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Control of Aflatoxin Biosynthesis in *Aspergilli*

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1. Introduction

Aflatoxins (AFs) are bisfurans that are polyketide-derived, toxic, and carcinogenic secondary metabolites produced by *Aspergillus flavus* on corn, peanuts, cottonseed, and tree nuts (Payne & Yu, 2010). While biosynthesis of these toxins has been extensively studied *in vitro*, much less is known about what causes the fungi to produce AFs under certain environmental conditions and only on certain plants. It is not yet known why wheat, soybean, and sorghum are resistant to AF contamination in the field whereas, under laboratory conditions *A. flavus* is able to colonize these plant tissues and produce AFs (Cleveland et al., 2009).

AF biosynthesis is a complicated process involving many levels of transcriptional and post-transcriptional control (Abbas et al., 2009; Chanda et al., 2009; Georgianna & Payne, 2009; Schmidt-Heydt et al., 2009). In this review we provide an overview of what is currently known about how environmental and nutritional factors stimulate or inhibit AF production. Environmental and nutritional signals interact directly with cell surface receptors or transport proteins. Usually the interaction sets up a cascade of cellular responses including activation of heat shock proteins or other chaperonin-like messengers as well as protein kinases or other protein modifying enzymes. These, in turn, activate transcription factors residing in the cytoplasm to allow them to cross the nuclear membrane boundary or, in some cases, to activate a DNA-binding protein already in the nucleus. The DNA-binding protein then has to find the correct location in the targeted gene's promoter and the region of chromatin containing the gene has to be in a transcriptionally competent (euchromatic) as opposed to an incompetent heterochromatic condensed state. Finding the correct location requires transcription-activating factors (TAFs) which either bind to chromatin and recruit the transcription factor to the DNA or bind to the transcription factor and guide it to the correct location. The stability of the transcription factor in the cell is another level of control of the transcriptional process. This stability is mediated by modification (ubiquitination or neddylation) (Busch et al., 2003). Recently, AF biosynthesis was shown to occur in dedicated peroxisomal vesicles (Roze et al., 2011). The ability to form and stabilize such vesicles could be critical to the coordination of the biosynthetic steps leading to AF formation. All of these processes are illustrated in Figure 1 and are discussed in more detail below.

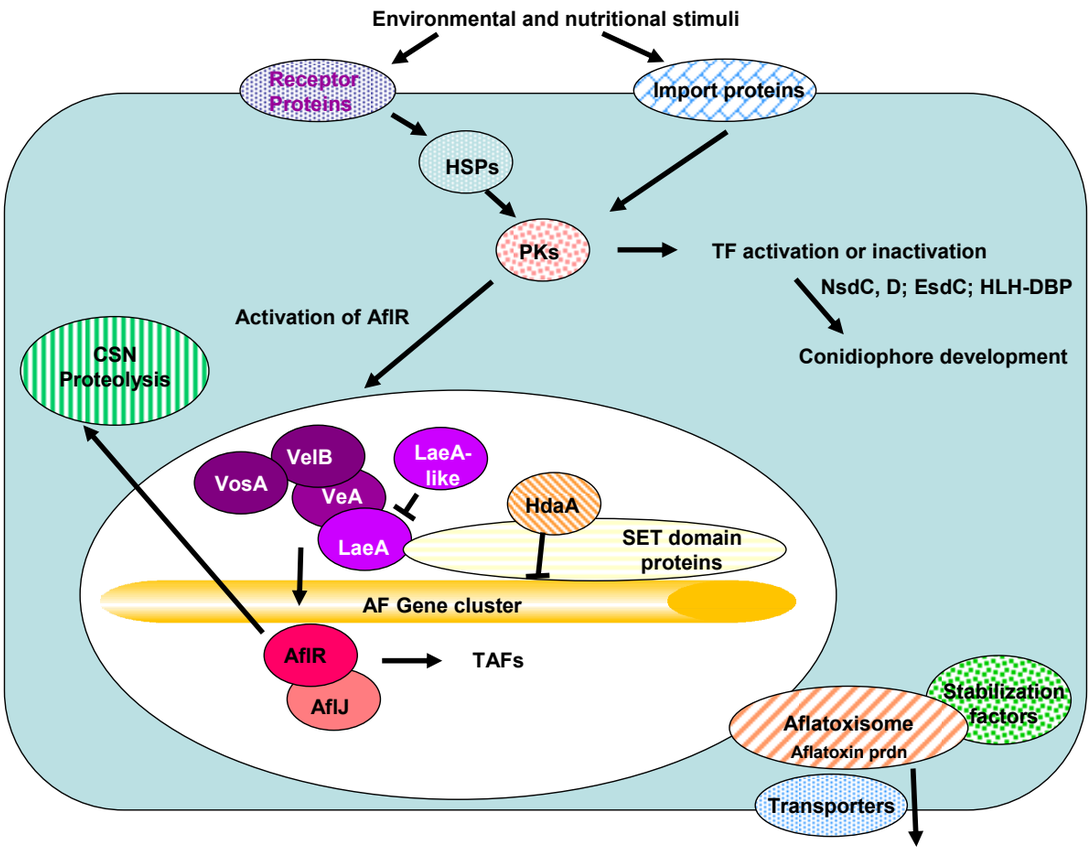

Fig. 1. Model showing factors involved in regulation of AF production. The cell is shown as a square and the nucleus as a large oval. Abbreviations: HSP-heat shock proteins, PK-protein kinase, CSN-COP9 signalosome, HLH_DBP-helix-loop-helix DNA-binding protein, TF-transcription factor, TAF-transcription activating factor, HdaA-histone deacetylase.

2. Transcriptional control of aflatoxin biosynthesis

2.1 The role of the pathway-specific transcription factor, AflR

Production of AF requires the coordinated transcription of about 30 clustered genes (Yu et al., 2004b). The genes for the 30 biosynthetic proteins are clustered within a 70-kb region of chromosome 3 (Fig. 2). In *A. flavus* the gene cluster is located 20 to 80 kb from the telomere depending on the *A. flavus* strain. The gene, *aflR,* encodes the sequence-specific Cys_6Zn_2 DNA-binding protein, AflR, which is responsible for transcriptional activation of most, if not all, AF structural genes (Chang et al., 1995; Ehrlich et al., 1998; Ehrlich et al., 1999b; Cary et al., 2000). AflR, as a typical Gal4-type transcription factor, has an N-terminal DNA binding domain and a C-terminal activation domain (Fig. 3). The DNA-binding domain recognizes the partially palindromic 11 bp double-strand motif, TCGSWNNSCGR (top strand only is shown, Fig. 3), in promoter regions of AF biosynthesis genes.

The strongest binding, based on electrophoretic mobility shift assays, is to sequences with a perfect 8 bp palindrome in an 11-bp motif, $TCG^{G}/_{C}NNN^{C}/_{G}CGA$. Footprinting studies showed that the preferred binding occurred to sequences in which the palindrome is flanked on the 5′-end by additional thymine or adenine residues. The binding motifs for

AflR almost always are within 200 bp of the gene's translational start site. In the *pksA* promoter region an additional AflR-binding site was found to be the AflR binding site for a short gene *hypC (*formerly *hypB1),* which encodes an enzyme necessary for oxidation of the AF precursor metabolite, norsolorinic acid anthrone.

Fig. 2. The AF cluster for *Aspergillus flavus, A. parasiticus and A. nomius*. Lettering on lower line is the current nomenclature for the genes using the *afl* designation(Yu et al., 2004a).

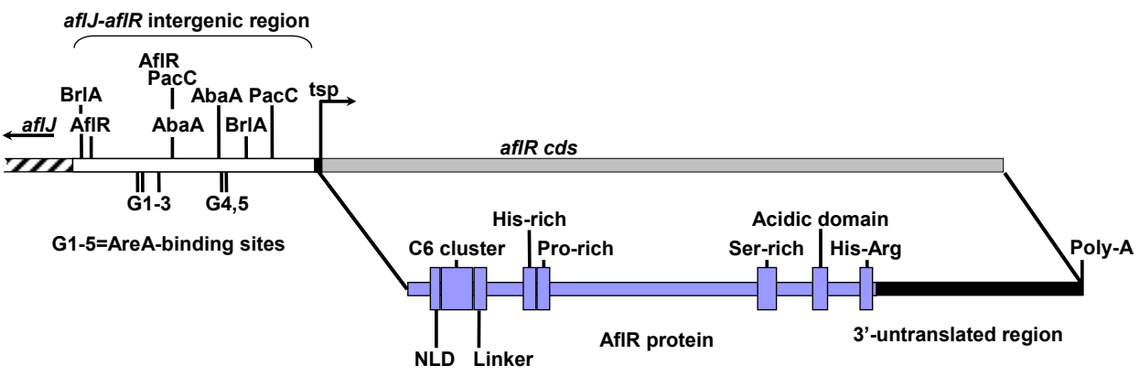

Fig. 3. Schematic depiction of the *aflR* gene. Consensus sequence elements for DNA-binding proteins are indicated on the *aflJ-aflR* intergenic region with their approximate positions. However, the *aflR* gene in each of the AF-producing species has fewer sites than those shown. Abbreviations: NL-nuclear localization; tsp-translational start point

In AflR a nuclear localization domain (RRARK) precedes the C6 cluster domain (CTSCASSKVRCTKEKPACARCIERGLAC) (Ehrlich et al., 1998). In many Gal4 type transcription factors the nuclear localization signal is within, not separate from, the C6 cluster as shown in Fig. 3). Furthermore many related C6 factors lack the underlined basic amino acids on the C-terminal side of the motif. A Blast search of *A. flavus* genome in the *Aspergillus* Comparative Database http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html with this sequence found only six other proteins with an E value=0.007 or lower (AFL2G_06146; AFL2G_11313; AFL2G_00473; AFL2G_02725; AFL2G_04045; AFL2G_08639). Since there are over 178 C6 transcription factor proteins in the database for *A. flavus* (Table 1), six is a low number for such proteins with this "AflR" type of DNA recognition domain. The amino acids immediately C-terminal to the C6-cluster, the "linker region", are presumed to determine DNA-binding specificity. This linker region (QYMVS**KR**MG**R**NP**R**) lacks basic amino acids at the N-terminal end of the region unlike the linker regions for many similar transcription factors, but possesses four basic amino acids in the C-terminal half of the motif. This set of amino acids may be a signature amino acid sequence that allows contact with the 11-bp $TCGN_5CGA$ DNA binding site recognized by AflR. A Blast search with this region gave only two close matches:

AFL2G_11313 and AFL2G_06146. These may also bind to a $TCGN_xCGA$ DNA motif. The C-terminal activation domain for AflR, like those of other C6-type transcription factors in fungi, has a high number (8/28) of acidic amino acids and (5/28) basic amino acids and is otherwise not distinctive. The C-terminal 38 amino acid region (residues 408–444) also has runs of His and Arg, and acidic amino acids (**HH**PASPFSLLGFSGLEANL**RHR**LRAVSSDIIDYL**HR**E) and these are also part of the transcription factor activation domain (Xie et al., 2000).

Some other features of the AflR protein may be important for its stability and its ability to interact with other proteins (Ehrlich et al., 2003). The AflR protein sequences of several different AF-producing Aspergilli (*A. flavus* AF70, *A. pseudotamarii,* and *A. parasiticus* contained a histidine-rich motif (HAHTQAHTHAHSH, aa 103-113 in *A. flavus*) on the carboxy-terminal side of the DNA-recognition domain. The length of this motif varies in size for the different species. Garnier plot analysis predicted that this region has a coiled rather than a helical or beta-sheet configuration. Such regions may be involved in pH-controlled protein-protein interactions unique to AflR. Similar His-rich regions are found in a number of eukaryotic transcription factors (Janknect et al., 1991). Differences in length of these repeats among AflRs could be important in modulating AflR's activity at different pHs.

In all AF-producing *Aspergillus* species, a proline-rich region was adjacent to this site on the C-terminal side. AflR in *A. nomius* isolates had 11-12 proline residues while *A. flavus* and *A. parasiticus* isolates had eight. A key distinguishing feature shared by *A. nomius* and *A. bombycis* isolates is a serine-rich sequence, NSSDSSGSSRSSSSSSNSP, approximately 100 amino acids from the AflR C-terminus and immediately preceding a conserved domain rich in acidic amino acids. Seven serine residues were present in the homologous region from *A. pseudotamarii* and four in *A. flavus* and *A. parasiticus*. The serine-rich region in AflRs from *A. nomius* and *A. bombycis* is a distinct PEST (proline, glutamine, serine, and threonine-rich region) sequence that may be a target for ubiquitin-mediated proteolysis (Rechsteiner, 1988). No comparable region exists in AflRs from other species. PEST scores for this region (RSSSSSSNSPTTVSEER) were +18 and +12 in *A. nomius* and *A. bombycis*, respectively, which are comparable to scores of +10 to +13 for known PEST domains in transcription factors (Suske, 1999). *A. nomius* isolates have a second PEST domain (HPPPPPQSDQPPH, PEST score = +15). Most proteins with PEST sequences are regulatory molecules that require fast turnover to avoid improper function. The PEST sequences in *A. nomius* and *A. bombycis* either may reflect regulatory mechanisms different from other AF-producing taxa or may be non-functional remnants of an ancestral mechanism to regulate cellular levels of AflR at the post-transcriptional level.

Analysis of the promoter region of AflR (Figure 3; the figure shows the whole *aflJ-aflR* intergenic region) revealed several possible regulatory elements (Ehrlich et al., 1999a). A region from -93 to -123 (CATTTAGGCCTAAGTGCGA**GGCAACGA**AAAG) upstream of the translational start site is important for promoter activity. A partial AflR-binding site is present in this region (underlined) and may allow *aflR* expression to be self-regulated. The *aflR* promoter lacks a detectable TATA-box or CCAAT-box binding domains found in the promoter regions of many eukaryotic genes. The gene has been shown to be expressed even under conditions not conducive to AF biosynthesis. Therefore, low levels of AflR may be present in the cell, but after induction the levels increase and are able to activate the other genes in the gene cluster. When *A. parasiticus* is transformed with a plasmid containing *aflR* expressed under the control of the glycerolphosphate dehydrogense "housekeeping"

promoter (*gpdA*), the fungi accumulate additional colored AF precursor metabolites, in particular, norsolorinic acid, indicating that higher levels of AflR cause increased expression of the early genes needed for production of the biosynthetic enzymes.

2.2 AflJ, a putative transcriptional co-activator of AF biosynthesis

The gene bidirectionally transcribed from *aflR* also is necessary for AF production (Meyers et al., 1998; Du et al., 2007). *aflJ* and *aflR* share a 737 bp intergenic region (Fig. 3). Knockout mutants of *aflJ* are unable to produce AF or its precursors (Meyers et al., 1998). Although AflJ has no recognized regions in the protein corresponding to enzymatic or regulatory domain, it has three putative membrane-spanning helices and a microbodies targeting signal. A microbody is a cytoplasmic organelle of a more or less globular shape that contains degradative enzymes bound within a single membrane. Microbody types include peroxisomes, glyoxisomes, glycosomes and Woronin bodies. A BlastP search of the GenBank database revealed some proteins having a methyltransferase domain (PFAM00891) but with certain regions missing (Fig. 4). PFAM00891 is a member of the superfamily cl10454 and includes O-methyltransferases that utilize S-adenosylmethionine as the substrate. In spite of the homology to known methyltransferases, it is unlikely that AflJ functions in this way. All of the AF-producing species of *Aspergillus*, the sterigmatocystin (ST)-producers, such as *A. nidulans*, and the dothistromin-producer, *Mycospaerella pini*, contain similar genes encoding an aflJ homolog. A tBlastN search of the *Aspergillus flavus* genome with the AflJ protein sequence revealed five hits (AFL2G_11558, E=-22; AFL2G_11312, E=-20; AFL2G_11323, E=-20; AFL2G_11580, E =-10; AFL2G_11922, E=-9). AFL2G_11323 has an OMT domain and AFL2G_11312 is a polyketide synthase with a methyltransferase domain.

There is still controversy about why Δ*aflJ* mutants fail to make AFs. In the original paper describing *aflJ* it was found that disruption of *aflJ* in *A. flavus* resulted in a failure to convert exogenously added pathway intermediates norsolorinic acid, sterigmatocystin, and *O*-methylsterigmatocystin to AF, indicating that these biosynthesis proteins were not made or were not active. The disrupted strain accumulated *pksA*, *nor1*, *ver1*, and *omtA* transcripts under conditions conducive to AF biosynthesis, but transcript levels for the early genes, *pksA* and *nor-1*, were significantly lower than in the parental strain (Du et al., 2007). Therefore, disruption of *aflJ* did not affect transcription of these genes. Although it was possible that Δ*aflJ* mutants failed to properly process RNA transcripts from AF cluster genes; recent studies found that the transcripts were processed normally (Du et al., 2007).

AflJ was shown to bind to AflR at a region within AflR's activation domain (Chang, 2003). Substitution of Arg429 and Arg431 in AflR with Leu residues abolished the binding. Deletions in targeted regions of AflJ, also prevented observable binding. AflJ stimulated the accumulation of AF and precursor metabolites in cultures when the transformant also contained a functional *aflR* gene. Transformants containing an extra copy of *aflR* but lacking an extra copy of *aflJ* had a reduced level of expression of *aflR* compared to transformants containing a second copy of both genes (Chang et al., 1995). Based on these results AflJ was classified as an AflR coactivator. From recent studies of Du, et al., *aflJ* was not found to be necessary for transcriptional activation of later genes (*ver1* and *omtA*) nor for *aflR*, but did upregulate expression of the polyketide synthase gene, *pksA* and *nor1*, two early genes necessary for the beginning steps in AF biosynthesis (Du et al., 2007). AflJ's transcription was regulated by AflR in *A. flavus* and *A. parasiticus*. An AflR-binding site near the

translational start site of *aflJ* (Fig. 2) may mediate this activation. One AF-producing species (*A. nomius*) lacks an AflR-binding site in this region and therefore transcription of *aflJ* may not be regulated by AflR in this species (Ehrlich et al., 2003).

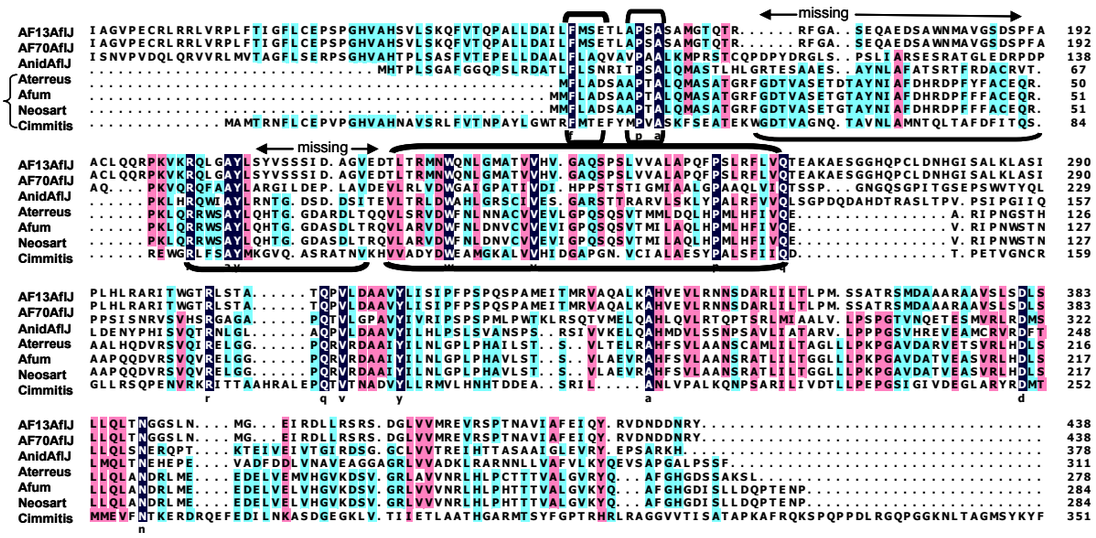

Fig. 4. Comparison of *A. flavus* AflJ sequence to those of non-aflatoxin producing species reveals partial homology to methyltransferase-2 family proteins. The bracketed proteins have *O*-methyltransferase domains. Regions bracketed at the bottom are the predicted methyltransferase domains.

One hypothesis to explain AflJ's role in transcription regulation is that, by binding to AflR, it allows AflR to interact with chromatin remodeling proteins such as LaeA (see below) (Ehrlich et al., 2011). Such interactions have been postulated to be necessary for opening up the chromatin region in which the AF cluster is located. Another possibility, that fits better with the likelihood that AflJ is a membrane-bound protein, as evidenced by the presence of transmembrane helices and its microbodies targeting signal, is that AflJ may be required for transmembrane transport of intermediates through intercellular compartments and, thereby, helps coordinate the localization of biosynthesis enzymes to a specialized organelle in the cell. The localization of AF biosynthesis has been postulated to occur in a dedicated peroxisomal vesicle, termed an aflatoxisome (Chanda et al., 2009). These two hypotheses will be discussed in more detail later in the paper.

2.3 Role of global transcriptional regulators

In concert with all eukaryotes, fungi make use of many different types of transcription factors to regulate cellular processes. Most of the important types that are relevant to control of development and secondary metabolite production are listed in Table 1. Transcription factors, such as AflR, which activate single genes or genes only in a related biosynthetic pathway generally act downstream of signaling cascades and can be activated in response to biological or environmental stimuli. These transcription factors are to be distinguished from globally acting factors which control the transcription of multiple sets of genes, sometimes in unrelated or more distantly related metabolic pathways. For fungi nutritional stimuli such as the carbon or nitrogen source as well as environmental stimuli such as temperature

and pH are especially important for control of AF biosynthesis (O'Brian et al., 2007; Wilkinson et al., 2007). These nutrients activate global transcription factors such as CreA (needed for control of expression of sugar utilization genes) and AreA (needed for control of expression of nitrate utilization genes) (Reverberi et al., 2010). PacC is a global transcription factor involved in pH regulation of transcription (Arst & Penalva, 2003). When AF is produced under conditions that stimulate the development of asexual reproductive structures (the conidiospore or sclerotium), the global transcription factors that are needed for activation of genes involved in formation of such structures (BrlA, AbaA, NsdC, NsdD, LreA, LreB) also can affect expression of genes in AF biosynthesis (Georgianna & Payne, 2009; Schmidt-Heydt et al., 2009). The globally acting transcription factors involved in AF synthesis recognize short GC-rich sequences in the promoters of key genes in the biosynthesis cluster. For AreA the consensus GC-rich recognition sequence is HGATAR, for CreA it is SYGGGG, for PacC it is GCCARG, for AbaA it is CATTCY, and for BrlA it is MRAGGGR. Usually more than one consensus domain is needed for strong transcriptional regulation (Gomez et al., 2003), but if the globally acting factor is close to the pathway specific transcription factor in the promoter it may be effective in activating transcription. Disruption of this type of transcription factor gene usually has a large effect on ability to utilize nutrients, morphology, or growth at certain pHs depending on the factor, but rarely results in complete loss of expression of the AF biosynthesis genes. However they may be strongly downregulated if the factor is missing. In the *aflJ-aflR* intergenic region (Figure 2), depending on the species, there are as many as five HGATAR sites, zero to two BrlA sites, one or more PacC sites, several AbaA sites, and one possible CreA site (not shown in Fig. 2).

Types of transcription factors	Approx. No. in *A. flavus* genome	PFAM designation	Examples
Cys2His2 zinc finger (C2H2)	40	PF00096	BrlA, NsdC, PacC
C6 transcription factor (Cys6Zn2)	178	PF00105	AflR, AlcR, NirA
Helix-loop-helix (HLH)	10	PF00010	DevR, PalcA
Helix-turn-helix	2	PF00046	AdaA, Rpc82
Basic leucine zipper (bZip)	17	PF00170	AtfA, NapA, CpcA
Homeodomain (C4HC3) ring finger)	1	PF00319	PHD
Winged helix	33	PF08279	Hpa3, GlcD, Sin3
GATA factors	6	PF00320	NsdD,AreA, LreA
ATTS factor	1	PF01285	AbaA

Table 1. Types of transcription factors in *Aspergillus flavus*. *A. flavus* genome size is 36.8 Mb (8 chromosomes) with 13,487 predicted genes.

2.4 Involvement of transcriptional coactivators in AF biosynthesis

Besides globally acting transcription factors, which act in concert with a pathway-specific transcription factor to activate gene expression, proteins other than AflJ, are required for AF biosynthesis and, like AflJ, probably function as coactivators (Lewis & Reinberg, 2003; Daniel & Grant, 2007). Some of these proteins have been mentioned briefly above. The most important of these for AF biosynthesis are listed in Table 2.

Factor	Putative conserved domains	Putative role	GenBank Accession #
VeA	Fibronectin attachment pfam 07174	Mediates AF activation by binding to LaeA	AFLA_066460
VosA	Topoisomerase related; pfam09770	Spore viability, possible transcription factor; similar motifs to VeA and VelB	AFLA_026900
VelB	Nucleoside diphosphatase	Similar role to that of VeA, forms heterodimer with VosA and LaeA	AFLA_081490
LaeA	SAM-depdt Methyltransferase	Global regulator of transcription of secondary metabolite genes; presumed to be involved in specific chromatin remodeling by methylating histones.	AFLA_033290
LaeA-like	SAM-depdt Methyltransferase	Modulates effect of LaeA	AFLA_035950
AflJ	Microbodies signal	Binds to AflR; possible co-activator	
Bre2 (CclA)	SPRY	Chromatin remodeling; histone lysine N-methyltransferase	AFLA_089250
HdaA	Histone deacetylase	Histone deacetylase; controls heterochromatin formation	AFLA_025220
DmtA	Cytosine DNA methyltransferase	Putative DNA cytosine methyltransferase, required for sexual development	AFLA_056340
FluG	glutamine synthetase	Regulates BrlA activity for conidiophore development	AFLA_101920
FlbA	Pleckstrin, RGS	Conidiophore development	AFLA_134030
FlbC	C2H2 transcription factor	Regulates BrlA activity for conidiophore development	AFLA_137320
FlbD	MYB family DNA binding	Regulates BrlA activity for conidiophore development	AFLA_080170
MedA	Transcriptional regulator	Medusa genes, necessary for correct metulae development; proper temporal expression of BrlA	AFLA_136410
RcoA (TupA)	WD repeat	Effects growth and sexual, asexual development & secondary metabolism; transcriptional repressor	AFLA_054810

Table 2. Transcription-activating factors (TAFs) involved in aflatoxin biosynthesis

2.4.1 Role of *velvet* complex genes

The *velvet* family of genes, so named because mutants have a velvet-like colony appearance, is involved in regulation of conidophore developmental and concomitantly secondary metabolite production (Calvo, 2008). The encoded proteins act upstream of BrlA, whose role is the regulation of transcription of genes needed for spore formation. *A. nidulans* has both a sexual and asexual stage (Note: *Aspergillus* species thought to only be able to reproduce asexually have now been shown to be capable of sexual reproduction as well). When grown in the light, the fungus reproduces asexually whereas in the dark it reproduces sexually. The *velvet* genes mediate the response to light and inhibit asexual development when the fungus is grown in the dark. Light activates a series of receptors including the red light receptor,

FphA, and the blue light receptors, LreA and LreB, as well as the near UV light receptor, CryA. When activated FphA, LreA and LreB form a complex with VeA, and presumably affect its activity or allow it to enter the nucleus. CryA acts differently and affects *veA* expression (Bayram et al., 2008a). Wild-type strains of *A. nidulans* display light-dependent conidiation; strains bearing a mutation (*veA1*) conidiate vigorously regardless of illumination conditions (Mooney & Yager, 1990). VeA likely acts as a negative regulator of asexual development. The majority of *A. nidulans* strains used are derived from the *veA1* mutant because of its increased conidiation. Whereas the *veA1* mutation still permits ST formation in *A. nidulans,* deletion mutants of *veA* in *A. nidulans* or in *A. flavus* are unable to produce ST or AFs, respectively. VeA and VelB with LaeA (see below) form a tripartite complex. VelB and VosA also form a complex, at least in *A. nidulans.* Light-dependent conidiation in *A. nidulans* is mediated via VeA nuclear translocation (Stinnett et al., 2007) together with interaction of VeA with the phytochrome photoreceptor FphA (Purschwitz et al., 2009). *A. flavus* conidiates well regardless of the presence or absence of light.

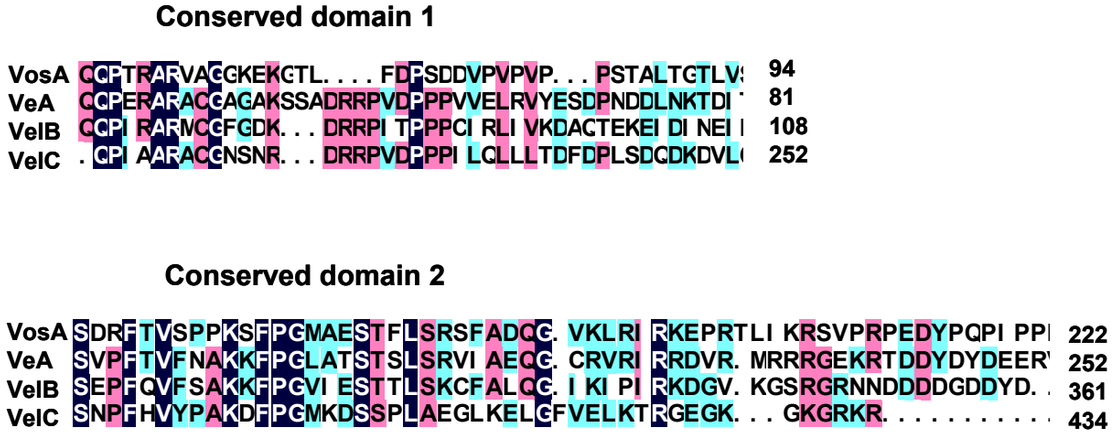

Fig. 5. Alignment of *Velvet* family proteins showing the two most conserved regions in the proteins.

The *velvet* family consists of four proteins with two regions of homology (Figure 5). These proteins are VeA, a 574 amino acid protein with a nuclear localization domain and a possible transmembrane domain, VelB, a 361 amino acid protein lacking both of these domains, but containing a cluster of Asp residues at its C-terminus, VelC a 434 amino acid protein of unknown function, and VosA, a 449 amino acid protein with conserved domains suggestive of its being a DNA-binding or chromatin-binding protein. A conserved domain search in GenBank suggests that these proteins have domains typically found in proteins that affect chromatin formation, in particular a region called Asc-1, identified in VelC and VosA. ASC-1 homology or ASCH domains are a beta-barrel domain found in proteins that interact with RNA and could mediate the interaction between a transcription factor and the basal transcriptional machinery (Iyer et al., 2006). VosA, (viability of spores) was identified as a protein essential for asexual spore maturation (Ni & Yu, 2007). It may have a novel winged helix transcription activation domain near its C-terminus. It forms a complex with VelB (Sarikaya Bayram et al., 2010). VosA is required for trehalose biogenesis (Ni & Yu, 2007). Trehalose is a compound that helps to protect the spore from stress. Spores, for their long-term survival require high amounts of trehalose. It has been suggested that VosA may

primarily control the activity of genes involved in the late process of sporulation, including trehalose biogenesis. VelB and VeA bind to LaeA ((Bayram et al., 2008b), see below). The competition for binding to VosA and LaeA makes VelB a partner in regulating the expression of genes involved in asexual sporulation and in secondary metabolite formation. The VeA-VelB heterodimeric complex of *A. nidulans* presumably forms in the cytoplasm and serves as the major pathway for VelB's entry into the nucleus. VelB and VosA predominantly interact in the nucleus. The same interactions of the *Velvet* genes presumably apply to the role of these proteins in transcription control of AF biosynthesis and sporogenesis in *A. flavus*.

2.4.2 Role of LaeA

Although there is much understanding from the literature on yeast of how Cys_6Zn_2 transcription factors function to activate gene expression, there is much less known about what activates the expression of these transcription factors. In a search for proteins that affect *aflR* expression in *A. nidulans*, a species that accumulates sterigmatocysin (ST), an AF precursor, a gene called *laeA* (Loss of AflR Expression), was isolated in which null mutants are unable to express AflR and lose the ability to make ST as well as other metabolites (Bok & Keller, 2004). *laeA* was predicted to encode a 375 amino acid protein with a S-adenosylmethionine-dependent methyltransferase domain typical of histone methyltansferases and argine methyltransferases. LaeA, however, lacked the SET and double loop domains typically found in such proteins and lacked a canonical nuclear localization signal, even though it was shown to reside in the nucleus. *laeA* expression, in *A. nidulans* was found to be downregulated by AflR, possibly because of AflR-binding sites in its promoter. No AflR-binding sites are present in the promoter of the *A. flavus* ortholog. Therefore, such regulation may be species-specific. The methyltransferase domain was shown to be required for LaeA's function. *laeA* in *A. nidulans* was also shown to be negatively regulated by protein kinase A and RasA, two signal transduction proteins shown to be involved in regulation of secondary metabolite gene activity and conidial development (see below). *laeA* null mutants showed little difference in spore production compared to the wild type, suggesting that the primary role of LaeA is to regulate expression of secondary metabolite gene clusters. It was proposed that LaeA may function as a unique, fungal secondary metabolite-specific regulator of chromatin organization necessary for activation of the genes in such clusters, including *aflR*.

In *ΔlaeA* mutants of *A. nidulans* silencing of *aflR* expression was found to be a consequence of the *aflR* being inside the cluster. When *aflR* was expressed in a locus outside the cluster, ST was produced even in the *ΔlaeA* mutants (Bok et al., 2006). Furthermore when a gene not associated with ST production was placed in the cluster, in the absence of functional LaeA, it was silenced. These results further suggested that chromosomal activity was mediated for secondary metabolite cluster genes by LaeA, and supported the hypothesis that LaeA plays a role in chromatin modification. In *A. parasiticus ΔlaeA* mutants, expression of *aflR* and other AF biosynthesis genes was also undetectable (Kale et al., 2007). Overexpression of *laeA* in strains having a functional copy of *aflR* increased *aflR*'s expression as well as the production of AFs. Furthermore, expression of *veA* was much lower in *ΔlaeA* mutants. Unlike *A. nidulans*, *A. flavus ΔlaeA* mutants showed decreased amounts of conidiation compared to the wild type and a complete absence of sclerotial production. In *ΔlaeA* mutants of *A. nidulans*, ST production was detected when a gene involved in heterochromatin maintenance encoding a histone deacetylase (*hdaA*), was disrupted. This

result supported the role of LaeA in chromatin remodeling at the site of secondary metabolite gene clusters (Shwab et al., 2007). The coordinated involvement of both VeA and LaeA in secondary metabolite production was established when it was found that these two factors interact with each other and with VelB in the *velvet* family of light responsive factors. (Bayram et al., 2008b). VelB interacts with the N-terminus of VeA, whereas LaeA interacts with the C-terminus of VeA.

It is assumed that VeA, with its functional nuclear localization domain, facilitates nuclear transport of VelB and LaeA, which lack such domains. Once in the nucleus they function as coactivators to specifically target genes at the secondary metabolite locus, presumably by either aiding in the chromatin remodeling preceding AflR binding to the promoters of cluster genes or by directly interacting with AflR or other pathway-specific DNA-binding proteins involved in secondary metabolite gene cluster activation. Since overexpression of AflR is able to relieve the block in gene expression caused by loss of LaeA, it is possible that AflR, alone or in conjunction with globally acting factors, initiates the chromatin remodeling necessary for expression of genes in the biosynthesis gene cluster.

2.5 Regulation by chromatin conformation

In eukaryotes, DNA with the help of histones forms compact particles, the nucleosomes, where about 147 DNA base pairs make two turns around the histone protein octamer. DNA within nucleosomes is inaccessible to many transcription factors. Some transcription factors, so-called pioneering factors are still able to bind to their DNA recognition sites on the nucleosomal DNA. For most other transcription factors, the nucleosome must be opened up by specific chromatin remodeling proteins (Clapier & Cairns, 2009). Alternatively, the nucleosome can be partially unwrapped by destabilizing factors to allow temporal access to the transcription factor binding site. In many cases a transcription factor needs to compete for binding to its DNA binding site with other transcription factors and histone- or non-histone chromatin proteins. Pairs of transcription factors and other proteins can play antagonistic roles (activator versus repressor) in the regulation of the same gene.

Many of the known gene clusters involved in secondary metabolite formation are located in subtelomeric chromosomal regions in which the chromatin is normally inactive (heterochromatic) due to di- or trimethylation of lysine-9 or lysine-27 of histone-3 (H3K9Me3; H3K27Me3) (Palmer & Keller, 2010). Trimethylation of lysine 4 in histone 3 is associated with active chromatin as is histone lysine acetylation. Generally histone deacetylases (HDAC's) are involved in the formation of heterochromatin whereas histone methyltransferases are associated with either formation of euchromatin (active) or heterochromatin depending on the position of methylation (Jenuwein & Allis, 2001). The proposed function of LaeA is to loosen the chromatin specifically at certain gene clusters, such as the AF cluster, to make it accessible to global and pathway specific DNA binding transcription factors (Strauss & Reyes-Dominguez, 2010). The reason subtelomeric chromosomal regions are associated mainly with inactive chromatin is they are often repositories for foreign DNA, including transposons and retrotransposons (Shaaban et al., 2010). It is in the interest of the cell to prevent activity of the foreign DNA because it could lead to undesirable mutations of genes (by transposon insertion) critical for function of the organism.

In *Aspergillus* species the specific chromatin remodeling factors besides LaeA, are ClrD, a H3K9 methyltransferase, Bre2 (CclA), a H3K4 methyltransferase (part of the COMPASS complex, a complex of proteins in yeast responsible for H3K4 methylation), and HdaA, a histone deacetylase (Palmer et al., 2008; Reyes-Dominguez et al., 2008). Another protein,

HepA, encodes a heterochromatin protein (HP1), one of a family of proteins that bind di- or trimethylated H3K9, leading to gene silencing and heterochromatin formation (Reyes-Dominguez et al., 2010). Deletion mutants of these proteins have generally been associated with increased production of ST in *A. nidulans* except that deletion of *laeA* is associated with the loss of secondary metabolite production. In agreement with these gene disruption results, treatment of *A. nidulans* with the HDAC inhibitors, valproic acid or trichostatin A, increased metabolite production. The role of LaeA in the chromatin remodeling is unclear since it is still uncertain whether or not LaeA acts as a methyltransferase. One possibility is that it, along with associated transcription factors, act as a bridge connecting the basal transcriptional elements (the equivalent of a TATA binding protein) to the pathway specific transcription factor (AflR). Such a complex would resemble the SAGA (Spt-Ada-Gcn5 acetyltranferase) complex in yeast (Traven et al., 2006). Such complexes not only stabilize the transcription machinery, like a Mediator complex in yeast, but also actively recruit the RNA polymerase II to the transcription initiation site (Biddick et al., 2008). A schematic of these proposed interactions is shown in Figure 6.

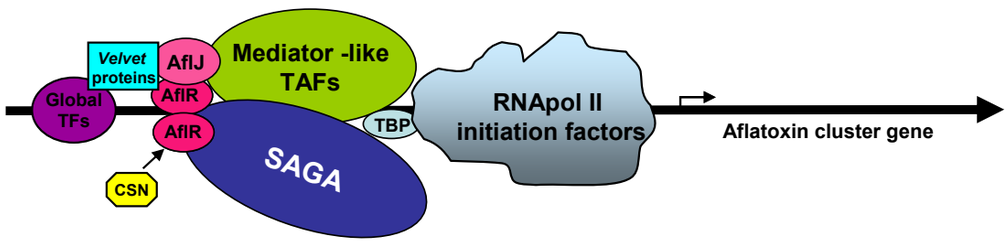

Fig. 6. Model of interactions of the aflatoxin cluster transcription regulatory factors with other proteins involved in the basal transcription machinery. Roles of SAGA and Mediator are described in a review by Traven, et al. (Traven et al., 2006).

In higher eukaryotes DNA methylation has been shown to affect gene expression (Clark & Melki, 2002). Because the level of DNA methylation in *Aspergillus* species is very low, it was considered unlikely to be involved in control of gene activity (Gowher et al., 2001). The loss of AF production and the induction of a *fluffy* phenotype in *A. parasiticus* clones treated with 5-azacytosine, a DNA methyltransferase inhibitor, as well as the presence in the genome of a DNA methyltransferase with predicted cytosine methylase function, suggested that DNA methylation may indeed play an unrecognized role in development and secondary metabolism (Tamame et al., 1983; Lee et al., 2008). DNA methyltransferases may function as chromatin remodeling agents in the absence of cytosine methylation. In support of this hypothesis, disruption of the gene for the DNA methyltransferase, *dmtA*, in *A. nidulans* yielded mutants unable to undergo sexual development (Lee et al., 2008). When gene expression by the nonaflatoxigenic *A. parasiticus* clones obtained by either 5-azacytosine treatment or by serial mycelial transfer (Chang et al., 2007) was compared to gene expression by the parental strain, the most highly downregulated genes were genes predicted to be involved in cell receptor recognition, signaling or transport (Wilkinson, et al., unpublished results). Surprisingly, the genes in the AF cluster were still transcribed at normal levels.

3. Signaling factors involved in AF biosynthesis

Transcription factors may be activated (or deactivated) through a signal-sensing domain by ligand binding, post-synthetic modifications, or proteolysis. The domain responsible for

binding to a hormone or an elicitor is called the ligand binding domain (LBD) (Gomperts et al., 2009). This domain participates in several activities including hormone binding, homo- and/or heterodimerization, formation of a complex with a heat-shock protein, and transcriptional activation and repression. The binding of the ligand may affect the conformation and concomitantly the transcription factor's ability to activate gene expression. Ligand binding and postsynthetic modifications, such as phosphorylation, myristylation, acetylation, also can influence where a transcription factor is located within a cell and whether the transcription factor is in its active state and is capable of binding to DNA, coregulatory proteins, or to other transcription factors, possibly as homo- or hetero-dimers.

3.1 Cell surface receptors –environmental signaling

A large number of organic compounds either produced by plants or derived from other sources, such as antimicrobial agents, pesticides, and herbicides have been shown to either induce or inhibit AF production by various mechanisms, some still only poorly understood. An extensive list of inhibitors has been given in an early review (Zaika & Buchanan, 1987). The list of inhibitors compiled today is considerable longer (Holmes et al., 2008). Plant metabolites, such as gallic acid, neem leaf and fruit extracts, certain spice oils , anthocyanins, and related flavonoids have been shown to be inhibitory to fungal toxin production. Low-molecular-weight aldehydes, ketones, and alcohols from cotton leaf, corn, and soybean are also inhibitory. These plant metabolites probably affect the activity or biosynthesis of key developmental regulatory proteins. The plant oxylipins, 13-hydroperoxylinoleic acid and 9-hydroperoxy linoleic acid, affect development and toxin synthesis, likely through conversion of these molecules into psi factor (Tsitsigiannis et al., 2005). Most of these inhibitors directly impact the cellular signaling and regulatory networks necessary for activation of genes required for growth, development and AF production.

3.2 G protein signaling and protein kinases

Development and secondary metabolism in fungi occurs in response to nutrient availability, light, oxidative stimuli, osmotic conditions or salt concentrations. These environmental signals are usually sensed at the cell surface and the signal transmitted to the nucleus by a series of secondary messengers such as diacylglycerol, inositol phosphates, divalent calcium, cyclicAMP or cyclic GMP, and chaperonins (heat shock factors that rapidly transmit stress signals). The secondary messenger transduces the signal from the membrane to cellular targets to mediate morphological changes necessary for reproduction or survival and production of secondary metabolites. Transduction of the activating or inhibiting signal depends on G-protein signaling.

A G-protein-mediated signaling cascade in *A. nidulans* was found to regulate both asexual sporulation and sterigmatocystin (ST) production (Yu & Keller, 2005; Brodhagen & Keller, 2006). FadA, a subunit of a heterotrimeric G-protein involved in developmental regulation, promotes vegetative growth and represses both sexual/asexual development and ST production in *A. nidulans*, as well as AF production in *A. flavus* and *A. parasiticus*. (Calvo et al., 2002). Inhibition of FadA involves FlbA, a regulator of G-protein signaling (RGS). FlbA acts as a positive regulator of ST production (Hicks et al., 1997). FlbA appears to act upstream of AflR because AflR is unable to activate ST or AF biosynthesis in a Δ*flbA* mutant (Shimizu et al., 2003).

The cAMP-dependent protein kinase PkaA functions downstream of FadA in this same signaling pathway. PkaA possibly prevents ST production in *A. nidulans* by inhibiting *aflR*

expression and as well as inactivating AflR (Shimizu & Keller, 2001; Shimizu et al., 2003). FluG is another protein in the *fluffy* protein family that is involved in control of asexual sporulation and ST production (Lee & Adams, 1994b; Lee & Adams, 1994a; Lee & Adams, 1996). The signal produced by FluG activates ST biosynthesis indirectly by activating *flbA* which represses FadA signaling (Seo et al., 2003; Seo et al., 2006). Another signaling protein involved in ST production is the GTP-binding protein RasA. RasA inhibits ST biosynthesis presumably by repression of *aflR* expression, although the molecular details of this repression are still unknown (Shimizu et al., 2003). Regulation of AflR by RasA may be at the post-transcriptional level and may be mediated by PkaA phosphorylation (Shimizu et al., 2003). A number of other protein kinases have been implicated in transduction of the environmental signals including the TOR kinase and MAP kinases. The TOR kinase, characterized in yeast as responsible for regulating nutrient mediated growth signaling, has recently been implicated in the biosynthesis of a fungal secondary metabolite (Teichert et al., 2006).

Nutrient regulation of AF and ST biosynthesis may be mediated by cAMP levels. cAMP has been shown to influence secondary metabolism in a number of species (Roze et al., 2004). Glucose and simple sugars in the growth medium act as inducers of AF biosynthesis, possibly by regulating levels of cAMP, and possibly by induction of the global transcription factor, CreA. Hormone-like signaling molecules, known as oxylipins, also contribute to regulation of secondary metabolites (Tsitsigiannis & Keller, 2006). These oxygenated lipid molecules mediate the balance of sexual to asexual spore production in aspergilli and are produced by fatty acid oxygenases encoded by ppo genes (Tsitsigiannis et al., 2004).

4. Specialized vesicles may be required for AF biosynthesis

AF and most secondary metabolites are hydrophobic organic compounds. Compartmentalization of hydrophobic substrates has been seen as a common feature of secondary metabolite production (Sirikantaramas et al., 2009). Recently, evidence has been obtained that the enzymes involved in AF biosynthesis are organized into a specialized peroxisomal vesicle where different oxidative steps occur after formation of the polyketide (Chanda et al., 2009). Development of aflatoxisomes was enhanced by conditions known to induce AF biosynthesis. Formation of the vesicle was inversely correlated with downregulation of a gene (*avaA*) required for vacuole formation (Ohsumi et al., 2002). Coordination of vesicle development was shown to be, in part, mediated by VeA and possibly by other proteins of the *Velvet* family. The organization and stability of such an organelle, obviously, is important for production of AF even if all of the biosynthetic genes are correctly transcribed, processed, and translated into proteins. The role of dedicated vesicles in the formation of AF could explain why in some cases, where the genes for AF biosynthesis are expressed and presumably the enzymes are made, no AF or precursor metabolites are formed. An example of this possibility is given below.

Previous research showed that 5-AC treatment and serial mycelial transfer in the absence of conidiation produced mutants with altered conidiophore development (a fluffy phenotype) (Tamame et al., 1983; Kale et al., 1994; Kale et al., 1996). Although these mutants were unable to produce AFs or precursor metabolites, as in some previous studies (Kale et al., 2007) they showed normal expression of most AF biosynthetic genes as well as normal expression of the aforementioned *laeA* and *veA*. A comparison of gene expression between these non-aflatoxigenic mutants and the parental strain by microarray analysis revealed that the most highly dysregulated genes were predicted to encode proteins targeted to cell membranes.

Among these are genes predicted to encode a protein kinase, an opsin homolog [a protein that may bind a photoreactive chromophore (Shichida & Matsuyama, 2009)], an integrin-repeat protein, and a calcium-binding protein. Since mycelial growth is unaffected in these mutants it is possible that the treatments affect proper formation of the vesicles necessary for AF biogenesis. Furthermore, other genes predicted to encode membrane-bound proteins are proteins involved in transport or exocytosis: a GPI (glycosylphosphatidylinositol)-anchored protein a GABA permease, a MFS transporter. Downregulation of expression of genes encoding membrane-bound proteins described above could explain the loss of normal development of asexual structures required for proper conidial formation and this phenotypic change in the mutant cells could prevent the formation of the vesicle needed for AF biogenesis (Chanda et al., 2009).

5. Protein turnover and its effect on AF biosynthesis

Another form of regulation that takes place post-translationally is control of transcription factor abundance by targeted degradation. Obviously if a transcription factor critical for a particular function is targeted for degradation, it no longer would be available for regulation of expression. As alluded to previously, some of the AflR proteins made by some *Aspergillus* species have PEST domains that may make these proteins destined for ubiquitin-mediated degradation (Rechsteiner, 1988). Ubiquitination is controlled by specialized ubiquitin ligases that reside in organelles called proteasomes. Another organelle related to the proteasome is called the COP9 Signalosome (CSN) (Busch et al., 2003). This multiprotein complex can both stabilize or destabilize other proteins by attaching or detaching a small protein (Nedd8) in a process called neddylation or deneddylation at the protein's ubiquitination site. The CSN complex also contains kinases that affect the activity of other regulatory factors.

In *A. nidulans* the COP9 signalosome was found to be a key regulator of light-dependent signaling and asexual and sexual development (Busch et al., 2003; He et al., 2005). The *Aspergillus* COP9 signalosome may control the abundance of the transcription factors that regulate these processes. Mutation of genes encoding *csnD* and *csnE* (components of CSN) (Busch et al., 2003) affects normal development in *A. nidulans* and pigmentation. In these mutants the abnormal mycelial pigmentation suggests that CSN regulates processes in both fungal development and secondary metabolism. When LaeA was used as the bait in a yeast two-hybrid assay with an *A. parasiticus* cDNA expression library as the prey, among the proteins binding to LaeA was COP9 signalosome complex subunit 5 (XM_001211499) (K. Ehrlich and B. M. Mack, unpublished data). This further suggests that the activity of LaeA may be modulated by specific interactions with CSN.

6. Conclusions

Expression of the genes in the AF biosynthesis cluster is mainly controlled by the pathway specific Cys_6Zn_2 DNA binding protein, AflR. While AflR appears to be necessary for the activation, a number of coactivators are important for fine-tuning of the timing of AflR's activity. These proteins, AflJ, LaeA, VeA, VelB and VosA, may form a complex in the nucleus to not only position AflR at the AF cluster genes but also alter the chromatin conformation in this locus in order to allow AflR and global transcriptional regulatory proteins to make contact with the basal transcription machinery. They may do this concomitantly with AflR binding or act to recruit AflR to the cluster. AflR expression is induced by simple sugars and inhibited by certain organic acids and aldehydes. Globally acting DNA-binding proteins are involved in

transmitting the nutritional or environmental signal to the activation of the AF gene cluster. A signaling cascade involving cAMP-dependent protein kinase A plays a role in the activation. The regulatory models shown in Figures 1 and 6 resemble that for expression of developmentally regulated genes in yeast and other fungi.

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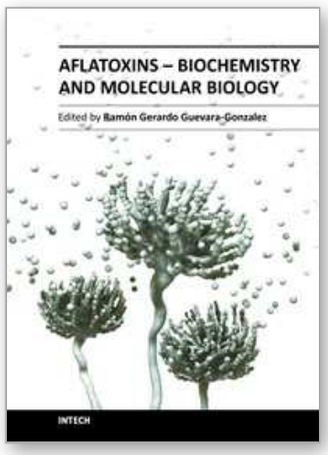

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Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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