Regulation and Role of Fungal Secondary Metabolites

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Abstract

Fungi have the capability to produce a tremendous number of so-called secondary metabolites, which possess a multitude of functions, e.g., communication signals during coexistence with other microorganisms, virulence factors during pathogenic interactions with plants and animals, and in medical applications. Therefore, research on this topic has intensified significantly during the past 10 years and thus knowledge of regulatory mechanisms and the understanding of the role of secondary metabolites have drastically increased. This review aims to depict the complexity of all the regulatory elements involved in controlling the expression of secondary metabolite gene clusters, ranging from epigenetic control and signal transduction pathways to global and specific transcriptional regulators. Furthermore, we give a short overview on the role of secondary metabolites, focusing on the interaction with other microorganisms in the environment as well as on pathogenic relationships.

INTRODUCTION

In nature, fungi are challenged by multiple biotic and abiotic stressors, ranging from other microorganisms to nutrient deprivation to pH and temperature. As one physiological response, they produce a vast number of secondary metabolites (SMs). The group of SMs is heterogeneous and consists of substances with low molecular weight characteristically produced by large multimodular polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) or enzymes such as prenyltransferases and dimethylallyl tryptophan synthases (19). The enormous structural diversity of these compounds also explains their broad spectrum of activities and functions. Of special interest is the role of SMs during the interaction with other organisms. Here, they fulfill very different functions, such as mediating communication within one species or between different species defense against competitors, nutrient acquisition, and even symbiotic interactions (137). For pathogenic interactions, SMs play crucial roles as virulence factors, which can be observed for fungi infecting animals as well as plants. Understanding the exact functions and mechanisms of action of those virulence factors might give an opportunity to effectively combat fungal infections. Not only does the role of SMs in pathogenicity make them interesting to study, but many SMs, including penicillins, statins, and cyclosporines, have been found to have medical applications (19). This is why the identification of new SMs is a fast growing research area. Advances in this field are often achieved by activating silent SM gene clusters because genomic approaches have revealed the presence of a high number of clusters in different fungal species, whereas only relatively few metabolites have been elucidated at present (19, 127). **Figure 1** illustrates this fact by showing the chromosomal location of SM gene clusters in the model ascomycete fungus *Aspergillus nidulans*. Sixty-eight clusters are distributed on the eight chromosomes, but products are known for only 20 of them.

For the activation of silent gene clusters and the identification of new SMs, a detailed knowledge of the mechanisms regulating SM gene clusters is essential. Many different strategies to induce the production of new compounds were developed and are extensively reviewed elsewhere (20, 84, 96, 126). Often they are based on mimicking the natural environment of the fungus by changing abiotic conditions, such as pH or temperature, or by confrontation with other microorganisms occurring in the habitat. Other approaches directly target regulatory mechanisms at different levels, such as signal transduction pathways, global regulators, cluster-specific transcription factors (TFs), or even epigenetic mechanisms.

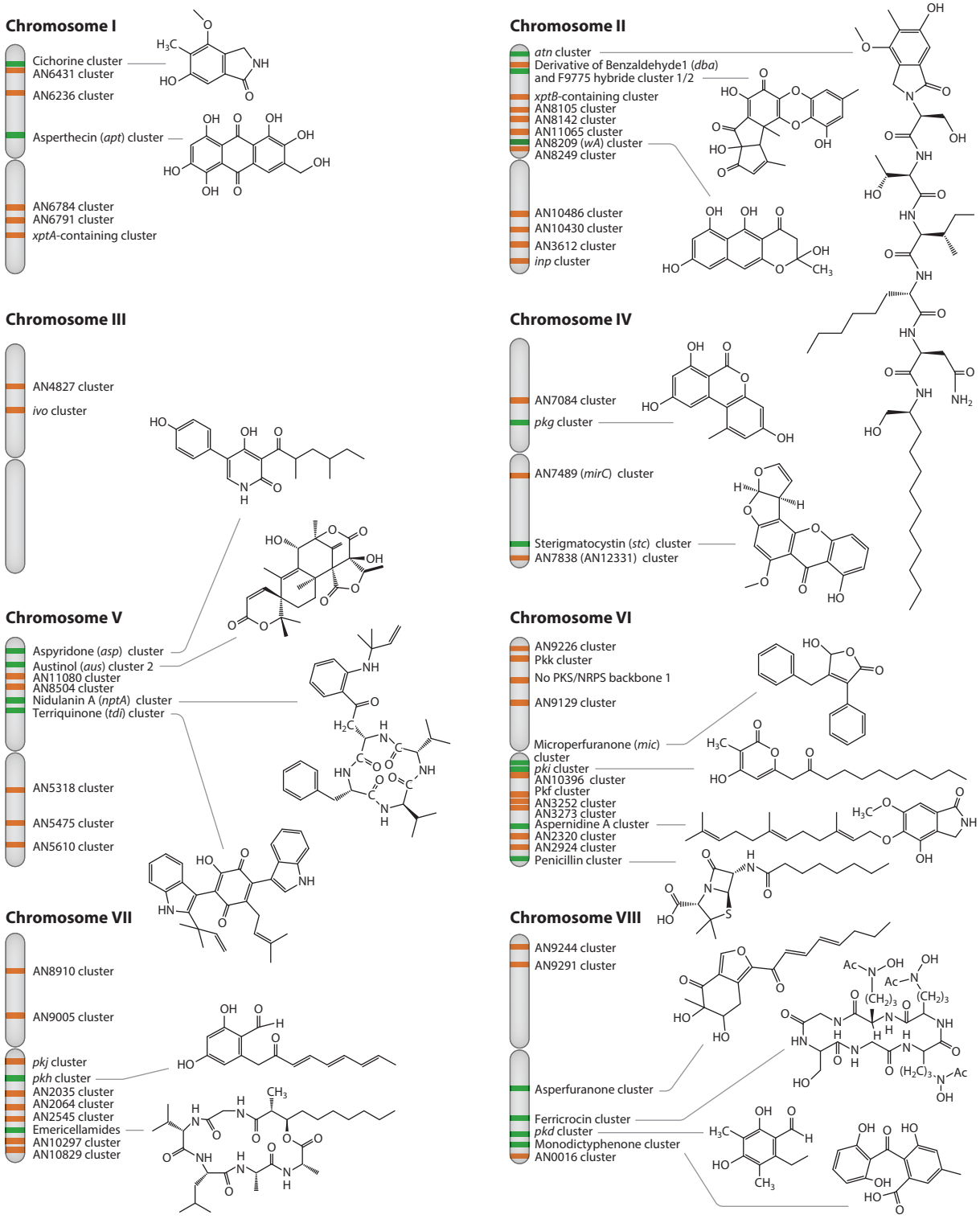
This review aims to deepen the understanding of the complex and multilayered regulation of fungal secondary metabolism and gives examples of the important roles that SMs play during interactions with other organisms.

REGULATION OF FUNGAL SECONDARY METABOLISM

The regulation of fungal secondary metabolism is very complex and operates on different regulatory levels, including pathway-specific and global regulators, signal transduction pathways, and epigenetic control. The following section explains these regulatory mechanisms in detail and highlights their impact on SM formation.

Figure 1

Location of *Aspergillus nidulans* secondary metabolite (SM) gene clusters on the fungal chromosomes (*gray*). Clusters with identified products are labeled in green, with the structure of the compound adjacent to them; all other predicted clusters are depicted in orange. Abbreviations: NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase.


Chromosome I
Cichorine cluster
AN6431 cluster
AN6236 cluster
Asperthecin (apt) cluster
AN6784 cluster
AN6791 cluster
xptA-containing cluster
Chromosome II
atn cluster
Derivative of Benzaldehyde1 (dba)
and F9775 hybride cluster 1/2
xptB-containing cluster
AN8105 cluster
AN8142 cluster
AN11065 cluster
AN8209 (wA) cluster
AN8249 cluster
AN10486 cluster
AN10430 cluster
AN3612 cluster
inp cluster
Chromosome III
AN4827 cluster
ivo cluster
Chromosome IV
AN7084 cluster
pkg cluster
AN7489 (mirC) cluster
Sterigmatocystin (stc) cluster
AN7838 (AN12331) cluster
Chromosome V
Aspyridone (asp) cluster
Austinol (aus) cluster 2
AN11080 cluster
AN8504 cluster
Nidulanin A (nptA) cluster
Terriquinone (tdi) cluster
AN5318 cluster
AN5475 cluster
AN5610 cluster
Chromosome VI
AN9226 cluster
Pkk cluster
No PKS/NRPS backbone 1
AN9129 cluster
Microperfuranone (mic) cluster
pki cluster
AN10396 cluster
Pkf cluster
AN3252 cluster
AN3273 cluster
Aspernidine A cluster
AN2320 cluster
AN2924 cluster
Penicillin cluster
Chromosome VII
AN8910 cluster
AN9005 cluster
pkj cluster
pkh cluster
AN2035 cluster
AN2064 cluster
AN2545 cluster
Emericellamides
AN10297 cluster
AN10829 cluster
Chromosome VIII
AN9244 cluster
AN9291 cluster
Asperfuranone cluster
Ferricrocin cluster
pkd cluster
Monodictyphenone cluster
AN0016 cluster

Global and Pathway-Specific Regulation

Fungal SM regulation does not follow a strict hierarchical regime. Interconnected and even overlapping pathways exist. Whereas global regulators translate environmental cues into SMs, pathway-specific transcription factors typically control transcription of their respective cluster biosynthetic genes.

Global regulators. Approximately half of the currently known SM gene clusters do not encode regulatory proteins. Instead, they are controlled by global regulators that, in addition to SM gene clusters, can also activate or inhibit genes not belonging to secondary metabolism (19, 127). Today, a number of such global TFs and their corresponding environmental signals have been identified: PacC and pH (147); CCAAT-binding complex (CBC) and iron (61); AreA and nitrogen (25); the velvet complex and light (9); and CreA and carbon (38).

One of the most studied examples of global regulators is PacC, the key factor for pH regulation in fungi. pH response by PacC is important for virulence of many plant and animal pathogens (110), e.g., in the opportunistic human pathogen *Aspergillus fumigatus* by influencing, e.g., gliotoxin biosynthesis (13). Moreover, PacC activates penicillin biosynthesis at alkaline pH (18) and, by contrast, represses aflatoxin biosynthesis in *Aspergillus parasiticus* (71). pH is also relevant for the biosynthesis of SMs by plant pathogens, e.g., fuminosins produced by *Fusarium verticillioides* (40), and bikaverin and fusaric acid synthesized by *Fusarium fujikuroi* (97, 162). Furthermore, ochratoxin A, produced by *Aspergillus ochraceus* (101), is a widespread food contaminant and has also been shown to be influenced by pH.

Redox status and iron are abiotic factors for which the response is regulated by the CBC, which consists of the three core subunits: HapB, HapC, and HapE. To regulate the response to iron availability, a fourth subunit, HapX, is necessary (53, 61, 146). SMs known as siderophores are needed for the uptake of iron from the environment, and their synthesis is tightly regulated by the presence of iron and the CBC (56). In response to the redox status of the cell, the CBC regulates penicillin biosynthesis in *Penicillium chrysogenum* (18) and aflatoxin production in *A. parasiticus* (116).

The key regulator of carbon metabolism is Cre1, which has been shown to play a role in aflatoxin biosynthesis. Aflatoxin biosynthesis is strongly upregulated when glucose is present (124), whereas in the case of bikaverin biosynthesis in *F. fujikuroi*, sucrose stimulates production (120). Interestingly, in cephalosporin biosynthesis, high concentrations of glucose repress the biosynthetic genes by CreA, the homolog of Cre1 (66, 67).

In fungi, the GATA TF AreA is the main regulator of nitrogen metabolism, thereby also affecting SM formation (150). The gibberellin (GA) biosynthesis gene cluster in *F. fujikuroi* is mediated by AreA through direct binding to the promoters of the GA biosynthesis genes (151). AreA is also required for the production of fumonisin B1 in *F. verticillioides* (74). SM response to varying nitrogen sources is not consistent between fungi. Opposite sterigmatocystin formation in *A. nidulans* and *A. parasiticus*, dependent on the nitrogen source, demonstrates species-specificity. Multiple GATA sequences in the aflatoxin and sterigmatocystin regulatory genes *aflR* and *aflJ* in *A. parasiticus* favor AreA as responsible for this characteristic (28, 90).

The second nitrogen-dependent GATA TF is AreB. It was shown that AreB regulates certain genes together with AreA, e.g., the gibberellin gene cluster (89), but also has specific targets, such as genes of apicidin F and fusaric acid biosynthesis in *F. fujikuroi* (97).

Responses to light in filamentous fungi are controlled by the heterotrimeric velvet complex, consisting of the proteins VelB and VeA and the nuclear methyltransferase-domain protein LaeA. The complex connects light-dependent fungal morphology and development to secondary metabolism, as was shown for *A. nidulans* (9). LaeA was found to affect approximately 50% of the

SM gene clusters in fungi, and loss of LaeA results in reduced SM formation (17). The tight regulation of light-dependent sexual development and SM formation shown in filamentous fungi (9) is accomplished by spatial compartmentalization of the velvet complex subunits. Both VelB and VeA can move between the nucleus and the cytoplasm (9). However, this switch is light-dependent only for VeA. In the absence of light, VeA migrates to the nucleus as a VeA-VelB heterodimer (139). The constitutive nuclear localization of LaeA (17) enables functional velvet complex assembly only under conditions of darkness. Consequently, light represses the velvet-dependent functions in development, sporulation, and secondary metabolism (19). This is supported by the finding that all examined Δ*laeA* mutants of pathogenic fungi display reduced virulence (47). Furthermore, it was suggested that LaeA might have an impact on SM gene cluster expression via epigenetic modification of the chromatin structure because of a methyltransferase domain in its protein sequence (111). Indirect evidence was found when it was discovered that an *A. nidulans* Δ*laeA* mutant displayed more heterochromatin protein A occupancy with increased repressive histone 3 lysine 9 trimethylation (H3K9me3) in genes of the sterigmatocystin cluster (118). Moreover, in *Trichoderma reesei*, LAE1 regulates several genes by inducing changes in the histone mark H3K4me3 (69). However, a substrate directly methylated by LaeA has not yet been identified.

No global regulator has been identified for temperature as another important extracellular stimulus, although an influence on SM production was shown. As an example, *A. fumigatus* can grow at many different temperatures, optimally at 37°C, but not all metabolites are produced at this temperature. The spore-bound metabolite endocrocin, for example, is produced only at temperatures below 35°C (12). The same is true for *Aspergillus flavus*, whose optimal growth temperature is 37°C, whereas aflatoxin biosynthesis takes place only at 30°C (103).

Cluster-specific regulators. Genes for cluster-specific regulators can be located outside or inside the cluster they regulate (19). Cluster-specific TFs often allow a direct connection to their role in the SM formation network. The most common and fungal-specific are sequence-specific DNA-binding proteins of the Zn_2Cys_6 type (27), but Cys_2His_2 TFs are also common in fungi (e.g., 149). Less frequently found in fungi are bZIP and winged helix proteins, which, as Cys_2His_2 TFs, are found in all eukaryotic organisms. bZIPs often link stress response and SM formation (60). The winged helix TF CPCR1, required for cephalosporin C production, is also involved in arthrospore formation, thus linking morphological development and SM production (129).

Among the well-known TFs of the Zn_2Cys_6 type are ApdR and AfoA in *A. nidulans* (11, 30), GliZ in *A. fumigatus* (16), LovE in *Aspergillus terreus* (72), and Bik5 in *F. fujikuroi* (162). A detailed overview of regulatory proteins in fungal secondary metabolism was recently published (75). The best-characterized cluster-specific activator is AflR, encoded in the aflatoxin and sterigmatocystin clusters in various *Aspergillus* spp. (22). A typical feature of cluster-specific TFs is that they are required for transcriptional activation of most, if not all, structural genes of the concomitant cluster. This holds true for AflR of the aflatoxin cluster in *A. flavus* and the sterigmatocystin cluster in *A. nidulans* (39, 164), and for ApdR, which regulates all genes of the aspyridone biosynthesis cluster in *A. nidulans* (11).

In recent years, the linear concept of one TF regulating its own gene cluster, thereby inducing the formation of the cluster-specific product, has changed, as more and more examples of cross talk and cluster interactions were found. In *A. nidulans*, the asperfuranone biosynthetic gene cluster is induced by expression of the SM cross-pathway regulator ScpR, which is encoded in a different SM gene cluster in the same fungus (10). Likewise, the above-mentioned TF AflR regulates both sterigmatocystin and asperthecin biosynthesis in *A. nidulans* (165). Another example of unusual cluster architecture and regulation is given by Wiemann et al. (161), who showed that a single supercluster in the subtelomeric region of chromosome eight of *A. fumigatus* encodes the proteins

for the biosynthesis of three SMs: fumitremorgin, fumagillin, and pseurotin. Interestingly, it turned out that the fumagillin and pseurotin gene clusters are intertwined and that the supercluster also contains genes that are not needed for product formation. Moreover, the cluster-specific TF FapR regulates only fumagillin and pseurotin biosynthesis but does not affect fumitremorgin production.

Interaction between fungal SM clusters may occur more often, not only adding another level of complexity in the regulatory network but again underlining the potential of a deeper understanding of the regulatory circuits for the discovery of potentially interesting new compounds.

Signal Transduction Pathways

Organisms need to adapt very quickly to environmental changes, which also influence SM formation in fungi. The translation from stimulus to response is normally based on signaling pathways, which regulate gene expression and activation of secondary metabolism.

Signaling pathways are highly conserved among fungi (119). The most studied pathways are cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), calcineurin/calmodulin, TOR, and mitogen-activated protein kinase (MAPK) (**Figure 2**). Many studies have associated the expression and accumulation of SMs with these pathways, disclosing a direct connection between

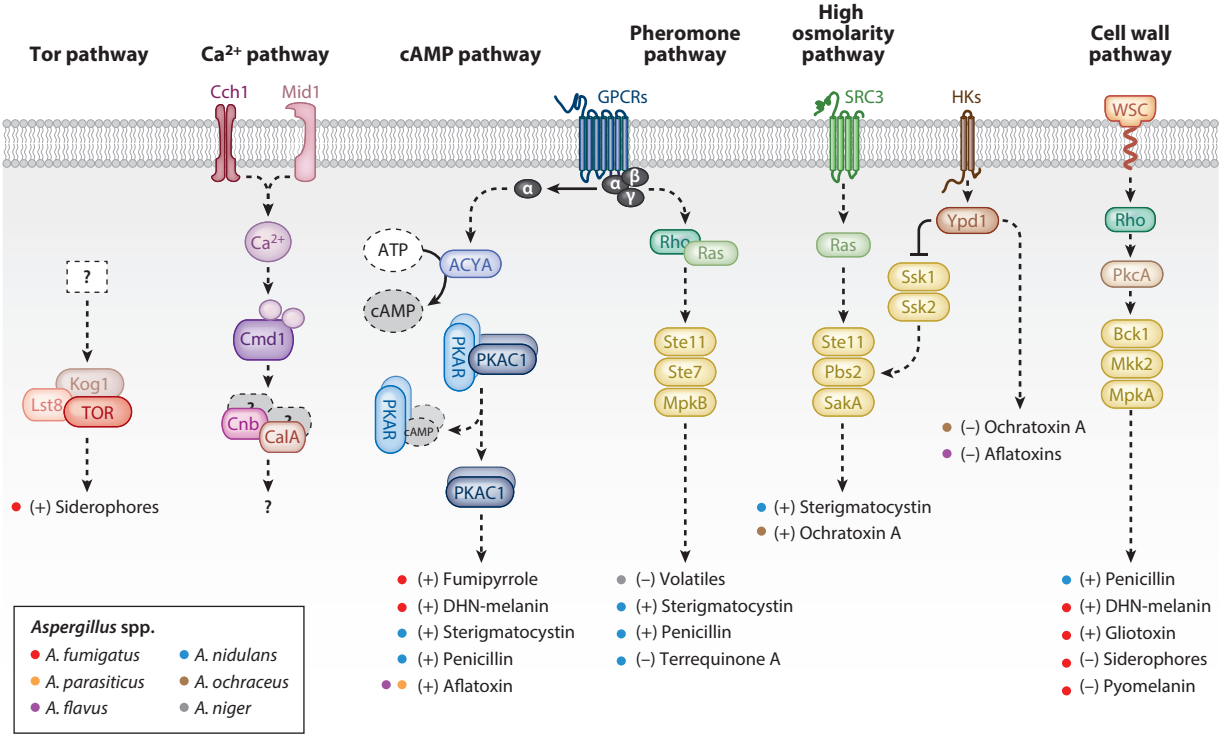


Figure 2

Representation of different signaling pathways in *Aspergillus* spp. and their connection to secondary metabolite production. The figure shows the different conserved signaling pathways involved in signal transduction in aspergilli. Compounds whose production is increased as a result of the respective signal transduction pathway are indicated with a plus sign (+), and compounds that increase their production after inhibition of the respective signal transduction pathway are indicated with a minus sign (–). The color code marks the different *Aspergillus* spp.

a certain signal and the production of specific active molecules. However, the majority of these studies focused mainly on transcriptomic approaches, and only a few were validated using chemical analyses.

Among the signaling pathways listed above, the cAMP pathway was one of the first associated with SM production in *A. fumigatus* (83). In particular, deletion of genes coding for important components of the pathway, such as the G-protein α subunit (GpaB) and adenylate cyclase (ACYA), led to a decrease of dihydroxynaphthalene (DHN)-melanin production in *A. fumigatus*, whereas the overexpression of the protein kinase A catalytic subunit (PkaC1) induced the expression of the DHN-melanin cluster genes (52). PKA was also associated with the production of other SMs, such as fumipyrrole in *A. fumigatus* (87).

Manipulation of the cAMP regulatory elements also affects SM production in other fungi. For example, increased PKA phosphorylation enhances the production of sterigmatocystin in *A. nidulans* (132), and the orthologous pathway regulates aflatoxin biosynthesis in *A. parasiticus* and *A. flavus* (122), whereas repression of the pathway by deletion of the gene coding for ACYA affects gibberellin and carotenoid production in *F. fujikuroi* (45). Activation of the cAMP pathway was also obtained by mutation of the upstream GpaB/FadA protein by allele modification, which promoted the sterigmatocystin accumulation in *A. nidulans* and also increased penicillin production in *A. nidulans* (145). The same approach was used in *P. chrysogenum*, where constitutively activated GpaB/FadA increased the production of penicillin, chrysogenin, and roquefortine (46), demonstrating that similar clusters in different species can be regulated in the same way.

In addition to being regulated by the cAMP signaling pathway, sterigmatocystin, penicillin, and DHN-melanin biosynthesis were found to be affected by fluctuation in the MAPK signaling pathways. MAPK signaling is conserved in all fungi (119) and is usually divided into three major signaling cascades: the cell wall integrity (CWI) signaling pathway, which is responsible for cell wall biosynthesis and repairing; the high osmolarity glycerol (HOG) pathway, which responds to osmostress; and the pheromone pathway, which is usually coordinating pseudohyphal formation and sexual crossing (155). Signals, which are normally perceived at the membrane level, are transduced through small GTPases to MAPKs that activate each other by phosphorylation (119). This phosphocascade ends up with the translocation of the final MAPK into the nucleus, where specific TFs are activated (65). However, these pathways strongly influence each other, showing a strong cross-talk interaction (4).

MAPK cross-talk interactions can be well correlated to SM formation. As an example, inhibition of the pheromone pathway in *A. nidulans* decreases the global levels of sterigmatocystin and penicillin (6, 8). In parallel, decreased levels of sterigmatocystin in *A. nidulans* were also observed after deletion of *sakA*, the main MAPK acting in the HOG pathway (159), whereas reduction of penicillin was detected when the CWI pathway was repressed by silencing the gene coding for protein kinase C (PkcA) (58).

Among the MAPK signaling cascades, the pheromone pathway was most associated with SM production. Inhibition of this cascade promotes the production of volatile terpenoids in *Aspergillus niger* (112), terrequinone A in *A. nidulans* (6), and, more strikingly, the expression of the major SM regulator *laeA* (6, 8, 77). This led to the association of this signaling pathway with different metabolites in a large variety of fungal species, suggesting a strong connection between pheromone signaling and the cAMP pathway (17).

The osmostress response pathway was also associated with SM formation. However, the majority of studies reported that the inhibition of the HOG signaling pathway has a negative effect on metabolite production. This was true for the production of the mycotoxins ochratoxin A in *Penicillium verrucosum*, *Penicillium nordicum*, and *Aspergillus carbonarius* (141) and aurofusarin and trichothecenes in *Fusarium graminearum* (102). The HOG pathway can be activated by deletion

of the bZIP family TF Yap1, which enhanced SakA phosphorylation and mycotoxin production in *Aspergillus* spp. and *Fusarium graminearum* (92, 115, 116).

The CWI pathway was widely studied in fungi as well, and its activity was mainly related to virulence studies (119). Nonetheless, a direct connection between CWI and SM production was demonstrated in *A. fumigatus* (155). Deletion of the central MAPK MpkA not only increases sensitivity to cell wall acting compounds but also decreases DHN-melanin and gliotoxin production (65, 95, 154). However, this mutant strain produces higher amounts of siderophores during iron starvation conditions (65). However, MpkA not only plays a role during cell wall stress but is also thought to balance energy consumption and stress response. In particular, it is important for nitrogen starvation and polyamine storage (65). Nitrogen starvation was previously associated to another signaling pathway, the so-called target of rapamycin (Tor) pathway (79). Silencing of the TOR kinase gene in *A. fumigatus* confirmed an opposite effect compared with that of MpkA, leading to highly reduced siderophore content during iron depletion conditions (7).

Studies on cell signaling revealed that many different pathways are involved in SM production. However, because genetic manipulation of signaling components often has drastic effects on cell physiology, it remains difficult to associate a signal cascade with the production of a compound. For instance, the production of pyomelanin, a melanin derived from tyrosine degradation in *A. fumigatus*, was associated with a general cell wall stress response that could be provoked by either deletion or hyperactivation of MpkA (154). This mechanism seems to be conserved in *Neurospora crassa*, where deletion of the *mpkA* ortholog enhances tyrosinase activity, thereby affecting the production of dihydroxyphenylalanine (DOPA)-melanin (109). Nonetheless, studies on signaling pathways demonstrate that we can use this knowledge to positively manipulate the determination of signals to activate cryptic gene clusters, providing the possibility of using synthetic biology tools to uncover information about unknown SMs.

Epigenetic Regulation

Remodeling of the chromatin landscape allows eukaryotic cells to tightly control nuclear processes and represents another mechanism for the regulation of SM production. The controlled assembly and disassembly of nucleosomes play a crucial role in regulating the accessibility of DNA, enabling transcription by RNA polymerase II. The landscape of packed DNA is greatly shaped by factors such as ATP-dependent chromatin remodelers, histone variants, and histone chaperones. This system is complemented by chromatin modifiers, which insert modifications on canonical histones and their variants, thereby changing chromatin structure and function as well as recruiting chromatin remodelers and other additional factors (82, 156).

In fungi, the clustered arrangement of SM genes suggests a coregulation mechanism by means of chromatin structure. Restructuring the nucleosome packaging, and thereby affecting the accessibility of genes encoded in that region, is a beneficial and presumably cost-effective mechanism for a cell to regulate larger genomic regions. This model was supported by a variety of studies with small inhibitor molecules that target lysine acetyltransferases (KAT), lysine deacetyltransferases (KDAC), and DNA methyltransferases (44, 107). In a pioneering study, Shwab et al. (134) treated *Alternaria alternata* and *Penicillium expansum* with trichostatin A (TSA), a bacteria-derived antifungal KDAC inhibitor, and observed an increase in the amount of several unidentified SMs. Inspired by this, the Chichewicz group performed similar studies and observed higher SM formation in fungal strains (163). 5-Azacytidine, a DNA-methyltransferase inhibitor, and suberoylanilide hydroxamic acid, a KDAC inhibitor, led to the production of a series of oxylipins and erylenequinones in *Cladosporium cladosporioides*. In another fungus, *Diatrype disciformis*, the addition of 5-azacytidine

resulted in the production of the new polyketides lunalide A and B (163). Similarly, the KDAC inhibitors valproic acid, TSA, and sodium butyrate increased the production of pseurotin A, patulin, cytochalasin E in *Aspergillus clavatus*, orsellinic acid in *A. nidulans*, cyclodepsipeptides in *Beauveria felina* and decreased biosynthesis of sterigmatocystin and several emericellamides in *A. nidulans* (2, 32, 131, 167).

KATs and KDACs of filamentous fungi have been characterized and analyzed for their role in the regulation of particular SM clusters. Thus far, Gcn5, NnaB, and Esa1 have been implicated in a regulatory role in SM induction (100, 128, 136). Gcn5 (GcnE in *A. nidulans*), as part of the multi-subunit complex SAGA/ADA, was responsible for the acetylation of H3K9 and K14 and thereby for the subsequent activation of the orsellinic acid gene cluster, as well as the activation of penicillin, sterigmatocystin, and terrequinone A biosynthesis in other culture conditions (99, 100). The same clusters were also regulated by Esa1, a KAT that carries out acetylation at histone H4 (136). Similarly, in *A. parasiticus*, active transcription of the aflatoxin cluster genes and acetylation of histone H4 were correlated, which could be correlated to the downregulation of a KAT under cluster-repressing conditions (121, 123). Deletion of the KAT-encoding gene *nnaB* in *A. nidulans* led to the overproduction and discovery of pheofungins and also implicated the same KAT in the production of orsellinic acid (128).

The first time chromatin regulatory mechanisms were linked to SM gene cluster regulation was in connection to the so-called eraser of histone acetylation (134). KDACs remove acetyl groups from lysine residues of proteins such as histones. Acetylation is mostly associated with active transcription; however, there are increasing numbers of publications showing opposing effects of KDACs involved in gene silencing as well as activation (36, 81, 142). In *A. fumigatus*, the deletion of the KDAC HdaA was found to increase the production of fumitremorgin B and pseurotin, but at the same time the knockout led to a downregulation of gliotoxin (81). Mutation of the homolog in *A. nidulans* led to a higher production of the well-studied SMs penicillin, sterigmatocystin, and terrequinone A (134). The sterigmatocystin cluster in *A. nidulans* was also found to be regulated by SirA, a NAD^+-dependent KDAC, and RpdA, a KDAC whose knockout was lethal (2, 133). Furthermore, RpdA was connected to the regulation of alternariol, the antibiotic 3127, emericellamides, austinols, and F9775A and B, two metabolites also found during the interaction of *A. nidulans* with *Streptomyces rapamycinicus* (2, 131). Interestingly, this fungal-bacterial interaction led to the activation of GcnE, a KAT involved in the regulation of many clusters also controlled by RpdA. Both GcnE and RpdA are found to also be responsible for development in *A. nidulans* (2, 100). This suggests that GcnE and RpdA might fulfill opposing functions in the regulation of some SM clusters as well as in development in this fungus.

Another well-studied modification, which is also known for its regulatory complexity toward gene expression, is methylation (21). One of the first studies in fungi examined the role of CclA in *A. nidulans* (15). CclA is a member of the COMPASS complex, a conserved multi-subunit complex that both activates and represses transcription through methylation of histone H3K4 (94). The deletion of the *cclA* gene in *A. nidulans* led to the production of monodictyphenone, emodin, and emodin analogs as well as to F9775A and B (15). The deletion of *A. fumigatus cclA* also resulted in an altered SM profile, increasing the biosynthesis of gliotoxin as well as other metabolites (106).

However, the formation of open or closed structured chromatin can influence not only the modification state of a histone but also the binding of regulatory factors. Interesting in this context is heterochromatin protein 1, a principal component of heterochromatin. Deletion of the respective gene in *A. nidulans* and *F. graminearum* led to an altered SM profile. In *A. nidulans*, the clusters encoding the biosynthesis of sterigmatocystin, penicillin, and terrequinone A were derepressed, and in *F. graminearum*, deletion resulted in the overproduction of aurofusarin and the reduced biosynthesis of deoxynivalenol (117, 118).

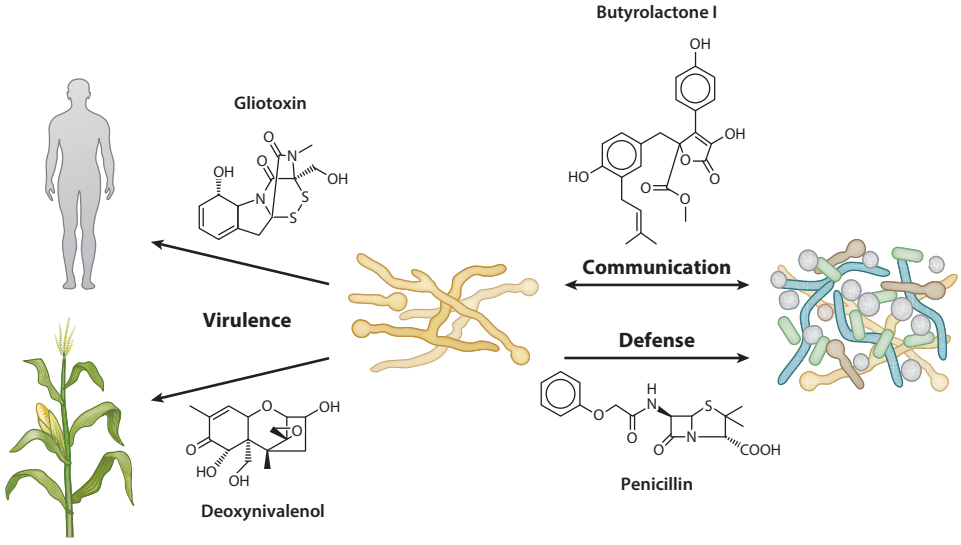


Figure 3

Ecological role of fungal secondary metabolites. Fungi produce secondary metabolites as virulence determinants for plant and animal infection, e.g., deoxynivalenol and gliotoxin, respectively. Furthermore, fungal secondary metabolites can serve as communication molecules, e.g., butyrolactone I, or as defense compounds against other microorganisms, e.g., penicillin, thereby structuring microbial consortia.

Consequently, the regulation of SM clusters requires the activation and repression of chromatin modifiers to support the introduction and removal of regulating chromatin marks. From the well-studied organism *A. nidulans*, we have learned that many clusters share common features but are also distinct from each other in their combinatorial network of chromatin regulation. Therefore, ongoing analysis of histone modifications and chromatin remodelers in diverse fungi in a multitude of conditions can give us a bigger picture of the chromatin regulatory network of SM gene clusters and represents a novel avenue to drug discovery for fungi not amenable to genetic engineering.

ROLE AND REGULATION OF FUNGAL SECONDARY METABOLISM DURING THE INTERACTION WITH OTHER ORGANISMS

SMs are low-molecular-weight compounds, which are not essential for growth. Since fungi share their habitat with a multitude of other organisms SMs may provide protection or serve as mediators for communication (**Figure 3**). SMs are also known as virulence factors for plant and animal pathogens. This role probably evolved to defend against amoebae, nematodes, and other invertebrates, which can feed on fungi (42). The following section clarifies the role and function of SMs in the interaction of fungi with microbes, plants, and animals.

Interaction with Other Microorganisms

In nature, microbes coexist in communities, which are diverse in species and genera. Each species constantly interacts with a multitude of different microorganisms, and SMs are often used as signals involved in competition and cooperation (19). This section focuses mainly on interactions between bacteria and fungi, which can be either of a chemical or physical nature (43).

The role of fungal SMs during polymicrobial interactions often remains unclear. Antibiotics, for instance, are mainly known for their importance in treatment of infectious diseases in humans, but little is known about their function in nature. For a long time, it was thought that bacteria use antibiotics as weapons to kill or inhibit competitors (35), as the most known antibiotic, penicillin, was developed based on the antibiosis of a *Penicillium* mold contaminating *Staphylococcus* spp. (41). However, recent studies have revealed their function as signals that coordinate interspecies interactions between coexisting bacterial strains (1). Abrudan et al. (1) clearly demonstrated enhanced antibiotic production by *Streptomyces* strains in response to signals received from neighboring microorganisms together with repressed antibiotic production in competitors. Their data showed that antagonistic relationships between coexisting microorganisms are dependent on the competing species as well as on the availability of nutrients (1, 152). Hence, microorganisms have developed different mechanisms to combat antimicrobial compounds secreted by antagonists. For example, the production of 2,4-diacetylphloroglucinol, which is the key factor in the antimicrobial activity of *Pseudomonas fluorescens* CHA0, is repressed by fusaric acid produced by *Fusarium oxysporum*. The mycotoxin reduces the expression of the responsible biosynthetic genes (98). In contrast, secreted antimicrobial compounds can also promote the growth of other microbes or act as chemoattractants, as was shown for fusaric acid and other metabolites that promote the colonization of *F. oxysporum* hyphae by *P. fluorescens* WCS365 (37).

Along with defending their habitats, microorganisms have developed a communication process, namely quorum sensing (QS), that correlates, dependent on cell densities, with several behaviors, such as secretion of virulence factors, biofilm formation, and bioluminescence (51). QS was extensively studied in gram-positive and gram-negative bacteria (160). Later, this phenomenon was described for eukaryotic organisms, e.g., *Candida albicans*, where mycelia formation is controlled by farnesol and tyrosol (3). In contrast to the bacterial QS systems no species-specific QS molecules have been described for eukaryotes until now. Very recently, Homer et al. (59) described the small peptide Qsp1 in *Cryptococcus neoformans*, which acts as a classical QS regulator by triggering virulence and morphology. The discovery of Qsp1 raises the question of whether other analogous systems exist in other fungal species. However, it is also known that the production of SMs can be a response to QS signals. *Penicillium sclerotiorum* produces the aldose reductase inhibitor sclerotiorin (31), whose production appears to be related to the QS molecule multicolic acid and derivatives thereof (113). Interestingly, these QS molecules contain γ-butyrolactone, which has been identified as a signaling molecule in actinomycetes like the A-factor (91) and SVB1 (166). The addition of a supernatant extract from *P. sclerotiorum*, containing γ-butyrolactone compounds, to a submerged *P. sclerotiorum* culture led to an enhanced production of sclerotiorin (113). Moreover, butyrolactones are present in many filamentous fungi. For instance, lovastatin production in *A. terreus* was increased by adding butyrolactone I to liquid cultures (108, 113). The authors hypothesize that butyrolactone I operates as a QS molecule in *A. terreus*, regulating lovastatin biosynthesis and, in parallel, acting in an autostimulatory way on its own production. Because filamentous fungi coexist with bacteria, it has been speculated that fungal SMs can interfere with bacterial QS communication to protect themselves against nearby growing bacteria. This led to the hypothesis that QS might be a new drug target to control bacterial diseases. Indeed, from a collection of 50 *Penicillium* spp. that were tested for their ability to produce QS inhibitory compounds, patulin and penicillic acid were found to target the QS response systems RhlR and LasR in *Pseudomonas aeruginosa*. Furthermore the authors showed that the *P. aeruginosa* biofilms, which are formed in presence of patulin, are more sensitive to antibiotic treatment (114). In this example, fungal SMs provide a way to prevent bacterial communication, thereby reducing their virulence; this could provide a mechanism to sense and respond to other microbes in their vicinity.

In addition, SMs assume a crucial role in microbial communication (19). Recent studies have led to the discovery that fungal interactions with other species trigger the production of new SMs (96). For example, the intimate interaction between the model fungus *A. nidulans* and the soil-dwelling bacterium *S. rapamycinicus* led to the discovery of new SMs (131). The induction of silent fungal gene clusters was investigated in a microarray-based approach. Out of 58 different actinomycetes, only *S. rapamycinicus* was able to activate the fungal orsellinic acid gene cluster (*ors*), which was accompanied by the production of orsellinic acid, lecanoric acid, and the cathepsin inhibitors F-9775A and F-9775B (131). Interestingly, physical contact between both organisms is needed to activate the silent gene clusters. Lecanoric acid is a typical lichen metabolite, and although it is usually found in symbiotic relationships (140), it had no obvious effect on *S. rapamycinicus*. Possibly, the fungus produces the compounds as an SOS signal to other microorganisms sharing the same habitat. Using the same streptomycete species, an otherwise silent fungal gene cluster could be activated in the human pathogen *A. fumigatus*, where the novel compounds fumicycline A and B were discovered (76).

In summary, fungal SMs can have multiple roles in microbial interactions and their gene expression can be induced by either direct contact or QS molecules. Nevertheless, the investigation of their ecological role has just begun and further studies are needed.

Secondary Metabolites as Virulence Factors in Plant-Fungal Interactions

The role of fungal SMs in plant-fungal interactions is also of scientific interest. Here, examples of SMs involved in pathogenic plant-fungal interactions are discussed (**Figure 3**). Fungal SMs were also shown to be involved in the interactions of symbiotic and mycorrhizal or endophytic fungi with plants (55), which are beyond the scope of this review and therefore not discussed in detail.

The interaction of the cereal pathogen *F. graminearum* and its main host wheat has been extensively investigated. It has been shown that the trichothecene deoxynivalenol (DON) can act as a virulence factor. In vitro studies revealed several abiotic factors, such as low pH, reactive oxygen species (ROS), and temperature as well as plant-based polyamines or sugars, to be able to activate DON biosynthesis (70).

Species of the genus *Cercospora* are the causative agents of leaf spot and blight diseases within a wide host range. During the initial stages of infection, the perylenequinone cercosporin, which belongs to the group of photosensitizers, is secreted. In the presence of light and oxygen, cercosporin generates ROS and causes local necrotic lesions on the plant surface (34).

The rice blast pathogen *Magnaporthe grisea* attacks its host with differentiated infection structures, the so-called appressoria. The invasion into the leaf tissue of the host is conducted by an enormous turgor pressure in the appressorium. To stabilize the high turgor pressure the fungus deposits the polyketide DHN-melanin into the cell wall of appressoria. Mutants of DHN-melanin biosynthesis are not able to invade the host, owing to impaired penetration (62). Interestingly, an unknown PKS-NRPS hybrid gene product encoded in *M. grisea* was shown to be recognized by specific rice cultivars resistant to appressorial invasion, and the lack of this gene reconstituted virulence (14). Recently, an attempt at heterologous expression of this SM cluster was unsuccessful, and the structure of the respective product could not be determined (135).

The genera *Alternaria* and *Cochliobolus* are known for their production of host-selective toxins. Different species synthesize distinct SMs that determine their host range and host-specific virulence. In the case of *A. alternata*, pathotypes with distinguishable host ranges show the production of different host-selective toxins, e.g., the NRPS-derived cyclic peptide host-specific toxins, AM toxins, are specifically produced by an *A. alternata* pathotype that infects apples, whereas a second pathotype causes black spots on strawberries, which are connected to the production of AF toxin, a

member of the epoxy-decatrienoic acid family. Along with their structural differences, both toxins have different target sites in the plant cell (see also 148). *Cochliobolus carbonum*, which causes leaf and ear symptoms on corn, produces the histone deactylase inhibitory cyclotetrapeptide HC toxin, which induces a change in gene expression in the host. Another mechanism of host-selective toxins is utilized by T toxin, a polyketide produced by *Cochliobolus heterostrophus*. T toxin is connected to high virulence against corn cultivars containing the Texas male sterility cytoplasm (*T-cms*), which encodes the mitochondrial protein URF13. The binding of T toxin to URF13 leads to a collapse of mitochondrial physiology and finally to plant cell death (138).

During infections with the gray mold *Botrytis cinerea*, the sesquiterpene botrydial and the polyketide botcinic acid are produced on plants. Both SMs were shown to have redundant roles in fungal virulence. Single deletion mutants of the genes essential for biosynthesis of both compounds show no differences in pathogenicity compared to the wild type. However, a double deletion mutant incapable of producing either SM was severely impaired in causing chlorosis and necrosis of plant leaves, although infection could still proceed (33).

Siderophores are common features of fungal iron acquisition and storage (56). In particular, extracellular siderophores were shown to be involved in virulence of several phytopathogenic fungi. For example, deletion of the *nps6* gene in *C. heterostrophus* affected iron uptake and increased the susceptibility to ROS, one of the defense mechanisms of the host (80, 104).

Secondary Metabolites as Virulence Factors in Animal Infection

The role of fungal SMs in the interaction with animals is diverse and in many cases not yet elucidated in detail. To narrow this extensive topic, the following section focuses on pathogenic fungal-animal interactions and SMs as possible virulence factors.

An extensively investigated fungal virulence factor is melanin. For *A. fumigatus*, the conidial pigment DHN-melanin, which is formed by a central PKS, plays an important role in pathogenicity. It not only reduces acidification of phagolysosomes after phagocytosis and thus killing of conidia but also prevents apoptosis of macrophages (57). Additionally, the opportunistic pathogenic yeast *Cryptococcus neoformans* produces DOPA-melanin during infection, and pigmentless strains of this fungus display reduced virulence (78).

Siderophores are also essential for fungal virulence, as iron availability is often limited in the host environment (56). To overcome iron limitation, fungi produce siderophores, which require the activity of NRPSs. For instance, *A. fumigatus* produces triacetylfusarinin C for extracellular iron acquisition and ferricrocin for intracellular iron storage. Biosynthesis of both siderophores is NRPS dependent, and deletion of one of the two NRPS encoding genes results in significantly reduced virulence (130). Similarly, deletion of certain siderophore biosynthetic genes in the entomopathogen *Metarhizium robertsii* also leads to reduced virulence in this fungus (49).

Apart from melanin and siderophores, the role of SMs during infection has been poorly investigated. To gain hints on which SMs might be important during infection, several transcriptomic or proteomic approaches have been applied. A well-characterized example for the regulation of secondary metabolism during the interaction with a human host is given by *A. fumigatus*. Many studies have been performed to monitor changes in gene expression upon interaction with immune cells or during infection. Using a microarray hybridization approach, it was shown that during initiation of murine infection several SM gene clusters of *A. fumigatus* were upregulated, among them the siderophore biosynthetic gene cluster and clusters encoding genes for the production of gliotoxin, fumitremorgin, fumagillin, and pseurotin (88). Siderophore biosynthesis was also upregulated when *A. fumigatus* was grown in human blood (64) or when interacting with human bronchial epithelial cells (105). In contrast, coincubation of the fungus with different human immune cell

types repressed or had no effect on the expression of SM gene clusters (93, 144). This leads to the assumption that it is not the host itself but rather the environmental conditions during infection that regulate secondary metabolism in *A. fumigatus*. Transcriptomic and proteomic studies that mimic infection conditions support this hypothesis. Gliotoxin and siderophore biosynthesis are both upregulated at 37°C reflecting the temperature of humans compared to 24°C more often found in nature (143), and pseurotin and fumagillin biosynthetic genes show increased expression under the hypoxic conditions that can be found in necrotic tissues (157). Furthermore, biofilm growth induces production of gliotoxin (23) and differentially regulates several SM gene clusters, e.g., fumitremorgin, pseurotin, fumagillin, and fumipyrrole (48). The exact role and importance of the above-mentioned products during infection are often not clear, although various functions have been described. Only gliotoxin has been shown to contribute to virulence in a mouse infection model (144).

Dermatophyte genomes also encode a huge number of SM gene clusters (24), implying a function of their products for survival and virulence of these fungi. For *Trichophyton rubrum*, several SM gene clusters were differentially regulated upon cultivation in the presence of human skin sections (85), and the pigment xanthomegnin was shown to be produced during growth of *T. rubrum* on skin and nail (54). Despite the toxicity of xanthomegnin toward liver and kidney cells (26), and a possible effect on the host immune response by inhibition of the nitric oxide synthase (5), its role for *T. rubrum* virulence is still unclear.

The interaction with insects also induces SM formation in fungi. Although different forms of fungus-insect interactions exist, secondary metabolism is best studied in entomopathogenic fungi, primarily in *Metarhizium* species and *Beauveria bassiana*. For *M. robertsii*, expression of the gene encoding the NRPS for destruxin biosynthesis was observed during infection of *Spodoptera exigua* larvae (50), indicating that this metabolite might be important for fungal virulence. Destruxins are cyclic depsipeptides that possess a multitude of effects, such as inhibiting V-ATPase (29), altering ion transport in gut and epithelial tissues (125), or even inducing behavioral changes (63). Therefore, they are considered virulence factors of *Metarhizium anisopliae* for the infection of different insect hosts (73). For *B. bassiana*, the biosynthetic genes for the well-characterized SMs tenellin, beauvericin, and bassianolide were expressed during infection of *Triatoma infestans* nymphs (86). Tenellin was shown to scavenge iron-mediated oxidative stress, which is caused by intracellular free iron under iron excess conditions by chelating iron ions (68). Not much is known about the mechanisms underlying beauvericin and bassianolide function, but both represent virulence factors of *B. bassiana* (153), and beauvericin shows a broad activity spectrum against insects, bacteria, fungi, viruses, and tumor cells (158).

CONCLUSION AND PERSPECTIVE

Secondary metabolism in fungi is a very broad topic, and this review can give only a short overview of the interesting aspects of the role and regulation of SMs produced by fungi. Because of their important roles, e.g., as virulence factors in pathogenic interactions, and their beneficial applications in medicine and plant protection, research on SMs will be further intensified, leading to the identification of new SMs and new regulatory mechanisms as well as to their physiological function in nature. As this review attempts to point out, multiple levels of regulation exist, including epigenetic mechanisms, signal transduction pathways, global regulators, and pathway-specific TFs. Only the combination of all mechanisms can guarantee that a certain SM is produced as a specific response to distinct environmental requirements, thereby providing a benefit to the fungus. Currently, discovery of new SMs is done by changing one regulatory element, but it is also conceivable/possible that modifications in several regulatory levels at the same time might

lead to the induction of silent SM gene clusters. Moreover, studies on cluster interactions, the recognition that different clusters can be regulated by the same TF, and the fact that gene clusters can be interwoven suggest that future regulatory mechanisms may be uncovered outside of the one cluster–one product paradigm. Elucidation of the molecular principles behind this complex regulatory network will not only provide a deeper insight into how fungi translate environmental signals into SM biosynthesis but will also ensure identification of novel SMs and a deeper understanding of their ecological role in the future.

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