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Spatial and Temporal Control of Fungal Natural Product Synthesis

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Abstract

Despite their oftentimes-elusive ecological role, fungal natural products have, for better or worse, impacted our daily lives tremendously owing to their diverse and potent bioactive properties. This Janus-faced nature of fungal natural products inevitably ushered in a field of research dedicated towards understanding the ecology, organisms, genes, enzymes, and biosynthetic pathways that give rise to this arsenal of diverse and complex chemistry. Ongoing research in fungal secondary metabolism has not only increased our appreciation for fungal natural products as an asset but also sheds light on the pivotal role that these once-regarded “metabolic wastes” play in fungal biology, defense, and stress response in addition to their potential contributions towards human mycoses. Full orchestration of secondary metabolism requires not only the seamless coordination between temporal and spatial control of SM-associated machineries (e.g. enzymes, cofactors, intermediates, and end-products) but also integration of these machineries into primary metabolic processes and established cellular mechanisms. An intriguing, but little known aspect of microbial natural product synthesis lies in the spatial organization of both pathway intermediates and enzymes responsible for the production of these compounds. In this highlight, we summarize some major breakthroughs in understanding the genes and regulation of fungal natural product synthesis and introduce the current state of knowledge on the spatial and temporal control of fungal natural product synthesis.

1. Introduction

Be it the caffeine driving our need for morning coffee or the antibiotics used to treat bacterial infections, natural products have taken a significant role in our lives for thousands of years. Known also as secondary metabolites (SMs), these low molecular weight compounds produced primarily by fungi, plants, and bacteria, have unique structural diversity, a plethora of bioactive properties, and provide a unique chemical fingerprint to a species. The earliest documentation of natural products were plant oils including those of *Cupressus sempervirens* (cypress), *Cedrus* spp. (cedar), and *Papaver somniferum* (opium poppy) written on clay tablets in cuneiform from Mesopotamia dating back to 2600 B.C.¹. Millennia later, the serendipitous discovery of penicillin by A. Fleming from the filamentous fungus, *Penicillium notatum*, marked the beginning of the so-called “Golden Age of

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Antibiotics”². Since then, many inroads have been made towards understanding the ecology, organisms, genes, enzymes, and biosynthetic pathways involved in creating SM chemical diversities, allowing for the discovery of numerous microbial-derived compounds. An intriguing, but little known aspect of microbial natural product synthesis lies in the spatial organization of both pathway intermediates and enzymes responsible for the production of these compounds. In this highlight, we summarize some major breakthroughs in understanding the genes and regulation of fungal natural product synthesis and introduce the current state of knowledge on the spatial and temporal control of fungal natural product synthesis.

2. Natural Products of Filamentous Fungi: Overview of Secondary Metabolite Cluster Architecture and Regulation

Filamentous fungi especially of the phylum Basidiomycota and Ascomycota produce a wide repertoire of bioactive SMs with both pharmaceutical importance and detrimental impacts on agriculture and human health³. The Janus-faced nature of fungal SMs ushered in a field of research dedicated towards understanding “molecular switches” that govern their biosynthesis so we can capitalize on this knowledge to increase pharmaceutical and curb mycotoxin production. Fungal SMs fall into a few major chemical classes including polyketides, nonribosomal peptides, ribosomal peptides, terpenes and hybrid metabolites (e.g. meroterpenoids, polyketide-nonribosomal peptide hybrids)³. These chemical classes are defined by the type of starter substrate(s) (e.g. acyl-CoA, amino acids, etc.) incorporated into their core structures by specialized class-defining (backbone) enzymes such as polyketide synthases (PKSs), nonribosomal peptides synthetases (NRPSs), terpene cyclases (TCs), dimethylallyl tryptophan synthetases (DMATs), and geranylgeranyldiphosphate synthases (GGPPs). The genes involved in a single biosynthetic pathway are typically clustered together within the fungal genome and are comprised of co-regulated structural genes (backbone enzymes flanked by various types of modifying enzymes), and sometimes cluster-specific transcription factors, self-protection genes (e.g. *gliT* in the gliotoxin cluster in *Aspergillus fumigatus*), and various types of transporters³⁻⁵.

As SMs are oftentimes localized to various developmental tissues, synthesized in response to specific abiotic and biotic confrontations, and utilize common elements derived from primary metabolism, filamentous fungi have evolved means to coordinate the expression and synthesis of SM-associated machineries in an orderly manner and control the flux of carbon and nitrogen from primary metabolite pools to secondary metabolism. As a result, fungal secondary metabolism is subjected to a complex system of multi-tier regulation. Many of the “molecular switches” involved in SM gene cluster regulation have been identified and extensively reviewed⁶⁻⁸. Briefly, fungi utilize established cellular regulatory elements such as signal transduction pathways (e.g. cAMP signaling, MAP kinase signaling, protein kinase A signaling, etc.), chromatin remodeling mechanisms, and global regulators involved in nutrient-utilization (AreA), and stress response (PacC) in addition to evolving novel regulators of secondary metabolism such as members of the Velvet complex (e.g. VeA, LaeA, etc.) and pathway-specific regulators (e.g. AflR, GliZ, etc.) to coordinate

expression of SM gene clusters. It is through analysis of some of these “molecular switches” that insights into spatial and temporal regulatory patterns have arisen as detailed below.

3. Reaching the Right Location: Tracking Natural Products Synthesis

3.1 Building Blocks of Fungal Secondary Metabolites: Usage and Subcellular Distribution

Fungal SM biosynthesis requires the use of substrates (e.g. acyl-CoAs, amino acids, nucleotides, carbohydrates, etc.), cofactors, and energy generated during primary metabolism as building blocks^{9, 10}. For example, polyketide synthases – similar to fatty acid synthases - utilize a variety of acyl-CoAs (e.g. acetyl-CoA, propionyl-CoA, and malonyl-CoA etc.) derived from primary metabolic pools to synthesize a diversity of polyketides (Fig. 1)¹⁰. Acetyl-CoA is also a building block for the synthesis of farnesyl phosphates, which are used in terpene biosynthesis (e.g. paxilline, trichothecenes, carotenoids, etc.) (Fig. 1). Both proteinogenic and non-proteinogenic amino acids on the other hand, are used as building blocks for a variety of peptides and amino acid-derived compounds (e.g. alkaloids, β -lactam antibiotics, siderophores, amatoxins, etc.) (Fig. 1)¹¹⁻¹⁴. Some gene clusters contain multiple backbone enzymes that are able to incorporate a combination of acyl-CoA- and amino acid-derived substrates to generate hybrid metabolites of higher complexity such as the biosynthesis of fumonisins. Fumonisins are mycotoxins produced by *Fusarium* spp. with high structural similarities to sphingolipids and involves the usage of acetyl-CoA and a combination of various proteinogenic amino acids such as glutamic acid, serine, methionine, and alanine¹².

The biogenesis, intracellular distribution, and flow of acyl-CoAs and amino acids through various organelles have been reviewed in detail¹⁵. Acetyl-CoAs for example, can be formed in the peroxisomes and mitochondria via β -oxidation of long- and short-chain fatty acids respectively and also in the cytosol through pyruvate decarboxylation¹⁶⁻¹⁸. Amino acids, on the other hand, generally accumulate in the vacuole as a result of protein degradation and turnover though both mitochondria and the cytosol have been shown to be sites for catabolism of branched chain amino acids (e.g. valine, leucine, isoleucine) that generate the building blocks (e.g. isovaleryl-CoA, isobutyryl-CoA, etc.) of various polyketide antibiotics^{19, 20}. The utilization of common substrates for primary and secondary metabolism suggests that the cell must control the flux of substrates between primary and secondary metabolic pathways in some fashion. In addition, enzymes such as PKSs, NRPSs, TCs, and DMATs that use these substrates and even hybrid enzymes (PKS-NRPS) that use a combination of fatty acid and amino-acid derived substrates have to either be synthesized at or translocated to organelles with the appropriate substrate availability.

3.2 Generating Structures of Increasing Complexity: Spatial Organization of SM-associated Machineries

There are growing evidences that different steps within a given biosynthetic pathway in fungi tend to occur in distinct subcellular compartments^{15, 21}. Table 1 summarizes all subcellular sites of SM-associated proteins known to-date. Such distribution of SM-associated machineries is reminiscent of plant secondary metabolism. In fact, many studies done on localization of plant natural products and their enzymes such as flavonoids^{22, 23}

and alkaloids²⁴ paved the way for studies on spatial organization of fungal secondary metabolism. With the complexity of biological and chemical functions incurred during natural product synthesis, the need for spatial organization is high and significance of compartmentalizing biosynthetic enzymes, substrates, cofactors, and pathway products are immense. Compartmentalizing enzymes, cofactors, and substrates not only allow for sequestration of all biosynthetic machineries necessary to perform a biosynthetic reaction into a cellular space conducive for that reaction to occur but also allows for the confinement of cytotoxic intermediates, end products, and by-products generated from a pathway, thus preventing self-toxicity. The role and significance of subcellular compartments and a detailed description on the spatial organization of highly studied pathways (e.g. aflatoxin, β -lactam antibiotics) have previously been reviewed^{15, 25-29}. Subsequent sections will expand on previously known aspects of spatial organization in secondary metabolism to highlight some of the recent findings in the field.

3.2.1 Peroxisomes—One of the earliest subcellular organelles identified to harbor enzymes and intermediates of a SM biosynthetic pathway are the peroxisomes. Peroxisomes, also known as microbodies, are single membrane-bound organelles (ranging from 0.1 – 1 μ M in diameter) found in all eukaryotes known initially to harbor hydrogen-peroxide-producing oxidoreductases and catalases that quench reactive oxygen species (peroxide detoxification) within the cell³⁰. These organelles, however, have now been implicated in various cellular functions including the glyoxylate cycle, β -oxidation of fatty acids, and biogenesis of Woronin bodies (protein dense organelles that plug septal pores of wounded fungal hyphae thereby preventing cytoplasmic bleeding)^{31, 32}. In plant pathogens such as the cucumber anthracnose pathogen, *Collectotricum orbiculare* and the rice blast pathogen, *Magnaporthe oryzae*, peroxisome function is implicated in maturation of the appressorium, an infection structure used to penetrate host plant cuticle, and the accumulation of the appressorium pigment (melanin) essential for the function of these infection structures; peroxisome function in these plant pathogens are essential for host plant infection^{25, 33-35}. Peroxisomes are also important for the biosynthesis of various fungal SMs (e.g. polyketides, terpenes, siderophores, and β -lactam antibiotics)^{16, 31, 36-40}. As β -oxidation of fatty acids in the peroxisomes result in accumulation of acetyl-CoA (the building block used in the biosynthesis of many polyketides and terpenes), localization of biosynthetic enzymes from such pathways (e.g. aflatoxins, sterigmatocystin, β -lactam antibiotics, AK-toxins, paxilline) to the peroxisome is reasonable.

3.2.1.1 AK-toxins: AK-toxin is a host-specific toxin produced by the Japanese pear pathotype of *Alternaria alternata*⁴¹. The biosynthesis of the host-selective toxin AK-toxin from the pear pathogen *Alternaria alternata* is strictly confined within the peroxisomes²⁵. An *A. alternata* strain deficient for AaPEX6, a peroxin protein essential for peroxisome biogenesis in eukaryotic cells, loses peroxisomal localization (cytosolic mislocalization) of all three AK-toxin biosynthetic genes (Akt1, Akt2, and Akt3), abolished AK-toxin production, and consequently loses pathogenicity on host pear leaves²⁵. This result suggests that appropriate localization of Akt proteins to the peroxisome is essential for mycotoxin production and the hence, pathogenicity of this pear leaf pathogen.

3.2.1.2 Siderophores: More recently, it is shown that the enzymes catalyzing the early steps in the biosynthesis of the fusarinine-type siderophore, triacetyl-fusarinine C (TAFC), in the human pathogen *Aspergillus fumigatus* and the model organism, *A. nidulans* are also localized to the peroxisomes³⁶. Siderophores are small non-ribosomally generated peptides part of the high-affinity ferric iron acquisition mechanism important for surviving iron-limiting conditions and have a significant impact on pathogenicity of this organism⁴²⁻⁴⁴. In *A. fumigatus*, the first committed step in siderophore synthesis is the conversion of ornithine to N⁵-hydroxyornithine by the siderophore biosynthetic enzyme, SidA⁴⁴. The pathway then splits to generate both an extracellular siderophore (TAFC) used for mobilizing and sequestering extracellular iron, and an intracellular siderophore (hydroxyferricrocin) used for iron storage. The first three enzymes committed to TAFC biosynthesis (SidI, SidH, and SidF) and responsible for the conversion of mevalonate and N⁵-hydroxyornithine to N⁵-anhydromevalonyl-N⁵-hydroxyornithine are found to localize to the peroxisome³⁶. These enzymes use distinct peroxisomal targeting signals namely PTS1 and PTS2 to achieve proper localization³⁶. In contrast to *A. alternata* in which peroxisomal function is required for biosynthesis of AK-toxin, TAFC production does not require the presence of a functional peroxisome³⁶. Gruendlinger *et al.* further demonstrated that cytosolic mislocalization of all three enzymes (SidI, SidH, and SidF) in *A. nidulans* did not abolish nor decrease TAFC biosynthesis³⁶. This shows that specific localization to the peroxisomes per se, is not essential for TAFC biosynthesis as long as all three enzymes (SidI, SidH, and SidF) are localized to the same subcellular compartment, presumably to increase the efficiency of substrate channeling between these pathway enzymes³⁶. The results from this study also suggest that use of distinct targeting signals for these three enzymes to the same organelle may provide the fungus with the ability to temporally and spatially regulate enzyme targeting to the peroxisomes.

3.2.2 Cytosol—Another common subcellular site shown to house SM biosynthetic machineries is the cytosol (e.g. aflatoxins, sterigmatocystin, β -lactam antibiotics). The cytosol functions as a hub for synthesis and trafficking of enzymes, substrates, and cofactors across various subcellular compartments; due to the intertwined hyphal network nature of fungal lifestyle, the cytosol inherently acts as a highway for organelle trafficking across a single hypha, across the hyphal network, and to various morphological forms of the differentiating fungus. Many SM-associated proteins that are localized to various subcellular organelles tend to undergo an initial transient localization in the cytosol and then subsequent trafficking to their targeted sites. As demonstrated in the aflatoxin biosynthetic pathway using time-fractionated fungal colony technique coupled with immunoelectronmicroscopy, biosynthetic enzymes involved in the early, middle, and late stages of the aflatoxin biosynthetic pathways (Nor-1, Ver-1, and OmtA) initially distribute throughout the cytoplasm at early growth during aflatoxin-inducing conditions (24-36 hours) and subsequently localize to vesicles and vacuoles at later growth (48-72 hours)⁴⁵⁻⁴⁷. Versicolorin B synthase (Vbs), a late-pathway enzyme involved in aflatoxin biosynthesis also showed similar localization patterns. Vbs, the only glycosylated enzyme in the aflatoxin pathway, is observed to initially localize throughout the cytosol and then to ER-derived vesicles via the classical secretion mechanism⁴⁸.

Aside from acting as a transient hub for enzyme translocation, the cytosol has also been shown in multiple biosynthetic pathways to house specific steps in a given pathway. IPN synthase (IPNS), an enzyme that catalyzes the second step of the penicillin and cephalosporin biosynthetic pathway responsible for converting δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N⁴⁹ is localized to the cytosol. Recently, two middle-pathway enzymes in fumiquinazoline biosynthesis (FmqB and FmqC) were shown to co-localize to the cytosol in *A. fumigatus*²¹ (See section 3.3 for more details). Both FmqB and FmqC act in tandem to complete a single biosynthetic step: the conversion of FqF to FqA²¹. Another biosynthetic pathway with enzymes localized to the cytosol is the diterpenoid phytohormone, gibberellin, produced by *Fusarium fujikuroi* (see section 3.2.3 for more details). In gibberellin biosynthesis, *ent*-copalyl diphosphate/ *ent*-kaurene synthase (Cps/Ks), a middle-pathway bifunctional enzyme that catalyzes conversion of geranylgeranyl diphosphate (GGDP) to *ent*-kaurene is found to localize to the cytosol⁵⁰. It may be noteworthy that unlike the peroxisomes that can either house the initial or terminal biosynthetic step(s) of a pathway, the cytosol as of to date, is reported to only house intermediate steps of a biosynthetic pathway.

3.2.3 Vesicles, Vacuoles, and Toxisomes—Vesicles and vacuoles constitute a member of an elaborate intracellular endomembrane network; these organelles have promiscuous yet pivotal role in spatial organization (e.g. trafficking, storage, export of SM-associated machineries) of secondary metabolism^{15, 21, 28}. Vesicles, a double membrane-bound organelle, form through the budding of various intracellular membranous organelles; secretory vesicles are formed via budding from the endoplasmic reticulum (ER) and Golgi apparatus; endosomes are formed via invagination of the cytoplasmic membrane; various types of vesicles are formed via budding from peroxisomes, mitochondria, and nucleus. Based on the nature of vesicle biogenesis, these organelles serve as excellent carriers for protein cargos to and from different subcellular compartments (e.g. secretory vesicles to vacuoles) and materials from the extracellular space (e.g. endosomes)^{28, 51}. Vacuoles on the other hand, (small and large) are single-membrane bound organelles formed from the fusion of endosomes and vesicles and are important sites for macromolecule recycling, storage of amino acids, organic and inorganic nutrients, and maintaining intracellular pH homeostasis⁵¹. Thus, vacuoles act like a hub for material exchange of both intracellular and extracellular origins. With such versatility, fungi have evolved to maximize its limited genome and utilize this established cellular membrane flow system to achieve spatial organization of their SM-associated machineries.

The aflatoxin biosynthetic machinery, starting most likely in the peroxisome¹⁵, is proposed to utilize different vesicle biogenesis mechanisms (e.g. secretory, cytoplasmic to vacuole transport) to segregate its pathway enzymes into distinct vesicles prior to consolidating these enzymes via vacuolar fusion to form a specialized multi-functional vesicle called toxisomes (e.g. aflatoxisomes)¹⁵. Aflatoxisomes are speculated to arise from the fusion of at least three types of vesicles/vacuoles of distinct subcellular origins, each containing enzymes involved in various stages of aflatoxin biosynthesis¹⁵. Of particular interest is the distinct localization of the middle-pathway enzyme, AflK (Vbs), from other pathway enzymes into ER-derived secretory vesicles. The authors speculate that the AflK-containing vesicle must

fuse with two other vesicles of distinct origins containing late pathway enzymes to bring all the aflatoxin-generating machineries together and complete the biosynthetic pathway¹⁵. As AflK catalyzes the formation of versicolorin B, the last non-toxic intermediate in the aflatoxin biosynthetic pathway, its distinct compartmentalization, in addition to sequestering toxic end products into specialized vesicles (toxisomes), allows the cell to safely store other non-toxic intermediates and elicit temporal control over the production of the pathway's toxic end products. More recently, toxisomes have also been implicated in trichothecene biosynthesis⁵².

3.3 New Insights into Tempo-Spatial Coordination of Secondary Metabolism through Fumiquinazoline Synthesis

The fumiquinazolines comprise a family of sequentially generated cytotoxic peptidyl alkaloids that are signature metabolites of *A. fumigatus*. Recent expression and localization studies on fumiquinazoline biosynthesis provide new insights into the temporal and spatial control of fungal secondary metabolism^{21, 53}. Fumiquinazoline C (FqC), the terminal metabolite of the cluster is selectively accumulated to the conidia²¹. FmqA (trimodular NRPS), the first biosynthetic enzyme in the pathway responsible for synthesizing fumiquinazoline F (FqF) is localized to punctate vesicles within the cell; the nature of this vesicle is yet to be determined²¹. FmqB (monooxygenase) and FmqC (monomodular NRPS) catalyze the intermediate reaction in the pathway are both co-localized to the cytoplasm²¹. Both FmqB and FmqC act in tandem to complete a single biosynthetic step: the conversion of FqF to fumiquinazoline A (FqA)²¹. The co-localization suggests a potential formation of a multienzyme complex to increase efficiency of substrate channeling. Formation of such multienzyme complex has been previously observed for the aflatoxin-associated machinery where a multienzyme complex called norsolorinic acid synthase (NorS) consists of two fatty acid synthases (Fas1 and Fas2) and one polyketide synthase (PksA)⁵⁴.

The final enzyme in fumiquinazoline biosynthesis, FmqD (oxidoreductase responsible for converting FqA to FqC) is of particular interest as it is the first secondary metabolite enzyme shown to localize to the cell wall matrix of both hyphae and spores of the fungus²¹. Localization studies of FmqD indicate the protein's passage through the classical secretory (ER-Golgi) pathway and subsequently to the cell wall matrix²¹. Observations to support passage of FmqD through the classical secretory system include i) predicted three surface-exposed glycosylation sites via homology modeling of the protein, ii) presence of an N-terminal signal peptide predicted to facilitate extracellular export, and iii) cell wall localization of FmqD is disrupted when the fungus is treated with Brefeldin A, an inhibitor of the ER-Golgi transport. Western blot analysis of non-covalent cell wall extracts and also the growth medium of the fungus indicate that FmqD can be secreted into the environment. As FmqD is responsible for synthesizing the terminal product FqC, localization of FmqD to the cell wall matrix potentially shed new light on metabolite export and compartmentalization. The enzymes responsible for synthesizing FqA (precursor to FqC) are localized in the cytosol and therefore, FmqD localized to the cell wall may present a new mechanism for enzyme-mediated metabolite delivery to the extracellular space. This spatial regulation of fumiquinazoline enzymes is further complicated by the temporal regulation by

the sporulation-specific transcription factor, BrIA. BrIA is required for spore development in the *Aspergilli* ^{21, 55}. Removal of either BrIA or cell wall localization signals of FmqD abolishes selective accumulation of fumiquinazoline C to the conidia. When the N-terminal signal sequence is truncated from FmqD, we observe that the enzyme mislocalize to the cytosol, demonstrating that proper localization of FmqD to the cell wall is essential for proper enzyme function and FqC accumulation in the spores. These observations imply that both temporal and spatial coordination between a known developmental regulator for asexual sporulation and the classical secretion machinery plays an important role to achieve selective localization of metabolites to specific developmental structures of the fungus.

3.4 Natural Products and the Fungal Spores

An inevitable part of the filamentous fungus lifecycle is the formation of an elaborated multicellular network of hyphae called the mycelia, which upon appropriate environmental and cellular signals can further differentiate into specialized developmental structures (e.g. conidiophores, cleistothecium, perithecium) that bear asexual (e.g. conidia) or sexual (e.g. ascospores) spores. In some species of fungi (e.g. *A. flavus* and *Claviceps purpurea*), the mycelial network can form masses of compact and dense overwintering structures called sclerotia; this overwintering structure contains nutrient reserves that the fungus needs to survive long periods of extreme environmental conditions (e.g. winter) and also generates ascospores under the right conditions. Sclerotia in particular accumulate natural products that have a repertoire of chemical and biological activity and act as defense molecules against various abiotic and biotic confrontations ^{56, 57} and deletion of SM genes have resulted in loss or aberrancies in sclerotial production in *Aspergillus flavus* ^{58, 59}. One of the earliest observations of fungal SM was the localization of specific metabolites to asexual spores. Melanin have been associated with spores of many fungi ⁶⁰ and some of the first SM biosynthetic genes cloned were those of the *A. nidulans* ⁶¹ and *A. fumigatus* ⁶² polyketide synthase genes responsible for melanin production. Ecological and pathogenicity studies have shown that spore SMs are often associated with resistance to UV radiation and/or enhanced resistance to host defense in pathogenic fungi ⁶³. Recently, endocrocin, a spore SM from the human pathogen, *A. fumigatus*, is shown to have immunosuppressive properties against neutrophils. Interestingly, it is discovered that one of the global regulators of secondary metabolism, LaeA, may mediate asexual spore SM production through activation of BrIA, the *Aspergillus/Penicillium* transcription factor required for conidiophore and hence conidial development ⁶⁴⁻⁶⁶. For example, the human pathogen *A. fumigatus* contains many asexual spore-associated SMs including endocrocin, alkaloids, fumiquinazolines, tryptacin ⁶⁷⁻⁷⁰, all of which are regulated by LaeA and BrIA. Although most SMs have been associated with pigmentation of asexual spores, several SMs are found only in the sexual spore or surrounding tissues such as fusarubins, a polyketide pigment synthesized by *Fusarium* spp. localized in the perithecium (sexual fruiting body) ⁷¹.

4. Conclusion

Historically, fungal natural products were viewed as by-products of primary metabolism with no known role in fungal biology. It is now apparent that fungal SMs have evolved ecological and biological functions to secure niches and ensure long-term survival and

adaptation to the producing organism. Various studies have shed light on the pivotal role SMs play in fungal development as signaling molecules, and in defense against various biotic and abiotic confrontations⁷²⁻⁷⁶. Some SMs are continuously produced and selectively accumulated to specific developmental structures (e.g. asexual spores, sexual spores, mycelia, fruiting bodies etc.) and oftentimes serve protective roles (e.g. UV damage, oxidative stress, etc.) for these structures; some SMs are induced only upon certain environmental challenge (e.g. production of siderophores in response to iron starvation). It is clear now that these fungal armaments not only play a major role in fungal fitness but also impact disease progression in pathogenic fungi^{77, 78} and provide the chemical framework for a multitude of pharmaceuticals^{79, 80}.

Full orchestration of secondary metabolism requires not only the coordinated control of SM biosynthetic gene clusters but also proper translocation of the SM-associated machineries (e.g. substrates, enzymes, cofactors, pathway intermediates, and terminal products) both within a single cell and between different specialized cells (e.g. deposition of certain natural products to specific developmental tissues)^{15, 21, 26, 28, 81}. To achieve that, fungi have “learnt” to exploit established cellular resources, processes, and protein trafficking machineries necessary to support life to generate a diversity of natural products that provide them with long-term ecological fitness^{15, 21}. Spatial organization via compartmentalization of biosynthetic machineries may provide new avenues for yet another tier of natural product regulation such as (i) coordinated production of a set of natural products and (ii) a mechanism for the simultaneous delivery of a set of compounds to a common target site or developmental structure to facilitate synergistic effects of these compounds. Co-compartmentalization of enzymes from pathways that produce structurally similar metabolites (e.g. anthraquinones produced by non-reducing PKSs) could potentially mediate crosstalk(s) between these structurally related but distinct biosynthetic pathways through enabling utilization of common intermediates. Vice versa, subcellular compartmentalization could be a means of segregating not only certain enzymes in a given pathway but also segregating enzymes of a given pathway from another presumably to prevent unwanted biochemical crosstalk between intermediates and enzymes of co-expressed gene clusters. Hence knowledge of where and how these metabolites are synthesized in the fungus are critical for future studies be they industrially, ecologically or medicinally motivated.

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Figure 1.

Generating Structures of Increasing Complexity. Figure 1 depicts the biosynthetic flow of basic building blocks (acyl-CoA, amino acids) to generate highly complex chemical structures of select natural products discussed in this review. Formation of polyketide-peptide and polyketide-terpene (meroterpenoids) hybrids further contributes to the increased structural complexity (not depicted). Abbreviations: DMAPP (dimethylallyl diphosphate); GPP (geranyl diphosphate); GGPP (geranylgeranyl diphosphate); DMAT (dimethylallyl tryptophan).

Table 1

Subcellular Localization of Secondary Metabolism Genes and Enzymes

Class	Biosynthetic Pathway	Enzymes	Function	Localization (Subcellular)	Reference
Polyketide	Aflatoxin	AflA (Fas-1), AflB (Fas-2), AflC (PksA)	fatty acid synthase α fatty acid synthase β PKS	Peroxisomes ^A	15, 16
		AflD (Nor-1)	ketoreductase	Cytoplasm, Vesicles, Vacuoles	45, 47
		AflK (Vbs)	cyclase; Versicolorin B synthase	Cytoplasm, Vesicles, Vacuoles	28, 48
		AflM (Ver-1)	dehydrogenase	Cytoplasm, Vesicles, Vacuoles	28, 46-48
		AflP (OmtA)	O-Methyltransferase A	Cytoplasm, Vesicles, Vacuoles	27, 45
		AflQ (OrdA)	oxidoreductase	Cytoplasm, Vesicles, Vacuoles	27
Peptides	Fumiquinazolines	FmqA ^B	NRPS	Vesicles, Vacuoles	21
		FmqB	monooxygenase	Cytoplasm	21
		FmqC	NRPS	Cytoplasm	21
		FmqD	oxidoreductase	Cell Wall	21
		FmqE ^B	MFS transporter	Punctate Organelles	21
	Siderophores	SidI	mevalonyl-CoA ligase	Peroxisomes	36
		SidH	mevalonyl-CoA hydratase		36
		SidF	androhydromevalonyl-CoA transferase	Peroxisomes	36
	Penicillin/Cephalosporin	ACVS ^C	NRPS	Vacuoles, Cytoplasm	49, 82-84
		IPNS	oxidoreductase	Cytoplasm	49, 84
		IAT/AT	CoA:6 amino penicillanic acid acyltransferase	Peroxisomes	38, 49, 85
		PCL	phenylacetyl CoA ligase	Peroxisomes	39
	Cyclosporin	Cyclosporin Synthase	NRPS	Vacuolar Membrane	86
	AK-toxin	Akt1	carboxyl-activating enzymes	Peroxisomes	37
		Akt2	estelase-lipase type enzymes	Peroxisomes	37
		Akt3	hydratase-isomerase type enzyme	Peroxisomes	37
	Amatoxins	POPB	prolyl oligopeptidase	Vacuoles	87
Terpenes	Trichothecenes	Hmr1p	hydroxymethylglutaryl (HMG)-CoA reductase	ER, Vesicle (toxisomes) Membrane	52
		Tri4p	cytochrome P450 moxygenase	Vesicles (toxisomes)	52

Class	Biosynthetic Pathway	Enzymes	Function	Localization (Subcellular)	Reference
		Tri1p	cytochrome P450 moxygenase	Vesicles (toxisomes)	52
		Tri12p	transporter	Vesicles, Vacuoles, Plasma membrane	52
	Gibberellic acids	HmgR	hydroxymethylglutaryl (HMG)-CoA reductase	ER-membrane	50
		Cps/Ks	<i>ent</i> -copalylidiphosphate/ <i>ent</i> -kaurene synthase	Cytoplasm	50
		Ggs2	geranylgeranyl disphosphate synthase	Punctate Organelles	50
	Paxilline	GgsA	geranylgeranyl disphosphate synthase	Punctate Organelles	40
		GgsB	geranylgeranyl disphosphate synthase	Peroxisomes	40

^AThe product of these enzymes, norsolorinic acid, is found in the peroxisomes and these enzymes are shown to co-localize in a multi-enzyme complex. However, direct evidence for enzyme accumulation in the peroxisomes is still lacking ¹⁶.

^BThe nature and origin of the vesicles and punctate organelles yet to be determined ²¹.

^CContradicting reports exist for vacuolar vs. cytosolic localization of ACVS ^{83, 84}.