

Chapter 2

Key Players in the Regulation of Fungal Secondary Metabolism

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Introduction

Beyond their environmental ubiquity and critical roles in nearly every ecological niche as primary decomposers, fungi are well known for producing a wealth of low molecular weight molecules called secondary metabolites, which are also known as natural products. Although the true ecological function of most secondary metabolites (SMs) is still unknown, their roles as biotic and abiotic protectants or defensive metabolites is emerging [1–4]. Furthermore, their significant impact on human well-being, both positive and negative, makes them attractive study targets.

With a broad spectrum of biological activity, SMs can have major influences on human health. For example, subsets of SMs known as mycotoxins are responsible for millions of dollars in crop loss annually just in the USA alone [5]. When mycotoxin contamination of consumables goes undetected, the resulting mycotoxicoses have additional health and economic consequences, which has been documented throughout recorded history [6]. Crop losses and health consequences are especially devastating in developing countries where testing for toxin contamination is either not well established or is nonexistent [7]. Conversely, the diverse pharmacodynamics of medically relevant SMs such as the β (beta)-lactam antibiotics penicillin and cephalosporin, and the popular cholesterol-lowering agent lovastatin, are examples of how fungal SMs have had positive impacts on human well-being.

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S. Zeilinger et al. (eds.), *Biosynthesis and Molecular Genetics of Fungal Secondary Metabolites*, Volume 2, Fungal Biology, DOI 10.1007/978-1-4939-2531-5_2

At the genomic level, one of the defining hallmarks of SM is the grouping of biosynthetic genes into discreet clusters [8] and general localization to subtelomeric regions of the chromosome [9]. Many, but not all, clusters contain cluster-specific transcription factors that regulate expression of the biosynthetic genes for their respective metabolites. This clustering, coupled with unique chromosomal location, allows for multiple regulatory layers giving the producing fungus precise spatial and temporal control over metabolite expression and likely contributes to intra-, and possibly inter-kingdom, horizontal cluster transfer [10–12]. Additionally, SM production is often tightly correlated with growth and development [13], and often-times disruption of one process will have a significant effect on the other.

The study of fungal SMs has established several model fungal systems and particular metabolites with facile laboratory characterization allowing clear study of their expression and regulation. Yet, as an increasing number of fungal genomes become available, it has become clear that the number of metabolites observed under routine laboratory conditions is a paltry representation of the theoretic yield based on predicted *in silico* genomic regions containing signature SM biosynthetic cluster motifs [14]. Resulting from these observations, endeavors to unlock these “cryptic” gene clusters have shed light on SM regulators working at the level of individual metabolites or at a larger, global scale.

In this chapter, we review the general mechanisms of key players in the regulation of fungal SM. As will become evident in the following paragraphs, fungal SM regulation does not follow a strict hierarchical regime and is composed of overlapping and interconnected pathways making regulatory classifications of regulators less amenable to simple progressional delineations. As such, we will group and present key players based on the largely cluster-specific $\text{Zn(II)}_2\text{Cys}_6$ family of transcription factors, then investigate additional families that possess more pleiotropic regulatory characteristics, and finish with global regulators and multiprotein complexes that respond to environmental cues.

$\text{Zn(II)}_2\text{Cys}_6$

The grouping of SM biosynthetic genes into discreet and contiguous clusters is a distinguishing feature of SM in fungi [8]. In-cluster transcription factors are commonly found that exhibit regulatory control over their respective cluster’s transcription of biosynthetic genes. As the production and regulation of SMs in fungi are replete with diversity, it is not surprising that multiple families of transcription factors regulate these processes. Therefore, we will begin with the largest cluster-specific family of fungal transcription factors (Table 2.1) [15–35].

Unique to fungi are the $\text{Zn(II)}_2\text{Cys}_6$ family of transcription factors. Some distinguishing features of these proteins are the binding of two zinc atoms to six cysteine residues and their ability to bind DNA as monomers, homodimers, or heterodimers [36]. Common nomenclatures for $\text{Zn(II)}_2\text{Cys}_6$ transcription factors include: C_6 , Zn_2C_6 , zinc binuclear cluster, as well as zinc cluster. Compared to other families of transcription factors, $\text{Zn(II)}_2\text{Cys}_6$ proteins are the most common family of dedicated cluster regulators for fungal SM.

Table 2.1 Examples of $\text{Zn(II)}_2\text{Cys}_6$ cluster-specific transcription factors (adapted from Yin and Keller 2011 [15])

Transcription factor	Target cluster	Species	Source
AflR	Aflatoxin/ sterigmatocystin	<i>Aspergillus nidulans</i> <i>Aspergillus flavus</i> <i>Aspergillus parasiticus</i>	Brown et al. 1996 [16] Woloshuk et al. 1994 [17] Chang et al. 1995 [18]
AfoA	Asperfuranone	<i>Aspergillus nidulans</i>	Chiang et al. 2009 [19]
ApdR	Aspyridone	<i>Aspergillus nidulans</i>	Bergmann et al. 2007 [20]
Bik5	Bikaverin	<i>Fusarium fujikuroi</i>	Wiemann et al. 2009 [21]
CTB8	Cercosporin	<i>Cercospora nicotianae</i>	Chen et al. 2007 [22]
CtnA	Citrinin	<i>Monascus purpureus</i>	Shimizu et al. 2007 [23]
CtnR	Asperfuranone	<i>Aspergillus nidulans</i>	Chiang et al. 2009 [19]
DEP6	Depudecin	<i>Alternaria brassicicola</i>	Wight et al. 2009 [24]
FapR	Fumagillin/pseurotin	<i>Aspergillus fumigatus</i>	Wiemann et al. 2013 [25]
FUM21	Fumonisin	<i>Fusarium verticillioides</i>	Brown et al. 2007 [26]
GIP2	Aurofusarin	<i>Gibberella zeae</i>	Kim et al. 2006 [27]
GliZ	Gliotoxin	<i>Aspergillus fumigatus</i>	Bok et al. 2006b [28]
LovE	Lovastatin	<i>Aspergillus terreus</i>	Kennedy et al. 1999 [29]
MdpE	Monodictyphenone	<i>Aspergillus nidulans</i>	Chiang et al. 2010 [30]
MlcR	Compactin	<i>Penicillium citrinum</i>	Abe et al. 2002b [31]
MokH	Monacolin K	<i>Monascus pilosus</i>	Chen et al. 2010 [32]
ORFR	AK-toxin	<i>Alternaria alternata</i>	Tanaka and Tsuge 2000 [33]
SirZ	Sirodesmin PL	<i>Leptosphaeria maculans</i>	Fox et al. 2008 [34]
ZFR1	Fumonisin	<i>Fusarium verticillioides</i>	Flaherty and Woloshuk 2004 [35]

To date, the most well-characterized cluster-specific transcription factor is the aflatoxin (AF)/sterigmatocystin (ST) regulator AflR. As the paradigm for $\text{Zn(II)}_2\text{Cys}_6$ -mediated cluster regulation, AflR has unequivocally established the importance of studying transcriptional regulators in the context of SM expression and regulation. Deletion of *aflR* in *Aspergillus nidulans* produced a condition in which ST production was suppressed at the transcriptional level, even under ST-stimulating conditions [37] and, conversely, increasing expression of *aflR* in normally non-AF producing conditions resulted in concomitant expression of AF biosynthetic genes [18].

Many of the SM clusters characterized in *A. nidulans* contain $\text{Zn(II)}_2\text{Cys}_6$ proteins including AfoA and CtnR, ApdR, and MdpE required for asperfuranone, aspyridone, and monodictyphenone gene expression, respectively [19, 20, 30].

Overexpression of $\text{Zn(II)}_2\text{Cys}_6$ proteins can be sufficient to activate silent SM clusters as was first reported for ApdR, thus representing a critical proof-of-principle for fungal genome mining in the postgenomic era [20]. This technique, however, does not always work, resulting in the development of alternative methods to bypass $\text{Zn(II)}_2\text{Cys}_6$ regulation altogether and target individual keystone SM genes [38]. Cluster-specific $\text{Zn(II)}_2\text{Cys}_6$ transcription factors regulating SM production in plant pathogenic fungi include CTB8 for cercosporin, DEP6 for depudecin, FUM21 and ZFR1 for fumonisin, GIP2 for aurofusarin, ORFR for AK-toxin, and SirZ for sirodesmin PL in *Cercospora nicotianae*, *Alternaria brassicicola*, *Fusarium verticillioides*, *Gibberella zeae*, *Alternaria alternata*, and *Leptosphaeria maculans*, respectively [22, 24, 26, 27, 33–35]. Additionally, Bik5 controls synthesis of the mycelial pigment bikaverin in *Fusarium fujikuroi* [21]. Understanding $\text{Zn(II)}_2\text{Cys}_6$ cluster regulation is also critical in biotechnologically relevant fungi given that this family of transcription factors can also control production of the important antihypercholesterolemic agents lovastatin and monacolin K, as well as the pravastatin sodium precursor compactin, regulated by LovE, MokH, and MlcR in *Aspergillus terreus*, *Monascus pilosus*, and *Penicillium citrinum*, respectively [29, 31, 32, 39]. Recently, an interesting new twist to the canonical view of a $\text{Zn(II)}_2\text{Cys}_6$ regulating a single SM came with the finding of the transcription factor FapR that simultaneously regulates biosynthetic genes of the intertwined fumagillin and pseurotin cluster in *Aspergillus fumigatus* [25]. Table 2.1 provides a few additional examples of these types of transcription factors [15–35].

CYS₂HIS₂

Common to fungi (Table 2.2) and all other eukaryotes are the Cys₂His₂ family of transcription factors [21, 40–69]. Defining features of this family include two or more of the conserved, repeating amino acid zinc finger units that bind a single zinc atom [36]. Cys₂His₂ proteins bind DNA as monomers and can also be referred to as C₂H₂ and classical zinc finger transcription factors.

For surviving a multitude of environmental challenges, fungi often require structural pigments for withstanding biotic and abiotic stresses [70]. A unique group of orthologous Cys₂His₂ transcription factors has been found in several plant pathogenic fungi, all positively regulating biosynthesis of the structural pigment melanin. The proteins Cmr1p, Pig1p, Cmr1, and BMR1 are found in *Colletrichum lagenarium*, *Magnaporthe grisea*, *Cochliobolus heterostrophus*, and *Bipolaris oryzae*, respectively [43, 45, 46]. In addition to two Cys₂His₂ motifs, these four proteins also possess a $\text{Zn(II)}_2\text{Cys}_6$ sequence. The earliest recorded examples of SM regulation by Cys₂His₂ proteins are MRTRI6 and Tri6 regulating trichothecene mycotoxin gene clusters in the plant pathogenic fungi *Myrothecium roridum* and *Fusarium sporotrichioides*, respectively [50, 58]. In one of the few cases of an SM-specific regulator gene lying outside of the cluster it controls, the Cys₂His₂ transcription factor ScpR on chromosome II was shown to activate the asperfuranone cluster in

Table 2.2 Transcription factor families and their regulated metabolite(s)

Regulator	Metabolite(s)	Species	Source
Cys ₂ His ₂			
AreA	AF gibberellin fumonisin	<i>Aspergillus parasiticus</i> <i>Gibberella fujikuroi</i> <i>Fusarium verticillioides</i>	Chang et al. 2000 [40] Mihlan et al. 2003 [41] Kim and Woloshuk 2008 [42]
BMR1	Melanin	<i>Bipolaris oryzae</i>	Kihara et al. 2008 [43]
BcYOH1	Botrydial/botcinic acid	<i>Botrytis cinerea</i>	Simon et al. 2013 [44]
Cmr1	Melanin	<i>Cochliobolus heterostrophus</i>	Eliahu et al. 2007 [45]
Cmr1p	Melanin	<i>Colletrichum lagenarium</i>	Tsuji et al. 2000 [46]
CreA	Flavipucine cephalosporin	<i>Aspergillus terreus</i> <i>Acremonium chrysogenum</i>	Gressler et al. 2011 [47] Jekosch and Kück 2000 [48, 49]
MRTR16	Trichothecene	<i>Myrothecium roridum</i>	Trapp et al. 1998 [50]
PacC	Penicillin ST/AF cephalosporin bikaverin fumonisins gluconic acid	<i>Aspergillus nidulans</i> <i>A. nidulans/A. parasiticus</i> <i>Acremonium chrysogenum</i> <i>Fusarium fujikuroi</i> <i>Fusarium verticillioides</i> <i>Penicillium expansum</i>	Bergh and Brakhage 1998 [51] Keller et al. 1997 [52] Schmitt et al. 2001 [53] Wiemann et al. 2009 [21] Flaherty et al. 2003 [54] Barad et al. 2013 [55]
Pig1p	Melanin	<i>Magnaporthe grisea</i>	Tsuji et al. 2000 [46]
Sda1	Fumonisin B ₁	<i>Fusarium verticillioides</i>	Malapi-Wight et al. 2013 [56]
ScpR	Asperguranone	<i>Aspergillus nidulans</i>	Bergmann et al. 2010 [57]
Tri6	Trichothecene	<i>Fusarium sporotrichioides</i>	Hohn et al. 1999 [58]
bZip			
Aoyap1	Ochratoxin	<i>Aspergillus ochraceus</i>	Reverberi et al. 2012 [59]
AtfB	AF	<i>Aspergillus parasiticus</i>	Roze et al. 2011 [60]
HapX	Ferricrocin	<i>Aspergillus nidulans</i>	Eisendle et al. 2006 [61]
MeaB	Bikaverin AF	<i>Fusarium fujikuroi</i> <i>Aspergillus flavus</i>	Wagner et al. 2010 [62] Amaike et al. 2013 [63]
RsmA	ST/asperthecin gliotoxin	<i>Aspergillus nidulans</i> <i>Aspergillus fumigatus</i>	Yin et al. 2012 [64] Sekonyela et al. 2013 [65]
ToxE	HC-toxin	<i>Cochliobolus carbonum</i>	Bussink et al. 2001 [66]; Pedley and Walton 2001 [67]
Winged helix			
AcFKH1	Cephalosporin C	<i>Acremonium chrysogenum</i>	Schmitt et al. 2004 [68]
CPCR1	Cephalosporin C	<i>Acremonium chrysogenum</i>	Schmitt and Kuck 2000 [69]

A. nidulans, likely through binding the promoter region of *afoA*, embedded in the asperfuranone cluster located on chromosome VIII [57].

Recently, the characterization of two new Cys₂His₂ transcription factors has shown regulation of other physiological processes beyond SM. Exhibiting positive regulation over the toxins botrydial and botcinic acid in *Botrytis cinerea*, BcYOH1 exhibited a more global regulatory role as it also affects mechanisms in detoxification, virulence, and carbohydrate metabolism [44], whereas *sda1* knockout strains of *F. verticillioides* had excessive fumonisin B₁ biosynthesis, reduced capacity to form conidia, and an inability to grow on selected carbon sources [56].

bZIP

Found in all eukaryotic organisms, basic leucine zipper (bZIP) transcription factors are characterized by basic and leucine zipper regions. The basic region dictates sequence-specific DNA-binding whereas the leucine zipper region mediates dimerization of the protein. As dimers, bZIPs target palindromic DNA sequences. Many fungal bZIPs (Table 2.2 [21, 40–69]) have been characterized as stress response transcription factors, responding to a variety of environmental stresses that appears to link them to SM production [60, 71, 72]. bZIPs associated with SM regulation and stress include RsmA regulating sterigmatocystin and asperthecin in *A. nidulans* and gliotoxin in *A. fumigatus* [64, 65], AtfB regulating aflatoxin in *Aspergillus parasiticus* [60], and Aoyap1 regulating ochratoxin in *Aspergillus ochraceus* [59]. The bZIP protein MeaB, involved in nitrogen regulation, has also been associated with regulation of SMs including bikaverin in *F. fujikuroi* and AF in *A. flavus* [62, 63].

In the plant pathogen *Cochliobolus carbonum*, HC-toxin biosynthetic genes are regulated by the hybrid bZIP/ankyrin repeat transcription factor ToxE [73]. Despite having the basic region characteristic of bZIPs, ToxE lacks the leucine zipper sequence but possesses four ankyrin repeats. Both the basic region and ankyrin repeat region mediate DNA binding to promoter regions of all HC-toxin biosynthesis genes [67]. ToxE, together with the putative transcription factor Bap1 from the tomato pathogen *Cladosporium fulvum*, represent a potentially novel class of fungal-specific hybrid transcription factors possessing bZIP and ankyrin repeat characteristics [66].

Winged Helix

Winged helix proteins are found in all organisms and belong within the general helix-turn-helix structural group of proteins. Broadly speaking, the structure of a winged helix protein consists of two wings, three α (alpha) helices, and three β (beta) strands [74]. The industrially relevant fungus *Acremonium chrysogenum* is well

known for production of the antibiotic cephalosporin C. Cluster-specific regulation of cephalosporin C production in *A. chrysogenum* was first shown to be controlled by the transcription factor CPC1, which belongs to the winged helix subfamily of regulatory factor X (RFX) proteins [69]. Another regulator of cephalosporin C, AcFKH1 belongs to a subfamily of winged helix proteins possessing a fork-head associated domain (FHA) and a forkhead DNA-binding domain (FKH) [68]. Consistent with the observation that SM production is often inextricably linked to morphological development, it was shown that CPC1 is not only required for cephalosporin C production, but also for the formation of arthrospores and whereas *AcFKH1* deletion strains still retained the ability to form arthrospores despite possessing swollen and highly septate hyphae [75]. To the best of our knowledge, cephalosporin C is the only fungal SM currently known to be regulated by winged helix proteins (Table 2.2 [21, 40–69]).

Global Regulators

AreA—Nitrogen

So far we have reviewed transcription factors largely characterized as cluster-specific and will now move into global regulators that translate environmental cues into SM and concomitant physiological responses. In *A. nidulans* an increase in ST, and an increase in the rate of sexual development, is observed when grown on nitrate, whereas an opposite response for both phenotypes is observed on ammonium media [76]. Conversely, ammonium stimulates AF biosynthesis in *A. parasiticus* and nitrate inhibits its production [77]. Comparing the SM response to varying nitrogen sources between these two *Aspergillus* species suggests a highly dynamic interplay between environment, SM adaptation, and developmental regime.

Among fungi, the highly conserved global transcription factor AreA is responsible for repression of nitrogen metabolism in the presence of glutamine or ammonium, and is a member of the GATA family of transcription factors, which are conserved among eukaryotes and are characterized by their Cys₂Hys₂ zinc finger DNA binding domains [78]. Beyond its regulatory role in primary metabolism, AreA modulates morphological development and SM regulation in filamentous fungi and is likely responsible for the aforementioned species-specific mycotoxin responses based on the observation of multiple GATA sequences in the aflatoxin and sterigmatocystin regulatory genes *aflR* and *aflJ*, and subsequent AreA binding to these regions in *A. parasiticus* [40]. The rice pathogen *Gibberella fujikuroi* exhibits decreased transcript levels for nearly all structural genes for the SM gibberellin in an *areA* deletion mutant, showing direct regulation of this cluster by AreA [41]. Additionally, AreA in the maize pathogen *F. verticillioides* is a positive regulator of the toxic SM fumonisin [42].

PacC—pH

Beyond nutritional requirements, another environmental parameter essential for growth and development is pH. The global regulator PacC is a conserved Cis_2His_2 zinc finger transcription factor among fungi [79], capable of regulating a suite of physiological processes, including SM production, in response to environmental pH [80]. Studies investigating PacC regulation of SM have shown that this alkali-activated transcription factor dynamically controls metabolites to be expressed in a pH environment most suitable for compound bioactivity and maximum niche exploitation [81]. In *A. nidulans*, penicillin production was shown to be increased in an alkaline environment [51, 82], possibly as an ecological adaptation by the fungus to outcompete increased bacterial competition in high pH environments [81]. Also in *A. nidulans*, and in contrast to penicillin production, ST synthesis was repressed by alkaline pH as was AF synthesis in *A. parasiticus* [52]. Expression of another important β (beta)-lactam, cephalosporin, by *A. chrysogenum* was shown to be regulated by PacC through binding to promoter regions of structural genes [53]. Expression of the mycelial pigment bikaverin from the rice pathogen *F. fujikuroi* also exhibits PacC regulation through inhibition of its synthesis in a high pH environment [21]. Production of the mycotoxin fumonisin is also downregulated under elevated pH conditions by the maize pathogen *F. verticillioides* [54]. Interestingly, a recent study provides evidence for a dynamic role of PacC in tree fruit spoilage by the phytopathogen *Penicillium expansum* such that mechanisms of acidification after initial colonization, via secretion of organic acids such as gluconic acid, are mediated by PacC to control subsequent expression of the mycotoxin patulin [55]. Like other strains deficient in global regulators of SM, PacC deletion strains exhibit developmental phenotypes such as reduced conidiation [79] or growth inhibitions [21, 54], illustrating a pivotal role for pH sensing and homeostasis in proper growth, development, and SM expression.

CreA—Carbon

When grown in media rich in glucose, filamentous fungi downregulate genes required for metabolizing other carbon sources via a phenomenon called carbon catabolite repression [83], mediated largely by the Cys_2His_2 zinc finger transcription factor CreA [84]. Some examples of CreA-mediated regulation of SM include an overproducing cephalosporin strain of *A. chrysogenum*, obtained by classical mutagenesis techniques, which appeared to have deregulation of the CreA homologue Cre1 compared to wild-type strains [48]. Additionally, putative Cre1 binding sites found within the promoter regions of two cephalosporin biosynthetic genes suggest carbon catabolite repression of this metabolite [49]. In *A. nidulans*, glucose represses antibiotic production independent of a mutated CreA binding site in the penicillin biosynthetic gene *ipnA* promoter region [82] and was still unaffected by a CreA mutant background [85]. Although the role of CreA in regulating SM is less

clear-cut compared to AreA and PacC, it is important to acknowledge the role of this global regulator in environmental sensing and the concomitant physiological response and consider how SMs might be affected. For example, the stringency of glucose repression, likely mediated by CreA, on a cryptic SM cluster in *A. terreus* was recently shown to be insurmountable even by overexpression of a putative in-cluster transcription factor [47].

Velvet Complex—Light

Like most organisms, filamentous fungi sense and respond to light. Conserved throughout the filamentous and dimorphic ascomycetes and possibly basidiomycetes [86] is the light-sensing heterotrimeric velvet complex consisting of LaeA, and the velvet proteins VeA and VelB [87]. The velvet complex links sexual development with SM production in response to light and accomplishes this through tightly regulated spatial compartmentalization of velvet complex components. VelB has nuclear and cytoplasmic localization regardless of illumination status [87], whereas VeA is cytoplasmic under light conditions and migrates to the nucleus in the absence of light [88] as a heterodimer with VelB, and LaeA is constitutively nuclear [89]. Consequently, the three velvet complex proteins are only colocalized to the nucleus under dark conditions, allowing formation of the fully functional heterotrimeric velvet complex. The mechanism of velvet complex regulation of SMs is best studied in *A. nidulans* but increasingly well known in other fungi [90–97]. Once assembled in the nucleus of *A. nidulans*, the velvet complex drives sexual development and production of SMs, whereas these processes are repressed under illuminating conditions resulting from a dissociated velvet complex [81]. Consequently, when localization of members of this complex is compromised, such as with VeA via interacting with the newly described LaeA-like methyltransferase LlmF, neither sexual development nor SM production can proceed normally [98]. The LlmF mechanism of VeA control appears conserved in other ascomycetes as well [99].

The finding that LaeA exhibited some similarity to methyltransferases involved in histone modification, coupled with the observation that SM clusters were most frequently found in subtelomeric regions of the genome [9], led to the hypothesis that SM clusters could be under epigenetic regulation [100]. This indeed has proved to be the case and for detailed coverage of histone modifications and their influence on SM production, we direct the reader to Chap. 3 for chromatin-based regulation of secondary metabolism.

CBC—Iron

Beyond the velvet complex, which is required for coordinating sexual development and SM production in response to light, another well-characterized global regulatory complex in filamentous fungi is the CCAAT-binding complex (CBC). Unlike velvet

components, which are only conserved among filamentous fungi, CBC complexes are found in all eukaryotic organisms [101]. AnCF, the CBC in *A. nidulans* formerly known as PENR1 [101] is composed of the proteins HapB, HapC, and HapE. The AnCF is a positive regulator of genes required for penicillin biosynthesis including *ipnA* and *aataA* [102], is a negative autoregulator of *hapB* [103], and coordinates physiological processes in response to cellular redox state [104] and environmental iron [105]. With respect to iron depletion, CBC-mediated upregulation of *sidC* in *A. nidulans* is dependent upon physical interaction with the bZIP protein HapX [105] with SidC being a core enzyme for synthesis of ferricrocin, an SM siderophore that is essential for intracellular iron homeostasis and morphological development [61].

Conclusion

The study of SM in fungi originated primarily for two reasons: (1) to understand the regulation of mycotoxin gene clusters with the goal of using this knowledge to ameliorate the deleterious costs of crop contamination with mycotoxins and (2) to identify compounds with novel bioactivities applicable for pharmaceutical, medicinal, and agricultural uses. Since then, affecting all aspects of fungal biology, the integrated and critical roles of SMs have emerged as being greater than anticipated. SM regulators originally characterized as cluster-specific transcription factors have later been shown to have regulatory functions beyond their native cluster. For example, the canonical AF/ST regulator and $\text{Zn(II)}_2\text{Cys}_6$ transcription factor *aflR* was shown in *A. parasiticus* to regulate several genes beyond the defined boundaries of the cluster [64, 106]. Additionally, the Cys_2Hys_2 transcription factor Tri6 from *F. graminearum* was shown to regulate 192 genes, ranging from central metabolism to virulence, beyond the known targets within the trichothecene gene cluster [107], leading the authors to propose characterizing Tri6 as a global regulator much like the other known global Cys_2Hys_2 regulators AreA, PacC, and CreA.

Beyond expanding the roles of well-established regulators, research aimed toward elucidating new biosynthetic pathways and compounds has also benefited from exploiting the tight interplay of primary and SM. In one particular study, carbon catabolite repression was so stringent over a cryptic cluster, the authors utilized a suite of growth media containing various carbon sources to define nutrient conditions favorable for cluster activation, eventually identifying the compounds hydroisoflavipucic acid and dihydroisoflavipucic acid [47].

One of the greatest challenges in studying fungal SM will be to make sense of how key regulators fit into an ever-expanding network of global interactions linking primary and secondary metabolism with growth and development and how this modulates in response to nutrient availability, biotic and abiotic stresses, and niche exploitation. Taken together, it is apparent that ongoing and future studies will necessarily approach fungal SMs not from the standpoint that they are “accessory” molecules, but rather integrated members of a complex metabolic network affecting every facet of fungal biology.

Biosynthesis and Molecular Genetics of Fungal

Secondary Metabolites, Volume 2

Zeilinger, S.; Martín, J.-F.; García-Estrada, C. (Eds.)

2015, XI, 256 p. 35 illus., 14 illus. in color., Hardcover

ISBN: 978-1-4939-2530-8