

Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge

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Received 13 July 2012; revised 27 September 2012; accepted 15 October 2012. Final version published online 20 November 2012.

DOI: 10.1111/1574-6976.12009

Editor: Pierre de Wit

Keywords

mycotoxins; transcriptional regulation; epigenetic regulation; environmental effects; nutritional regulation; food safety.

Abstract

Plant pathogenic fungi *Aspergillus flavus*, *Fusarium verticillioides*, and *Fusarium graminearum* infect seeds of the most important food and feed crops, including maize, wheat, and barley. More importantly, these fungi produce aflatoxins, fumonisins, and trichothecenes, respectively, which threaten health and food security worldwide. In this review, we examine the molecular mechanisms and environmental factors that regulate mycotoxin biosynthesis in each fungus, and discuss the similarities and differences in the collective body of knowledge. Whole-genome sequences are available for these fungi, providing reference databases for genomic, transcriptomic, and proteomic analyses. It is well recognized that genes responsible for mycotoxin biosynthesis are organized in clusters. However, recent research has documented the intricate transcriptional and epigenetic regulation that affects these gene clusters. Significantly, molecular networks that respond to environmental factors, namely nitrogen, carbon, and pH, are connected to components regulating mycotoxin production. Furthermore, the developmental status of seeds and specific tissue types exert conditional influences during fungal colonization. A comparison of the three distinct mycotoxin groups provides insight into new areas for research collaborations that will lead to innovative strategies to control mycotoxin contamination of grain.

Introduction

Mycotoxins, secondary metabolites produced by fungi on foods and foodstuffs, pose significant food safety risks and health hazards and ultimately limit the marketability of grain supply worldwide. Fungal plant pathogens producing these secondary metabolites have been found wherever the host crops are grown. Although hundreds of mycotoxins have been identified, only relatively few are known to impact global agriculture (Bennett & Klich, 2003). The importance of mycotoxin research is reflected in the numerous review articles written each year and the multitude of annual conferences that document scientific progress. In this review, we will focus on the three key mycotoxins that have enormous impact on the quality of grain: aflatoxins, fumonisins, and trichothecenes.

Aspergillus flavus (teleomorph: *Petromyces flavus*) and *Fusarium verticillioides* (teleomorph: *Gibberella moniliformis*) are pathogens of maize that cause ear and kernel rots (Fig. 1) and produce aflatoxins and fumonisins,

respectively (Fig. 2; Table 1; Desjardins, 2003; Munkvold, 2003; Klich, 2007). The toxicity and numerous acute and chronic disorders caused by these mycotoxins in humans and animals have been thoroughly documented (Bennett & Klich, 2003). Aflatoxins are produced by other *Aspergillus* species, including *A. parasiticus*, and fumonisins are also produced by *Fusarium proliferatum*. In addition, *Aspergillus nidulans*, which produces the aflatoxin precursor sterigmatocystin, has served as a model fungal system for studies on the molecular regulation of aflatoxin biosynthesis. *Fusarium graminearum* (teleomorph: *Gibberella zeae*) causes an ear rot disease of maize and head blight of wheat and barley (Fig. 1; Munkvold, 2003; Starkey *et al.*, 2007). In addition to causing yield losses, *F. graminearum* is one of several *Fusarium* species that produce trichothecene mycotoxins. Over 150 structurally related trichothecenes have been identified, including deoxynivalenol (DON; also known as vomitoxin; Fig. 2), nivalenol, and T-2 toxin (Trucksess, 2001). Trichothecenes are potent inhibitors of protein synthesis in mammalian systems. When



Fig. 1. Disease symptoms caused by (a) *Aspergillus flavus* (Aspergillus ear rot of maize), (b) *Fusarium verticillioides* (Fusarium ear rot of maize), (c) *Fusarium graminearum* (Gibberella ear rot of maize), and (d) *F. graminearum* (head scab of wheat/barley).

Table 1. Major fungal producers and the toxic effects of aflatoxin B1, fumonisin B1, and DON

Mycotoxins	Major producers	Host	Toxin Effects
Aflatoxins (B1)	<i>A. flavus</i> <i>A. parasiticus</i>	Maize, cottonseed, tree nuts, peanuts	Hepatotoxicity, cancer, immunosuppression
Fumonisin (FB1)	<i>F. verticillioides</i> <i>F. proliferatum</i>	Maize	Hepatotoxicity, cancer, pulmonary edema, leukoencephalomalacia
Trichothecenes (DON)	<i>F. graminearum</i> <i>F. culmorum</i>	Maize, Wheat, Barley	Gastrointestinal toxicity, inflammation of central nervous system

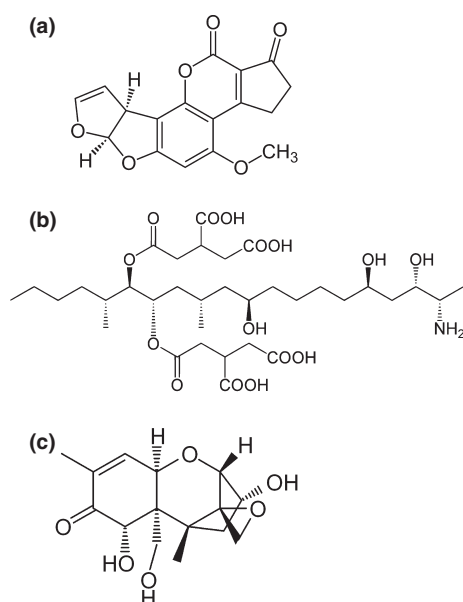


Fig. 2. Chemical structure of (a) aflatoxin B1, (b) fumonisin B1, and (c) deoxynivalenol.

consumed in contaminated foods, trichothecenes are neurotoxic, immunosuppressive, and nephrotoxic (Rotter *et al.*, 1996; Richard, 2007).

The genes involved in the biosynthesis of each of aflatoxins, fumonisins, and trichothecenes occur in clusters within the respective fungi. *Aspergillus flavus* has an estimated genome size of 36.8 Mb with nearly 12 197 predicted genes on eight chromosomes (Payne *et al.*, 2006). A total of 30 genes responsible for the biosynthesis of aflatoxins

reside in the 75-kb cluster, which is near the telomere on chromosome 3 of *A. flavus* (Amaike & Keller, 2011). The genome size of *F. verticillioides* is 41.7 Mb with an estimated 14 179 genes on 12 chromosomes (Ma *et al.*, 2010). The 23 genes involved in fumonisin biosynthesis are clustered in an 80-kb region residing on chromosome 1. The genome size (36.2 Mb) and gene number (13 332) of *F. graminearum* are similar to those of *F. verticillioides*. However, the pathogen has only four chromosomes (Cuomo *et al.*, 2007). Interestingly, only 15 genes are known to be involved in trichothecene biosynthesis, and these are distributed on all four chromosomes (Lee *et al.*, 2008), with 12 genes clustered on one locus on chromosome 2.

For a multitude of reasons, separate research communities have investigated the fungal genomics, functional genetics, and host–pathogen interactions that are associated with the production of aflatoxins, fumonisins, and trichothecenes. The level of knowledge about these different mycotoxins has reached a status that allows us to now examine the similarities and differences in molecular mechanisms that regulate mycotoxin biosynthesis. We will discuss the various levels of molecular regulations, summarized in Table 2 and Fig. 3. Our goal is to encourage new questions and enhance collaborations.

Regulators of transcription

Pathway-specific activators

Essential to the biosynthesis of each mycotoxin is a specific regulatory gene encoding a protein that binds to cis-elements in the promoters of biosynthetic pathway genes. These

Table 2. Summary of the major genes that impact the biosynthesis of aflatoxins fumonisins and trichothecenes

	Aflatoxins	Fumonisins	Trichothecenes
Pathway-specific activators	<i>AFLR</i>	<i>FUM21</i>	<i>TRI6</i>
RNA polymerase II complex	nd	<i>FCC1</i> , <i>FCK1</i>	<i>CID1</i> , <i>FgFRB10</i> , <i>FgCTK1</i>
Epigenetic regulators	<i>LAEA</i> , <i>HDAA</i> , <i>VEA</i> , <i>VELB</i>	<i>FLAE1</i> , <i>FvVE1</i>	<i>FgVEA</i> , <i>FgVE1</i> , <i>FLT1</i> , <i>HDF1</i> , <i>HDF3</i>
Light responsive	<i>VEA</i>	<i>FUM1</i> , <i>FUM21</i> , <i>FvVE1</i>	nd
Nitrogen regulators	<i>AREA</i>	<i>AREA</i>	nd
pH regulators	<i>PACC</i>	<i>PAC1</i>	<i>PAC1</i>
Carbon regulators	nd	<i>HXX1</i> , <i>ZFR1</i>	nd
Host environment	nd	<i>FST1</i>	nd

nd, not determined.

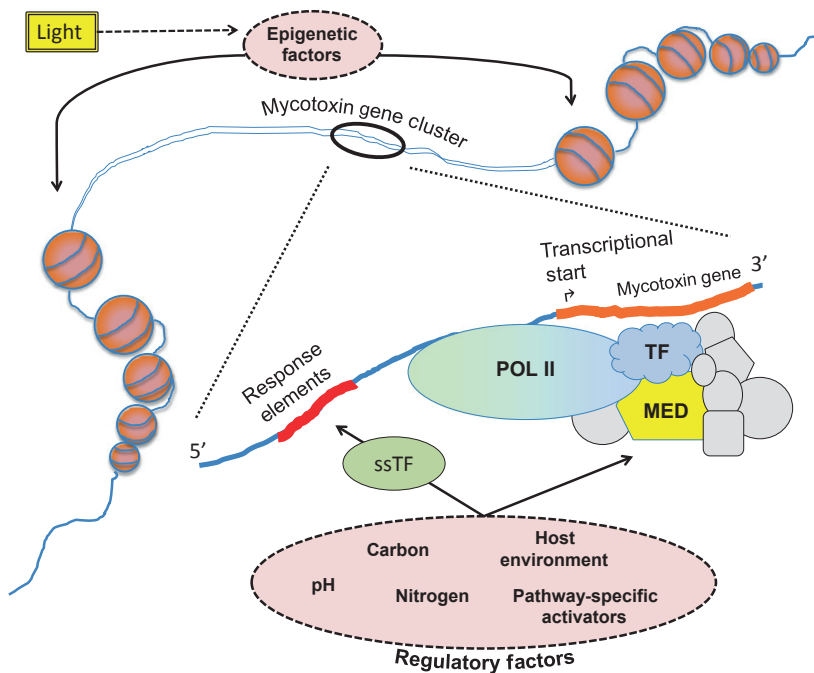


Fig. 3. Schematic overview of the regulatory components involved in the transcription of mycotoxin biosynthetic genes. A variety of regulatory factors (in dotted oval) ultimately influence RNA polymerase II complex for the transcription of genes in the mycotoxin gene cluster. Epigenetic factors also play a critical role in structural modification of chromatin, which ultimately promotes the expression of the mycotoxin gene. POLII, polymerase II; TF, general transcription factors; MED, mediator complex; ssTF, sequence-specific transcription factors.

transcription factors act as positive regulators that assist in recruiting RNA polymerase II to initiate transcription. The aflatoxin gene cluster contains the *cis*-regulator gene *AFLR*, which is essential for aflatoxin production (Chang *et al.*, 1993; Payne *et al.*, 1993). *AflR* belongs to a class of transcription factors referred to as zinc cluster proteins, found only in fungi and primarily in the Ascomycetes (MacPherson *et al.*, 2006). The most extensively studied zinc cluster protein is Gal4 in yeast, which is responsible for activating genes involved in galactose metabolism (Traven *et al.*, 2006). Zinc cluster proteins have six cysteine residues conserved in a motif that binds with two zinc molecules (Macpherson *et al.*, 2006). The amino acid sequence of *AflR* contains the C-X₂-C-X₆-C-X₆-C-X₆-C that aligns perfectly with the Gal4 motif (Woloshuk *et al.*, 1994). *AflR* binds to the consensus DNA sequence TCG(N₅)CGA, which is found in the promoters of many afla-

toxin biosynthetic genes (Yu *et al.*, 1996; Fernandes *et al.*, 1998). The fumonisin gene cluster in *F. verticillioides* also contains a gene (*FUM21*) encoding a zinc cluster protein that regulates transcription (Brown *et al.*, 2007). Unlike *AFLR*, which lacks introns, *FUM21* remained unrecognized in the cluster until Brown *et al.* (2007) discovered that the gene contains multiple introns that are alternatively spliced, giving rise to a family of transcripts, many not encoding the complete zinc cluster motif. The authors demonstrated that strains without a functional *FUM21* lacked transcripts from two biosynthetic genes (*FUM1* and *FUM8*) and failed to produce fumonisin B1 (FB1), the major form of fumonisin produced in nature. There are no reports describing the putative *cis*-element in the promoters of the fumonisin pathway genes that is recognized by *Fum21*. In *F. graminearum*, the transcription factor gene (*TRI6*) is located in the gene cluster on

chromosome 2 (Proctor *et al.*, 1995; Hohn *et al.*, 1999). Tri6 is a member of the Cys2-His2 zinc finger family of regulators, which are common in all eukaryotes. This class of proteins associates with a single zinc molecule when binding to promoter sequence DNA (MacPherson *et al.*, 2006). Disruption of *TRI6* reduces transcription of genes involved in trichothecene biosynthesis (Proctor *et al.*, 1995; Hohn *et al.*, 1999). A consensus *cis*-promoter element to which Tri6 binds was identified as YNAGGCC (Proctor *et al.*, 1995). Recent microarray and ChIP-Seq experiments indicate that in addition to regulating trichothecene biosynthesis, Tri6 has a more global impact on gene transcription (Seong *et al.*, 2009; Nasmith *et al.*, 2011).

RNA polymerase II complex and transcription

One function of the specific transcription regulators such as *AFLR*, *FUM21*, and *TRI6* is to recruit the RNA polymerase holoenzyme complex to the gene promoter. This complex is composed of RNA polymerase II, general transcription factors, and Mediator (Holstege *et al.*, 1998; Lewis & Reinberg, 2003). Studies in yeast have provided fundamental understanding of how these various components interact with the gene-specific regulators to initiate transcription (Mitsuzawa & Ishihama, 2004). It is also apparent that many of the signal transduction pathways eventually influence RNA polymerase function through the Mediator complex (Myers & Kornberg, 2000; Borggrefe *et al.*, 2002; Lewis & Reinberg, 2003). With respect to mycotoxin biosynthesis, only a few studies provide insight into the transcription process. During the screening of REMI mutants of *F. verticillioide*s, Shim & Woloshuk (2001) identified a strain with reduced FB1 production on autoclaved maize kernels. The mutant also accumulated a scarlet metabolite in the medium, grew more slowly, and produced very little aerial hyphae and fewer conidia than the wild type (Shim & Woloshuk, 2001). Acidic growth conditions restored conidia production and FB1 biosynthesis. The REMI insertion occurred in the gene *FCC1*, which encodes a protein similar to the Mediator protein Ssn8 in yeast (also known as Srb11 and Ume3; Myers & Kornberg, 2000; Shim & Woloshuk, 2001). Ssn8p is a C-type cyclin that pairs with a kinase encoded by *SSN3* (also known as *SRB10* and *UME5*; Myers & Kornberg, 2000). Bluhm & Woloshuk (2006) cloned the homologue of *SSN3* in *F. verticillioide*s (*FCK1*) and demonstrated by yeast two-hybrid analysis that Fck1 and Fcc1 associate with each other. The phenotype of the *FCK1*-disruption strain was similar to that of *fcc1::REMI* strain (Bluhm & Woloshuk, 2006). In yeast, Ssn8 and Ssn3p participate in the regulation of genes in response to environmental stress and nutrient availability. As yeast grows on a fermentable sugar, Gal4 and the C-terminus

of polymerase II are phosphorylated by Ssn8, resulting in accelerated transcription of the *GAL* genes. Under conditions of stress, that is, nonfermentable carbon, Ssn8 and Ssn3 are degraded and Gal4 is not phosphorylated, resulting in increased expression of 173 genes (Cooper *et al.*, 1997; Holstege *et al.*, 1998; Rohde *et al.*, 2000). The impact of *FCC1* on gene expression was examined by subtractive hybridization with RNA from the wild type and *fcc1::REMI* strain, resulting in the identification of 658 unique ESTs (Shim & Woloshuk, 2001). When a microarray constructed with the ESTs was probed with RNA isolated from wild type and *fcc1::REMI* grown on autoclaved maize kernels, 116 ESTs were found to be differentially expressed (Pirttila *et al.*, 2004). Significant differences were also measured for 166 ESTs during growth at low (3) and high (8) pH conditions.

A mutant of *F. graminearum* (*Acid1*) disrupted in the *FCC1* homologue *CID1* exhibited many of the same developmental phenotypes as *fcc1::REMI* strain in *F. verticillioide*s, including severe mycotoxin (DON) reduction (Zhou *et al.*, 2010). The authors demonstrated that a functional *CID1* is required for pathogenicity on wheat and maize and resistance to environmental stresses (Ni⁺, Cd²⁺, H₂O₂; Zhou *et al.*, 2010). More recently, the *FCK1* homologue was characterized as part of a larger study investigating the 116 protein kinase genes in *F. graminearum*. Referred to as *FgSRB10* (Fg04484), the disruption mutant (Fgsrb10) grew poorly, exhibited reduced conidiation, and was non-pathogenic on wheat (Wang *et al.*, 2011). Also among the kinase genes examined was *FgCTK1* (Fg06793), a homologue of yeast *CTK1*, which is another cyclin-dependent kinase that phosphorylates the C-terminus of RNA polymerase II and is involved in transcript elongation (Ahn *et al.*, 2009). Disruption of *FgCTK1* resulted in reduced growth, conidiation, ascospore production, pathogenicity, and DON production (Wang *et al.*, 2011). The cumulative results from these studies suggest that transcription of FB1 and DON biosynthetic genes involves similar Mediator components. No studies have examined the components involved in the RNA polymerase holoenzyme complex in *A. flavus*. However, studies in *A. nidulans* (Shimizu & Keller, 2001; Shimizu *et al.*, 2003) demonstrated that phosphorylation of AflR by the cAMP-dependent protein kinase PkaA negatively impacts the function of this regulator. This modification appears to be part of the signaling mechanism that regulates the functional activity of AflR, but it is not known whether this serves as a signal to Mediator or the RNA polymerase II complex.

Epigenetic regulation

Epigenetic regulation broadly refers to the changes in gene expression regulated by mechanisms not linked to

mutations in the gene sequence (Vaquero *et al.*, 2003). Such changes result in a variety of phenotypes induced in the same genotype often in response to environmental or metabolic cues. The mechanisms most recognized as epigenetic are DNA methylation, histone modifications, RNA interference (RNAi), and chromosome position effects. Current evidence indicates that histone modification is involved in the epigenetic regulation of mycotoxin biosynthesis, but not DNA methylation (Liu *et al.*, 2012). Furthermore, the epigenetic phenomenon affecting mycotoxin biosynthesis is also global across the genome, consequently impacting the expression of many other genes and pathways.

Loss of AFLR expression (*LAEA*) has provided the clearest evidence for epigenetic regulation of mycotoxin biosynthesis. Butchko *et al.* (1999) used a creative visual screen to identify mutations that affected sterigmatocystin biosynthesis in *A. nidulans*. In this screen, a total of 176 isolates failed to produce the orange-colored metabolite norsolorinic acid of the sterigmatocystin pathway, which accumulated in the mutagenized strain. Three mutants that appeared normal with respect to growth and development exhibited no *AFLR* expression. Bok & Keller (2004) characterized *LAEA* as the mutation in one of three strains lacking *AFLR* expression. The gene is located on chromosome 8 in *A. nidulans*, whereas its homologue in *A. flavus* is on chromosome 2 (Bok & Keller, 2004). Without a functional *LAEA*, production of secondary metabolites is repressed, while growth and conidiation are not affected (Bok & Keller, 2004; Georgianna *et al.*, 2010). Recent evidence indicates a clear link between *LaeA*, chromatin modification, and transcription of the genes involved in sterigmatocystin biosynthesis (Reyes-Dominguez *et al.*, 2010). In addition, over-expression of *LAEA* revealed gene clusters responsible for the synthesis of other secondary metabolites (Bok *et al.*, 2006). Histone modification, particularly the N-termini of the core histone proteins, is a major mechanism of epigenetic regulation. Modifications, such as methylation, acetylation, phosphorylation, and ubiquitylation, result in the reversible switching between heterochromatin and euchromatin states (Vaquero *et al.*, 2003). Reyes-Dominguez *et al.* (2010) demonstrated that histone subunit H3 with methylation at lysine 9 (K9) of the N-terminus functions in maintaining a heterochromatin state surrounding the gene cluster responsible for sterigmatocystin biosynthesis, thus rendering the region inaccessible to the RNA polymerase II complex. When conditions are suitable to trigger sterigmatocystin production, the level of methylated H3 subunits decreases, resulting in euchromatin state at the gene cluster locus (Reyes-Dominguez *et al.*, 2010). Although *LaeA* has a role in this transition, the exact mechanism remains unresolved.

The *LAEA* homologue (*FfLAE1*) was recently identified in *Fusarium fujikuroi*, which causes Bakanae disease of rice (Wiemann *et al.*, 2010). This pathogen is primarily known as the model for studying gibberellin biosynthesis, but it is also capable of producing small amounts of fumonisins (Proctor *et al.*, 2004; Stepien *et al.*, 2011). The $\Delta Fflae1$ mutant of *F. fujikuroi* exhibited reduced gibberellin production, but measurements of fumonisin production were not presented (Wiemann *et al.*, 2010). Significantly, *FfLAE1* restored the production of the sterigmatocystin pathway when expressed in the *laeA* mutant of *A. nidulans* (Wiemann *et al.*, 2010), demonstrating functional conservation in two fungal species.

Acetylation of the core histone proteins reduces the affinity of histone to DNA due to effects on the charge, whereas deacetylation results in more condensed chromatin structure (Vaquero *et al.*, 2003). The effect of the histone deacetylase gene *HDAA* on sterigmatocystin biosynthesis in *A. nidulans* was examined by Shwab *et al.* (2007). Deletion of *HDAA* caused the accumulation of *STCU* and *AFLR* transcripts 6–12 h sooner than in the wild-type strain, but did not impact fungal growth. Different results were obtained in *F. graminearum* in which a pair of genes (*FLT1* and *HDF1*) was identified as important components for histone deacetylation (Ding *et al.*, 2009; Li *et al.*, 2011). Deletion of these genes reduced conidia production, pathogenicity, and histone deacetylase activity; however, the mutation of *HDF1* was solely responsible for more than 30% reduction in DON production (Li *et al.*, 2011). Two other putative histone deacetylase genes (*HDF2* and *HDF3*) were also studied. The $\Delta hdf3$ strain exhibited wild-type growth, conidiation, and pathogenicity, but produced 60% less DON (Li *et al.*, 2011). This difference observed in *A. nidulans* and *F. graminearum* is intriguing. Shwab *et al.* (2007) suggested that *HDAA* affects the gene cluster near the telomere, but not those at more proximal locations. Deletion of the *HDAA* homologue in *Aspergillus fumigatus* up-regulated the production of secondary metabolites whose genes were not located near telomeres (Lee *et al.*, 2009). The deletion also reduced conidia germination and the production of gliotoxin. These results illustrate the complexity of epigenetic regulation.

In *F. verticillioides*, application of the histone deacetylase inhibitor chostatin A resulted in increased transcription of *FUM1* and *FUM21* (Visentin *et al.*, 2012). Although not statistically significant, FB1 levels were consistently higher in the chostatin A-treated cultures. Co-precipitation assays with antibodies specific for acetylated histone H4 yielded more promoter DNA from *FUM1* and *FUM21* under conditions conducive for FB1 production than under normal culture conditions. The results indicated that histone acetylation has an important role in

the transcriptional regulation of fumonisin genes (Visentin *et al.*, 2012).

A few recent studies have examined nonhistone proteins that are involved in chromatin modification. The heterochromatin protein HP1 can bind to the K9 methylated H3, and the HP1 ortholog in *A. nidulans* (*HEPA*) has a major role in silencing transcription of the sterigmatocystin genes (Reyes-Dominguez *et al.*, 2010). To determine whether HP1 is important for DON production, Reyes-Dominguez *et al.* (2012) examined a *HEP1* mutant of *F. graminearum*. Growth of the mutant was similar to that of the wild-type strain, but DON production and transcription of *TRI5* and *TRI6* were significantly reduced (Reyes-Dominguez *et al.*, 2012).

Much attention has been focused on the Velvet (VE) complex and its role in the epigenetic regulation of mycotoxin production. The *VE* gene family, which was first described in *A. nidulans*, is major regulator of light response, development, and secondary metabolism (Bayram & Braus, 2012). In *A. nidulans*, VeA and VelB interact with LaeA to facilitate the epigenetic activity of LaeA (Bayram *et al.*, 2008; Yin & Keller, 2011). The presence of light greatly impacts this interaction resulting in less sterigmatocystin, and the converse is also true (Stinnett *et al.*, 2007; Bayram *et al.*, 2008). In *F. verticillioides*, mutations in *FvVE1*, a homologue of *VEA*, abolish FB1 production and transcription of *FUM21* and *FUM8* (Myung *et al.*, 2009). Furthermore, *FvVE1* appears to be required for disease symptom development in inoculated maize seedlings (Myung *et al.*, 2012). The *FvVE1* mutants grew endophytically in maize tissues and failed to produce the necrotic leaf symptoms observed with wild-type strains. Two research groups have recently published the results of their studies on the *VeA* homologue (*FgVEA* and *FgVE1*) in *F. graminearum* (Jiang *et al.*, 2011; Merhej *et al.*, 2012). As with the *F. verticillioides* gene, mutation of *FgVEA* (*FgVE1*) reduces mycotoxin (DON) production and pathogenicity. Even though *FgVEA* and *FgVE1* designate the same gene (FGSG_11955) in *F. graminearum* genome, the authors reported contrasting effects on conidia production and germination in their mutant strains. Deletion of the gene (*FgVEA*) resulted in a significant increase in conidia production, but the time needed for germination was twice that of the wild-type strain PH-1 (Jiang *et al.*, 2011). In contrast, an insertional mutation (*FgVE1*) resulted in a severe reduction in the number of conidia produced, but spore germination was not affected (Merhej *et al.*, 2012). In *F. verticillioides*, Fanelli *et al.* (2012) showed that expression of *FvVE1* is affected by light, as observed with *A. nidulans*. The study demonstrated that fumonisin production and expression of *FvVE1*, *FUM1*, and *FUM21* are greater under individual color (red to blue) exposure and pulsed light than under continuous white light or dark conditions.

Epigenetic effects also were hypothesized to explain the silencing of aflatoxin production in parasexually derived diploids of *A. flavus* strain 649 (Woloshuk *et al.*, 1995; Smith *et al.*, 2007). Papa (1979, 1980) described strain 649 as a nonproducer of aflatoxin that exhibits dominant repression of aflatoxin in diploids. He also mapped the mutation (*aff1*) to the same locus that was later determined as the aflatoxin gene cluster (Foutz *et al.*, 1995). Once the genomic sequence of *A. flavus* became available, Smith *et al.* (2007) probed strain 649 by Southern analysis and discovered that the strain has a 317-kb deletion in chromosome 3, which includes the entire aflatoxin gene cluster. The deleted chromosome end was replaced with a duplicated region (939 kb) from chromosome 2. The silencing observed in diploids was hypothesized as a transvection phenomenon (Woloshuk *et al.*, 1995; Smith *et al.*, 2007). First described in *Drosophila melanogaster*, transvection (or trans-sensing) is the inactivation of alleles due to somatic chromosome pairing and often arises due to genomic rearrangements (Wu & Morris, 1999; Duncan, 2002). Because fungi are haploid, this type of silencing effect is rare. In *Neurospora crassa*, transvection was described for *ASM-1*, a gene required for ascospore maturation (Aramayo & Metzberg, 1996; Aramayo *et al.*, 1996). Deletion of *ASM-1* has a recessive effect on growth in the haploid fungus; however, during the diploid phase of meiosis, the mutation exerts a dominant effect resulting in aborted ascospores (Aramayo & Metzberg, 1996). In strain 649, the repression of gene expression in diploids is confined to the aflatoxin biosynthetic genes with normal expression of genes outside the cluster (Smith *et al.*, 2007). Also, ectopic insertion of *AflR* restores aflatoxin production in diploids (Smith *et al.*, 2007). In *D. melanogaster*, several epigenetic mechanisms have been suggested as explanations for transvection (Wu & Morris, 1999). It is reasonable to propose that in diploids formed with strain 649, the heterochromatin state at the aflatoxin cluster is not affected by the expression of *LAEA*. It remains unclear how the expression of *AFLR* at another locus restores transcription of the genes in the aflatoxin cluster.

Impact of environmental factors

Nitrogen repression

Fungi can utilize a wide variety of nitrogen sources (Marzluf, 1997). Most preferentially, fungi utilize ammonium and glutamine over other sources, such as nitrate, nitrite, and proteins (Arst & Cove, 1973). Global regulators control the expression of the genes for nitrogen utilization – *AREA* and *NIT2* in *A. nidulans* and *N. crassa*, respectively (Caddick *et al.*, 1986; Fu & Marzluf, 1990;

Kudla *et al.*, 1990). Expression of *AREA* and *NIT2* is repressed when sufficient amounts of ammonium or glutamine are available. However, once these are removed from the environment, derepression leads to the activation of the other nitrogen utilization pathways. One step in the activation is the binding of *AreA* to GATA sequences in the gene promoters. Many of the genes in the fumonisin cluster have GATA sequences in their promoter (Kim & Woloshuk, 2008). Also, addition of ammonium phosphate to *F. verticillioides* cultures actively producing FB1 results in abrupt cessation in toxin production (Shim & Woloshuk, 1999). Strains lacking a functional *AREA* fail to grow and do not produce FB1 on nitrate-containing medium. In contrast, strains with constitutive expression of *AREA* produce FB1 under conditions of nitrogen repression (Kim & Woloshuk, 2008).

Aspergillus flavus has an *AREA* gene, and the promoters of the aflatoxin biosynthetic genes have GATA sequences (Chang *et al.*, 2000). Interestingly, while *AreA* binds to the promoter of *AFLR* (Chang *et al.*, 2000), nitrogen effects on aflatoxin production are quite different from effects on FB1 production by *F. verticillioides*. Media containing ammonium salts support aflatoxin production, whereas nitrate inhibits (Niehaus & Jiang, 1989). At the transcriptional level, expression of the aflatoxin pathway genes *PKSA* and *NOR1* is repressed during growth in medium containing sodium nitrate, but not when the medium contains ammonium chloride (Feng & Leonard, 1998). Flaherty & Payne (1997) also confirmed the inhibitory effects of nitrate-containing media and demonstrated that the pH of the medium was not alkaline, a condition that inhibits aflatoxin production. They also showed that constitutive expression of *AFLR* did not alleviate the inhibition on aflatoxin biosynthesis. Both northern analysis and expression of a promoter reporter (*ver::GUS*) indicated that aflatoxin pathway genes were expressed in the strain constitutively expressing *AFLR*, suggesting that reduced transcription of pathway genes is not completely responsible for the inhibitory effects of nitrate (Flaherty & Payne, 1997). This interpretation differs from that of Chang *et al.* (1995) who showed that an extra copy of *AFLR* in *A. parasiticus* suppressed the inhibitory effects of nitrate on expression of the aflatoxin pathway genes. These authors concluded that the nitrate effects are caused by insufficient levels of *AFLR* (Chang *et al.*, 1995). Unfortunately, they did not include analyses of aflatoxin production, leaving open the possibility that aflatoxin production remained inhibited as observed by Flaherty & Payne (1997). Nevertheless, Chang *et al.* (1999) pursued the hypothesis that inhibition is manifested by an interaction with a putative negative regulatory protein that binds to *AflR* and that the expression of an extra copy of *AFLR* titrates out the repressor. Their premise was that the

repressor interacts with C-terminus of *AflR* similar to the interaction of *Nmr* with *Nit2* in *N. crassa*. Under nitrogen-repressive conditions, *Nmr* binds to a specific C-terminus element of *Nit2*, thus rendering it ineffective (Feng & Leonard, 1998). Support for a similar mechanism was observed in *A. parasiticus*, in which a strain expressing the C-terminus of *AflR* produced aflatoxin in nitrate medium (Chang *et al.*, 1999). The identity and function of the putative negative regulator remain unresolved. Adding to this complexity regarding the effects of nitrogen is the observation that ammonium chloride inhibits the expression of sterigmatocystin genes in *A. nidulans*, whereas sodium nitrate does not. It remains to be determined how constitutive expression of *AREA* affects aflatoxin production.

Miller & Blackwell (1986) demonstrated that production of 3-acetyldeoxynivalenol (ADON) by *F. graminearum* is induced by a limitation of nitrogen in the culture medium. These authors observed that nearly complete depletion of ammonia (7 mM) within 3 days after inoculation was coincident with the onset of ADON accumulation, suggesting that nitrogen metabolite repression (NMR) is a key regulatory mechanism for ADON production (Miller & Blackwell, 1986). DON production in culture medium containing 200 mM ammonium sulfate was found to be about 12-fold greater after 4 days in ammonium medium containing calcium nitrate (Ilgen *et al.*, 2009). Expression data also showed that *TRI4* and *TRI5* were repressed in the nitrate medium, but expression of *TRI6* and *TRI10* was unaffected. Band intensities of the rtPCR products were similar to those of *TRI4* and *TRI5* in a medium containing ammonium sulfate. Similar results were reported for DON production by *Fusarium culmorum*, which produced 143-fold higher DON with ammonium sulfate than with sodium nitrate (Covarelli *et al.*, 2004). A time-course experiment, which followed the expression of *TRI5* and *TRI6* during growth on ammonium sulfate medium, indicated the expression of *TRI6* 72 h after inoculation and *TRI5* at 96 h, coincident with the earliest detection of DON (Covarelli *et al.*, 2004). Although ammonium concentrations were not measured in the studies by Ilgen *et al.* (2009) and Covarelli *et al.* (2004), it is likely that by 5 days after inoculation, the ammonium concentration was sufficiently low enough to promote DON production. It is also possible that increased pH may be responsible for the inhibition of DON during growth on the nitrate medium. Recent studies also indicate that polyamines are excellent sources of nitrogen and the best inducers of DON production (Gardiner *et al.*, 2009a; Gardiner *et al.*, 2010). Polyamines are important fungal metabolites that impact fungal growth and development (Ruiz-Herrera, 1994), and they play an important role in plant development and host-

pathogen interactions (Walters, 2003; Alcazar *et al.*, 2010). In the study by Gardiner *et al.* (2009a), DON production and expression of *TRI5* were greater in media containing polyamines than in media with either ammonium salt or nitrate. Combining nitrate with various polyamines repressed DON production. Thus, it is possible that the mechanism responsible for nitrate inhibition of aflatoxin biosynthesis may also operate in *F. graminearum*.

pH effects

The impact of pH on the production of aflatoxins, fumonisins, and trichothecenes appears simple: Acidic conditions are conducive and alkaline conditions are repressive. However, the molecular aspects of pH regulation are complex and our understanding remains incomplete. The repression by alkaline conditions has been demonstrated for all three mycotoxin groups (Keller *et al.*, 1997b; Shim & Woloshuk, 2001; Gardiner *et al.*, 2009b; Merhej *et al.*, 2011). Molecular studies have focused on homologues of PACC, the pH-responsive transcription factor in *A. nidulans* (Tilburn *et al.*, 2010). PacC is a Cys₂-His₂ zinc finger protein that becomes functionally active after specific proteolysis, which occurs under alkaline conditions. The processed PacC protein is transported into the nucleus where it binds to promoter DNA, resulting in the expression of alkaline pH-induced genes and repression of acidic pH-expressed genes. Evidence suggests that PACC homologues repress the expression of genes involved in aflatoxin, fumonisin, and trichothecene production. Several PACC mutations in *A. nidulans* (referred to as *pacC^C*) affect the regulatory activities of PacC (Tilburn *et al.*, 2010). For instance, the mutant PacC^{C202} produces a truncated PacC protein and exhibits a phenotype that mimics growth under alkaline conditions (Tilburn *et al.*, 2010). Under acidic conditions, sterigmatocystin production in this strain was 10-fold less than in the wild type (Keller *et al.*, 1997a). Transcripts of the pathway gene *STCU* were also reduced compared to the wild type (Keller *et al.*, 1997a).

Shifting the pH from 3.6 to 6.5 is sufficient to inhibit the production of DON and 15ADON in *F. graminearum* (Merhej *et al.*, 2011). Deletion of the *PAC1* gene resulted in poor growth at pH 8, but had no effect on growth or mycotoxin production under acidic culture conditions. A strain of *F. graminearum* was engineered with a truncated *PAC1* (*Pac1^C*) similar to the *A. nidulans* PacC^{C202} mutant. Although growth was not affected by this mutation, trichothecene production and expression of *TRI5* in *Pac1^C* were severely reduced compared to the wild type (Merhej *et al.*, 2011).

In *F. verticillioides*, disruption of *PAC1* resulted in higher FB1 production than in the wild type when grown

on autoclaved maize kernels (Flaherty *et al.*, 2003). The expression of *FUM1* was 14-fold higher when the mutant was grown in medium buffered at pH 4.5. Although growth of the mutant was severely inhibited at alkaline pH (8.4), FB1 and *FUM1* expression was detectable, indicating that Pac1 represses fumonisin production (Flaherty *et al.*, 2003).

Carbon sources

Vegetative growth and aflatoxin production are optimal in media containing glucose, ribose, xylose, or glycerol (Davis & Diener, 1968). The addition of sugar to a culture of *A. flavus* has a dramatic effect on aflatoxin biosynthesis. When mycelia of *A. flavus* are transferred from a medium such as the peptone mineral salts to the same medium containing glucose, aflatoxin production is measurable in the medium between 12 and 18 h, and the amount of mycotoxin substantially increases over the next 18 h (Woloshuk *et al.*, 1994; Flaherty *et al.*, 1995). Similarly, shifting *A. parasiticus* mycelia from yeast extract medium to a medium containing sucrose results in aflatoxin production after 12-h incubation (Wilkinson *et al.*, 2007). The minimal concentration of glucose needed to induce aflatoxin is 100 mM (Wiseman & Buchanan, 1987). The results obtained from a *VER1(p)::GUS* reporter indicate that transcription from the *VER1* promoter was sequentially elevated with increasing amounts of glucose (1–200 mM) added to the medium (Woloshuk *et al.*, 1997). Yu *et al.* (2003) examined the effects of oil (soybean and peanut) on aflatoxin production by *A. flavus* and *A. parasiticus* and found that the addition of oil to a noninductive medium (PMS) resulted in 60% and 10% of the aflatoxin produced by *A. flavus* and *A. parasiticus*, respectively, when they were grown in glucose-containing medium (GMS; Yu *et al.*, 2003).

Trichothecene production by *F. graminearum* is supported by a variety of carbon sources (Miller *et al.*, 1983; Miller & Greenhalgh, 1985; Jiao *et al.*, 2008; Zhang & Wolf-Hall, 2010). Clear differences exist in responses to a particular carbon source, and the specific effects are influenced by the nitrogen source and pH. Observations by Miller *et al.* (1983) suggested that trichothecene production is repressed by high sugar concentrations. Carbon catabolite repression (CCR) is a global regulatory system, similar to NMR, in which expression of genes involved in the utilization of alternative carbon sources is repressed when adequate levels of preferred carbon, such as glucose, are available. In *A. nidulans*, the *CREA* gene is responsible for CCR and, unlike AreA, CreA is a negative regulator of gene expression (Dowzer & Kelly, 1989). Jiao *et al.* (2008) addressed the question of whether or not glucose exerts CCR on DON production in *F. graminearum*. Strain H3

grew equally well on both glucose and sucrose as carbon sources. DON production was poor when grown on glucose, but slightly more DON was produced at the lower glucose concentrations. In contrast, sucrose supported DON production without a notable concentration effect. Expression of *TRI4* and *TRI5* was higher with sucrose in cultures than with glucose, but no differences were detected in *TRI6* and *TRI10* expression. Increasing amounts of glucose combined with sucrose did not affect DON production, suggesting that CCR is not a key regulating mechanism for DON.

There is no clear evidence to indicate that CCR regulates fumonisin production in *F. verticillioides*. Shim & Woloshuk (1999) demonstrated that different concentrations of sucrose in culture did not influence the level of FB1 production. A few studies have reported on the effects of carbon sources on fumonisin production by *F. verticillioides* (Jimenez *et al.*, 2003; Bluhm & Woloshuk, 2005). Bluhm & Woloshuk (2005) also found that a medium containing amylopectin supported the production of more FB1 than a medium with glucose or maltose. Jimenez *et al.* (2003) found that fructose supported more FB1 production than five other carbon sources, including glucose, sucrose, and maltose. A mutant containing a disruption of *HXK1*, a putative hexose kinase, failed to grow on a fructose-containing medium, suggesting that *HXK1* is required for fructose metabolism (Kim *et al.*, 2011). FB1 production was also reduced by 80% when grown on glucose as the carbon source.

Impact of host environment

Aflatoxins, fumonisins, and trichothecenes are produced in seeds of infected cereals, all three on maize and DON also on wheat and barley. Production of mycotoxins in the seed environment has several layers of complexity, including the effects associated with the stage of seed development at the time of infection, the tissues colonized by the pathogen, and host responses to infection. Furthermore, the mycotoxigenic fungi elicit changes in the microenvironment within the infected seeds that influence the molecular regulation of mycotoxin biosynthesis. Here, we discuss mycotoxin production during the different stages of seed development and within specific seed tissues.

Kernel development and mycotoxin production

Depending on when infection occurs, the pathogen may encounter a nutritional environment that is changing within the developing seed (kernel). At pollination, the double fertilization of the female gametophyte results in the diploid zygote, which gives rise to the embryo (germ),

and the triploid endosperm nucleus, which produces the endosperm and aleurone layer (Evers & Millar, 2002; Sabelli & Larkins, 2009; Sreenivasulu *et al.*, 2010; Becraft & Gutierrez-Marcos, 2012). Nuclear divisions in the endosperm are more rapid than the zygote, proceeding through many rounds of synchronous nuclear division followed by cellularization. By 6–10 days after pollination, these cells will have differentiated into transfer cells, aleurone cells, starch-storage endosperm cells, and the specialized cells surrounding the embryo. The maturation of the seed is a dynamic process that can be separated into distinct developmental stages (Abendroth *et al.*, 2011). At the earliest stage (R2 in maize), amino acids and sugar flow through the transfer cells into the endosperm. At this time, the seed is characteristically watery in appearance. Once starch begins to accumulate, the endosperm becomes milky (R3 stage in maize). Subsequently, as solid starch accumulates, the seed enters the dough stage (R4 in maize) and through the dent stage (R5 in maize). During this period, nutrients are provided to the developing embryo by the specialized endosperm cells. Once starch accumulation begins, the cells of the endosperm initiate a progressive process of programmed cell death. At maturation, only the cells of the aleurone layer remain alive. The final stage of seed development involves the loss of moisture. Thus, considering the developmental stages, the pathogen infecting the seed early in development will experience a different environment (nutrient and moisture) than one infecting in the later stages.

Inoculation of the various development stages (R2–R5) of maize seeds with *F. verticillioides* indicated that the pathogen could colonize these stages equally well (Bluhm & Woloshuk, 2005). However, significant FB1 production occurred only in the R5 (dent)-stage kernels. Expression of *FUM8* and *FUM12* as well as low amounts of FB1 was detected in the R3 (milk) and R4 (dough) stages. In contrast, no FB1 or *FUM* gene expression was detectable in the R2 (blister) stage. Subsequent experiments revealed that the fungus produced fourfold more FB1 on amylopectin than on glucose. The branched molecule of amylopectin was also superior to the linear amylose in supporting FB1 production. The fungus grows poorly on amylose medium and on maize (ae1) mutants that accumulate amylose. Also, FB1 production is greatest in the amylopectin-rich endosperm (Shim *et al.*, 2003; Flaherty & Woloshuk, 2004). Significantly, during growth on various kernel developmental stages, *F. verticillioides* experiences changes in the pH of the kernels (Bluhm & Woloshuk, 2005). By 4 days after inoculation, the pH of the R2 and R3 kernels becomes increasingly alkaline. At this same time point, the R5 kernels are markedly acidic and the R4 kernels are unchanged. Such changes in pH indicate that only the R5 kernels provide the acidic

conditions that are most conducive for FB1 production. Kim & Woloshuk (2008) also demonstrated that the environment of the R2 kernel is repressive to *AREA* expression, likely due to the abundance of free amino acids. They hypothesized that metabolism of the amino acids leads to an alkalization of the extracellular environment. In fact, addition of amylopectin to the inoculated R2 kernels reduced the pH, eliminated repression of *AREA* expression, and induced FB1 production (Kim & Woloshuk, 2008).

The different development stages of maize kernels were found to support the growth of *A. flavus* (Reese *et al.*, 2011). Aflatoxin was produced in kernels at all stages, and it was consistently higher in the R2 seeds 5 days after inoculation. However, microarray analyses indicated that significant expression of the aflatoxin biosynthetic genes occurred in the R5 kernels. Although not measured, pH in the colonized seeds may have changed similar to the changes observed with *F. verticillioides*. At 5 days, pH conditions in the R2, R3, and R4 stages may have become nonconductive to maintain transcription of the aflatoxin genes relative to the R5 kernels.

Induction of DON in *F. graminearum* is different. Because DON has a role as a virulence factor, most studies have focused on the initial stages of infection of wheat and barley at anthesis (Ilgen *et al.*, 2009; Gardiner *et al.*, 2010; Boenisch & Schafer, 2011; Hallen-Adams *et al.*, 2011). Microarray analysis of RNA collected between 24 and 190 h after inoculation indicated that expression of the genes involved in DON production increased significantly as early as 48 h (Guldener *et al.*, 2006). The accumulation of DON could be measured after this time point and continued to increase with time (Gardiner *et al.*, 2010). With *F. graminearum* strains expressing the green fluorescent protein (GFP) under the control of the *Tri5* promoter, Ilgen *et al.* (2009) demonstrated GFP expression in the colonized developing seed 4 days after inoculation. The production of DON and the spread of the fungus in the spikes correlate well with the presence of several polyamine compounds that accumulate as infection progresses through the spike (Gardiner *et al.*, 2010). Hallen-Adams *et al.* (2011) also used microarrays to measure the expression of the trichothecene pathway genes during colonization of seeds by *F. graminearum*. When fungal inoculum was sprayed on barley heads, expression of the trichothecene genes was observed within 24 h and continued to increase beyond 7 days postinoculation. Based on these studies, we can conclude that the environment within the earliest stage of seed development is conducive for DON production. However, very little information is available on how the later stages of seed development impact DON production and the expression of trichothecene pathway genes. One study examined the

expression of *TRI5* during colonization of individual barley kernels (Hallen-Adams *et al.*, 2011). Six days after inoculation of a single spikelet, *TRI5* expression was maximal in the adjacent seed. Expression of *TRI5* progressively decreased with time, but was still measurable at 21 days postinoculation. A similar pattern of expression was observed for the other seeds as the pathogen spread up and down the rachis from the initial inoculation site.

Tissue-specific activation/suppression

In *F. verticillioides*, *ZFR1* encodes a protein that is a member of the $Zn(II)_2Cys_6$ zinc cluster family. The gene was identified among ESTs in a cDNA subtraction library derived from the wild type and *fcc1* mutant of *F. verticillioides* (Flaherty & Woloshuk, 2004). Deletion of *ZFR1* resulted in a mutant strain ($\Delta zfr1$) that produced only trace amounts of FB1 (Flaherty & Woloshuk, 2004). In liquid medium and on autoclaved maize kernels, $\Delta zfr1$ grew similar to the wild-type strain. Further examination revealed that growth of $\Delta zfr1$ on germ tissue was twice that of the wild type, but only 40% of the wild type in the endosperm tissues (Bluhm *et al.*, 2008). Expression of *ZFR1* also was greater in the endosperm tissues than in germ (Flaherty & Woloshuk, 2004). These results suggest that *Zfr1* activates the genes involved in tissue-specific growth. The de-repression in the germ tissue that leads to enhanced growth has not been investigated. Radial growth of $\Delta zfr1$ on solid media containing glucose, maltose, amylopectin, or dextrin was inhibited compared to the wild type (Bluhm *et al.*, 2008). The colonies had a distinct morphology with minimal extension beyond the margin of the colonies. The mutant had no defect in amylase production (Bluhm *et al.*, 2008) or in sugar uptake in liquid medium (B.H. Bluhm and C.P. Woloshuk, unpublished data). Furthermore, *Zfr1* impacts the expression of several putative sugar transporters. In the wild-type strain, these transporter genes were expressed significantly higher in the endosperm tissue than in the germ, and expression was severely reduced in $\Delta zfr1$ strain. Deletion of one of these sugar transporter genes (*FST1*) reduced FB1 production and decreased the rate of colonization of kernels, but the mutation did not affect the growth on various carbon media or on autoclaved maize kernels (Kim & Woloshuk, 2011). *FST1* is highly expressed in the endosperm, and the protein appears to localize to the plasma membrane before being turned over in vacuolar structures. Expression of *FST1* in a yeast strain lacking hexose transporter genes failed to promote the growth on media containing glucose, fructose, or mannose. Thus, the specific function of *FST1* remains unresolved.

Infection of wheat seeds in early development by *F. graminearum* most often results in shriveled kernels

referred to as tombstones. Microscopic examination of the tombstones indicates that damage is so severe that definitive tissue structures are lacking (Chelkowski *et al.*, 1990). On a weight basis, tombstones contain more DON than kernels that are able to fully mature (Reid *et al.*, 1996; Sinha & Savard, 1997). The light-weight, diseased maize kernels also contain the highest amount of mycotoxin (Schaafsma *et al.*, 2004), and the lighter-weight kernels can be removed from the marginally diseased and healthy kernels by density separators. Analysis of the denser, less diseased kernels indicates that DON contamination is distributed in all kernel fractions, that is, endosperm, germ, and pericarp (Hart & Braselton, 1983; Chelkowski *et al.*, 1990; Schaafsma *et al.*, 2004). On a percent basis, the highest concentrations of DON are in the pericarp, the tissue that originates from the carpel wall (Chelkowski *et al.*, 1990). It is perhaps significant that other parts of the inflorescence support higher concentrations of DON than the seed tissue (Reid *et al.*, 1996; Savard *et al.*, 2000).

Keller *et al.* (1994) followed colonization and aflatoxin production in maize kernel tissues with strains of *A. flavus* and *A. parasiticus* that accumulate the orange-colored, pathway metabolite norsolorinic (NOR), which accumulate due to a partial block in the aflatoxin biosynthetic pathway. They demonstrated that aflatoxins are preferentially produced in germ tissues. When inoculated to wounded kernels, the embryo was colonized by the fungus within 4 days, and nearly all the NOR and aflatoxin accumulated in the embryo tissues. Invasion through wounds to the endosperm region resulted in NOR production in the aleurone layer, but little in the starchy endosperm (Keller *et al.*, 1994). During colonization, little degradation of starch and storage proteins occurs (Mellon *et al.*, 2005). The germ appears to be a good source of sugar and triglycerides. Killing the embryo by heat prior to inoculation results in excellent fungal growth but less aflatoxin production compared to the nontreated kernels. Sugars (sucrose and raffinose) were found to be more available in the living embryo than in the heat-killed, whereas triglyceride availability was similar in both treatments (Mellon *et al.*, 2005). Because these studies were carried out with rehydrated, mature seeds, the results may not account for all of the events that occur under preharvest conditions. Rehydration of dormant seeds activates the metabolic activity of the embryo and aleurone cells, which likely impacts the environment encountered by the pathogen. Regardless of any potential differences, studies have clearly shown that when contaminated maize, harvested from the field, is fractionated into its milling components, the majority of aflatoxin is in the germ fraction (Pietri *et al.*, 2009). In the developing kernel, the embryo and the specialized endosperm cells that

surround the embryo would provide *A. flavus* with a lipid and sugar mixture, which together support greater aflatoxin production than each of the components alone (Yu *et al.*, 2003).

Summary and future directions

In this review, we have provided an overview of the current knowledge pertaining to the molecular regulation of aflatoxin, fumonisin, and trichothecene biosynthesis. Transcription of the pathway genes is critical for mycotoxin biosynthesis (Fig. 3). The question is how this activity is meticulously controlled, particularly in three mycotoxigenic fungal species. We described the similarities in the genes involved in the epigenetic regulation, which controls transcriptional access to the mycotoxin gene clusters by the transcriptional machinery and other regulators. Each mycotoxin group has a specific activator that binds to response elements in the promoters of the pathway genes. Furthermore, a variety of molecular factors and signals interact with the response elements or through Mediator to regulate the RNA polymerase. Table 2 summarizes these different levels of regulation and the corresponding genes. Clearly, more research is needed to fill in specific gaps in knowledge.

Researchers in the mycotoxin community will continue to investigate the issues that are most exploitable to their specific fungal systems. With respect to converging knowledge, we anticipate a couple of exciting and fruitful research areas. First, an understanding of the molecular networks involved in mycotoxin production and their connection with phenotypic networks (Loscalzo & Barabasi, 2011) will help provide a clearer understanding of the complex regulations affecting growth, reproduction, pathogenicity, and mycotoxin production. Son *et al.* (2011) used this approach with *F. graminearum*. They generated mutant strains for 657 of the 709 putative transcription factors and evaluated each mutant for 17 phenotypic characters, including growth, development, pathogenicity, and mycotoxin production. A network showing the interaction between phenotypic responses and the underlying genes was derived by statistical comparisons between the phenotypic data and gene expression data obtained from microarrays (Son *et al.*, 2011). The results provide a foundational network to be expanded with further research. Similar approaches in *A. flavus* and *F. verticillioides* will likely be published soon.

A second exciting area of research is the subcellular-level trafficking of the enzymes and pathway intermediates involved in mycotoxin biosynthesis. Special membrane-bound vesicles have been observed in *A. parasiticus*, and aflatoxin biosynthesis is hypothesized to occur in

these vesicles and is transported to the cell surface for exocytosis (Chanda *et al.*, 2009; Linz *et al.*, 2012). Although there remains much to learn about how subcellular organelles are involved in the regulation of aflatoxin biosynthesis in *A. flavus*, recent evidence indicates that similar mechanisms may function in other mycotoxigenic fungi. In *F. verticillioides*, organelles and vesicles associated with the endoplasmic reticulum are implicated in pathogenicity and secondary metabolism (Shin & Shim, 2009; Kim *et al.*, 2010; Wang *et al.*, 2010). The prospect of discovering how this network of cellular organelles regulates the complex mechanism of mycotoxin biosynthesis is intriguing.

Acknowledgements

We thank Dr Larry Dunkle for his review of the manuscript. The photograph of head scab symptoms (Fig. 1d) was taken by Dr Kiersten Wise. Funding to C.P.W. was provided by USDA/NIFA/AFRI, award number 10-65108-20567.

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