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Transcriptional Regulatory Elements in Fungal Secondary Metabolism

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Abstract

Filamentous fungi produce a variety of secondary metabolites of diverse beneficial and detrimental activities to humankind. The genes encoding the enzymatic machinery required to make these metabolites are typically clustered in fungal genomes. There is considerable evidence that secondary metabolite gene regulation is, in part, by transcriptional control through hierarchical levels of transcriptional regulatory elements involved in secondary metabolite cluster regulation. Identification of secondary metabolism regulatory elements could potentially provide a means of increasing production of beneficial metabolites, decreasing production of detrimental metabolites, aid in the identification of 'silent' natural products and also contribute to a broader understanding of molecular mechanisms by which secondary metabolites are produced. This review summarizes regulation of secondary metabolism associated on transcriptional regulatory elements from a broad view as well as tremendous advances in discovery of cryptic or novel secondary metabolites by genomic mining in the basis of this knowledge.

Keywords

transcriptional regulatory elements; velvet complex; $Zn(II)_2Cys_6$; bZIP; StuA; genome mining

I. Introduction

Secondary metabolites (SMs) are a vast array of small molecules produced by microbes and plants. These molecules are not essential for normal growth of an organism but play important roles, for example by functioning as defense compounds or signaling molecules in ecological interactions. Many SM often possess pharmacological activities and have been exploited in medicine as antibiotics, anticancer and anti-infective agents and for a broad range of other applications (Fox and Howlett 2008b; Osbourn 2010). Considering this importance, much effort has focused on studying the genetic regulation of SM gene expression. Fungi are among the most prolific SM producers. One significant hallmark of fungal SM biosynthetic genes is that genes for any particular metabolite (e.g. penicillin) are clustered and act as a single genetic locus (Keller *et al.,* 2005; Yu and Keller 2005). Furthermore, the SM clusters are not randomly distributed in the genome but most often subtelomerically located (Palmer and Keller 2010; Perrin *et al.,* 2007). These findings have provided a convenient platform to elucidate the regulatory mechanisms of SM biosynthetic gene expression.

The co-regulation of SM biosynthetic genes can be in part explained by transcriptional control through hierarchical levels of transcriptional regulatory elements. One group consists of bonefide transcription factors ranging from pathway specific transcription factors (e.g. AflR regulating the sterigmatocystin (ST)/aflatoxin (AF) SM clusters, Brown *et al.,* 1996;

Fernandes *et al.,* 1998) to broad domain transcription factors (e.g. PacC mediating fungal responses to changes in pH, Tilburn *et al.,* 1995) and bZip proteins involved in cluster activation (e.g. MeaB, Polley and Caddick 1996 and RsmA, Shaaban *et al.,* 2010). Another class consists of heteromeric protein complexes participating in global regulation of multiple SM clusters coupled with other developmental processes (e.g. the Velvet complex, Bayram *et al.,* 2008a) and the CCAAT-binding complex AnCF/PENR1 (Brakhage *et al.,* 1999).

In this short review, we focus primarily on these and associated transcriptional regulatory elements of secondary metabolism and how this knowledge, in turn, has led to tremendous advances in genomic mining of the fungal secondary metabolism.

II. Cluster Specific Regulatory Elements

Fungal secondary metabolic gene clusters often contain one or more transcription factors that are required for expression of the genes for the biosynthetic enzymes (Hoffmeister and Keller 2007; Osbourn 2010). AF is one of the best-characterized fungal secondary metabolites and, along with the related metabolite ST, among the first compounds found to have all of their biosynthetic genes organized within a DNA cluster. The biosynthetic gene clusters were found to include *ca.* 25 genes arranged in about 70 kb clusters in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Brown *et al.,* 1996; Yu *et al.,* 1995). Two pathway specific regulatory genes, *aflR* and *aflJ*, located divergently adjacent to each other within the AF/ST cluster are involved in the regulation of AF/ST gene expression. These two genes are now referred to as *aflR* and *aflS* in both *A. flavus* and *A. parasiticus*, respectively (Yu *et al.,* 2004).

a) Transcription factors

AflR, encoding a sequence-specific DNA-binding binuclear zinc cluster ($Zn(II)_2Cys_6$) protein, is required for AF and ST activation by binding to a palindromic sequence, called AflR binding motif, 5′-TCG(N_5)GCA. It is found in most promoters of the AF/ST biosynthetic genes in *A. flavus*, *A. parasiticus* and *A. nidulans* (Brown *et al.,* 1996; Chang 2003; Fernandes *et al.,* 1998; Georgianna and Payne 2009). A second binding site, 5′-TTAGGCCTAA, is reported as important in auto-regulation of *aflR* transcript in *A. flavus* and *A. parasiticus* (Chang *et al.,* 1995a). Disruption or mutation of *aflR* abolishes expression of AF/ST cluster genes (Cary *et al.,* 2000; Woloshuk *et al.,* 1994; Yu *et al.,* 1996). Conversely, overexpression of *aflR* in the genome increases biosynthetic gene expression and SM production (Bok *et al.,* 2006c; Chang *et al.,* 1995b). Despite clear differences in the sequence of AflR between *A. nidulans* and *A. flavus*, function is conserved. AflR from *A. flavus* is able to drive expression of the ST cluster in an *A. nidulans aflR* deletion strain (Yu *et al.,* 1996).

To more carefully understand the role of AflR in gene regulation, Price *et al.*(2006) assessed gene expression in wild-type and *aflR* deletion strains of *A. parasiticus* using microarrays. The results indicated that AflR regulates genes not only in the biosynthetic cluster but also outside it. This implies that AflR has a complex binding motif. Ehrlich *et al.,* (1999) thoroughly examined promoters for AflR binding in eleven different genes from AF cluster by using electrophoretic mobility shift assays (EMSA). All of these genes demonstrate some degree of AflR binding in EMSA assays except three genes: *aflR*, *aflJ* and *avnA* having sites that deviate from the predicted AflR binding motif. Moreover, all of these genes were differentially expressed between WT and the Δ*aflR* mutant, suggesting AflR is required to activate their expression (Price *et al.,* 2006). However, the differences in AF gene expression do not always correspond with the differences in AF production as seen in the comparison (Price *et al.,* 2006). This suggests that there are other regulatory factors modulating AF gene cluster expression. AflJ (also called AflS) is another regulator which resides next to AflR.

The mechanism of AflJ regulation of AF biosynthesis remains elusive although data suggest AflJ may act as an enhancer and it is required for full AflR activity and modulates AF expression through its interaction with AflR (Chang 2003). Over-expression of *aflJ* in the aflatoxin-producing strains resulted in increased AF (Du *et al.,* 2007). Inactivation or mutation of *aflJ* gives a phenotype similar to an *aflR* deletion, i.e., a great reduction in AF production (Meyers *et al.,* 1998). The dual presence of AflR and AflJ-like proteins is present in several SM clusters such as the *A. nidulans* monodictyphenone cluster where both *mdpA* (a putative *aflJ* homolog) and *mdpE* (a putative *aflR* homolog) are required for activation of *mdp* biosynthetic genes (Chiang *et al.,* 2010). In fact, evolutionary evaluations of the AF biosynthetic cluster suggest *aflR/aflJ(aflS)* exists as a conserved regulatory module in SM clusters (Carbone *et al.,* 2007).

Zinc binuclear ($Zn(II)_2Cys_6$) proteins like AflR are the most common type of in-cluster pathway regulators and are unique to fungi (Table 1). *A. fumigatus* GliZ required for expression of the gliotoxin cluster (Bok *et al.,* 2006a). Gliotoxin is an epipolythiodioxopiperazine (ETP) metabolite and has been shown to play a significant role in enabling the virulence of *A. fumigatus* (Fox and Howlett 2008c). Due to its role in invasive aspergillosis, there has been much focus on the biosynthesis of gliotoxin and its regulation mechanism (Schrettl *et al.,* 2010b; Sugui *et al.,* 2007). The putative gliotoxin (*gli*) cluster spans about 28 kb in the genome of *A. fumigatus* (Gardiner and Howlett 2005). Deletion of *gliZ* resulted in the complete inhibition of all gliotoxin cluster gene expression and effective diminution of gliotoxin production (Bok *et al.,* 2006a). Placement of multiple copies of *gliZ* in the genome increased gliotoxin production. SirZ a $Zn(II)_2Cys_6$ DNA binding protein present in the ETP sirodesmin cluster in *Leptosphaeria maculans* regulates sirodesmin production in a manner similar to GliZ regulation of gliotoxin (Fox *et al.,* 2008a).

Other characterized SM $Zn(II)_2Cys_6$ proteins include MclR, which is required for compactin biosynthesis by *Penicillium citrinum* (Abe *et al.,* 2002a), the fumonisin regulators and FUM21 found in *Fusarium verticillioides* (Brown *et al.,* 2007; Flaherty and Woloshuk 2004). ZRF1, reported as a positive regulator of fumonisin production requiring a cyclin-like (C-Type) gene FCC1 for function, is an example of a regulatory gene found outside of the SM cluster it regulates (Flaherty and Woloshuk 2004). Little or no fumonisin production in FUM21 deletion mutants also indicates its positive role in the biosynthesis of fumonisin (Brown *et al.,* 2007). CTB8 was identified to regulate the transcription of seven clustered genes responsible for the biosynthesis of cercosporin in *Cercospora nicotianae* (Chen *et al.,* 2007). CtnA is a major activator of citrinin biosynthesis in *Monascus purpureus*. A strain of *M. purpureus* in which *ctnA* was disrupted exhibited a decrease in citrinin production (Shimizu *et al.,* 2007), whereas heterologous expression of an additional copy of *ctnA* in *A. oryzae* results in 400-fold higher citrinin production than that of the parental strain (Sakai *et al.,* 2008). LovE regulates biosynthesis of lovastatin in *A. terreus* (Huang and Li 2009; Kennedy *et al.,* 1999). GIP2 regulates aurofusarin biosynthetic gene cluster in *Gibberella zeae* (Kim *et al.,* 2006) and *mokH* is required for monacolin K synthesis (Chen *et al.,* 2010). ApdR activates the biosynthesis of the PKS-NRPS metabolites aspyridones (Bergmann *et al.,* 2007) and CntR is the transcriptional regulator of asperfuranone. As mentioned earlier, *mdpE* (a putative *aflR* homolog) activates monodictyphenone biosynthesis (Chiang *et al.,* 2010). Cmr1p and Pig1p, which regulate melanin synthesis in *Colletotrichum lagenarium* and *Magnaporthe grisea* respectively, contain a Cys_2His_2 zinc finger motif as well as a $Zn(II)_2Cys_6$ binuclear cluster motif (Tsuji *et al.,* 2000). Aside from the few mentioned in this review, there are numerous examples of $Zn(II)_2Cys_6$ –like proteins in SM clusters which have not yet been characterized (e.g. the solanapyrone gene cluster recently found in *Alternaria solani* (Kasahara *et al.,* 2010)). Commonly, $Zn(II)_2Cys_6$ DNA binding domains interact with DNA binding sites consisting of conserved terminal trinucleotides, which are

usually in a symmetrical configuration and are spaced by an internal variable sequence of defined length, for example, $CCG(N)_XCGG$ (MacPherson *et al.,* 2006).

Other types of pathway specific transcription factors have been found to be important in cluster transcriptional regulation. These include several Cys_2His_2 zinc finger proteins such as Tri6 and MRTRI6 for trichothecene production in *Fusarium sporotrichiodes* (Hohn *et al.,* 1999; Trapp *et al.,* 1998), and an ankyrin repeat protein ToxE for HC-toxin production in *Cochliobolus carbonum* (Pedley and Walton 2001). Hohn and coworker determined the Tri6 binding sequence motif to be YNAGGCC in the promoter regions of nine other *tri* genes. Loss of this protein leads to decreased expression of *tri* cluster genes and accumulation of low levels of the trichothecene precursor trichodiene (Hohn *et al.,* 1999). Tri6 in turn is regulated by the master regulatory gene Tri10 (Tag *et al.,* 2001). Recently, two more putative Cys_2His_2 transcription factors ScpR and AfoA were found to interact with each other where ScpR not only regulated expression of two adjacent NRPS genes *inpA* and *inpB* but also AfoA, the specific regulator of the asperfuranone gene cluster (Bergmann *et al.,* 2010). The proposed product of *inp* cluster is a tripeptide with a reduced C-terminus. The final product of this cluster is unknown. Furthermore, two transcription factors CPCR1 (cephalosporin C regulator 1) and AcFKH1 (forkhead transcription factor 1) required for cephalosporin C production were characterized from *Acremonium chrysogenum* (Schmitt and Kuck 2000). They are members of subfamilies of winged helix transcription factors (Hoff *et al.,* 2005; Schmitt *et al.,* 2004). The CPCR1 protein belongs to the conserved family of eukaryotic regulatory factor X (RFX) transcription factors. Transcription factor AcFKH1 belongs to the family of forkhead proteins and is characterized by two conserved domains, the forkhead-associated domain (FHA), which might be involved in phospho-protein interactions, and the C-terminal forkhead DNA-binding domain (FKH). CPCR1 and AcFKH1 regulate cephalosporin C biosynthesis by binding to regulatory sequences in the promoter region of the cephalosporin C biosynthesis genes *pcbAB-pcbC* (Schmitt *et al.,* 2004; Schmitt and Kuck 2000).

b) Alternative in-cluster regulation

In addition to pathway specific transcription factors, other members in SM clusters can affect expression of the remaining cluster genes. For example, the disruption of *gliP*, encoding a multimodular nonribosomal peptide synthetase (NRPS) in the gliotoxin cluster, resulted in the inhibition of expression of other *gli* genes (Sugui *et al.,* 2007). On the other hand, *gliT* encoding a gliotoxin reductase required for self-protection against gliotoxin, is independently regulated from other *gli* genes; that is, it is still expressed in a *A. fumigatus* Δ*gliZ* background (Schrettl *et al.,* 2010b). Another interesting case of in-cluster regulation has been reported in the fungus *Leptosphaeria maculans* (Gardiner *et al.,* 2005). Here, the disruption of a putative ABC transporter, *sirA*, in the sirodesmin cluster resulted in increased expression of *sirP*, the sirodesmin NRPS (Gardiner *et al.,* 2005). Observations in our lab suggest such in-cluster regulation may be fairly common (Lim and Keller unpublished data).

III. Transcriptional linkage to environmental programs

In addition to pathway-specific regulators, fungal secondary metabolite production is also responsive to general environmental factors called broad domain transcription factors. Carbon and nitrogen sources, temperature, light, and pH are well known parameters which greatly affect metabolite output. Fungi are able to turn the energetically costly process of SM production off and on under certain environmental conditions presumably as an advantage to the producing fungus. Many studies have indicated that these environmental signals influencing SM production are mediated through Cys_2His_2 zinc finger global transcription factors such as CreA for carbon signaling (Dowzer and Kelly 1989; Dowzer and Kelly 1991), AreA for nitrogen signaling (Hynes 1975), and PacC for pH signaling

(Tilburn *et al.,* 1995). There are several reports of all three proteins affecting both negative and positive regulation of gene clusters.

For example, carbon catabolite repression by the CreA-transcriptional repressor is widespread in filamentous fungi (Dowzer and Kelly 1989). The best known example is ethanol metabolism in *A. nidulans* (Felenbok *et al.,* 2001). In the ethanol utilization pathway, CreA directly represses the expression of *alcA* and *aldA* encoding alcohol and aldehyde dehydrogenases, respectively, and also directly represses *alcR*, encoding a transcriptional activator required for *alcA* and *aldA* expression (Felenbok *et al.,* 2001). Although penicillin production is carbon catabolite repressed (Brakhage *et al.,* 1992), extensive investigations in *A. nidulans* support only a minimal role for negative regulation by CreA in this species (Espeso *et al.,* 1993; Espeso and Penalva 1992). AreA is a GATA transcription factor that mediates nitrogen metabolite repression in response to NH_4^+ or glutamine (Wilson and Arst 1998). Its binding sites are found in promoter regions throughout the AF biosynthetic cluster in *A. parasiticus*. This indicates that AreA may play a role in the regulation of AF biosynthesis by nitrogen (Chang *et al.,* 2000). More concrete data comes from studies in *Gibberella fujikuroi* where AreA is directly involved in positively regulating gibberellin (GA) gene expression (Mihlan *et al.,* 2003; Tudzynski *et al.,* 1998). PacC is a zinc finger transcription factor that regulates gene expression depending on ambient pH (Tilburn *et al.,* 1995). Penicillin production is positively regulated by PacC but appears to negatively regulate ST gene expression (Espeso *et al.,* 1993; Keller *et al.,* 1997; Martin 2000). Additionally, transcriptional studies have shown that ochratoxin A biosynthetic genes are down regulated in alkali pH, possibly an indication of PacC regulation (O'Callaghan *et al.,* 2006). Some evidence also exists for possible negative regulation of fumonisin by PacC in *F. verticillioides* (Flaherty et al., 2003). Sometimes SM clusters are regulated by two or more of these broad domain factors such as the case of bikaverin in *F. fujikuroi* where the *bik* gene cluster is repressed by both AreA and PacC (Wiemann *et al.,* 2009).

IV. Transcriptional Complexes

a) Velvet Complex

In fungi, SM production is regulated not only by pathway specific transcription factors or broad domain transcription factors but also heteromeric complexes encoded by genes unlinked to the SM biosynthetic gene clusters. A significant advance in our understanding of SM cluster regulation was the finding of a conserved fungal complex known as the Velvet complex linking light regulation of both secondary metabolism and sexual development (Bayram *et al.,* 2008a) (Figure 1). This heterotrimeric complex is composed of at least three proteins, LaeA, VeA and VelB. Of these, VeA has been known for several decades but it was LaeA that first captured the attention of the SM community.

LaeA (loss of *aflR* expression-A) was first identified in *A. nidulans* through a genetic mutagenesis strategy to find regulators of AflR (Bok and Keller 2004; Butchko *et al.,* 1999). The conserved LaeA sequence led quickly to identification of orthologs in other Aspergilli including *A. terreus*, *A. fumigatus* (Bok and Keller 2004) and subsequently *A. flavus* (Kale *et al.,* 2008). Overexpression of *laeA* greatly increases penicillin and lovastatin production in *A. nidulans* and *A. terreus* respectively. Loss of LaeA results in inactivation of numerous secondary metabolite gene clusters, including ST, penicillin and terrequinone A production in *A. nidulans*, lovastatin production in *A. terreus*, AF and aflatrem production in *A. flavus* and gliotoxin production in *A. fumigatus* (Bok *et al.,* 2006c; Kale *et al.,* 2008; Keller *et al.,* 2005). Deletion of *laeA* in *A. fumigatus* decreases the virulence of this human pathogen (Bok *et al.,* 2005; Dagenais and Keller 2009). A similar effect was observed in *A. flavus laeA* deletants which are reduced in virulence on host seed (Amaike and Keller 2009). LaeA

is also used as a genomic mining tool providing a novel method for identifying new SMs (Bok *et al.,* 2006b) including terrequinone A (Bouhired *et al.,* 2007). Microarray analysis of wild-type and Δ*laeA* strains of *A. fumigatus* indicated that LaeA controls up to 9.5% of the transcriptome and up to 13 of its 22 secondary metabolite gene clusters (Perrin *et al.,* 2007). LaeA orthologs have since been identified in other filamentous fungi. For example, PclaeA was found from *P. chrysogenum* where it controls penicillin biosynthesis, pigmentation and sporulation (Kosalkova *et al.,* 2009), *P. citrinum* where it is proposed to regulate mevastatin synthesis (Xing *et al.,* 2010), *M. pilosus* where it is associated with monicolin J production (Zhang and Miyake 2009) and FflaeA1 where it both positively (GAs, fumonisins and fusarin C) and negatively (bikaverin) regulates secondary metabolism (Wiemann *et al.,* 2010). It has now been shown that all *laeA* mutants are also aberrant in sporulation as well as secondary metabolism (Dagenais *et al.,* 2010; Kosalkova *et al.,* 2009; Shaaban *et al.,* 2010; Wiemann *et al.,* 2010).

LaeA was found to interact with VeA and VelB in 2008 (Bayram *et al.,* 2008a) and, as a complex, this heterotrimeric unit, called the Velvet complex, jointly links SM production to sporulation processes (Figure 1). Although little is known about VelB function, VeA, also called *velvet*, was first described in 1965 (Kafer 1965) and early on associated with differentiation of asexual and sexual development. Recently, VeA has been found to function as a key global metabolic regulator in the biosynthesis of many SMs concomitant with sexual development (Calvo 2008). Kato *et al.,* (2003) found that *A. nidulans veA* regulates the expression of ST and penicillin genes. *veA* is necessary for activation of the ST-specific transcription factor *aflR* expression leading to the production of this mycotoxin (Calvo *et al.,* 2004). The deletion of *veA* decreased production of numerous SMs (Dreyer *et al.,* 2007; Kato *et al.,* 2003; Myung *et al.,* 2009). Similarly, *A. parasiticus* and *A. flavus veA* genes were necessary for the transcription of both *aflR* and *aflJ* (Calvo 2008). Furthermore, VeA can activate production of other secondary metabolites including CPA and aflatrem in *A. flavus* (Amaike and Keller 2009; Duran *et al.,* 2007). Interestingly, although VeA is required for penicillin biosynthesis, over-expression of VeA represses expression of penicillin synthesis genes (Brakhage *et al.,* 2004). Chanda *et al.* (2009) reported a novel role of VeA in *A. parasiticus* where it mediates not only AF gene expression (shown earlier in Kato *et al.*, 2003) but also accumulation of vesicles known as aflatoxisomes which contain AF and AF precursors. Orthologs of VeA have been found in other fungal genera, such as FvVe1 in plant pathogen *F. verticillioides* (Li *et al.,* 2006), AcveA in *Acremonium chrysogenum* (Dreyer *et al.,* 2007), *velA* in *Trichoderma virens* (Mukherjee and Kenerley 2010) and *veA-1* in *N. crassa* (Bayram *et al.,* 2008b) and MVE1 in *Mycosphaerella graminicola* (Choi and Goodwin 2010).

It is now clear that the overlapping properties of *laeA* and *veA* mutations are due to the formation of the Velvet complex (Bayram *et al.,* 2008a). In light conditions, nuclear levels of VeA are low, asexual development (asexual sporulation) is induced and genes involved in ST biosynthesis minimally expressed. By contrast, in dark conditions VeA nuclear levels are high and this protein acts as a bridge between LaeA and VelB to form a heterotrimeric complex that triggers induction of genes involved in SM and sexual development (Bayram *et al.,* 2008a) (Figure 1). Although it is unknown how the Velvet complex identifies, binds to and/or activates SM gene clusters, biochemical analysis of *laeA* and heterochromatin mutants (e.g. histone deacetylase and histone methyltransferase mutants) in *A. nidulans* reveal SM gene expression is associated with removal of heterochromatin marks, which in part require LaeA activity (Bok *et al.,* 2009; Palmer and Keller 2010; Reyes-Dominguez *et al.,* 2010). The Velvet complex has recently been characterized in other fungal species such as *F. fujikuroi* (Wiemann *et al.,* 2010) and *P. chrysogenum* (Hoff *et al.,* 2010). In all genera examined so far (*Aspergillus*, *Penicillium* and *Fusarium*), the Velvet complex couples sporulation processes with SM. Examination of the *F. fujikuroi* Velvet-like complex

revealed that it can simultaneously act as a positive (GAs, fumonisins and fusarin C) or negative (bikaverin) regulator of secondary metabolism.

b) HAP-like CCAAT-binding complex

Another important regulatory complex is the HAP-like CCAAT-binding complex which is evolutionary conserved in eukaryotic organisms (Kato 2005). Different names have been designated for the homologous complexes in various eukaryotic organisms: Hap in *Saccharomyces cerevisiae* (Mcnabb *et al.,* 1995; Pinkham and Guarente 1985), *Kluyveromyces lactis* (Mulder *et al.,* 1994), and *Arabidopsis thaliana* (Edwards *et al.,* 1998), AnCF/PENR1 in *Aspergillus* species (Brakhage *et al.,* 1999), CBF in *Xenopus laevis*, and NF-Y in mammals (Li *et al.,* 1998; Vanhuijsduijnen *et al.,* 1990), respectively. The *A. nidulans* CCAAT-binding factor (AnCF, formerly called PENR1) consists of the subunits HapB, HapC and HapE. All of the three subunits are necessary for DNA binding. AnCF can activate the expression of numerous genes including the anabolic penicillin biosynthesis genes *ipnA* and *aatA* and is necessary for penicillin production in a number of fungal species (Bergh *et al.,* 1996; Litzka *et al.,* 1996; Litzka *et al.,* 1998). Like the Velvet complex, AnCF-mediated repression of gene expression has also been described, e.g. the homoaconitase-encoding *lysF* and the *hapB* gene are repressed by AnCF (Steidl *et al.,* 2001; Weidner *et al.,* 2001). In addition, there is growing evidence that the expression of certain CCAAT-regulated genes requires additional proteins to complex with AnCF. For example, in *A. nidulans*, AnCF represses the transcription of genes that encode iron-containing proteins during iron-replete growth; and during iron-depleted conditions it interacts with the iron-sensing bZIP protein HapX (Hortschansky *et al.,* 2007). The HapX ortholog in *A. fumigatus* is required for full virulence, in part as it regulates siderophore and ribotoxin AspF1 production (Schrettl *et al.,* 2010a).

c) bZIPs

The finding that the bZIP factor HapX is necessary for siderphore regulation by AnCF is intriguing, especially considering two recent findings of two other bZIP proteins involved in SM. Basic leucine zipper (bZIP) proteins are transcription factors that contain a basic region mediating sequence specific DNA-binding which is followed by a leucine zipper region required for bZIP dimerization. In fungi, bZIP transcription factors regulate stress responses, development and morphology as well as multiple metabolic processes.

MeaB, a bZIP transcription factor, was first characterized in *A. nidulans* as affecting nitrogen metabolite repression (Polley and Caddick 1996). Later MeaB was found to tie into AreA function by transcriptionally activating NmrA, an AreA repressor (Wong *et al.,* 2007). As noted above, AreA is associated with regulation of several SM. Wong *et al.,* (2007) showed that MeaB binds the promoter region of *nmrA*, TTGCACCAT, and activates its expression in both nitrogen-sufficient and starved conditions. A direct role for MeaB in regulating a SM cluster comes from work in *F. fujikuroi* where it is negatively associated with the production of bikaverin and GAs in *F. fujikuroi* (Wagner et al., 2010). Deletion of MeaB increases the expression of nitrogen-regulated genes under nitrogen-limiting conditions, such as the GA and bikaverin biosynthesis genes (Wagner et al., 2010). MeaB expression also appears to impact SM expression in *A. nidulans* (Amaike, Yin and Keller unpublished data).

Recently, a putative bZIP protein, RsmA (remediation of secondary metabolism), was identified by using a multicopy-suppressor genetics approach in *A. nidulans* (Shaaban *et al.,* 2010). Overexpression of *rsmA* partially remediates secondary metabolism in Δ*laeA* and Δ*veA* backgrounds and greatly increases SM in strains of *A. nidulans* with an intact Velvet complex. The finding that both RsmA and MeaB affect expression of multiple metabolites

suggests that like Velvet and AnCF, bZIP proteins may be involved in global SM regulation (Shaaban *et al.,* 2010).

d) StuA

The complexes described above also impact sporulation processes of the fungi; it is not surprising therefore to find that transcriptional activators first identified as sporulation regulators affect SM. In particular, the transcriptional activator StuA, first identified in *A. nidulans* as a spatial modifier of conidiphore morphology (Miller *et al.,* 1991), has now been associated with concomitant regulation of SM in several fungal genera. StuA orthologs associated with both developmental processes and SM activation include those in *A. fumigatus* (Sheppard et al., 2005; Twumasi-Boateng et al., 2009), *P. chrysogenum* (Sigl *et al.,* 2010), *Stagonospora nodorum* (Ipcho *et al.,* 2010) and *Fusarium graminearum* (Lysoe *et al.,* 2011). StuA activation is closely allied with BrlA activity, another transcription factor first identified in the regulation of *A. nidulans* conidiphore formation (Adams *et al.,* 1988). In the above studies, BrlA as been found to be important in a subset of StuA regulated SM clusters in *A. fumigatus* but not *P. chrysogenum* (Sigl et al., 2010; Twumasi-Boateng et al., 2009).

V. Genome mining

With the identification of pathway-specific transcription factors, global transcription regulators as well as transcriptional complexes coupled with available genomic sequences, several studies have recently focused on application of this knowledge to mine genomes for SM. Many promising approaches based on chimeric regulation have been developed for the discovery of SMs, principally using variations of microarray analysis as the basis of genomic mining and/or engineering silent SM cluster expression through chimeric promoters, inter-organismal confrontations or chromatin remodeling (Brakhage and Schroeckh 2011; Cichewicz 2010).

a) Microarray approach

More and more available fungal genome sequences make it possible to analyze SM-regulating gene clusters at a genomic level using microarray approach. LaeA-based genomic mining of microarrays analysis from *A. nidulans* Δ*laeA* mutants showed that many SM gene clusters are down-regulated containing the best-characterized fungal SM gene clusters ST and PN clusters as well as some indole alkaloid biosynthesis (Bok *et al.,* 2006b). Conversely, microarray analysis of an overexpression *laeA* strain identified many up-regulated SM clusters. This led to the identification of the anti-tumor compound terrequinone A (Bok *et al.,* 2006b). Consistently, genomic profiling of *laeA* mutants in *A. fumigatus* showed similar regulation effect on SM gene clusters (Perrin *et al.,* 2007). Microarray data revealed that 13 SM gene clusters was influenced by LaeA. Ten of them with a majority of genes within these clusters are significantly down-regulated in Δ*laeA* background. In addition, three additional clusters have at least one gene encoding a critical enzyme such as a nonribosomal peptide synthetase (NRPS) or a polyketide synthase showing decreased expression in Δ*laeA* strain (Perrin *et al.,* 2007). Microarray analysis of *A. flavus* Δ*laeA* and *OE::laeA* strains revealed LaeA to regulate up to 50% of the 55 putative SM clusters in this species (Georgianna *et al.,* 2010). Furthermore, microarray technology was employed for the first time to monitor the selective induction of silent fungal SM genes through bacterial–fungal interactions (Schroeckh *et al.,* 2009). Very recently, a functional genomic profiling of *A. fumigatus* biofilm was developed. From proteome and transcriptome studies, genes and proteins involved in the biosynthesis of the mycotoxin gliotoxin were upregulated in biofilm-grown cultures (Bruns *et al.,* 2010).

b) Promoter activation

As an inducible promoter, the *alcA* promoter (*alcA(p)*) has been broadly applied to activate cryptic gene clusters (Scherlach and Hertweck 2009). Bergmann *et al.,* (2007) found a silent hybrid PKS-NRPS gene cluster in the genome of *A. nidulans* and activated the cluster by driving the cluster transcription factor *apdR* with the *alcA* promoter. This resulted in the production of aspyriones. Northern blot analyses revealed that five other predicted pathway genes (*apdB*, *apdC*, *apdD*, *apdE* and *apdG*) are only expressed upon the *alcA* induction condition (Bergmann *et al.,* 2007). By replacing the original promoter of the regulatory gene *ctnR* with the *alcA* promoter, Chiang *et al.,* (2009b) activated a cryptic gene cluster containing two polyketide synthases (PKS) for asperfuranone biosynthesis. Another silent gene cluster containing two NRPS genes *inpA* and *inpB* was induced by the chimeric *alcA(p)-scpR* construct (Bergmann *et al.,* 2010). The proposed product of *inp* cluster is a tripeptide with a reduced C-terminus. As mentioned earlier, the *alcA(p)-scpR* construct also activated the asperfuranone cluster (Bergmann *et al.,* 2010). Similarly, the *mdp* gene cluster for monodictyphenone biosynthesis was activated by expression of regulatory gene *mdpE* with *alcA(p)* (Chiang *et al.,* 2010).

gdpA(p), a strong constitutive promoter, firstly characterized in *A. nidulans* (Punt *et al.,* 1990) has also been used to increase or activate SM genes. Flaherty and Payne (1997) examined the effects of *aflR* overexpression with *gpdA* promoter on AF production, and expression of AF biosynthetic genes in *A. flavus*. The results showed that AF production greatly increased in *gpdA*(p)::*aflR* strain and transcriptional activities of AF pathway genes *nor-1* and *pksA* also increased dramatically. Chen *et al.,* (2010) expressed the monacolin K transcription factor *mokH* with the *gpdA(p)* promoter in *M. pilosus*. The transcripts of *mokH* in the transformants were expressed at a much higher level than those of the wild-type strain. Consistently, the production of monacolin K in the transformant showed a 1.7-fold higher than the wild-type strain (Chen *et al.,* 2010). Similarly, expression of the bZip protein RsmA under *gpdA(p)* control conditions greatly increases ST production in *A. nidulans* (Shaaban *et al.,* 2010).

c) Fungus-microbe confrontation

Fungi and bacteria co-inhabit a wide variety of environments and their interactions constitute a driving force for SM production (Daniel 2004). Simulating these habitatsby culturing two or more strains together should be a rational way to obtain new SM through their cross-talk. Co-cultivation of two marine microorganisms (Oh *et al.,* 2007), the fungus *Emericella sp.* and the actinomycete *Salinispora arenicola* greatly enhanced the expression of the emericellamids biosynthesis gene cluster which identified in *A. nidulans* by Chiang *et al.* (2008). In addition, Schroeckh *et al.* (2009) co-cultivated *A. nidulans* with a collection of 58 actinomycetes. Using microarray analyses of both *Aspergillus* secondary metabolism and full-genome arrays, in combination with Northern blot and quantitative RT-PCR analyses, they identified a bacterium, *Streptomyces rapamycinicus* that selectively triggered the expression of silent gene clusters under standard laboratory conditions. Another interesting example is identification of biosynthetic gene cluster of mutanobactin A from the oral pathogen *Streptococcus mutans.* Mutanobactin A is capable of suppressing the morphological transition of *Candida albicans* from yeast to mycelium under co-culture conditions (Joyner *et al.,* 2010). Losada *et al.* (2009) demonstrated that co-culture of different *Aspergillus* spp. together resulted in the up-regulation of different SM than when each species was grown alone. Consequently, the concept of interspecies cross-talk leading to regulatory complex and chemical diversity provides a conceptual framework for the elucidation of secondary metabolites and the understanding their cross-talk regulation.

VI. Conclusions

Filamentous fungi are well known prolific producers of low-molecular weight molecules with various biological activities (Hoffmeister and Keller 2007). However, bioinformatic analyses of the sequenced fungal genomes indicate that secondary metabolism gene clusters are silent under standard laboratory conditions and it appears that many “cryptic” SM await discovery (Hertweck 2009). In order to customize the biosynthesis of natural products or activate silent gene clusters we must understand how SMs are regulated. Undoubtly, the research of transcriptional regulatory elements and their functional mechanism plays a major role in secondary metabolism. This review provides a broad view of our understanding of SM regulation mechanism from transcriptional regulatory elements and cluster specific regulatory elements to global regulators and transcription complexes. This would be very helpful to the development of new approaches for discovering new natural products. In combination with genomic mining and microarray approaches, genome sequence availability has led to rapid progress in understanding fungal secondary metabolism regulation by genomic mining or microarray approaches. Many successful approaches in the basis of understanding SM gene regulation have been developed to activate silent or cryptic genes (Brakhage and Schroeckh 2010; Chiang *et al.,* 2009a). These include overexpression of transcription factors or other pleiotropic regulators, promoter exchange and simulation of the natural habitat by co-cultivation of microorganisms. A major future goal is to investigate the molecular mechanisms that interconvert the transcriptional regulatory elements into active and inactive forms and yield unexpected discoveries and significant new insights.

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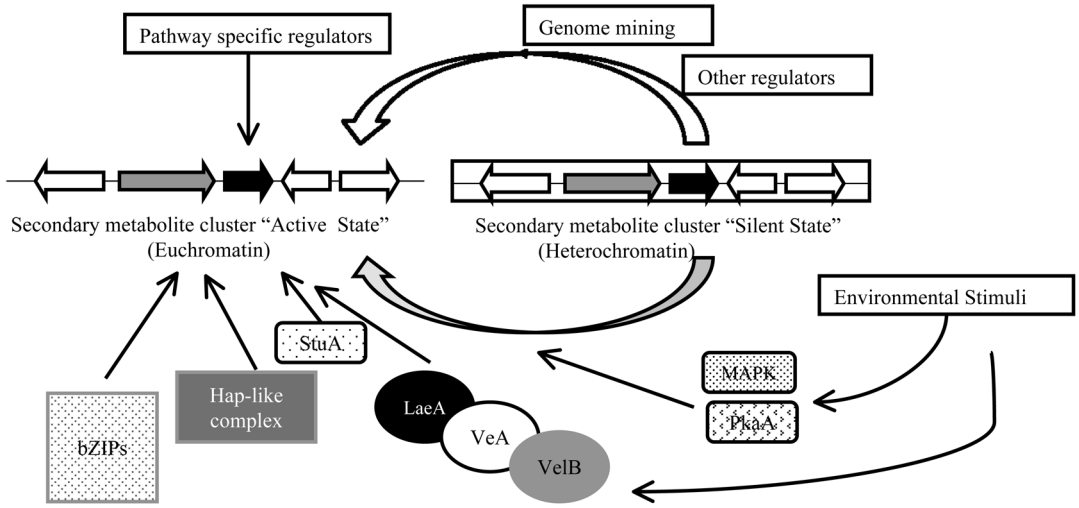

Figure 1.

Regulation of fungal secondary metabolism by transcriptional regulatory elements. SM gene clusters are often silent in laboratory conditions. Activation of SM clusters is complex and involves several layers of transcriptional regulators from pathway specific regulators to more global regulators such as bZip proteins, StuA and Velvet (LaeA, VeA and VelB) and Hap-like complexes. This activation is associated with conversion of heterochromatin marks (silent cluster) to euchromatin marks (active cluster). In addition, environmental stimuli are translated by signal transduction cascades, including MAPK and PkaA, to activate SM synthesis.

Table 1

Examples of identified $Zn(II)_2Cys_6$ transcription factors (TFs) involvement in secondary metabolism in fungi

Protein	Regulated gene cluster	Regulatory mode	Species	Reference
AflR	AF/ST	Positive	*A. nidulans*, *A. flavus A. parasiticus,*	(Brown *et al.,* 1996; Chang *et al.,* 1995b; Ehrlich *et al.,* 1999; Fernandes *et al.,* 1998; Yu *et al.,* 1996)
GliZ	gliotoxin	Positive	*A. fumigatus*	(Bok *et al.,* 2006a)
SirZ	sirodesmin PL	Positive	*Leptosphaeria maculans*	(Fox *et al.,* 2008)
MlcR	compactin	Positive	*Penicillium citrinum*	(Abe *et al.,* 2002b)
Bik5	bikaverin	Positive	*Fusarium fujikuroi*	(Wiemann *et al.,* 2009)
DEP6	depudecin	Positive	*Alternaria brassicicola*	(Wight *et al.,* 2009)
ZFR1 FUM21	fumonisin	Positive	*Fusarium verticillioides*	(Brown *et al.,* 2007; Flaherty and Woloshuk 2004)
CTB8	cercosporin	Positive	*Cercospora nicotianae*	(Chen *et al.,* 2007)
GIP2	aurofusarin	Positive	*Gibberella zeae*	(Kim *et al.,* 2006)
CtnA	citrinin	Positive	*Monascus purpureus*	(Shimizu *et al.,* 2007)
LovE	lovastatin	Positive	*A. terreus*	(Huang and Li 2009; Kennedy *et al.,* 1999)
ApdR	aspyridone	Positive	*A. nidulans*	(Bergmann *et al.,* 2007)
CtnR	asperfuranone	Positive	*A. nidulans*	(Chiang *et al.,* 2009b)
MdpE	monodictyphenone/ emodin analogs	Positive	*A. nidulans*	(Chiang *et al.,* 2010)
Cmr1p	melanin	Positive	*Colletotrichum lagenarium*	(Tsuji *et al.,* 2000)
Pig1p	melanin	Positive	*Magnaporthe grisea*	(Tsuji *et al.,* 2000)
MokH	monacolin K	Positive	*Monascus pilosus*	(Chen *et al.,* 2010)