275–2277.

Fennell, D.I., Bothast, R.J., Lillehoj, E.B., Peterson, R.E., 1973. Bright greenish-yellow fluorescence and associated fungi in white corn naturally contaminated with aflatoxin. Cereal Chem. 50, 404.

Fernandes, M., Keller, N.P., Adams, T.H., 1998. Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. Mol. Microbiol. 28, 1355–1365.

Field, M.C., 2005. Signalling the genome: the Ras-like small GTPase family of trypanosomatids. Trends Parasitol. 21, 447–450.

Flaherty, J.E., Payne, G.A., 1997. Overexpression of *aflR* leads to upregulation of pathway gene transcription and increased aflatoxin production in *Aspergillus flavus*. Appl. Environ. Microbiol. 63, 3995–4000.

Frisvad, J.C., Skouboe, P., Samson, R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-*O*-methylsterigmatocystin, *Aspergillus rambellii* sp. *Nov*.. Syst. Appl. Microbiol. 28, 442–453.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.J., Wortman, J.R., et al., 2005. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. Fumigatus* and *A. oryzae*. Nature 438, 1105–1115.

Georgianna, D.R., Hawkridge, A.M., Muddiman, D.C., Payne, G.A., 2008. Temperature-dependent regulation of proteins in *Aspergillus flavus*: whole organism stable isotope labeling by amino acids. J. Proteome Res. 7, 2973–2979.

Groopman, J.D., Johnson, D., Kensler, T.W., 2005. Aflatoxin and hepatitis B virus biomarkers: a paradigm for complex environmental exposures and cancer risk. Cancer Biomark. 1, 5–14.

Gupta, S.K., Maggon, K.K., Venkitasubramanian, T.A., 1977. Regulation of aflatoxin biosynthesis. 2 Comparative study of tricarboxylic acid cycle in aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*. Microbios 19, 7–15.

Han, K.H., Seo, J.A., Yu, J.H., 2004. A putative G protein-coupled receptor negatively controls sexual development in *Aspergillus nidulans*. Mol. Microbiol. 51, 1333–1345.

He, Z., Price, M.S., O'Brian, G.R., Georgianna, D.R., Payne, G.A., 2007. Improved Protocols for the functional analysis in the pathogenic fungus *Aspergillus flavus*. BMC Microbiol. 7, 104.

Hicks, J., Shimizu, K., Keller, N.P., 2002. Genetics and biosynthesis of aflatoxins and sterigmatocystin. In: Bennet, K.A. (Ed.), The Mycota. Spring-Verlag, Berlin, pp. 55–69.

Hicks, J.K., Bahn, Y.S., Heitman, J., 2005. Pde1 phosphodiesterase modulates cyclic AMP levels through a protein kinase A-mediated negative feedback loop in *Cryptococcus neoformans*. Eukaryot. Cell 4, 1971–1981.

Hicks, J.K., Yu, J.H., Keller, N.P., Adams, T.H., 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G alpha protein-dependent signaling pathway. EMBO J. 16, 4916–4923.

Hsieh, D.P., Mateles, R.I., 1970. The relative contribution of acetate and glucose to aflatoxin biosynthesis. Biochim. Biophys. Acta 208, 482–486.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., et al., 1991. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350, 427–428.

Jayashree, T., Subramanyam, C., 2000. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. Free Radic. Biol. Med. 29, 981–985.

Kachholz, T., Demain, A.L., 1983. Nitrate repression of averufin and aflatoxin biosynthesis. J. Nat. Prod. 46, 499–506.

Kale, S.P., Cary, J.W., Bhatnagar, D., Bennett, J.W., 1996. Characterization of experimentally induced, nonaflatoxigenic variant strains of *Aspergillus parasiticus*. Appl. Environ. Microbiol. 62, 3399–3404.

Kato, N., Brooks, W., Calvo, A.M., 2003. The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. Eukaryot. Cell 2, 1178–1186.

Keller, N.P., Hohn, T.M., 1996. Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. 21, 17–29.

Keller, N.P., Nesbitt, C., Sarr, B., Phillips, T.D., Burrow, G.B., 1997. PH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp.. Phytopathology 87, 643–648.

Keller, N.P., Turner, G., Bennett, J.W., 2005. Fungal secondary metabolism—from biochemistry to genomics. Nat. Rev. Microbiol. 3, 937–947.

Kim, J.H., Campbell, B.C., Yu, J.J., Mahoney, N., Chan, K.L., et al., 2005. Examination of fungal stress response genes using *Saccharomyces cerevisiae* as a model system: targeting genes affecting aflatoxin biosynthesis by *Aspergillus flavus* Link. Appl. Microbiol. Biotechnol. 67, 807–815.

Kim, J.H., Yu, J., Mahoney, N., Chan, K.L., Molyneux, R.J., et al., 2008. Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. Int. J. Food Microbiol. 122, 49–60.

Kobayashi, T., Abe, K., Asai, K., Gomi, K., Juvvadi, P.R., et al., 2007. Genomics of *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 71, 646–670.

Kramer, C., Loros, J.J., Dunlap, J.C., Crosthwaite, S.K., 2003. Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*. Nature 421, 948–952.

Lafon, A., Han, K.H., Seo, J.A., Yu, J.H., d'Enfert, C., 2006. G-protein and cAMP-mediated signaling in *Aspergilli*: a genomic perspective. Fungal Geneti Biol. 43, 490–502.

Lee, C.Z., Liou, G.Y., Yuan, G.F., 2006. Comparison of the *aflR* gene sequences of strains in *Aspergillus* section *Flavi*. Microbiology 152, 161–170.

Lee, J.W., Roze, L.V., Linz, J.E., 2007. Evidence that a wortmannin-sensitive signal transduction pathway regulates aflatoxin biosynthesis. Mycologia 99, 562–568.

Lillehoj, E.B., Garcia, W.J., Lambrow, M., 1974. *Aspergillus flavus* infection and aflatoxin production in corn: influence of trace elements. Appl. Microbiol. 28, 763–767.

Liu, B.H., Chu, F.S., 1998. Regulation of *aflR* and its product, AflR, associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. 64, 3718–3723.

Luchese, R.H., Harrigan, W.F., 1993. Biosynthesis of aflatoxin—the role of nutritional factors. J. Appl. Bacteriol. 74, 5–14.

Mahoney, N., Molyneux, R.J., 2004. Phytochemical inhibition of aflatoxigenicity in *Aspergillus flavus* by constituents of walnut (*Juglans regia*). J. Agric. Food Chem. 52, 1882–1889.

Mann, G.E., Codifer, L.P., Dellear, F.G., 1967. Effect of heat on aflatoxin in oil seed meals. J. Agric. Food Sci. 15, 1090–1092.

Marsh, P.B., Simpson, M.E., Trucksess, M.W., 1975. Effects of trace metals on the production of aflatoxins by *Aspergillus parasiticus*. Appl. Microbiol. 30, 52–57.

Mateles, R.I., Adye, J.C., 1965. Production of aflatoxins in submerged culture. Appl. Microbiol. 13, 208–211.

McDonald, T., Brown, D., Keller, N.P., Hammond, T.M., 2005. RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. Mol. Plant Microbe Interact. 18, 539–545.

Mellon, J.E., Dowd, M.K., Cotty, P.J., 2005. Substrate utilization by *Aspergillus flavus* in inoculated whole corn kernels and isolated tissues. J. Agric. Food Chem. 53, 2351–2357.

Meyers, D.M., O'brian, G., Du, W.L., Bhatnagar, D., Payne, G.A., 1998. Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. Appl. Environ. Microbiol. 64, 3713–3717.

Nesbitt, B.F., O'Kelly, J., Sargeant, K., Sheridan, A., 1962. *Aspergillus flavus* and turkey X disease. Toxic metabolites of *Aspergillus flavus*. Nature 195, 1062–1063.

Niehaus, W.G., Jiang, W.P., 1989. Nitrate induces enzymes of the mannitol cycle and suppresses versicolorin synthesis in *Aspergillus parasiticus*. Mycopathologia 107, 131–137.

Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., et al., 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438, 1151–1156.

Nyikal, J., Misore, A., Nzioka, C., Njuguna, C., Muchiri, E., Njau, J., Maingi, S., Njoroge, J., Mutiso, J., Onteri, J., Langat, A., Kilei, I.K., Nyamongo, J., Ogana, G., Muture, B., Aflatoxin Task Force, Kenya Ministry of Health, Tukei, P., Onyango, C., Ochieng, W., Kenya Medical Research Institute, Tetteh, C., Likimani, S., Nguku, P., Galgalo, T., Kibet, S., Manya, A., Dahiye, A., Mwihia, J., Mugoya, I., Kenya Field Epidemiology and Laboratory Training Program/Kenya Ministry of Health, Onsongo, J., Ngindu, A., World Health Organization Kenya Country Office, K.M. DeCock, Lindblade, K., Slutsker, L., Amornkul, P., Rosen, D., Feiken, D., Thomas, T., CDC Kenya, Mensah, P., Eseko, N., Nejjar, A., World Health Organization Regional Office for Africa, Onsongo, M., Kessel, F., Foreign Agricultural Svc, US Dept. of Agriculture, Njapau, H., Park, D.L., Center for Food Safety and Applied Nutrition, Food and Drug Administration, Div. of International Health, Epidemiology Program Office, Lewis, L., Luber, G., Rogers, H., Backer, L., Rubin, C., National Center for Environmental Health, Gieseker, K.E., Azziz-Baumgartner, E., Chege, W., Bowen, A., EIS officers, CDC, 2004. Outbreak of Aflatoxin Poisoning—Eastern and Central Provinces, Kenya, January–July 2004, MMWR. 53, 790-793.

O'Brian, G.R., Georgianna, D.R., Wilkinson, J.R., Yu, J., Abbas, H.K., et al., 2007. The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. Mycologia 99, 232–239.

Osherov, N., May, G., 2000. Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. Genetics 155, 647–656.

Papa, K.E., 1979. Genetics of *Aspergillus flavus*: complementation and mapping of aflatoxin mutants. Genet. Res. 34, 1–9.

Park, J.I., Grant, C.M., Dawes, I.W., 2005. The high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae* is the major determinant of cAMP levels in stationary phase: involvement of different branches of the Ras-cyclic AMP pathway in stress responses. Biochem. Biophys. Res. Commun. 327, 311–319.

Parton, R.M., Fischer, S., Malho, R., Papasouliotis, O., Jelitto, T.C., et al., 1997. Pronounced cytoplasmic pH gradients are not required for tip growth in plant and fungal cells. J. Cell Sci. 110, 1187–1198.

Payne, G.A., Brown, M.P., 1998. Genetics and physiology of aflatoxin biosynthesis. Annu. Rev. Phytopathol. 36, 329–362.

Payne, G.A., Hagler Jr., W.M., 1983. Effect of specific amino acids on growth and aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus* in defined media. Appl. Environ. Microbiol. 46, 805–812.

Payne, G.A., Nierman, W.C., Wortman, J.R., Prictchard, B.L., Brown, D., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Mashida, M., Yu, J.J., 2006. Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. Med. Mycol. 44, 9–11.

Payne, G.A., Nystrom, G.J., Bhatnagar, D., Cleveland, T.E., Woloshuk, C.P., 1993. Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl. Environ. Microbiol. 59, 156–162.

Penas, M.M., Hervas-Aguilar, A., Munera-Huertas, T., Reoyo, E., Penalva, M.A., et al., 2007. Further characterization of the signaling proteolysis step in the *Aspergillus nidulans* pH signal transduction pathway. Eukaryot. Cell 6, 960–970.

Perrin, R.M., Fedorova, N.D., Bok, J.W., Cramer, R.A., Wortman, J.R., et al., 2007. Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. PLoS Pathog. 3, e50.

Price, M.S., 2005 Classical and modern genetic approaches reveal new gene associations with aflatoxin biosynthesis in *Aspergillus parasiticus* and *A. flavus*. Ph.D. Thesis.

Price, M.S., Conners, S.B., Tachdjian, S., Kelly, R.M., Payne, G.A., 2005. Aflatoxin conducive and non-conducive growth conditions reveal new gene associations with aflatoxin production. Fungal Genet. Biol. 42, 506–518.

Price, M.S., Yu, J., Nierman, W.C., Kim, H.S., Pritchard, B., et al., 2006. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol. Lett. 255, 275–279.

Prieto, R., Yousibova, G.L., Woloshuk, C.P., 1996. Identification of aflatoxin biosynthesis genes by genetic complementation in an *Aspergillus flavus* mutant lacking the aflatoxin gene cluster. Appl. Environ. Microbiol. 62, 3567–3571.

Purschwitz, J., Muller, S., Kastner, C., Schoser, M., Haas, H., et al., 2008. Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. Curr. Biol. 18, 255–259.
Reuther, G.W., Der, C.J., 2000. The Ras branch of small GTPases: Ras family members don't fall far from the tree. Curr. Opin. Cell Biol. 12, 157–165.
Reverberi, M., Fabbri, A.A., Zjalic, S., Ricelli, A., Punelli, F., et al., 2005. Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. Appl. Microbiol. Biotechnol. 69, 207–215.
Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., Camera, E., et al., 2008. Modulation of antioxidant defence in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for *ApyapA* gene. Eukaryot. Cell 25, 25.
Richard, J.L., Payne, G.A., 2003. Mycotoxins: Risks in Plant and Animal Systems. Council for Agricultural Science and Technology.
Rokas, A., Payne, G., Fedorova, N.D., Baker, S.E., Machida, M., et al., 2007. What can comparative genomics tell us about species concepts in the genus *Aspergillus*? Stud. Mycol. 59, 11–17.
Roze, L.V., Arthur, A.E., Hong, S.Y., Chanda, A., Linz, J.E., 2007a. The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Mol. Microbiol. 66, 713–726.
Roze, L.V., Beaudry, R.M., Arthur, A.E., Calvo, A.M., Linz, J.E., 2007b. *Aspergillus* volatiles regulate aflatoxin synthesis and asexual sporulation in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 73, 7268–7276.
Roze, L.V., Beaudry, R.M., Keller, N.P., Linz, J.E., 2004a. Regulation of aflatoxin synthesis by FadA/cAMP/protein kinase A signaling in *Aspergillus parasiticus*. Mycopathologia 158, 219–232.
Roze, L.V., Calvo, A.M., Gunterus, A., Beaudry, R., Kall, M., et al., 2004b. Ethylene modulates development and toxin biosynthesis in *Aspergillus* possibly via an ethylene sensor-mediated signaling pathway. J. Food Prot. 67, 438–447.
Roze, L.V., Mahanti, N., Mehigh, R., McConnell, D.G., Linz, J.E., 1999. Evidence that MRas1 and MRas3 proteins are associated with distinct cellular functions during growth and morphogenesis in the fungus *Mucor racemosus*. Fungal Genet. Biol. 28, 171–189.
Roze, L.V., Miller, M.J., Rarick, M., Mahanti, N., Linz, J.E., 2004c. A novel cAMP-response element, CRE1, modulates expression of *nor-1* in *Aspergillus parasiticus*. J. Biol. Chem. 279, 27428–27439.
Schindler, A.F., Palmer, J.G., Eisenberg, W.V., 1967. Aflatoxin production by *Aspergillus flavus* as related to various temperatures. Appl. Microbiol. 15, 1006–1009.
Schmidt-Heydt, M., Magan, N., Geisen, R., 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiol. Lett. 284, 142–149.
Schroeder, H.W., 1966. Effect of corn steep liquor on mycelial growth and aflatoxin production in *Aspergillus parasiticus*. Appl. Microbiol. 14, 381–385.
Schroeder, H.W., Hein Jr., H., 1967. Aflatoxins: production of the toxins in vitro in relation to temperature. Appl. Microbiol. 15, 441–445.
Seo, J.A., Yu, J.H., 2006. The phosducin-like protein PhnA is required for Gbetagamma-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in *Aspergillus nidulans*. Eukaryot. Cell 5, 400–410.
Shantha, T., Murthy, V.S., 1981. Influence of tricarboxylic acid cycle intermediates and related metabolites on the biosynthesis of aflatoxin by resting cells of *Aspergillus flavus*. Appl. Environ. Microbiol. 42, 758–761.
Shih, C.H., Marth, E.H., 1974. Some cultural conditions that control biosynthesis of lipid and aflatoxin by *Aspergillus parasiticus*. Appl. Microbiol. 27, 452–456.
Shimizu, K., Hicks, J.K., Huang, T.P., Keller, N.P., 2003. Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in *Aspergillus nidulans*. Genetics 165, 1095–1104.
Shimizu, K., Keller, N.P., 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics 157, 591–600.
Shwab, E.K., Bok, J.W., Tribus, M., Galehr, J., Graessle, S., et al., 2007. Histone deacetylase activity regulates chemical diversity in *Aspergillus*. Eukaryot. Cell 6, 1656–1664.
Smith, C.A., Robertson, D., Yates, B., Nielsen, D.M., Brown, D., et al., 2008. The effect of temperature on natural antisense transcript (NAT) expression in *Aspergillus flavus*. Curr. Genet. 54, 241–269.
Smith, C.A., Woloshuk, C.P., Robertson, D., Payne, G.A., 2007. Silencing of the aflatoxin gene cluster in a diploid strain of *Aspergillus flavus* is suppressed by ectopic *aflR* expression. Genetics 176, 2077–2086.
Som, T., Kolaparthi, V.S., 1994. Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. Mol. Cell Biol. 14, 5333–5348.
Tag, A., Hicks, J., Garifullina, G., Ake Jr., C., Phillips, T.D., et al., 2000. G-protein signalling mediates differential production of toxic secondary metabolites. Mol. Microbiol. 38, 658–665.
Thevelein, J.M., de Winde, J.H., 1999. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 33, 904–918.
Tice, G., Buchanan, R.L., 1982. Regulation of aflatoxin biosynthesis: effect of exogenously supplied cyclic nucleotides. J. Food Sci. 47, 153–157.
Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., et al., 1995. The *Aspergillus* PacC zinc-finger transcription factor mediates regulation of both acid-expressed and alkaline-expressed genes by ambient Ph. EMBO J. 14, 779–790.
Tsitsigiannis, D.I., Keller, N.P., 2006. Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. Mol. Microbiol. 59, 882–892.
Tsitsigiannis, D.I., Keller, N.P., 2007. Oxylipins as developmental and host–fungal communication signals. Trends Microbiol. 15, 109–118.
Tsitsigiannis, D.I., Kowieski, T.M., Zarnowski, R., Keller, N.P., 2005. Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. Microbiology 151, 1809–1821.
van Egmond, H.P., Schothorst, R.C., Jonker, M.A., 2007. Regulations relating to mycotoxins in food: perspectives in a global and European context. Anal. Bioanal. Chem. 389, 147–157.
Varga, J., Rigo, K., Molnar, J., Toth, B., Szencz, S., et al., 2003. Mycotoxin production and evolutionary relationships among species of *Aspergillus* section *Clavati*. Antonie Van Leeuwenhoek 83, 191–200.
Wilkinson, J.R., Yu, J., Abbas, H.K., Scheffler, B.E., Kim, H.S., et al., 2007. Aflatoxin formation and gene expression in response to carbon source media shift in *Aspergillus parasiticus*. Food Addit. Contam. 24, 1051–1060.
Williams, R.B., Henrikson, J.C., Hoover, A.R., Lee, A.E., Cichewicz, R.H., 2008. Epigenetic remodeling of the fungal secondary metabolome. Org. Biomol. Chem. 6, 1895–1897.
Wiseman, D.W., Buchanan, R.L., 1987. Determination of glucose level needed to induce aflatoxin production in *Aspergillus parasiticus*. Can. J. Microbiol. 33, 828–830.
Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E., et al., 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60, 2408–2414.
Woloshuk, C.P., Yousibova, G.L., Rollins, J.A., Bhatnagar, D., Payne, G.A., 1995. Molecular characterization of the *afl-1* locus in *Aspergillus flavus*. Appl. Environ. Microbiol. 61, 3019–3023.
Yabe, K., Chihaya, N., Hamamatsu, S., Sakuno, E., Hamasaki, T., et al., 2003. Enzymatic conversion of averufin to hydroxyversicolorone and elucidation of a novel metabolic grid involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 69, 66–73.
Yazdanpanah, H., Mohammadi, T., Abouhossain, G., Majid Cheraghali, A., 2005. Effect of roasting on degradation of aflatoxins in contaminated pistachio nuts. Food Chem. Toxicol. 43, 1135–1139.
Yu, J.H., Butchko, R.A., Fernandes, M., Keller, N.P., Leonard, T.J., et al., 1996a. Conservation of structure and function of the aflatoxin regulatory gene aflR from *Aspergillus nidulans* and *A. Flavus*. Curr. Genet. 29, 549–555.
Yu, J.H., Butchko, R.A.E., Fernandes, M., Keller, N.P., Leonard, T.J., et al., 1996b. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. Curr. Genet. 29, 549–555.
Yu, J.H., Keller, N., 2005. Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol. 43, 437–458.
Yu, J.H., Wieser, J., Adams, T.H., 1996c. The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J. 15, 5184–5190.
Yu, J.J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., et al., 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70, 1253–1262.
Zhang, Y.Q., Keller, N.P., Tsitsigiannis, D., Wilkinson, J.R., 2004. In: An, Z. (Ed.), Handbook of Industrial Mycology. Marcel Dekker, New York, pp. 355–385.