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Fungal secondary metabolism: regulation, function and drug discovery

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Abstract

One of the exciting movements in microbial sciences has been a refocusing and revitalization of efforts to mine the fungal secondary metabolome. The magnitude of biosynthetic gene clusters (BGCs) in a single filamentous fungal genome combined with the historic number of sequenced genomes suggests that the secondary metabolite wealth of filamentous fungi is largely untapped. Mining algorithms and scalable expression platforms have greatly expanded access to the chemical repertoire of fungal-derived secondary metabolites. In this Review, I discuss new insights into the transcriptional and epigenetic regulation of BGCs and the ecological roles of fungal secondary metabolites in warfare, defence and development. I also explore avenues for the identification of new fungal metabolites and the challenges in harvesting fungal-derived secondary metabolites.

Fungi have a long and intimate connection with humankind, particularly at the chemical level. The realization that fungi were the source of both harmful and beneficial compounds was brought to light by the aflatoxin poisoning event Turkey X disease in the 1960s[1] and the discovery of the first broad-spectrum antibiotic, penicillin, considered the ‘wonder drug’ of World War II[2]. These bioactive molecules, termed secondary metabolites (also known as natural products), are produced by specific fungal taxa, predominately by filamentous fungi that belong to the Pezizomycotina Ascomycete class, and several Basidiomycete classes (for example, Agaricomycetes and Exobasidiomycetes), as well as by unexpected taxa such as *Kluyveromyces lactis*, in which the pulcherrimin gene cluster was recently discovered[3].

Secondary metabolites are derived from central metabolic pathways and primary metabolite pools, with acyl-CoAs being the critical initial building blocks that feed into the synthesis of polyketide (for example, aflatoxin) and terpene (for example, carotene) secondary metabolites and amino acids being used for the synthesis of non-ribosomal peptide secondary metabolites (for example, penicillin) (FIG. 1a). In contrast to genes that are required for the synthesis of a primary metabolite that are dispersed throughout the fungal

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genome, the genes encoding the enzymatic activities to produce any secondary metabolite are arranged in a contiguous fashion as a biosynthetic gene cluster (BGC), such as the aflatoxin BGC[4] (FIG. 1b). Secondary metabolites are crucial players in fungal development and actively shape interactions with other organisms. Indeed, genes within a BGC are often co-regulated in accordance with the ecological function of their encoded secondary metabolite. For example, the BGCs that encode pigments in *Aspergillus fumigatus* are induced during spore synthesis in this fungus[5], the BGC that encodes the *Fusarium graminearum* virulence factor trichothecene is upregulated during colonization of plants[6] and the *Fusarium* spp. BGC that encodes the antibacterial compound bikaverin is expressed during confrontations with the bacterium *Ralstonia solanacearum*[7].

An often cited literature survey[8] showed that of the 1,500 compounds that have been isolated from fungi between 1993 and 2001, more than half displayed antibacterial, antifungal or antitumour activity. A newer review[9] covering fungal natural products that were discovered between 2009 and 2013 confirms the enormous potential of the fungal secondary metabolome. Although the underlying interest in fungal secondary metabolites is multivariate, these reviews highlight the predominate interest in fungal secondary metabolites — drug discovery. This interest has escalated in the past 10 years as a consequence of advances in genome sequencing, bioinformatic algorithms, phylogenetic sleuthing and increasing ease in fungal genome manipulations[10].

In this Review, I present a universal view of key discoveries in the field of fungal secondary metabolites that have led to the prevailing worldwide focus of extracting the fungal secondary metabolome for profit and understanding of microbial communication. I describe the classification and genetics of secondary metabolites and BGCs, the transcriptional and epigenetic regulation of clusters and the ecological roles of secondary metabolites in defence, warfare and development. Finally, I explore avenues for the identification of new fungal metabolites with potential pharmaceutical application and the challenges in characterizing BGCs.

Chemistry and genetics

The synthesis of secondary metabolites primarily involves the polymerization of primary metabolites by dedicated enzymes (often referred to as backbone or core enzymes). The metabolites generated by the backbone enzymes are further ‘decorated’ by additional enzymes that can vastly alter the bioactivities of the metabolites. The backbone enzyme defines the chemical class of the generated secondary metabolite. For example, polyketide synthases (PKSs) produce polyketides from acyl-CoAs, non-ribosomal peptide synthetases (NRPSs) generate non-ribosomal peptides from amino acids and terpene synthases and terpene cyclases (TSs and TCs, respectively) generate terpenes from activated isoprene units. Some secondary metabolites are hybrids that are synthesized from two synthases and/or synthetases such as fumagillin (PKS–TC hybrid) or echinocandin (PKS–NRPS hybrid). The PKS–NRPS-derived hybrid secondary metabolites are often referred to as lipopeptides, but care is needed in assuming the origins of lipopeptides as the term also refers to molecules formed from ribosomally derived peptides attached to fatty acids or isoprenoids (such as canonical fungal mating pheromones)[11]. The two enzymes may be separate (for example,

NRPS and PKS, generating valactamide)[12] or fused (NRPS–PKS, generating tenuazonic acid)[13]. These backbone enzymes define the 'typical' classes of secondary metabolites, and the reader is referred to reviews focusing on the interesting chemistry involved in the synthesis of these metabolites[14–17]. Fungal secondary metabolites that are not generated by the synthases or synthetases discussed above include the ribosomally derived peptide ustiloxin[18], fatty-acid-derived oxylipins[19] and the recently identified isocyanide xanthocillin, which requires an isocyanide synthase[20].

The genes involved in the biosynthesis of fungal secondary metabolites are typically arranged in BGCs, a chromosomal architecture that has facilitated the development of algorithms to predict BGCs that encode conserved synthases and/or synthetases in fungal genomes. Of note, algorithms are merely predictive and may underpredict or overpredict inclusion of genes within a BGC. True association with a cluster can be confirmed only through gene deletion. BGCs can range from two genes (for example, the valactamide BGC)[12] to over 20 genes (for example, the aflatoxin BGC)[21]. The smaller clusters (two to four genes) contain genes encoding synthases or synthetases and tailoring enzymes that decorate initial synthase or synthetase products of the secondary metabolite. Larger BGCs contain not only synthases and/or synthetases and many tailoring genes but also frequently a gene that encodes a cluster-specific transcription factor as well as several genes inconsistent with chemical structure formation and/or hypothetical in nature. For example, although several genes in the sterigmatocystin BGC are co-regulated during the production of sterigmatocystin, there is no defining phenotype following gene deletion (for example, *stcC*, *stcM* and *stcR*)[22]. Many of these uncharacterized genes encode hypothetical, fungal and even species-specific proteins such as Cpur_05425 and Cpur_05426 in the ergochrome BGC[23] and AFLA_022990, AFLA_023060 and AFLA_023090 in the aspergillic acid BGC[24]. By contrast, some of the proteins encoded by these other incongruous genes that were once overlooked are now known to provide protection from — or localization and/or destination of — toxic BGC natural products[25] (see below).

Although most natural products are produced from a series of contiguous genes, there are notable exceptions to this arrangement. *A. fumigatus* possesses a 'supercluster' in which the genes encoding the secondary metabolites fumagillin and pseurotin are intertwined in one genomic locus[26]. Moreover, in *Aspergillus nidulans*, the synthesis of nidulanin A requires the enzymatic activity encoded on two chromosomes[27] and in various *Fusarium* species, trichothecene genes are located in a primary *tri5* BGC, but at least three genes are located outside of this cluster[28]. Dothiostromin, a phytotoxin structurally related to aflatoxin, is encoded by genes that are fragmented into three mini-c lusters on a single chromosome of the pine pathogen *Dothistroma septosporum*[29].

Cluster regulation

Environmental signals.

The transcriptional and epigenetic activation of BGCs is a consequence of environmental stimuli and is dependent on the developmental stage of the producing fungus[30,31]. Nutritional input has long been realized to be important as reflected in the one strain-many compounds (OSMAC) approach to metabolite mining[32]. Temperature and light have been

observed to induce or repress the synthesis of natural products since studies of aflatoxin production in *Aspergillus flavus* decades ago[33]. The regulatory pathway in response to changes in temperature has been shown to be dependent on the Velvet complex (see below) in *A. fumigatus*[34] and may have an impact on virulence (for example, the production of at least two *A. fumigatus* spore secondary metabolites, the toxin trypacidin and the immunomodulator endocrocin is temperaturedependent)[35,36] or mycotoxin contamination (exemplified by the temperature-dependent production of the terpene T-2 toxin in *Fusarium* species)[37].

A considerable number of research groups have identified roles for red and blue light photoreceptors and/or their respective signal transduction pathways during the synthesis of fungal secondary metabolites[38]. The aflatoxin and related sterigmatocystin mycotoxin BGCs are among the well-known clusters that are repressed by white light[39] (FIG. 2), whereas the *Alternaria alternata* mycotoxins alternariol and altertoxin are stimulated by white light (specifically blue light)[40]. The photoresponse pathways can intersect with known transcription factors such as CreA and/or Cre1 (the carbon catabolite regulator in fungi, called CreA in some species and Cre1 in other species) to regulate the synthesis of natural products such as the polyketide dihydrotrichotetro-nine[41]. The finding that the *A. nidulans* phytochrome FphA forms a complex with both blue light receptors and VeA, which is a member of the Velvet complex, provides a mechanistic model for how light sensing and the synthesis of secondary metabolites are conjoined[42,43]. The transcriptional responses of BGCs to changing environmental stress pathways, particularly oxidative stress, support the notion that secondary metabolites (for example, aflatoxin) function as protective agents from reactive oxygen species[44,45]. Taken together, published data clearly demonstrate that environmental signals that affect the synthesis of secondary metabolites are interdependent[46].

Transcriptional regulation.

Our understanding of the regulation of BGCs[47] continues to expand at a fast pace. Insights that have been gained from several studies present a hierarchical genetic circuitry from cluster-specific regulators to global transcriptional complexes (FIG. 2). Approximately, up to 50% of fungal BGCs contain a cluster-specific transcription factor, most commonly a C6-zinc cluster protein that recognizes palindromic motifs in cluster gene promoters[48] (FIG. 1b). Occasionally a BGC contains more than one transcription factor[49,50]. Although originally thought to solely (and positively) regulate the genes within a given BGC, it is clear that some C6 transcription factors regulate genes within other BGCs and several metabolic pathways[51,52]. These studies suggest that a deeper analysis of the circuitry regulated by cluster-specific transcription factors could reveal unexpected insights into metabolic programmes that link BGC product formation with primary metabolism. For example, RNA sequencing (RNA-seq) and chemical analysis of overexpression of HasA, the C6 transcription factor that positively regulates the ironbinding secondary metabolite hexadehydroastechrome encoded within the *has* BGC, showed that HasA is a key component of a metabolic feedback circuitry that balances iron pools in *A. fumigatus*. This study exposed the concept that metal availability, in this case iron, could be a trigger for the production of secondary metabolites[51]. Metal starvation, specifically copper insufficiency,

induces the synthesis of xanthocillin, presumably through the regulation of the *xan* BGC C6 transcription factor XanC[20].

A number of 'broad-domain' transcription factors contribute to both positive and negative regulation of several BGCs, including the pH regulator PacC, CreA, the nitrogen regulator AreA and the CAAX basic leucine zipper protein HapX[47]. However, by far the most influential transcriptional complex that affects global regulation of secondary metabolites across every fungal genera studied thus far is the Velvet complex. This complex was first described in *A. nidulans*[39] and is composed of LaeA (in *Aspergillus* spp., also known as Lae1 in most other fungi), VeA (or Vel1) and VelB (or Vel2). Detailed studies have primarily focused on LaeA (Lae1)[53] and VeA (Vel1), which is postulated to be the DNA-binding partner of the complex. In general, these proteins are associated with global positive regulation of BGCs with some exceptions[54]. In contrast to the Velvet complex, McrA seems to be a global negative regulator of BGCs in *Aspergillus* and *Penicillium* species[55].

Follow-up studies of microarray and RNA-seq cascades of regulators involved in the production of secondary metabolite have yielded unpredictable insights into BGC regulation. BrlA is a C_2H_2 transcription factor required for conidiophore development in *Aspergillus* and *Penicillium* species. Although several studies over the past 10 years have shown a contribution of BrlA to the production of secondary metabolites, particularly the regulation of metabolites involved in spore formation[56,57], the magnitude of this regulation was only recently revealed in a study linking LaeA-mediated regulation of BrlA to the genome-wide control and biosynthesis of BGCs in *A. fumigatus*[5]. BrlA-dependent regulation of BGCs showed remarkable concordance with LaeA-dependent regulation of BGCs active during spore formation and during the non-developmental vegetative state.

Epigenetic regulation.

Efforts to remodel the fungal chromosome landscape by altering transcriptional accessibility to BGCs have been particularly fruitful in identifying metabolites from cryptic clusters[58]. The idea that heterochromatin and euchromatin could be important features in BGC accessibility arose from the gene cluster architecture, which suggested a co-regulation mechanism of expression associated with chromosome structure. This hypothesis was supported by a study showing a correlation between histone H4 acetylation of aflatoxin BGC promoters and the transcriptional order of their expression[59]. In 2007, the first study to address the potential of epigenetic manipulation in fungal secondary metabolite discovery[60] reported that deletion of *hdaA*, which encodes a histone deacetylase, resulted in the transcriptional activation and consequent increase in the expression of multiple BGCs and their products in *A. nidulans*. Since then, a plethora of studies have expanded the concept of epigenetic regulation of BGCs, either by characterizing histonemodifying enzymes involved in activation or silencing of chromatin tracks or by treatment of fungal cultures with chemical inhibitors of these enzymes[58,61–63]. Most of these studies identified cryptic metabolites that were synthesized by deleting or overexpressing genes encoding histone acetylases, deacetylases, methylases and demethylases[61,64]. However, it is likely that an equal number of compounds are dampened through epigenetic regulation. For example,

downregulation of the *A. nidulans* histone deacetylase RpdA resulted in equal numbers of metabolites being upregulated and downregulated by 100-fold[65].

Several studies have coupled secondary metabolite regulatory networks with the epigenome. In 2010, a study[66] showed that during the early-phase growth, the *A. nidulans* sterigmatocystin BGC is silent with methylation marks on histone H3 on residue K9 (indicative of heterochromatin), and those chromatin marks are reversed (indicative of euchromatin) as the fungus switches to the stationary, secondary metabolitepromoting growth phase; this reversal requires the global secondary metabolite regulator LaeA. Lae1, the functional homologue of LaeA, is also involved in regulating chromatin marks in *Trichoderma reesei*[67], and in *Fusarium fujikuroi*, overexpression of the histone acetyltransferase HAT1 histone acetyltransferase could restore secondary metabolism in a Δ*lae1* background[68]. A pioneering study aimed at uncovering cryptic BGCs[69] showed that the bacterium *Streptomyces rapamycinicus* induced the expression of the silent orsellinic acid BGC in *A. nidulans* through the activation of the histone acetyltransferase GcnE, a member of the histone acetyltransferase SAGA–ADA (Spt–Ada–Gcn5–acetyltransferase–ADA) complex. The SAGA–ADA complex also induced the expression of the sterigmatocystin BGC (FIG. 2). This work thus tied interspecies communication to the epigenetic machinery[70]. The LaeA partner VeA has also been connected to GcnE activity in suppression of the silent orsellinic acid BGC[71].

Functions of secondary metabolites

There are several lines of evidence supporting ecological fitness roles for fungal secondary metabolites, including the assessment of regulatory cascades, genetic studies of BGC mutants and interaction studies with other organisms[25,30,47]. These studies showed that many genes that encode secondary metabolites are regulated in a manner congruent with fungal development or in response to stressors (both abiotic and biotic) and that loss or overproduction of specific secondary metabolites can alter fungal development, survival or interkingdom and intrakingdom encounters.

Protection from UV damage.

Melanin is a natural pigment typically found in spores or hyphae, and it is derived from polyketide or L-3,4-dihydroxyphenylalanine (L-DOPA) pathways. Melanin biosynthesis genes are frequently arranged in BGCs[72,73]. One of the first ecologically minded studies to show a protective role of spore melanin reported that an albino mutant of the maize pathogen *Cochliobolus heterostrophus* (formerly *Bipolaris maydis*) was unable to survive in the field[74]. Since this initial discovery, many studies have demonstrated the role of melanins and other aromatic natural products in photoprotection and/or protection from antioxidant chemicals[75,76] (FIG. 3a). The same properties that protect from oxidizing radiation also have been shown to provide protection from host defence molecules in pathogenic fungi[77] and have provided the foundation for the application of melanin as ultraviolet protectants for human skin and in food colouring, bioelectronics and cosmetics[78].

Defence and weapons.

Protective and weaponized natural products help fungi obtain land rights in highly competitive ecological niches. Although hundreds of studies note the antibiotic properties of fungal metabolites, most studies are correlative and consist of testing extracts against a multitude of bacteria and fungi. Additionally, tests of extracts are biased in the form of concentrations relative to ecological concentrations, and it may well be that physiologically relevant concentrations of a metabolite could function as a signal rather than as a toxin. This possibility is illustrated by the gradient-dependent effects of *Pseudomonas aeruginosa* phenazine on *Aspergillus*, whereby a high concentration of phenazine is antifungal but a moderate concentration induces prolific sporulation in the fungus[79]. Similarly, the literature has dozens of examples of induction of fungal metabolites when fungi are confronted by other microorganisms and insects. Genetic, ecological and mechanistic studies now provide indisputable evidence that fungal chemicals compose the core element of microbial 'language' (fungi and microorganisms[80]; fungi and plants[81]; and fungi and insects[82]). Informative insect–fungal and bacterial–fungal chemical encounters illustrate the sophistication of this language as detailed below.

The production of either fungal or bacterial natural products has ecological outcomes on each microorganism. A series of elegant studies have addressed the curious symbiosis of the fungus *Rhizopus microsporus* with the endosymbiotic bacterium *Burkholderia rhizoxinica.R. microsporus* causes rice seedling blight, which is exacerbated by the microbial toxin rhizoxin. Although originally thought to be synthesized by the fungus, it is the endosymbiont that actually produces the polyketide macrolide[83]. To add further complexity to the system, rhizoxin is further chemically altered by some host fungi to enhance phytotoxicity in a mutually beneficial outcome for the two microorganisms[80,84].

In what seems to be a more confrontational interaction, a succession of studies have unveiled a critical chemical role in the fraught interaction of the soil phytobacterium *R. solanacearum* with agricultural fungi. The lipopeptide ralsolamycin produced by *R. solanacearum* induces fungal development of a bacterial 'housing' chamber (chlamydospore)[85] and is also the signal for either suppression (for example, imizoquin synthesis in *A. flavus*)[86] or induction (for example, bikaverin in *Fusarium* spp.)[7] of antibacterial fungal secondary metabolites. Intriguingly, induction of bikaverin by ralsolamycin is conserved in the fungus *Botrytis cinerea*, which horizontally acquired the bikaverin BGC from *Fusarium* spp.[87,88]. This study raises the concept that horizontally acquired BGCs may also transfer conserved regulatory responses, possibly as fitness responses (in this case production of an antibacterial compound), which promotes success in polymicrobial conflicts (FIG. 3b).

Another example of bacterial–fungal interaction is the unique multi-kingdom symbiosis of the Attine (fungus-farming) ant system. The ants are colonized by antibiotic-producing actinobacteria that protect the fungal garden from colonization by mycoparasites, including the fungus *Escovopsis* sp. The symbiotic bacterium *Pseudonocardia* sp. produces various antifungals (for example, dentigerumycin) that specifically target *Escovopsis* but not the garden fungus *Leucoagaricus gongylophorus*[89]. *Escovopsis* in turn also produces natural products that target both the actinobacteria and *L. gongylophorus*[90].

Fungal–bacterial interactions may also be more subtle. The above interactions illustrate responses of Ascomycete fungi to various bacteria. Basidiomycete fungi are also known to alter their metabolic profile in bacterial encounters. Pigment synthesis is induced in the brown rot fungus *Serpula lacrymans* and one of the induced pigments, variegatic acid, inhibited swarming and biofilm spreading of *Bacillus subtilis*[91,92].

The entomopathogenic pathogen *Beauveria bassiana* not only kills its insect prey — with toxic secondary metabolites being part of the arsenal — but also then poisons the cadaver with the antibacterial polyketide oosporein to limit microbial competition for its food supply[93]. Aflatoxin produced by *A. flavus* is another secondary metabolite with toxic properties towards insects, and a recent study provided evidence that aflatoxin provides a fitness advantage to *A. flavus* when the fungus encounters insects[82,94]. Together, these studies found that fungal fitness increased 26-fold in competition tests with the insect in an aflatoxin-rich environment and that aflatoxigenic strains of the fungus caused higher mortality in *Drosophila melanogaster* and held a measurable fitness advantage over non-toxigenic strains of the fungus during competition.

Protection from toxic natural products.

If so many of the BGC products are antifungal, how do fungi protect themselves from their own weapons? Many BGCs carry a mechanism to ensure self-protection against toxic BGC products (FIG. 3c). The three primary in-cluster self-protection strategies include efflux transporters (for example, GliA, an efflux pump important in resistance to gliotoxin[95], and Tri12, which provides protection to trichothecenes[28]) or cellular BGC intermediate transporters (for example, CefM, which translocates the intermediate penicillin N from a microbody to the cytosol, where it is converted into the end product cephalosporin C)[96]; detoxifying enzymes that alter the chemical structure of the secondary metabolite to reduce critical target-binding properties (for example, GliT, an oxidoreductase that modifies the gliotoxin structure)[97]; and duplicated copies of the target protein (for example, incluster 3-hydroxy-3-methylglutarylCoA (HMG-CoA) reductase in the lovastatin BGC)[98]. And if the word clever can be applied to fungi, certainly the finding of duplicated, resistant target genes within a BGC fits this definition[99]. This genetic arrangement is particularly propitious for drug discovery as the duplicated function reveals an immediate natural-product eukaryotic target. Fungal BGCs containing known or postulated target proteins, in addition to the extra copy of HMG-CoA reductase in the statin BGCs, include the fumagillin BGC (target methionine aminopeptidases)[26], fetullamide (the experimental validation of the protective value was shown by deleting *inpE*, the in-cluster copy of the proteasome subunit targeted by fetullamide)[99], echinocandin (target β−1,3-d-glucan synthase)[100], aspterric acid (target dihydroxy-acid dehydratase)[101] and mycophenolic acid (target inosine-5′-monophosphate dehydrogenase)[102]. All of these metabolites are antifungal; indeed, echinocandin derivatives are widely used as one of the few effective treatments of invasive human pathogens and pathogenic biofilms[103].

Development.

Fungi produce many natural products that are either incorporated into developmental structures or function as signals to initiate developmental programmes. In addition to the

1,8-dihydroxynaphthalene (DHN) melanin spore metabolites, several BGCs encode pigments or toxic metabolites that provide protection for fungal sexual structures from environmental extremes or insect predation. Fusarubins are required for pigmentation of the perithecia in *Fusarium* species[104], the perithecial toxin furocoumarin (neurosporin A) deters fungivore feeding on *Neurospora crassa* sexual stage[105] and deletion of a PKS in the fungus *Sordaria macrospora* inhibits perithecial maturation, whereas its overexpression resulted in malformed fruiting bodies[106] (FIG. 3d). Similarly, deletion of several BGC backbone genes results in aberrant sclerotial development in *A. flavus*[43]. Natural products are also important in spore germination, although most commonly in an offensive move whereby a fungus or bacterium will secrete a secondary metabolite that inhibits the spore germination of another fungus such as the polyketide GKK1032A from *Penicillium* that inhibits germination of the rice blast pathogen *Magnaporthe oryzae*[107]. By contrast, imizoquins are endogenous metabolites that are required for normal germination in *A. flavus*, in which loss delays and overproduction accelerates germination. Interestingly, the imizoquin BGC is repressed by the *R. solanacearum* lipopeptide ralsolamycin, and subsequently germination is delayed during *A. flavus* co-culture with the wild-type bacterium compared with co-culture of the fungus with a ralsolamycin-deficient mutant[86].

Drug discovery

Since ancient times, health practices have incorporated the medicinal properties of fungi. Several potent fungal secondary metabolites (for example, penicillin, statins, cyclosporin and mycophenolic acid) were knowingly used in large-scale efforts to extend human life starting in the 20th century. However, progress is slow in identifying new fungal metabolites that can be advanced to the clinical stage, in part owing to an inability to identify the BGCs and/or express the desired secondary metabolites to high enough levels. However, the recent advances in genetic tools that enable the exploitation of the fungal metabolome (TABLE 1) coupled with the astounding numbers of BGCs revealed by fungal genome sequences[108] (BOX 1) has created great optimism towards our ability to lucratively harvest fungal-derived pharmaceuticals. This ability is reflected not only in an avalanche of publications but also in the establishment of companies that focus solely on capturing these fungal treasures.

Tools and techniques to access the fungal secondary metabolome.

The ability to capture, produce and characterize fungal secondary metabolites requires use of algorithms to identify BGCs, further bioinformatic tools to assist in structure prediction, genetic expression and induction techniques, host fungi for expression and instrumentation to detect and elucidate structure (FIG. 1; TABLE 1). Several recent reviews provide extensive details in the technological development of mining capabilities[109–111].

The initial algorithms to identify BGCs were published in 2010 (REF.[112]) (SMURF) and 2011 (REF.[113]) (AntiSMASH), coming well after the 2005 publications of three *Aspergillus* spp. genomes, which alerted the research community to the vast number of (cryptic) BGCs in fungi[114–116]. Both algorithms are based on identifying conserved synthase and/or synthetaseencoding genes that are aligned next to genes that are likely to encode decorating enzymes within specified locational parameters. As these programmes do not capture all

BGCs nor address dereplication concerns, additional programmes have been developed such as the new programme DEREPLICATOR + [117] (TABLE 1).

Once a BGC of interest is identified, the genes must be activated, which can be achieved in either the producing fungus (endogenous activation) or heterologous hosts. Endogenous activation is largely limited to fungi with accessible genetic systems (such as *Aspergillus*, *Fusarium*, *Trichoderma*, *Penicillium* and *Pestalotiopsis* spp.) by using knowledge of the regulatory systems (see above). Success in 'turning on' cryptic clusters in these species has been mostly attributed to the overexpression of genes encoding transcription factors[86,118], promoter exchange[99], manipulation of global regulatory complexes[55,119], epigenetic manipulation[61,64], chemical induction (for example, quorum molecule)[120] and co-culture (see above and REF.[121]).

However, many fungi are recalcitrant to genetic manipulation, and the best method is to place and express their BGCs in heterologous systems, most commonly *Saccharomyces cerevisiae* or *Aspergillus* species[110,122,123] (FIG. 4). Various synthetic approaches were successful, often taking advantage of the yeast recombination system to 'stitch' together BGCs using appropriate promoters for the host model[124]. These methods lead to the expression of a single BGC. A pioneering and scalable approach used a variation of the bacterial artificial chromosome system to design the FAC-MS (fungal artificial chromosome-metabolic scoring) system, which enabled the placement of 56 BGCs yielding 15 new compounds in an *A. nidulans* host[12]. Another scalable system, termed HEx, has been described using the host *S. cerevisiae* and led to the detection of 22 compounds from 41 BGCs[125] (TABLE 1).

Limitations to characterizing fungal BGCs.

One challenge of 'picking' the right BGC is that it may already have been characterized. The wealth of sequenced fungal genomes has enabled advanced bioinformatic studies that have defined evolutionary trends in BGC maintenance, formation and decay[126]. Many BGCs and their variants are conserved in disparate fungi, often as a result of horizontal transfer events[88,127,128], thus a bioinformatic search for a cluster homologue is a critical factor in BGC selection. What is not yet achievable is knowing whether a cryptic cluster will actually produce a metabolite given the availability of all techniques at hand. What is rarely addressed — at least in public — are the numerous failures despite successful application of these genetic tools. In some cases, all genes in one BGC are heterologously expressed but no product is formed. Certainly post-transcriptional mechanisms, including intron splicing, misfolding of peptide, lack of precursors and cellular trafficking, could contribute to difficulties in execution, perhaps more so in *S. cerevisiae*, which has not evolved to synthesize complex natural products. But can there be other reasons? Are some BGCs on an evolutionary dead end or, alternatively, on a path to synthesis?

The BGCs of *A. fumigatus* provide a framework for speculation on this matter. This fungus is an important human pathogen, and genome sequences of over 100 isolates have been completed and substantial efforts have been made to identify its (potentially toxic) BGC products. Currently, over 50% of the secondary metabolites encoded in the known BGCs are assigned[20,129,130] (FIG. 5). Detailed examination of BGC sequences of 66 isolates of *A.*

fumigatus revealed seven types of BGC variation, including SNP, gene loss, gene gain, cluster loss, cluster mobility (transfer to another chromosome), fusion of clusters and idiomorphic (multi-allelic) clusters[131]. Of the 36 BGCs, only 20 showed no or little variation and consist primarily of characterized clusters, including the two siderophore clusters (ferricrocin and fusarinine C), fumigaclavine, hexadehydroastechrome, endocrocin, fumisoquin, DHN melanin, gliotoxin, fumiquinazoline, trypacidin, pyripyropene A, neosartoricin and the intertwined fumagillin–pseurotin supercluster. For the remaining 16 clusters, variation ranged from loss to multiple polymorphisms among the 66 strains of *A. fumigatus*, and only 2 of these BGCs are characterized (fumitremorgin and helvolic acid). It is instructive to look at the remaining 14 BGCs. For example, one of uncharacterized BGCs, called the fusarielin-like BGC, has five genes with homology to the six-gene fusarielin cluster in *F. graminearum*[132]. The cluster is absent in 4 of the 66 strains and has SNPs in 43 other strains, most of which, upon inspection, would lead to dysfunction of one or more gene products. Perhaps this cluster represents a death event caught in evolutionary time and no efforts to genetically manipulate the cluster would result in product formation in at least 43 of the strains. The fusarielin-like BGC may be reflective of an ancient BGC horizontal transfer from *Fusarium* to *B. cinerea*, where the bikaverin BGC is repeatedly decayed in numerous isolates of *B. cinerea*[88,133].

Outlook

Nearly 30 years have passed since the discovery of the penicillin BGC. What was then considered a rare event — the contiguous arrangement of genes dedicated to a single metabolite — is now known to be a common motif in the filamentous fungal genome, providing a bounty of wealth for the expansion of the pharmaceutical repertoire. The excitement and consequent investment of funds in fungal drug discovery opportunities are expected to continue unabated owing to the advanced and fairly inexpensive genomic tools available for fungal research that have successfully led to many elegant studies and the discovery of fascinating molecules[12,125].

Existing challenges include avoiding replication (re-discovery) of known metabolites, expression of cryptic BGCs and the identification of BGCs composed of unexplored gene composition. Whereas mass spectrometry can attend in part to the dereplication issue[117,134] and use of dereplication host strains can boost detection of novel compounds[135], it is wise to address this potential concern before extensive genetic efforts. Phylogenetic analyses and bioinformatic programmes (TABLE 1) offer assistance in replication issues and insight into birth and death events in BGC evolution. Avoidance of characterizing synthases and/or synthetases of high identity to known enzymes could help in reducing re-discovery of known compounds, although consideration of the tailoring enzymes within a BGC is important as desired bioactivities can be attributable to modifying steps of the initial polyketide, non-ribosomal or terpene backbone. Additionally, metabolites derived from less common and, as yet, unknown chemistry most assuredly sidestep the replication issue but will require additional programming and machine learning efforts to identify this potential chemical space.

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Box 1 |

How many biosynthetic gene clusters do fungi contain?

In 1990, two independent research groups identified the first secondary metabolite cluster, the penicillin biosynthetic gene cluster (BGC), from the fungus *Penicillium chrysogenum*[136,137]. the clustering motif was considered uncommon at the time. By the mid-1990s, a half dozen additional secondary metabolite clusters had been identified, including the trichothecene, aflatoxin and sterigmatocystin mycotoxin clusters and several melanin BGCs[138]. However, at that time, the research community was not aware of the vast number of BGCs waiting to be discovered. indeed, the first sequenced fungal genome published in 1996 (*Saccharomyces cerevisiae*) was devoid of natural-product BGCs[139]. in 2005, an awakening to the incredible wealth of the fungal secondary metabolome arose from the genome sequences of three *Aspergillus* species[114–116]. Now algorithms suggest between 30 and 70 BGCs per species of fungi that are rich in secondary metabolites[140]. there are no studies of precisely how many species contain BGCs, but just considering *Aspergillus* and *Penicillium* alone, these genera are estimated to contain 339 and 354 species, respectively[141,142]. averaging the number of clusters to a conservative 50 BGCs per species, that would be 34,650 BGCs. even assuming that up to 25% of the clusters are duplicates, this brings the number to approximately 25,000 in these 2 genera. adding secondary-metabolite-rich classes such as Dothideomycetes (for example, *Alternaria*, *Cochliobolus*, *Cercospora* and *Cladosporium*) and sordariomycetes (for example, *Fusarium*, *Tolypocladium*, *Magnaporthe* and *Pestalotiopsis*) with conservatively 20,000 species[143,144] and estimating that half of this number of species contain substantial chemical wealth adds another 500,000 BGCs. Lichenized fungi (which include 20,000 species) such as those in the class Lecanoromycetes (for example, *Cyanodermella*) are predicted to hold the equivalent if not more secondary metabolome space[145]. Considering the additional BGC-rich ascomycete taxa and Basidiomycetes, there is no doubt that the numbers of fungal BGCs lie over several million, a potentially several-fold underestimation.

Metabolome

The total number of small molecules in a biological sample.

Primary metabolites

Metabolites that are produced by many unrelated taxa and are required for normal growth, development and reproduction.

Tailoring enzymes

Enzymes that modify non-ribosomal peptides, polyketide backbones and/or terpenoid backbones after chain elongation from respective synthetases, synthases or cyclases.

Velvet complex

A conserved transcriptional complex in filamentous fungi that is critical for the regulation of fungal secondary metabolism and reproduction in response to light and other environmental signals.

Phytochrome

A red-light photoreceptor found in fungi, bacteria and plants.

Conidiophore

The asexual spore (called conidium) bearing structure that is produced by many filamentous fungi. Specific secondary metabolites are associated with asexual spore formation.

Heterochromatin

Highly condensed chromatin tightly wound around histones and less available to the transcriptional machinery. The heterochromatin state is dependent on specific post-translational histone modifications, such as deacetylation.

Euchromatin

Lightly packed chromatin with looser arrangement around histones and accessible to the transcriptional machinery. The euchromatin state is dependent on specific post-translational histone modifications, such as acetylation and methylation.

Perithecia

Sexual fruiting bodies containing sexual spores of some Ascomycete fungi.

Dereplication

A screen in secondary metabolite analysis to eliminate already-known compounds from the discovery process.

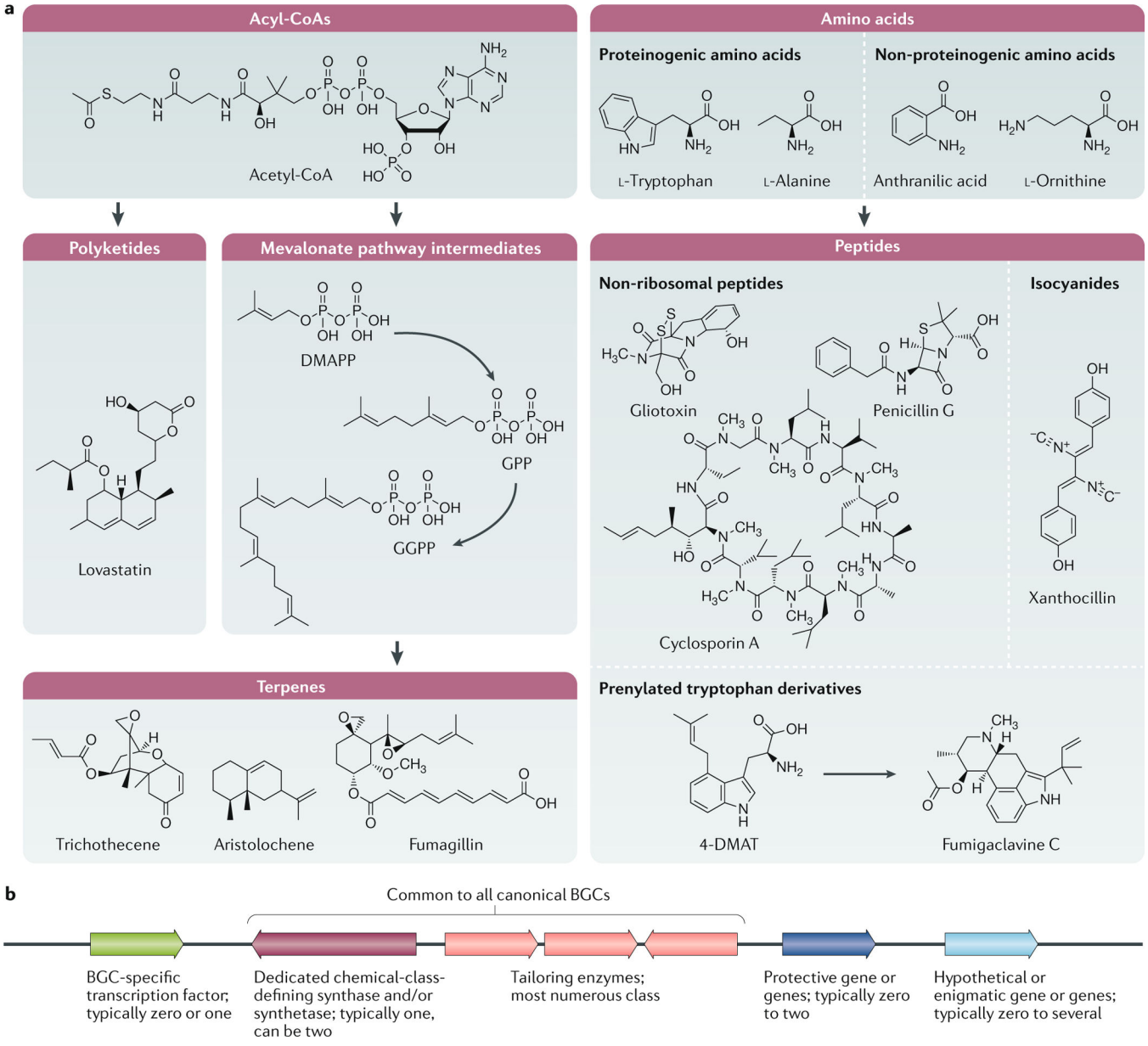

Fig. 1 |. The typical building blocks of secondary metabolites and a schematic overview of a biosynthetic gene cluster.

a | Most secondary metabolites can be grouped into three chemical categories: polyketides derived from acylCoAs, terpenes derived from acyl-CoAs and small peptides derived from amino acids. Hybrid molecules (polyketide– terpene, non-ribosomal peptide–polyketides and polyketide–fatty acid) are not shown. Fatty acid synthases (not shown) can occasionally contribute to the biosynthesis of secondary metabolites (for example, aflatoxin and sterigmatocystin are polyketide–fatty acid hybrids). **b** | Biosynthetic gene clusters (BGCs) are minimally composed of a chemically defining synthase and/or synthetase (polyketide synthase, terpene synthase and/or cyclase, non-ribosomal synthetase and isocyanide synthase) that use primary metabolites to form carbon backbones that are further modified by tailoring enzymes (for example, methyltransferases, p450 monooxygenases, hydroxylases

and epimerases). Some BGCs contain cluster-specific transcription factors that typically positively regulate the other genes within the BGC; genes that encode proteins that mitigate the toxic property of the BGC secondary metabolite; and incongruous genes with hypothetical functions that are not obviously involved in the production of secondary metabolites or protection from the encoded metabolite. DMAPP, dimethylallyl diphosphate; DMAT, dimethylallyl tryptophan; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate. Part **a** adapted with permission from reF.[30], Springer Nature Limited.

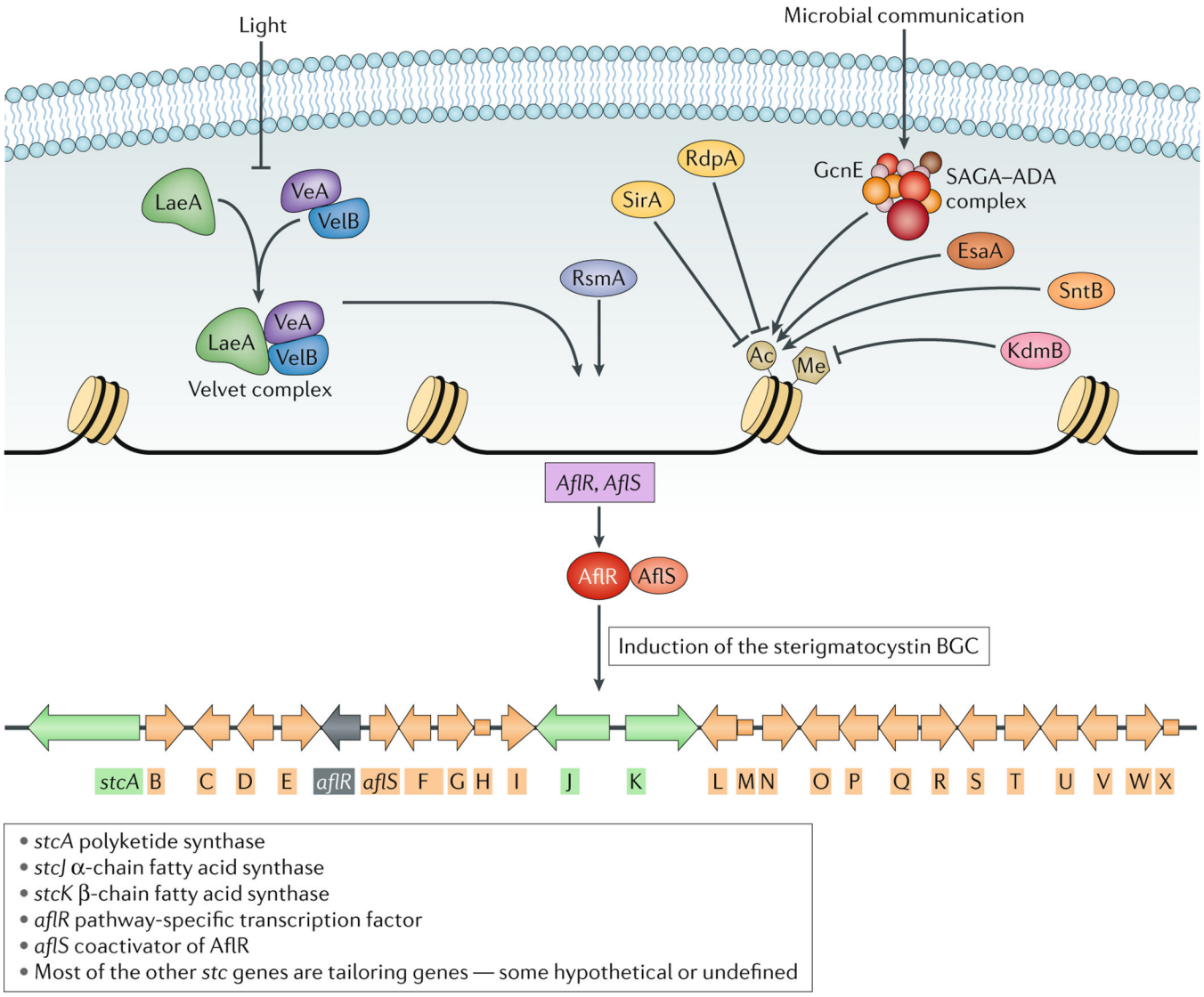

Fig. 2 |. Regulation of the sterigmatocystin biosynthetic gene cluster.

The *Aspergillus nidulans* sterigmatocystin biosynthetic gene cluster (BGC) is one of the most thoroughly studied BGCs at the regulatory level. The pathway-specific regulatory transcription factor, AflR, and its partner, AflS, are induced by specific proteins (for example, RsmA, a basic leucine zipper transcription factor[146]) and are epigenetically regulated by the Velvet complex[66] and chromatin modifiers, including the histone 3 demethylase KdmB[63], the histone 4 acetylase EsaA[147], the histone deacetylases RdpA[65] and SirA[148] and the histone reader SntB[149]. Environmental factors such as light and interactions with other microorganisms or insects also affect the induction of the sterigmatocystin BGC. For example, fungus–bacteria interactions induce the cluster through the histone acetyltransferase GcnE, a member of the histone acetyltransferase SAGA–ADA (Spt–Ada–Gcn5–acetyltransferase–ADA) complex[69], whereas white light can repress expression of some BGC-encoded genes[39]. A schematic of the sterigmatocystin BGC details the structure and encoded genes. Adapted with permission from reF.[150], Springer Nature Limited.

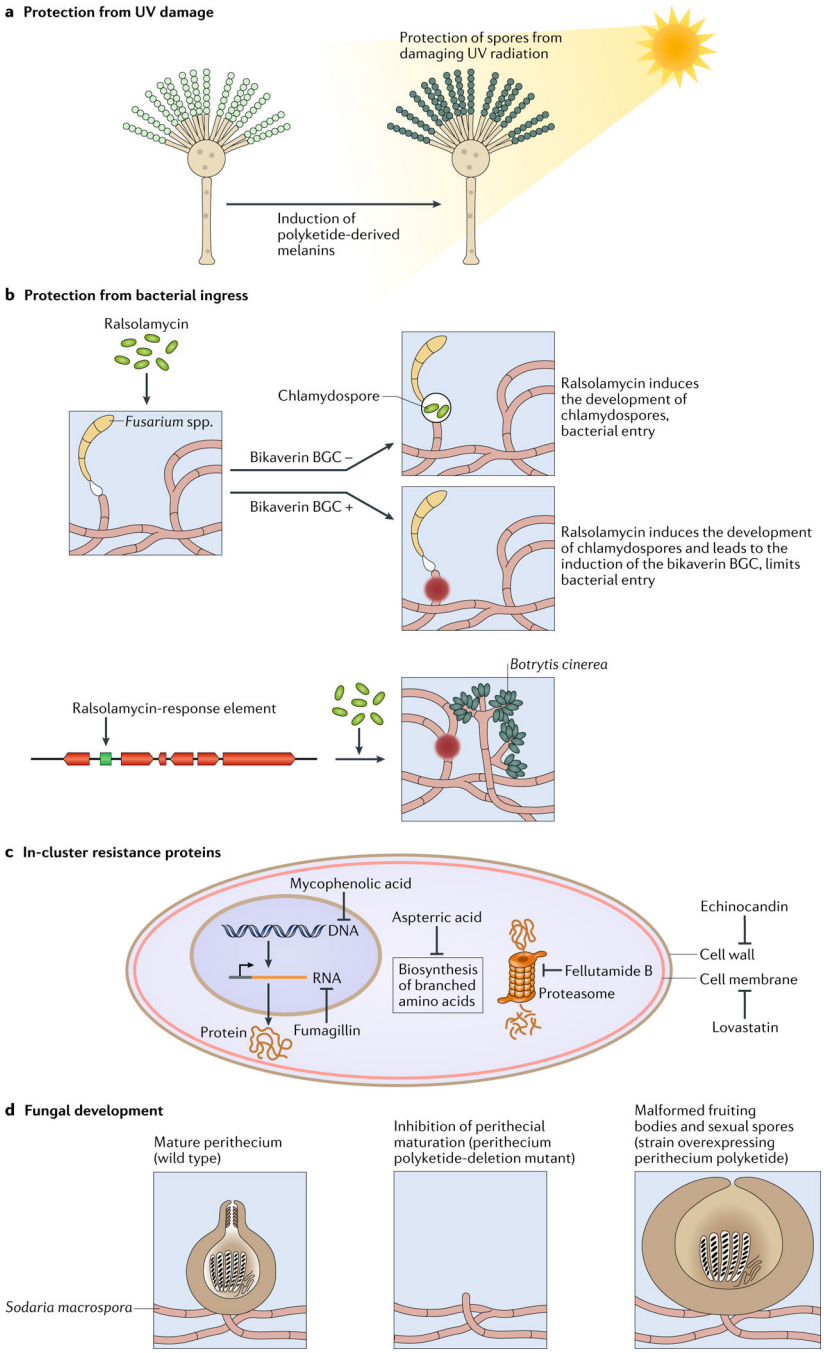

Fig. 3 |. The ecological roles of secondary metabolites.

a | Many fungi produce polyketide-derived melanins, a natural pigment that protects spores from damaging ultraviolet (UV) radiation. **b** | The bacterium *Ralstonia solanacearum* secretes the lipopeptide ralsolamycin that induces chlamydospore formation in fungi and expression of the bikaverin gene cluster in *Fusarium* spp.[7,85]. Bikaverin reduces bacterial entry and growth. Both the bikaverin biosynthetic gene cluster (BGC) and ralsolamycin response (that is, protection from bacterial ingress) have been transferred to some *Botrytis* species. **c** | For fungi to protect themselves from their own BGC-encoded antifungal

secondary metabolites, they have evolved various self-protection strategies, including duplicated, resistant copies of target proteins within a BGC. The diagram of a fungal cell shows the cellular processes that are targeted by six secondary metabolites containing in-cluster resistant genes. Lovastatin interferes with ergosterol biosynthesis and thus cell membrane integrity by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase[98] (not shown), echinocandin targets cell wall synthesis by inhibiting β−1,3-d-glucan synthase[100] (not shown), fellutamide is a proteasome inhibitor targeting the proteasome subunit C5 (REF.[99]), aspterric acid interferes with protein synthesis by targeting the branched chain amino acid synthesis enzyme dihydroxy-acid dehydratase[101] (not shown), fumagillin inhibits RNA synthesis by targeting methionine aminopeptidase[26] (not shown) and mycophenolic acid interferes with purine synthesis by inhibiting inosine-5′-monophosphate dehydrogenase[102] (not shown). **d** | Secondary metabolites can affect developmental processes in fungi. Deletion of a polyketide synthase in the fungus *Sordaria macrospora* inhibits perithecial formation, whereas its overexpression results in malformed fruiting bodies that lack the usual perithecial neck[106].

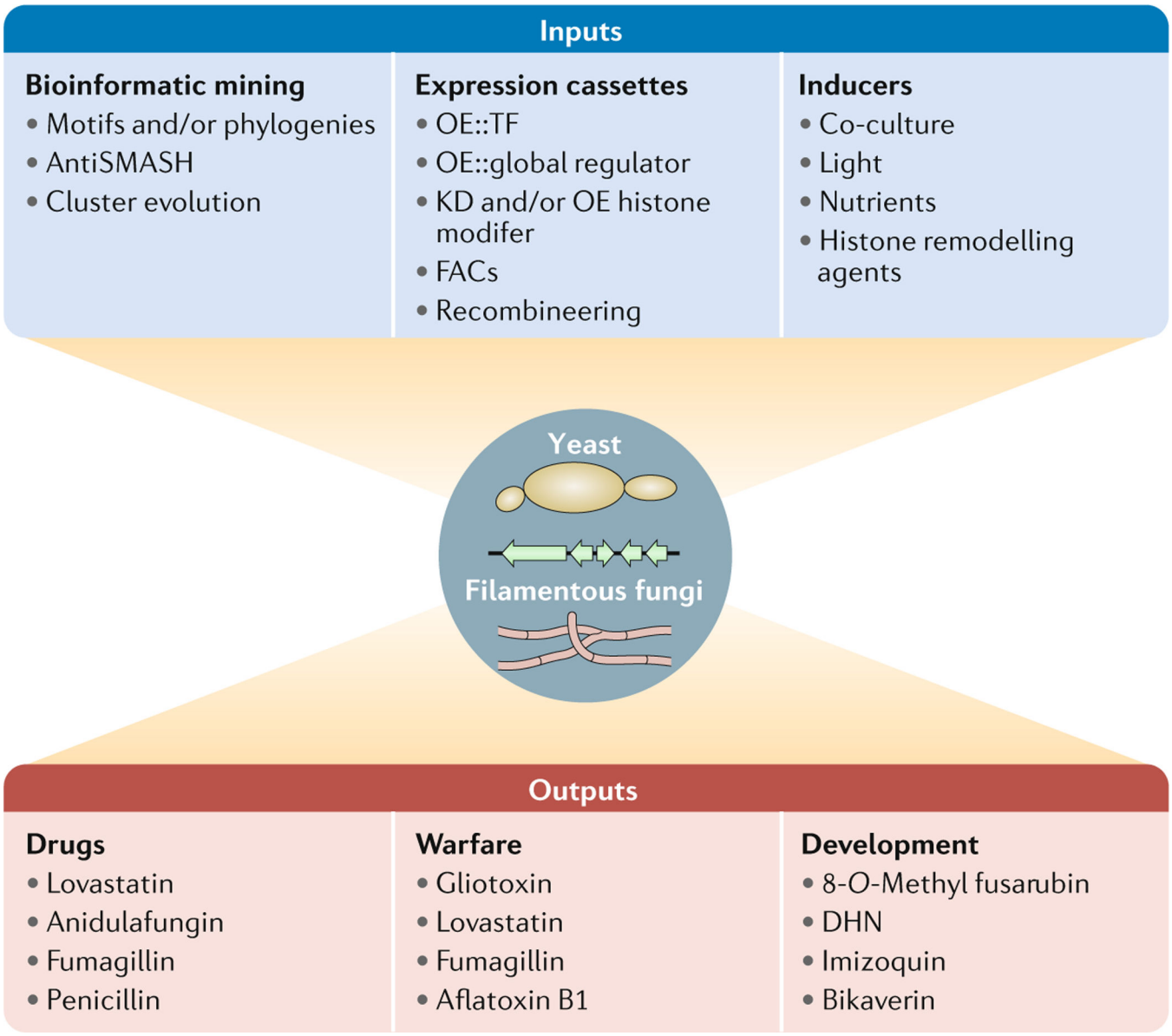

Fig. 4 |. Integration of genome mining with fungal biology yields valuable secondary metabolites. Biosynthetic gene clusters (BGCs) can be expressed in either heterologous hosts (typically yeast and *Aspergillus* spp.) or endogenous filamentous hosts (middle circle). Key input features to select BGCs of interest start with bioinformatic mining of sequenced genomes to eliminate replication and identify uniquegenes. Expression cassettes can be used for gene overexpression, gene deletion, yeast recombineering and fungal artificial chromosome (FAC) construction. Inducers that can activate cryptic BGCs include abiotic stress, epigenetic chemicals, nutrients and co-cultures. Outputs include new drugs that may overlap with endogenous function of the fungus, including warfare and developmental signals. DHN, 1,8-dihydroxynaphthalene; KD, knockdown; OE, overexpression; TF, transcription factor.

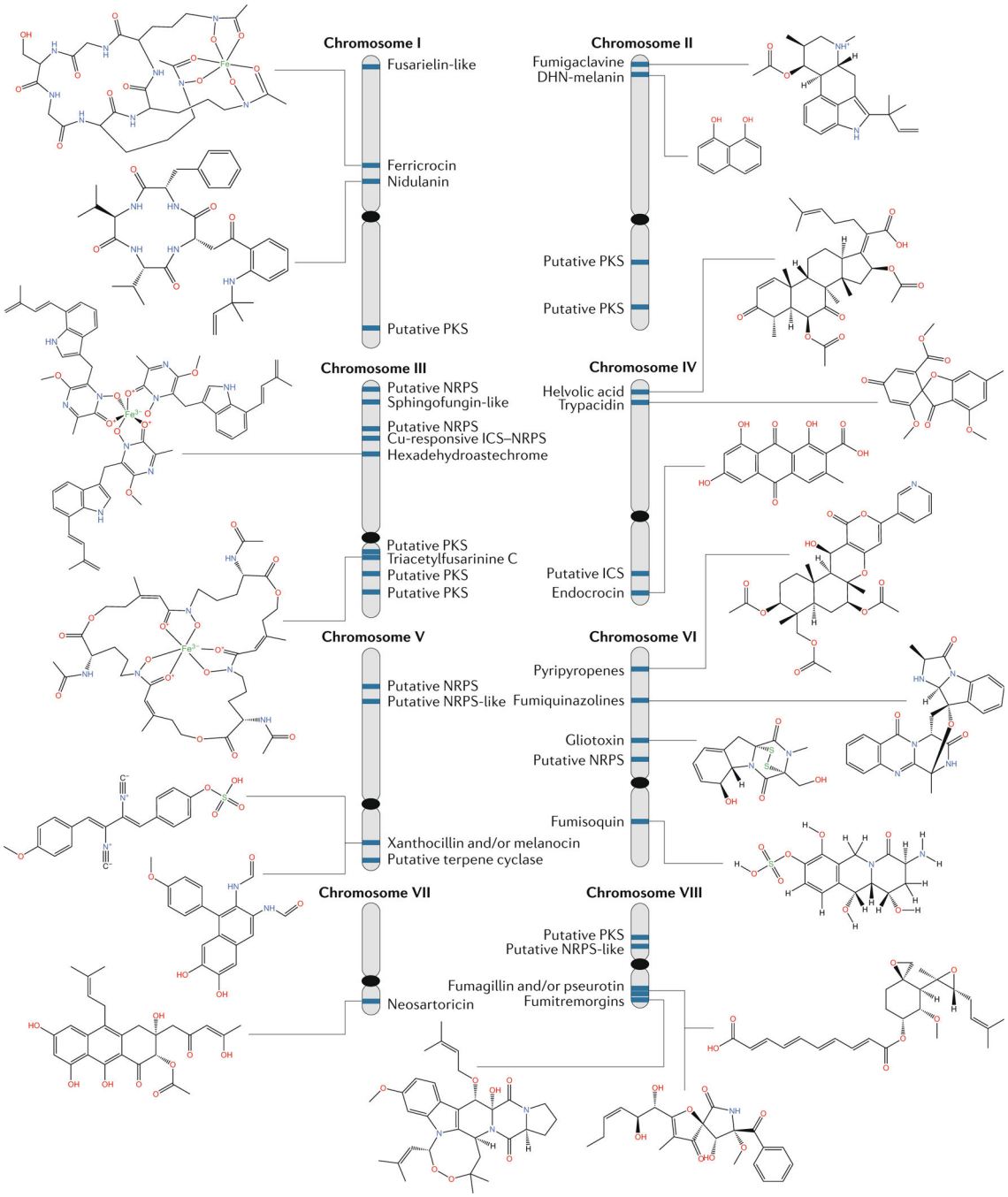

Fig. 5 |. Chromosomal position of *Aspergillus fumigatus* biosynthetic gene clusters and their known or predicted products.

The predicted product nidulanin is based on a near-identical copy of the *Aspergillus nidulans* nidulanin A biosynthetic gene cluster (BGC)[27] in *Aspergillus fumigatus*. Details of other clusters can be found in REFS[20,129]. DHN, 1,8-dihydroxynaphthalene; ICS, isocyanide synthase; NRPS, non-ribosomal synthetase; PKS, polyketide synthase. Adapted with permission from reF.[47], Annual Reviews.

Table 1 |

Tools and techniques for mining the fungal secondary metabolome

Genetic and Bioinformatic tool	Feature	Refs
Endogenous expression systems		
Targeted promoter exchange	Synthetic promoter	99
Transcription factor OE	Synthetic promoter	50,118,149
Epigenetic remodelling	Chromatin in repressed state	58,60,62–66,147,148,151–153
Global regulator OE	Synthetic promoter	55,119
Expression methods for heterologous hosts		
Yeast stitching	Synthetic BGC, universal host	122
FAC	Scalable expression in *Aspergillus*	12
CoIN	Co-induction of sterigmatocystin promoters	154
Hex	Scalable expression in *Saccharomyces*	125
BGC mining algorithms		
SMURF	Synthases (NRPS, PKS, DMATS and NRPS–PKS) and coordinates of genes	112
AntiSMASH	Synthases and substrate predictors	155
PRISM	NRPS, PKS and NRPS–PKS dereplication	156
SMIPS and CASSIS	DNA regulatory site	157
MIDDAS	Gene annotation, proteins and transcriptome data	18
FunGeneClusterS	NRPS, PKS, DMATS and co-expression data	158
SeMPI	Modular PKS	159
DEREPLICATOR+	Dereplication strategies	117
Databases with fungal and bacterial BGCs		
ClusterMine360	297 BGCs	160
IMG-ABC	2,489 BGCs	161
MIBiG	1,393 BGCs	109
Substrate predictors		
AntiSMASH	Incorporates NRPS predictor	161
NP.searcher	Bacteria, output chemical structure	100
Pep2Path	MassSpec guided peptidic natural products	162
PRISM	NRPS, PKS and NRPS–PKS dereplication	156
GRAPE	Works with PRISM, retrobiosynthesis PKs and NRPs	163
GARLIC	Compares PRISM and GRAPE outputs for likelihood of backbone prediction	163
SeMPI	Modular PKS	159
NRPS predictor	A domain specificity	164

BGC, biosynthetic gene cluster; CASSIS, cluster assignment by islands of sites; CoIN, co-inducible nitrate; DMATS, dimethylallyl tryptophan synthase; FAC, fungal artificial chromosome; NRP, non-ribosomal peptide; NRPS, non-ribosomal peptide synthetase; OE, overexpression; PK; polyketide; PKS, polyketide synthase; SMIPS, secondary metabolites by InterProScan.