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Advances in molecular and genomic research to safeguard food and feed supply from aflatoxin contamination

D. Bhatnagar[1*], K. Rajasekaran[1], M. Gilbert[1], J.W. Cary[1] and N. Magan[2]

[1]US Department of Agriculture, Agricultural Research Service, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA; [2]Applied Mycology Group, Cranfield University, MK45 4DT, Cranfield, United Kingdom; deepak.bhatnagar@ars.usda.gov

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Abstract

Worldwide recognition that aflatoxin contamination of agricultural commodities by the fungus *Aspergillus flavus* is a global problem has significantly benefitted from global collaboration for understanding the contaminating fungus, as well as for developing and implementing solutions against the contamination. The effort to address this serious food and feed safety issue has led to a detailed understanding of the taxonomy, ecology, physiology, genomics and evolution of *A. flavus*, as well as strategies to reduce or control pre-harvest aflatoxin contamination, including (1) biological control, using atoxigenic aspergilli, (2) proteomic and genomic analyses for identifying resistance factors in maize as potential breeding markers to enable development of resistant maize lines, and (3) enhancing host-resistance by bioengineering of susceptible crops, such as cotton, maize, peanut and tree nuts. A post-harvest measure to prevent the occurrence of aflatoxin contamination in storage is also an important component for reducing exposure of populations worldwide to aflatoxins in food and feed supplies. The effect of environmental changes on aflatoxin contamination levels has recently become an important aspect for study to anticipate future contamination levels. The ability of *A. flavus* to produce dozens of secondary metabolites, in addition to aflatoxins, has created a new avenue of research for understanding the role these metabolites play in the survival and biodiversity of this fungus. The understanding of *A. flavus*, the aflatoxin contamination problem, and control measures to prevent the contamination has become a unique example for an integrated approach to safeguard global food and feed safety.

Keywords: *Aspergillus flavus*, secondary metabolites, preharvest control, postharvest control, aflatoxin

1. Introduction

Aflatoxin B_1 (AFB_1) is a human carcinogen and acutely toxic to humans, pets, livestock and wildlife. Aflatoxins are secondary metabolites produced by the fungus *Aspergillus flavus* in many commodities, both prior to harvest and during storage after harvest, thereby greatly reducing their value and marketability due to the toxic effects of these compounds. Aflatoxins are among the most carcinogenic natural toxins known, and ingestion by animals and human beings can result in diseases, such as liver cancer. Children are particularly affected by aflatoxin exposure, which leads to stunted growth and delayed development (CAST, 2003). In animal models, activation of AFB_1 by mammalian microsomal cytochrome P450 is required for its carcinogenic effect. Cytochrome P450 monooxygenases in the liver convert AFB_1 to a variety of metabolites of increased polarity, including AFB_1-8,9-epoxide, the ultimate carcinogen, which binds covalently to guanine residues in DNA resulting in DNA damage, mutations, and ultimately carcinoma (Egner *et al.*, 2003). Aflatoxin contamination is a worldwide problem, especially in the warmer parts of the world. The US Food and Drug Administration prohibits interstate commerce of feed grain containing more than 20 g/kg AFB_1 and prohibits the sale of milk and eggs containing more than 0.5 g/kg aflatoxin M_1. Researchers from around the world have attempted to measure the impact of this contamination on the economics of agriculture, as well as the food and feed safety issues (reviewed in a special issue of *World Mycotoxin Journal* by Brown *et al.*, 2015). So far, no completely effective control strategies are available to prevent aflatoxin accumulation in

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the field when conditions are favourable for the fungus. A complete understanding of the ecology of the fungus, host plant-*A. flavus* interaction, and aflatoxin contamination processes will help in the development of new control measures aimed at interrupting the mechanisms responsible for preharvest aflatoxin contamination with the goal of producing a safer, economically viable food and feed supply. Numerous investigations, as reviewed in this article, have attempted to gain a better understanding of the relationship between the host plant and the invading fungus, *A. flavus*, because this relationship affects the aflatoxin contamination process. It is now hypothesised that several host plant and fungal genes are probably involved in determining the degree of aflatoxin contamination of the crop, as outlined in this review. More recently, use of biotechnology has enabled researchers to decipher and find solutions much more rapidly to the aflatoxin contamination problem. Even the best laid crop production efforts cannot avoid all pre-harvest aflatoxin contamination, and proper post-harvest management techniques are required to avoid fungal growth and toxin production. Sorting, drying, shelling, aeration in storage, as well as control of temperature, moisture, and pests all contribute to lower aflatoxin levels and improved food quality at the household, village, and industrial levels. Such a concerted effort to minimise or control aflatoxin contamination will economically benefit the agricultural industry in developed countries, where strict regulatory guidelines prevent the sale of contaminated commodities, but also will increase food safety for populations in developing countries where regulations, if they exist, may not be enforced. Recent research advances to understand biodiversity and ecology of the fungus, host plant-pathogen interaction, genomic and environmental aspects of production of various secondary metabolites, including aflatoxins, and finally the importance of continued postharvest efforts to minimise the aflatoxin contamination to keep the food and feed supply safe for consumption are summarised in this review.

2. Biodiversity of *Aspergillus flavus*

A. flavus is a soil fungus found in temperate parts of the world. *A. flavus* produces aflatoxins B_1 and B_2 and cyclopiazonic acid (CPA), but is incapable of producing aflatoxins G_1 and G_2, which are produced by another related *Aspergillus* species, *A. parasiticus.* Only half of the *A. flavus* isolates found in the field are toxigenic. The populations of this fungus are very diverse and are comprised of many vegetative compatibility groups (VCGs), which prevent hyphal fusion across VCGs, but allow genetic exchange within the same VCG. New VCGs are created by chance mutations or through the genetic recombination. As a consequence, isolates of *A. flavus* from different VCGs differ from each other in many ways including enzyme production, virulence characteristics, and aflatoxin-producing ability. *A. flavus* was thought to exclusively propagate vegetatively through asexual spores (conidia). However, recent studies (Horn *et al.*, 2009) have demonstrated that another type of spore, the ascospore, is produced by the sexual telemorph (*Petromyces flavus*) of *A. flavus*. Long history of genetic recombination in this fungus is being attributed to sexual recombination in laboratory and limited field studies (Moore *et al.*, 2013). The diversity of this fungus is also described in terms of the sclerotia size these strains produce; the two morphotypes being S (small sclerotia) and L (large sclerotia). Apart from sclerotia size, the S-group isolates produce more sclerotia and less conidia than the L-group; the two types of isolates respond differently to pH and grow differently in light and dark conditions. An unnamed taxon isolated from West Africa called S_{BG}, which is phylogenetically divergent from, but morphologically similar to the S-type *A. flavus,* that produces small sclerotia and large amounts of both B- and G-aflatoxins has been identified (Atehnkeng *et al.*, 2008). However, further genomic analysis of S_{BG} strains should clarify their exact taxonomic or possible hybrid status.

3. Ecology of *Aspergillus flavus*

The ecology of *A. flavus* is directly related to the life cycle of the fungus and the influence that the environment has on the saprophytic and pathogenic phases. The most important factors for the former phase are the soil water potential and temperature, the relative organic matter content and the presence of crop residue. These all govern the inoculum potential of this soil-borne fungus. *A. flavus* survives as sclerotia and mycelium on crop debris, and under conducive conditions this facilitates mycelial growth and sporulation from either sclerotia or asexual conidial formation. The conidia from these sources are spread by insects and wind to ripening crops, e.g. maize, where pathogenic infection phase is initiated on ripening maize cobs during silking. These phases are all influenced by the prevailing water availability (water potential in soil or water activity [a_w] in crop debris and ripening crops) and temperature. pH optimum for colonisation of crop debris is in the range of 5 to 6.5 (Griffin *et al.*, 2001). In addition, soil nutritional content will interact with environmental conditions. Thus, a significant positive correlation between organic matter content of soil and the isolation of *A. flavus* populations was demonstrated (Zablotowicz *et al.*, 2007). In the life cycle of *A. flavus*, the optimum and range of interacting environmental conditions necessary for sporulation, germination, and fungal growth are thus important. This needs to be compared with those which allow aflatoxin production.

Germination of conidia of *A. flavus* in terms of germination rate and percentage viable germinating spores is significantly influenced by a_w × temperature conditions. Marín *et al.* (1998) showed that *A. flavus* conidia can germinate rapidly and linearly at between 0.995-0.90 a_w at 25-30 °C. At ≤0.85 a_w this is much slower. Overall, germination of conidia was

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maximum at 35-40 °C with practically freely available water, but this was slower at 0.95 and 0.90 a_w (25% germination) and <20% at 0.85 a_w based on a 40 day incubation period on a maize-meal based medium.

In terms of mycelial colonisation, studies by Nesci *et al.* (2004) and Giorni *et al.* (2008a) showed that growth of strains of *A. flavus* was more sensitive to matric water stress where soil conditions were simulated than to solute stress. Thus, growth was optimum at -2.8 to -7.0 MPa water potential (= 0.98 to 0.95 a_w at 25/30 °C) in ionic and non-ionic solute potential, while this was optimum at -1.4 to -2.9 MPa (= 0.99-0.98 a_w) matric potential. *A. flavus* was significantly more sensitive to matric stress with no growth at -14.0 MPa (= 0.90 a_w) while in solute stress this was at -21.0 MPa (= 0.85 a_w; Giorni *et al.*, 2008b). The sporulation of *A. flavus* from sclerotia under different a_w conditions *in vitro* and on maize stalks has been quantified (Giorni *et al.*, 2012). Comparison of artificial (Czapek Dox Agar) and natural (maize stalks) substrates under different temperatures (5-45 °C) and a_w levels (0.50-0.99) showed that on maize stalks the number of spores/sclerotium was log 4.62 with production initiated after 24 h. The optimal temperature was 20-25 °C on maize stalks (log 4.79 spores/sclerotium). Water stress imposition only reduced sporulation at ≤0.90 a_w. With more available water, no significant differences were found in the range 0.90 to 0.99 a_w (Figure 1). Asexual conidial production on maize-based medium suggested that optimum a_w × temperature conditions were 30-35 °C at 0.95 a_w (log 7.05 to 7.16 spores/cm^2 colony) where glycerol was used to amend the a_w levels. At 20 °C there was no sporulation at 0.90 a_w although there was >log 5.75 spores/cm^2 colony at >0.95 a_w (Abdel-Hadi, 2011).

The effect of interacting conditions of a_w × temperature is critical in determining the effects on colonisation and AFB_1 contamination of food matrices such as ripening maize, peanut, tree nuts or spices (Sanchis and Magan, 2004). Recent studies have examined the effect of a_w × temperature conditions on growth and AFB_1 production by a type strain of *A. flavus* (NRRL 3357 strain; Abdel-Hadi *et al.*, 2012) (Figure 2). The boundary conditions of germination, growth and toxin differed with the widest/broadest a_w × temperature ranges; being wider for germination, than growth and toxin production. Such data were used to develop contour maps of the profiles which indicate the optimum and marginal interacting conditions for growth and for AFB_1 production. This clearly shows that the optimum conditions for growth and for AFB_1 production may be slightly different. At marginal conditions of <20 °C and <0.90 a_w minimal growth and toxin are produced. Of course the interface with the food plant species is critical. For example, in maize there is a period during silking when the a_w of the ripening maize cobs are ideal for colonisation by *A. flavus*. This is particularly relevant during the dough or R4 stage (0.976 a_w), and the dent or R5 stage (0.958 a_w) of maize kernels. Thus any damage to maturing maize cobs during these periods via insects, carrying *A. flavus* spores would lead to rapid colonisation and AFB_1 contamination. Similarly, drought stress in peanut ripening represents conditions where ecologically *A. flavus* is at a competitive advantage, which facilitates colonisation and contamination with the toxin. These types of data sets on the ecology of the life cycle of *A. flavus* can be used to develop models to predict the relative potential risk from toxin contamination based on prevailing weather conditions (Battilani *et al.*, 2013). *A. flavus* is very resilient ecologically and this suggests that under future changes in climate conditions it will perhaps cause more problems in staple food production systems in areas where it was not considered important previously (Medina *et al.*, 2017a).

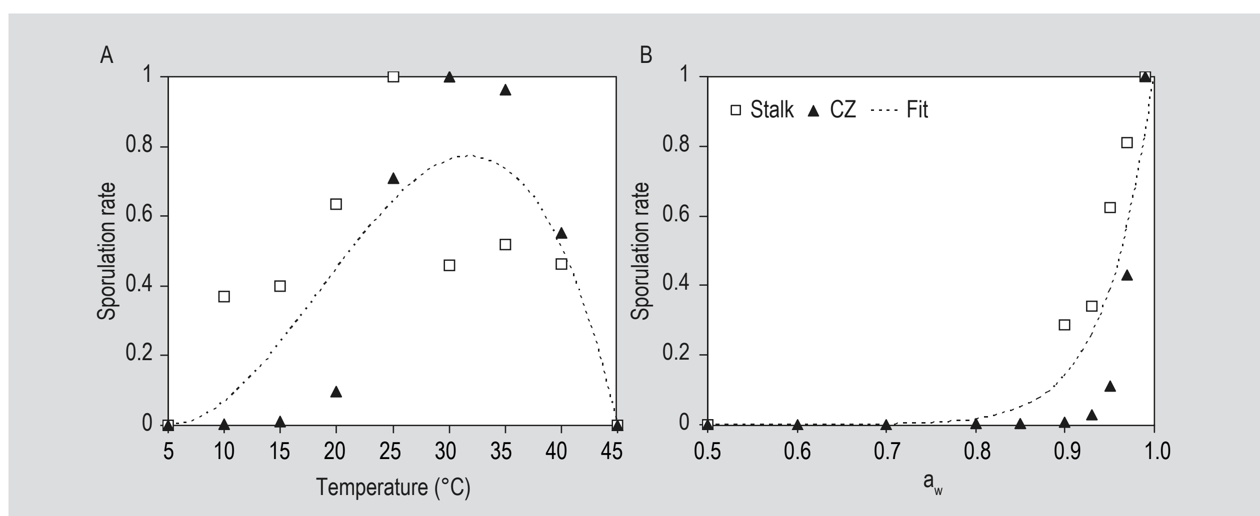

Figure 1. Sporulation rate of *A. flavus* (MPVP 2062) grown on Czapek agar (CZ) (▲) and maize stalks (□) at (A) different temperatures and (B) at different a_w levels. The dotted line is the best fit line (from Giorni *et al.*, 2012).

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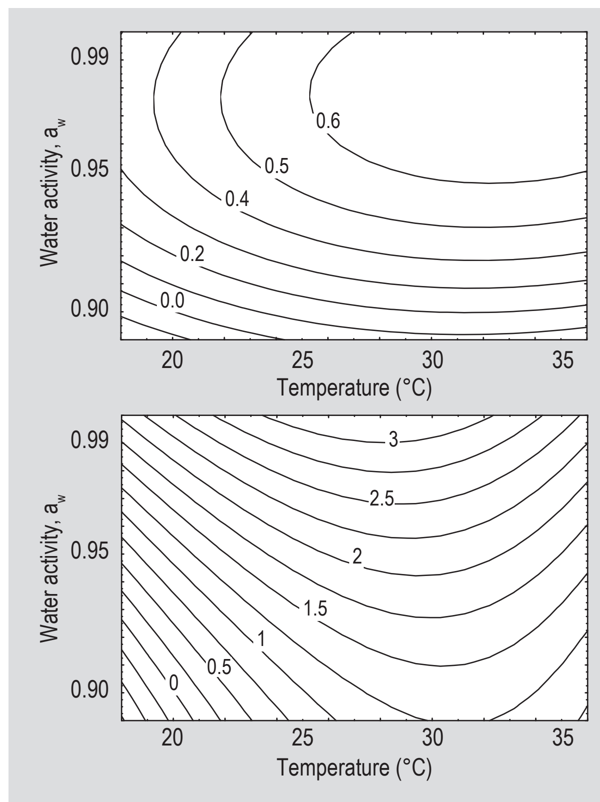

Figure 2. (A) Contour plot for temperature versus water activity on the growth rate (in mm/d). Numbers on the isopleth lines join conditions of the same growth rate. (B) Contour plot for effect of temperature versus water activity conditions on aflatoxin B_1 (AFB_1) production (in µg/g). The isopleth lines join conditions at which similar quantities of AFB_1 were produced (from Abdel-Hadi *et al.* 2012).

4. Genomics of *Aspergillus flavus* and aflatoxin production

Genome sequences and comparative genomics

Comparative genomics, comparing multiple genomes, combined with phenotypic or transcriptomic data has been used in *A. flavus* isolates to elucidate gene function, define evolutionary relationships, secondary metabolite gene function, biotechnology applications, and factors related to pathogenicity (Ehrlich and Mack, 2014; Horn *et al.,* 1996; Moore *et al.,* 2016; Payne *et al.,* 2006). Strain NRRL 3357 (ATCC 200026, GenBank assembly accession: GCA_000006275.2) was the first fully sequenced *A. flavus* genome (Nierman *et al.,* 2015; Payne *et al.,* 2006) and has been developed as a model organism from studies examining secondary metabolite production (Ehrlich and Mack, 2014; Kim *et al.,* 2008; Rank *et al.,* 2012), proteomics (Pechanova *et al.,* 2013), pathogenesis (Chitarrini *et al.,* 2014; Mylroie *et al.,* 2016), and biological control (Donner *et al.,* 2010). The second major strain sequenced, AF70, was originally isolated from soil obtained from a cotton field in Yuma Valley, AZ, USA; and due to high AFB_1 levels produced during infection of maize kernels (Cotty 1989) has been used in characterising secondary metabolite production (Ehrlich and Mack, 2014), fungal development (Cary *et al.,* 2015a; Gilbert *et al.,* 2016), pathogen-host interaction (Hruska *et al.,* 2014), genome profiling (Moore *et al.,* 2015), and oxidative stress (Baidya *et al.,* 2014). Recently 14 additional isolates have been sequenced and made publicly available on the National Center for Biotechnology Information website (Faustinelli *et al.,* 2016; Weaver *et al.,* 2017). Nine of these isolates were obtained from peanut seeds in Georgia, USA, three of which do not produce aflatoxin. Eight of these isolates were sequenced as part of a larger project that demonstrated how fingerprinting by genomic insertion/deletion events (indels) across the aflatoxin cluster was used to cluster isolates, which corresponded with aflatoxin production and potentially the amount of aflatoxin produced (Faustinelli *et al.,* 2017).

Comparative genomics of *A. flavus* has been reviewed and reported previously (Cleveland *et al.,* 2009; Georgianna and Payne, 2009; Payne *et al.,* 2006; Rokas *et al.,* 2007; Yu *et al.,* 2007). These early studies revealed the high genomic identity between *Aspergillus oryzae* and *A. flavus*, and that *A. flavus* is enriched in secondary metabolic biosynthesis genes relative to other sequenced Aspergilli. More recent genomic comparison of these strains indicated that some *A. flavus* strains are even more phenotypically and genetically similar to *A. oryzae* than other *A. flavus* strains, considering factors such as amylase production, secondary metabolic profiles, and some secondary metabolic gene cluster sequences (Gibbons *et al.,* 2012). A highly comprehensive comparative genome analysis of 34 ascomycete strains covering 19 Aspergilli strains (De Vries *et al.,* 2017) was recently conducted, which also incorporated extensive phenotypic and expression analysis. These analyses showed that *A. flavus* has distinctly low levels of cellulose-degrading enzymes, and a relatively high number of xylan-degrading enzymes produced, although the presence of regulatory sequences between species is nearly constant. In addition, *A. flavus* exhibits differences in the molecular regulation of conidiation than some other Aspergilli, and the authors report an absence or negligible effect on *A. flavus* spore production under illumination; however, this particular observation is not consistent with previous reports (Calvo *et al.,* 1999).

Transcriptomics

Early functional genomics technologies characterising *A. flavus* included microarray analysis of natural antisense transcripts (Smith *et al.,* 2008), transcriptional regulation (Cary *et al.,* 2007), and aflatoxin production (Guo *et al.,* 2003; O'Brian *et al.,* 2003; Wilkinson *et al.,* 2007; Yu; *et al.,* 2006, 2007). Later, studies looking at additional secondary metabolic clusters were conducted

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that illustrated the transcriptomic response to a range of media and environmental conditions (Georgianna *et al.*, 2010; Reverberi *et al.*, 2013) and the influence of the global regulatory factor *veA* (Cary *et al.*, 2014, 2015a). These studies aided in the identification or further characterisation of gene clusters for piperazines, aflatrem, leporins, aflavarin, asparasone, and aflatoxin, and several of these gene cluster products have yet to be determined (discussed in detail later).

Given the comprehensive and increasingly affordable nature of RNA-sequencing (RNA-seq) technologies, it is not surprising that RNA-seq has replaced many previous genome-wide transcriptomic profiling strategies (Goodwin *et al.*, 2016; 't Hoen *et al.*, 2008; Zhao *et al.*, 2014). A relatively recent report (Ehrlich and Mack, 2014) was among early attempts to utilise RNA-seq to characterise *A. flavus*, where several putative gene clusters were identified through transcriptomic comparison. The use of knockout technology combined with RNA-seq was conducted on the large sclerotia producer *A. flavus* strain AF70 to examine the effects of knocking out the global regulatory zinc-finger transcription factor *nsdC*, which resulted in decreased aflatoxin and aflatrem production, and altered expression of the genes responsible for penicillin, asparasone, and ustiloxin B biosynthesis (Gilbert *et al.*, 2016). Clusters 5, 20, 41, 44 (Georgianna *et al.*, 2010) and others were also affected in the *nsdC* knockout strain. Recently, treatment with chemicals in conjunction with RNA-seq technology has successfully characterised various gene clusters and developmental processes in *A. flavus*, including the aflatoxin cluster. Treatment of *A. flavus* in culture by the naturally occurring phytoalexin and antifungal compound resveratrol resulted in decreased aflatoxin production, and of the 30 genes the authors identify as aflatoxin cluster genes, *aflA* and *aflB* were the only significantly downregulated genes. The regulatory gene *aflR* was not significantly affected, suggesting individual cluster genes could be subjected to independent regulation (Wang *et al.*, 2015). Treatment of *A. flavus* with decanal, another plant volatile, caused 26 of the 55 gene clusters to be affected, a decrease in growth, conidia and sclerotia production, and increase in expression of most aflatoxin cluster genes (Chang *et al.*, 2014). It has been suggested the DNA methyltransferase inhibitor 5-azacytidine interrupts toxisome formation (Wilkinson *et al.*, 2011), and treatment of *A. flavus* with such an inhibitor resulted in down regulation of genes in the aflatoxin cluster (Lin *et al.*, 2013). However, it has been shown that *A. flavus* does not undergo DNA methylation (Liu *et al.*, 2012), indicating the potential role of a feedback regulatory mechanism in aflatoxin production.

Finally, a 2016 RNA-seq study characterising oxidative stress in *A. flavus* was conducted whereby different strains were subjected to H_2O_2 exposure. A subset of genes within the aflatoxin cluster (*aflG, I, K, M, Ma, O, P, Q, V, W, X*, and *Y;* Figure 3) responded in a dosage dependent manner in two *A. flavus* strains, AF13 and Strain 3357. In addition, some genes in the aflatrem and kojic acid (KA) clusters were affected (Fountain *et al.*, 2016). This again suggests cluster genes can be subject to individual regulation, as *aflR* remained highly expressed in all samples.

Bioinformatics and aflatoxin production

Analysing RNA-seq data to characterise the regulation of metabolite production, their interaction with the host transcriptome, and broader impacts on fungal gene expression, an array of bioinformatics tools have been applied in functional genomics analysis of *A. flavus* (Cary *et al.*, 2014; Gilbert *et al.*, 2016; Lin *et al.*, 2013; Wang *et al.*, 2015), the most common of which include KEGG (Kyoto Encyclopedia of Genes and Genomes) (Aoki-Kinoshita and Kanehisa, 2007), gene co-expression network analysis (Langfelder and Horvath, 2008), and gene ontology (GO) enrichment analysis (Young *et al.*, 2010). Examples of these strategies applied to *A. flavus* and aflatoxin production include Fountain *et al.* (2016) whereby *A. flavus* was treated with H_2O_2 to induce oxidative stress and a weighted co-expression analysis identified a network of oxidative stress, fungal growth, and secondary metabolic genes, including aflatoxin production, describing a series of coordinated processes facilitating pathogenesis. In another study, Musungu *et al.* (2016) also applied co-expression analysis, this time to maize kernels infected with *A. flavus*, which were subjected to a dual-genome RNA-seq. They found *aflS* was co-regulated with multiple ROS-related maize genes, suggesting its functioning as a possible 'monitor' for host ROS levels. Finally, the emergence of secondary metabolite and genomic databases, such as ClusterMine360, IMG-

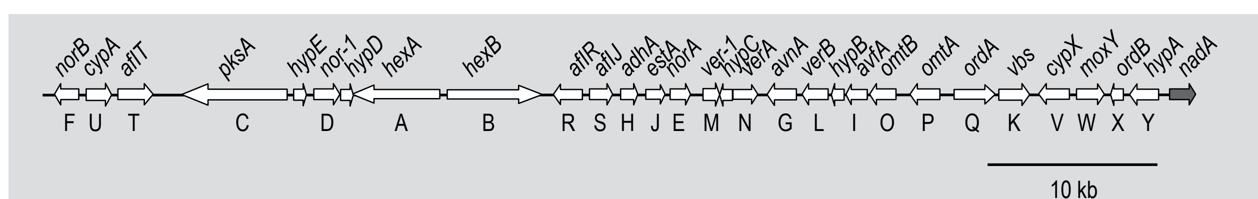

Figure 3. Schematic of the aflatoxin gene cluster. Genomic nomenclature at top of aflatoxin cluster represents functionality of genes, while the single letter *afl* nomenclature is also presented (below the horizontal line). The *nadA* gene (grey arrow) is part of the adjacent sugar cluster. Arrows denote direction of transcription.

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ABC, MIBiG, and FungiDB, combined with computational strategies like those described here using comparative and computational genomics, transcriptomics and secondary metabolic gene cluster prediction is resulting in more complex data sets providing meaningful insights into fungal and crop biology (Andersen *et al.,* 2013; Nagano *et al.,* 2016; Vesth *et al.,* 2016; Weber and Kim 2016). Tools, such as these applied specifically to *A. flavus*, will continue to aid in understanding aflatoxin regulation, with a goal towards identifying novel targets and strategies for mitigation.

5. *Aspergillus flavus* secondary metabolite gene clusters

This section briefly summarises the genetics and biochemistry of secondary metabolite (SM) gene clusters in *A. flavus* with particular emphasis on the aflatoxin biosynthetic gene cluster and those regulatory factors known to be involved in aflatoxin production. In addition, information will be provided on other SM gene clusters and their associated metabolites that have been experimentally identified and characterised in *A. flavus* and their potential biological functions.

Secondary metabolites play many important roles in fungal ecology, such as virulence factors, chemical defence agents, developmental regulators and as compounds that are toxic to humans and livestock. Identification of the form and function of the products of these SM gene clusters will undoubtedly provide invaluable insights into the pathobiology of *A. flavus* as well as other fungal plant pathogens harbouring similar gene clusters. Web-based tools such as the SMURF (Secondary Metabolite Unique Regions Finder) (Georgianna *et al.,* 2010) and antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) (Medema *et al.,* 2011) algorithms have shown that *A. flavus* has the potential to produce numerous other SMs, many whose biological activities are unknown. SMURF analysis predicted that the *A. flavus* genome harbours up to 55 SM gene clusters [56 when including the KA gene cluster identified originally in *A. oryzae* by Marui *et al.* (2011)]. Identification of these clusters is based on the presence of cluster 'backbone' genes, such as non-ribosomal peptide synthases (NRPSs), polyketide synthases (PKSs), PKS-NRPS hybrids, geranylgeranyl pyrophosphate synthases (GGPSs) and dimethylallyltryptophan synthases (DMATs), as well as flanking genes encoding decorating enzymes, transcription factors, and transporters. Although about 40 SMs have been identified from *A. flavus* cultures (Cole *et al.,* 2003; Turner and Aldridge, 1983), metabolites have only been experimentally assigned to 12 *A. flavus* gene clusters. Brief descriptions of the physical, genetic, and biological properties of these twelve SM gene clusters and their associated metabolites are provided below and in Table 1.

Aflatoxin cluster

The aflatoxin (AF) gene cluster is probably the most extensively studied fungal SM gene cluster and has been the subject of numerous comprehensive reviews (Bhatnagar *et al.,* 2003; Kumar *et al.,* 2016; Roze *et al.,* 2013; Yu, 2012). The AF gene cluster (~70 kb) is located near the telomere on the right arm of chromosome 3 and is composed of 25 genes (Yu *et al.*, 2004; Figure 3). Significant contributions to the elucidation of aflatoxin biosynthesis and its regulation can be attributed to studies in *Aspergillus nidulans*, a model fungus that produces the aflatoxin precursor sterigmatocystin (STE) (Brown *et al.,* 1996; Calvo *et al.,* 2002). Many of the ground-breaking enzymatic and genetic studies on aflatoxin biosynthesis were also performed in the closely related aflatoxin producer, *Aspergillus parasiticus*. Prior to elucidation of the physical and functional genetics of the AF biosynthetic gene cluster, many of the enzymes involved in aflatoxin biosynthesis were identified and characterised (reviewed in Yu, 2012). The genetic makeup of the AF gene cluster was fully characterised before sequencing and annotation of the *A. flavus* genome in large part through genetic complementation of AF biosynthetic mutants using *A. flavus* and *A. parasiticus* cosmid libraries (Yu *et al.,* 1995).

Regulation of aflatoxin biosynthesis is controlled by pathway-specific and globally-acting regulatory factors as well as epigenetic factors (reviewed in Amare and Keller, 2014; Brakhage, 2013; Macheleidt *et al.*, 2016). Annotation of the *A. flavus* genome indicates that about 50% of SM gene clusters do not encode a cluster-specific transcriptional activator suggesting that regulation of these clusters occurs at the global level (Ehrlich and Mack, 2014). A genetic linkage governing both development and secondary metabolism was demonstrated in *A. nidulans* (Adams and Yu, 1998; Hicks *et al.,* 1997) and this is also true for regulation of aflatoxin biosynthesis in *A. flavus*. Discussion of only a select few regulators of aflatoxin biosynthesis will be presented here with emphasis on those not presented in the latest review (Macheleidt *et al.*, 2016).

Pathway-specific regulatory factors

A Gal4-type binuclear cluster Zn(II)2-Cys6 transcriptional activator encoded by *aflR* regulates expression of most AF/STE cluster genes (Flaherty *et al.,* 1995; Woloshuk *et al.,* 1994; Yu *et al.,* 1996). *A. flavus aflR* deletion mutants do not make aflatoxins and demonstrate loss of AF cluster gene expression while *aflR* overexpressing strains demonstrated increased levels of AF gene cluster transcripts and aflatoxin production. AflR binds to the palindromic sequence 5'-TCGN5CGA-3' present in the promoter region of most AF biosynthetic genes (Ehrlich *et al.,* 1999; Fernandes *et al.,* 1998). Interestingly, AflR was also shown to control expression of the *nadA*, *hlyC*, and

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Table 1. Features of experimentally characterised *Aspergillus flavus* secondary metabolite gene clusters.

Cluster metabolite[1]	Cluster size (kb)/ chromosome	Backbone gene classification[2]	Cluster-specific transcriptional regulator	Number of genes in cluster[3]	Putative biological function	References
Aflatoxin	66.7/3	PKS	yes	25	antiinsectan/oxidative stress resistance?	Kumar *et al.* (2017); Roze *et al.* (2013)
Aflatrem	9.7/7 and 6.9/5	DMATs/GGPPs	no	5 and 3	antiinsectan/antifeedant	Nicholson *et al.* (2009); Tang *et al.* (2015)
Aflavarin	15/3	PKS	no	5	antiinsectan/sclerotial development	Cary *et al.* (2015a)
Asparasone A	15.8/1	PKS	yes	4	antiinsectan/sclerotial pigment/abiotic stress resistance	Cary *et al.* (2014)
Cyclopiazonic acid	22.9/3	PKS-NRPS	no[4]	5	mammalian antifeedant?	Chang *et al.* (2009a); Kato *et al.* (2011)
Ditryptophenaline	14.3/4	NRPS	no	3	?	Saruwatari *et al.* (2014)
Kojic acid	5.8/5	oxidoreductase	yes	3	insect antifeedant/ antioxidant?	Terabayashi *et al.* (2010)
Leporin	31.6/6	PKS-NRPS	yes	9	insect antifeedant/sclerotial development	Cary *et al.* (2015b)
Piperazine	15.1/6 and 14.0/8	NRPS-like	no	6 and 6	sclerotial development	Forseth *et al.* (2013)
Ustiloxin B	27/5	RiPS	yes	16	?	Umemura *et al.* (2014); Nagano *et al.* (2016)

[1] Some clusters produce more than one metabolite.
[2] NRPS = non-ribosomal peptide synthases; PKS = polyketide synthases; GGPS = geranylgeranyl pyrophosphate synthases; DMAT = dimethylallyltryptophan synthases; RiPS = ribosomal peptide synthetic pathway.
[3] As defined experimentally or as defined in Georgianna *et al.* (2010).
[4] The cyclopiazonic acid (CPA) cluster does contain a gene encoding a putative C6 transcription factor, *cpaR*, but the role of this gene in regulation of CPA cluster gene expression has not been experimentally determined.

niiA genes located outside of the AF gene cluster (Price *et al.,* 2006). Aflatoxin biosynthesis is also regulated by *AflS* (*aflJ*) located adjacent to and divergently transcribed from *aflR* (Meyers *et al.,* 1998). AflS does not present similarity to other proteins of known function and the exact role of *AflS* in aflatoxin biosynthesis is not clear despite intense investigation. Deletion of *aflS* in *A. flavus* did not appear to affect expression of AF cluster genes, however, *aflS* deletion mutants did not produce aflatoxins (Meyers *et al.,* 1998). An *A. flavus* strain overexpressing *aflS* did not elevate transcription of late AF pathway genes *aflM* (*ver-1*) and *aflP* (*omtA*) nor did it increase expression of *aflR* (Du *et al.,* 2007). However, an increase in expression of early and mid-AF pathway genes was observed when overexpressing *aflR* and *aflS*, even more so than when overexpressing *aflR* alone. This suggested that *AflS* modulates the expression of AF early and mid-pathway genes in conjunction with *aflR* (Chang *et al.,* 2002). This is in agreement with earlier yeast two-hybrid assays that showed AflS interacts with AflR suggesting that an intimate association of AflS and AflR is required to fully activate expression of some AF structural genes (Chang, 2003).

Globally-acting regulatory factors

A heterotrimeric, nuclear-localised regulatory complex consisting of the proteins VeA, LaeA and VelB has been shown to coordinate fungal development and secondary metabolism in a light-dependent manner (Bayram *et al.,* 2008). VeA, was originally characterised as a positive regulator of sexual development and STE production in *A. nidulans* (Kim *et al.,* 2002). Studies in *A. flavus* demonstrated that VeA is required for production of both sclerotia and aflatoxin as well as numerous other secondary metabolites including CPA and aflatrem (Duran *et al.,* 2007). LaeA, is a putative chromatin-modifying protein containing a methyltransferase domain (Reyes-Dominguez *et al.,* 2010). Similar to *veA*, deletion of *laeA* results in abrogation of sclerotia and aflatoxin production and transcriptomic analysis has indicated regulation by LaeA of as many as 24 SM gene clusters (Kale *et al.,* 2008). The

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velvet-like protein B (VelB) forms a heterodimer with VeA in the cytosol, migrates into the nucleus in the absence of light, and then interacts with LaeA (Bayram and Braus, 2012). Deletion of *velB* results in reduced conidiation and loss of sclerotia and aflatoxin production (Chang *et al.*, 2013). In *A. flavus*, VeA appears to take a dominate role to velB in control of conidiation. Interestingly, yeast two-hybrid assays showed that both LaeA and VelB interact with FluG, a positive regulator of conidiogenesis, but negative regulator of sclerotial formation in *A. flavus* (Chang *et al.*, 2012). This study showed that FluG does not control secondary metabolism in *A. flavus* though it was shown to be required for STE production in *A. nidulans* (Hicks *et al.*, 1997).

The global transcription factor genes *nsdC* (C_2H_2-type) and *nsdD* (GATA-type) control both sclerotial, conidial and aflatoxin production in *A. flavus* (Cary *et al.*, 2012). Inactivation of *nsdC* or *nsdD* in *A. flavus* resulted in loss of sclerotial production while aflatoxin biosynthesis was severely reduced or totally lost in Δ*nsdD* and Δ*nsdC* mutants, respectively. A subsequent RNA-seq study comparing gene expression in an *A. flavus* Δ*nsdC* mutant compared to a control revealed differential expression of genes associated with SM production including down-regulation of genes involved in aflatrem biosynthesis but upregulation of penicillin biosynthetic cluster genes (Gilbert *et al.*, 2016). A recently identified global regulator, *hbx1*, encoding a homeobox transcription factor was shown to be required for production of aflatoxins, CPA and aflatrem in *A. flavus* (Cary *et al.*, 2017). Hbx1 was also shown to be essential for conidial and sclerotial production making it the first transcription factor identified in *A. flavus* required for production of conidia, sclerotia and aflatoxins.

Lipid metabolism especially that of fatty acid-derived oxylipins, has been shown to play a role in *A. flavus* virulence, development and aflatoxin production (Amaike and Keller, 2009; Burow *et al.*, 1997). Recently, Luo *et al.* (2016) identified *far* transcription factor genes that regulate fatty acid beta-oxidation required for aflatoxin biosynthesis. Aflatoxin production was significantly decreased in a Δ*farB* mutant. A putative RNA-Pol II transcription elongation factor gene, *rtfA*, was shown to regulate development and SM production in *A. flavus* possibly through an epigenetic mechanism (Lohmar *et al.*, 2016). A Δ*rtfA* mutant exhibited a significant decrease in sclerotial and aflatoxin production as well as other uncharacterised SMs. Another epigenetic factor controlling development and aflatoxin production in *A. flavus* is the histone-modifying methyltransferase, RmtA (Satterlee *et al.*, 2016). RmtA was shown to be a positive regulator of sclerotial production while suppressing conidiation and aflatoxin production. Upon forced overexpression, a globally-acting C_2H_2 zinc-finger transcription factor, MtfA, was shown to be required for normal development and aflatoxin production in *A. flavus* (Zhuang *et al.*, 2016).

6. Other *Aspergillus flavus* secondary metabolite gene clusters

Of the 56 predicted SM gene clusters in the *A. flavus* genome, only twelve clusters have been associated with a specific metabolite (Figure 4). A number of metabolites from uncharacterised gene clusters have been identified utilising comparative metabolomics of cluster gene knockout and overexpressing mutants to wild-type strains (Cary *et al.*, 2014; Forseth *et al.*, 2013; Saruwatari *et al.*, 2014). However, elucidation of the products of some SM gene clusters is confounded by the fact that many are transcriptionally silent, inactive due to gene mutations, or expressed at extremely low levels thus making identification of their respective products problematic under laboratory growth conditions. These types of gene clusters are termed cryptic or orphan and identification of their metabolite(s) cannot be achieved using classical deletion or overexpression of a cluster gene followed by comparative metabolite analysis by LC-MS. Analysis of RNA-Seq data from a number of *A. flavus* strains growing on artificial media indicates that approximately half of the SM gene clusters are silent or expressed at very low levels (Ehrlich and Mack, 2014). A number of methods have been used to overcome silent gene clusters thereby enabling identification of some previously unknown cluster metabolites (Brakhage, 2013; Brakhage and Schroeckh, 2011; Strauss and Reyes-Dominguez, 2011). In addition to the AF gene cluster, the metabolites produced by the following clusters have been experimentally identified in *A. flavus*.

Cyclopiazonic acid cluster

CPA is an indole-tetramic acid produced by the activity of a hybrid PKS-NRPS in *A. flavus*. The toxicology and biosynthesis of CPA has been reviewed by Chang *et al.* (2009a). In addition to being an inhibitor of calcium-dependent ATPases present in the sarcoplasmic reticulum of animals (Riley *et al.*, 1992), CPA has also been suggested to function as a pathogenicity factor that can induce cell death in plants (Chalivendra *et al.*, 2017). The CPA cluster is located in the subtelomeric region adjacent to the AF gene cluster and can be absent in some *A. flavus* strains (Chang *et al.*, 2009b). Five genes are thought to comprise the *A. flavus* CPA cluster; a monoamine oxidase, *maoA*; dimethylallyl tryptophan synthase, *dmtA*; hybrid PKS-NRPS, *pks/nrps*; MFS transporter, *mfs1*; and a putative C6-type transcription factor, *ctfR*1. Disruption of any of these genes except the *ctrR1* and *mfs1* resulted in loss of CPA production. A CPA-producing *A. oryzae* strain was shown to have two additional genes, *cpaH* and *cpaM*, located between *maoA* (*cpaO*) and *mfs1* (*cpaT*) (Kato *et al.*, 2011). While no function was attributed to *cpaM*, *cpaH* encodes a

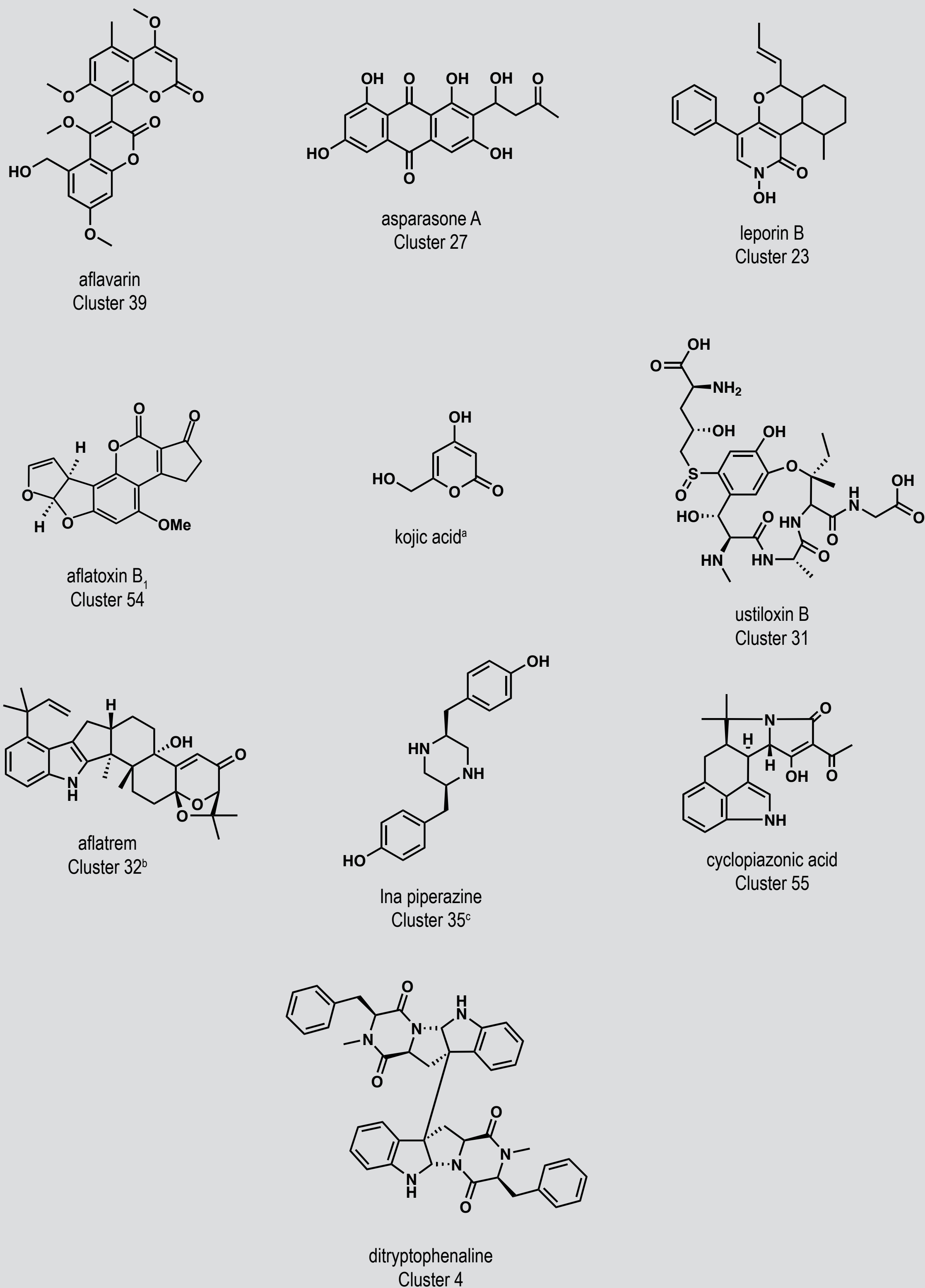

Figure 4. Representative structures of secondary metabolites that have been experimentally linked to a gene cluster in *Aspergillus flavus*. Cluster designations are based on the numbering system of Georgianna *et al.* (2010).

[a] The kojic acid cluster was characterised in *Aspergillus oryzae* and was not identified by SMURF analysis of *A. flavus* so a cluster number was not designated.

[b] Genes required for aflatrem biosynthesis are also present on Cluster 15.

[c] In addition to the Ina gene cluster (#35) a highly homologous Inb piperazine gene cluster (#48) was also present in *A. flavus*.

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cytochrome P450 that mediates the conversion of CPA to 2-oxoCPA, the less toxic end product of the CPA pathway in *A. oryzae*. The absence of homologs of *cpaH* and *cpaM* in *A. flavus* 3357 suggests that they were lost during the evolution of *A. flavus* and may reflect the need for the more toxic form of CPA in the field.

Aflatrem clusters

Aflatrem and its isomer, β-aflatrem, are examples of indole-diterpenes (IDTs), a class of structurally-diverse secondary metabolites (Saikia *et al.,* 2008). Aflatrem was shown to be a potent mammalian tremorgen (Gallagher and Wilson, 1979). Identification of the genes required for aflatrem biosynthesis (*atm*) in *A. flavus* was made possible through comparative genomics with *Penicillium paxilli* genes (*pax*) required for the production of the IDT, paxilline (Saikia *et al.,* 2006; Zhang *et al.,* 2004). Unlike most SM gene clusters, the genes required for the synthesis of aflatrem are located on two different chromosomes (5 and 7) in *A. flavus* (Nicholson *et al.,* 2009). In addition to aflatrem, *A. flavus* also produces a similar class of IDTs termed aflavinines that demonstrate anti-insectan properties. Both aflatrems and aflavinines are derived from a common core structure consisting of a tetracyclic diterpene originating from the activities of a geranylgeranyl diphosphate synthase (AtmG) and geranylgeranyl transferase (AtmC). The diterpene is subsequently converted to either aflatrem or aflavinine by the action of unique IDT cyclases (IDTCs) (Tang *et al.,* 2015). Interestingly, Tang *et al.* (2015) identified a standalone IDTC (not associated with the aflatrem/aflavinine gene cluster) that was responsible for production of aflavinine but not aflatrem. It is unclear how *A. flavus* coordinates the expression of the IDTCs responsible for production of aflatrems or aflavinines (mainly present in sclerotia) but it may depend on the type of biotic pressure present in the particular ecological niche inhabited by the fungus.

Piperazine clusters

Similar to aflatrem, two separate gene clusters were identified termed lna (chromosome 6) and lnb (chromosome 8) that are involved in the biosynthesis of piperazines and associated derivatives in *A. flavus* (Forseth *et al.,* 2013). Both clusters contain genes encoding putative NRPS-like proteins (*lnaA/lnbA*), as well as decorating enzymes, such as an NMR-like protein (*lnaB/lnbB*), cytochrome P450s (*lnaC/lnbC*), alcohol dehydrogenase/NADH:flavin oxidoreductase (*lnaE/lnbE*), and a transporter (*lnaF/lnbF*). The lna cluster also contains a second putative P450 enzyme gene (*lnaD*) while the lnb cluster contains a gene encoding a hypothetical protein not present in the lna cluster.

Comparative metabolomics of *A. flavus* wild-type, deletion, and overexpression (OE) strains allowed identification of two major piperazine metabolites and eight other lna cluster-derived metabolites that had not been previously reported in aspergilli or other fungi (Forseth *et al.,* 2013). Both LnaA and LnbA proteins activate tyrosine, but because they lacked condensation domains, there was no peptide bond formation. It was postulated that LnaA and LnbA C-terminal reductase domains reduce L-Tyr to its aldehyde suggesting that these enzymes more closely resemble amino acid reductases characteristic of Lys2-type reductases involved in fungal L-lysine biosynthesis (Ehmann *et al.,* 1999). The resulting L-Tyr aldehyde subsequently dimerizes and is further reduced to piperazine and morpholine-like compounds. Of particular interest was the finding that LnaB can participate in the reduction of LnaA-derived intermediates indicating that the lna and lnb clusters encode partially redundant biosynthetic pathways capable of sharing biosynthetic intermediates. Down-regulation of both of *lnaA* and *lnbA* expression in *A. flavus* resulted in significant reduction in sclerotial formation compared to the wild type suggesting a role for the secondary metabolite production of these clusters in fungal development (Forseth *et al.,* 2013).

Ditryptophenaline cluster

The ditryptophenaline (DTP) cluster is responsible for the production of the dimeric diketopiperazine alkaloid, (–)-ditryptophenaline, that is derived from two molecules of tryptophan and phenylalanine (Saruwatari *et al.,* 2014). Comparative metabolomics of putative *A. flavus* DTP cluster gene (*dtp*) mutants with a control strain enabled determination of the function of the cluster genes. The cluster was found to be composed of three genes encoding a NRPS (*dtpA*) that condenses L-tryptophan and L-phenylalanine together to form the diketopiperazines core; an N-methyltransferase (*dtpB*) that methylates the core structure; and a cytochrome P450 (*dtpC*) that is likely responsible for cyclization and dimerization of the methylated diketopiperazine to form DTP. Though DTP has been shown to have weak activity as an inhibitor of the neurotransmitter, substance P, in animal models, its function in the biology of *A. flavus* is not known (Barrow and Sedlock, 1994).

Ustiloxin B cluster

Ustiloxins are a diverse class of tetrapeptides with ustiloxin B consisting of the amino acids Tyr-Ala-Ile-Gly (YAIG) (Koiso *et al.,* 1994). Ustiloxin B is derived from enzymes encoded by a cluster of 18 genes needed to cyclise the tetrapeptide at the side chains of Tyr and Ile and catalyse side chain modifications and link a non-proteinogenic amino acid, norvaline, to the Tyr moiety (Umemura *et al.,* 2014). Ustiloxin B was initially thought to be the product of a NRPS-like enzyme, but no NRPS-like genes were present in or near the gene cluster. The presence of a gene (*ustA*)

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in the cluster encoding a precursor protein harbouring 16 short YAIG repeat peptide regions and an N-terminal signal peptide suggested that ustiloxin B is biosynthesised through a ribosomal peptide synthetic pathway (RiPS) not previously described in *A. flavus* or other ascomycetes (Umemura *et al.,* 2014).

Screening of *Aspergillus* genome sequences by Nagano *et al.* (2016) for the presence of the *ustYa*/*ustYb* and *ustA* gene homologs indicated that there are as many as 94 ribosomal peptide precursor candidate genes displaying a variety of core peptide sequences in addition to YAIG. From this computational analysis a novel bicyclic peptide designated asperipin-2a was experimentally identified in *A. flavus*. Ustiloxin B was shown to inhibit polymerisation of purified porcine microtubule proteins as well as mitosis in human cell lines, however the role of ustiloxin B in the biology of *A. flavus* remains to be determined (Koiso *et al.,* 1994).

Kojic acid cluster

Though not originally confirmed in *A. flavus*, the KA gene cluster was experimentally identified in its close relative, *A. oryzae* (Terabayashi *et al.,* 2010). Comparative transcriptomics of *A. oryzae* cultures grown under KA-conducive and non-conducive conditions, allowed identification of two genes responsible for KA biosynthesis. It was determined from analysis of the *A. oryzae* genome sequence that these two genes mapped within 3 kb of one another. One of the genes designated *kojA*, encodes a predicted FAD-dependent oxidoreductase while the other, *kojT* encodes a major facilitator superfamily transporter. KojA is believed to oxidise glucose to KA, although other as of yet unidentified gene products may be required for complete conversion of glucose to KA (Terabayashi *et al.,* 2010). Located between *kojA* and *kojT* is a gene designated *kojR*, encoding a Zn(II)2-Cys6 transcription factor that when deleted caused loss of KA production while its overexpression led to significantly higher levels of KA (Marui *et al.,* 2011).

Kojic acid may function as an insect anti-feedant and it has also been suggested that it functions as an antioxidant that inhibits the ability of insect oxidative enzymes to detoxify AFB_1 (Dowd, 1988). While KA was shown to be relatively non-toxic in mouse assays, it appeared to act synergistically with AFB_1 in insect feeding studies causing increased mortality compared to feeding of either metabolite alone (Dowd, 1988).

Asparasone cluster

Asparasone A, a PKS-derived anthraquinone, is responsible for the formation of the dark pigment associated with sclerotia (Cary *et al.,* 2014). Asparasone A was first described in *A. parasiticus* and demonstrated to possess anti-insectan properties (Sobolev *et al.,* 1997). The asparasone A gene cluster consists of four genes, a polyketide synthase (*pks27*), a Zn(II)2-Cys6-type transcription factor (*znf27*) and putative high-affinity glucose (*mfs1*) and MFS transporters (*mfs2*). Deletion mutants of *A. flavus pks27* produced greyish-yellow sclerotia rather than the wild-type dark brown pigment. Gene expression analysis showed a significant reduction in expression of *pks27, mfs1 and mfs2* in the *Δznf27* mutant compared to the control indicating that *znf27* functions as a pathway-specific transcription factor. Expression of *pks27* was shown to be dependent on VeA.

Cary *et al.* (2014) revealed that the dark brown pigments present in *A. flavus* sclerotia are the product of Pks27-derived anthraquinones rather than typical tetrahydronapthalene-based melanins. The asparasone A-based anthraquinone pigment in sclerotia was shown to provide resistance to both biotic and abiotic stressors.

Leporin cluster

Leporins were originally identified in *Aspergillus leporis* as 2-pyridones that exhibited anti-insectan properties (TePaske *et al.,* 1991). Cary *et al.* (2015b) described a silent gene cluster in *A. flavus* that consisted of a hybrid PKS-NRPS (*lepA*) and six additional genes responsible for production of leporin B and the novel metabolite, leporin C. Overexpression of a putative cluster-specific transcriptional regulatory gene, *lepE*, resulted in transformants producing a distinctive orange-red pigment identified as a highly stable leporin B trimer complexed with an atom of iron (trioxyleporin B). Such a trimeric iron chelate had not been previously described for a 2-pyridone nor had there been any reports of siderophore-like molecules produced by a hybrid PKS-NRPS in fungi.

While leporin A was shown to have anti-insectan properties there were no previous reports on the insecticidal properties of leporins B or C. Insect feeding studies performed with a preparation of the leporin B-iron chelate (LIC) compound showed a significant reduction in growth of first instar larvae of Fall armyworms (*Spodoptera frugiperda*) and Corn earworms (*Helicoverpa zea*) indicating that the LIC was acting as an antifeedant, but not an anti-insectan agent (Cary *et al.,* 2015b). A 10-fold reduction in conidia production was also observed in the *lepE* overexpressing strain (OE::*lepE*) and sclerotial production was either reduced or delayed compared to the control.

Aflavarin cluster

The anti-insectan secondary metabolite, aflavarin, was first isolated and characterised from sclerotia of *A. flavus* (TePaske *et al.,* 1992). Comparative transcriptomics of an *A. flavus* Δ*veA* mutant and isogenic control strain led to the identification of the aflavarin gene cluster (Cary *et al.,*

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2015a). The aflavarin gene cluster is composed of genes encoding a PKS (*afvB*), O-methyl transferase (*afvC*), methyl transferase (*afvD*), cytochrome P450 (*afvE*) and a NADH oxidase (*afvA*). Comparative analysis of the metabolomes of an *A. flavus* control and Δ*afvB* (PKS) mutant identified a series of bicoumarins that were present in the control and absent in the mutant (Cary *et al.,* 2015a). The major metabolite identified had a calculated mass of 455, which was identical to the mass of aflavarin.

In addition to its anti-insectan properties, aflavarin was required for normal levels of sclerotial production in *A. flavus* (Cary *et al.,* 2015a). While no cluster-specific transcriptional activator gene was present in the aflavarin cluster, a number of putative binding sites for stress-responsive DNA binding proteins as well the aflatoxin cluster-specific transcriptional activator, AflR were identified. This suggested that there may be regulatory cross-talk between these two clusters (Cary *et al.,* 2015a).

7. Environmental impacts on *Aspergillus flavus* and aflatoxin production

Environmental conditions in agriculturally relevant areas will likely undergo dramatic changes that are expected to affect precipitation, temperature, and the composition of atmospheric gasses within the next 100 years. Data from the authoritative UN Panel on Climate Change indicate that global temperatures will increase approximately 0.2-0.4 °C per decade at increasing rates (IPCC, 2014) depending on mitigation efforts. Forecasts further indicate increases in carbon dioxide (CO_2), methane, nitrous oxide, aerosol, and other gases, with CO_2 reaching as high as 1000 ppm, nearly tripling current levels. Periods of heavier rainfall and periods of longer drought are also forecast (IPCC, 2013, 2014). Not surprisingly, many of these factors are known to impact fungal growth, secondary metabolite production, and host-pathogen interaction (Giorni *et al.,* 2008a,b; Marín *et al.,* 1998; Medina *et al.,* 2015; Sultan, 2010; Wu *et al.,* 2011). Gaining insight into how the combination of climate change-related weather variables will influence *A. flavus* pathogenicity is vital for the continued development of strategies aimed at preserving global food security.

Both laboratory and farming experiences have provided examples of drought conditions and increased temperature causing increased aflatoxin production (Diener *et al.,* 1987; Giorni *et al.,* 2007; Kos *et al.,* 2013; Payne *et al.,* 1988; Piva *et al.,* 2006). A particularly illustrative example occurred in 2012 in Serbia when daily temperatures (30-40 °C) and the number of hot days increased significantly from previous years and precipitation decreased to drought levels. That year, 68% of maize samples tested were positive for aflatoxin contamination whereas no contamination was reported during the previous years (Kos *et al.,* 2013). A similar incident in 2004-2005 was also documented in northern Italy (Giorni *et al.,* 2007). While these examples provide adequate evidence for concern, it is essential for researchers to focus on exacerbating circumstances and identify means of mitigation.

The influence of temperature and water on fungal growth and pathogenicity has been studied for decades (Anderson and Smith, 1972; Holmquist *et al.,* 1983; Northolt *et al.,* 1977; Sabburg *et al.,* 2015; Schmidt-Heydt *et al.,* 2010). Data from our laboratory and that of others (Marín *et al.,* 1998; O'Brian *et al.,* 2007; Yu *et al.,* 2011) have demonstrated optimal conditions for the expression of aflatoxin cluster genes and aflatoxin production between 28 °C and 30 °C. Aflatoxin biosynthesis increases as temperatures increase between 20 °C and 30 °C, where it can remain in production until temperatures reach 37 °C (Abdel-Hadi *et al.,* 2012; Schmidt-Heydt *et al.,* 2010). This is significant in the context of crop contamination and especially that of maize, which typically grows when air temperatures are between 10 °C and 30 °C (Cross and Zuber, 1972; Greaves, 1996). Greenhouse and field studies have characterised this interaction in the context of pathogenesis. In one study maize grown at 32 to 38 °C had 73% of kernels infected, whereas at 21-26 °C, only 2.5% of kernels were infected (Jones *et al.,* 1980). Drought stress has also been shown to induce higher levels of aflatoxin contamination (Davis *et al.,* 1986), and the irrigation of maize fields during periods of low precipitation has been shown to reduce fungal infection and aflatoxin contamination (Jones *et al.,* 1981).

Recently, functional genomics tools, i.e. microarray technologies, quantitative proteomics, and RNA-sequencing, have examined the effect of abiotic factors on fungal development and plant-fungus interactions (Abdel-Hadi *et al.,* 2012; Georgianna *et al.,* 2008; Lanubile *et al.,* 2010; Luo *et al.,* 2010; Zhang *et al.,* 2014). Microarray analysis demonstrated a complex regulation of genes within the aflatoxin cluster in response to temperature and water. In general, the aflatoxin cluster genes exhibit lower expression at 37 compared to 28 °C (O'Brian *et al.,* 2007). It was further determined by Schmidt-Heydt *et al.* (2009) that different temperature and water combinations induce increased expression of the regulatory gene *aflS*, which interacts with *aflR*, and is correlated with high aflatoxin production. Further, a high *aflS*:*aflR* gene expression ratio (above 1) was also highly correlated with aflatoxin production. Subsequent microarray analysis and predictive modelling done by Abdel-Hadi *et al.* (2012) extended this by establishing that *aflD* and *aflM* are also highly subjected to temperature and water availability, and the relative expression of *aflD* or *aflM* to that of *aflR* and *aflS*, are related to temperature, water activity levels and aflatoxin production. An RNA-seq study of *A. flavus* interacting with maize kernels at various temperature and water activity levels corroborated previous observations, and identified

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an interactive effect of temperature and water activity on *aflR* and *aflS* expression (Medina *et al.,* 2017b).

Research has recently begun addressing the impact of increased carbon dioxide levels on *A. flavus*, as research in other host-pathogen systems has indicated that CO_2 levels can exacerbate host infection (Vary *et al.,* 2015). In the first attempt we know of to characterise the interaction of three environmental variables (temperature, water and CO_2) on *A. flavus* growth and aflatoxin production, Medina *et al.* (2015) demonstrated that growth of *A. flavus* was unaffected by CO_2 levels at 1000 ppm. However, there was markedly increased expression of aflatoxin cluster genes and AFB_1 levels in response to high CO_2 levels, especially at high temperature (37 °C) and low water activity levels (0.92-0.95_{aw}). Similar but modest effects were observed at the lower temperature tested (34 °C). Ongoing work in our laboratory and from others will address this important research area of interest.

The possible combined effects of drought stress, temperature, and carbon dioxide levels on the fungus-plant interaction and aflatoxin contamination level have not yet been extensively studied. It has been suggested that elevated CO_2 may increase crop metabolism resulting in increased crop yields (Driever and Kromdijk, 2013). However, several researchers have further speculated that higher temperatures and higher CO_2 levels will still likely result in conditions further accommodating fungal growth (DaMatta *et al.,* 2010; Paterson and Lima, 2011; Porter and Semenov, 2005). It has also been shown that drought-stressed maize exhibits suppression of several genes related to pathogen resistance such as pathogenesis-related proteins, heat shock proteins, and cell wall degrading enzymes (Luo *et al.,* 2010; Yu and Setter, 2003). Thus, there remains a pressing need to examine the effects of CO_2 on plant processes during fungal infection.

The effects of temperature and water activity on small non-coding RNA's (Bai *et al.,* 2015), development and the production of other secondary metabolites have also been examined (Medina *et al.,* 2017b; Yu *et al.,* 2011; Zhang *et al.,* 2014). Yu *et al.* (2011) demonstrated that 10 of the 55 gene clusters contained genes with increased expression at 30 °C as compared to 37 °C (cluster 1, 10, 11, 19, 20, 21, 24, 45, 54, and 55) and Cluster 3 decreased at the lower temperature examined. Medina *et al.* (2017b) also examined the secondary metabolic gene cluster response, where changes in water activity or temperature affected genes in the CPA, ustiloxin B, and penicillin clusters. Because secondary metabolites are intricately involved in *A. flavus* growth, development, and have toxigenic potential, characterising their expression levels in addition to aflatoxin is also of importance.

8. Genetic improvement of crops to control aflatoxin contamination

Pre-harvest aflatoxin contamination is a very complex problem affected by a multitude of biotic and abiotic factors (Bhatnagar-Mathur *et al.,* 2015; CAST, 2003; Gressel and Polturak, 2017). Therefore, a multi-pronged approach may need to be employed to control aflatoxin contamination when conditions in the field are favourable for fungal infection. Prominent among these approaches is the development of preharvest host plant-resistance through classical or molecular breeding (Bhatnagar *et al.,* 2008a,b). Less contamination during preharvest stage is very beneficial for reduced postharvest contamination. With the knowledge available so far on aflatoxin biosynthesis and natural and synthetic antifungal genes, it is now possible to impart innate host plant resistance using a combination of approaches.

Classical breeding

Although classical breeding is a proven approach to develop resistance, it is slow and is hampered by the lack of available resistance genes in germplasm in some susceptible crops. Several maize lines have been identified and developed with increased resistance to *A. flavus* infection and aflatoxin contamination and this has enabled the identification of natural resistance traits (Brown *et al.,* 2004, 2006, 2010; Campbell and White, 1995). However, these investigations have indicated that resistance to aflatoxin contamination is polygenic. Therefore, attempts to move resistance from inbred lines into commercial varieties with desirable agronomic characteristics have been a slow process due to the lack of availability of biomarkers to facilitate the transfer of resistance genes (Menkir *et al.,* 2008; Williams *et al.,* 2015). However, the roles of some antifungal proteins in maize have been validated (Chen *et al.,* 2010; Majumdar *et al.,* 2017b) and mapped to the genome (Chen *et al.,* 2016). More research is needed to elucidate the biochemical mechanisms that manifest the resistance phenotype in maize kernels or other sources so that they can be utilised to enhance resistance through marker-assisted breeding in maize or genetic engineering in cotton (Brown *et al.,* 2009, 2010; Cary *et al.,* 2009; Rajasekaran *et al.,* 2006, 2009a,b; Williams *et al.,* 2015).

Recent breeding efforts towards the development of aflatoxin-resistant maize lines have resulted in a number of germplasm release as IITA-SRRC TZAR inbreds. In 2008, TZAR 101-106, derived from a combination of African and southern-adapted US lines have been field-tested in different parts of the Southern USA with promising results (Menkir *et al.,* 2008). Recently, these lines were also determined to be resistant to both aflatoxin and fumonisin contamination (A. Menkir, unpublished data). They also exhibited resistance to lodging and common foliar diseases.

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GT-603 was released in 2011, after having been derived from GT-MAS:gk (Guo *et al.,* 2011), while Mp-718 and Mp-719 were released as southern adapted resistant lines which are both shorter and mature earlier than previous Mp lines (Williams and Windham, 2012). These lines are also being tested as inbreds and in hybrid combinations in the Southern USA.

Unlike maize, cotton or peanut have a limited diversity in their germplasm and no defined mechanism or well-characterised resistance proteins/genes have been identified to date for natural resistance to *A. flavus* (Holbrook *et al.,* 2000; Moore *et al.,* 2017). For this reason, it is critical that a seed-based resistance be developed using either genetic engineering or molecular breeding approaches in these crops.

Molecular breeding

Molecular breeding through biotechnological means is very attractive because it is less time consuming. Only a limited success has been demonstrated in the development of antifungal transgenic crops (Parisi *et al.,* 2016). Development of aflatoxin-resistant crops is further exacerbated by the fact that the causal fungus is an opportunistic saprophyte and does not follow typical host-pathogen interaction. However, success in this area will depend in large part on the following factors:

- Identification of resistance proteins, native or foreign, that express inhibitory activity against the mycotoxigenic fungi. A number of potential maize resistance-associated proteins (RAPs) and the genes encoding them have been identified (Table 2) and some of these have been introduced into other crops for evaluation (Chen *et al.,* 2006, 2007, 2010; Rajasekaran *et al.,* 2005a, 2008). Proteomic analyses of resistant maize kernels revealed existence of many resistance associated proteins and they have been shown to play an active role in resistance to *A. flavus* contamination (Chen *et al.,* 2002, 2015; Majumdar *et al.,* 2017b). Unlike maize, there are no well-characterised resistance proteins/genes available in cotton germplasm that demonstrate enhanced resistance to *A. flavus* infection and aflatoxin contamination. A sample of maize antifungal proteins that are inhibitory to *A. flavus* are given in Table 3.
- Development of efficient transformation methods that can be readily adapted to agronomically superior crop varieties (Cary *et al.,* 2011; Holbrook *et al.,* 2000; Rajasekaran *et al.,* 2005b).
- Selection of gene promoters that will provide the desired spatial and temporal regulation of antifungal transgenes and result in efficacious levels of production of the antifungal protein/peptide in targeted tissue or organelle (DeGray *et al.,* 2001; Rajasekaran *et al.,* 2012).

Table 2. Resistance-associated proteins identified through comparative proteomics (adapted from Brown *et al.,* 2010).

Antifungals
Zeamatin
Trypsin inhibitor 14kDa (TI)
Ribosome inactivating protein (RIP)
β-1,3-glucanase
Pathogenesis-related protein 10 (PR10)
PR10.1
Trypsin inhibitor 10 kDa (ZmTI)
Stress-related
Aldose reductase (ALD)
Cold-regulated protein (ZmCORp)
Water stress inducible protein (WSI)
Anionic peroxidase
Small heat shock protein 16.9/17.2 kDa
Glyoxalase I (GLX I)
Peroxiredoxin 1 (PER1)
Storage
Globulin I
Globulin II
Cupin domain containing protein (Zmcup)
Late embryogenesis abundant protein (LEA III)
LEA 14
Other
Serine/threonine protein kinase
Translation initiation factor 5A

Molecular breeding has become a useful rapid technology for development of crops resistant to aflatoxin contamination. Prominent among them is heterologous expression of foreign antifungal gene(s), either natural or synthetic, to provide resistance to the fungus and toxin production (Rajasekaran *et al.,* 2005b; Ruhlman *et al.,* 2014; Schubert *et al.,* 2015; Sharma *et al.,* in press). RNA-interference (RNAi)-mediated host induced gene silencing (HIGS) of key fungal genes critical for fungal pathogenesis and aflatoxin production has been successfully employed to incorporate *A. flavus* resistance in susceptible maize or peanut varieties (Arias *et al.,* 2015; Bhatnagar-Mathur *et al.,* 2015; Majumdar *et al.,* 2017a; Masanga *et al.,* 2015; Power *et al.,* 2017; Sharma *et al.,* in press; Thakare *et al.,* 2017). HIGS technology does not require that the host plant express a foreign protein so food and feed produced from resistant lines of transgenic maize should be more acceptable to regulatory agencies and consumers. Another set of novel methods that is available for crop improvement includes several gene-editing methods such as site-specific nucleases (SSNs), such as meganucleases (MNs), also known as homing nucleases (HNs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided nuclease (RGN) systems. The most widely used RGN is the clustered regularly interspaced short palindromic repeat (CRISPR)/

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Table 3. Natural and synthetic proteins/peptides with antifungal activity against *Aspergillus flavus*.

Protein/peptide	Protein family	Source	Mode of action	Reference
Haloperoxidase	peroxidase	*Pseudomonas pyrrocinia*	produce antimicrobial compounds – peracetic acid and hypohalites	Rajasekaran *et al.* (2000); Jacks *et al.* (2000)
β 1-3 glucanase	glycosyl hydrolase	tobacco	hydrolysis of fungal cell wall components	Sundaresha *et al.* (2010)
Ib-AMP_3	defensin	sweet potato	lytic	De Lucca *et al.* (1999)
AILP	lectin	hyacinth bean	inhibits germination and hyphal growth	Fakhoury and Woloshuk (2001)
Chitinase	glycosyl hydrolase	maize inbred Tex6	hydrolysis of fungal cell wall components	Moore *et al.* (2004)
ZmCORp	lectin	maize kernels	hemagglutination activity against fungal conidia	Baker *et al.* (2009)
Mod-1/RIP-1	ribosome-inhibiting protein	maize	inhibits hyphal tip growth	Nielsen *et al.* (2001); Weissinger *et al.* (2007)
Zeamatin	PR-5	maize	inhibits hyphal tip growth	Guo *et al.* (1997)
ZmPR10	PR-10	maize	RNAse activity	Chen *et al.* (2006)
ZmPRms	PR	maize kernels	defence regulator to modulate resistance	Majumdar *et al.* (2017b)
Thanatin	antimicrobial	Spined soldier bug	inhibits cellular respiration	Schubert *et al.* (2015)
Trypsin inhibitor	protease inhibitor	maize	trypsin/amylase inhibition	Chen *et al.* (1998)
Purothionin/ hordothionin	thionin	barley/wheat	lytic	K. Rajasekaran, unpublished data
D4E1	cecropin	synthetic peptide	lytic	Cary *et al.* (2000); Rajasekaran *et al.* (2005b)
D5C/D5C1		synthetic peptide	lytic	Weissinger *et al.* (2000)
AGM	tachyplesin	synthetic peptide	lytic	Rajasekaran *et al.* (2018)
D2A21		synthetic peptide	lytic	Weissinger *et al.* (2000)
MSI99	magainin	synthetic peptide	lytic	DeGray *et al.* (2001)

CRISPR-associated system 9 (CRISPR/Cas9), and DNA-guided nuclease (DGN) system, i.e. NgAgo (an acronym for *Natronobacterium gregoryi* Argonaute) (Miglani, 2017). CRISPR/Cas9/sgRNA system has been applied successfully to improve cultivated crops with agronomic traits. It is an inexpensive, easy, most user friendly, and rapidly adopted genome editing tool that enables precise genomic modifications in many different organisms including higher plants (Khatodia *et al.*, 2016; Wolt *et al.*, 2016; Zhang *et al.*, 2017). Development of genetically edited crops is similar to those developed by conventional or mutation breeding and this potential technique is useful in providing sustainable productive agriculture for better feeding of rapidly growing population in a changing climate. Once the target host plant genes are identified through comparative transcriptomic and gene network analyses in susceptible crops (Bedre *et al.*, 2015; Clevenger *et al.*, 2016; Lee *et al.*, 2012; Musungu *et al.*, 2015) these modern tools can be applied for generating aflatoxin resistant crops.

9. Post-harvest aflatoxin control strategies

Control of preharvest aflatoxin contamination has been addressed in the previous sections; however, it is important to consider aspects of postharvest control as well. Harvested produce with minimal or no fungal growth or aflatoxin levels is a good starting point to avoid postharvest aflatoxin contamination problems. Raw commodities prone to aflatoxin contamination (maize, groundnuts, tree nuts, spices, dried figs) must be effectively dried to facilitate short and medium term storage. Often in tropical countries aflatoxin is formed pre-harvest because of the conducive environmental conditions during ripening. However, to minimise subsequent toxin contamination effective drying and storage regimes are required for food and feed use. For example, maize is often harvested at 17-20% moisture content (m.c.) and the cereal must be dried effectively to around 15% m.c. (= 0.70 a_w) to avoid continued and increased contamination with aflatoxins. Some of these commodities, e.g. ground nuts and some trees nuts are very hygroscopic and can absorb moisture during storage from the atmosphere. They need to be stored at <7.5% m.c. (= 0.70 a_w). Otherwise, it can result in water and temperature conditions conducive to growth of xerophilic fungi such as *A. flavus* and aflatoxin contamination. Effective solar drying or in more developed systems heated drying is necessary for reaching the target safe m.c. levels. Table 4 shows the relationship between safe m.c. for these commodities and the equivalent water availability level for these commodities for safe short and medium term storage and those which represent marginal conditions and represent a risk of aflatoxin contamination.

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Table 4. The relationship between storage moisture content (m.c.) for safe storages and those representing a risk from aflatoxin contamination.

Commodity	Safe m.c. (% wet weight basis) = water activity (a_w)	At risk m.c. conditions
Maize	15.0% (= 0.70 a_w)	>16.5-17.0%
Peanut	9% (unshelled; = 0.70 a_w)	>10%
	7.5% (shelled; = 0.65 a_w)	
Pistachio	6.5-7.0% (unsalted; = 0.65-0.70 a_w)	>8.5-9.0%
Chillies	8-12% (= 0.60-0.70 a_w)	>12%

Molecular approaches have been used to monitor and control aflatoxin contamination post-harvest, especially in stored peanuts. For example, Abdel-Hadi *et al.* (2010) showed that the structural *aflD* gene in the biosynthetic cluster could be effectively used to monitor changes in *A. flavus* populations in peanuts stored under different storage conditions. Passone *et al.* (2010) found a correlation between populations of *Aspergillus* section *Flavi* (colony forming units) and RT-PCR for the *aflD* gene (r=0.613; P<0.0001). Changes in key structural and regulatory genes (e.g. *aflR*; *aflS*) can also be utilised effectively to monitor relative aflatoxin risk post-harvest.

There have also been studies which suggest that real time monitoring of CO_2 production, temperature and relative humidity could be used as good indicators of whether moulding and mycotoxin contamination may be initiated. Indeed, it has been shown that this approach can give an early warning system of relative risks of mycotoxin contamination. For example, for fumonisin contamination of maize it was shown that <0.8-0.9% dry matter loss would result in maize being contaminated with toxin at above the EU legislative limits (Mylona *et al.,* 2014). Recently, it has also been shown that in brown rice the risk of aflatoxin contamination post-harvest may be much higher than in unhusked paddy rice (Martin Castaño *et al.,* 2017).

A recent study by Waliyar *et al.* (2015) showed that effective drying of peanut pods or heated dryers and shellers can reduce post-harvest losses and reduce aflatoxin contamination of peanut. Storage at <7.5% m.c. at 10 °C can result in safe storage for between 6-12 months. However, at higher temperatures this time frame may be shortened as this may allow some reabsorption of moisture from the atmosphere and allow initiation of spoilage mould activity and an increase in aflatoxin contamination.

An integrated approach in Argentina encompassing pest control with entomogenous fungi + antioxidants in stored peanut and maize has shown effective control of aflatoxin contamination (Barra *et al.,* 2013, 2015a,b). This approach has optimised the use of entomogenous strains of *Purpureocillium lilacinum* for control of storage pests in silos, as well the use of antioxidants for controlling *A. flavus* colonisation. This has achieved significant reductions in AFB_1 contamination. This post-harvest integrated pest management strategy may be an effective way forward for controlling both pests and *A. flavus*, especially where hygiene practices are not effective. Again the cost-effectiveness of this approach and formulation of this type of application needs to be evaluated.

Gaseous treatment of stored commodities with either sulphur dioxide (SO_2) or ozone (O_3) has been examined for control of aflatoxins and other mycotoxins (Magan, 1993; Mylona *et al.*, 2014). Indeed, O_3 has been suggested as a method for control of aflatoxins in Brazil nuts (Giordana *et al.*, 2012). However, the economics of this approach combined with the associated deleterious health and safety issues limited its use. In addition, effects on organoleptic properties may also need to be considered as thresholds to prevent quality deterioration may be very low. The recent report by WHO (2017) also suggests that where pre- and post-harvest treatments are considered the potential for co-occurrence of different mycotoxins needs to be considered. Certain post-harvest control strategies may result in a switch to another mycotoxin, which could subsequently pose problems (e.g. aflatoxins to CPA).

10. Conclusions

Based on the severity and the extent of aflatoxin contamination of various commodities prior to harvest, and many more in storage worldwide, the health implications of this contamination as well as the very significant economic impact to many economies (discussed in WHO, 2017), *A. flavus* has probably become one of the most thoroughly studied microorganism, both at the macro level as well at the molecular level. The purpose of all these studies is to find meaningful and lasting strategies to control this contamination. Biological control has been demonstrated to be a strategy that is consistently functional in various parts of the world to significantly reduce pre-harvest aflatoxin contamination, which may be carried over to storage as well. However, biocontrol has its limitations and will not completely eliminate toxin contamination. Therefore, this strategy must be complemented with enhancement of host-resistance either by transgenic expression of antifungal genes or through host induced silencing of fungal genes. Identification of resistance associated markers, combined with other molecular tools to turn off aflatoxin production during the fungal-host interaction has now become reliable means of developing crops resistant to aflatoxin contamination. Moreover, with significant information being currently developed on the genomics and transcriptomics of various commodities affected by toxin contamination, additional knowledge of host-fungal

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interactions at the molecular level will identify control strategies that will be most effective in eliminating aflatoxin contamination worldwide. In this regard, availability of modern targeted gene editing in susceptible plants should pave the way for incorporating resistance to aflatoxin contamination. To improve the overall food and feed safety free of dangerous aflatoxin levels, it is not only necessary to minimise or eliminate the preharvest contamination levels but also extend constant postharvest monitoring to avoid fungal growth and toxin production during storage and handling. Although all the control measures, one at a time, discussed in this review will significantly minimise the occurrence of aflatoxin contamination in susceptible crops, it is unlikely to result in complete elimination of this problem because of (1) the adaptability of the fungus to broad-range of environments during crop production and during postharvest handling and storage, and (2) the complex role of the ubiquitous, opportunistic, saprophytic fungus in host plant-fungal pathogen relationship. Thus, it is essential to adopt multiple, yet coordinated, and complementary processes to control *A. flavus* and aflatoxin contamination using agronomic, genetic, biological, biotechnological, and molecular approaches.

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