FUNGAL JEWELS: SECONDARY METABOLITES

EDITED BY: Nancy Keller, Jonathan Palmer and Ozgur Bayram
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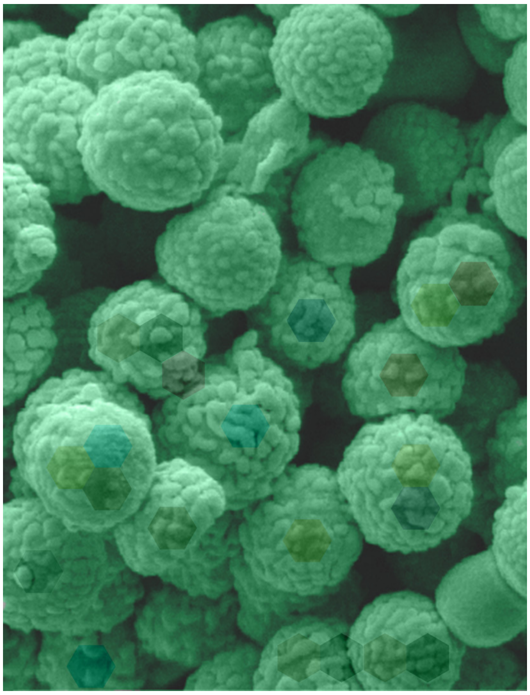
FUNGAL JEWELS: SECONDARY METABOLITES

Topic Editors:
Nancy Keller, University of Wisconsin-Madison, USA
Jonathan Palmer, US Forest Service Madison, USA
Ozgur Bayram, Maynooth University, Ireland



Scanning electron microscopic (SEM) image of asexual spores of model fungus of secondary metabolism, Aspergillus nidulans. Pseudocolored SEM image merged and scattered with hexagonal shapes representing various secondary metabolites produced by this fungus.
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Fungal natural products are friends and foes of humans such as deleterious mycotoxins, cytotoxic, carcinogenic compounds or beneficial compounds such as antibiotics, fungicides, insecticides, antiviral and antitumor metabolites. Understanding fungal diversity and estimation of fungal species on our planet poses a great challenge to researchers. This complexity is further multiplied by secondary metabolite diversity of fungi, which requires interdisciplinary studies. It is extremely important to understand the fungal secondary metabolism to stop human, animal and plant diseases caused by fungi and harvest their valuable metabolites. Furthermore, many secondary metabolite gene clusters are silenced under laboratory conditions. It is vital to develop effective methods to activate those clusters in order to discover novel potent metabolites. This e-book is a compilation of original review articles contributed by leading fungal secondary metabolite researchers with a wide range of expertise. Important aspects of fungal secondary metabolism, including regulation, genome mining, evolution, synthetic biology and novel methods have been discussed. This book will be a great source to those people, who are interested in understanding overall structure, diversity and regulation of production of these tiny but precious chemicals.

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One Juliet and four Romeos: VeA and its methyltransferases

Özlem Sarikaya-Bayram*[1]*, Jonathan M. Palmer*[2]*, Nancy Keller*[3]*, Gerhard H. Braus*[4] *and Özgür Bayram*[1]*

[1] *Department of Biology, Maynooth University, National University of Ireland, Maynooth, Ireland*
[2] *Center for Forest Mycology Research, Northern Research Station, United States Forest Service, Madison, WI, USA*
[3] *Department of Medical Microbiology and Immunology, University of Wisconsin at Madison, Madison, WI, USA*
[4] *Department of Molecular Microbiology and Genetics, Georg-August Universität Göttingen, Göttingen, Germany*

Edited by:
Biswarup Mukhopadhyay, Virginia Tech, USA

Reviewed by:
Barbara G. Turgeon, Cornell University, USA
Miaoying Tian, University of Hawaii at Manoa, USA

****Correspondence:***
Özgür Bayram, Department of Biology, Maynooth University, National University of Ireland, Maynooth, Ireland
e-mail: ozgur.bayram@nuim.ie

Fungal secondary metabolism has become an important research topic with great biomedical and biotechnological value. In the postgenomic era, understanding the diversity and the molecular control of secondary metabolites (SMs) are two challenging tasks addressed by the research community. Discovery of the LaeA methyltransferase 10 years ago opened up a new horizon on the control of SM research when it was found that expression of many SM gene clusters is controlled by LaeA. While the molecular function of LaeA remains an enigma, discovery of the velvet family proteins as interaction partners further extended the role of the LaeA beyond secondary metabolism. The heterotrimeric VelB–VeA–LaeA complex plays important roles in development, sporulation, secondary metabolism, and pathogenicity. Recently, three other methyltransferases have been found to associate with the velvet complex, the LaeA-like methyltransferase F and the methyltransferase heterodimers VipC–VapB. Interaction of VeA with at least four methyltransferase proteins indicates a molecular hub function for VeA that questions: Is there a VeA supercomplex or is VeA part of a highly dynamic cellular control network with many different partners?

Keywords: secondary metabolism, methyltransferases, velvet family, LaeA, VelB–VeA–LaeA, VosA, LlmF, VapA–VipC–VapB

INTRODUCTION

Small bioactive chemicals, also named secondary metabolites (SMs), are produced by several groups of organisms, including fungi, bacteria, and plants (Bok and Keller, 2004; Yu and Keller, 2005; Brakhage, 2013). Fungi are incredibly diverse and recent efforts in sequencing fungal genomes have demonstrated that they are rich in genes involved in production of SM. Additionally, it has been conservatively estimated that there are at least five million fungal species, of which there are only about 100,000 described species (O'Brien et al., 2005; Blackwell, 2011), thus the majority of fungi and their bioactive SMs remain unstudied. Genes encoding the enzymes responsible for the biosynthesis of SMs are often clustered in fungal chromosomes reminiscent of bacterial operons and are therefore often referred to as gene clusters. SM gene clusters tend to be transcriptionally co-regulated by a variety of different genetic mechanisms that range from specific regulation by DNA binding transcription factors to global regulation via changes in chromatin structure (Palmer and Keller, 2010; Strauss and Reyes-Dominguez, 2011; Gacek and Strauss, 2012). Interestingly, global regulatory protein complexes involved in fungal differentiation processes in response to environmental signals including light, nutrient deprivation or pH have also been shown to regulate SM gene clusters; solidifying the link between SM and fungal development.

Undoubtedly one of the few known conserved global regulators of SM in fungi is the putative methyltransferase LaeA (Bok and Keller, 2004). LaeA has been shown to control a large percentage of SM clusters as well as several aspects of fungal development in several genera (Kamerewerd et al., 2011; Butchko et al., 2012; Wu et al., 2012; Karimi-Aghcheh et al., 2013). Furthermore, LaeA forms a heterotrimeric protein complex with the two members of the fungal-specific velvet domain transcription factor family [VeA & a velvet-like protein B (VelB)] that coordinate fungal development and SM production (Bayram et al., 2008). Interestingly, in addition to LaeA, three more methyltransferases have recently been discovered to interact with VeA (Palmer et al., 2013; Sarikaya-Bayram et al., 2014). Given the fact that these complexes control many processes in fungal biology and it is the 10th anniversary of the discovery of LaeA, here we summarize recent insights into the role of the velvet complex and associated methyltransferases.

VeA AS A MOLECULAR HUB FOR PROTEIN–PROTEIN INTERACTIONS

The *veA* gene encodes a protein of 574 amino acid long, which is required for sexual development and SM production in *Aspergillus nidulans* (Kato et al., 2003; Bok et al., 2013). Deletion of *veA* leads to complete loss of sexual fruiting bodies, whereas an N-terminally truncated protein encoded by *veA1* results in a reduced cleistothecia and increased conidia production (Kim et al., 2002). With the availability of whole genome sequences and reverse-genetics, several other velvet-like proteins have been identified, which include VeA, VelB, VosA, and VelC (Bayram and Braus, 2012). The velvet family proteins are conserved in filamentous fungi, however they are absent in yeasts. VeA forms a heterotrimeric protein complex with VelB and the methyltransferase LaeA, which is required for the coordination of fungal development with SM production (Bayram et al., 2008). The trimeric velvet complex, VelB–VeA–LaeA, is predominantly formed in the nucleus when the fungus is grown without light, as VeA is translocated into the nucleus in the dark (Stinnett et al., 2007). Light is inhibitory to VeA expression as well as its nuclear entry through a currently unknown mechanism. VeA–VelB primarily enters into the nucleus together, where they meet the methyltransferase LaeA. Nuclear entry of the VeA–VelB heterodimer is driven by the α-importin KapA (**Figure 1**). VelB has additional functions in spore viability and trehalose biogenesis, which requires VelB–VosA heterodimer formation (Sarikaya Bayram et al., 2010).

While the velvet domain does not have amino acid similarity to other DNA binding domains, the different combinations of dimerization in the velvet proteins are reminiscent of the bZip family of transcription factors and as such were hypothesized to be transcription factors themselves. Biochemical characterization with the partial crystal structure of VosA–VelB heterodimer suggested that the velvet domain possesses a DNA binding fold similar to NF-κB transcription factors of mammals (Ahmed et al., 2013), and thus is potentially a transcription factor. NF-κB proteins in mammals are responsible for many cellular responses including antiviral, antibacterial responses of inflammation. VosA was shown to bind to an 11-nucleotide consensus sequence (CTGGCCAAGGC) found in the promoters of asexual regulators such as *brlA*, *wetA*, *vosA* as well as of trehalose biosynthetic genes *tpsA* and *treA*. Similarly, it was also found in the human pathogenic ascomycete *Histoplasma capsulatum* that VosA and VelB homologs (Ryp2 and Ryp3), are required for the dimorphic switch from hyphal form to a more pathogenic yeast form (Webster and Sil, 2008). Both Ryp2 and Ryp3 bind to a 10 nucleotide consensus sequence (A/T)CCA(T/C)GG(T/A)(T/A)(C/A) that is present in the promoters of the genes responsible for temperature-dependent dimorphic switch (Beyhan et al., 2013). VeA–VelB heterodimer presumably binds to DNA as well, however, they probably recognize a different consensus element than VelB–VosA because there are significant differences in the interaction surfaces of the velvet domains of VelB–VosA versus VeA–VelB. In analogy to Rel family proteins of NF-κB group, possible hetero- and homodimers among the velvet family proteins are likely to form within the nucleus, where they control different responses by binding to different consensus sequences. There is little known how the fourth velvet family protein VelC contributes to the development. VelC was originally described as a positive regulator of sexual development as VeA and VelB because deletion of *velC* gene in *A. nidulans* causes decreased number of cleistothecia and increased conidiation and might serve as an auxiliary factor of development in *A. nidulans* (Park et al., 2014). Interestingly, VelC was found to interact with VosA in yeast two-hybrid and *in vitro* studies, but, this interaction has not yet been confirmed *in vivo*. Although it is not known where VelC localizes, the VosA–VelC dimer might control development by binding to alternative promoter sequences (**Figure 1**). VelB forms homodimers *in vivo* and *in vitro* (Sarikaya Bayram et al., 2010). However, the function of these homodimers and their target consensus is currently unknown.

One of the proteins that VeA interacts with is a mitogen activated protein kinase (MAPK) AnFus3 (MpkB) that is a homologue of yeast Fus3 involved in the pheromone response pathway (Bayram et al., 2012). AnFus3 interacts with the VeA protein in the nucleus and phosphorylates VeA *in vitro*, which promotes formation of VeA–VelB heterodimer. In contrast, the VeA–LaeA interaction is not influenced by VeA phosphorylation (Bayram et al., 2012). Red light receptor phytochrome protein FphA also interacts with VeA protein, which together with the blue light receptors LreA–LreB forms the light complex (Purschwitz et al., 2008). In particular, VeA is in physical contact with the histidine kinase domain of the FphA, but VeA is not phosphorylated by the FphA protein (Purschwitz et al., 2009). It is currently unknown how the VeA–FphA–LreA–LreB complex acts at the molecular level to control light-dependent development and SM production.

These examples underscore the hypothesis that VeA acts as a molecular scaffold, thereby integrating several signals into the coordination of development and SM production ranging from MAPK signaling pathways to light receptors. However, an additional interesting facet of the VeA interacting proteins is that several of them are S-adenosyl L-methionine (SAM)-dependent methyltransferases, such as LaeA, LaeA-like methyltransferase F (LlmF), velvet interacting protein C (VipC), and VipC associated protein B (VapB). Given the central importance of the LaeA methyltransferase for secondary metabolism and development regulation, the link to these methyltransferases cannot be ignored, and thus these four interaction partners of the VeA will be further discussed.

LaeA, A CRYPTIC AND ENIGMATIC METHYLTRANSFERASE

LaeA was the first methyltransferase discovered to associate with VeA. The *laeA* (*l*oss of *aflR* *e*xpression *A*) gene was originally identified through a forward genetics screen in *A. nidulans* looking for mutants that were unable to make precursors of the sterigmatocysin (ST; Butchko et al., 1999; Bok and Keller, 2004). Several of the isolated mutants had also lost expression of *aflR*, the ST cluster specific transcription factor. One of these mutants was then complemented with a genomic DNA cosmid library, which led to identification of the putative SAM-dependent methyltransferase LaeA (Bok and Keller, 2004). Subsequent generation of *laeA* null mutants (*ΔlaeA*) in *A. nidulans*, *A. fumigatus*, and *A. flavus*, demonstrated that LaeA was responsible for regulation of approximately 50% of the SM clusters in these species (Bok and Keller, 2004; Bok et al., 2005, 2006; Perrin et al., 2007; Kale et al., 2008; Georgianna et al., 2010). As noted above, this regulation of large number of SMs is conserved in other genera. The conserved regulation of SM clusters is also associated with reduced hyphal pigmentation as well as reduction in sporulation capacity. Besides SM production, LaeA also influences development by controlling the expression and posttranslational modifications (PTMs) of the

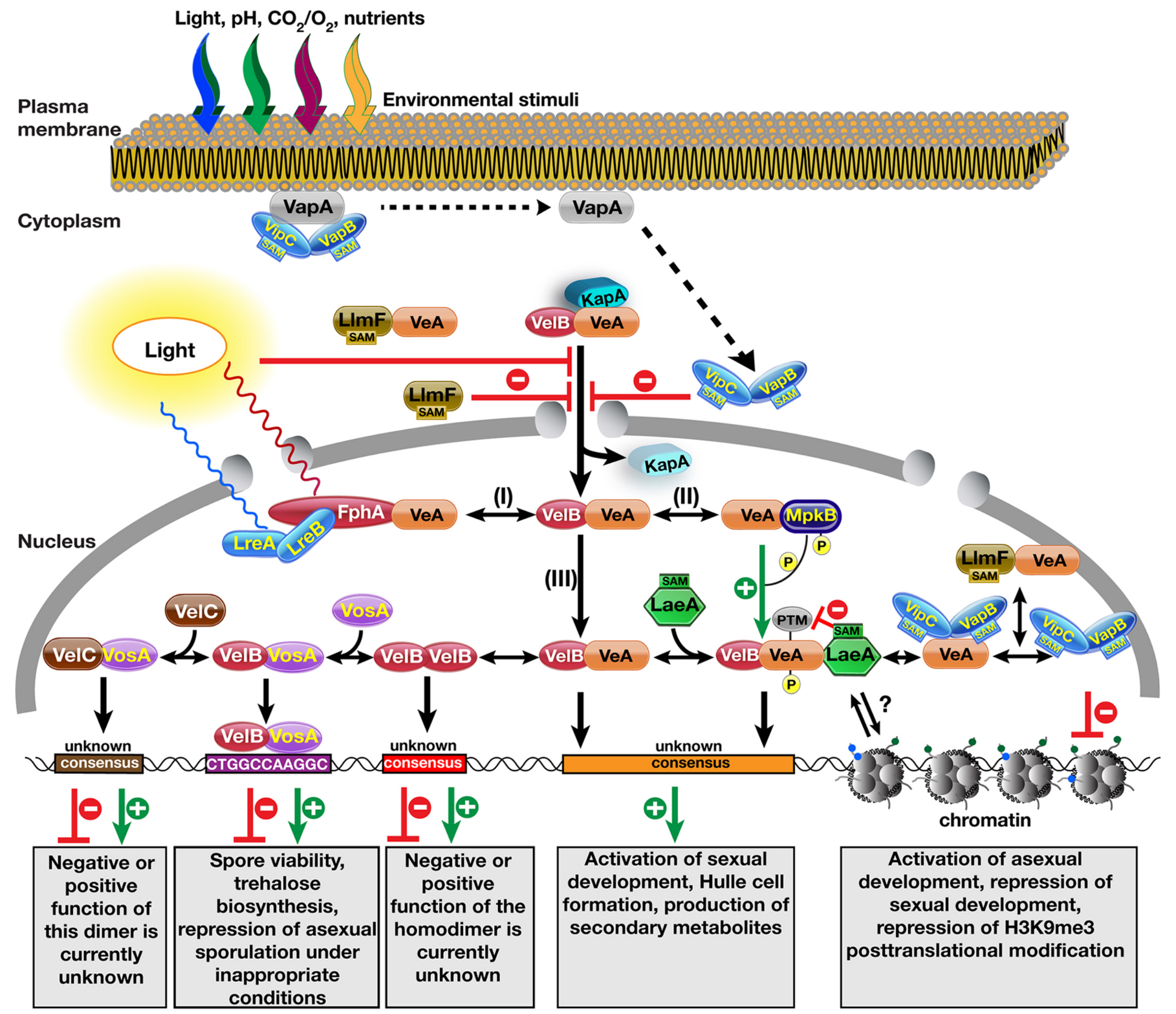

FIGURE 1 | Molecular complexes formed by the velvet family proteins and the methyltransferases on the control of fungal development and secondary metabolite production. Nuclear entry of the VeA–VelB heterodimer is operated by the α-importin KapA protein. Light reduces nuclear entry of the heterodimer by an unknown mechanism. VapA tethers the two SAM-dependent methyltransferases to the plasma membrane. Reception of an unknown signal (e.g., light, pH, CO_2, O_2, starvation) triggers the release of VipC–VapB methyltransferase heterodimers, which are targeted to the nucleus. During their translocation to the nucleus, they inhibit the nuclear import of the VeA protein. Another LaeA-like SA\ependent methyltransferase LlmF also hinders the nuclear entry of the VeA protein and forms a complex with VeA. After entering the nucleus, the VelB–VeA dimer follows different options: (I) VeA interacts with the red light receptor protein phytochrome FphA, which together with blue light receptors LreA–LreB forms a tetrameric VeA–FphA–LreB–LreA complex. (II) VeA interacts with MAPK MpkB that phosphorylates VeA, which makes VeA more interactive for VelB. (III) VeA–VelB dimer is a part of more dynamic system where addition of methyltransferase LaeA results in the trimeric VelB–VeA–LaeA velvet complex. VelB–VeA or VelB–VeA–LaeA might bind to a consensus sequence to activate sexual developmental genes as well as SM gene clusters. Molecular function of LaeA between the chromatin and VelB–VeA heterodimer function is still unclear. VeA might recruit two methyltransferases VipC–VapB. Either VeA–VipC–VapB or VipC–VapB influence the histone posttranslational modifications (PTMs) and activates asexual genes. LlmF–VeA also forms a complex with VeA in the nucleus, whose function needs to be resolved. VelB component of VelB–VeA heterodimer dissociates from VeA by forming VelB–VelB homodimer. Free VelB also attracts the third velvet family protein VosA to form active transcription factor heterodimers that bind to target sequence of trehalose biosynthetic genes and asexual genes. Furthermore, VosA might recruit VelC, VosA–VelC heterodimer might activate the genes controlling sexual development and spore viability. Velvet family proteins might form more combinations of hetero and homodimer complexes as the Rel homology domain (RHD) proteins of NF-κB family in mammals.

velvet family proteins VeA, VelB, and VosA. In the absence of LaeA, protein levels of the velvet family elevate and VeA undergoes an unknown PTM, which results in drastically reduced Hülle cell formation and small cleistothecia (Sarikaya Bayram et al., 2010; Shaaban et al., 2010).

To date, all examined *ΔlaeA* mutants of pathogenic fungi have been found to display reduced virulence. This was first described in the human pathogen *A. fumigatus* (Bok et al., 2005) and later for plant pathogenic fungi including *A. flavus* (Kale et al., 2008; Amaike and Keller, 2009), *Cochliobolus heterostrophus*

(Wu et al., 2012), and several *Fusarium* species (Wiemann et al., 2010; Butchko et al., 2012; Lopez-Berges et al., 2013). Several of the SMs regulated by LaeA in these species contribute to virulence or cellular damage as described most thoroughly in studies of *A. fumigatus* where helvolic acid, gliotoxin, endocrocin, pseurotin, hexadehydro-astechrome, fumagillin, and fumitremorgin have all been found to be regulated by LaeA (reviewed in, Jain and Keller, 2013; Wiemann et al., 2013).

Thus far, all the research done on LaeA in filamentous fungi is suggestive of an integral role in mediating fungal development and SM production, however the obvious question remains, "what is the molecular function of LaeA?" Due to LaeA harboring a required methyltransferase domain and coupled with gene expression studies illustrating the regulation of the contiguous gene clusters, several groups have hypothesized that LaeA could be regulating SM clusters epigenetically through modification of chromatin structure, either directly or indirectly. The first evidence for this comes from a microarray study in *A. fumigatus* (Perrin et al., 2007), where Perrin et al. (2007) showed that LaeA influences the expression of 9.5% of the *A. fumigatus* genome, where 13 out of 22 SM gene clusters were strongly down regulated in the absence of *laeA*. Further clues regarding the LaeA involvement in epigenetic control come from a study where it was shown that lack of LaeA leads to more heterochromatin (HepA) occupancy with increased repressive histone 3 (H3) lysine 9 (K9) trimethylation (H3K9me3) in the ST gene cluster (Reyes-Dominguez et al., 2010). In addition to increased HepA and H3K9me3 accumulation, the levels of canonical activation marks decreased (H3K9/14 acetylation). Similar to the study in *A. nidulans*, but at a less pronounced level, 75 genes of *Trichoderma* showed a LAE1-regulated pattern for their expression correlating with changes in histone marks (H3K4me3; Karimi-Aghcheh et al., 2013). While these data suggest that chromatin structure is changed in *ΔlaeA* mutants, they do not directly link LaeA to modification of histones and subsequent epigenetic control. Significant efforts have been made to find a methylation substrate of LaeA, however a recent biochemical study was not able to identify a substrate *in vivo* nor *in vitro* (Patananan et al., 2013). Interestingly, a novel automethylation reaction producing S-methylmethionine was discovered in LaeA, however subsequent mutagenesis of this residue resulted in a functional LaeA protein (Patananan et al., 2013). Thus, if LaeA has another methylation substrate besides itself, whether it might be a demethylase and requires methylated substrates or how LaeA controls SM cluster regulation remains an enigma.

LaeA-LIKE METHYLTRANSFERASE LlmF

While the molecular function of LaeA remains unknown, there are several other putative methyltransferases that have homology to LaeA in fungal genomes. These proteins have recently been studied in *A. nidulans* and named LaeA-like methyltransferases (LlmA–LlmJ; Palmer et al., 2013). These nine genes were systematically deleted to determine their roles in development and SM production. Interestingly, LlmF was found to control production of ST as well as influence sexual development. Moreover, LlmF was shown to interact with VeA in a yeast-two-hybrid assay and that interaction was confirmed *in vivo*. While LaeA is constitutively localized in the nucleus, LlmF exhibited a nucleo-cytoplasmic distribution. LlmF is hypothesized to function by directly interacting with VeA and influencing the nuclear/cytoplasmic ratio of VeA. In the absence of LlmF, VeA accumulates in the nucleus, which results in increased production of ST and increased sexual development. Consistently, overproduction of LlmF results in VeA accumulation in the cytoplasm and thus decreased sexual development and ST production (Palmer et al., 2013). Biochemical analysis showed that the SAM-binding motif is required for LlmF function, however none of the proteins known to be involved in nuclear transport of VeA (VeA, VelB, or KapA) could be identified as a methylation substrate. Although it was proposed that LlmF–VeA interaction takes place in the cytoplasm, it is still possible that LlmF might also interact with VeA within the nucleus since LlmF is not completely excluded from the nuclear fraction (**Figure 1**).

A homologue of LlmF was also studied in the maize pathogen *C. heterostrophus* that produces the host-selective T-toxin. In contrast to the *laeA1* and *vel1* mutants where T-toxin production decreases, deletion of *llm1* gene in this fungus leads to increased T-toxin production, suggesting a repressive role for Llm1 in the production of this toxin in *C. heterostrophus* (Bi et al., 2013). This repressor role of Llm1 on T-toxin is similar to the role of LlmF on ST production in *A. nidulans*. In agreement with deletion studies, overexpression of *llm1* represses T-toxin production and similar to *A. nidulans*, *llm1* is not epistatic to *laeA1* and *vel1*. Furthermore, overexpression of *llm1* results in individuals that cannot act as fertile females and de-repression of asexual sporulation during sexual as well as vegetative growth.

THE HETEROTRIMERIC ZINC FINGER-METHYLTRANSFERASE COMPLEX VapA–VipC–VapB

An experimental strategy followed by Sarikaya-Bayram et al. (2014) led to the discovery of the third VeA interacting methyltransferase, VipC, also named LlmB. Detailed biochemical analysis of the VipC interaction partners resulted in discovery of two VipC associated proteins (VapA, VapB) with VapB representing the fourth VeA interacting methyltransferase. The heterotrimeric VapA–VipC–VapB complex controls the appropriate morphogenic responses to environmental stimuli such as light.

VapA is a FYVE zinc finger protein that acts as a negative regulator of asexual conidiation and as positive regulator of sexual development. Deletion of *vapA* leads to increased conidiation and reduced cleistothecia production, and consistently the transcripts of asexual regulators, BrlA and AbaA, are substantially increased in the *vapA* deletion strain. The VapA protein is localized to the plasma membrane as the other FYVE type zinc finger proteins (Hayakawa et al., 2007). The major function of VapA protein is to recruit the two methyltransferase components of the complex, VipC and VapB, to the plasma membrane and not to release them until an environmental signal triggers their release.

VipC–VapB heterodimer is a negative regulator of sexual and positive regulator of asexual development. This function of VipC–VapB is similar to the function of LlmF on development. Both VipC and VapB proteins are found at the plasma membrane as well as in the nuclear fraction. bimolecular fluorescence complementation (BIFC) showed that VipC–VapB interacts with the zinc finger VapA at the plasma membrane, but VipC–VapB heterodimer is also found in the nucleus.

Interestingly, similar to LaeA and LlmF, this small methyltransferase heterodimer also interacts with VeA protein and this interaction takes place around and inside the nucleus. Moreover, they influence the developmental responses of the fungus in part by modulating nuclear accumulation of the VeA protein, which is similar to the LlmF effect whereas the VipC–VapB heterodimer negatively influences VeA nuclear entry, the antagonistically acting VapA positively affects VeA nuclear accumulation. This positive impact on VeA nuclear localization is not a direct result of VapA because zinc finger VapA and transcription factor VeA do not interact *in vivo*. VapA supports VeA localization by keeping VipC–VapB heterodimers at the plasma membrane and preventing their membrane-cytoplasm-nucleus migration. Recently, it has been shown by Bok et al. (2013) that VeA acts as a repressor of the polyketide orsellinic acid (OA) gene cluster. In the absence of *veA*, the metabolites OA and its derivatives, F9775A-B are produced in higher amounts and mycotoxin ST is not produced. Confirming these results, overexpression of *vapB* methyltransferase leads, similarly, to high OA levels and lack of ST, which is caused by the negative effect of VapB methyltransferase on VeA nuclear accumulation as well as transcription.

How does the VapA–VipC–VapB complex control asexual gene expression? VapA protein does not show DNA binding zinc finger features. Furthermore, it is not found to influence transcriptional machinery in the nucleus. However, the VipC–VapB heterodimer is found in the nucleus and has the potential to control gene expression. The N-terminus of histone proteins is highly conserved in eukaryotes and undergoes various PTMs such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitinylation. Histone 3 is the most intensively modified histone protein among the other histones. Especially, acetylation of histone 3 lysine 9 (H3K9ac) leads to gene activation whereas methylation of the same residue H3K9me2 (dimethylated) or me3 (trimethylated) results in gene silencing by attracting the heterochromatin protein responsible for heterochromatin formation. First hints for the nuclear function of the VipC–VapB methyltransferases come from their influence on histone PTM. Particularly, VapB overexpression leads to almost 50% reduction in H3K9me3 levels, suggesting that VapB is involved in control of gene expression by epigenetic interference. In connection with the reduced H3K9me3 marks, nuclear distribution of the heterochromatin protein (HepA) drastically alters. Furthermore, repressive H3K9me3 increases in the *abaA* promoter when VipC–VapB are absent but diminishes in *vapA* mutant strain. These data draw a picture where VipC and particularly VapB counteracts against repressive H3K9me3 with an unknown mechanism. Similarly to the effect of LaeA on chromatin, it is currently unknown whether this is a direct influence of VapB, e.g., as demethylase or the result of indirect interactions of VapB with several regulators such as the VeA protein.

VapA–VipC–VapB orthologs are highly conserved in the fungal kingdom as are the LaeA and LlmF methyltransferases, which suggests that this heterotrimeric complex might possess major regulatory roles in fungal development, virulence, and SM production in the other members of the fungal kingdom. There are already hints for the similar complexes in fungi such as *Fusarium graminearum* where several Vips were found as FgVeA interaction partner in a yeast two-hybrid screen in this fungus (Jiang et al., 2011). One out of the six identified Vip proteins is a LaeA homolog protein, the other five Vips are all methyltransferase domain containing proteins similar to LlmF and VipC (LlmB). However, deletions of these genes do not lead to serious consequences on virulence or the SM production of *F. graminearum*.

Interaction of the VeA protein with at least four methyltransferases (LaeA, LlmF, VipC, VapB) suggests that VeA should have an affinity domain for these methyltransferases. However, such an affinity domain has not been shown except for the LaeA–VeA interaction where LaeA binds to C-terminus of VeA (Bayram et al., 2008). Furthermore, detailed multiple alignments of the four methyltransferases exhibit only conserved regions around the SAM binding domain whereas the N as well as C terminal domains differ from each other. These data indicate that perhaps a tertiary domain found in VeA provides the interaction between the VeA protein and the methyltransferases. Future structural studies will show whether this assumption is correct or whether there are completely different mechanisms how VeA attracts methyltransferases.

CONCLUSION

The fungal kingdom has an estimated five million species and thus represents an enormous amount of potential in terms of discovering pharmaceutically important small molecules. With the advances in whole genome sequencing, the number of SM identified from fungi has been rapidly increasing. The regulatory complexes governing the SM clusters are key to our ability to understand the biological roles of SM. Whereas many regulatory players of development and secondary metabolism have been identified, the exact molecular mechanisms that drive the fungal growth and SM production are still in their infancy. While the molecular function of LaeA remains an enigma, research on this topic has greatly contributed to our understanding of gene cluster regulation as well as the link between development and secondary metabolism. The founding member of the velvet family, VeA, appears to be immersed in the core of the fungal transcriptional response to environmental stimuli, which is evidenced by its plethora of interacting proteins. Novel players such as LlmF, VipC, and VapB, which were discovered to be in connection with the velvet complex, have been increasing the complexity of the general picture and suggesting the presence of a VeA supercomplex. Understanding the mechanistics of these complicated but highly orchestrated transcriptional response is crucial to strengthen our knowledge on SM production and fungal development, which will allow us to use the power of fungi in medicine, agriculture, and biotechnology.

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Association of fungal secondary metabolism and sclerotial biology

Ana M. Calvo[1]* **and Jeffrey W. Cary**[2]

[1] Department of Biological Sciences, Northern Illinois University, DeKalb, IL, USA
[2] Southern Regional Research Center, United States Department of Agriculture – Agricultural Research Service, New Orleans, LA, USA

Edited by:
Ozgur Bayram, Maynooth University, Ireland

Reviewed by:
Geromy G. Moore, United States Department of Agriculture – Agricultural Research Service, USA
Jae-Hyuk Yu, University of Wisconsin, USA

***Correspondence:**
Ana M. Calvo, Department of Biological Sciences, Northern Illinois University, 155 Castle Road, DeKalb, IL 60115, USA
e-mail: amcalvo@niu.edu

Fungal secondary metabolism and morphological development have been shown to be intimately associated at the genetic level. Much of the literature has focused on the co-regulation of secondary metabolite production (e.g., sterigmatocystin and aflatoxin in *Aspergillus nidulans* and *Aspergillus flavus*, respectively) with conidiation or formation of sexual fruiting bodies. However, many of these genetic links also control sclerotial production. Sclerotia are resistant structures produced by a number of fungal genera. They also represent the principal source of primary inoculum for some phytopathogenic fungi. In nature, higher plants often concentrate secondary metabolites in reproductive structures as a means of defense against herbivores and insects. By analogy, fungi also sequester a number of secondary metabolites in sclerotia that act as a chemical defense system against fungivorous predators. These include antiinsectant compounds such as tetramic acids, indole diterpenoids, pyridones, and diketopiperazines. This chapter will focus on the molecular mechanisms governing production of secondary metabolites and the role they play in sclerotial development and fungal ecology, with particular emphasis on *Aspergillus* species. The global regulatory proteins VeA and LaeA, components of the *velvet* nuclear protein complex, serve as virulence factors and control both development and secondary metabolite production in many *Aspergillus* species. We will discuss a number of VeA- and LaeA-regulated secondary metabolic gene clusters in *A. flavus* that are postulated to be involved in sclerotial morphogenesis and chemical defense. The presence of multiple regulatory factors that control secondary metabolism and sclerotial formation suggests that fungi have evolved these complex regulatory mechanisms as a means to rapidly adapt chemical responses to protect sclerotia from predators, competitors and other environmental stressors.

Keywords: secondary metabolism, genetic regulation, sclerotia, VeA, *velvet*, *Aspergillus*, gene cluster, morphogenesis

INTRODUCTION

Fungal species are able to develop specialized structures allowing them to disseminate and survive adverse environmental conditions. Aspergilli differentiate by forming conidiophores, structures that produce conidiospores. Some *Aspergillus* species, such as the model species *Aspergillus nidulans*, also produce sexual fruiting bodies known as cleistothecia where meiospores (i.e., ascospores) are generated. Both reproductive processes, asexual and sexual development, are controlled by temporal and spatial genetic regulation (Adams and Yu, 1998; Calvo et al., 2002; Fischer and Kues, 2006). Other species, such as *Aspergillus flavus* or *Aspergillus parasiticus*, form resting structures capable of surviving environmental extremes termed sclerotia that represent vestiges of fruiting bodies incapable of producing ascospores (Coley-Smith and Cooke, 1971; Malloch and Cain, 1972; Wicklow, 1987). Initial evidence presented by Geiser et al. (1996) supported that asexual Aspergilli are often derived from meiotic lineages and postulated for the first time that sclerotia might be vestigial cleistothecia that lost the capacity to produce ascospores. In more recent years, the complementary alpha- and HMG-domain MAT genes have been characterized from *A. flavus* and *A. parasiticus* (Ramirez-Prado et al., 2008). Presence and functionality of mating type genes in *Aspergillus oryzae* was also found, supporting a possible heterothallic breeding system in this fungus (Wada et al., 2012). Furthermore, Horn et al. (2009b, 2014) reported ascospore-bearing ascocarps embedded within sclerotia of *A. flavus* and *A. parasiticus.* The proposed common origin between cleistothecia and sclerotia suggested that conserved genetic regulatory pathways controlling cleistothecia formation could also control sclerotial production. Rapid progress on studies of the cleistothecium-producing model fungus *A. nidulans* and other related fungi [i.e., Dyer and O'Gorman (2012) and references therein] has facilitated uncovering regulatory pathways controlling sclerotial production in other fungi, particularly in *A. flavus.*

Studies have found that a number of genetic regulators controlling the formation of developmental structures, including sclerotia, also govern the production of secondary metabolites (Calvo et al., 2002; Calvo, 2008). While some of these compounds, also termed natural products, are beneficial (e.g., penicillin and lovastatin), other secondary metabolites are deleterious, such as mycotoxins [reviewed in Gloer (2007)]. Among fungal secondary metabolites, aflatoxins (AFs) are probably the most well known and studied. These compounds were discovered after the United Kingdom's outbreak of Turkey X disease in 1962, caused by consumption of *A. flavus*-contaminated feed and resulted in the deaths of numerous turkey poults (Bennett and Klich, 2003). *A. flavus* is capable of colonizing economically important crops such as peanut, cotton, maize and other oilseed crops both pre- and post-harvest. In the U.S. alone, *A. flavus* costs 100s of millions USD annually due to market losses from AF contaminated crops (Wu, 2004). In addition to AFs, *A. flavus* produces other secondary metabolites and many of them have been found in sclerotia (Gloer, 1995). Genetic regulation of development and secondary metabolism has been intensely studied in the Aspergilli, and in particular *A. flavus* and *A. nidulans.* In this review we focus on the association between secondary metabolism and sclerotial formation in this fungal genus, including genetic co-regulatory patterns leading to the activation of the secondary metabolic gene clusters and formation of sclerotia. Important components of this shared regulatory mechanism are the global regulatory proteins VeA and LaeA, part of the *velvet* complex (Bayram et al., 2008a; Bayram and Braus, 2012). Additionally, we also discuss the possible roles of secondary metabolites associated with sclerotia, particularly in Aspergilli.

SECONDARY METABOLITES PRESENT IN FUNGAL SCLEROTIA

It is difficult to determine just how many species of fungi exist, but estimates have suggested that the fungal kingdom is very diverse having anywhere from 1.0 to 2.7 million species with only a fraction of these having been isolated and described Hawksworth and Rossman (1997) and Mueller and Schmit (2007). One common theme of many of the described species is that they are prolific producers of biologically active secondary metabolites. The diversity of these natural products rivals that of the fungal kingdom. Fungal secondary metabolites have garnered much attention for their beneficial impact as therapeutic agents (e.g., lovastatin and penicillin) and continue to be mined as a source of important end products and building blocks for pharmaceutical development. On the other hand, secondary metabolites have also received considerable attention for their adverse impact of humans and animals due to their widespread occurrence as mycotoxins (e.g., AFs and fumonisins) on food and feed crops as well as indoor environments. Fungi produce a number of structural classes of secondary metabolites including polyketides (PKs), non-ribosomal peptides (NRPs), hybrid PK-NRPs, indole alkaloids, and terpenes (Keller et al., 2005). In almost all cases the genes responsible for the production of these classes of secondary metabolites are organized as a gene cluster (discussed below). Secondary metabolites of this type that have been identified in sclerotia will be the main focus of this section.

Though many of the recognized biological activities of important secondary metabolites relate to their direct influence on humans and other vertebrates, it is generally accepted that these natural products play key roles in the ecology of the fungus as well. Over the course of evolution, secondary metabolites have been fashioned for numerous biological functions in microorganisms, as chemical messengers between microbes and as a means of defense from predation and competing microbes (Wicklow, 1988; Yim et al., 2007; Rohlfs and Churchill, 2011; Yin et al., 2012). Fungi are much like plants in that; in general, they are static organisms incapable of readily escaping from encroaching predators and competing microbes. In spite of this, fungi are quite successful at inhabiting and surviving for long periods of time in highly competitive environments. It has been hypothesized that these competitive environments have provided considerable selective pressure for fungi to produce an array of antagonistic secondary metabolites as part of their "chemical" defense against numerous fungivores and competitors (Gloer, 2007; Rohlfs et al., 2007; Rohlfs and Churchill, 2011). A recent study showed that arthropod grazing induces a "resistance" phenotype in *A. nidulans* to fungivory that coincided with elevated levels of secondary metabolite and sexual fruiting body formation (Rohlfs and Churchill, 2011). Plants also tend to concentrate secondary metabolites in reproductive structures (e.g., seeds) as a means of defense against herbivores; as well, herbivores tend to avoid feeding or oviposition on plants or plant tissues that contain high levels of secondary metabolites (Rhoades, 1985). In an analogous fashion, various fungi are known to sequester secondary metabolites in asexual conidia and sexual fruiting structures that are critical to survival and which often results in reduced incidences of insect fungivory (Doll et al., 2013).

In addition to conidia and fruiting bodies, numerous fungi also produce structures termed sclerotia. Sclerotia are compacted mats of hyphae produced by certain fungi that allow survival for long periods of time under adverse environmental conditions (Coley-Smith and Cooke, 1971). Upon onset of favorable conditions, sclerotia can germinate to produce large quantities of either hyphae or conidia, and as such they represent a primary source of fungal inoculum in the field. Sclerotia are commonly produced on plant tissues during fungal invasion and eventually end up in soil, or on decaying plant tissues, in the field where they are exposed to predation by insects. In addition to serving as survival structures, in many *Aspergillus* species (e.g., *A. flavus* and *A. nomius*), with proper environmental conditions and mating pair interactions, sclerotia can serve as a substrate (termed stromata) for the formation of sexual structures (Horn et al., 2009a, 2011). The stromata harbor ascospore-bearing cleistothecia, similar to cleistothecia of other ascomycetous species that have a sexual cycle (Horn et al., 2009a, 2014). Many of the genetic mechanisms that connect secondary metabolism to morphogenesis of sexual fruiting bodies have also been shown to control sclerotial production (discussed below). Production of sclerotia represents a substantial metabolic investment by the fungus that is warranted based on the critical role of these structures in reproduction and survival. The importance of sclerotia to fungal biology combined with their high nutrient value to insects would justify the existence of considerable selective pressure on the fungus to produce antiinsectan/antifeedant secondary metabolites as part of their chemical defenses. In fact, this appears to be the case as numerous studies have shown sclerotia to be veritable storehouses of a diversity of secondary metabolites with antiinsectan properties [reviewed in Wicklow (1988) and Gloer (1995, 1997, 2007)]. The fungus' need for a diverse array of defensive secondary metabolites may be a reflection of the ability of the target organism to develop resistance to specific inhibitory agents. One would predict that the presence of a number of secondary metabolites in sclerotia, many of which may have different modes of action, would make it more difficult for the target organism to evolve resistance either through mutation or acquisition of resistance genes than if it were faced with having to overcome just one inhibitory metabolite.

Perhaps the quintessential example of sclerotia-based chemical defense is that of *Claviceps purpurea.* This ascomycetous fungus produces a group of indole-derived secondary metabolites known as ergot alkaloids (EAs) during growth on a number of plants including many cereal crops (Haarmann et al., 2009). Consumption of food and feeds contaminated with the alkaloid-containing sclerotia (ergot) resulted in vast epidemics of human and animal disease that were reported as early as 600 BC. In addition to *C. purpurea*, a number of chemically diverse EAs are produced by other fungi including many grass endophytes, as well as strains of *Penicillium* and *Aspergillus*, though most of these strains are not known to produce sclerotia (Gloer, 2007). The proposed ecological role of EAs is to protect the fungus by reducing consumption of the host crop by herbivores or from direct consumption by fungivorous insects (Schardl et al., 2006). The remainder of this section will focus on secondary metabolites identified in sclerotia, primarily of *Aspergillus* species, though a few examples will be provided for other fungi. A list of secondary metabolites found in sclerotia from *Aspergillus* species is presented in **Table 1**. Reports by Gloer (1995, 1997, 2007) provide an excellent source of information on the chemistry and biological function of fungal metabolites associated with sclerotia. This review will only touch on new findings since the (Gloer, 2007) publication and provide a few examples of interest. In many cases, previous literature on fungal secondary metabolites describe whole culture extracts and fail to specify if the metabolite(s) was present in sclerotia. In some instances, the investigators report on secondary metabolites that were extracted from isolated sclerotia but fail to indicate if they were also present in other fungal structures such as mycelia and conidia. This review is focused on secondary metabolites of sclerotial origin, but in some cases information will be presented on metabolites that are present in sclerotia as well as other fungal structures, or secreted outside of the cell.

One of the most intensely studied fungal genera with respect to production of secondary metabolites is *Aspergillus.* Members of this genus of fungi are ubiquitous in nature and are capable of living as saprophytes in soils or as opportunistic pathogens of humans, plants and animals. With well over 250 identified species of *Aspergillus* (Geiser et al., 2007), probably the best known members of this genus are *A. flavus* and *A. parasiticus,* that produce carcinogenic and toxigenic AFs. Many species of *Aspergillus* produce both sclerotia and the polyketide-derived AFs, however, the majority of the literature has focused on AF production in *A. flavus* as it is most commonly associated with contamination of food and feed crops (Payne and Brown, 1998; Cary et al., 2000; Bhatnagar et al., 2002). AFs are produced during growth of the fungus on oilseed crops such as corn, peanuts, cottonseed, and treenuts and they can also contaminate many additional crops during storage. Production of AFs in *A. flavus* and *A. parasiticus* is known to occur in specialized endosomes of mycelia and subsequently secreted into the environment (Chanda et al., 2009). AFs have also been found in all fungal cell structures including mycelia, conidia, and sclerotia (Wicklow and Cole, 1982; Wicklow and Shotwell, 1983). Though the exact role of AFs in the biology of producing species remains elusive, evidence suggests that they are produced in response to oxidative stress and may also be endowed with antiinsectan properties (Chinnici and Bettinger, 1984; Narasaiah et al., 2006; Grintzalis et al., 2014). In addition, AF production and sclerotial development may be closely related, as increased production of AF precursors was associated with a decrease in sclerotial size (Chang et al., 2002). It was suggested that this may be due to common substrates like acetate being directed toward AF production resulting in lowered availability for biogenesis of sclerotia.

Cyclopiazonic acid (CPA) is an indole-tetramic acid mycotoxin that is produced by a number of species of *Aspergillus* and *Penicillium* (Burdock and Flamm, 2000; Vinokurova et al., 2007). It is a common contaminate of a number of food commodities and is often present as a co-contaminate with AFs (Martins and Martins, 1999). CPA has been found in sclerotia of *A. flavus*, however, it was also detected in mycelia (Wicklow and Cole, 1982). Though its role in the ecology of the fungus is not known, CPA has been shown to be an inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum with exposure in some animals leading to organ necrosis and death (Riley et al., 1992).

Another mycotoxin of importance to human health is ochratoxin A (OTA; El Khoury and Atoui, 2010). OTA is produced by several species of *Aspergillus* and *Penicillium* via a pentaketide that is derived from a dihydrocoumarin coupled to β-phenylalanine. It is detected worldwide in various food and beverage sources. OTA can have several toxicological effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic, and immunotoxic. OTA has been isolated from sclerotia of *Aspergillus ochraceus*, *Aspergillus sclerotioniger*, and *Aspergillus carbonarius*, with OTA isolated from the latter shown to have antiinsectan properties (Paster et al., 1984; Wicklow et al., 1996; Frisvad et al., 2014). Only a few strains of *Aspergillus* section *Nigri* have been reported to produce sclerotia, but when cultured in artificial media supplemented with natural substrates such as fruits and grains, sclerotial production was induced along with numerous sclerotial secondary metabolites (Frisvad et al., 2014). In addition to detection of OTA, some isolates were found to produce apolar indoloterpenes of the aflavinine type and okaramines (Frisvad et al., 2014; Petersen et al., 2014).

A number of fungi produce polyketide-derived melanins which are the black or near-black pigments formed by oxidative polymerization of phenolic compounds produced by the dihydroxynaphthalene (DHN)-melanin pathway (Wheeler, 1983; Butler and Day, 1998). Melanin has been shown to be a virulence factor in plant, animal, and human pathogenic fungi and it also functions in survival and longevity in nature of fungal propagules such as sclerotia (Butler and Day, 1998). Sclerotial DHN-melanins have been reported as a component of black sclerotia of *Sclerotinia sclerotiorum* and *S. trifoliorum* (Butler et al., 2009). Recently, an *A. flavus* gene cluster was found to be responsible for the production of

Table 1 | Secondary metabolites associated with sclerotia of *Aspergillus* species.

Fungus	Metabolite	Structural class	Reference
A. alliaceus	Isokotanins	Polyketide	Laakso et al. (1994)
	Nominine	Indole diterpene	Laakso et al. (1994)
	Paspaline	Indole diterpene	Laakso et al. (1994)
A. arenarius	Arenarins	Prenylated terphenyl	Oh et al. (1998)
A. auricomus	Variecolactol	Sesterterpene lactone	De Guzman et al. (1999)
	Penicillic acid	Polyketide	De Guzman et al. (1999)
	Dihydropenicillic acid	Polyketide	De Guzman et al. (1999)
A. carbonarius	Ochratoxin A	Polyketide	Frisvad et al. (2014)
	Carbonarin A	Naphthopyrone	Gloer (1997)
	Aurasperone	Naphthopyrone	Gloer (1997)
A. flavus	Aflatoxins	Polyketide	Wicklow and Cole (1982)
	Aflatrems	Indole diterpene	Wicklow and Cole (1982)
	Asparasone	Polyketide	Cary et al. (2014)
	Cyclopiazonic acid	Indole tetramic acid	Wicklow and Cole (1982)
	Aflavarin	Polyketide	Gloer (1995)
	Aflavinines	Indole diterpene	Gloer (1995)
	Aflavazole	Indole diterpene	Gloer (1995)
	Kotanin	Polyketide	Gloer (1995)
A. leporis	Leporin A	2-pyridone	Gloer (1995)
A. melleus	Bis-indoyl benzenoids	Bis-indoyl benzenoid	Gloer (1995)
	Variecolin	Sesterterpenoid	Gloer (1995)
A. variecolor	Variecolin	Sesterterpenoid	Gloer (1995)
A. nomius	Nominine	Indole diterpene	Gloer (1995, 1997)
	Aspernomine	Indole diterpene	Gloer (1995, 1997)
	Paspalinine derivatives	Indole diterpene	Gloer (1995, 1997)
A. ochraceus	Ochratoxin A	Polyketide	Paster et al. (1984)
	Diketopiperazines	Diketopiperazine	Gloer (1995)
	Ochrindoles	Bis-indoyl benzenoid	Gloer (1995)
A. sclerotiorum	Sclerotiamide	Diketopiperazine	Gloer (1997)
	Scleramide	Cyclic hexapeptide	Whyte et al. (2000)
	Oxoasterriquinol D	Bis-indoyl benzenoid	Whyte et al. (2000)
A. sulphureus	Penitrem analogs	Indole diterpene	Gloer (1995)
	Radarins	Indole diterpene	Gloer (1995)
	Sulpinines	Indole diterpene	Gloer (1995)
Aspergillus section Nigri [a]	Aflavinines	Indole diterpene	Gloer (1997), Frisvad et al. (2014)
	Ochratoxin A	Polyketide	Frisvad et al. (2014)

[a] *Aspergillus section Nigri is composed of 15 related black-spored species of Aspergillus.*

a sclerotia-specific pigment identified as the polyketide, asparasone (discussed below; Cary et al., 2014). Sclerotia produced by mutants of the asparasone polyketide synthase (PKS) lacked dark pigmentation, were significantly less resistant to insect predation than wild-type sclerotia and were more susceptible to the deleterious effects of ultraviolet light and heat. Fungal sclerotia and conidia were previously thought to be mostly resistant to this type of damage due to the presence of DHN-melanins. The study of Cary et al. (2014) showed that the dark brown pigments in *A. flavus* sclerotia derive from anthraquinones produced by the asparasone cluster rather than from the typical DHN-melanin pathway.

GLOBAL GENETIC REGULATORY MECHANISMS GOVERNING PRODUCTION OF SECONDARY METABOLITES THAT INFLUENCE SCLEROTIA

The global regulatory proteins VeA and LaeA, components of the *velvet* nuclear protein complex, control both development and secondary metabolism in numerous fungi, including *Aspergillus* species. The characterization of the *veA* gene began more than 60 years ago, when Kafer (1965) generated the first *veA* mutant in *A. nidulans*, *veA1*, a mutant with partial loss-of-function. However, its characterization was delayed for many years due to the fact that the VeA predicted protein did not demonstrate homology with any other proteins of known function. Further studies with *veA* deletion mutants in *A. nidulans* and in other fungi provided valuable insight into the role of this regulator. *veA* is known to have a role in activating sexual development and inhibiting asexual development (Champe et al., 1981; Yager, 1992; Kim et al., 2002). Interestingly the role of *veA* in the regulation of morphogenesis is light-dependent; light reduces and delays cleistothecial formation and promotes conidiation in *A. nidulans* strains with a *veA* wild-type allele, while in the dark the fungus develops fruiting bodies (Yager, 1992; Stinnett et al., 2007). Deletion of *veA* in *A. nidulans* resulted in hyperconidiating strains unable to produce cleistothecia (Kim et al., 2002; Kato et al., 2003). Similarly, deletion of *veA* increases conidiation and completely blocks sclerotial formation in *A. flavus* and *A. parasiticus* (Calvo et al., 2004; Duran et al., 2007).

Another major breakthrough contributing to the understanding of *veA* function was the discovery of its regulatory role in secondary metabolism in *A. nidulans* (Kato et al., 2003). *veA* was shown to control the biosynthesis of several compounds including antibiotics and mycotoxins, specifically sterigmatocystin (ST), the penultimate intermediate in the AF biosynthetic pathway (Kato et al., 2003). Further studies revealed this regulatory role to be conserved in many other fungi. Importantly, *veA* was demonstrated to be required for the production of AFs in *A. parasiticus* and *A. flavus*, as well as CPA and aflatrem in *A. flavus* (Calvo et al., 2004; Duran et al., 2007, 2009). Studies of the *veA*-dependent transcriptome in *Aspergillus fumigatus* indicated that *veA* affects the expression of 100s of genes (Dhingra et al., 2013), while studies in *A. flavus* and *Fusarium verticillioides* demonstrated a role for *veA* in response to oxidative stress (Baidya et al., 2014; Lan et al., 2014) and hydrolytic activity (Duran et al., 2014). However, in this review we will mainly focus on the role of *veA* and known *veA*-related regulatory factors in the control of morphogenesis, particularly in the formation of sclerotia and in the biosynthesis of secondary metabolites.

Numerous putative *veA* homologs have been identified in other fungal species and many of them have been experimentally characterized (Li et al., 2006; Dreyer et al., 2007; Bayram et al., 2008b; Chettri et al., 2012; Dhingra et al., 2012; Laskowski-Peak et al., 2012; Myung et al., 2012; Lopez-Berges et al., 2013). In *A. nidulans* the study of possible *veA*-interacting proteins revealed that VeA forms complexes with other proteins (Bayram et al., 2008a; Calvo, 2008; Purschwitz et al., 2008; **Figure 1**). After its transport to the nucleus by the alpha-importin KapA (Stinnett et al., 2007; Araujo-Bazan et al., 2009) VeA forms a complex with the red phytochrome FphA (Purschwitz et al., 2008). This interaction is dependent on the presence of the tetrapyrrole chromophore. LreA and LreB, blue sensing proteins, do not interact directly with VeA, but through FphA association; the FphA protein was found to interact with LreB, which interacts with LreA. Deletion of either *fphA* or *lreA*/*lreB* genes affected sexual development as well as secondary metabolism in *A. nidulans* where they play antagonistic functions (Purschwitz et al., 2008). FphA also negatively affects VeA transport to the nucleus in the presence of light. It is likely that a similar regulatory output of the light-sensing proteins is also conserved in *A. flavus*.

Additional studies in *A. nidulans* showed that VeA also interacts with LaeA, forming part of the *velvet* complex (Bayram et al., 2008a). LaeA encodes a putative methyl transferase involved in chromatin remodeling (Keller et al., 2005; Bok et al., 2006b; Reyes-Dominguez et al., 2010). In addition, LaeA influences VeA post-translational modifications and inhibits sexual development in *A. nidulans* in response to light (Sarikaya Bayram et al., 2010). Moreover, *laeA* has been shown to be a positive regulator of gene clusters involved in secondary metabolism in this model organism (Keller et al., 2005; Bok et al., 2006a). In *A. flavus* the *laeA* homolog is necessary for production of AFs and sclerotial formation (Kale et al., 2008). Additionally, it has been shown that *laeA* is a negative regulator of *veA* expression in *A. flavus*. Transcriptome analysis of *A. flavus* wild-type and *laeA* deletion strains indicated that *laeA* not only regulates AF production but also controls the expression of other secondary metabolic gene clusters (Georgianna et al., 2010). Similar to FphA, an *A. nidulans* LaeA-like putative methyltransferase, designated LlmF, also interacts with VeA, negatively affecting VeA transport to the nucleus and acting as negative regulator of ST production and sexual development (Palmer et al., 2013).

Another component of the *velvet* complex interacting directly with VeA is VelB (Bayram et al., 2008a; Park et al., 2012), a member of the *velvet* protein family together with VosA and VelC (Ni and Yu, 2007; Park et al., 2014). In *A. nidulans*, VelB binds to VeA in the cytoplasm and they are co-transported to the nucleus (Bayram et al., 2008a). Similar to the *veA* deletion mutant, deletion of *velB* results in a strain unable to display a light-dependent developmental pattern and it is unable to form cleistothecia (Bayram et al., 2008a). However, dissimilar from the *veA* deletion, absence of *velB* only showed reduced and delayed production of ST. VelB also interacts with VosA (Bayram et al., 2010). The *velvet* domain in these two proteins has been shown to bind DNA in *A. nidulans* (Ahmed et al., 2013) and in *Histoplasma capsulatum*, where there are involved in the activation of the yeast-phase specific gene expression program (Beyhan et al., 2013). In addition, the VelC *velvet* protein functions as a positive regulator of sexual development in *A. nidulans* (Park et al., 2014). Homologs of *A. nidulans* VelB, and VelC have also been characterized in *A. flavus* (Chang et al., 2013). Deletion of *A. flavus velB* (but not *velC*), similar to the case of *veA* (Duran et al., 2007), prevents sclerotial formation and AF biosynthesis.

In addition to the interaction between *A. flavus* LaeA and VelB with VeA, Chang et al. (2013) also described interactions between LaeA and VelB with FluG (**Figure 1**), a known developmental regulator previously characterized in *A. nidulans*. FluG contributes to the inactivation of the FadA G-protein signaling pathway in the model fungus, leading to ST production and allowing sexual and asexual development. Mutations in *A. nidulans fluG* result in proliferation of undifferentiated vegetative hyphae that yield fluffy cotton-like colonies lacking the capacity to produce ST (Adams et al., 1992; Wieser et al., 1994). FadA function was also conserved in the AF-producer *A. parasiticus* (Hicks et al., 1997).

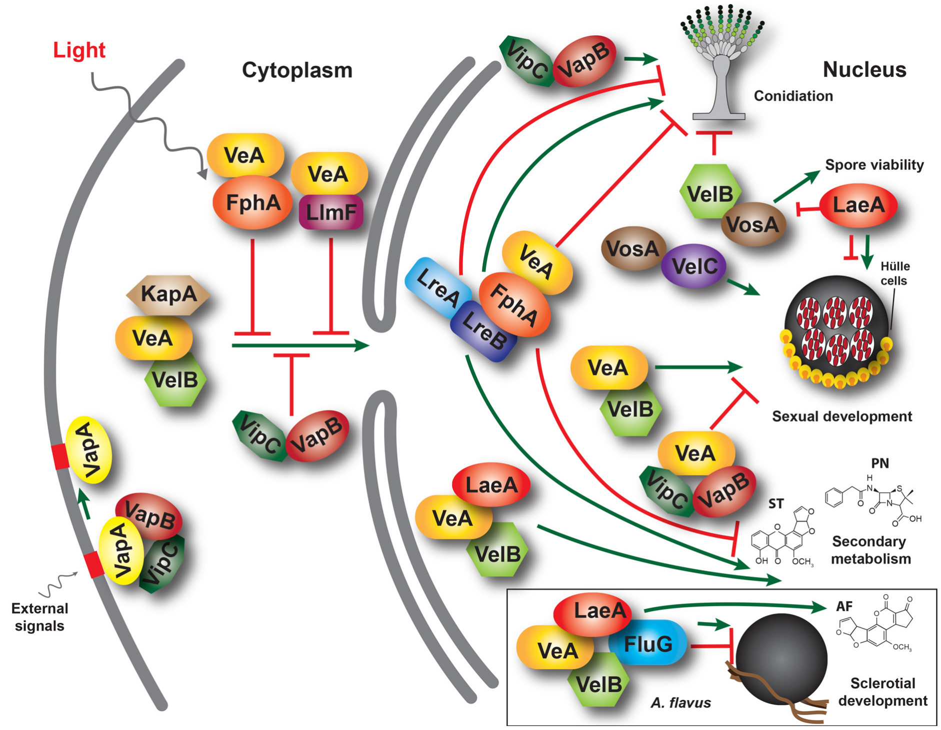

FIGURE 1 | A model illustrating interactions between *velvet* family proteins, LaeA and other associated proteins in the model fungus *Aspergillus nidulans*. The α-importin KapA transports the VeA-VelB dimer from the cytoplasm to the nucleus, particularly in the dark. This transport is negatively influenced by other proteins, such as FphA, LlmF and Vip-VapB dimer in the light. In the nucleus, VelB-VeA activates sexual development and can interact with LaeA, forming the *velvet* complex. VeA also interacts with FphA, which is associated with LreB-LreA forming a light-sensing protein complex. VelB, repressor of asexual development, also forms homodimers and heterodimers with VosA, a protein required for spore viability activating trehalose biosynthesis. VosA also interacts with VelC, which positively regulates sexual development. Additionally, VipC and VapB associate with VeA in the nucleus repressing cleistothecial formation. These complexes regulate development and secondary metabolism in a coordinated manner. VeA, LaeA, and VelB have also been shown to control sclerotial and AF production in *A. flavus*, where they also form a protein complex, together with FluG (box). ST, sterigmatocystin. PN, penicillin; AF, aflatoxin B1.

Evidence for a connection between *fluG* and *veA* was previously provided by Mooney and Yager (1990) and Yager et al. (1998). Mooney et al. (1990) found three extragenic *veA1* suppressor mutations that restored light-dependent conidiation in *A. nidulans* corresponded to different *fluG* alleles. This suggested that *veA* light-dependent activities are related to *fluG* function. *fluG* is involved in the synthesis of a diffusible compound that triggers the FluG signaling pathway directing conidiation and mycotoxin biosynthesis while reducing vegetative growth (Lee and Adams, 1996). The diffusible molecule was determined to be dehydroaustinol (Rodriguez-Urra et al., 2012). Two gene clusters in *A. nidulans* have been found to encode the complete dehydroaustinol pathway (Lo et al., 2012). However, co-culturing experiments did not show a similar diffusible secondary metabolite produced by *A. flavus*. These results suggest that the function of *fluG* and the signaling pathways related to conidiation might be different in these two related Aspergilli (Chang et al., 2012). Based on *A. flavus* studies, Chang et al. (2013) postulated that a delicate balance in the interaction between VeA, VelB, FluG, and LaeA is necessary to maintain normal sclerotiogenesis, conidiogenesis and secondary metabolism, where FluG plays an antagonistic role with respect to VeA, VelB, and LaeA regarding sclerotia formation (Chang et al., 2012). Deletion of *fluG* resulted in a notable increase in sclerotial formation but did not affect AF production. This also differs from the role of *fluG* in *A. nidulans*, where this gene is necessary for ST biosynthesis.

Other characterized VeA-interacting proteins include VipC-VapB methyltransferases, released from the VapA-VipC-VapB membrane-bound complex (Sarikaya Bayram et al., 2014). Presence of VipC-VapB reduces the abundance of the nuclear VelB-VeA-LaeA complex resulting in decreased sexual development. Additionally, VapB also decreases histone 3 lysine 9 trimethylation favoring asexual development.

Post-translational modification of VeA, such as that detected in LaeA-dependent modification in *A. nidulans*, could also have an effect on the *velvet* complex function (Sarikaya Bayram et al., 2010). Purschwitz et al. (2009) demonstrated that VeA is phosphorylated. Later Bayram et al. (2012) showed that MpkB phosphorylates VeA. The MAP-kinase *mpkB*, homolog of *FUS3* in *Saccharomyces cerevisiae*, was first characterized in *A. nidulans* by Paoletti et al. (2007) and Atoui et al. (2008). MpkB transcription increased during sexual development and deletion of the *mpkB* gene resulted in sterility (Paoletti et al., 2007), as well as in a decreased in the expression of ST biosynthetic genes and concomitant ST biosynthesis (Atoui et al., 2008). Furthermore, the absence of *mpkB* also decreased the expression of genes in the penicillin and terrequinone A gene clusters (Atoui et al., 2008). *mpkB* is also necessary for normal expression of *laeA*, that as discussed above, is a global regulator of secondary metabolism (Atoui et al., 2008). The *mpkB* homolog is present in the *A. flavus* genome, however, its possible function in sclerotial development and secondary metabolism has not yet been characterized experimentally in this AF producer.

Both sclerotial and conidial development and secondary metabolism have been shown to be modulated by *A. flavus* oxylipins as well as by endogenous plant oxylipins that interact with the infecting fungus (Burow et al., 1997; Calvo et al., 1999; Brown et al., 2008; Affeldt et al., 2012; Scarpari et al., 2014). The *A. flavus* genome harbors four dioxygenase genes, *ppoA*, *ppoB*, *ppoC*, and *ppoD*, and one lipoxygenase gene, *loxA* (Brown et al., 2008, 2009). In the model fungus *A. nidulans* it has been shown that *veA* is important for *ppo*-dependent regulation of development. For instance, *veA* regulates *ppoA* expression (Tsitsigiannis et al., 2004). Furthermore, the triple mutant *ppo*A/B/C showed an increase in *veA* expression suggesting a regulatory loop between *ppo* genes and the master regulator *veA* (Tsitsigiannis et al., 2005). Absence of these genes results in alteration in morphological and chemical development in *A. flavus* [review by Amaike and Keller (2011)]. For example, strains with deletion of these five genes showed high levels of AF production and sclerotial formation (Brown et al., 2009). The antagonistic roles of different types of oxylipins appear to contribute to a balance between conidiation and sclerotial formation.

The necessity of *veA* for sclerotial production and AF biosynthesis could also be related to the requirement of *veA* for a proper oxidative stress response in *A. flavus* (Baidya et al., 2014). Using modulators that inhibit oxidative stress as well as thiol redox state, Grintzalis et al. (2014) demonstrated that oxidative stress regulates both AF biosynthesis and sclerotial development. Several research groups have also provided evidence of the association between AF production and oxidative stress in Aspergilli (Chang et al., 2011; Reverberi et al., 2012; Hong et al., 2013; Roze et al., 2013).

Recently other regulatory genes have been found to affect development and secondary metabolism in *A. flavus*, specifically *nsdD* and *nsdC* (Cary et al., 2012). The *nsdD* gene, first described in *A. nidulans*, encodes a GATA-type zinc finger transcription factor necessary for cleistothecia formation (Han et al., 2001), while *nsdC* encodes a C_2H_2 zinc finger-type transcription factor shown to negatively regulate asexual sporulation (Kim et al., 2009). *veA* only slightly affects *nsdD* expression (Kato et al., 2003), and has no effect on *nsdC* expression (Kim et al., 2009), suggesting that the role of these genes is independent of *veA* in *A. nidulans*. In *A. flavus*, both *nsdC* and *nsdD* are necessary for sclerotial production and normal levels of AF biosynthesis (**Figure 2**; Cary et al., 2012).

Functional genomic analysis is a powerful approach that has helped to elucidate the genetic connections between sclerotial formation and secondary metabolism. For instance, Wu et al. (2014) compared the transcriptome of mycelium and sclerotium developmental stages and found that backbone genes of 38 secondary metabolite pathways were transcribed in both the mycelial and sclerotial cultures, including the AF biosynthetic pathway. A transcriptome study by Lin et al. (2013) of *A. flavus* cultures treated with 5-azacytidine, an inactivator of DNA methyltransferase, provided further evidence that secondary metabolism and development are co-regulated. Addition of 5-azacytidine altered the expression of backbone genes of two identified secondary metabolite gene clusters, #35 and also #27, both of which have been demonstrated experimentally to be associated to sclerotial biology in either a *veA*- or *laeA*-dependent manner (Forseth et al., 2012; Cary et al., 2014). Additionally, Chang et al. (2014) studied the transcriptome of cultures treated with decanal and observed that this volatile compound halted development at the vegetative state rendering the fungus unable to produce sclerotia. This coincided with early transcriptional activation of AF and kojic acid biosynthesis gene clusters as well as subtle altered timing of other secondary metabolite gene clusters.

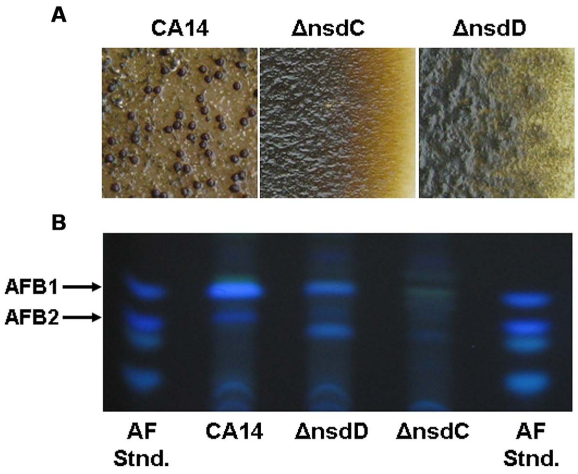

FIGURE 2 | Production of sclerotia and aflatoxins in *A. flavus* CA14 Δ*nsdC* and Δ*nsdD* mutants. (A) Surface of colonies demonstrating sclerotial production after 14 days growth in the dark. Sclerotia were absent in the Δ*nsdC* and Δ*nsdD* mutants and were produced in the wild-type CA14 (dark structures). **(B)** TLC analysis of aflatoxin production from the wild-type CA14, Δ*nsdC*, and Δ*nsdD* mutants. Extracts (5 ul) were spotted onto 250 um silica gel TLC plates and metabolites were separated in ethyl acetate: methanol: water (40:1:1). Aflatoxin standards were also spotted on the plate. Adapted and modified from Cary et al. (2012).

GENE CLUSTERS PRODUCING SECONDARY METABOLITES ASSOCIATED WITH SCLEROTIA

Rapid progress in sequencing of fungal genomes, coupled with bioinformatics, has provided researchers with an *in silico* approach for identifying potential secondary metabolic gene clusters (Bergmann et al., 2007; Winter et al., 2011; Ehrlich and Mack, 2014). Many of the prediction algorithms (e.g., SMURF, antiSMASH, and MIDDAS-M) in use are based on identification of core or "backbone" genes that encode enzymes such as a PKSs, NRPSs, or dimethylallyltryptophans (DMATs) as well as closely allied genes encoding "decorating" enzymes (e.g., dehydrogenases, methyltransferases, and oxidases), transcription factors and transporters (Khaldi et al., 2010; Medema et al., 2011). The MIDDAS-M algorithm has been used to identify potential secondary metabolic gene clusters that may not contain common core genes such as that for ustiloxin B, an *A. flavus* secondary metabolite produced by a ribosomal peptide synthetic pathway (Umemura et al., 2014). These types of algorithms have resulted in the identification of numerous putative secondary metabolic gene clusters in fungi, typically between 30 and 40 in *Aspergillus* species sequenced (Brakhage and Schroeckh, 2011; Andersen et al., 2013; Inglis et al., 2013) including as many as 55 in *A. flavus* (Georgianna et al., 2010). Some of the products of these clusters have been verified based on prior knowledge of genes and metabolites that constitute the cluster (e.g., AF and CPA). In other cases, the products have been predicted in one species based on homology to genes known to produce the metabolite in another fungal species; for example, the identification of the penicillin gene cluster in *A. flavus* based on amino acid identity to the known penicillin biosynthetic genes from *A. nidulans*. However, in most instances, the identity of the metabolites encoded by predicted secondary metabolic gene clusters remains unknown. In these instances the clusters have been termed "orphans." In a number of cases, these orphan clusters can be "cryptic" or silent when the conditions required to activate expression of the cluster genes have not been determined (Brakhage and Schroeckh, 2011; Brakhage, 2013). Once a putative secondary metabolic gene cluster has been identified, a number of techniques can then be utilized to aid in identification of the cluster metabolite (Brakhage and Schroeckh, 2011; Sanchez et al., 2012).

When genes from orphan clusters are actively transcribed under laboratory growth conditions, standard gene-inactivation techniques can be applied, coupled with comparative metabolic profiling of the pathway mutant and the control strain using LC–MS. A common method used for the identification of cryptic gene cluster metabolites is to overexpress the pathway-specific transcriptional activator (if known) by placing it under the control of a strong inducible or constitutive promoter. For example, normally silent genes of the *A. nidulans* aspyridone (apd) gene cluster were activated by coupling of the *apdR* transcriptional activator to the inducible alcohol dehydrogenase promoter of *A. nidulans*, thus allowing identification of aspyridones A and B (Bergmann et al., 2007). In the absence of a pathway-specific transcription factor, it may be possible to activate gene expression of cryptic clusters by overexpressing global regulatory factors. This is exemplified by the use of overexpressing and deletion mutants of the global regulator, *laeA*, to identify the terrequinone A gene cluster in *A. nidulans* (Bok et al., 2006a). In keeping with epigenetic regulation of secondary metabolite production, a number of chemical agents (e.g., histone deacetylase or DNA methyltransferase inhibitors) or genes (e.g., inactivation of a histone deacetylase or sumoylation gene) that modulate chromatin structure have been used to successfully induce expression of cryptic clusters [reviewed in Sanchez et al. (2012) and Brakhage (2013)].

A recent study indicated the presence of secondary metabolite-mediated crosstalk between two separate gene clusters (Forseth et al., 2012). Comparative metabolomics of gene knockout, knockdown (RNAi-based), and overexpression strains of *A. flavus* were used to identify a group of secondary metabolites derived from two *laeA*-regulated orphan gene clusters, designated lna and lnb. The lna cluster is located on chromosome 6 and lnb on chromosome 8. The two clusters harbor non-canonical NRPS genes (*lnaA* and *lnbA*) with high sequence identity as well as associated genes encoding tailoring enzymes that are involved in the production of a group of piperazines. It was shown that addition of the one of the piperazine metabolites, produced almost exclusively by the lnaA cluster, to wild-type cultures greatly increased expression of the lnaB NRPS. The apparent "sensing" of a metabolite produced by a separate but related gene cluster may represent another layer in the complex regulation of secondary metabolism in fungi. Interestingly, loss of these *lnaA*- and *lnaB*-derived piperazines resulted in a significant reduction in sclerotial formation in the mutant strains, thereby demonstrating a role of these secondary metabolites in fungal development.

Lastly, the most ecologically based of all secondary metabolite induction techniques, is the simulation of interactions in nature between the fungus and other resident microbes. This technique is based on the premise that microorganisms share ecological niches; and as such, produce secondary metabolites as a means of intra- and interspecies communication or as defense mechanisms. By simulating these interactions in culture, using two or more organisms, there is a chance that the organism of interest will respond by eliciting production of a secondary metabolite. For example, utilizing microarray technology with co-cultivation techniques, the interaction of *A. nidulans* with the soil-dwelling actinomycete, *Streptomyces rapamycinicus*, induced the expression of a cryptic gene cluster in *A. nidulans* involved in the production of the polyketide, orsellinic acid (Schroeckh et al., 2009).

Sclerotia represent a means by which fungi maintain a quiescent viable state in the absence of a suitable host or of conditions favoring active growth (Coley-Smith and Cooke, 1971). As such, mature sclerotia are essentially dormant metabolically, and therefore would not be amenable to any of the methods discussed above for activation of cryptic secondary metabolic pathways. However, it is probable that many of the secondary metabolites present in sclerotia are produced in the hyphae that coalesce during the early phases of sclerotial morphogenesis. Most sclerotial metabolites identified so far in fungi have been identified from extracts of sclerotia generated under laboratory conditions on artificial media. It is likely that sclerotia found in nature harbor many additional as of yet unidentified secondary metabolites. Below we describe a number of genetically and biochemically well characterized secondary metabolite gene clusters whose products have been found in sclerotia of filamentous fungi. As most of these clusters have been thoroughly reviewed in the literature, only a brief synopsis with references will be provided here.

ERGOT ALKALOIDS

Ergot alkaloids represent a complex family of indole derivatives with diverse structures and broad biological and pharmacological activities. The genetics and enzymology of EA biosynthesis is detailed in reviews by Wallwey and Li (2011) and Jakubczyk et al. (2014). Chemically, EAs can be divided into three groups: ergoamides, ergopeptines, and clavines. The biosynthetic gene clusters responsible for the production of each of these types of EAs have been identified in a number of fungal species. The gene cluster in *C. purpurea* leading to the formation of complex ergopeptines consists of 14 genes spanning about 68.5 kb of the genome. The *Claviceps fusiformis* cluster is responsible for the production of the clavines, agroclavine and elymoclavine, that lack the peptide moieties present on the ergoline ring of ergopeptines. The *C. purpurea* and *C. fusiformis* gene clusters are homologous with the exception of three genes that are lacking in *C. fusiformis.* These genes (*lpsA1, lpsA2,* and *lpsC*) encode NRPSs that are responsible for biosynthesis of the peptide moieties present in the ergopeptines. *A. fumigatus* also produces the clavine-type metabolites, fumigaclavines, but these have only been associated with conidiation. No genes encoding a putative pathway-specific transcriptional activator or transporter have been identified in EA gene clusters.

The genes involved in EA biosynthesis are divided into early and late pathway steps. The first step of the early pathway is catalyzed by the dimethylallyl prenyltransferases (DmaW) that prenylates L-tryptophan in the presence of dimethylallyl pyrophosphate (DMAPP) to form DMAT. Subsequent methylation (EasF) and two successive oxidations (EasC and EasE) produce chanoclavine-I, the ergoline ring C structure. Chanoclavine-I is then oxidized by EasD to generate the aldehyde form which in *Claviceps* is subsequently cyclized by EasA and EasG reductases to form the unsaturated ergoline ring D structure, agroclavine, that represents the last intermediate of the early pathway. The late step pathway genes encode an oxidase (CloA) responsible for the formation of paspalic acid which, either spontaneously or via an isomerase, forms lysergic acid. Three NRPSs (Lps1, Lps2, and lpsC) activate lysergic acid and form the tripeptide moiety of the ergopeptine end products.

ASPARASONE A

Expression of genes present in an *A. flavus* cluster, designated #27 based on SMURF analysis by Georgianna et al. (2010) was found to be significantly downregulated in a *veA* mutant (Cary et al., 2014). The cluster was predicted to consist of a Zn(2)-Cys(6)-type transcription factor, PKS, two putative transporters and a gene encoding a hypothetical protein. A schematic depiction of the cluster is shown in **Figure 3A**. Expression of the *pks27* gene was first observable at 48 h, was maximal at 120 h, and decreased by 144 h (Cary et al., 2014). Transcription paralleled sclerotial development and pigmentation which appeared to be maximal at 120 h in wild-type *A. flavus.* The transcription factor, *znf27,* was required for wild-type levels of expression of the other three cluster genes but not for the gene encoding the hypothetical protein. The putative high-affinity glucose (*mfs1*) and MFS transporter (*mfs2*) genes showed an expression profile similar to that observed for the *pks27* and the *znf27* genes. qRT-PCR of RNA isolated from mycelia, conidia, and sclerotia of the *A. flavus* wild-type showed that expression of *pks27* and *znf27* was specific to the sclerotium.

Inactivation of *pks27* resulted in *A. flavus* colonies that produced only grayish-yellow sclerotia, instead of the characteristic dark brown color of the wild-type, indicating that the mutational defect was only in pigmentation and not in sclerotial maturity (**Figure 3B**). Comparison of extracts of the wild-type and the Δ*pks27* mutant by ultra-high performance liquid chromatography and mass spectrometry revealed a metabolite of mass 358 Da that was present in the wild-type but missing in the mutant. Based on this mass, the metabolite was putatively identified as the anthraquinone asparasone A [1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)-anthraquinone; $M = 358$ Da; **Figure 3C**]. It was previously reported to be produced by *A. parasiticus* which is a close relative of *A. flavus* (Sobolev et al., 1997). The identification of the 358 Da metabolite as asparasone A was confirmed by LC–MS comparison with an authentic asparasone A standard. It was hypothesized that dehydration of asparasone would result in conjugated olefins which, like styrene, might rapidly polymerize in the presence of metal dioxygenases such as laccases to form the dark sclerotial pigment (Cary et al., 2014).

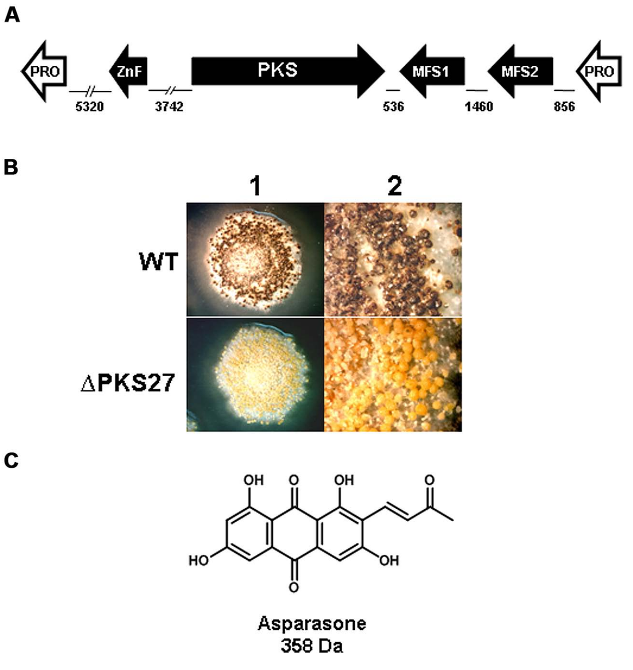

FIGURE 3 | (A) Schematic representation of the *A. flavus* asparasone gene cluster. The cluster is composed of four genes [putative Gal4-type transcription factor (ZnF); polyketide synthase (PKS); putative high-affinity glucose transporter (MFS1); and a second putative MFS transporter (MFS2)]. The two hypothetical protein encoding non-cluster genes (Pro) flanking the asparasone cluster are shown in white. The size in bp of intergenic regions are shown. Arrowheads denote direction of transcription. **(B)** Microscopic examination of sclerotia. Sclerotium production in Af70 pyrG-1 (WT) and an Af70 Δ*pks27* mutant was observed using an Olympus SZH10 stereomicroscope and Nikon DS-Qi1 camera. Panel: (1) colony surface, X10 magnification; (2) colony surface, X25 magnification. **(C)** Chemical structure of asparasone A. Adapted and modified from Cary et al. (2014).

AFLATOXIN

The genetics, molecular biology, and biochemistry of AF biosynthesis in *A. flavus* and *A. parasiticus* have been the focus of a number of reviews, and we direct the reader to these references for more detailed information (Yu et al., 2004; Ehrlich et al., 2005; Georgianna and Payne, 2009; Yu, 2012). Many of the contributions to our understanding of AF biosynthesis and its regulation have come from studies in the model fungus, *A. nidulans*, on the biosynthesis of ST. ST a precursor of AF, and the genes for ST biosynthesis in *A. nidulans* are highly homologous to those required for the production of ST in the AF gene cluster. The AF biosynthetic gene cluster of *A. flavus* spans ~70 kb of chromosome 3 and consists of 28 genes including two regulatory factors, *aflR* and *aflS* (*aflJ*). AflR is a Zn(2)-Cys(6)-type, pathway-specific transcriptional activator while AflS does not share any significant identity with other fungal proteins but has been shown to be required for AF production. AflS has been shown to interact with AflR and facilitate the activation of other AF biosynthetic genes (Du et al., 2007). AflR protein binds to the palindromic sequence 5′-TCGN$_5$CGA-3′ (or deviations thereof) found in the promoter region of all AF biosynthetic genes. The AF gene cluster in *A. flavus* is under the control of the global regulators, VeA, NsdD, NsdC, and LaeA (Duran et al., 2007; Kale et al., 2008; Cary et al., 2012).

Aflatoxins are a group of polyketide-derived furanocoumarins that are produced from acetate via a PKS (AflC) and two fatty acid synthetases (AflA and AflB), and a number of tailoring enzymes. AF biosynthesis requires at least 18 known enzymatic reactions to catalyze synthesis of the four major AFs, AFB1, AFB2, AFG1, and AFG2 found in *A. parasiticus*. In general, *A. flavus* only produces AFB1 and AFB2. Just outside of the distal end of the AF gene cluster in *A. parasiticus* and *A. flavus* is a conserved sugar utilization gene cluster. However, the genetic composition at the proximal end (the end closest to the telomere) of the AF cluster is not conserved in these two species. *A. flavus* strains contain a deletion at the proximal end of the cluster that result in functional loss of *aflU* (*cypA*) and *aflF* (*norB*) genes. The inability of *A. flavus* to produce the G toxins is due to the partial deletions of *aflU* and *aflF*, which encode a P450 monooxygenase and a putative aryl alcohol dehydrogenase, respectively, and are required for conversion of hydroxymethyl-ST to AFG1 in *A. parasiticus* (Zeng et al., 2011).

Aspergillus flavus as a species contains two morphotypes that differ in sclerotial size and in their ability to produce AFs. Large (L) and small (S) sclerotial strains are often found in soils from agricultural fields, and the S strains are generally found to produce higher levels of AF than L strains (Zhang and Cotty, 2006; Horn, 2007). *A. flavus* is a genetically diverse species and, unlike other aflatoxigenic *Aspergillus* species, a portion of *A. flavus* populations has lost the ability to produce AFs (Cotty and Bhatnagar, 1994). A survey of 38 non-aflatoxigenic *A. flavus* strains, isolated from across the Southern United States, identified eight patterns of gene deletion present in the AF gene cluster (Chang et al., 2005). There is evidence that gene loss in the AF gene cluster of non-aflatoxigenic *A. flavus* isolates is irreversible, and that balancing selection maintains non-aflatoxigenicity and lineage-specific gene loss in *A. flavus* populations (Moore et al., 2009, 2011).

AFLATREMS

Both aflatrem and its isomer, β-aflatrem (502 Da), are indole-diterpenes that have been isolated from the sclerotia of *A. flavus* (TePaske et al., 1992). Aflatrems are tremorigenic mycotoxins that have been shown to cause neurological disorders in mammals, including muscle tremors and hyperexcitability in livestock, that have consumed feed contaminated with *A. flavus* (Valdes et al., 1985). β-aflatrem displayed significant activity against corn earworm in feeding studies (TePaske et al., 1992). Biosynthesis of aflatrems proceeds much like that of paxilline in *Penicillium paxilli* (Parker and Scott, 2004), in that aflatrem consists of a paxilline-like core, with an additional prenyl group on the indole moiety and an acetyl group on the diterpene skeleton (Nicholson et al., 2009). Utilizing sequence information for genes involved in paxilline biosynthesis in *P. paxilli* and the genome sequence of *A. flavus*, the genes required for aflatrem biosynthesis were found to be present on two separate loci in *A. flavus* (Zhang et al., 2004; Nicholson et al., 2009). Expression of aflatrem cluster genes and concomitant production of aflatrem has been shown to require the presence of *veA* and *laeA* (Duran et al., 2007; Georgianna et al., 2010).

Two gene clusters involved in aflatrem biosynthesis have been described. The ATM1 locus, present on chromosome 5 in *A. flavus*, harbors a gene cluster consisting of the *atmG, atmC,* and *atmM* genes. These encode the geranylgeranyl pyrophosphate (GGPP) synthase, prenyltransferases, and monoxygenase, respectively, that are involved in synthesis of paspaline, the first stable intermediate in paxilline and aflatrem biosynthesis. The ATM2 locus, located on chromosome 7, contains *atmD*, encoding an aromatic prenyltransferse; *atmQ* and *atmP,* both encoding P450 monooxygenases; and *atmA* and *atmB*, both encoding predicted membrane proteins believed (but not proven) to be transporters required for paxilline biosynthesis. The exact functions of *atmG*, *atmC*, *atmM*, and *atmB* and their pax orthologs in paspaline biosynthesis are not clear. It is believed that AtmG catalyzes the condensation of indole-3-glycerol phosphate and DMAPP to generate GGPP, which is then epoxidated by AtmM and cyclized by AtmC to form paspaline (Saikia et al., 2006). AtmP converts paspaline to 13-desoxypaxilline via removal of the C-30 methyl group and oxidation at C-10. AtmQ catalyzes the oxidation of 13-desoxypaxilline at C-7 then C-13 to form paspalinine. Finally, AtmD prenylates paspalinine on the indole moiety to form aflatrem. No pathway-specific transcription activator gene was identified in the clusters.

CYCLOPIAZONIC ACID

It was noted that *A. flavus* strains unable to form AFs, due to deletions that extended from the adjacent subtelomeric region to within the AF gene cluster, were often unable to produce CPA (Chang et al., 2009). A region spanning about 30 kb from the subtelemeric end of the AF cluster was shown to harbor genes encoding a putative DMAT (*dmtA*), PKS-NRPS (*pks-nrps*), and FAD-dependent oxidoreductase (*moaA*) that were considered candidates for CPA production based on enzymes identified in biosynthesis of EAs. Inactivation of these three genes resulted in loss of CPA production. Orthologous genes (*cpaD* = *dmtA*; *cpaA* = *pks-nrps*; *cpaO* = *moaA*) have been identified in *A. oryzae* and also shown, by gene disruption, to be required for biosynthesis of CPA (Shinohara et al., 2011). Interestingly, the CPA cluster in both of these fungi also contained a putative transcription factor (*cpaR* = *ctfR1*), however, disruption of this gene in both *A. flavus* and *A. oryzae* did not affect CPA production. Production of CPA has been shown to require the presence of the *veA* gene (Duran et al., 2007).

In the initial step in CPA biosynthesis, the PKS-NRPS catalyzes the condensation of L-tryptophan and two molecules of acetyl-CoA to generate cycloacetoacetyl-L-tryptophan (cAATrp) which is then converted by the DMAT to β-CPA. The FAD-dependent oxidoreductase is then responsible for the cyclization of β-CPA to CPA (Shinohara et al., 2011). Interestingly, *A. oryzae* RIB40, which does not make CPA, was found to have a truncated version of the PKS-NRPS (*cpaA*) gene.

CONCLUSION AND FUTURE PERSPECTIVES

It will be difficult to ascertain the exact role of individual sclerotial secondary metabolites in fungal biology. However, observations such as the induction of orsellinic acid production in *A. nidulans* upon co-culture with a soil microbe provide strong support for a role of these natural products in cross-species communication or defense against competing microbes. The potential role of sclerotial secondary metabolites as a chemical defense against insect predators is supported by the plethora of studies that have demonstrated their antiinsectan/antifeedant properties (Gloer, 1995, 2007). The finding of Cary et al. (2014) of preferential feeding by insects on sclerotia collected from a mutant *A. flavus* no longer producing asparasone A represents the first *in vivo* experimental evidence of the contribution of a secondary metabolite to sclerotial chemical defense. These types of gene knockout experiments should prove invaluable in identifying the contribution of individual secondary metabolites to fungal chemical defense. This will be important as many of the secondary metabolites identified in *A. flavus* have not been assigned to a predicted gene cluster, and it is highly probable that in the near future many of these orphan clusters will be found to produce compounds that are associated with the sclerotium. Advances in functional genomics and metabolomics will invariably accelerate the pace in the identification of secondary metabolic gene clusters associated with the synthesis of sclerotial compounds. Accordingly, these studies will also provide relevant information on the genetic regulatory networks governing activation and modulation of secondary metabolic gene clusters that play a role in sclerotial biology as well as other cellular processes. In this regard, continued structural and comparative analyses of sequenced fungal genomes, coupled with ever-increasing understanding of the molecular and functional biology of secondary metabolites in the Aspergilli, will undoubtedly accelerate the identification and functional characterization of secondary metabolite gene clusters in other filamentous fungi.

The majority of studies on the biological functions of sclerotial secondary metabolites have focused on their role in chemical defense against insect predators and other competing organisms. More research is needed to investigate other possible roles for these metabolites in sclerotial biology. For example, no information exists as to why *A. flavus* produces two morphotypes of sclerotia and if there is any difference in the secondary metabolic profiles of these morphotypes. If a metabolite(s) is consistently present in one sclerotial morphotype versus the other this may indicate a role for the metabolite(s) in sclerotial morphogenesis. Not only are S morphotype sclerotia smaller than L morphotype, but they are almost always produced in greater numbers. The ability to produce greater numbers of S morphotype sclerotia may represent an adaptive response by the fungus to survive insect predation compared to that of L strains. A correlation between selective pressure, due to predation, and sclerotial size has been suggested (Wicklow, 1988), in which long-term survival of a fungus is improved by the production of larger, better chemically defended sclerotia compared to those of fungi that produce numerous small sclerotia. However, it can also be argued that smaller size may increase the ease with which S morphotype sclerotia are damaged/consumed by predators and therefore the fungus has evolved to produce increased numbers as a means to ensure dissemination and survival of the species. During the course of evolution, selective pressure from increased predation on S morphotype sclerotia may have led to an increase in the levels/classes of antiinsectan secondary metabolites present in small sclerotia. The study of Chang et al. (2002) demonstrated that an increase in AF intermediates led to smaller sclerotial size in *A. flavus*. A similar correlation may be used to explain the existence of the S morphotype. If sclerotia of S morphotype *A. flavus* strains have increased levels of secondary metabolites compared to L morphotype, the increased demand for carbon building blocks (e.g., acetate) for biosynthesis of the additional secondary metabolites would result in less availability of carbon for sclerotial biogenesis, resulting in the observed small sclerotial morphotype. Chemical analysis of sclerotial extracts coupled with insect feeding studies should be able to shed some light on the relationship of sclerotial morphotype and fungivory.

Another unexplored area is the potential role of secondary metabolites in mating of normally asexual species of Aspergilli. It will be of interest to determine if secondary metabolite profiles differ in the stromata generated from the mating of two *A. flavus* strains compared to that present in the sclerotia produced during normal growth of each strain. Perhaps a chemical signal produced by hyphae of the interacting mating types can induce production of novel secondary metabolites in the sexual stromata that are not present in sclerotia of the individual mating partners. The chemical signal itself could be the product of a secondary metabolic gene cluster that is activated upon interaction of hyphae of opposite mating types. The presence of novel secondary metabolites in stromata would indicate that these compounds may play a role in the recognition and initiation of sexual reproduction by strains of opposite mating type, or they may be produced as a means of expanding the chemical arsenal of antiinsectan agents present in the fruiting structures.

Sclerotia are very important to the survival and dissemination of fungi in nature, and as such should be the target of strategies for control of fungal contamination of food and feed crops. As presented in this review, a number of global regulators that control production of secondary metabolites also control sclerotial formation. Novel technologies such as host-induced gene silencing can take advantage of host plant-derived siRNAs that target expression of these global regulators in the invading fungus. For example, maize can be transformed with RNAi-based constructs that generate siRNAs targeting *veA* or *nsdC* gene transcripts of *A. flavus*. This approach, in theory, would reduce both AF and sclerotial production in the invading fungus (Nunes and Dean, 2012). The soundness of this concept has already been demonstrated for control of several cereal pathogens, including barley powdery mildew (Nowara et al., 2010), wheat leaf rust (Panwar et al., 2013) and maize ear-rot caused by *F. verticillioides* (Tinoco et al., 2010). Continued study of the biogenesis and function of fungal secondary metabolites and their association with development, as well as elucidation of the regulatory mechanisms controlling production of these natural products, will facilitate the design of additional strategies to reduce the detrimental effects of pathogenic fungi.

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Cellular compartmentalization of secondary metabolism

H. Corby Kistler* and Karen Broz

United States Department of Agriculture-Agricultural Research Service, Cereal Disease Laboratory, University of Minnesota, Saint Paul, MN, USA

Edited by:
Ozgur Bayram, Maynooth University, Ireland

Reviewed by:
Jae-Hyuk Yu, University of Wisconsin, USA
Wilhelm Schäfer, Universität Hamburg, Germany
John Edgar Linz, Michigan State University, USA

****Correspondence:***
H. Corby Kistler, United States Department of Agriculture-Agricultural Research Service, Cereal Disease Laboratory, University of Minnesota, 1551 Lindig Street, Saint Paul, MN 55108, USA
e-mail: hckist@umn.edu

Fungal secondary metabolism is often considered apart from the essential housekeeping functions of the cell. However, there are clear links between fundamental cellular metabolism and the biochemical pathways leading to secondary metabolite synthesis. Besides utilizing key biochemical precursors shared with the most essential processes of the cell (e.g., amino acids, acetyl CoA, NADPH), enzymes for secondary metabolite synthesis are compartmentalized at conserved subcellular sites that position pathway enzymes to use these common biochemical precursors. Co-compartmentalization of secondary metabolism pathway enzymes also may function to channel precursors, promote pathway efficiency and sequester pathway intermediates and products from the rest of the cell. In this review we discuss the compartmentalization of three well-studied fungal secondary metabolite biosynthetic pathways for penicillin G, aflatoxin and deoxynivalenol, and summarize evidence used to infer subcellular localization. We also discuss how these metabolites potentially are trafficked within the cell and may be exported.

Keywords: aflatoxin, deoxynivalenol, mycotoxin, non-ribosomal peptide, polyketide, penicillin, terpene

INTRODUCTION

Filamentous fungi produce a diverse range of low molecular mass natural products (NPs) often associated with unique bioactive properties. Prominent among these fungal secondary metabolites (SMs) are compounds beneficial to human society such as antibiotics, pigments, fragrances or pharmaceuticals (Demain, 2014). Conversely, some fungal SMs have had marked negative impact on food safety and human health in the form of mycotoxins or molecules potentiating fungal pathogenesis. Fungal SMs are structurally diverse and principally categorized as polyketides, terpenoids, alkaloids or small non-ribosomal peptides (Keller et al., 2005). Recent progress in large scale DNA sequencing from a wide range of filamentous fungi has led to the discovery that fungal genomes possess a broad genetic potential to produce SMs. A remarkably large number of enzymes that produce SMs—polyketides synthases (PKSs), non-ribosomal peptide synthases (NRPSs) and terpene synthases (TSs)—have been described, often as part of biosynthetic gene clusters predicted to be responsible for the synthesis of one or more NP (e.g., Nierman et al., 2005; Wiemann et al., 2013). However, the NPs synthesized by these predicted gene clusters are mostly unknown. Even the environmental conditions under which many of these cryptic SM gene clusters may be expressed has yet to be determined (Wiemann and Keller, 2014).

While knowledge of the enzymology and genetics of fungal SM production has advanced greatly in recent years, scant information is available on the cell biology of their biosynthesis. Where are SM compounds assembled within the cell? Since pathways for fungal primary and secondary metabolism often draw upon the same chemical starting materials, how do cells channel and apportion the supply of shared molecular precursors to primary and secondary metabolic pathways? The answer to these questions will require greater understanding of the cellular and developmental processes that define the fungal metabolome.

For this review we have chosen three well studied SMs synthesized by separate fungal species: penicillin G produced by *Penicillium chrysogenum*, aflatoxin produced by *Aspergillus parasiticus* and deoxynivalenol (DON) produced by *Fusarium graminearum*. Each compound represents a separate class of fungal NPs: peptide (penicillin), polyketide (aflatoxin) or terpene (DON). Each pathway for biosynthesis of these SMs involves enzymes targeted to multiple subcellular locations. Genetically engineering fungi so that enzymes for SMs may be physically co-localized or localized to particular organelles can have significant impact on flux through pathways and ultimate biosynthetic yield of NPs (Albertsen et al., 2011; Herr and Fischer, 2014). Therefore, it is reasonable to assume that evolutionary forces may have shaped the expression and regulation of fungal NP output by directing subcellular localization of SM biosynthetic enzymes.

Our goal is to summarize evidence for the subcellular location of enzymes and transporters associated with the biosynthesis of the three featured NPs. We also will summarize the types of evidence used to establish each cellular location. One difficulty for such localization studies is that the examined proteins likely are translocated within the cell as part of their normal synthesis, maturation and ultimate turn-over. Early on, proteins will be localized to ribosomes and endoplasmic reticulum (ER) where they are synthesized and modified whereas, with time, defective or aged proteins ultimately may be targeted to the vacuole or proteasome for proteolysis. Deciding on the subcellular location of a catalytically active protein therefore requires judgment and may be inferred based on the timing of peak enzyme activity or NP accumulation or by co-purification of organelles with active enzymes.

CYTOSOL

Determining whether an enzyme is located in the fungal cytosol often has been a process of elimination. For example, if no discernible localization pattern within the cell is detected using electron microscopic immunolocalization (e.g., Lee et al., 2004) a cytoplasmic location may be inferred. Likewise if fluorescently tagged proteins demonstrate little or no selectivity for cellular location (e.g., Hong and Linz, 2008), a protein may be inferred to be cytoplasmic. Fractionation of cellular lysates by centrifugation is another approach; proteins that remain soluble upon high speed centrifugation may be deemed cytoplasmic (e.g., van der Lende et al., 2002). In support of these direct observations are bioinformatic tools. If a protein lacks a predicted transit peptide leader sequence suggestive of secretion, has no predicted membrane anchoring or multiple transmembrane domains indicative of integral membrane proteins or lacks other amino acid motifs suggesting localization to particular organelles, the protein in question may be cytosolic.

These approaches to determining cytosolic localization are illustrated by studies of enzymes involved in the first two steps of penicillin synthesis in *P. chrysogenum*: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and isopenicillin N synthase (IPNS). Transmission electron microscopy coupled with immunogold labeling of either ACVS or IPNS under penicillin producing conditions seems to show non-specific dispersal of electron dense gold particles throughout hyphal cross sections although largely excluded from vacuoles (van der Lende et al., 2002). Moreover, western blot analysis of the pellet vs. supernatant of high speed ultracentrifugation of cell lysates suggests enrichment for ACVS and INS in the soluble fraction compared to the pelleted (100,000 × g) fraction. Van der Lende. argue from these observations (and the alkaline pH optimum for ACVS activity) that both enzymes are likely cytosolic and unlikely to be functional within the acidic vacuolar lumen. By this assertion they refute the findings of Lendenfeld et al. (1993) who, by density gradient centrifugation and gel filtration, found that ACVS (but not IPNS) co-purified with biochemical markers for the vacuole and apart from an enzymatic marker for the cytoplasm (glucose-6-phosphate dehydrogenase). Van der Lende et al. suggest that ACVS is likely in an inactive form while in the vacuole; yet the relative activity of cytosolic and vacuolar ACVS was not directly measured in either study. We and others (Lim and Keller, 2014) consider the results of these localization studies equivocal and further work is warranted. Nevertheless, it is perhaps naïve to assert that ACVS must exist solely in a single cellular location. It is possible that the enzyme is cytoplasmic with an affinity for association with the outer surface of the vacuolar membrane, as suggested by Lendenfeld et al. (1993), and so may be found in both free cytosolic and vacuole associated forms (**Figure 1A**).

Cytosolic enzymes may be re-directed to other subcellular locations upon induction of SM biosynthesis. Several enzymes in the aflatoxin biosynthetic pathway including Nor-1, Ver-1 and Vbs initially were proposed to be cytoplasmic based on their immunodetection by transmission electron microscopy (Chiou et al., 2004; Lee et al., 2004) and distribution based on fluorescent protein tagging (Hong and Linz, 2008, 2009) (**Figure 1B**). Further work using fractionation of cell lysates as well as genetic and biochemical inhibitors now suggest that these proteins additionally may be found in small vesicular bodies within the cytoplasm (Chanda et al., 2009a,b; Roze et al., 2011). Cytoplasm to vacuole transport may play a role in movement of cytoplasmic enzymes to the subcellular location where they are presumably active. Repositioning of Nor-1, Ver-1, and OmtA from the cytosol to vesicles is correlated with the timing of aflatoxin biosynthesis and protein modifications indicative of cytoplasm to vacuole transport (CVT) (Lee et al., 2004; Hong and Linz, 2008, 2009; Roze et al., 2011; Linz et al., 2014). Aflatoxin biosynthetic enzyme Vbs is also thought to move from the cytoplasm to vesicles via the ER-Golgi secretory pathway during aflatoxin biosynthesis (Chiou et al., 2004). Further evidence for the localization of these enzymes in vesicles will be discussed below.

To date no enzymes involved in trichothecene biosynthesis have been definitively localized to the cytoplasm (**Figure 1C**). Nevertheless, based on their physical properties and structure both trichodiene synthase (Rynkiewicz et al., 2001) and trichothecene 3-O-acetyltransferase (Garvey et al., 2008) might be expected to be cytosolic proteins. Investigations to determine their cellular localization using fluorescent protein tagging are underway (Broz, unpublished).

PEROXISOMES

Peroxisomes are organelles specialized for both anabolic and catabolic metabolism in fungi and most other eukaryotic organisms. As their name implies, they are the cellular location for generation of hydrogen peroxide and its turnover by the enzyme catalase, as well as the site of other enzymes involved in the conversion of reactive oxygen species to less toxic compounds. These organelles thus may provide protection to the cell by sequestering these potentially toxic metabolites. Peroxisomes also are the site of acetyl CoA generation by β-oxidation of fatty acids and contain the enzymes isocitrate lyase and malate synthase specific to the glyoxylate cycle for the capture of acetyl CoA for gluconeogenesis or to provide intermediates for the TCA cycle within mitochondria (van der Klei and Veenhuis, 2013). Peroxisomes have been imaged by appending peroxisomal targeting sequences (such as the amino acids -SKL) to the C-terminus of fluorescent proteins (e.g., Meijer et al., 2010) or by tagging integral membrane proteins of the peroxisome such as PEX3 (e.g., Menke et al., 2013; **Figure 2**).

The final enzymatic steps of penicillin synthesis in *P. chrysogenum* occur within the peroxisome (**Figure 1A**). The enzyme isopenicillin N-acyltransferase (IAT) converts isopenicillin N (IPN) to penicillin G by exchange of the α-amino adipyl side chain of IPN with CoA-activated phenylacetic acid (van der Klei and Veenhuis, 2013); for other forms of penicillin, other activated carboxylic acid substrates are exchanged (Koetsier et al., 2009). Müller et al. (1992) demonstrated that a peroxisomal targeting sequence at the C terminus of IAT was required for synthesis of penicillin. A mutant strain producing an IAT protein lacking the putative C-terminal peroxisome signal sequence (-ARL) produced no penicillin. Whereas the wild type enzyme was localized to peroxisomes ("microbodies") as determined by immunolocalization in transmission electron microscopy, the mutant protein showed no similar localization pattern but rather appeared in the

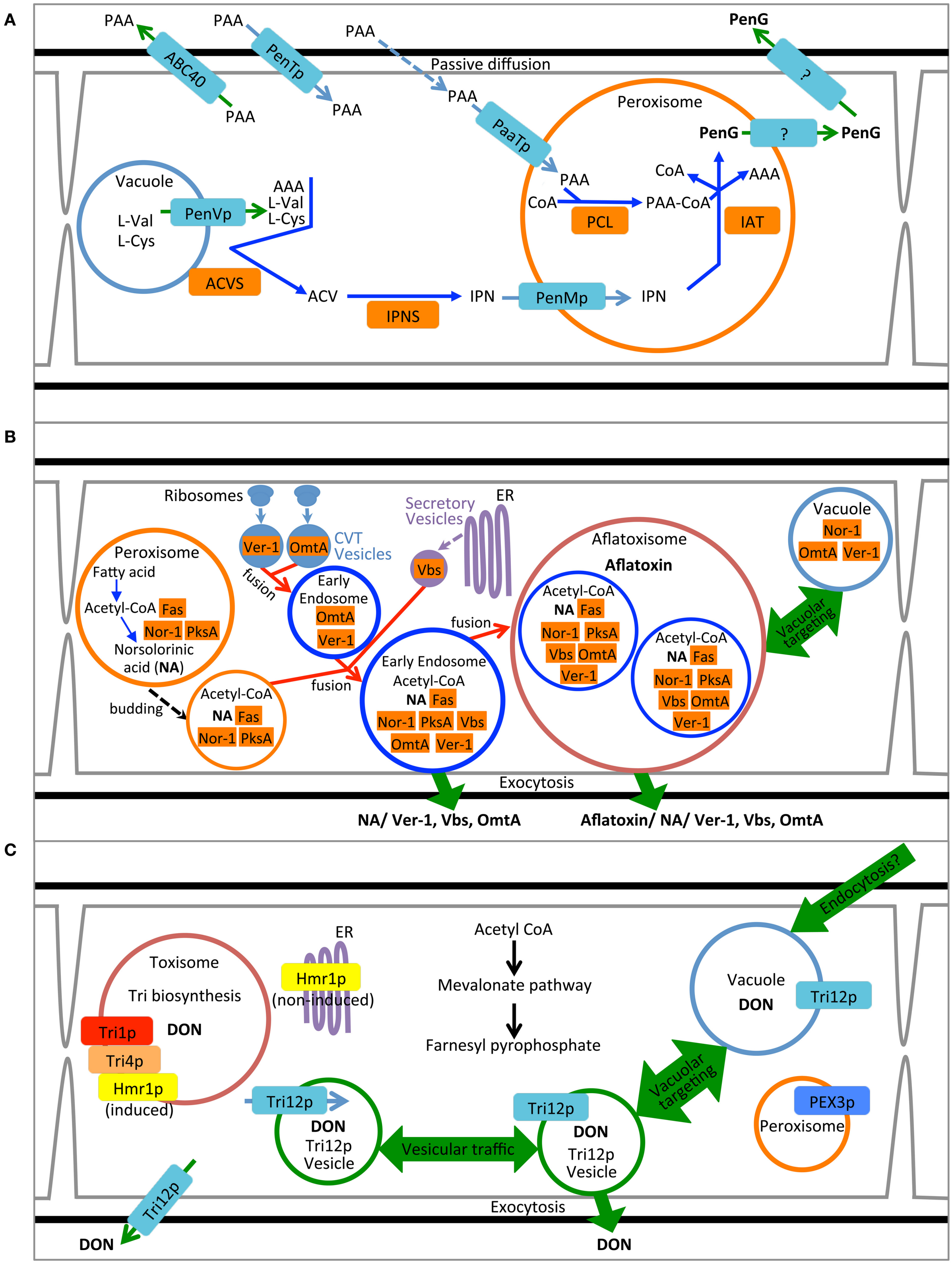

FIGURE 1 | Compartmentalization models for biosynthesis of penicillin, aflatoxin and trichothecene. (A) Synthesis of penicillin in *P. chrysogenum*. Pathway precursors and intermediates are abbreviated: AAA, L-α-aminoadipic acid; L-Cys, L-cysteine; L-Val, L-valine; PAA, phenylacetic acid; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; IPN, isopenicillin N; PenG, penicillin G. Enzymes are abbreviated: ACVS, ACV synthetase; IPNS; isopenicillin N synthase; PCL, Phenylacetyl-CoA ligase; *(Continued)*

FIGURE 1 | Continued
and IAT, IPN acyltransferase. **(B)** Synthesis of aflatoxin in *A. parasiticus*. Pathway intermediate abbreviation NA is norsolorinic acid. Enzymes are abbreviated: Nor-1, NA reductase; PksA, polyketide synthase A; Fas, Fatty acid synthase; Ver-1, versicolorin A dehydrogenase; OmtA, dihydro-sterigmatocystin O-methyltransferase; Vbs, versicolorin B synthase. **(C)** Synthesis of trichothecene in *F. graminearum*. Product abbreviation DON is deoxynivalenol. Enzymes are abbreviated: Tri1p, calonectrin oxygenase; Tri4p, trichodiene oxygenase, Hmr1p, 3-hydroxy-3-methyl-glutaryl-CoA reductase.

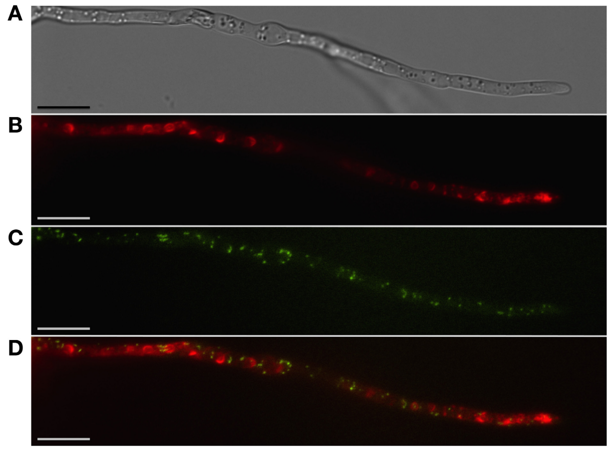

FIGURE 2 | Localization of peroxisomes and toxisomes in *F. graminearum*. Shown is a strain of *F. graminearum* having a GFP-tagged Pex3 protein and a TagRFP-T-tagged trichodiene oxygenase grown under trichothecene-inducing conditions. **(A)** Hypha visualized using differential interference contrast (DIC) microscopy. **(B)** TagRFP-T visualized by epifluorescence reveals the trichodiene oxygenase in spherical toxisomes in the subapical cells and in reticulate pattern toward the hyphal tip. **(C)** GFP fluorescence from Pex3 revealing puntate structures corresponding to peroxisomes. **(D)** Overlay of GFP and TagRFP-T fluorescence showing that peroxisomes are distinct from toxisomes. Bar = 10 μm. Results presented in Menke et al. (2013); figure generated for Weber (2013).

cytosol and vacuole. While these results suggest that correct targeting of IAT to the peroxisome is critical for normal pathway function it should be noted that the mutant also appeared to produce less IAT protein and showed reduced IAT activity compared to wild type (Müller et al., 1992). Therefore, it is unclear if peroxisomal localization, or wild type enzyme titre and activity, or both are responsible for the mutant phenotype.

The enzyme responsible for transfer of CoA to phenylacetic acid is phenylacetyl CoA ligase (or PCL) that catalyzes the penultimate step in penicillin biosynthesis; this enzyme also may be localized to peroxisomes. PCL labeled at the N-terminus with cyan fluorescent protein co-localized with yellow fluorescent protein labeled IAT in dual tagged strains of *P. chrysogenum* (Koetsier et al., 2010). Fluorescence for both proteins was entirely contained within punctate structures consistent in size and distribution with peroxisomes. As many as eight other acyl-CoA ligases related to synthesis of naturally occurring penicillins have been proposed for *P. chrysogenum* (Martín et al., 2012) each with a range of different substrate specificities (e.g., Koetsier et al., 2010) and all but one have well defined C-terminal peroxisome targeting sequences (Martín et al., 2012).

In addition to the enzymes of the penicillin biosynthetic pathway, two predicted peroxisome membrane associated proteins have been implicated in penicillin G synthesis in *P. chrysogenum* (**Figure 1A**). PaaT protein has been linked to phenylacetic acid transport and PenM protein associated with IPN uptake at the peroxisome membrane (Fernández-Aguado et al., 2013b, 2014). Both proteins were DS-Red labeled and shown to be peroxisomal by co-localization with GFP-SKL constructs. Silencing PaaT resulted in overall reduction in penicillin G accumulation while levels of IPN remained nearly the same; over-expression of PaaT increased penicillin G accumulation and resistance to the inhibitory effects of added phenyl acetate. Because the amino acid sequence of PaaT is a predicted MFS transporter, it is suggested that its location in the peroxisome membrane may allow it to translocate phenylacetic acid to the peroxisomal lumen where it may be activated with acetyl CoA by the peroxisomal enzyme PCL thereby creating the side chain for penicillin G (Fernández-Aguado et al., 2013b). Silencing the gene for PenM, predicted to encode another MFS transporter, also caused reduction in penicillin accumulation and, in general at 48 h, increased intracellular levels of IPN. Together these results suggest that in *P. chrysogenum* PenM promoted translocation of IPN from the cytosol to the peroxisomal lumen where it could be further metabolized to penicillin G (Fernández-Aguado et al., 2014).

A case also has been made for peroxisomes as the site for the initial steps of aflatoxin biosynthesis (Roze et al., 2011) (**Figure 1B**). Efforts to directly localize the polyketide synthase associated with the synthesis of the first step in aflatoxin synthesis (PksA) have been unsuccessful (Maggio-Hall et al., 2005). Nevertheless, direct localization of the fluorescent metabolite resulting from this enzymatic step, norsolorinic acid, has been localized to peroxisomes based on co-occurrence with isocitrate lyase-GFP. Either the enzyme itself occurs in the peroxisome or the product of the enzymatic reaction, norsolorinic acid, is imported to the peroxisome after synthesis in the cytoplasm or another organelle (Maggio-Hall et al., 2005; Lim and Keller, 2014).

Peroxisomes also generate acetyl CoA via β-oxidation that normally may be used to fuel the glyoxylate cycle and the anabolic pathway to gluconeogenesis. Alternatively, peroxisomal acetyl CoA may be used as a shared precursor for aflatoxin (polyketide) synthesis. Indeed, Maggio-Hall et al. (2005) demonstrated that acetyl CoA generated via β-oxidation in the peroxisome contributes to the ability of *A. nidulans* to form sterigmatocystin, as evidenced by deletion of the peroxisome localized enzyme *FoxA*. *FoxA* encodes a protein in *Aspergillus* necessary for β-oxidation of very long chain fatty acids (Maggio-Hall and Keller, 2004), and when deleted the ability of *A. nidulans* to accumulate sterigmatocystin in fatty acid amended medium is reduced. The organelle thus may be a critical central locus for both primary and secondary metabolism, with acetyl CoA serving as a common link for these divergent pathways.

Peroxisomes are not known to be important for trichothecene biosynthesis in *F. graminearum* and have been shown to be distinct from the "toxisome" itself (Menke et al., 2013; **Figure 2**). (Full explanation of the *Fusarium* toxisome is in the section below). Nevertheless, peroxisomes are a potential source of acetyl CoA which is the precursor of both the mevalonate and trichothecene pathways. In *F. graminearum* peroxisomes are highly motile in cells during the process of toxisome formation. But in cells with fully formed toxisomes, peroxisomes may establish close stationary contact with the toxisomes (**Video S1**).

ENDOPLASMIC RETICULUM AND GOLGI

The endoplasmic reticulum (ER) is a conserved organelle of eukaryotic cells consisting of a double membrane structure. Its membranous network may be found throughout the cell but is most closely associated with nuclei since it adjoins the nuclear membrane (perinuclear ER). The ER is highly dynamic and also may form tubular or reticulate structures within the cell often more highly concentrated toward the hyphal tip and septa (Maruyama et al., 2006). The ER is the site of isoprenoid and lipid synthesis as well as calcium storage. Additionally, the ER is where proteins destined for secretion are folded, processed and glycosylated during anteriograde transport to the fungal Golgi. The ER is also the site for protein quality control in the form of ERAD (endoplasmic-reticulum-associated protein degradation) whereby misfolded or otherwise defective proteins are ubiquitinated and targeted for degradation at the proteasome (Jo and Debose-Boyd, 2010). The ER may be visualized by fluorescent protein tagging ER resident proteins such as the molecular chaperone BiP or by using "ER Tracker" (Wedlich-Söldner et al., 2002; Maruyama et al., 2006) containing fluorescently labeled glibenclamide that binds to conserved sulphonylurea receptors of ER membrane associated potassium channels (Hambrock et al., 2002).

SM biosynthetic enzymes may pass through the ER-Golgi for modification before reaching their final subcellular destination. Vbs, an enzyme in the middle of the aflatoxin pathway, is glycosylated at the N-terminus and has been shown to localize to the ER as well as the cytoplasm (Chiou et al., 2004). Vbs has also been identified in vesicular bodies where other aflatoxin biosynthetic enzymes are located (discussed below), presumably being delivered there via the ER-Golgi secretory pathway (Chiou et al., 2004; Roze et al., 2011).

The synthesis of trichothecene and other terpene secondary metabolites in fungi utilizes farnesyl diphosphate, the product of the ER-localized mevalonate pathway (Koning et al., 1996). A key enzyme of this pathway is hydroxymethylglutaryl (HMG) CoA reductase that has been shown to accumulate at the perinuclear or peripheral ER in yeast based on co-localization with Sec61p, an ER integral membrane protein or Kar2p found in the ER lumen (Koning et al., 1996). In fungi that produce terpene SMs, HMG CoA reductase labeled with fluorescent proteins localized to reticulate endomembrane structures that have been interpreted as the fungal ER (Albermann et al., 2013; Menke et al., 2013).

Strong induction of trichothecene mycotoxins in the fungus *F. graminearum* is accompanied by a remarkable shift in the pattern of HMG CoA reductase localization in fluorescently tagged strains (**Figure 3**). While initially fluorescence is found throughout the cell in structures consistent with peripheral and perinuclear ER, after induction fluorescence shifts to numerous intensely fluorescent spherical structures that are asymmetrically labeled at their periphery. These structures appear similar to lamellar proliferations of the perinuclear ER similar to "karmellae" observed in yeast by Koning et al. (1996) in HMG CoA reductase over-producing strains.

Two inducible cytochrome P-450 oxygenases responsible for catalyzing early and late steps in the trichothecene biosynthetic pathway also localize to $\sim 3\,\mu m$ spherical structures (**Figure 4**). Both trichodiene oxygenase (Tri4p) and calonectrin oxygenase (Tri1p) contain single predicted membrane anchor sequences and co-localize with each other and to the membrane of structures containing Hmg CoA reductase under inducing conditions (Menke et al., 2013). Because the structures contain both studied toxin biosynthetic enzymes, they have been called "toxisomes" and are the presumptive site of trichothecene assembly. However, it remains unclear whether the structures represent a novel organelle or rather a profoundly reorganized ER responsive to trichothecene induction.

The three enzymes of the toxisome show the identical asymmetrical pattern of localization suggesting that regions of the membrane may be specialized for particular biosynthetic pathways as in yeast (Koning et al., 1996) presumably by direct

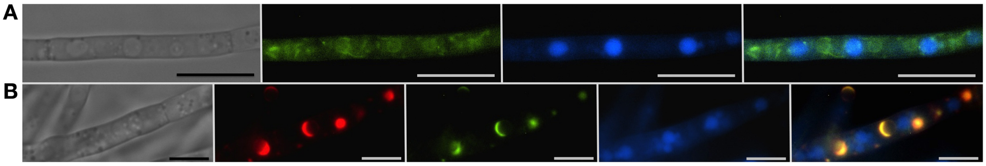

FIGURE 3 | Vacuoles, the ER and toxisomes in *F. graminearum*. Organelles are visualized in a strain of *F. graminearum* having a GFP-tagged HMG CoA reductase and a TagRFP-T-tagged trichodiene oxygenase. **(A)** Strain grown in minimal medium which does not induce trichothecene synthesis. (Left to right) Hypha visualized using differential interference contrast (DIC) microscopy; GFP fluorescence showing HMG CoA reductase consistent with localization to reticulate and perinuclear ER; CMAC fluorescence used to visualize vacuoles; combined GFP and CMAC fluorescence showing both ER and vacuoles. Bar = $10\,\mu m$. **(B)** Strain grown in medium which induces trichothecene synthesis. (Left to right) Hypha visualized using DIC; TagRFP-T florescence showing trichodiene oxygenase within the membrane of spherical toxisomes; GFP fluorescence showing HMG CoA reductase within the membrane of spherical toxisomes; CMAC fluorescence used to visualize vacuoles; combined GFP, RS-Red and CMAC fluorescence showing co-localization of HMG CoA reductase and trichodiene oxygenase in toxisomes and vacuoles. Bar = $5\,\mu m$. (Note that the TagRFP-T tagged trichodiene oxygenase is expressed only under toxin inducing conditions). Results presented in Menke et al. (2013); figure generated for Weber (2013).

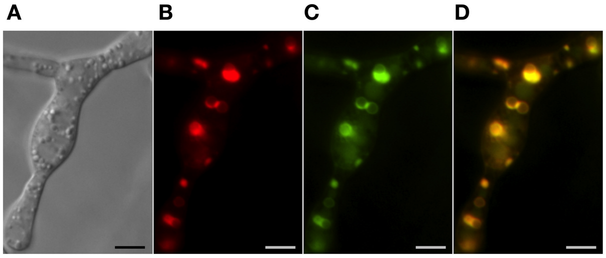

FIGURE 4 | Co-localization of trichothecene biosynthetic enzymes calonectrin oxygenase and trichodiene oxygenase in toxigenic cells. Toxisomes are visualized in a strain of *F. graminearum* having a GFP-tagged calonectrin oxygenase and a TagRFP-T-tagged trichodiene oxygenase. **(A)** Toxigenic cells visualized using DIC microscopy. Note swollen cells with prominent vacuoles; **(B)** TagRFP-T-tagged trichodiene oxygenase fluorescence at the periphery of toxisomes; **(C)** GFP calonectrin oxygenase fluorescence at the periphery of toxisomes; **(D)** combined GFP and TagRFP-T florescence showing toxisome co-localization. Results presented in Menke et al. (2012); figure generated for Menke (2011).

protein-protein interactions. If so, this would bring in physical proximity enzymes functioning in primary and secondary metabolic pathways, perhaps channeling pathway intermediates and promoting pathway efficiency. HMG CoA reductase is subject to protein level regulation mediated by ERAD in a broad range of organisms (Theesfeld and Hampton, 2013) and so, in *Fusarium*, may bring trichothecene synthesis under the same post-translational control. Finally, because the catalytic sites of the trichothecene oxygenases are predicted to be within the lumen of the toxisome, toxic intermediates of the biosynthetic pathway presumably would be sequestered within, protecting the cell from potentially damaging effect. How the toxic trichothecenes may safely exit the toxisome and the cell is discussed below.

THE VACUOLE

Vacuoles are multifunctional acidified organelles most often associated with storage and protein turnover, but increasingly recognized as the site of specialized biosynthetic processes. Vacuolar systems may be highly dynamic and can differ dependent upon the age of hyphae, hyphal function, and growth conditions (Shoji et al., 2006). Several approaches have been used to identify fungal vacuoles and vacuolar enzymes (Richards et al., 2012). First, vacuoles have a distinct "hollow" appearance in cells viewed by differential inference contrast (DIC) microscopy likely due to the distinct refractive index imparted by the contents of the vacuolar lumen (**Figure 4**). Several markers also are used to identify vacuoles, most notably CMAC (7-amino-4-chloromethylcoumarin) which conjugates glutathione and other thiols concentrated within the vacuolar lumen. The pH indicator Neutral Red which is taken up and colors acidified cellular compartments, primarily vacuoles, also has been used as has the fluorescent dye FM4-64 which enters the cell via endocytosis and progressively labels the plasma membrane, endosomes and ultimately the vacuolar membrane (Fischer-Parton et al., 2000). Identifying vacuoles also is achieved by localization of commonly recognized vacuolar proteins including subunits of the conserved vacuolar ATPase complex for the vacuolar membrane and carboxypeptidase Y for the vacuolar lumen (Richards et al., 2012).

The *P. chrysogenum* protein PenVp has been suggested to be an integral membrane protein of the vacuole facilitating penicillin G biosynthesis (Fernández-Aguado et al., 2013a). Silencing PenV gene expression greatly reduces penicillin G accumulation under inducing conditions as well as decreases concentration of intermediates IPN and ACV. A DS-Red tagged PenVp localizes to structures consistent with the vacuole (similar structures are seen in separately prepared FM4-64 labeled cells). Based on these results and the predicted amino acid sequence of the protein, Fernández-Aguado et al. postulate that PenVp may be an amino acid permease transporting L-cysteine and L-valine from the vacuole to the cytoplasmic side of the vacuole where they may serve as substrates for ACVS to synthesize ACV (**Figure 1A**).

The *Aspergillus* gene Ver-1encodes a NADPH dependent reductase catalyzing the reaction converting versicolorin A to demethylsterigmatocystin, a step toward the middle of the aflatoxin biosynthetic pathway. Based on fluorescent protein tagging of the Ver-1 protein Hong and Linz (2009) suggested that it is transported from the cytoplasm to the vacuole in an active state. However, more recent work has reinterpreted the results, suggesting that synthesis actually occurs within motile vesicles termed aflatoxisomes (Chanda et al., 2009a,b, 2010) (**Figure 1B**).

Likewise, the predicted integral membrane protein and trichothecene transporter, Tri12p has been demonstrated by GFP-tagging to localize to the vacuolar lumen. However, the vacuolar form of the protein may represent a degraded state since the predicted membrane associated protein (and presumed functional form) is present in the plasma membrane and in small motile vesicles (Menke et al., 2012).

VESICLES, VESICLE TRAFFIC AND INTRACELLULAR TRANSPORT

Vesicles are small, membrane delimited organelles specialized for intracellular transport. Several different types of transport vesicles exist within fungal cells, each having different composition, biogenesis, means for translocation and function (Shoji et al., 2014). Vesicles participate in the well-studied, Golgi mediated exocytosis at the hyphal tip and also at the septum (Riquelme and Sánchez-León, 2014). Endosomes are vesicles arising from endocytosis at the plasma membrane (PM) that may translocate cargo for degradation to the vacuole or recycle materials back to the PM and cell surface (Steinberg, 2014). Other novel vesicular export processes, termed unconventional secretion (Shoji et al., 2014), bypass the Golgi, and have been shown to be important for delivery of protein effectors (e.g., Giraldo et al., 2013).

Aflatoxin and aflatoxin biosynthetic enzyme activity in *A. parasiticus* were localized to a membrane rich fraction (the "V fraction") of cell lysates derived from protoplasts resulting from cultures induced to synthesize aflatoxin (Chanda et al., 2009b). The V fraction, obtained by density centrifugation contained vesicles, as determined by light and electron microscopy, as well as vacuolar components as determined by MDY-64 and CMAC fluorescence (Chanda et al., 2009a,b). This fraction was capable of converting pathway intermediates versicolorin A (VerA) and sterigmatocystin (ST) to aflatoxin (Chanda et al., 2009a;

Linz et al., 2012). This suggests that enzymes for the middle and late steps of aflatoxin synthesis are found within this fraction. Western blot analysis also indicates the proteins Nor-1, Vbs, and Ver-1 are present in the V fraction (Chanda et al., 2009a). These data support the hypothesis that the enzymes and presumably pathway intermediates may be co-localized within vesicles.

The proteome of the V fraction obtained from aflatoxin induced cells indeed contains many, but not all, of the enzymes of the aflatoxin biosynthetic pathway. Nevertheless, the fraction also is made up of a complex mixture of proteins including those associated with peroxisomes (catalase, superoxide dismutase, Woronin body protein), the ER (calnexin), and mitochondria (F1 ATPase). Biochemical assays of the V fraction for the presence of mitochondrial and cytoplasmic enzymes were negative (Chanda et al., 2009b). Linz et al. (2012) interpret the content of the V fraction as being primarily transport vesicles, endosomes and vacuoles and suggest that proteins seemingly from other organelles may in fact be found within transport vesicles captured in route to those organelles.

In support of this interpretation, a variety of microscopic, genetic and biochemical data have been presented. Cells induced to produce aflatoxin have an increased ratio of small vesicles ($<2.5\ \mu m$) to vacuoles ($\geq 2.5\ \mu m$) compared to non-induced cultures as determined using light microscopy (Chanda et al., 2009a). Mutants (avaA) and a chemical treatment (Sortin3) that disrupt fusion of late endosomes to vacuoles also increase the number of small vesicles, and increase aflatoxin levels in mycelium and in the culture medium (Chanda et al., 2009a). Linz et al. (2014) propose that two alternative pathways for aflatoxin traffic occur: one leading to the vacuole and resulting in the turnover of biosynthetic enzymes and reduced levels of the toxin and a second pathway mediated by the small mobile vesicles (termed "aflatoxisomes"), that contain the enzymes that synthesize aflatoxin and mediate its export (**Figure 1B**). Inhibition of aflatoxisome traffic to the vacuole in avaA mutants or by Sortin3 therefore is proposed to increase the alternative pathway toward continued toxin synthesis and export.

THE PLASMA MEMBRANE AND BEYOND: TRANSPORTERS, METABOLITE TRAFFIC AND EXPORT

How fungal cells transport secondary metabolites between cellular compartments or facilitate export remains largely unknown. Gene clusters for the synthesis of secondary metabolites typically encode predicted transporter proteins especially major facilitator superfamily (MFS) transporters (Coleman and Mylonakis, 2009). These membrane-spanning proteins may promote traffic of the product of the biosynthetic pathway between organelles or participate in their export.

Industrial strains of *P. chrysogenum* can accumulate up to 50 g l^{-1} penicillin in batch culture yet the mechanism by which the compound, produced within the peroxisome, is exported from the organelle and from the cell, remains to be fully explained (Martín et al., 2010). The genome sequence of *P. chrysogenum* contains over 800 potential export proteins (Van den Berg et al., 2008), but functional analysis leading to the discovery of penicillin G transporters is still lacking.

Aspergillus parasiticus contains a predicted MFS transporter within the aflatoxin biosynthetic gene cluster (Yu et al., 2004). *AflT* encodes a predicted 14-membrane spanning domain MFS transporter (Chang et al., 2004) but the cellular localization for the AflT protein has yet to be determined. Deletion mutants of *aflT* still synthesize and export aflatoxins, similar to wild type strains (Chang et al., 2004), although the methods used to draw this conclusion were considered "semi-quantitative" and so may not accurately detect modest but statistically significant changes in aflatoxin accumulation.

Upon induction to produce aflatoxin, vesicles may be detected within *A. parasiticus* cells as observed by fluorescent light microscopy using dyes FUN-1, MDY-64, and CMAC and show repositioning such that they are adjacent to the plasma membrane. Remarkably these vesicles also appear to transit the membrane and cell wall, forming vesicular structures outside of the cell (**Figure 5**). Fluorescent antibodies toward aflatoxin and norsolorinic acid indicate accumulation of these compounds in discrete patches at the hyphal surface. These results have been explained by proposing that exocytosis of aflatoxisomes allows for export of both toxin and toxin biosynthetic enzymes (Chanda et al., 2010; Linz et al., 2014). The exact mechanism by which exocytosis may occur, whether by conventional exocytosis, Golgi-independent secretion, MVB-mediated secretion or by other non-canonical pathways (Shoji et al., 2014) is not known at this time.

The trichothecene biosynthetic gene cluster of *F. sporotrichioides* and *F. graminearum* each contain a gene (*Tri12*) encoding a predicted 14-membrane spanning domain MFS transporter associated with trichothecene synthesis and resistance (Alexander et al., 1999; Menke et al., 2012). Tri12p is a member of the DHA2 family of drug:proton antiporters conferring multidrug resistance

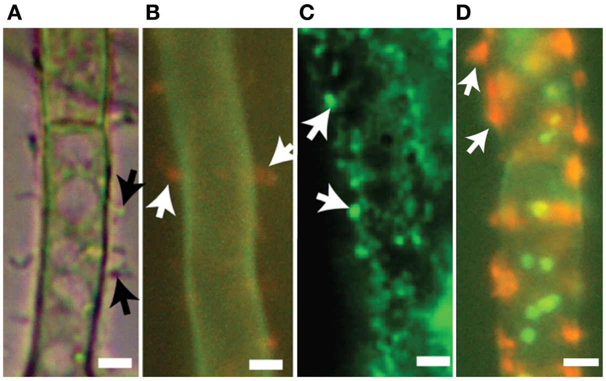

FIGURE 5 | Export vesicles and localized secretion of aflatoxin and norsolorinic acid. Toxigenic cells of *A. parasiticus*. **(A)** Visualized using bright-field microscopy, toxigenic cells show pronounced extracellular protuberances (arrows). **(B)** Similar toxigenic cells treated with the fluorescent vital dye FUN-1 visualizing orange extracellular bodies (arrows) normally associated with cylindrical intravacuolar structures (Millard et al., 1997). **(C)** Detection of foci (arrows) on surfaces of toxigenic cell by immunofluorescence using anti-aflatoxin antibodies. **(D)** Detection of norsolorinic acid (NA) on the cell surface (arrows) using fluorescent anti-NA antibodies. Photos reprinted from Chanda et al. (2010) by permission from the publisher, the American Society for Microbiology.

or participating in the uptake of amino acids into the cell or the vacuole (Dias and Sá-Correia, 2013). Drug transport is made possible by pH and electrochemical gradients which occur across the plasma membrane or the tonoplast.

In *F. sporotrichioides*, disruption of the *Tri12* gene resulted in a 97% reduction in the trichothecene accumulation compared to wild type (Alexander et al., 1999); *tri12* deletion mutants in *F. graminearum* had a more moderate phenotype with trichothecene content reduced about 31% *in planta* (Menke et al., 2012). Expression of the *F. sporotrichioides Tri12* gene in yeast allows for enhanced uptake of the trichothecene pathway intermediate 15-decalonectrin but did not confer greater tolerance to the externally added trichothecene diacetoxyscirpenol (Alexander et al., 1999). This suggests that while Tri12p may facilitate transport of trichothecene metabolites across a membrane barrier, it may not necessarily confer toxin resistance. Nevertheless, *tri12* mutants of both species grow more slowly than the wild type under trichothecene biosynthesis-inducing conditions suggesting that in their native cellular context, the transporter confers a small but significant degree of metabolite tolerance.

Although the cellular localization of Tri12p from *F. sporotrichioides* has not been determined, in *F. graminearum*, GFP-tagged Tri12p largely localizes to the plasma membrane (Menke et al., 2012). Further study has shown Tri12p also localizes to the vacuole/late endosome and small ($\sim 1\,\mu m$) motile vesicles (Menke et al., 2013). Organelles containing Tri12p are not randomly arranged but rather have a striking alternating arrangement with the spherical "toxisomes" containing the trichothecene biosynthetic enzymes (**Figure 6**). Vacuoles in toxigenic cells chiefly are found adjacent to toxisomes with the smaller, motile Tri12p-linked vesicles displaying motion between the two (**Video S2**) a Tri12p vesicles may pause in close proximity to the toxisome (**Figure 6B**) and fuse with the vacuole or plasma membrane. We propose that acidified organelles containing Tri12p such as these vesicles and the vacuole may then accumulate trichothecenes (**Figure 1**). Induction of trichothecene biosynthesis *in vitro* causes a rapid acidification of the culture medium (Gardiner et al., 2009), potentially providing the pH gradient across the plasma membrane that would potentiate trichothecene export by plasma membrane imbedded Tri12p.

The processes of (1) trichothecene biosynthesis and (2) trichothecene traffic and export thus may involve different cellular compartments. We have proposed a model (**Figure 1C**) whereby the synthesis of trichothecenes occurs in a spherical structure made up of repatterned ER (toxisomes) while the transport of trichothecenes occurs by motile structures arising from endosomes. The toxisomes may play a part in sequestration of the trichothecene product and intermediates, thus protecting the cell from their toxic activity and promoting pathway efficiency. The Tri12p system may allow for the accumulation of trichothecenes within acidified vesicles and transport to the vacuole or plasma membrane for storage within the vacuole or export via exocytosis. As in the case of aflatoxisomes, the exact mechanism by which exocytosis may occur is currently unknown.

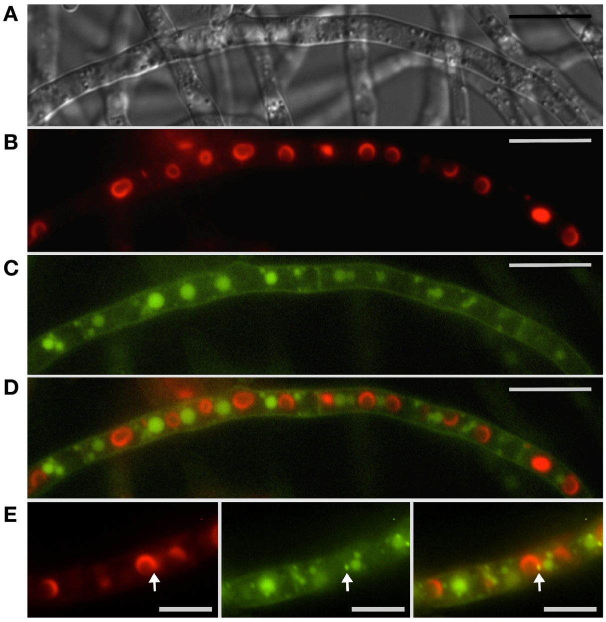

FIGURE 6 | Interaction between toxisomes and motile Tri12p containing vesicles. Toxisomes, vacuoles and motile vesicles are visualized in a strain of *F. graminearum* having a GFP-tagged Tri12p and a TagRFP-T-tagged trichodiene oxygenase. **(A)** Hyphae visualized using DIC microscopy. **(B)** TagRFP-T-tagged trichodiene oxygenase fluorescence at the periphery of toxisomes. **(C)** GFP-tagged Tri12p localizes to vacuoles and smaller motile vesicles (see also Supplemental Video S2). **(D)** Overlay of B and C showing alternate arrangement of vacuoles and toxisomes. Bar = $10\,\mu M$. **(E)** Closer view of (left to right) TagRFP-T-tagged trichodiene oxygenase, GFP-tagged Tri12p and overlay. Bar = $5\,\mu m$. Note transient co-localization of motile vesicle and toxisome (arrow). Movement of Tri12p vesicles between the toxisome and vacuole as well as fusion of vesicles with the plasma membrane and vacuoles are illustrated in the Supplemental **Video S2**. Results presented in Menke et al. (2013); figure generated for Menke (2011).

SUMMARY AND FUTURE DIRECTIONS

Based on the three examples of cellular compartmentalization examined in this review, each has a separate developmental pattern associated with localization of their unique SM pathways. The enzymatic components of the penicillin, aflatoxin and trichothecene pathways are variously localized to peroxisomes, endosomes, the cytosol, the ER or perhaps more specialized vesicular structures.

In each case, induction results in clear cytological repatterning reflective of their subcellular specialization for SM biosynthesis. Intact peroxisomes are important for synthesis of penicillin G in *P. chrysogenum* and conditions that induce penicillin synthesis increase the number of peroxisomes per cell (Meijer et al., 2010) and up-regulate genes for peroxisome function (Van den Berg et al., 2008). Strains of *P. chrysogenum* selected for high penicillin production have increased numbers of peroxisomes (Van den Berg et al., 2008; Meijer et al., 2010). Growth of *Aspergillus* in aflatoxin inducing medium results in pronounced changes in the vesicular content of the cell with greater numbers of smaller vesicles ($<2.5\,\mu m$) relative to vacuoles ($>2.5\,\mu m$) in toxigenic cells

(Chanda et al., 2009a). Vesicles contain enzymes for aflatoxin synthesis (Linz et al., 2012) and may facilitate export of the enzymes and metabolites by way of exocytosis (Chanda et al., 2010). Remarkable cellular and subcellular differentiation also occurs in *F. graminearum* under conditions conducive to trichothecene biosynthesis (Menke et al., 2012). Within 12 h of induction, subapical hyphal swelling occurs with increased vacuole formation. By 36 h, hyphae thicken, branch extensively and become increasingly vacuolated (**Figure 3**). During this time period the fluorescently tagged ER protein HMG CoA reductase largely transitions from a reticulate endomembrane localization to the membrane of distinct ∼3 μm toxisomes that also are the site of trichothecene biosynthetic enzymes (Menke et al., 2013). A separate vesicular structure containing Tri12p may facilitate trichothecene traffic and export.

In the future, studies should concentrate on whether the compartmentalization of biosynthetic pathways, represented in this review by examples of peptide, polyketide and terpene compounds, are part of conserved evolutionary adaptations for synthesis of these compound classes (**Figure 1**). For example, are all fungal polyketides produced within small, endosome related, vesicular structures like aflatoxisomes? Are all fungal terpene secondary metabolites produced within ER related structures like the *Fusarium* toxisomes? Each exemplifies an adaptation of the preexisting machinery of primary metabolism for the more specialized task of synthesis, traffic and export of secondary metabolites. Additionally, are terpene SMs like trichothecenes produced within the same structures as terpene primary metabolites such as sterols? Both the fungal sterol and trichothecene biosynthetic pathways carry out oxygenation reactions catalyzed by cytochrome P-450 oxygenases. Is there a common site within the ER for all cytochrome P-450s? Each might be expected to share a common NADPH:cytochrome P-450 reductase and rely upon the electrochemical gradient across the ER membrane.

Co-compartmentalization of SM biosynthetic enzymes might be expected to allow certain types of selective advantage. Colocalization of consecutive enzymes in a SM biosynthetic pathway may promote pathway efficiency through proximity. Localization to the site where pathway precursor metabolites also occur may serve to channel metabolites toward SM synthesis. For example, both polyketides and terpenes ultimately are derived from acetyl CoA, but acetyl CoA may be synthesized in several locations within the cell, for example, as a result of β-oxidation of fatty acids in mitochondria or in peroxisomes or by pyruvate decarboxylation in the cytosol. Do the terpene and polyketide pathways utilize the same or different subcellular sources of acetyl CoA?

Compartmentalization of SM biosynthetic pathways not only brings together enzymes as a functional unit but also sets the pathway apart from the rest of the cell. This is especially important if products and intermediates may be toxic to the producing cell. Compartmentalization also may facilitate export of products by exocytosis although the exact mechanisms of SM export have yet to be determined for any filamentous fungus.

Certainly it is clear that relatively little is known about the cellular compartmentalization of secondary metabolic pathways in filamentous fungi. Given the importance of these compounds to industry, food safety and public health, and the importance of understanding the regulation and expression of the fungal secondary metabolome, this research doubtlessly will be a fertile area of exploration for years to come.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00068/abstract

Video S1 | Potential interactions of peroxisomes and toxisomes during trichothecene biosynthesis in *F. graminearum*. Spinning disc confocal microscopy of a *F. graminearum* strain with GFP-tagged Pex3 protein and a TagRFP-T-tagged trichodiene oxygenase grown under trichothecene-inducing conditions. Peroxisomes (green) and toxisomes (red) are shown in developing (top) and mature (bottom) toxigenic cells. Dashed arrows indicate areas where toxisomes appear to be forming from a diffuse reticulate endomembrane matrix. Peroxisomes are highly motile in this developing cell. Solid arrows indicate fully formed toxisomes in a mature toxigenic cell adjacent to less motile peroxisomes. Arrow heads indicate co-fluorescent punta indicating mature toxisomes closely juxtaposed with peroxisomes. Bar = 10 μm. Results presented in Menke et al. (2013); video generated for Weber (2013).

Video S2 | Interaction between toxisomes and motile Tri12p containing vesicles. Spinning disc confocal microscopy of a *F. graminearum* strain with GFP-tagged Tri12 protein and a TagRFP-T-tagged trichodiene oxygenase grown under trichothecene-inducing conditions. Toxisomes (red) and Tri12p-containing motile vesicles and vacuoles (green) appear to interact. Solid arrows indicate co-fluorescent punta (yellow) suggesting transient interaction between the toxisome and the motile vesicles. Dashed arrows indicate areas where motile vesicles appear to be interacting with the plasma membrane. Data presented in Menke et al. (2012); video generated for Menke (2011).

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REVIEW
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Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters

Tina Netzker [1,2], Juliane Fischer [1,2], Jakob Weber [1,2], Derek J. Mattern [1,2], Claudia C. König [1,2], Vito Valiante [1], Volker Schroeckh [1] and Axel A. Brakhage [1,2]**

[1] Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Jena, Germany, [2] Department of Microbiology and Molecular Biology, Institute of Microbiology, Friedrich Schiller University Jena, Jena, Germany

Edited by:
Nancy Keller,
University of Wisconsin-Madison,
USA

Reviewed by:
Mikael R. Andersen,
Technical University of Denmark,
Denmark
Christopher L. Schardl,
University of Kentucky, USA

****Correspondence:***
Axel A. Brakhage and
Volker Schroeckh, Department
of Molecular and Applied
Microbiology, Leibniz Institute
for Natural Product Research
and Infection Biology – Hans Knöll
Institute, Adolf-Reichwein-Straße 23,
07745 Jena, Germany
axel.brakhage@hki-jena.de;
volker.schroeckh@hki-jena.de

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Microorganisms form diverse multispecies communities in various ecosystems. The high abundance of fungal and bacterial species in these consortia results in specific communication between the microorganisms. A key role in this communication is played by secondary metabolites (SMs), which are also called natural products. Recently, it was shown that interspecies "talk" between microorganisms represents a physiological trigger to activate silent gene clusters leading to the formation of novel SMs by the involved species. This review focuses on mixed microbial cultivation, mainly between bacteria and fungi, with a special emphasis on the induced formation of fungal SMs in co-cultures. In addition, the role of chromatin remodeling in the induction is examined, and methodical perspectives for the analysis of natural products are presented. As an example for an intermicrobial interaction elucidated at the molecular level, we discuss the specific interaction between the filamentous fungi *Aspergillus nidulans* and *Aspergillus fumigatus* with the soil bacterium *Streptomyces rapamycinicus*, which provides an excellent model system to enlighten molecular concepts behind regulatory mechanisms and will pave the way to a novel avenue of drug discovery through targeted activation of silent SM gene clusters through co-cultivations of microorganisms.

Keywords: co-culture, secondary metabolite gene cluster activation, natural products, intermicrobial communication, posttranslational histone modifications, chromatin, acetyltransferases, mass spectrometry

Introduction

Secondary metabolites (SMs) are low-molecular-mass organic compounds that, unlike primary metabolites, are not directly involved in growth, development or reproduction of the producing organism. Up until 2014 ∼170,000 natural products have been characterized from both marine and terrestrial organisms (Seyedsayamdost and Clardy, 2014; Chapman and Hall, 2015). Microorganisms are able to synthesize a large number of SMs, but the exact number is not known. Furthermore, mining of microbial genomes revealed the presence of numerous SM gene clusters, displaying a discrepancy between the number of putative genes involved in secondary metabolism and the known SMs in a single microbe (Bergmann et al., 2007; Sanchez et al., 2012; Craney et al., 2013). For example, the model fungus *Aspergillus nidulans* is potentially able to produce 32 polyketides, 14 non-ribosomal peptides and two indole alkaloids (Brakhage et al., 2008; Rank et al., 2010), with little more than 50% of the produced SMs being identified. Furthermore, SMs

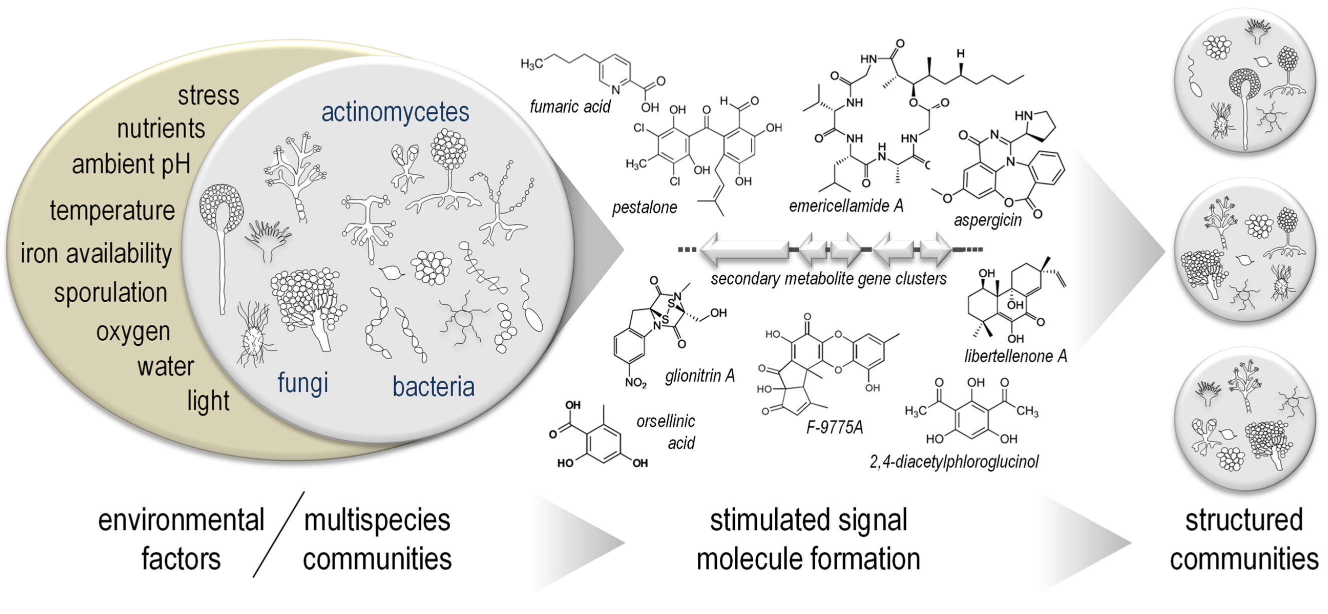

FIGURE 1 | Microorganismic multispecies communities form secondary metabolites that contribute to the stabilization/changes in these companionships. In nature, microorganisms process signals from both abiotic and biotic environments. The latter represent secondary metabolites or natural products whose formation is often suppressed in pure cultures under standard conditions in the laboratory. Microbial mixed cultivation is therefore a suitable means to exploit their potential for natural product discovery and to study the molecular concepts behind the regulatory interactions.

can be found in diverse environments and even chemical biogeographic distribution maps for biomedically valuable families of natural products in the environment have been created (Charlop-Powers et al., 2014). A number of these compounds have important pharmacological applications and are used as antibiotics/antibacterial drugs (Brakhage, 2013). Unfortunately, antibiotic resistance is spreading faster than the development of new antibiotics. As a consequence, there is the need for a constant provision of new compounds for the antibiotic development pipeline (Bbosa et al., 2014; Nathan and Cars, 2014). This is contrasted with a continuous rise in re-isolation of already known natural products (Strand et al., 2014). To manage this conflict, a more targeted natural product search is necessary. This effort directs SM research incrementally to a deeper understanding of the physiological relevance and ecological significance of SMs. It is generally accepted that in nature a substantial benefit to the SM producers must exist, simply arising from the fact that these very energy consuming biosynthetic pathways were maintained through evolution. An early postulated explanation for the role of SMs in nature was its function to defend the habitats of the producers by inhibiting the growth of its competitors (Davies, 1990; Brakhage et al., 2005; Galán et al., 2013). Another more recent hypothesis postulates an association between epibiotic predation and antibiotic production due to widespread predatory abilities in the genus *Streptomyces* (Kumbhar et al., 2014). At low, therefore non-inhibitory concentrations, such molecules are believed to function as signaling molecules (Aminov, 2009; Andersson and Hughes, 2014). This is supported by the assumption that over millions of years the evolution of SMs happened because microorganisms used them as chemical signals for communication between cells of the same species, different species (**Figure 1**) or between host cells, e.g., as endophytes in other microorganisms (Partida-Martinez and Hertweck, 2005) or plants (Brader et al., 2014).

As reported above, the majority of computationally identified SM gene clusters are silent under standard laboratory growth conditions. Successful methods to induce the formation of new metabolites include genetic engineering (Bergmann et al., 2007), mutagenesis, the OSMAC approach (Bode et al., 2002) or treatment with epigenetic modifiers (Cichewicz, 2010; Nützmann et al., 2011; Brakhage, 2013). In contrast to these classical methods, co-cultivation of bacteria–bacteria, fungi–fungi or of bacteria and fungi represent a naturally driven approach mimicking physiological conditions, involving competition and communication. Furthermore, co-cultures are highly relevant for drug research because they allow not only for the identification of new compounds, but can also monitor drug effects on synthetic microbial consortia. Up to now, various co-cultivation strategies have been applied. A summary with the focus on synthetic biology was given by Goers et al. (2014), while successful strategies with a special emphasis on SM formation in co-culture experiments were recently reviewed by Bertrand et al. (2014b).

The regulatory mechanisms of SM biosynthetic gene clusters are poorly understood. Unraveling both production conditions and signal transduction in nature, e.g., by identifying global regulators, will help to understand their function and support new possibilities to further explore SMs. Only a few examples on the gene regulatory network during SM formation in co-cultures have been reported. One is given by the specific interaction between *A. nidulans* and *Streptomyces rapamycinicus*. Thereby, activation of a fungal silent gene cluster and production of novel compounds was mediated by manipulating the chromatin-based regulation in the eukaryotic partner by the bacteria (Schroeckh et al., 2009; Nützmann et al., 2011). This review focuses on the communication between microorganisms, which has led to the activation of silent gene clusters and the formation of (novel) SMs by at least one of the involved species. Of particular focus is the bacteria-triggered activation of silent SM gene clusters in fungi and the role of chromatin remodeling in SM formation. Furthermore, methodical perspectives for the analysis of natural products are also discussed.

Microbial Communication as an Inducer of Silent Secondary Metabolite Gene Clusters

Microbes and their SMs are known as one of the best resources for new drugs (Brakhage, 2013; Luo et al., 2014). Microorganisms form diverse multispecies communities within the natural environment. Here, they are subjected to intra- and interspecies interactions, which may result in beneficial or even harmful outcomes for the species involved. The real triggers leading to the activation of natural product biosynthesis in these communities are as diverse as the products themselves. They range from environmental signals, such as pH, carbon and nitrogen sources, to organisms living in the same habitat (**Figure 1**; Yu and Keller, 2005; Brakhage, 2013). Several recent reviews on mixed microbial cultivation and SMs have been published (Scherlach and Hertweck, 2009; Tarkka et al., 2009; Bertrand et al., 2014b; Marmann et al., 2014; Schroeckh et al., 2014), which clearly support co-cultivations of two (or even more) organisms on solid/liquid cultures as an adequate way to trace new metabolites. Additionally, such cultivations allow a tremendously enhanced production of already known natural compounds.

Starting in 1982, when Watanabe et al. (1982) discovered the formation of the antibacterial polyketide enacyloxin by *Gluconobacter* sp. W-315 during a co-cultivation with the fungi *Neurospora crassa* or *Aspergillus oryzae*, the number of publications dealing with mixed fermentations has drastically increased. The vast majority have been published within the last 5–7 years and nowadays co-cultivation of microbial species has turned into a key method in the discovery of new natural products with certain relevance to pharmaceutical or agricultural applications (Schroeckh et al., 2009; Brakhage, 2013; Moody, 2014).

The typical motivation for co-cultivation experiments is the identification of new bioactive compounds by unlocking cryptic SMs present in the genomes of the microorganisms in use. This has been shown for many microbial combinations, i.e., bacterium-bacterium, bacterium-fungus and fungus-fungus. Examples for SMs produced in fungus-fungus co-cultures are the acremostatins A-C, formed by *Acremonium* sp. in mixed culture with *Mycogone rosea* (Degenkolb et al., 2002), aspergicin, derived from a culture of two *Aspergillus* species (Zhu et al., 2011) or cyclo-(l-leucyl-trans-4-hydroxy-l-prolyl-d-leucyl-trans-4-hydroxy-l-proline), produced in the co-culture broth of two mangrove fungi *Phomopsis* sp. K38 and *Alternaria* sp. E33 (Li et al., 2014). Additionally, screening of fungal co-cultures in solid media led to the identification of an unusual long-distance growth inhibition between *Trichophyton rubrum* and *Bionectria ochroleuca* (Bertrand et al., 2013b). Analytical methods, such as LC-MS-based metabolomics (see below), identified five *de novo* induced compounds, and the structure of one was successfully achieved (4′-hydroxysulfoxy-2,2′-dimethylthielavin P).

Bacterial mixed cultures that led to the synthesis of previously unknown SMs mostly involve gram-positive bacteria, such as streptomycetes, which form the largest genus in the actinomycetes order and represent an unlimited source of novel compounds, including many therapeutic molecules with anti-tumor, anti-cancer, antibiotic, and antifouling properties (Subramani and Aalbersberg, 2012; Doroghazi et al., 2014). As reported, *Streptomyces lividans* TK23 produces a red pigment after the direct interaction with the mycolic acid-containing bacterium *Tsukamurella pulmonis* TP-B0596 (Onaka et al., 2011). In parallel, *T. pulmonis* TP-B0596 is also able to induce natural product synthesis or, at least, to enhance their production in other *Streptomyces* strains. Accordingly, a novel antibiotic named alchivemycin A was isolated from the culture broth of the co-cultivation between *T. pulmonis* and *Streptomyces endus* (Onaka et al., 2011).

Streptomycetes are not only soil microorganisms, but are also widespread throughout marine ecosystems and have been isolated from various seaweed and marine sediments. Co-cultivation of marine streptomycetes was also successfully used to encrypt silent gene clusters. They have also been found to represent a promising source of antifoulants (Xu et al., 2010). Biofouling, the accumulation of microorganisms, algae and plants on wet surfaces, is one of the most serious problems encompassed in various marine industries. The active antifouling diterpene lobocompactol was rapidly induced and isolated from the marine actinomycete *Streptomyces cinnabarinus* (PK209) after co-cultivation with the lobocompactol-resistant bacterium KNS-16 (*Alteromonas sp.*; Cho and Kim, 2012), leading to the isolation of an extremely valuable compound for both marine ecology and industry.

Induction of Fungal Silent Secondary Metabolite Gene Clusters by Co-Cultivation with Bacteria

In nature, interactions between bacteria and fungi are commonly present. Physical contact between these microorganisms can be assumed in various environments, such as soil, food or even patients (e.g., cystic fibrosis) where organisms can live in close contact and compete for different resources (Frey-Klett et al., 2011). Already in 2001, the production of pestalone, a potent antibiotic against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*, was obtained in the co-culture of a marine-derived gram-negative bacterium of the genus *Thalassopia* sp. (CNJ-328) and the marine fungus *Pestalotia* (Cueto et al., 2001). Although fungi-bacteria consortia exist in both terrestrial and marine environment, the majority (>90%) of the currently known microbial natural compounds are derived from terrestrial microorganisms (Subramani and Aalbersberg, 2012). *Streptomyces*, *Pseudomonas*, and *Bacillus* are reported to be the most commonly found bacteria in soil and the rhizosphere (Bouizgarne, 2011) and play the most important role as fungal partners. The gram-positive model organism *Bacillus subtilis* is one of the most frequently found microorganisms in the rhizosphere. It can also induce SM production in fungi, as proved by the formation of macrocarpon C, 2-(carboxymethylamino)benzoic acid and (−)-citreoisocoumarinol in *Fusarium tricinctum* (Ola et al., 2013). Compared to the fungal monoculture, the production of lateropyrone, cyclic depsipeptides of the enniatin type, and fusaristatin A were up to 78-fold higher (Ola et al., 2013). Also marine-derived fungal-bacterial communities have been found to be a promising origin of novel SMs (Marmann et al., 2014). Oh et al. (2007) observed that co-cultivation of a marine fungus identified as *Emericella parvathecia* and the actinomycete *Salinispora arenicola* led to a 100-fold production of emericellamides A and B by the fungus. Both metabolites showed a slightly increased activity against MRSA. *Emericella* is the teleomorph (sexual form) of many *Aspergillus* spp. (Geiser, 2009). In fact, the emericellamide biosynthetic gene cluster, which contains a PKS and a NRPS, was also described in the model organism *A. nidulans* (Chiang et al., 2008). Co-cultivation of the marine α-proteobacterium *Thalassopia* sp. (CNJ-328) and the fungus *Libertella* sp. led to the production of libertellenones A-D by the fungus. A direct physical contact appears to be important for libertellone production, as the diterpenoids were neither produced in a *Libertella* monoculture nor by adding supernatant or extract of the bacterial culture (Oh et al., 2005). Libertellenones showed an increased cytotoxic activity against human adenocarcinoma cell line (HCT-116), but no antibiotic properties.

Aspergillus fumigatus, the most common airborne fungal pathogen, has been reported to produce at least 226 potentially bioactive SMs (Frisvad et al., 2009) including well studied metabolites like gliotoxins, pseurotins, and fumagillins. Again, most of the biosynthetic gene clusters are silent under laboratory conditions. Zuck et al. (2011) co-cultured *A. fumigatus* with *Streptomyces peucetius* which led to the formation of formyl xanthocillin analogs, named fumiformamide, and *N,N′*-((1Z,3Z)-1,4-bis(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide. *A. fumigatus* co-cultured with *Streptomyces bullii* produced ergosterol and numerous new metabolites, including seven metabolites of the diketopiperazine alkaloids, brevianamide F, spirotryprostatin A, 6-methoxy spirotryprostatin B, fumitremorgin C and its 12,13-dihydroxy derivative, fumitremorgin B as well as verruculogen, 11-*O*-methylpseurotin A and a new isomer 11-*O*-methylpseurotin A_2 (Rateb et al., 2013). *A. fumigatus* is also part of a microbial interaction in another unusual habitat—coal mine drainages where such interactions may be helpful for survival. Co-cultures of two coalmine drainage-derived organisms, a *Sphingomonas* strain and an *A. fumigatus* strain led to the detection of glionitrin A, which is a new diketopiperazine (Park et al., 2009). Glionitrin A shows significant antibiotic activity against both MRSA as well as increased cytotoxic activity against four human cancer cell lines. Further potential microbial interactions were revealed in the genus *Fusarium*, which are also filamentous fungi widely distributed in the soil. Analysis of the interaction between *Fusarium pallidoroseum* and *Saccharopolyspora erythraea* resulted in three new decalin-type tetramic acid analogs related to equisetin (Whitt et al., 2014).

Functional Analysis of Microbial Communication

The various examples presented above illustrate that mixed microbial fermentations are an emerging field in microbiology. They can be seen as a strategy to mimic the physiological conditions in the different microbial consortia. The better understanding of the native bacterial-fungal interactions will not only expand our possibilities to identify interesting new SMs (e.g., lead structures), but also affect our knowledge on how these consortia are structured by the signals derived from the involved species. In a recent study it was shown how SMs contribute to the structure of microbial communities (Donia et al., 2014). The biosynthetic capacity of the human microbiome was explored by systematic analysis of its biosynthetic gene clusters and identified the thiopeptide lactocillin, which is produced by the vaginal commensal *Lactobacillus gasseri*. Interestingly, lactocillin is active against several pathogens like *S. aureus* and *Corynebacterium aurimucosum*, but inactive against commensals thus influencing the microbial composition of this specific habitat. Metatranscriptomic data analysis revealed that the corresponding thiopeptide biosynthetic gene cluster is indeed expressed *in vivo* in human samples (Donia et al., 2014). Something similar was shown in other kingdoms. In another example the effect of SMs produced by endophytic fungi on the cohabitating host plant was shown to provide benefits to the host. In mixed microbial cultures the endophytic fungus, *Alternaria tenuissima*, significantly increased the production of several polyketides, including the antifungal stemphyperylenol, which is active against another endophytic fungus, *Nigrospora sphaerica*, a well-known leaf pathogen.

True symbioses between microorganisms have even shown a fruitful source for new SMs. A very special kind of interaction between a fungus and a bacterium is that of the zygomycete *Rhizopus microsporus* harboring endosymbiotic bacteria of the species *Burkholderia rhizoxinica*, a novel species discovered by Partida-Martinez et al. (2007a). Together with its symbiont the fungus is an important plant pathogen causing rice seedling blight. For more than two decades, it was thought that the fungus produces the causal agent for the plant disease. As shown, the endosymbiont is the actual producer of the phytotoxin, rhizoxin, that binds to the β-tubulin of the rice plant cells and causes mitotic arrest (Partida-Martinez and Hertweck, 2005, 2007; Partida-Martinez et al., 2007b). This, in turn induces the typical symptoms of swelling of the seedling tips and finally resulting in the death of the plants' offspring (Scherlach et al., 2006). Additionally, it has also been shown that the endobacterium is obligatory for sporulation of its host fungus (Partida-Martinez et al., 2007b). Elucidation of the underlying molecular mechanisms of this interaction (Leone et al., 2010) led to the discovery of "self" resistance mechanisms of the fungus against the mycotoxin (Schmitt et al., 2008) and of factors essential for symbiosis (Leone et al., 2010; Lackner et al., 2011). Recent data revealed that a type 2 secretion system (T2SS) is also required for the formation of the endosymbiosis between the fungus and the endobacterium (Moebius et al., 2014). By use of comparative proteome analysis, it was shown that chitinolytic enzymes and chitin-binding proteins were released by the secretion system of the bacterium. Further experiments (e.g., targeted gene-knock-outs, sporulation assays) clearly showed that a chitinase is essential for the bacteria to enter the hyphae (Moebius et al., 2014). More recently, the biosynthesis of antifungal and antibacterial polyketides by *Burkholderia gladioli* in co-culture with *R. microsporus* has been investigated (Ross et al., 2014). Conditions emulating tempe bongkrek production, a type of fermented soybeans made with the addition of coconut, resulted in the formation of novel members of the enacyloxin family of antibiotics and to enhanced production of the toxin, bongkrekic acid, by the tempe contaminant *B. gladioli*.

Overall, the underlying mechanisms of SM biosynthetic gene cluster regulation are emerging, but are still poorly understood. Only few studies reported the gene regulation mechanisms involved in SM formation during microorganism interaction. One of them is the antibiotic concanamycin A production by *Streptomyces halstedii*. Concanamycin A alters the proteomic profile of *A. nidulans* and probably plays an active role in defense-related pathways (Melin et al., 2002). Another example, which will be extensively described below, is the specific interaction between *A. nidulans* and *S. rapamycinicus*. During this mutual interplay, the activation of silent gene clusters, and subsequent production of novel compounds, is transduced by affecting the chromatin-based regulation in the eukaryotic partner (Schroeckh et al., 2009; Nützmann et al., 2011).

The Interaction of *Aspergillus* with *Streptomyces rapamycinicus*

It was discovered that the intimate physical contact of *A. nidulans* with a distinct soil-dwelling bacterium, *S. rapamycinicus*, identified from a collection of 58 species of actinomycetes, led to the selective activation of silent PKS and NRPS gene clusters in the fungus (Schroeckh et al., 2009). One induced cryptic PKS gene encodes the long sought-after orsellinic acid synthase, thus the corresponding cluster was named the *ors* gene cluster. In addition to this archetypal polyketide orsellinic acid, three derivatives (lecanoric acid and two cathepsin K inhibitors F-9775A and F-9775B) were produced by *A. nidulans*. Lecanoric acid is a typical lichen metabolite that is usually found in a fungal/bacterial mutualism (Stocker-Worgotter, 2008), and thus likely plays a role in microbial communication. Indeed, the inducing bacterium was not affected by lecanoric acid. As mentioned above, a physical contact between both partners is needed for the activation of this silent gene cluster (Scherlach and Hertweck, 2009; Schroeckh et al., 2009). It is conceivable that a symbiotic relation between the fungus and the bacterium exists to defend against other microorganisms. One explanation on how the bacterium can trigger SM formation in *Aspergillus* would have been that rapamycin produced by the streptomycete could activate the cluster, either *via* the inhibition of the TOR pathway (Fitzgibbon et al., 2005) or due to its more general antifungal activity. Alternatively, another bacterial metabolite, the fungistatic antibiotic trichostatin A (TSA), which is produced by *Streptomyces hygroscopicus* could be responsible *via* its respective histone deacetylase (HDAC) inhibiting activity (Tsuji et al., 1976; Kouraklis and Theocharis, 2002). However, neither the addition of rapamycin nor TSA led to the activation of the *ors* gene cluster, therefore making both compounds unlikely to play a role in the interaction. When *S. rapamycinicus* was co-cultivated with *A. fumigatus*, this fungus also displayed an altered SM profile showing a group of similar new SMs (König et al., 2013). In a microarray approach, a SM gene cluster was identified that was up-regulated only in the co-culture. Deletion of the PKS of the identified cluster, correlated with the lack of the corresponding natural products. Two metabolites of this group were isolated and named fumicycline A and B and the corresponding PKS was designated as FccA. It was shown that again a direct physical contact was necessary to induce the *fcc* gene cluster. An ortholog of the *fcc* gene cluster was identified in *Neosartorya fischeri* (Chooi et al., 2013). Overexpression of the transcription factor gene of the cluster led to the production of neosartoricins. These metabolites demonstrate high similarity to fumicyclines and showed T-cell antiproliferative activity, suggesting a physiological role as an immunosuppressive agent (Chooi et al., 2013).

Studies of various chemical inhibitors led to the striking finding that the interaction between *S. rapamycinicus* and *A. nidulans* relies largely on the activity of chromatin remodelers. Supplementation of the co-culture with a TSA-like HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), and with the histone acetylase (HAT) inhibitor anacardic acid led to the activation and inhibition of the transcription of the *ors* gene cluster, respectively. These findings indicated that chromatin remodeling can play an essential role in the regulation of SM clusters and that the targeted activation or inactivation of the respective chromatin modifiers can alter the SM production of the fungus. Nützmann et al. (2011) demonstrated that acetylation plays an essential role for mediating the interaction. Therefore, a comprehensive deletion library of all putative HATs in *A. nidulans* was generated and systematically screened for the ability of mutants to activate the *ors* gene cluster during co-incubation with *S. rapamycinicus* (Nützmann et al., 2011). Thereby, the HAT GcnE was identified as being essential for the cluster induction. This HAT is the catalytic subunit of the Saga/Ada complex (see **Figure 2**), a conserved multi-subunit complex also found in other eukaryotic organisms (Baker and Grant, 2007; Govind et al., 2007). Furthermore, it was shown that the acetylation of histone H3 lysines 9 and 14 is needed for the onset of the *ors* gene cluster transcription and product formation (Nützmann et al., 2013). However, SAGA not only seemed to play a role during the interaction with *S. rapamycinicus*, but also for the regulation of other well-known natural products such as penicillin, sterigmatocystin and terrequinone A in *A. nidulans* (Nützmann et al., 2011, 2013). Due to the intimate contact of *S. rapamycinicus* with *A. nidulans* the question arose whether there is a common mechanism by which the bacterium might interact with other members of the *Aspergillus* family, e.g., with *A. fumigatus*. It is possible to postulate different ways that can lead to the activation of such clusters in the fungus. As shown in **Figure 2**, an unknown, possibly membrane-bound compound or a protein can modulate the Saga/Ada complex directly (**Figures 2**a,b). Alternatively, the signal could be induced either by the physical contact between the two organisms (**Figure 2**c), or by a protein or compound secreted by the bacterium, and specifically sensed by receptors of *A. nidulans* (**Figure 2**d). These components need to be specific for

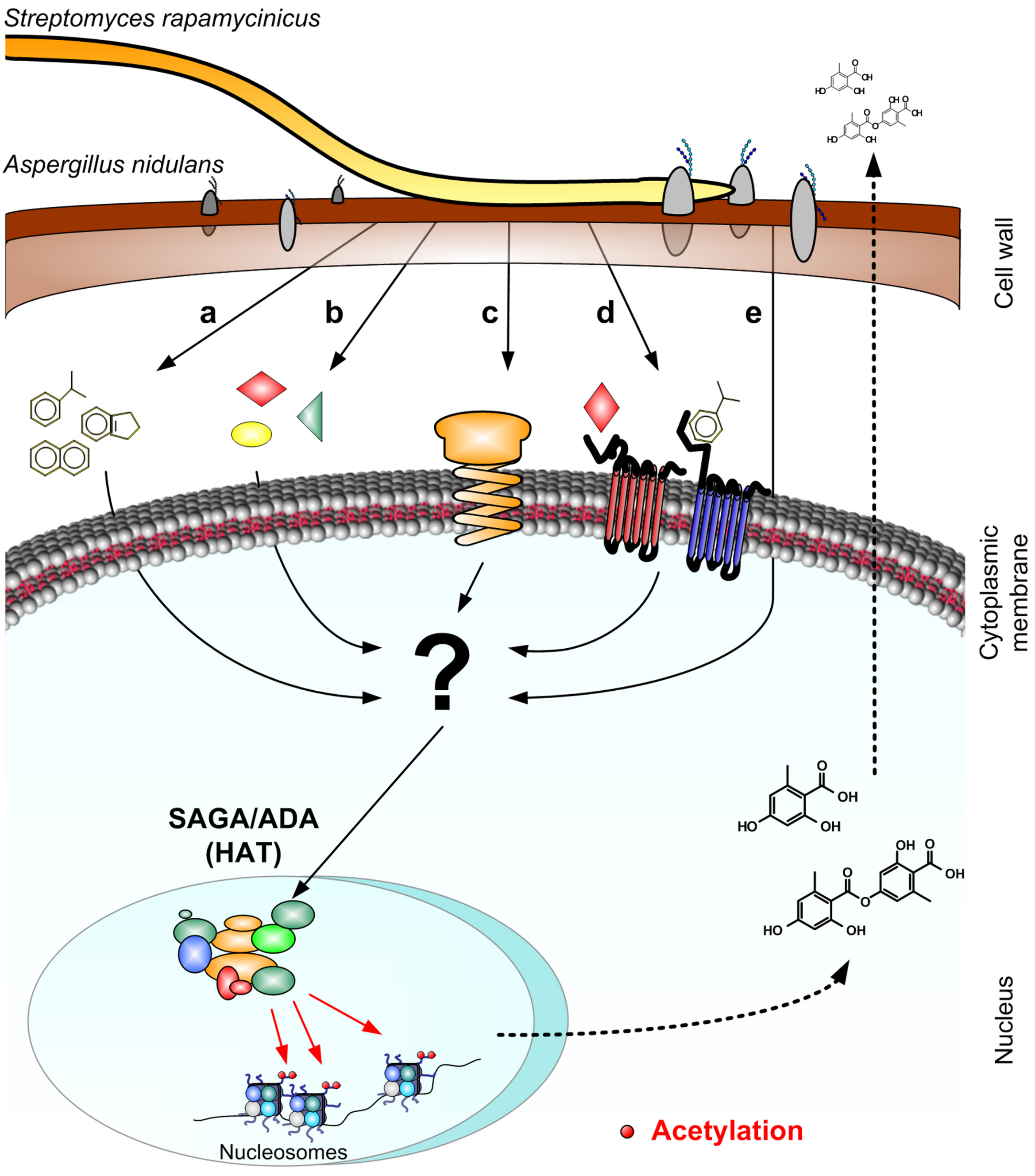

FIGURE 2 | Model of the interaction between *A. nidulans* and *S. rapamycinicus*. The figure presents hypotheses about different stimuli that could be responsible for the activation of SM gene clusters during the interaction between *A. nidulans* and *S. rapamycinicus*. The signal that finally results in the Saga/Ada complex activation could derive from at least five possible events: possibly membrane-bound compounds (a), or peptides (b), could reach the cytosol of the fungus and indirectly activate the Saga/Ada complex. Alternatively, a specific fungal receptors could recognize either the attachment of the bacterium (c), or compounds secreted during the interaction (d). A further hypothesis could be that fungal surface proteins could recognize the streptomycete directly triggering a signaling cascade (e). The internal signal that should lead to the activation of the Saga/Ada complex is unknown.

S. rapamycinicus and must not be found in other actinomycetes. Convincible is also that the recognition of a fungal surface protein by the streptomycete, could directly lead to the activation of a signaling cascade triggering the SAGA complex (**Figure 2**e). In the interaction of *A. nidulans* with *S. rapamycinicus* the *ors* gene cluster regulation relies largely on the activity of GcnE and its acetylation activity of lysine 9 and 14 at histone H3. This in turn is induced upon physical contact with the bacterium leaving room for speculation on the key influence of the streptomycete on the fungus. Regarding the signaling pathway behind this interaction, it is known that LaeA as a global SM regulator has no influence on GcnE and therefore on histone H3 acetylation (Nützmann et al., 2011). This means that there must be an alternative pathway and a transcriptional regulator responsible for the recruitment of the HAT and the multi-subunit complex, SAGA, to the respective loci.

For some clusters, such as penicillin, it has already been shown that some general regulators are required like the major pH regulator PacC, which activates penicillin biosynthesis at alkaline pH in *A. nidulans*, or the CCAAT binding complex (Tilburn et al., 1995; Litzka et al., 1998; Then Bergh and Brakhage, 1998). For the *ors* gene cluster, however, the key regulators still remain to be discovered.

Modulation of Gene Expression by Interaction Partner Induces Chromatin Remodeling

The impact of chromatin remodeling on the communication between organisms or the control of host gene expression has gained attention in the last years. There are several examples of bacterial pathogens interfering with the host regulatory system of gene expression. Fewer are known about the regulatory mechanisms of interactions involving fungi, especially when it comes to those leading to the expression of SM gene clusters. However, some light has been shed on the impact of chromatin remodeling on natural product regulation in fungi. By now, a variety of chromatin modifiers have been discovered, which regulate SM biosynthesis in filamentous fungi (Gacek and Strauss, 2012). Most knowledge so far has been gained on acetyltransferases (HATs), which are grouped in diverse families, comprising amongst others the MYST-family, p300/CBP-family, and the Gcn5-related acetyltransferases (GNATs) (Carrozza et al., 2003). The latter includes Gcn5, the catalytic subunit of the SAGA complex, also referred to as GcnE in *Aspergillus* species. As mentioned before, a distinct deletion mutant of the knock-out library of HATs in *A. nidulans* led to an altered SM biosynthesis pattern. Hence, it was speculated that the systematic screening of the deletion library allows for the identification of novel metabolites. Indeed, a drastically altered metabolic profile was detected in the Δ*nnaB* (nidulans *N*-acetyltransferase B) deletion mutant. Aside from a number of orsellinic acid derivatives, there was also a new class of compounds identified as pheofungins, which are heterocyclic molecules with cytotoxic activity (Scherlach et al., 2011). HAT modification led the production of SMs also in other fungi. The aflatoxin biosynthetic gene cluster in *Aspergillus parasiticus* was also shown to be co-regulated by a MYST-type family member of the HATs. Furthermore, the transcription of the aflatoxin cluster genes coincides with the acetylation of histone H4, showing that a HAT is involved in the regulation of this cluster (Roze et al., 2007, 2011). Soukup et al. (2012) obtained similar results by overexpressing *esaA*, a gene encoded for a MYST-type HAT. The overexpression of this gene affected the production of penicillin, sterigmatocystin, terrequinone A, and the *ors* gene clusters in *A. nidulans* (Soukup et al., 2012).

The example of *S. rapamycinicus*, which mediates cluster regulation in *A. nidulans via* an increased histone acetylation upon contact, shows how bacteria can interfere with the eukaryotic histone modification system (epigenetic regulation). Other bacteria were also reported to have a similar impact on eukaryotic cells. *Listeria monocytogenes*, the producer of the toxin listeriolysin O, is a bacterium causing foodborne infections leading to sepsis, miscarriages during pregnancy, and meningitis, and is largely found in immunocompromised patients. Hamon et al. (2007) were able to show that listeriolysin O caused a dramatic modulation of the host gene expression. This was caused by deacetylation of histone H4 but also drastic dephosphorylation of serine 10 on H3 thus leading to a downregulation of substantial immunity factors in the host cells. Similar observations have been made for *Clostridium perfringens* and *Streptococcus pneumoniae*, the producers of perfringolysin and pneumolysin, respectively (Maekita et al., 2006; Hamon et al., 2007). Both toxins also led to dephosphorylation of the host's chromatin. Thus, different toxins secreted by bacteria appear to manipulate and control chromatin remodeling and thereby transcription of targeted genes of eukaryotic hosts.

The so-called erasers of acetylation are the HDACs that fulfill the opposing reaction of the HATs by removing the acetyl group from lysine residues of the histone proteins. HdaA, a class II HDAC, was one of the first discovered to play a role in SM cluster regulation. The deletion of *hdaA* in *A. nidulans* not only led to reduced growth of the fungus during oxidative stress, but also resulted in a higher production of SMs such as penicillin, sterigmatocystin, and terrequinone A (Tribus et al., 2005; Shwab et al., 2007). Consistently, HdaA had a significant impact on SM produced in *A. fumigatus*, such as fumitremorgin B, pseurotin, and gliotoxin. Interestingly, however, was the finding that gliotoxin production was down-regulated upon deletion of *hdaA* in *A. fumigatus* (Shwab et al., 2007; Lee et al., 2009). Garcia-Garcia et al. (2009) were able to connect the activity of the human HDAC1 with the infection process of *Anaplasma phagocytophilum* in THP-1 cells (granulocyte model). Hereafter, the infection process led to an increased activation of HDAC1 leading to a reduced histone H3 acetylation and to the silencing of host defense genes. In accordance, the inhibition of HDAC1 by siRNA led to a significant drop of the bacterial load. This shows that the epigenetic control of the host cell by the bacterium promotes the disease by increased survival of the pathogen (Garcia-Garcia et al., 2009).

Besides the widely studied acetylation of histones, there is a multitude of other chromatin modifications which have been found to regulate expression of SM gene clusters (Strahl and Allis, 2000). Methylation of lysine is regarded as one of the most complex modifications found so far with diverse impacts on gene transcription depending on its conformation (Rolando et al., 2013). Reyes-Dominguez et al. (2010) showed that upon growth arrest the methylation of lysine 9 was subsequently reduced, but affected only genes located inside the sterigmatocystin cluster, leading to its activation. Furthermore, H3 K9 methylation marks were associated with heterochromatin protein A (HepA), a protein responsible for heterochromatin formation. Consistently, the deletion of HepA led to the activation of the *stc* gene cluster (Reyes-Dominguez et al., 2010; Brakhage, 2013). However, the combination of both the *hepA* and the *laeA* deletions reduced the sterigmatocystin production to wild-type levels (Shaaban et al., 2010). The global SM regulator LaeA was indirectly found to be involved in histone methylation by influencing the methylation of H3 K9 and the occupancy of the respective locus by HepA. The deletion of this gene was also found to constrain the expression of the prominent natural product gene clusters penicillin, sterigmatocystin and the cholesterol lowering agent lovastatin (Bok and Keller, 2004; Reyes-Dominguez et al., 2010). In another study, the heterochromatin protein, HP1 of the fungus *Leptosphaeria maculans*, could be implicated in the pathogenicity process responsible for plant infection. The fungus harbors effector genes with low expression during axenic cultivation, while being highly transcribed upon co-cultivation with plants. In an infection model with *Brassica napus* the effect of histone H3K9me3 on the respective effector genes was investigated. HP1 as well as the DNA-methylase DIM-5 were silenced by RNAi and analyzed concerning the transcription level of the effector genes in axenic cultures. Interestingly, the effector genes were actively transcribed in the mutant strains outside of the co-cultivation leading to the conclusion that HP1 as well as DIM-5 must be somehow involved in the repression of the effector genes during the non-infective life cycle (Soyer et al., 2014). Additionally, in a symbiotic interaction of the endophyte *Epichloë festucae* and *Lolium perenne*, the fungus produced ergot alkaloids and lolitrems when cohabitating with a plant. Production of these bioprotective substances was repressed during axenic cultures. Comparing levels of H3K9me3 and H3K27me3 between co-cultivation and non-symbiotic cultivation of the fungus, the methylation marks were reduced upon growth in the plant. Furthermore, the deletion of the responsible methyltransferases ClrD and EzhB resulted in an activation of the alkaloid and lolitrem gene clusters also in the axenic cultures of the fungus (Chujo and Scott, 2014).

Methylation of lysines 4 at histone H3 by CclA was found to be important for SM biosynthesis as well as for conidiation in *A. nidulans* and *A. fumigatus* (Palmer et al., 2008, 2013; Bok et al., 2009). The deletion of the respective genetic locus in *A. nidulans* not only led to the production of F-9775A and F-9775B, which are also produced upon contact with the bacterium *S. rapamycinicus*, but led also to the activation of a novel monodictyphenon gene cluster. Another very interesting study was published by Rolando et al. (2013). They showed that pathogens can also introduce, so far, unknown modifications on host nucleosomes and thereby influence gene expression. In their study they elegantly revealed that *Legionella pneumophila* is able to tri-methylate lysine 14 on histone H3 of its host by a factor called RomA. This protein is a SET-domain containing methyltransferase which is secreted by *Legionella* during the infection process. Genome-wide ChIP analysis showed that approximately 4870 promoters were target of this modification by RomA (Rolando et al., 2013). The cause for this drastic modulation of the host genome by *Legionella* is not fully understood yet. One possible explanation might be that the switch to a methylated histone leads to down-regulation of the target genes due to a mutual exclusion of the acetylated lysine 14, which was found to coincide with actively transcribed genes (Cheung et al., 2000).

In summary, the impact of post-translational regulation on SM cluster expression and the interaction of organisms have revealed their great potential for future studies in natural product research. Prokaryotes are able to modify their host's gene expression in multiple ways. Bacterial toxins were shown to be useful tools during the infection process by reducing levels of acetylation and phosphorylation of histones and thereby influencing the expression of their target genes. Often, chromatin modifying complexes are mediators of those interactions, specifically targeting host defense genes and modulating their expression. Interestingly, this is not only achieved by host derived remodelers but also by proteins introduced by the interaction partner itself, which in turn can lead to unknown modification on the host genome. Taken together, recent studies have shown the great potential of bacteria and fungi to modulate gene expression of organisms during co-cultivation experiments. Seeing this, it is convincible that the investigation at the molecular basis of multispecies interaction has great potential. The more we understand about communication between species, the better we can trigger the discovering of unknown natural products in microorganisms.

Perspectives for the Analysis of Natural Products

In the search for SMs in co-cultivations, one must also determine which analytical method to use for the detection of these compounds. This topic has already been extensively reviewed by other groups (Scherlach and Hertweck, 2009; Tarkka et al., 2009; Bertrand et al., 2014b; Marmann et al., 2014; Schroeckh et al., 2014), but the most interesting new studies will be covered here. Thus far, the methods for natural product analysis have ranged from simply the extraction of co-cultures in liquid/solid media to the use of quite novel techniques such as imaging/real-time mass spectrometry that can be carried out in solid-state cultures. The former technique has shown to be of value resulting in the discovery of many new SMs or in the study of the regulation of different products, which cannot be found in monocultures. This technique commonly entails the extraction of the natural products from the culture broth, which are then subjected to a form of liquid chromatography-mass spectrometry (LC-MS). In a further step, potential new SMs can be purified and isolated for structural elucidation by nuclear magnetic resonance (NMR) spectroscopy (**Figure 3**). This workflow was applied to the discovery of new SMs from the co-cultivation of *S. rapamycinicus* with *A. nidulans* and *A. fumigatus*, respectively. In both cases new fungal products were detected by LC-MS when the streptomycete was added (see above). Other examples for LC-MS detection of co-culture-derived products are the new antibiotic alchivemycin A in *S. endus* by the mycolic acid-containing bacteria *T. pulmonis* (Onaka et al., 2011; see above), as well as the co-culture of *Streptomyces coelicolor* with myxobacterium *Corallococcus coralloides*, where the streptomycete increased the production of the biologically active compound undecylprodigiosin 60-fold (Schäberle et al., 2014). Because of its potential for the discovery of new natural products, there is also a need for high-throughput methods to encompass large-scale co-cultivations. This question was addressed for the study of more than 600 different fungal strains. With the help of automated data analysis, new molecular masses were observed which were not found in natural product databases (Bertrand et al., 2013a). In a further example of high-throughput screening,

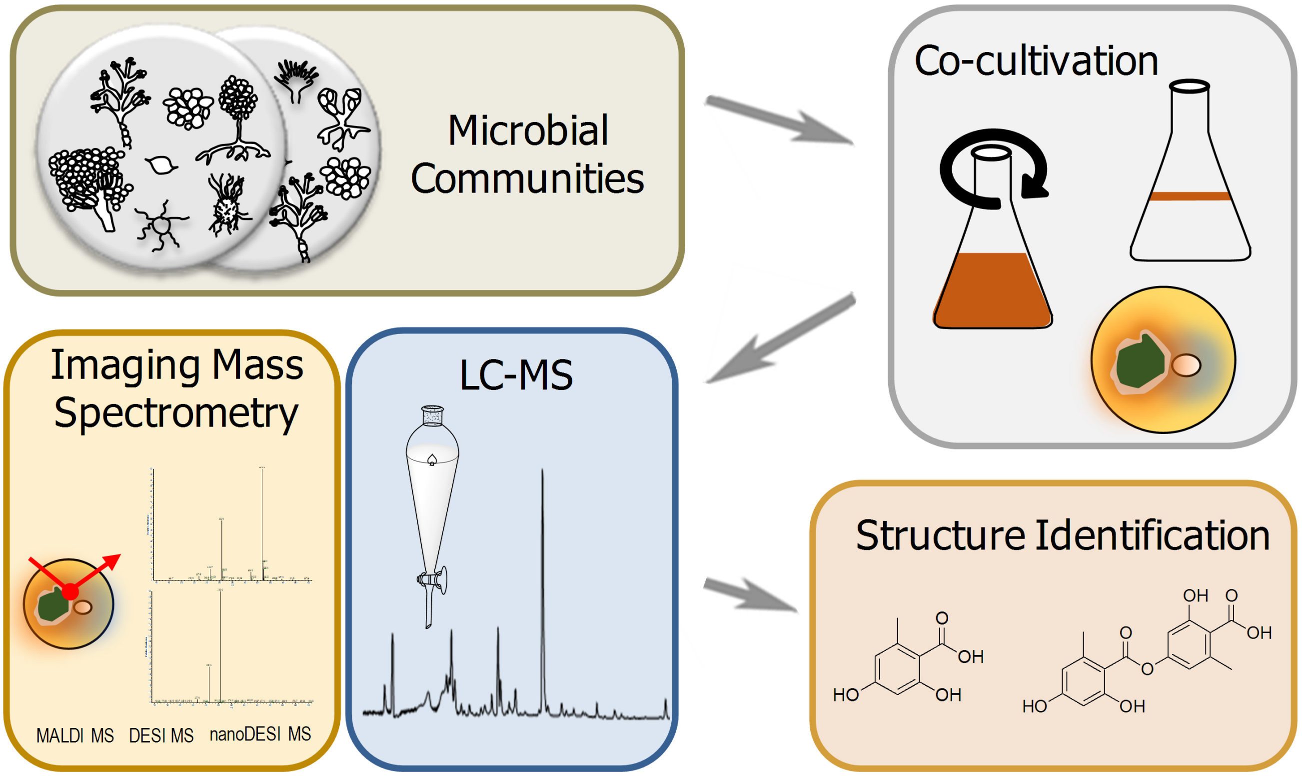

FIGURE 3 | Proceeding of natural product analysis from microbial co-cultivations. Microbial communities are co-cultured in flasks (static or planktonic) or on plates (solid-state culture). Primary analysis of natural products via extraction and LC–MS methods or directly evaluated by imaging mass spectrometry based methods. Subsequent structure identification of unknown masses can be conducted using NMR techniques.

fungal co-cultures were cultivated with very small culture volumes. A big advantage of small culture volumes is that sample preparation can be completed in less time and the number of different cultures can be increased (Bertrand et al., 2014a). These are just few examples to show that LC-MS analysis of co-cultivations can be a very practical tool, and because of the constant problem to obtain enough product for structure elucidation, scale-up of these cultures can usually be accomplished.

Besides the well-established exploration of co-cultures for new bioactive compounds by LC–MS, there has also been advancements in the field of imaging and real-time mass spectrometry (Fang and Dorrestein, 2014), where metabolites can be detected by their spatial distribution. Imaging mass spectrometry (IMS) has, for the most part, been associated with matrix-assisted laser desorption/ionization (MALDI), which is then coupled to a mass spectrometer, for which images can be produced depicting the spatial organization of natural products (Cornett et al., 2007; Esquenazi et al., 2009; Watrous and Dorrestein, 2011; Bouslimani et al., 2014; Shih et al., 2014). This technique has shown to be useful in observing the role of natural products in the interaction between different microorganisms, such as *A. fumigatus* with *Pseudomonas aeruginosa* (Moree et al., 2012), *B. subtilis* with *S. coelicolor* (Yang et al., 2009), *B. subtilis* with *S. aureus* (Gonzalez et al., 2011), and the cannibalism of *B. subtilis* (Liu et al., 2010), just to name a few. Additionally, two promising examples, which aided in the discovery of novel natural products by IMS will be discussed. The first demonstrated that the infection of the button mushroom, *Agaricus bisporus*, with the soft rot-causing bacterium *Janthinobacterium agaricidamnosum* and the brown blotch disease-causing bacterium *Pseudomonas tolaasii*. *J. agaricidamnosum* infected mushrooms revealed the presence of a novel virulence factor, jagaricin when analyzed by MALDI-MS at the sites of infection. This substance was shown to play an important role in soft rot of mushrooms and also appeared to be a potent antifungal (Graupner et al., 2012). The second example, also using IMS, investigated the infection of *A. bisporus* with *P. tolaasii* and could show that the tolasin metabolites, which were observed at the site of infection, are responsible for this disease (Scherlach et al., 2013).

Similarly, a more recently developed technique, real-time mass spectrometry, encompassing the techniques of desorption electrospray ionization (DESI) or nanospray desorption electrospray ionization (nanoDESI), is also a sufficient option in detecting natural products in co-cultivations. An advantage of this method compared to MALDI, is that it does not depend on the formation of the matrix and also has little to no sample preparation. Measurements can be taken directly from the plate and can also be used for IMS. Moreover, the method is usually termed ambient mass spectrometry because ionization takes place at atmospheric conditions and room temperature. For further information the following reviews are recommended (Bouslimani et al., 2014; Fang and Dorrestein, 2014; Hsu and Dorrestein, 2015). Furthermore, Watrous et al. (2013) have also shown a methods paper using nanoDESI IMS with little sample preparation of different microbial monocultures and co-cultures directly from a Petri dish. nanoDESI IMS has even led to the discovery of new desferrioxamine derivatives in co-cultures of *S. coelicolor* with other actinomycetes (Traxler et al., 2013). The use of IMS for the detection of natural products is an ever-evolving field and new techniques are constantly being discovered and older techniques optimized. One recent ionization method, direct analysis in real time mass spectrometry (DART-MS), could also be used in studying the role of SMs in co-culture (Gross, 2014).

Conclusion

Secondary metabolites are of major interest due to their applicability as therapeutic agents. To satisfy the constant need for new SMs, and to come up against the continuous emerging of bacterial resistant strains, it would be advantageous to understand the SMs' physiological relevance and their ecological significance. In this context, mixed microbial cultivations have become a powerful method to induce previously unexpressed biosynthetic pathways, leading to the production and identification of new SMs (Schroeckh et al., 2009; Bertrand et al., 2014b; Marmann et al., 2014). A greater understanding of the underlying molecular mechanisms driving microbial co-cultivations would be important for deriving general mechanisms. This knowledge could be used specifically to induce silent SM biosynthesis gene cluster in laboratory conditions. However, a deeper understanding of the SM biosynthetic gene cluster regulation alone will not be sufficient. Due to the often very tiny amounts of SMs produced by the microorganisms, the development of analytic tools is getting more and more important. At the same time, multidisciplinary collaborations are necessary ensuring a careful analysis and validation process of the collected data from any MS method in terms of dereplication (Hufsky et al., 2014). Therefore, data collection and processing could be applied in global libraries, as seen for genome and transcriptome data, and used to help the scientific community in the constant race between the discovery of new antibiotics and the continuous emergence of resistance mechanisms.

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Mycotoxins are a component of *Fusarium graminearum* stress-response system

Nadia Ponts *

UR1264 - MycSA, Institut National de la Recherche Agronomique, Centre de Bordeaux-Aquitaine, Villenave d'Ornon, France

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Edited by:
Jonathan Palmer,
US Forest Service, USA

Reviewed by:
JinRong Xu,
Purdue University, USA
Kathryn Bushley,
University of Minnesota, USA

****Correspondence:***
Nadia Ponts
nadia.ponts@bordeaux.inra.fr

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SECONDARY METABOLITES IN THE CONTEXT OF PHYTOPATHOGENIC FUNGAL LIFE

Fusarium graminearum is a phytopathogenic Ascomycota that can cause Fusarium head blight in wheat and other cereals worldwide, leading to important yield losses as well as reduced grain quality. The analysis of the *F. graminearum* genome sequence revealed the presence of 20 non-ribosomal peptide synthases, 15 polyketide synthases, and 17 terpenoid synthases, all potentially involved in the production of a panel of secondary metabolites, including yet unknown ones, such as the mycotoxins deoxynivalenol (DON) and other type B trichothecenes (Cuomo et al., 2007). The toxicity of DON to humans and animals upon ingestion has been extensively illustrated (see Bonnet et al., 2012; Awad et al., 2013; Pinton and Oswald, 2014 for reviews), and the presence of this mycotoxin in cereal-derived food and feeds represents a serious threat for public health (reviewed in Marin et al., 2013; Sirot et al., 2013; Wu et al., 2014).

Secondary metabolites are a structurally diverse family of compounds that are often qualified as unessential for short-term development but important for long-term survival (see Roze et al., 2011a for a review). Secondary metabolism is tightly linked to primary metabolism,starting with the fact that primary metabolites "feed" secondary metabolite biosynthetic pathways (reviewed in Audenaert et al., 2014; Sheridan et al., 2015). For example, the biosynthesis of type B trichothecenes by *F. graminearum* derives from the isoprenoid pathway, an essential metabolic pathway involved in various cellular processes. Remarkably, the genes involved in the biosynthesis of isoprenoids are positively regulated by the transcription factor Tri6, which also regulates the genes involved in the trichothecenes biosynthetic pathway (Seong et al., 2009). Moreover, Menke et al. (2013) proposed that type B trichothecenes are produced in specific cellular vesicles, so-called "*toxisomes,*" and found pieces of evidence that the HMG-CoA reductase of the isoprenoid pathway also localizes to toxisomes when the production of toxins is induced (Menke et al., 2013). Whether these toxisomes are neo-formed for toxin biosynthesis or derive from vesicles hosting elements of the primary metabolism remains to be investigated. Nonetheless, the production of mycotoxins by *F. graminearum* could be the result of a tight coordination with primary metabolism.

Fungi are known to produce diverse families of secondary metabolites, biological functions of which are not yet fully understood. In the context of host-pathogen interactions, DON was shown to be a virulence factor for *F. graminearum* infecting wheat by promoting the spreading of the pathogen (Jansen et al., 2005). During infection and colonization, *F. graminearum* is particularly exposed to plant metabolites that can be constitutive components of the host or molecules produced as a consequence of the presence of the pathogen. Some of these metabolites can trigger fungal stress-response pathways. There are accumulated evidences showing that the production of fungal secondary metabolites, DON in particular, could be an element of the general stress response in *F. graminearum*.

SECONDARY METABOLITES ARE PARTS OF STRESS RESPONSE PATHWAYS

Stresses caused by variations of the environment can be biotic (the surrounding microbiome) or abiotic (*e.g.*, heat, pH, light, *etc.*), and both types can lead to either adaptation and survival, or cell death. Here, cell death does not necessary mean a failure to implement an effective stress response but can be the result of the response pathway itself when a destructive response is implemented. The effects of various stresses that phytopathogenic fungi are likely to encounter in the context of host invasion have been the subjects of numerous works. In particular, oxidative stress, as the result of the early defensive "oxidative burst" triggered in the host plant upon infection, has been intensively examined.

Oxidative Stress

Oxidative stress has been extensively studied regarding its interaction with secondary metabolism in fungi. Secondary metabolites can counteract or, conversely, enhance the deleterious effects of oxidative stress, and fungi may use reactive oxygen species (ROS) as signals that initiate/modulate biosynthesis (reviewed in Hong et al., 2013a; Montibus et al., 2015; Sheridan et al., 2015). Response to oxidative stress and fungal secondary metabolism are indeed intertwined. Many of the regulators involved in such response described to date belong to the basic leucine zipper (bZIP) family of transcription factors as illustrated below.

In *Aspergillus nidulans*, the Yap-like bZIP factor *NapA*, was shown to be involved in tolerance to oxidative stress (Asano et al., 2007). Yin et al. (2013) showed that treatment with the pro-oxidant tert-butyl hydroperoxide (tBOOH) is associated with increased accumulation of the mycotoxin sterigmatocystin, an effect counteracted by over-expressing *NapA* (Yin et al., 2013). A similar trend was observed in the ochratoxin producer *Aspergillus ochraceus* and the *NapA* orthologue *AoYap1* (Reverberi et al., 2012). Not only is ochratoxin production no longer stimulated by tBOOH in a mutant disrupted for *AoYap1* but toxin accumulation is also enhanced in untreated conditions (Reverberi et al., 2012).

On the mechanistic side, mobility shift assays provided experimental evidence that antioxidant and aflatoxin biosynthetic genes in *Aspergillus parasiticus* have binding sites for the same bZIP transcription factor *AtfB* that is activated upon oxidative stress *via* MAPK signaling (Hong et al., 2013a,b). In addition, chromatin immunoprecipitation assays showed that the binding of AtfB on the promoters of aflatoxin genes occurs only when *A. parasiticus* is grown in toxin-inducing medium (Roze et al., 2011b), which supports the concept that toxin biosynthesis and oxidative stress are tightly linked. Considering the interconnectivity between oxidative stress response and mycotoxin production illustrated above, mycotoxin production as a way to cope with endogenous oxidative stress has been previously proposed in *Aspergillus* species (see Reverberi et al., 2010 for a review). In the gray mold fungus *Botrytis cinerea*, the *BcAtf1* factor was also shown to positively regulate the production of secondary metabolites (Temme et al., 2012).

In *F. graminearum*, previous results indicate that oxidative stress with H_2O_2 could be a pre-requisite for the biosynthesis of the mycotoxin DON, which may suggest that DON production and endogenous oxidative stress could be connected (Ponts et al., 2006, 2007; Montibus et al., 2013). In this case, stress response and the regulation of DON synthesis are mediated by the transcription factor *FgAP1* that activates the transcription of antioxidant enzymes (Montibus et al., 2013). Possible sources of endogenous oxidative stress in fungi include NAPDH oxidases (Nox) that generate superoxide anions. However, gene deletion experiments showed that the characterized NADPH oxidases NoxA and NoxB do not seem to play a role in the production of trichothecenes (Wang et al., 2014). Other sources of ROS such as mitochondrial respiration or monoamine oxidase could be involved. Similarly, the regulator of transcription FgSKN7 seem to be involved in both oxidative and cell wall stress response, as well as DON biosynthesis (Jiang et al., 2015). All together, it seems likely that the production of DON and its acetylated derivatives is part of an adaptive response to oxidative stress (reviewed in Audenaert et al., 2014; Montibus et al., 2015). In this context, the ROS produced by the host plant as an early defense mechanism in response to infection are used by *F. graminearum* to its own benefit. Recent data may bring clues as of the mechanism of activation of DON biosynthesis upon oxidative stress. A glycogen synthase kinase GSK3 was shown to be essential for both virulence and DON production *F. graminearum*, and to be up regulated upon oxidative stress by H_2O_2 (Qin et al., 2015). These considerations strongly suggest that mycotoxin biosynthesis and response to oxidative stress are intertwined.

Other Stresses

The pH of the environment was shown to be particularly critical for the initiation of trichothecene B biosynthesis in *F. graminearum*. An acidic pH is a prerequisite for DON production (Merhej et al., 2010). The biosynthesis of type B trichothecenes has been shown to be negatively controlled by the transcription regulator *FgPac1*, homologous to the member of the pH regulator system *PacC* in *A. nidulans* (Merhej et al., 2011). It is noteworthy that oxidative stress and acidification have been described to occur together in various organisms including fungi (see examples in Jia et al., 2014; Pérez-Sampietro and Herrero, 2014). The relationship between these two stresses is however not clear.

Different response pathways can be triggered to counteract stresses that modify the organization and stability of the fungal cell wall. For example, the cell wall integrity pathway is responsive to changes in osmotic pressure and oxidative stress (see Hayes et al., 2014 for a review). Typically, the activation of the cell wall integrity pathway leads to the activation of cell wall biogenesis genes. In *F. graminearum*, the cell wall integrity pathway involves the MAP kinase FgMgv1 (Hou et al., 2002), itself activated by the MAP kinase FgMkk1 (Yun et al., 2014). When the pathway is altered by gene deletion, the production of DON is drastically reduced (Hou et al., 2002; Yun et al., 2014). Once more, the production of DON seems linked to a stress response pathway. It is noteworthy that the production of the secondary metabolite aurofusarin, a pigment, is also diminished in the absence of FgMgv1 (Hou et al., 2002). Such observation provides another example of the association between the activation of secondary metabolites pathways and stress response pathways. Remarkably, the MAP kinase FgOS-2 of the high osmolarity glycerol (HOG) response pathway is also activated by FgMkk1 (Yun et al., 2014), which illustrates the coupling between cell wall stress response and osmotic stress response. The HOG response pathway was initially described in the budding yeast as required for osmoadaptation (reviewed by Hohmann et al., 2007). In *F. graminearum*, FgHOG1 of the osmoregulation MAP kinase pathway, mediated by the FgOS-2 kinase, is involved in hyperosmotic as well as cell membrane stress responses. FgHOG1 also plays a role in ROS-mediated signaling. Its deletion causes a drastic reduction of DON accumulation, also caused by hyperosmotic conditions, as well as other developmental defects (Van Thuat et al., 2012; Zheng et al., 2012). Similarly, gene deletion experiments targeting FgOS-2 showed that DON production is strongly reduced *in planta* (Van Thuat et al., 2012).

A SECONDARY METABOLISM REGULATORY PATHWAY PART OF ENVIRONMENTAL STRESS RESPONSE

Regulators of fungal secondary metabolite biosynthesis that play a role in regulating other aspects of fungal life, including response to stress, have been described. The example of the *F. graminearum*, transcription factor Tri6 has been evoked above (Seong et al., 2009). In *Aspergilli*, LaeA has been described as a secondary metabolism-specific regulator involved in switching from inactive heterochromatin to the transcriptionally permissive euchromatic state. In *F. graminearum*, FgLae1 is a regulator of secondary metabolism that activates the production of DON (Kim et al., 2013). The deletion of *FgLae1* has pleiotropic effects in *F. graminearum*, also affecting sexual development for example (Kim et al., 2013). Typically LaeA is a member of the Velvet complex, involved in the response to light stress. In the absence of light, LaeA is available in the nucleus and chromatin at secondary metabolite gene clusters is active (and DON is produced for example). On the contrary, when light is applied, chromatin inactivates and secondary metabolite biosynthesis is shut down. Such a "secondary metabolism switch" could be conveniently associated with general stress response pathways to extend them to the production of secondary metabolites.

Previous observations made about *F. graminearum*'s response to oxidative stress, *i.e.*, defensive plant-produced H_2O_2 may serve as a signal to produce DON (Ponts et al., 2006, 2007; Montibus et al., 2013), also fit with this theory. Other examples of such hijacking have been proposed, for example the use of the plant carbon and nitrogen metabolisms by *F. graminearum* for its own development and secondary metabolism (Audenaert et al., 2014). Polyamines such as agmatine or putrescine are, indeed, excellent activators of DON biosynthesis (Gardiner et al., 2010). From the evolutionary point of view, the general components of oxidative, osmotic, and cell wall stress pathways appear well conserved among fungi (Nikolaou et al., 2009). However, specific sensors and regulators involved in those pathways are diverse and seem to have fairly recently rapidly evolved to adapt to fungal specific life traits (Nikolaou et al., 2009). In a general manner, plant pathogens are more sensitive to oxidative stress than human pathogens for example (Nikolaou et al., 2009). Along the same line, stress response pathways in fungal plant pathogens, especially oxidative, osmotic and cell wall stress, are typically involved in tolerance to fungicides targeting the fungal cell wall (*e.g.*, caspofungin, nikkomycin Z, tunicamycin, fluconazole; see Hayes et al., 2014 for a review). Such aspects must definitely be considered and addressed by strategies aiming at controlling mycotoxin occurrence in cereals.

The hypothesis of a coupling between stress response and mycotoxin production is reinforced by recent evidence that proteins involved in secondary metabolite pathways, including the aflatoxin one in *A. parasiticus*, co-localize with stress response proteins to the endosome/transport vesicles/vacuoles fraction of a fungal cell extract (Linz et al., 2012). In *F. graminearum*, previous work showed that the endoplasmic reticulum stress response and oxidative stress are tightly linked (Malhotra et al., 2008). Remarkably, recent work found that vesicular sequestration of enzymes of the DON-producing pathway co-localize with the endoplasmic reticulum (Menke et al., 2013; Boenisch et al., 2015). When all elements are taken into consideration, *F. graminearum* secondary metabolism and stress response pathways are indisputably very closely interconnected. Although further investigation is required, an attractive hypothesis is that secondary metabolism pathways could be part of the fungus' stress-response system. Under this scenario, more than a coupling of pathways, the production of DON and other secondary metabolites would be integral part of the fungus' arsenal to cope and adapt to its always-changing environment, including in the context of host-pathogen exchanges.

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Individual and combined roles of malonichrome, ferricrocin, and TAFC siderophores in *Fusarium graminearum* pathogenic and sexual development

Shinichi Oide[1,2], Franz Berthiller[3], Gerlinde Wiesenberger[4], Gerhard Adam[4] and B. Gillian Turgeon[1]*

[1] Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, USA
[2] The Research Institute of Innovative Technology for the Earth (RITE), Kizugawa-Shi, Japan
[3] Department of Agrobiotechnology (IFA-Tulln), Center for Analytical Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria
[4] Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria

Edited by:
Nancy Keller, University of Wisconsin, USA

Reviewed by:
Hubertus Haas, Innsbruck Medical University, Austria
Daren W. Brown, United States Department of Agriculture, USA

***Correspondence:**
B. Gillian Turgeon, Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, 334, Plant Science Bldg., Ithaca, NY 14853, USA
e-mail: bgt1@cornell.edu

Intra- and extracellular iron-chelating siderophores produced by fungal non-ribosomal peptide synthetases have been shown to be involved in reproductive and pathogenic developmental processes and in iron and oxidative stress management. Here we report individual and combined contributions of three of these metabolites to developmental success of the destructive cereal pathogen *Fusarium graminearum*. In previous work, we determined that deletion of the *NPS2* gene, responsible for intracellular siderophore biosynthesis, results in inability to produce sexual spores when mutants of this homothallic ascomycete are selfed. Deletion of the *NPS6* gene, required for extracellular siderophore biosynthesis, does not affect sexual reproduction but results in sensitivity to iron starvation and oxidative stress and leads to reduced virulence to the host. Building on this, we report that double mutants lacking both *NPS2* and *NPS6* are augmented in all collective phenotypes of single deletion strains (i.e., abnormal sexual and pathogenic development, hypersensitivity to oxidative and iron-depletion stress), which suggests overlap of function. Using comparative biochemical analysis of wild-type and mutant strains, we show that *NPS1*, a third gene associated with siderophore biosynthesis, is responsible for biosynthesis of a second extracellular siderophore, malonichrome. *nps1* mutants fail to produce this metabolite. Phenotypic characterization reveals that, although single *nps1* mutants are like wild-type with respect to sexual development, hypersensitivity to ROS and iron-depletion stress, and virulence to the host, triple *nps1nps2nps6* deletion strains, lacking all three siderophores, are even more impaired in these attributes than double *nps2nps6* strains. Thus, combinatorial mutants lacking key iron-associated genes uncovered malonichrome function. The intimate connection between presence/absence of siderophores and resistance/sensitivity to ROS is central to sexual and pathogenic development.

Keywords: siderophores, malonichrome, *Fusarium graminearum*, virulence, sexual development, HPLC/MS

INTRODUCTION

The evolutionary diversity and array of genes in filamentous fungi encoding megaenzymes for biosynthesis of secondary metabolites underscore the importance of these factors in fungal biology. While many secondary metabolites are best known for their favorable (e.g., medicinals) or unfavorable (e.g., toxins) effects on other organisms such as humans and agricultural crops, these attributes are largely corollaries to their principal functions in the fungal cells themselves. For example, intra- and extracellular iron-chelating siderophores produced by non-ribosomal peptide synthetases (NRPSs) are centrally, but differentially, involved in developmental processes.

Iron is an essential component of aerobic metabolism and numerous enzymes have Fe-ions as a cofactor. Yet, intracellular free iron (labile iron), in the presence of hydrogen peroxide or superoxide, generates highly cytotoxic reactive oxygen species (ROS), i.e., hydroxyl radicals, from less toxic hydrogen peroxide H_2O_2 through the Haber-Weiss/Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$). Tight regulation of the labile iron pool is thus critical for aerobic organisms. Paradoxically, though essential and abundant in the earth's crust, bio-available iron is limited due to low solubility. To solve this dilemma and increase iron solubility, fungi and bacteria produce siderophores, low-molecular weight organic compounds with strong iron-chelating activity biosynthesized by NRPSs (Lee et al., 2005; Oide et al., 2006).

Most fungal siderophores are of the hydroxamate type, produced through condensation of N^5-acyl-N^5-hydroxy-L-ornithine (AHO) units by dedicated NRPSs. Fungal hydroxamate-type siderophores tend to be one of three types, ferrichrome, coprogen, or fusarinine. Members of the latter two groups are produced simply through condensation of AHO units by the NRPSs encoded by the conserved *NPS6/sidD* genes (Oide et al., 2006; Schrettl et al., 2007) while the ferrichrome-type is produced through condensation of AHO units plus amino acids such as glycine, serine, or alanine (Bushley et al., 2008). Genes encoding ferrichrome-type siderophore synthetases are conserved across the fungal kingdom and phylogenetic analyses have provided evidence that they can be divided into two lineages, one corresponding to *Cochliobolus heterostrophus NPS2* homologs and the other to *Aspergillus nidulans sidC* homologs (Bushley et al., 2008). Some species, including *Gibberella zeae* (hereafter, *Fusarium graminearum* http://www.ncbi.nlm.nih.gov/pubmed/23379853), carry orthologs of both lineages in their genomes. The metabolites produced by Nps2 and SidC are indispensable for sexual development in heterothallic *C. heterostrophus*, and homothallic *F. graminearum* and *A. nidulans*, indicating functional conservation of these metabolites (Eisendle et al., 2006; Oide et al., 2007). However, functional divergence/overlap between the metabolites produced by the ferrichrome-type siderophore synthetases with distinct evolutionary histories has not been evaluated in a single species.

In our previous work with *F. graminearum*, we identified two NRPSs, one encoded by the *NPS2* gene and the other by the *NPS6* gene, that are involved in siderophore production (Oide et al., 2006, 2007). Biochemical characterization of *nps2* mutants revealed that the Nps2 protein is responsible for biosynthesis of ferricrocin, which acts as an intracellular iron-capturing metabolite in this species. Deletion of *NPS6*, on the other hand, abolished production of the extracellular siderophore, triacetyl fusarinine C (TAFC) and its derivatives. *nps2* mutants are greatly impaired in sexual development, whereas *nps6* mutants show pleiotropic defects including attenuated virulence to wheat, hypersensitivity to ROS, increased sensitivity to iron depletion and reduced asexual sporulation (Oide et al., 2006). Notably, *nps2* mutants are not affected in any of the characteristics found for *nps6* mutants and *vice versa*. *nps6* mutants are thus wild-type (WT) in terms of sexual reproduction, highlighting distinct contributions of intra- and extracellular siderophores to pathogenic, vegetative, and reproductive development of *F. graminearum*.

In addition to *NPS2* and *NPS6*, we (and others) have noted that the genome of *F. graminearum* carries another *NPS* gene likely involved in siderophore biosynthesis (Oide et al., 2007; Tobiasen et al., 2007; Hansen et al., 2012; Adam et al., 2015; Sieber et al., 2014) *NPS1* is an ortholog (Bushley et al., 2008) of *A. nidulans sidC* whose encoded NRPS accounts for ferricrocin synthesis in this species (Eisendle et al., 2006). Our previous attempt to identify the metabolite biosynthesized by Nps1 was unsuccessful, and phenotypic characterization of *nps1* mutants failed to link Nps1 to iron metabolism (Oide et al., 2007). Much earlier work described a siderophore named malonichrome that is produced by a *Fusarium roseum* strain (ATCC 12822) (Emery, 1980). The structure established for malonichrome is that of a ferrichrome-type compound, a cyclic hexapeptide with one alanine, two glycines, and three AHOs in which the hydroxylamino groups are acylated with malonic acid. More recently, a study on *Fusarium oxysporum* (strain FGSC 9935) reported that this fungus produces three different ferrichrome-type siderophores, ferricrocin, ferrichrome C, and malonichrome (Lopez-Berges et al., 2012). Like *F. graminearum*, *F. oxysporum* has corresponding orthologs of *NPS1* (FOXG_17422), *NPS2* (FOXG_06448), and *NPS6* (FOXG_09785).

In this study, we report characterization of strains of *F. graminearum* in which *NPS1*, *NPS2,* and *NPS6* genes are deleted in all possible combinations. Double *nps2nps6* mutant strains completely lack ability to make the intracellular siderophore ferricrocin and the extracellular fusarinine siderophores, and are augmented in the phenotypes identified in each single *nps*-deletion strain (i.e., hypersensitivity to iron depletion and oxidative stress, reduction in virulence, and abnormal sexual development), rather than the sum of each phenotype, which suggests overlap in function. We also formally demonstrate that *NPS1* is responsible for the production of the previously described second extracellular ferrichrome-type siderophore, malonichrome (Emery, 1980). Thorough characterization of the *nps1nps6* and *nps1nps2nps6* strains uncovered roles of Nps1 in stress tolerance, sexual development, and virulence to the host.

MATERIALS AND METHODS

FUNGAL STRAINS AND FUNGAL AND PLANT CULTURE CONDITIONS

The *F. graminearum* (*G. zeae*) wild-type strain Gz3639 was used for all experiments (**Table 1**). Unless otherwise mentioned, all cultures were grown on complete medium [CM; (Leach et al., 1982)] at 24 C under continuous fluorescent light (Watt-Miser F34 WW/RS/WM, Warm White, General Electric). Wheat cultivar Norm was grown (four plants per #6 pot) in a greenhouse in 14 h light/10 h dark at 25 C.

DNA MANIPULATIONS AND FUNGAL TRANSFORMATIONS

Fungal genomic DNA was prepared as described previously (Oide et al., 2006, 2007). Unless otherwise mentioned, all PCR reactions were carried out with PCR master mix (Promega) following the manufacturer's recommendations. Transformation of *F. graminearum* was carried out as described previously (Oide et al., 2006, 2007). All transformants were purified by two rounds of single asexual spore isolation. All mutants used in this report are listed in **Table 1**.

CONSTRUCTION OF *F. GRAMINEARUM* nps1nps6, nps2nps6 DOUBLE AND nps1nps2nps6 TRIPLE MUTANT STRAINS

Single deletion *nps1*, *nps2,* and *nps6,* and double deletion *nps1nps2* mutants were constructed previously (Oide et al., 2006, 2007). Double deletion mutants *nps1nps6* and *nps2nps6* were constructed by deleting *NPS6* in the hygromycin B resistant strains Gznps1-5-1 and Gznps2-6-1 (Oide et al., 2007), respectively (**Table 1**). Triple *nps1nps2nps6* mutant strains were constructed by deleting *NPS6* in the hygromycin B and G418 resistant *nps1nps2* mutant strain Gznps2-6-1nps1-5-1 (Oide et al., 2007). For this, the *NPS6* ORF was partially replaced with the *Streptomyces griseochromogenes BSD* gene, which confers resistance to the antibiotic blasticidin S (BS, Invitrogen). The *BSD* gene, under the control of the *A. nidulans TrpC* promoter and terminator was PCR amplified from pBF101 (Kimura et al.,

Table 1 | Strains used in this study.

Strain[a]	Genotype	Designation in text	Designation in figures	Comments and/or References
Gz3639(NRRL 29169)	WT[b] (*NPS1*;*NPS2*;*NPS6*)	WT	WT	WT, (Bowden and Leslie, 1999)
Gznps1-5-1	*nps1*Δ::*hygB*	*nps1*	*1*	Oide et al., 2007
Gznps2-6-1	*nps2*Δ::*hygB*	*nps2*	*2*	Oide et al., 2007
Fgnps6G-1	*nps6*Δ::*nptII*	*nps6*	*6*	Oide et al., 2006
Gznps2-6-1Δnps1-5-1	*nps1*Δ::*hygB*; *nps2*Δ::*nptII*	*nps1nps2*	*1;2*	Oide et al., 2007
Gznps1-5-1Δnps6-3-1	*nps1*Δ::*hygB*; *nps6*Δ::*nptII*	*nps1nps6*	*1;6*	This study, Gznps1-5-1 bkg[c]
Gznps1-5-1Δnps6-5-1	*nps1*Δ::*hygB*; *nps6*Δ::*nptII*	*nps1nps6*	*1;6*	This study, Gznps1-5-1 bkg
Gznps2-6-1Δnps6-6	*nps2*Δ::*hygB*; *nps6*Δ::*nptII*	*nps2nps6*	*2;6*	This study, Gznps2-6-1 bkg
Gznps2-6-1Δnps6-17	*nps2*Δ::*hygB*; *nps6*Δ::*nptII*	*nps2nps6*	*2;6*	This study, Gznps2-6-1 bkg
Gznps2-6-1Δnps1-5-1 Δnps6-3-2	*nps1*Δ::*hygB*; *nps2*Δ::*nptII*; *nps6*Δ::*BDS*	*nps1nps2nps6*	*1;2;6*	This study, Gznps2-6-1nps1-5-1 bkg
Gznps2-6-1Δnps1-5-1 Δnps6-11-1	*nps1*Δ::*hygB*; *nps2*Δ::*nptII*; *nps6*Δ::*BDS*	*nps1nps2nps6*	*1;2;6*	This study, Gznps2-6-1nps1-5-1 bkg

[a] *Nomenclature: Gz, Gibberella zeae—Fusarium graminearum; Gznps1-5-1 is single conidial isolate 1 of transformant 5 in which NPS1 was deleted from WT; Gznps2-6-1nps1-5-1 is single conidial isolate 1 of transformant 5 of Gznps2-6-1Δnps1-5-1, in which the NPS1 gene was deleted in strain Gznps2-6-1.*

[b] *WT carries wild-type copies of NPS1, NPS2, and NPS6.*

[c] *bkg, genetic background.*

1994) with the primer pair M13F/M13R. A linear construct for transformation was prepared in the same way as described for partial deletion of *NPS6* with the gene (*bar*) for bialaphos resistance (Oide et al., 2006). Screening of transformants was carried out on complete medium without salts (CMNS) with 300 μg/ml BS. Deletion of *NPS6* was confirmed by PCR using methods described earlier (Oide et al., 2006; Inderbitzin et al., 2010).

STRESS SENSITIVITY ASSAYS

Each strain was grown on solid minimal medium (MM) with or without the stress agent. Sensitivity to each stress was scored by measuring the colony radius of 5 day-old cultures on plates with the stress agent. Sensitivities to H_2O_2, the superoxide-generator, KO_2, and the membrane-permeable iron chelator, 2-2′-dipyridyl (2DP), were examined by determining minimal inhibitory concentration (MIC) of each stress agent, as described previously (Oide et al., 2006, 2007). Briefly, a fresh stock solution of each stress agent was prepared for each experiment (1 M KO_2 and 10 mM 2DP, in water) and the stress agents were added to MM after autoclaving (MM at approximately 48°C). Fresh MM plates with the stress agents were prepared for each experiment. All experiments were carried out in the dark. For determination of MIC of H_2O_2 to *F. graminearum*, MM plates with 0, 3, 6, and 12 mM H_2O_2 were prepared. For $KO_{2,two}$ different sets of MM plates with 0, 6, 12, and 24 mM KO_2, or with 0, 3.5, 7, 14, and 28 mM KO_2, were prepared. For 2DP, MM plates with 0, 100, 200, and 400 μM 2DP were prepared.

To test sensitivity to iron depletion, growth of WT and different mutant strains was examined on MM and MM with 200/400 μM ferric citrate. Growing tips of mycelia were transferred from 3 day-old cultures on CM plates to fresh plates of MM with/without ferric citrate with a cork borer (3 mm diameter). The plates were incubated for 5 days under standard culture conditions. Five replicates were set up for each strain and for each condition. Average colony diameters of 5 day-old cultures were determined for each strain, and the data were analyzed by one-way analysis of variance (ANOVA) using Excel 2007.

In addition to determination of MIC of 2DP, sensitivity to 2DP of mutant strains was examined. Growing tips of mycelia were transferred by taking plugs with a cork borer (3 mm diameter) from 3 day-old cultures on CM plates to fresh plates of MM with 0, 50, and 100 μM 2DP. MM with 2DP was prepared as described above. The plates were incubated in the dark at 24°C for 4 days. Five replicates were set up for each strain and for each condition. Average colony diameters of 4 day-old cultures were determined for each strain, and the data were analyzed by ANOVA.

EVALUATION OF FERTILITY

F. graminearum self matings were set up on carrot juice (CJ) medium, as described previously (Oide et al., 2007). For mating, growing mycelial tips were placed in the centers of CJ agar plates, the plates were sealed with parafilm and incubated under continuous black light at 24°C for 7 days. Approximately 1 ml of sterile 2.5% Tween 60 solution was applied to the plate, and mycelia growing on the plate were knocked down with a rubber policeman, which induced sexual development. Excess Tween 60 solution was discarded and the unsealed plates were incubated under the original conditions for an additional 7 days (Oide et al., 2007).

Fertility was examined based on the number of perithecia per plate and the number of asci per perithecium. At least, five replicates were set up for each self and the number of perithecia per plate was recorded for each plate. At least 20 perithecia were opened for each self and the number of asci was recorded for each perithecium. All data were analyzed by ANOVA.

Fertility of *F. graminearum* selfs supplied with iron was examined in the same way as described above and previously (Oide et al., 2007). Briefly, a fresh stock solution of ferric citrate (10 mM in water) was applied to carrot juice medium before autoclaving, so that the final concentration was 100, 125, 200 or 250 μM.

EVALUATION OF VIRULENCE

Virulence assays of *F. graminearum* were carried out as described previously (Oide et al., 2006). At least five independent spikes were inoculated per strain and per assay. In point-inoculation of wheat spikes, local infection, as well as systemic infection, was evaluated. The time required for completion of local infection after inoculation was recorded for each spike. When an inoculated spikelet became completely bleached, local infection was considered finished. At least five spike replicates were set up for each strain, and the average time for completion of local infection was determined for each strain. The data were statistically analyzed by ANOVA. Experiments were repeated at least three times.

ISOLATION, IDENTIFICATION, AND SEMI-QUANTITATIVE ANALYSIS OF *F. GRAMINEARUM* SIDEROPHORES

Strains were pregrown under iron replete conditions in Fusarium MM made according to the Fusarium Laboratory Manual (Leslie and Summerell, 2006), containing the standard amount of iron (100 ml flasks, 22°C, 150 rpm). Mycelia from dense 4 day-old cultures were harvested by vacuum filtration using sterile Buchner funnels fitted with sterile filter paper. The mycelia were washed with about 25 ml of iron-free MM, then scraped off the filter paper and transferred to empty Petri dishes. After determination of the wet weight the mycelia were transferred into fresh Fusarium MM without iron. Initially, in all cases, a volume of 6 ml/g wet weight was added during the medium replacement, resulting in cultures with a volume of about 50 ml (±10%). The resuspended cultures were incubated in this iron starvation medium on a rotary shaker at 150 rpm at 22°C for 3, 6, 11, and 15 days. At harvest, the cultures were first filtered through glass wool to remove the mycelium then clarified by centrifugation to remove any remaining mycelium, including conidiospores formed during cultivation in iron-free medium. $FeCl_3$ was added to the cleared supernatant (10 mM final), which was centrifuged for 10 min at 12,000 rpm. The supernatants were analyzed with LC-UV-MS. HPLC grade methanol was purchased from J. T. Baker (Deventer, The Netherlands) and formic acid (analytical grade) was obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Pure ferricrocin, as well as a mixture containing fusigen, dimerum acid, coprogen, neocoprogens I and II and triacetylfusarinine C (TAFC) were purchased from EMC Microcollections (Tübingen, Germany) and used as qualitative standards.

HPLC separation was carried out on a Gemini-C18 column (4.6 × 150 mm, 5 μm) equipped with a 4 × 3 mm security guard cartridge (both from Phenomenex, Torrance, CA) on an 1100 Series HPLC system (Agilent, Waldbronn, Germany). Eluent A consisted of 5% aqueous methanol with 1% formic acid, while eluent B was 99% methanol with 1% formic acid. After a hold time of 1.5 min at 100% A, the proportion of B was increased linearly to 100% within 10 min, followed by a hold time of 2.5 min at 100% B and 6 min column re-equilibration at 100% A. Total runtime was 20 min at a flow rate of 750 μl/min. The column was kept at 25°C and an injection volume of 25 μl was used. The diode array detector was set to a wavelength of 435 nm. After the UV detector, the column effluent was transferred via a six-port valve either to the mass spectrometer (between 4 and 14 min) or to the waste. Full scan mass spectra (using Q1) were recorded from m/z 500 to 1200 on a QTrap LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ESI source in positive ion mode using Analyst version 1.4.1. The following settings were used for the MS: source temperature 400°C, curtain gas 25 psi, gas 1 (sheath gas) 30 psi, gas 2 (drying gas) 75 psi, ion spray voltage 5000 V, declustering potential 30 V, scan time 1 s.

High resolution mass spectrometric measurements were performed on a 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany) operated in MS and MS/MS mode. Chromatographic separation was achieved using a Gemini C6-Phenyl C-6 column (2.0 × 50 mm, 3 μm, Phenomenex) using a linear gradient from 5 to 100% methanol, containing 40 mM formic acid and 20 mM ammonium formate.

RESULTS

METABOLITE ANALYSIS

Three and a half decades ago, Emery described the siderophore, malonichrome, produced by a *F. roseum* strain (ATCC12822) (Emery, 1980). While *F. roseum* is generally considered as a synonym of *F. graminearum*, this strain is, in fact, most closely related to *F. equiseti* based on the nucleotide sequence available for the LSUrDNA D1D2 from the equivalent strain NBRC 8502 (Culture Collection Division of the Biological Resource Center, Japan). The established structure for malonichrome is that of a ferrichrome-type compound, specifically, a cyclic hexapeptide with one alanine, two glycines and three AHOs, in which the hydroxylamino groups are acylated with malonic acid (Emery, 1980).

A big advantage of iron-chelating compounds is their UV absorbance at 435 nm, which renders analytical detection straightforward. A LC-UV chromatogram of the culture supernatant of WT *F. graminearum*, grown under iron starvation for 6 days, is provided as **Figure 1**. Concurrent MS analyses of the peak at 8.00 min identified compounds with the mass and sum formula corresponding to malonichrome (after iron supplementation) and desferri-malonichrome (in the absence of iron) (**Figures 2A,B**). The enhanced resolution scan of the compound with the mass of malonichrome shows clearly that this compound

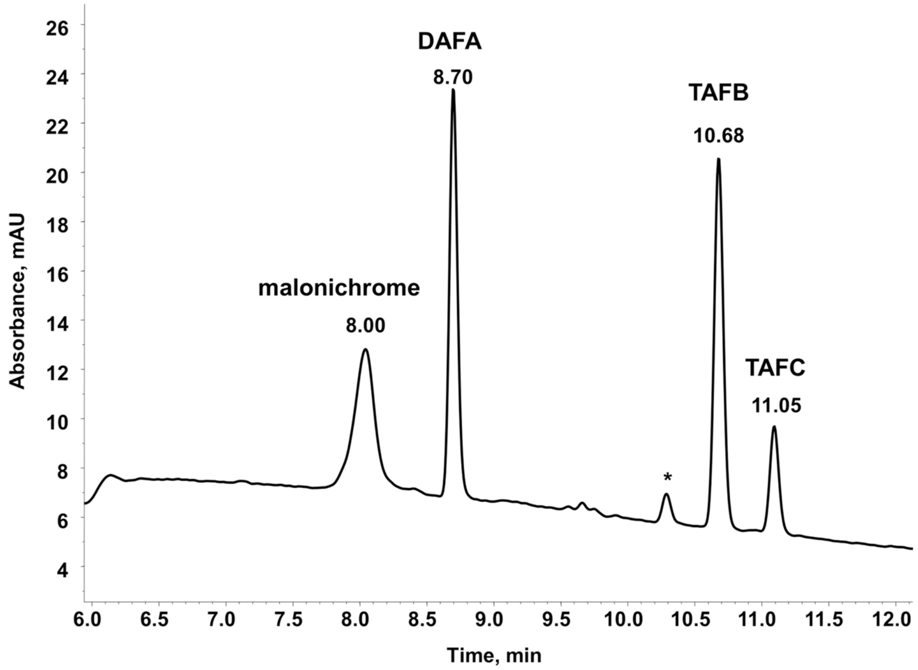

FIGURE 1 | LC-UV chromatogram of wild-type *F. graminearum*. The fungus was cultivated in liquid MM without iron for 6 days. $FeCl_3$ was added before measurement of the supernatant at 435 nm. Siderophores present are DAFA, malonichrome, TAFB, and TAFC. * denotes a small interfering peak, also visible in pure solvent.

is able to bind iron. The signal with *m/z* 885 originates from binding of ^{54}Fe (making up 6% in naturally occurring isotope mixtures) whereas the main peak with *m/z* 887 is derived from ^{56}Fe (92%) and the peak with *m/z* 888 from ^{57}Fe (2%). LC-HR-MS measurements yielded the highest intensity at *m/z* 887.2244 for the $[M+H]^+$ ion of the ferri-form. This confirms the sum formula of $C_{31}H_{44}FeN_9O_{18}$ with a deviation from the theoretical mass of +1.4 ppm (**Figure 2A**). For the desferri-form *m/z* values of 834.3105 (Δ +0.9 ppm), 851.3375 (Δ +0.2 ppm) and 856.2925 (Δ +0.7 ppm) have been found, which match the protonated ion, the ammonium adduct and the sodium adduct of $C_{31}H_{47}N_9O_{18}$, respectively (**Figure 2B**). Also the collision induced dissociation pattern in LC-MS/MS experiments (**Figure 2C**) is consistent with the structure proposed by Emery (1980). Cleavage of carbon dioxide from the three malonic acid ester (MA) groups, cleavage of the respective MA groups as well as cleavage of one or both AHO (including MA) groups from the molecule can be explained from the MS/MS spectrum. We therefore conclude that the compound identified in *F. graminearum* is malonichrome. As evident from **Figure 3**, malonichrome is missing in samples of all mutants lacking *NPS1* (*nps1*, *nps1nps2*, *nps1nps6* and the triple mutant *nps1nps2nps6*), demonstrating, formally, that the *NPS1* gene is required for malonichrome biosynthesis.

In agreement with our previous publication (Oide et al., 2007), ferricrocin was missing in samples of all mutants carrying a deletion of *NPS2* (**Figure 3**). Unexpectedly, however, we detected ferricrocin in the culture supernatant of WT, at later time points (after 11 days of iron starvation; not visible in **Figure 1**), indicating that this "intracellular siderophore" is not as strictly intracellular as previously thought. Furthermore, the supernatant of the *nps1nps6* mutant which is expected to produce only ferricrocin, had an approximately 2X increase in ferricrocin accumulation compared to WT (**Figure 3**). **Figure 3** also shows early accumulation of TAFC and transient formation of its derivatives triacetyfusarinine B (TAFB) and diacetylfusarinine A (DAFA) with hydrolyzed ester bonds. As noted by Emery for *F. roseum* (Emery, 1980), we observed a switch from production of the ester type fusarinines to the peptide type siderophore malonichrome as the major extracellular siderophore for WT *F. graminearum* after extended culture. Interestingly, production of TAFC and the hydrolysis product TAFB was maintained longer in the mutant lacking the other two siderophores (*nps1nps2*).

GROWTH AND SENSITIVITY TO ROS AND IRON DEPLETION

Single, double, and triple mutant strains (**Table 1**) were used to perform phenotypic tests. Single gene deletions of either *NPS1* or *NPS2* do not affect tolerance to oxidative stress mediated by H_2O_2 and KO_2 as described previously (Oide et al., 2007). Likewise, *nps1nps2* strains show WT tolerance. Deletion of *NPS6*, however, compromises tolerance to both types of ROS (Oide et al., 2006). *nps1nps6* strains showed *nps6*-like sensitivity to ROS, while double deletion of *NPS2* and *NPS6* further increased sensitivity to H_2O_2 and KO_2 compared to *nps6* strains (**Table 2**). Triple *nps1nps2nps6* mutant strains were indistinguishable from double *nps2nps6* mutant strains in terms of sensitivity to H_2O_2 (see **Table 2** for MICs). Similarly, there was no difference in MIC of KO_2 to *nps2nps6* and *nps1nps2nps6* strains, when sensitivity to 3, 6, 12, and 24 mM KO_2 was examined. A subtle reduction in

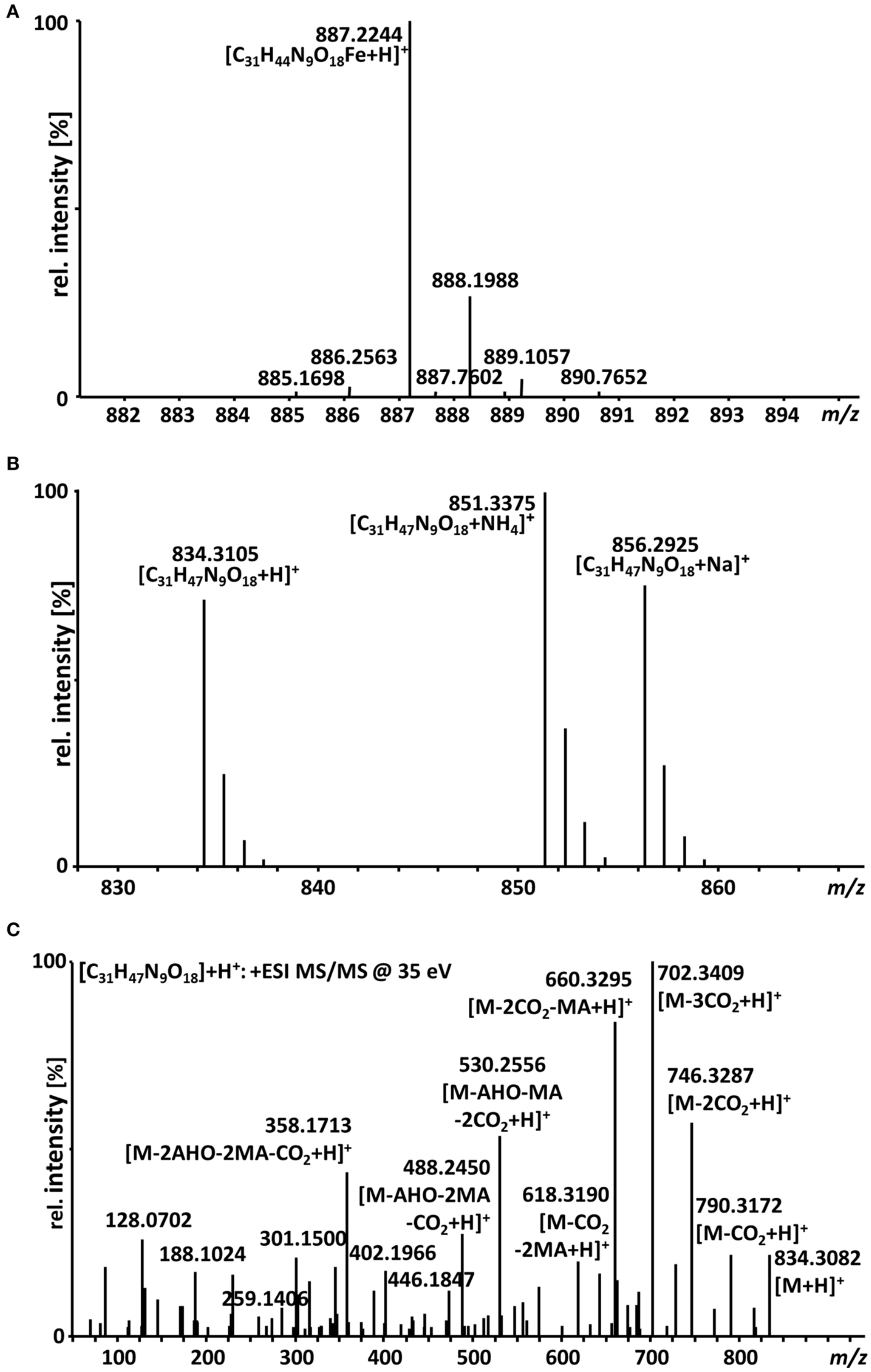

FIGURE 2 | LC-HR-MS spectra of malonichrome. (A) Isotope pattern of the $[M+H]^+$ ion cluster of malonichrome (ferri-form). **(B)** LC-HR-MS spectrum of malonichrome (desferri-form), showing $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions. **(C)** LC-HR-MS/MS spectrum of malonichrome (desferri-form) at a collision energy of 35 eV.

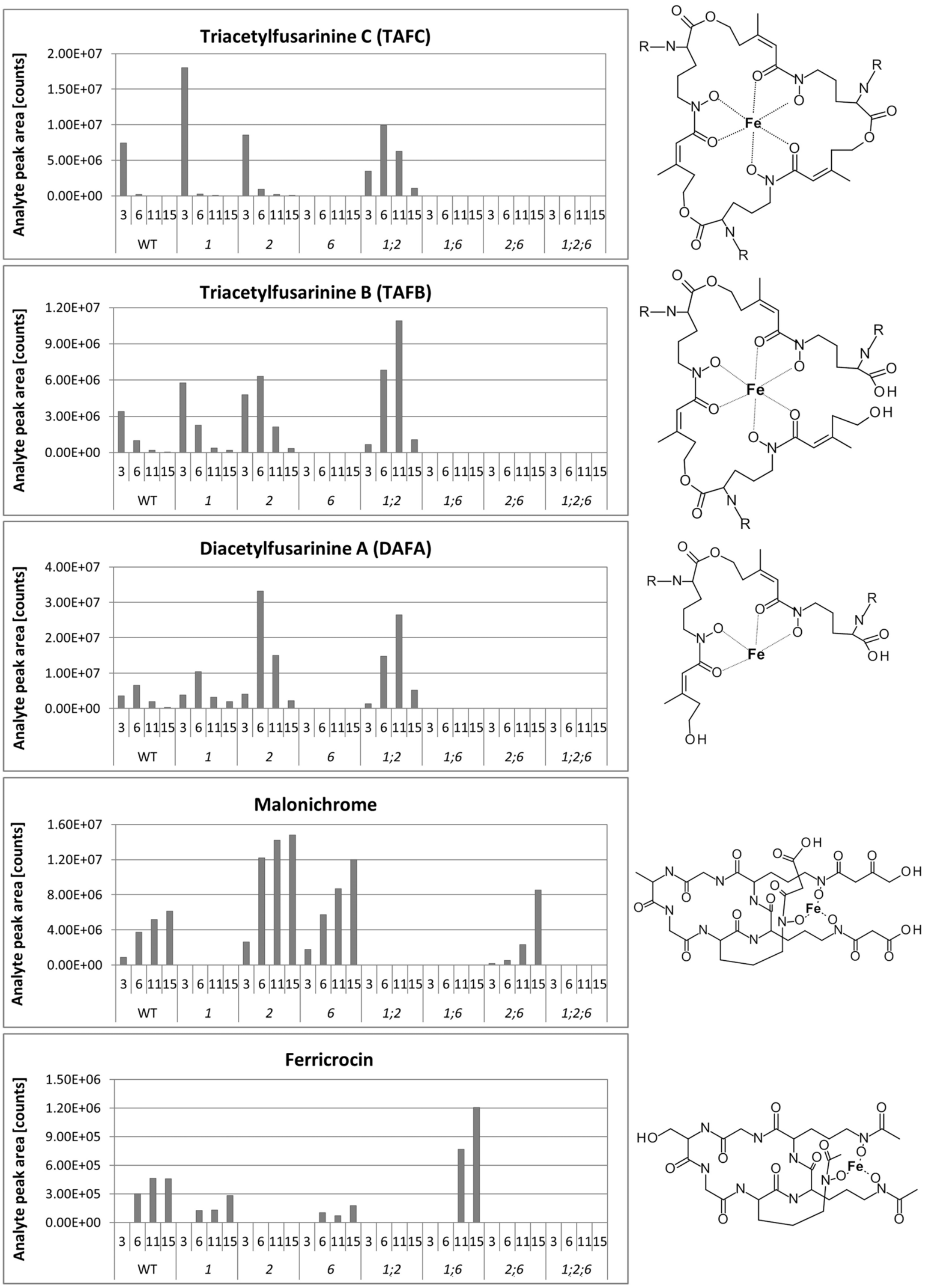

FIGURE 3 | Siderophores produced by wild-type and mutant strains. Siderophores produced by WT Gz3639 and mutant strains (see **Table 1**) cultivated in MM and subsequently starved in iron-free MM as described in Materials and Methods. Samples were taken 3, 6, 11, and 15 days after shift to iron-starvation medium. Supernatants were analyzed for siderophores by HPLC as described in the Materials and Methods. X-axis: upper row, days after shift to iron-free medium, lower row, strains investigated. Y-axis: relative concentrations (arbitrary units), R (TAFC, TAFB, DAFA): acetyl. Note, metabolites are missing in all strains with deletions of corresponding genes, as expected. Malonichrome is present in strains with a WT *NPS1* gene and absent from strains deleted for this gene.

Table 2 | MIC of H_2O_2, KO_2, and 2DP to WT and mutant strains.

Strain	Stress		
	H_2O_2 (mM)	KO_2 (mM)[b]	2DP (μM)
WT	12 >[a]	24/28	400
nps6	6	12/7	200
nps2nps6	3	6/7	200
nps1nps6	6	12/7	200
nps1nps2nps6	3	6/3.5	200

[a] *"> " indicates that growth of a strain was observed on MM with the maximal concentration of a stress agent tested in this study. Thus, MIC of the stress agent to that strain is higher than the maximal concentration tested (e.g., MIC of H_2O_2 to WT is higher than 12 mM).*

[b] *MIC of KO_2 to F. graminearum strains was examined in two different scales. In the first series of experiments (left of forward slash), sensitivity to 3, 6, 12, and 24 mM KO_2 was examined. In the second series, sensitivity to 3.5, 7, 14, and 28 mM KO_2 was examined.*

growth, however, was observed for the latter strain on MM with 3 mM KO_2 compared to the former (data not shown). When examined on MM with 3.5, 7, 14, and 28 mM KO_2, MIC of KO_2 to *nps1nps2nps6* strains was 3.5 mM, while that of *nps2nps6* strains was 7 mM (**Table 2**), indicating that deletion of *NPS1* enhances the sensitivity of *nps2nps6* strains to KO_2. These observations allow the conclusion that *NPS1* and *NPS2* contribute to oxidative stress tolerance.

nps1, *nps2*, as well as *nps1nps2* strains show the same tolerance to iron depletion as the WT strain, whereas *nps6* strains are compromised in growth under low iron conditions. Judged by MICs of 2DP, no difference in sensitivity to iron depletion was observed among *nps6*, *nps1nps6*, *nps2nps6*, and *nps1nps2nps6* strains (**Table 2**). Nevertheless, a reduction in growth on MM with 100 μM 2DP was observed for the latter three compared to *nps6* strains (**Figure 4A**), and growth of *nps1nps2nps6* strains was most severely affected. As previously published (Oide et al., 2006), *nps6* strains have a growth defect on MM due to starvation for iron, and exogenous application of iron restores WT growth to *nps6* strains (**Figure 4B**). When cultured on MM, growth of *nps1nps6*, *nps2nps6*, and *nps1nps2nps6* strains was reduced to the same extent as observed for *nps6* strains (**Figure 4B**). Iron-dependent restoration of growth was observed for *nps2nps6* strains but not for *nps1nps6* strains. Moreover, increasing concentrations of iron further attenuated growth of *nps1nps2nps6* strains, suggesting that the strains are hypersensitive to iron overload. Overall, these results indicate that *NPS1* is involved in iron metabolism of *F. graminearum*.

SEXUAL REPRODUCTION

Earlier we reported that *nps2* strains of self-compatible *F. graminearum* (*G. zeae*) are defective in ascus/ascospore development, although they show WT perithecium development. In contrast, *nps1* and *nps6* deletion strains are not affected in sexual development. *nps1nps2* strains are like *nps2* strains in terms of fertility (Oide et al., 2007).

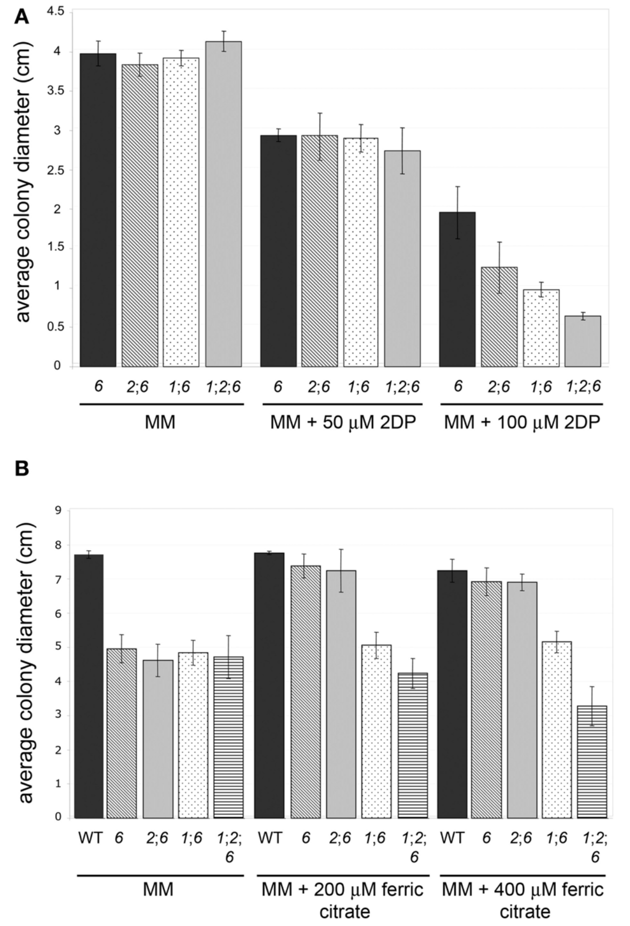

FIGURE 4 | Sensitivity to iron depletion of WT and mutant strains. (A) *nps2nps6*, *nps1nps6*, and *nps1nps2nps6* strains show further increased sensitivity to 2DP, compared to the *nps6* strain. Average colony diameters of 4 day-old cultures on MM with/without 2DP are shown. Strains used are as in **(B)**. Error bars indicate 95% confidence intervals. A statistically significant reduction in growth on MM with 100 μM 2DP was observed for the *nps2nps6* and *nps1nps6* strains, compared to the *nps6* strain. The *nps1nps2nps6* strains show further reduced growth on MM with 100 μM 2DP, compared to the *nps2nps6* or *nps1nps6* strains. No significant difference in growth was observed on MM among all strains. **(B)** Application of iron enhances growth of *nps6* and *nps2nps6*, but not *nps1nps6* and *nps1nps2nps6* strains. Average colony diameters of 5 day-old cultures of WT (strain Gz3639), *nps6* (strain Fgnps6G-1), *nps2nps6* (Gznps2-6-1nps6-17), *nps1nps6* (Gznps1-5-1nps6-3-1), and *nps1nps2nps6* (Gznps2-6-1nps1-5-1nps6-3-2) strains on MM with/without iron are shown. Error bars indicate 95% confidence intervals. A statistically significant reduction in growth on MM was observed for all mutant strains compared to WT. Application of 200 or 400 μM ferric citrate restored WT growth to the *nps6* and *nps2nps6* strains, indicating that the growth defect of these strains on MM is due to iron deficiency. In contrast, application of iron did not enhance growth of the *nps1nps6* strains. Application of 400 μM ferric citrate reduced growth of the *nps1nps2nps6* strains, implying that they are hypersensitive to iron overload.

When the *nps1* deletion was combined with the *nps6* deletion, *nps1nps6* strains were as fertile as WT strains, indicating that *NPS1* and *NPS6* are dispensable for sexual development in the presence of *NPS2* (**Figures 5A,B**). Double *nps2nps6* mutants formed perithecia that were WT in number and in morphology when selfed (**Figure 5A** top, left) but, like *nps2* strains, showed defects in ascus development (**Figure 5A** top, right). However, in contrast to perithecia developed by *nps2* strains, in which immature ascus-like structures are often observed (note **Figure 5B**, top right, arrows), perithecia developed by *nps2nps6* strains were devoid of any ascus-like structures (**Figure 5B** bottom, left). Application of iron restored ability to develop asci and ascospores to both types of strain, but there were fewer asci per perithecium in the *nps2nps6* compared to the *nps2* selfs (**Figures 5C,D**). Taken together, these results indicate that iron metabolism during sexual development is impaired more severely in *nps2nps6*, compared to *nps2* strains.

Although single *nps1* or double *nps1nps6* strains did not show any defect in sexual reproduction, triple deletion *nps1nps2nps6*

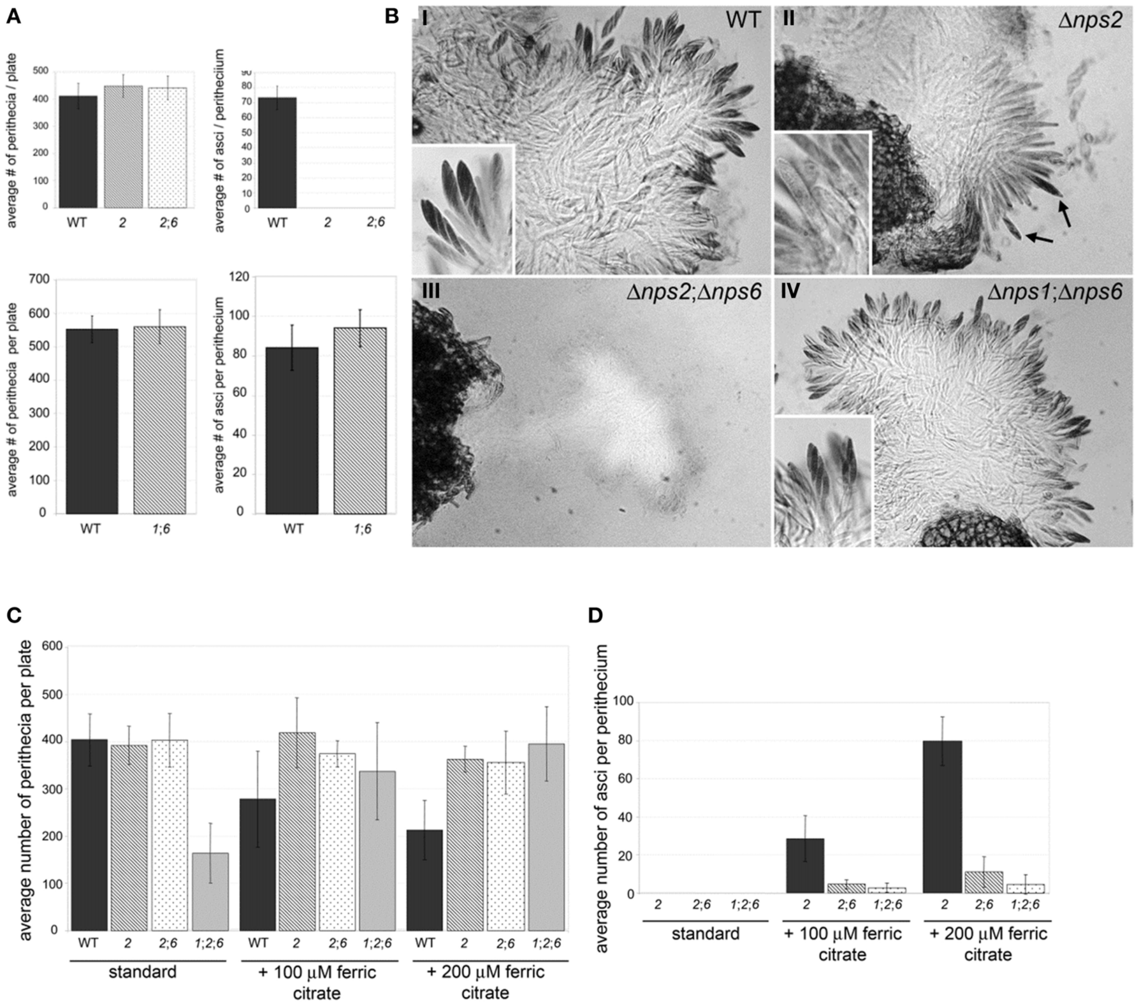

FIGURE 5 | Nps2 is required for sexual reproduction of self-compatible strains but Nps6 plays a role when Nps2 is absent. (A) *nps2nps6* and *nps1nps6* strains form perithecia as well as WT strains do. Top and bottom, left, average number of perithecia per plate is shown for WT, *nps2* (*2*), *nps2nps6* (*26*), and *nps1nps6* (*16*) selfs. Ten replicates were set up for each strain. Error bars indicate 95% confidence intervals. No significant difference was observed in the number of perithecia per plate between WT, and any of the mutant strains. Top, right, *nps2nps6* strains fail to develop asci and ascospores. WT, *nps2,* and *nps2nps6* selfs are shown. As observed in *nps2* strains, *nps2nps6* strains fail to develop asci and ascospores. Bottom, right, *nps1nps6* strains are as fertile as WT. Average number of asci per perithecium is shown for WT and *nps1nps6* selfs. Thirty perithecia were opened for each self. Error bars indicate 95% confidence intervals. No significant difference was observed in the number of asci per perithecium between WT and *nps1nps6* selfs. **(B)** *nps2* strains which develop immature ascus-like structures (II, arrows), however no ascus-like structures were found in perithecia developed in the *nps2nps6* selfs (III, arrows), indicating that sexual development of the *nps2nps6* strain is more severely affected than that of the *nps2* strain. *nps1nps6* strains are as fertile as WT (compare I and IV). Magnification is × 200, inserts × 500. **(C)** *nps1nps2nps6* strains are more affected in sexual development than *nps2nps6* strains. Application of iron restores ability to develop asci and ascospores to *nps2*, *nps2nps6*, and *nps1nps2nps6* strains. Five replicates were set up for each self and for each condition. A statistically significant reduction in the number of perithecia per plate was observed in the *nps1nps2nps6* selfs, compared to WT, *nps2*, and *nps2nps6* selfs. Exogenous application of iron enhanced perithecium development of the *nps1nps2nps6* selfs, indicating that reduced perithecium development is due to iron deficiency. When iron is applied, a reduction in perithecium development was observed in WT selfs. **(D)** Twenty perithecia were opened for each self and for each condition. Error bars indicate 95% confidence intervals. Although no statistically significant difference was observed in the number of asci per perithecium between the *nps2nps6* and *nps1nps2nps6* selfs, the number tended to be smaller in the *nps1nps2nps6* compared to the *nps2nps6* selfs. A statistically significant difference in the number of asci per perithecium was observed between the *nps2* and *nps2nps6* strains, further demonstrating that *nps2nps6* strains are more affected in sexual development.

strains showed a reduction in the number of perithecia developed in selfs, compared to the WT, *nps2*, or *nps2nps6* selfs (**Figure 5C**; **Table 3**). As observed for the *nps2nps6* selfs, the perithecia developed in the *nps1nps2nps6* selfs had WT morphology but lacked any ascus-like structures (not shown). When iron was applied exogenously, the *nps1nps2nps6* selfs formed as many perithecia as the *nps2* or *nps2nps6* selfs (**Figure 5C**), indicating that reduced perithecium development of the *nps1nps2nps6* selfs is attributable to iron deficiency. Application of iron also restored ability to form asci and ascospores to *nps1nps2nps6* strains (**Figure 3D**), but the number of asci/perithecium of the *nps1nps2nps6* selfs was less than that of the *nps2* selfs (**Figure 3D**). No statistically significant difference between the *nps2nps6* and *nps1nps2nps6* selfs was observed in the number of asci/perithecium, although the number of asci tended to be less in the latter compared to the former. The observations indicate that *NPS1* plays a role in maintaining iron homeostasis during sexual development.

VIRULENCE

Deletion of *NPS6* impairs virulence of *F. graminearum* to wheat (Oide et al., 2006), whereas *nps1*, *nps2*, *nps1nps2* strains retain WT virulence to the host (data not shown). A subtle reduction in virulence was observed for *nps2nps6* strains, compared to *nps6* strains (**Figure 6**, compare second from left wheat head sets with second from right sets). Generally, *nps2nps6* strains took longer to complete local infection than did *nps6* strains, although variation was observed among individual infection events (**Figure 7**). Reduced virulence of *nps2nps6* strains was also observed in systemic infection. In general, the spikes challenged by *nps2nps6* strains were less damaged than those challenged by *nps6* strains (**Figure 6A**). Similarly, kernels in spikes infected by *nps2nps6* strains (**Figure 6B**, second from right) were less damaged compared to those in spikes infected by *nps6* (**Figure 6B**, second from left) or WT strains (**Figure 6B**, left). Again, some variation in severity of symptom was observed among individual spikes challenged by the *nps6* and *nps2nps6* strains. Like *nps6* strains, *nps1nps6* strains showed reduced virulence to wheat (**Figures 6A,B**, **7**), however, no significant difference was observed between these strains in either local or systemic infection of wheat.

The triple mutant *nps1nps2nps6* strain almost completely lost virulence to wheat. In point-inoculation assays of spikes, *nps1nps2nps6* strains developed moderate symptoms only occasionally (**Figures 6A,B**, right image sets). Generally, about 80% of the spikes inoculated with the *nps1nps2nps6* strain were asymptomatic, even though the spikes were incubated up to 16 days post-inoculation. These data demonstrate that simultaneous deletion of *NPS1*, *NPS2* and *NPS6* in *F. graminearum* leads to further attenuated virulence on wheat, compared to *nps2nps6* strains.

Table 3 | Selfs set up in this study.

Self	Strains[a]
WT	Gz 3639
nps2	Gznps2-6-1
nps1nps6	Gznps1-5-1nps6-3-1 Gznps1-5-1nps6-5-1
nps2nps6	Gznps2-6-1nps6-6 Gznps2-6-1nps6-17
nps1nps2nps6	Gznps2-6-1nps1-5-1nps6-3-2 Gznps2-6-1nps1-5-1nps6-11-1

[a] *For strains, see **Table 1**.*

DISCUSSION

MALONICHROME PRODUCTION REQUIRES NPS1

We demonstrate here that, in addition to the ferricrocin (intracellular) and TAFC (extracellular) trihydroxamate siderophores biosynthesized by the Nps2 and Nps6 proteins, *F. graminearum* produces a second extracellular siderophore, malonichrome, described in 1980 as an extracellular siderophore of *F. equiseti (F. roseum)*. Biosynthesis of this siderophore did not occur in mutants with disruptions of the *NPS1* gene (**Figure 3**), thus the function of Nps1 can definitively be assigned. Previously, we proposed that the *F. graminearum* Nps1 protein may be partially functional and able to produce dihydroxamates, but not trihydroxamates (Oide et al., 2007). This hypothesis was falsified by our current experiments.

MALONICHROME PLAYS A ROLE IN IRON HOMEOSTASIS

Phenotypic characterization of *nps1nps6* and *nps1nps2nps6* strains implicates malonichrome in iron management in *F. graminearum*. Although no difference was observed in terms of MIC of 2DP, a reduction in growth was observed for *nps1nps6* strains compared to *nps6* strains in the presence of 100 μM 2DP. When cultured on MM, growth of *nps6* and *nps1nps6* strains was reduced to a similar extent compared to WT. In contrast to *nps6* strains, where WT growth is restored by iron (Oide et al., 2006), growth of *nps1nps6* strains was unaffected by exogenous application of iron, suggesting that malonichrome contributes to iron acquisition of *F. graminearum* in the absence of *NPS6*. This idea is further supported by the observation of *nps1nps2nps6* strains, which showed a subtle but reproducible increase in sensitivity to 2DP compared to *nps2nps6* strains. A sexual development phenotype unique to *nps1nps2nps6* strains was also observed. In selfs of these strains, a reduction in the number of perithecia was observed, in addition to complete sterility in terms of ascus/ascospore development. The reduction in numbers of perithecia was not observed in any other mutant strain, including *nps2*, *nps1nps2* (Oide et al., 2007), and *nps2nps6* strains. Application of iron facilitated perithecium formation of *nps1nps2nps6* strains, indicating that starvation for iron accounts for the developmental defect. Overall, our findings provide evidence that malonichrome produced by Nps1 is involved in iron metabolism during reproductive as well as vegetative development of *F. graminearum*.

EXTRACELLULAR SIDEROPHORES PRODUCED BY *NPS6* ARE OF PRIMARY IMPORTANCE FOR IRON ACQUISITION

Here we show that the primary extracellular siderophore, which depends on an intact *NPS6* gene, is TAFC, a cyclic peptide

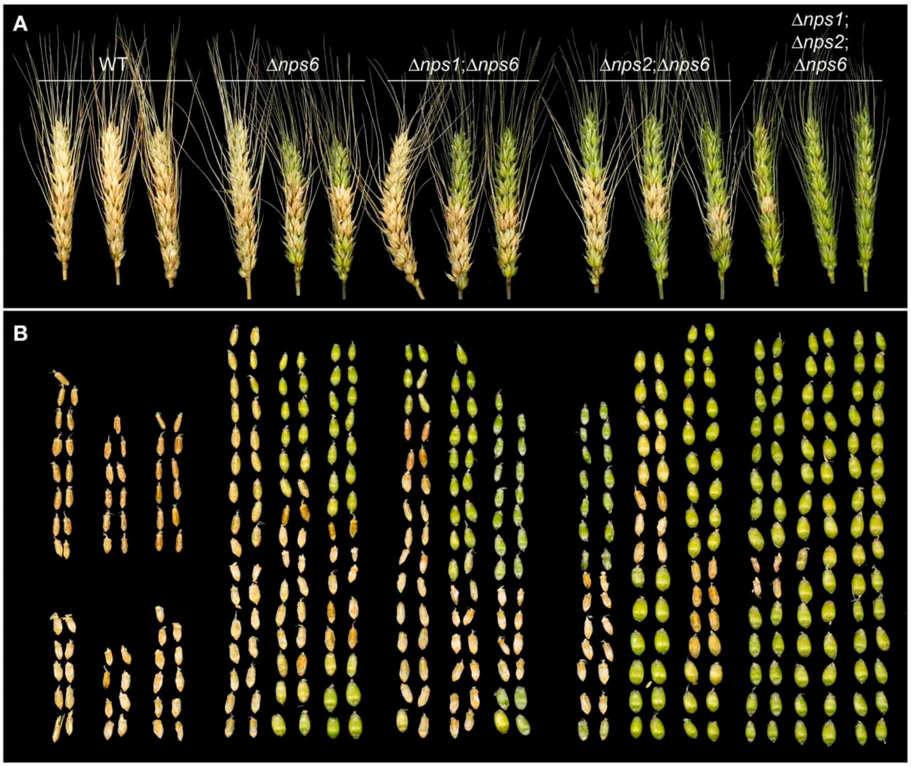

FIGURE 6 | Virulence of WT, *nps6, nps1nps6, nps2nps6,* and *nps1nps2nps6* strains to wheat. (A) Wheat spikes challenged by WT (strain Gz3639), *nps6* (strain Fgnps6G-1), *nps1nps6* (strain Gznps1-5-1nps6-3-1), *nps2nps6* (strain Gznps2-6-1nps6-17), and *nps1nps2nps6* (strain Gznps2-6-1nps1-5-1nps6-3-2), strains, 16 days after inoculation. Spikes inoculated with the *nps2nps6* strain (second set from right) are less damaged than those inoculated with the *nps6* strain (second set from left). Spikes inoculated with the *nps1nps6* strain (middle set) were as severely damaged as those inoculated with *nps6* strains. Spikes inoculated with the *nps1nps2nps6* strain (right set) show further reduced virulence compared to the *nps2nps6* strain and were almost intact. **(B)** Kernels taken from the spikes shown in **(A)**. Relative positions of kernels correspond to those of the spikes shown in **(A)**. Generally, spikes challenged by *nps2nps6* strains contained more healthy, green kernels (note tops of spikes) than did those challenged by *nps6* strains, demonstrating that the *nps2nps6* strain is more reduced in virulence for systemic infection of wheat spikes, than the *nps6* strain is. Kernels in the spikes infected by the *nps1nps6* strains were as severely damaged as those in the spikes infected by the *nps6* strain. Most of the kernels in the spikes infected by the *nps1nps2nps6* strains appeared undamaged. Overall, these data demonstrate that the *nps1nps6* strain is as virulent to wheat spikes as the *nps6* strain is, and that the *nps1nps2nps6* strain is further attenuated in virulence to wheat spikes, compared to the *nps2nps6* strain.

consisting of three N^5-cis-anhydromevalonyl-N^5-hydroxy-N^2-acetyl-l-ornithine residues linked by ester bonds. TAFC binds iron with very high affinity. In order to be able to transfer the tightly bound iron to other carriers (including the intracellular siderophore), the molecule has to be successively destroyed by hydrolysis of the ester bonds (Haas, 2014). We detected the linearized derivative (TAFB) generated by opening of one ester bond in TAFC, and the cleavage product (DAFA) generated by opening two ester bonds and loss of one N^5-cis-anhydromevalonyl-N^5-hydroxy-N^2-acetyl-l-ornithine unit (see **Figure 3**). In agreement with the proposed product/precursor relationship, all of these metabolites depended on the presence of the *NPS6* gene (**Figure 3**). With increasing incubation time under iron depletion conditions, TAFC and its hydrolysis products were replaced by malonichrome in WT and even more pronounced in *nps2* strains. The biological significance of this shift remains a matter of future research. As already pointed out by Emery (1980), malonichrome is less efficient in mediating iron uptake than TAFC in *F. roseum/equiseti*. Given that the impact of *nps1* deletion is apparent only in the absence of *NPS6*, this seems to be the case also for *F. graminearum*.

FERRICROCIN AN EXTRACELLULAR SIDEROPHORE?

The ferrichrome type siderophore, ferricrocin is generally considered to be an intracellular siderophore. Yet, we observed ferricrocin in culture filtrates of WT as well as *nps1nps6* mutants that still carried the WT *NPS2* gene. Thus, designation of ferricrocin as strictly intracellular appears to be an oversimplification. Although ferricrocin is generally recognized as an intracellular siderophore in *A. fumigatus*, trace amounts can be detected in culture supernatants (Schrettl et al., 2007). The mutualistic grass endophyte *Epichloë festucae sidN* gene encodes a siderophore synthetase producing a novel extracellular ferrichrome-type siderophore, epichloënin A (Koulman et al., 2012). Also for *S. pombe*, which contains only one siderophore biosynthesis gene in its genome (Bushley et al., 2008), the resulting hydroxamate-type

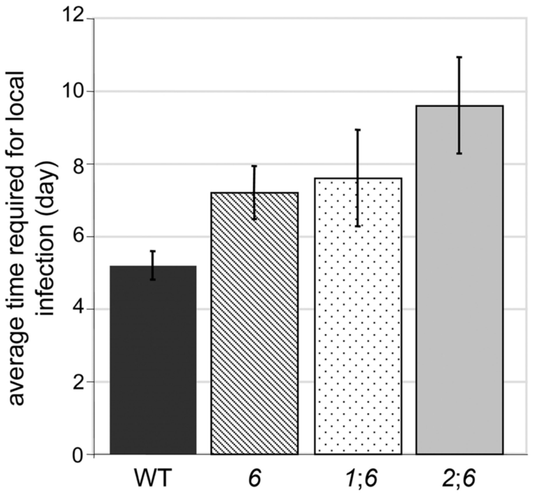

FIGURE 7 | Average time required for completion of local infection of wheat spikelets by WT, *nps6* (*6*), *nps1nps6* (*16*), and *nps2nps6* (*26*) strains. Note that the *nps1nps2nps6* strain was not included, as most of the spikelets inoculated with the *nps1nps2nps6* strains were asymptomatic until the end of infection assays (i.e., 16 days after inoculation). No significant difference was observed in the time for local infection between the *nps6* and *nps1nps6* strains, indicating that the *nps1nps6* strain is as virulent for local infection of wheat spikes as the *nps6* strain is. Generally, it takes longer for the *nps2nps6* strain to complete local infection, compared to the *nps6* strain.

siderophore, ferrichrome, is accumulated both intracellularly and excreted (Schrettl et al., 2004).

An obvious question is if the *F. graminearum* secreted ferricrocin can act as an extracellular siderophore or not. Exogenous application of ferricrocin restores the WT fertility to *nps2* strains (Oide et al., 2007) indicating that *F. graminearum* can take up ferricrocin from extracellular spaces. However, our observation on *nps1nps6* strains casts doubt on the contribution of extracellular ferricrocin to iron uptake. Although this mutant accumulates increased amounts of extracellular ferricrocin compared to other strains, iron supplied exogenously failed to mitigate the growth defect of the *nps1nps6* mutant on MM. Iron needs to be released from siderophores after entering fungal cells. Due to its property as an iron storage molecule, ferricrocin iron release may be strictly regulated, and therefore iron bound to ferricrocin may not be a good source of nutritional iron.

Overall, these findings confirm the previous conclusion that TAFC and its derivatives, the primary extracellular siderophores, play a pivotal role in iron acquisition and infection of the plant host.

EXTRACELLULAR SIDEROPHORES PLAY A ROLE IN IRON METABOLISM DURING SEXUAL DEVELOPMENT

nps1, *nps6*, and *nps1nps6* strains are like WT in terms of sexual development, demonstrating that fusarinines and malonichrome are dispensable for sexual reproduction in the presence of ferricrocin. When ferricrocin synthesis is abolished by deletion of *NPS2*, extracellular siderophore can partly compensate for the lack of intracellular siderophores, as evidenced by the more pronounced defect in ascus/ascospore development and reduced perithecium formation of *nps1nps2nps6* strains compared to *nps2* strains. For this compensatory role for ferricrocin, fusarinines are apparently more important than malonichrome. *nps2nps6* strains are more severely impaired in ascus/ascospore development compared to *nps2* strains, whereas *nps1nps2* strains are indistinguishable from *nps2* strains.

HOW ARE INTRACELLULAR SIDEROPHORES INVOLVED IN FUNGAL VIRULENCE TO PLANT HOSTS?

Loss of intracellular siderophore biosynthesis alone does not affect virulence of *F. graminearum*, *C. heterostrophus*, *A. brassicicola*, or *U. maydis* to each host (Yuan et al., 2001; Oide et al., 2007). A study on *Magnaporthe oryzae*, on the other hand, demonstrated the essential role of intracellular siderophores in virulence to rice (Hof et al., 2007). In the present study, we found that intracellular siderophores play a role in fungal infection of wheat in the absence of *NPS6*. *nps1nps2nps6* strains are more severely impaired in virulence to the host than are *nps2nps6* strains and both are more severely impaired in virulence than *nps6* and *nps1nps6* strains. How are siderophores inside fungal cells involved in virulence to plants? As discussed, ferricrocin by itself is not likely to contribute to acquisition of extracellular iron nutrient, though it is detectable in extracellular spaces.

Previous studies, including our own, demonstrated that intracellular siderophores have roles in certain types of fungal development such as asexual and sexual sporulation (Eisendle et al., 2003, 2006; Oide et al., 2007). Hence, intracellular siderophores may play a role in pathogenesis-related development. WT virulence of *nps2* strains to wheat, however, questions the hypothesis. It has been well documented that iron can be a toxic substrate as well as an important nutrient. In the presence of H_2O_2 or superoxide, intracellular free iron (labile iron) generates highly cytotoxic hydroxyl radicals through the Haber-Weiss/Fenton reaction, and thus, tight regulation of the labile iron pool is critical for aerobic organisms. Iron bound to siderophores does not readily participate in this reaction, hence intracellular siderophores have been proposed to play a role in control of intracellular labile iron pool, in addition to their role in storage of iron nutrient. A study on the *F. graminearum sid1* mutant, however, negates this proposed role (Greenshields et al., 2007). Deletion of *SID1*, encoding a L-ornithine N^5-oxygenase, leads to reduction in labile iron pool under iron-replete conditions compared to WT, indicating that loss of intracellular siderophore synthesis is not likely to promote the Haber-Weiss/Fenton reaction. The finding is in agreement with recent work on *A. fumigatus*, which reports that the major iron detoxification mechanism of this fungus is vacuolar iron storage mediated by the iron transporter CccA but not siderophore-dependent iron sequestration (Gsaller et al., 2012). These observations suggest that the role of ferricrocin as a virulence determinant of *F. graminearum* is unrelated to regulation of labile iron pool.

In *A. fumigatus*, blockage of extracellular siderophore synthesis at different steps has distinct consequences in terms of tolerance to iron depletion and virulence to mice (Schrettl et al., 2007). Deletion of the *NPS6* ortholog *SidD* results in more pronounced defects compared to the *sidF* mutant, in which acylation of N^5-hydroxy-L-ornithine is hampered. We found a similar discrepancy between *C. heterostrophus nps2nps6* and *sidA* mutants, both of which completely lack siderophore synthesis. The *nps2nps6* mutant is more compromised in stress tolerance and virulence to maize compared to the *nps6* mutant (Condon et al., 2014), whereas deletion of *SidA*, encoding a L-ornithine N^5-oxygenase, leads to the *nps6*-like phenotype (Oide and Turgeon, unpublished). A subtle difference in virulence to wheat is suggested also between *F. graminearum nps1nps2nps6* and *sid1* strains. According to the report, the *sid1* mutant is able to initiate WT-like infection within the inoculated spikelet, but fails to spread from spikelet to spikelet (Greenshields et al., 2007). In the case of *nps1nps2nps6* strains, most attempts of local infection are unsuccessful. Although direct comparison between these two strains has not been carried out, the observations suggest that virulence of the *nps1nps2nps6* mutant is more severely affected than the *sid1* mutant. Schrettl et al. (2007) proposed possible toxicity of intermediate metabolites as an interpretation of their observation on *A. fumigatus*. As external iron supply can cure the defects of both *sidD* and *sidF* mutants, the toxicity is likely related to iron deficiency. A study on *A. fumigatus sidC* shows that loss of intracellular siderophore synthesis provokes accumulation of extracellular siderophore breakdown products, which can chelate iron *in vivo* (Gsaller et al., 2012), indicating that intracellular siderophores play a role in sequestration of iron from siderophore breakdown products. In analogy, ferricrocin may retard binding of the precursors of siderophore synthesis to iron resulting in iron

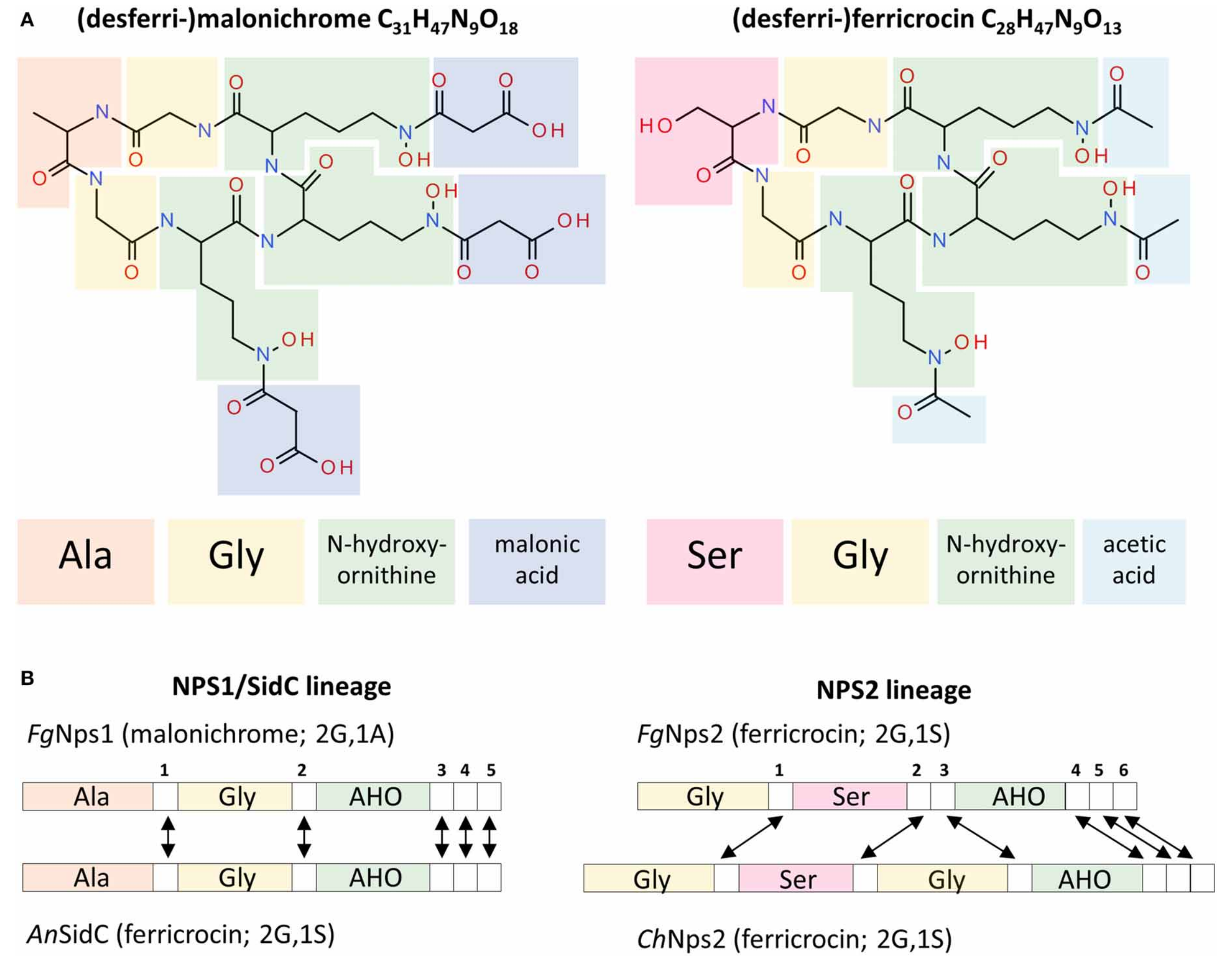

FIGURE 8 | Structures of the malonichrome and ferricrocin siderophores and their associated biosynthetic Nps1 and Nps2 proteins, respectively. (A) Structural features of the desferri-forms of malonichrome and ferricrocin. Building blocks are highlighted in different colors. **(B)** Domain structures of the corresponding Nps1 and Nps2 proteins. Adenylation domains are colored according to the entities introduced into the respective molecules **(A)**. Although the domain structures of *F. graminearum* (*Fg*) Nps1 and *A. nidulans* (*An*) SidC proteins are very similar, different products are generated (malonichrome and ferricrocin, respectively). In contrast, the domain structures of *F. graminearum* and *C. heterostrophus* (*Ch*) Nps2 proteins are different, but the same metabolite is formed (ferricrocin). White numbered boxes symbolize condensation domains. Arrows indicate orthologous domains (Bushley et al., 2008).

deficiency in *F. graminearum*, under the assumption that these metabolites show affinity to iron. The more pronounced defect in virulence to wheat of the *nps2nps6* mutant compared to the *nps1nps6* mutant is consistent with the hypothesis. The increased level of fusarinines and malonichrome in *nps2* strains compared to WT suggests that iron chelation by the metabolic intermediates is compensated by enhanced iron uptake, accounting for WT virulence of *nps2* strains to the host.

STRUCTURAL ASPECTS OF THE *NPS1* AND *NPS2* PROTEINS

Both ferricrocin and malonichrome are produced by combining three AHO units with amino-acids to produce a cyclic hexapeptide (**Figure 8A**). The amino acid between the two glycines in the structure is different; in malonichrome, it is alanine, and in ferricrocin it is serine. A major difference in structure is also found in the decoration of *N*-hydroxy-ornithine by *N*-acylation. Malonichrome contains *N*-hydroxy-ornithine residues aminoacylated with malonate, while in ferricrocin these are acetylated (**Figure 8A**). An N^5-hydroxyornithine:acetyl-CoA-N^5-transacylase (*SidL*) has been identified in *Aspergillus* (Blatzer et al., 2011). The corresponding *F. graminearum* gene FGSG_10426 is not part of the ferricrocin cluster (C33 cluster (FGSG_16474 - FGSG_05374). Interestingly, a gene, FGSG_11027, immediately upstream of *NPS1* (FGSG_11026) is a conserved hypothetical protein with a predicted acyl-CoA *N*-acyltransferase interpro motif (IPR016181). It is co-regulated with *NPS1* as part of the secondary metabolite gene cluster C63 (Sieber et al., 2014). The hypothesis that this may be the relevant malonyltransferase for malonichrome biosynthesis remains to be tested. *NPS1*, in contrast to *NPS2*, is clustered with putative transporter proteins, in agreement with its role as an extracellular siderophore.

From an evolutionary perspective, the genes encoding NRPSs for biosynthesis of ferrichrome type siderophores are among the most conserved *NPS* genes across the fungi (Bushley and Turgeon, 2010; Ohm et al., 2012). Despite this conservation, the *NPS1* and *NPS2* genes though related, are in distinct evolutionary lineages, likely due to an ancestral gene duplication (Bushley et al., 2008). While each encodes an NRPS protein with 3 adenylation domains, Nps2 has 6 condensation domains while the Nps1 protein has 5 (**Figure 8B**). Interestingly, although there are differences in the predicted order of domains (Bushley et al., 2008) between the *C. heterostrophus* and the *F. graminearum* Nps2 proteins, both catalyze the production of ferricrocin. In contrast, the *F. graminearum* Nps1 and the *A. nidulans* SidC proteins are structurally very similar with a conserved domain order, yet produce malonichrome and ferricrocin, respectively. The differences in amino-acids (alanine vs. serine) were predicted from protein modeling in the first two adenylation domains. The AHO domains were predicted by size of substrate considerations (Bushley et al., 2008) and it is not apparent that one would accept exclusively the acidic malonate conjugate or the neutral acetylated N-hydroxy-ornithine derivative.

SIDEROPHORES AS PAMPs?

As evident from the nearly complete loss in virulence of triple mutants, siderophores are needed to fetch iron from the host. The host strategy of withholding iron to limit pathogen growth is well documented in animal systems (Cassat and Skaar, 2013) and also can be an important aspect of plant defense in some cases (Expert, 1999; Nairz et al., 2010). Obviously reductive iron transport which sustains life of the triple *nps1nps2nps6* mutants is insufficient to support growth of the pathogen *in planta*.

It is unclear what the advantage of having two different types of extracellular siderophores is for *F. graminearum*, and whether the shift to a less efficient siderophore (Emery, 1980) can provide a selective advantage. Evolutionary conserved pathogen-derived proteins/molecules, so called pathogen-associated molecular patterns (PAMPs), serve as targets for host surveillance systems (Segonzac et al., 2011; Monaghan and Zipfel, 2012). Studies on the phytopathogenic bacterium *Erwinia chrysanthemi* report that siderophores produced by the bacterium trigger host defense responses (Dellagi et al., 2005). Recent work shows that a fungal siderophore from the hemibiotroph *Colletotrichum graminearum* also activates plant immune responses (Albarouki et al., 2014). One hypothesis is that TAFC, an evolutionary optimized and conserved siderophore is recognized as a PAMP by host plants (Adam et al., 2015). Biosynthesis of a different type of extracellular siderophore might be an outcome of the host-pathogen evolutionary "arms" race, in the same way evolving/acquiring novel effectors is. Yet, even if this scenario is valid, loss of the *NPS6* gene and of ability to produce the high affinity TAFC has an associated high fitness price that can at best be partly compensated by malonichrome.

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Endogenous cross-talk of fungal metabolites

Kevin J. Sheridan, Stephen K. Dolan and Sean Doyle*

Department of Biology, Maynooth University, Maynooth, Ireland

Edited by:
Nancy Keller, University of Wisconsin–Madison, USA

Reviewed by:
Philipp Wiemann, University of Wisconsin–Madison, USA
Daniel Panaccione, West Virginia University, USA

Correspondence:
Sean Doyle, Department of Biology, Maynooth University, Maynooth, Kildare, Ireland
e-mail: sean.doyle@nuim.ie

Non-ribosomal peptide (NRP) synthesis in fungi requires a ready supply of proteogenic and non-proteogenic amino acids which are subsequently incorporated into the nascent NRP via a thiotemplate mechanism catalyzed by NRP synthetases. Substrate amino acids can be modified prior to or during incorporation into the NRP, or following incorporation into an early stage amino acid-containing biosynthetic intermediate. These post-incorporation modifications involve a range of additional enzymatic activities including but not exclusively, monooxygenases, methyltransferases, epimerases, oxidoreductases, and glutathione S-transferases which are essential to effect biosynthesis of the final NRP. Likewise, polyketide biosynthesis is directly by polyketide synthase megaenzymes and cluster-encoded ancillary decorating enzymes. Additionally, a suite of additional primary metabolites, for example: coenzyme A (CoA), acetyl CoA, *S*-adenosylmethionine, glutathione (GSH), NADPH, malonyl CoA, and molecular oxygen, amongst others are required for NRP and polyketide synthesis (PKS). Clearly these processes must involve exquisite orchestration to facilitate the simultaneous biosynthesis of different types of NRPs, polyketides, and related metabolites requiring identical or similar biosynthetic precursors or co-factors. Moreover, the near identical structures of many natural products within a given family (e.g., ergot alkaloids), along with localization to similar regions within fungi (e.g., conidia) suggests that cross-talk may exist, in terms of biosynthesis and functionality. Finally, we speculate if certain biosynthetic steps involved in NRP and PKS play a role in cellular protection or environmental adaptation, and wonder if these enzymatic reactions are of equivalent importance to the actual biosynthesis of the final metabolite.

Keywords: NRPS, gliotoxin, natural products, secondary metabolites, redox homeostasis, siderophores, systems biology, PKS

INTRODUCTION

Non-ribosomal peptides (NRP) and polyketides are produced by both fungi and bacteria, via non-ribosomal peptide synthesis (NRPS) and polyketide synthesis (PKS), respectively, and are often associated with aiding the organisms to adapt to, or survive in, a hostile environment such as the presence of competing microorganisms, nutrient limitation, and protection against insect immune systems (Meier and Burkart, 2009; Wiemann and Keller, 2014). Although many NRPs and polyketides have known functions [e.g., siderophores (iron acquisition) and antibiotics (bacterial inhibition)] and biomedical applications (e.g., cyclosporin, lovastatin, and mycophenolic acid), the true biological function of many NRPs and polyketides identified to date is unknown, and abrogation of the biosynthesis of certain NRPs, or polyketides, may be without major consequence for the organism (O'Hanlon et al., 2011; Hansen et al., 2012; Steinchen et al., 2013).

With respect to the actual processes, NRPS in fungi is mediated by a combination of large multi-functional enzymes known as NRP synthetases, which include adenylation, thiolation, condensation, and sometimes tailoring domains. NRP synthetases require post-translational modification via 4′-phosphopantetheinylation, mediated by 4′-phosphopantetheinyl transferase (4′-PPTase), to yield the active holo-NRP synthetase. This modification, which requires coenzyme A (CoA) as the 4′-phosphopantetheine source, requires that each thiolation domain within an NRP synthetase is modified at a specific serine residue (Stack et al., 2007). In addition, so-called decorating enzymes or domains are responsible for the modification of NRP biosynthetic intermediates, which may be either tethered to, or released from, the NRP synthetase during the modification process. Likewise, polyketides are biosynthesized from acyl CoA precursors (e.g., malonyl CoA) by multi-modular enzymes consisting of essential ketosynthase, acyl carrier protein and acyltransferase, amongst other, domains (Weissman, 2014). As shown for *Streptomyces* spp. intracellular ATP levels may also affect NRP production in fungi (Li et al., 2008). Unlike PK synthases, NRP synthetases require ATP for NRPS.

Both proteogenic and non-proteogenic amino acids (e.g., ornithine) may be essential precursor substrates for NRP formation and during NRPS are conjugated together generally via peptide bond formation. Moreover, substrate amino acids can be modified either prior to, or during, NRPS (Thykaer and Nielsen, 2003; Chocklett and Sobrado, 2010). In addition, many other cellular components more commonly associated with primary metabolism are required for NRPS and PKS. Amongst others, these include *S*-adenosylmethionine (SAM), isoprenyl, and mevalonyl moieties, nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH), malonyl CoA, and acetyl CoA (Davis et al., 2011; Scharf et al., 2011; Haas, 2012; Yasmin et al., 2012). This suggests significant interplay between what is currently considered to be primary and secondary metabolism and that essential re-consideration must be given to the integration of these two historically defined discrete systems.

In fungi, the biosynthesis of a specific NRP is generally encoded by genes located within a gene cluster, which are often located in the sub-telomeric regions of chromosomes (McDonagh et al., 2008). The activity of these secondary metabolite (SM) gene clusters is controlled by local chromatin structure, which is effected via histone post-translational modification (e.g., methylation or acetylation). The influence of histone modifying enzymes on SM production was first reported for sterigmatocystin (ST) production and gene regulation in *A. nidulans*. The authors uncovered that histone deacetylase mutant Δ*hdaA* bypassed the requirement for the general SM activator LaeA, resulting in overproduction of the subtelomeric metabolites ST and penicillin (Shwab et al., 2007). In the endophytic filamentous fungus *Epichloë festucae* the gene clusters responsible for the production of bioprotective lolitrems and ergot alkaloids were shown to be derepressed following deletion of the genes encoding either the H3K9- (ClrD) or H3K27- (EzhB) histone methyltransferases. A further enhancement of cluster expression was evident when both of these methyltransferases were deleted (Chujo and Scott, 2014). Indeed, the production of many SM in *Aspergillus spp.* is controlled by a methyltransferase, *laeA*, and orthologs of this gene are found in many fungi (Bok and Keller, 2004). Interestingly, LaeA has been shown to counteract the establishment of heterochromatic marks thus activating SM production. Deletion of *laeA* in *A. nidulans* resulted in highly elevated H3K9 methylation levels suggesting that LaeA counteracts H3K9 trimethylation and thus heterochromatin formation at the ST locus (Reyes-Dominguez et al., 2010). The Velvet complex, which is comprised of the light-dependent regulators VeA and VelB, also regulates SM production, and serves to coordinate SM production and fungal development. It is now clear that the VelB–VeA–LaeA complex coordinates fungal sexual development and secondary metabolism (Bayram et al., 2008).

Thus, it appears that a truly phenomenal degree of cross-talk exists between so-called primary and secondary metabolism, also involving genetic regulation, to facilitate NRP and polyketide, and indeed other SM, production (**Figure 1**). Primary metabolism is required to provide the essential biosynthetic precursors for NRPS, many NRPs share substrate amino acids and additional co-substrates, chromatin structure apparently controls much SM gene cluster expression, and evidence is also emerging that NRPS pathways interact such that alterations in the biosynthesis of specific NRPs may impact on the production of apparently unrelated metabolites (O'Keeffe et al., 2014; Wiemann et al., 2014). This review is intended to highlight some of the metabolite cross-talk which has been identified to date, and to suggest possible future directions which may further illuminate our understanding of this complex molecular vista- which may, in part, necessitate the development of new models to augment current paradigms of distinct levels of cellular metabolism.

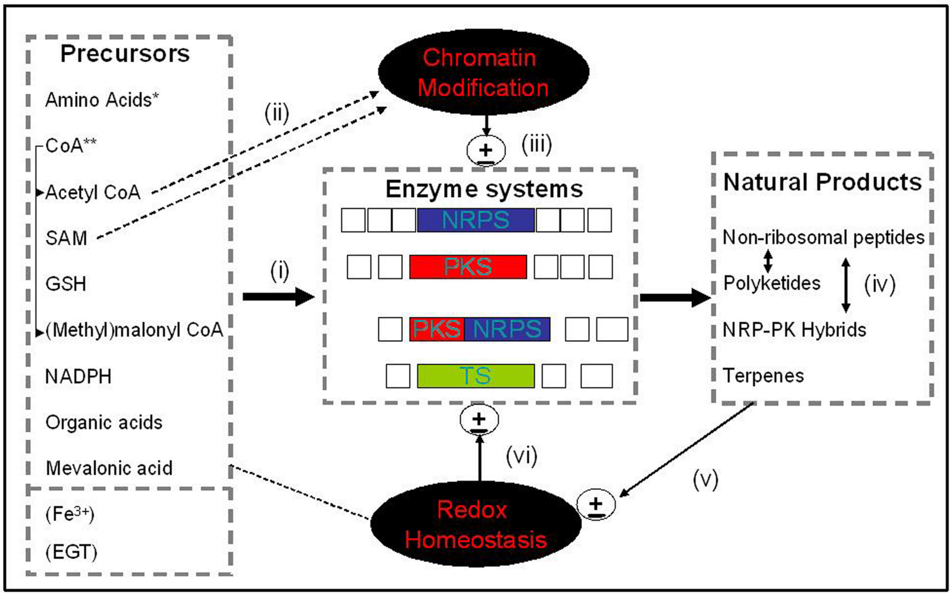

FIGURE 1 | Systems overview. An integrated depiction of how precursors from 'primary metabolism' (i) feed into enzyme systems [NRPS, PKS, NRPS-PKS, and terpene synthases (TS)] which effect natural product/secondary metabolite biosynthesis and, in part, (ii) affect both chromatin structure (e.g., Acetyl CoA and SAM), and cellular redox status (e.g., GSH, EGT, and Fe^{3+}). Chromatin structure, in turn, can (iii) regulate gene cluster expression and metabolite biosynthesis. (iv) Resultant natural products can interact synergistically to enable cellular function (e.g., iron uptake, antioxidant adaptation), or (v) influence cellular redox homeostasis which in turn can (vi) impact on gene cluster expression. Note: Fe^{3+} and EGT are not 'precursors' *per se*, however, they either bind to (e.g., Fe^{3+}-siderophores), or their biosynthesis is influenced by, natural products. *amino acids, proteogenic or non-proteogenic; **CoA, Coenzyme A, a substrate or co-factor for natural product biosynthesis; EGT, Ergothioneine; Open boxes, ancillary enzymes involved in PKS, NRPS, or TS.

PRIMARY METABOLITE INVOLVEMENT IN SM BIOSYNTHESIS

CoA AND ACETYL CoA

It is essential to consider the sources of primary metabolites used during fungal NRPS and PKS in order to begin to see why there may be interactions between apparently independent biosynthetic pathways (**Figure 1**). CoA is somewhat unique, as it functions (i) as a direct substrate which facilitates NRP synthetase and PK synthase activation via 4′-PPTase, and (ii) as malonyl CoA in PKS, and, as acetyl CoA, as the source of acetyl groups for NRPS and histone modification (Stack et al., 2009; Jeon and Lee, 2014; Weissman, 2014). To enable CoA biosynthesis, L-valine and L-aspartate are initially converted to pantoate and β-alanine, respectively, followed by condensation to form pantothenate (vitamin B5), which in turn is converted to CoA by the action of a multi-step biosynthetic pathway involving condensation to ADP (Spry et al., 2008; Chen et al., 2014a; **Figure 1**). Cytosolic acetyl CoA is provided by β-oxidation of fatty acids, from acetate through the action of acetate synthetase (Acs1), and via the ATP-citrate lyase (Acl1) which uses citrate as a substrate (Griffiths et al., 2012). Indeed it has been demonstrated that Acl1 and 2 function to regulate cytosolic acetyl CoA levels in *A. niger* which in turn affects growth and development (Chen et al., 2014a). Thus, it is clear that provision of two essential biomolecules (CoA and acetyl CoA) for fungal NRPS and PKS requires close interplay with primary metabolic processes. Interestingly, although studies directly pertaining to NRPs appear rare, it has been demonstrated that biosynthesis of the polyketide lovastatin in *A. terreus* is enhanced by media supplementation with calcium D-pantothenate, amongst other B vitamins (Bizukojc et al., 2007).

SAM

S-adenosylmethionine is the major source of methyl groups for cellular reactions involving methylation, and is directly synthesized via the action of SAM synthetase (Sauter et al., 2013). In fungi, the methionine cycle enables SAM biosynthesis, whereby L-homocysteine is converted to L-methionine via methionine synthase, SAM is formed in turn via SAM synthetase which requires ATP (Sieńko et al., 2009). Cellular methylation reactions (e.g., NRPS and PKS) then consume SAM to produce *S*-adenosylhomocysteine (SAH), which in turn is converted to L-homocysteine, thereby completing the methionine cycle (Liao et al., 2012). In *A. nidulans*, a SAM synthetase, SasA, has been demonstrated to affect SAM availability which is important for the production of ST, but also may play a role in coordinating fungal secondary metabolism and development (Gerke et al., 2012). These authors demonstrated SasA interaction with histone 2B via TAP-tag technology and LC-MS, and suggested that this interplay may facilitate epigenetic control via methylation. SAM availability is essential for the biosynthesis of a range of polyketides and NRPs including gliotoxin, where it acts as a methyl source for the *N*-methyltransferase GliN (Scharf et al., 2014), and the negative regulator of gliotoxin biosynthesis GtmA, which attenuates gliotoxin biosynthesis via SAM-dependent *bis*-thiomethylation of dithiolgliotoxin (Dolan et al., 2014). SAM is also the provider of methyl groups for the biosynthesis of many other NRPs and polyketides such as butyrolactone III (Guo et al., 2013a). Forseth et al. (2011) identified a plethora of methylated biosynthetic intermediates/shunt metabolites associated with gliotoxin biosynthesis which allowed the authors to speculate about the complex reactions required within this metabolic pathway.

Methionine and SAM are sulfur-containing metabolites, and it has recently been demonstrated that the bZIP transcription factor MetR plays an important role in sulfur assimilation in *A. fumigatus* (Amich et al., 2013). While these authors elegantly demonstrated that MetR functionality was essential for growth on inorganic sulfur sources, it was observed to be less important for regulating genes involved in methionine metabolism. Nonetheless, they identified a key interaction with iron homeostasis, whereby under sulfur-limiting conditions, genes involved in siderophore biosynthesis (*sidA*), siderophore transport (*mirB*), and iron regulation (*hapX*) underwent increased expression in *A. fumigatus* Δ*metR* – although sufficient Fe^{3+} was available in the media. Moreover, significantly elevated levels of the intracellular siderophore, ferricrocin, a NRP, were also observed which was necessary to chelate elevated intracellular Fe^{3+} to prevent cellular damage in Δ*metR*. Given the essential role of Fe–S cluster proteins in energy generation, this regulatory cross-talk, which involved altered iron-sensing due to defective sulfur assimilation, and led to altered NRP [ferricrocin (FC)] levels significantly underpins the integrated nature of cellular metabolism in fungi.

GSH

The biosynthesis of gliotoxin, acetylaranotin, and related epipolythiodioxopiperazine (ETP) compounds requires biosynthetic intermediate sulfurization and it has been established that GSH is this sulfur source (Davis et al., 2011; Scharf et al., 2011; Guo et al., 2013b). Furthermore, Atanasova et al. (2013) have noted elevated expression of genes predicted to be involved in GSH formation, possibly as a precursor to enable the biosynthesis of gliotoxin in *Trichoderma virens*. In any case, during gliotoxin biosynthesis a gene cluster-encoded glutathione *S*-transferase (GST) mediates GSH conjugation to a highly reactive acyl imine intermediate which results in a *bis*-glutathionylated product, which is subsequently processed by a suite of enzymes, initially by a γ-glutamyl cyclotransferase (GliK in the case of gliotoxin biosynthesis) to the final product (Davis et al., 2011; Gallagher et al., 2012; Scharf et al., 2011, 2013). Thus, a key cellular reductant, which can also undergo oxidation to the GSSG form to maintain cellular redox homeostasis, is an essential substrate for NRP biosynthesis. Indeed, it is interesting to speculate how, and why, fungi utilize an antioxidant (GSH) to generate an alternative metabolite, gliotoxin, with redox-active properties. Perhaps the ability of fungi to effect gliotoxin (and related ETP) but not GSH secretion is in part responsible for this apparent puzzling situation.

ERGOTHIONEINE

Ergothioneine (EGT) is a sulfurized and tri-*N*-methylated L-histidine derivative, and functions as an antioxidant due to its high redox potential (Fahey, 2013). It is produced by non-yeast fungi, with the gene EGT-1 found in all fungal phyla, except the Saccharomycotina subphylum (Genghof, 1970; Jones et al., 2014).

EGT exists as a tautomer between its thione and thiol forms, however, it is predominantly found in the thione form at physiological pH (Carlsson et al., 1974). While most naturally occurring thiols have a redox potential ranging from −0.2 V and −0.32 V, EGT has a redox potential of −0.06 V, which means that it is less susceptible to auto-oxidation compared to GSH (Jocelyn, 1972). In *Neurospora crassa*, ergothioneine has been proposed to protect conidia against oxidative stress (Bello et al., 2012). Although not strictly a NRP, recent work has revealed further requirements for provision of SAM and γ-glutamylcysteine (and molecular O_2) to enable ergothioneine biosynthesis in Mycobacteria (Seebeck, 2010). In *A. fumigatus*, Gallagher et al. (2012) observed that EGT levels increased significantly when production of the NRP gliotoxin was abrogated. The significance of this correlation, if any, has yet to be elucidated. In other fungi (*Schizosaccharomyces pombe*), the multi-functional enzyme Egt-1, which comprises a methyltransferase domain and a hercynylcysteine sulfoxide synthase activity, requires both SAM and L-cysteine to enable ergothioneine biosynthesis – whereby SAM provides methyl groups and L-cysteine is the sulfur source (Pluskal et al., 2014). As noted, Gallagher et al. (2012) were the first to demonstrate that deletion of *gliK* from *A. fumigatus* not only abrogated gliotoxin biosynthesis, but also resulted in significantly elevated levels of intracellular ergothioneine. Although these authors noted a significantly elevated sensitivity to H_2O_2-induced oxidative stress, the precise mechanistic details of the interaction between these redox-active metabolites is presently unclear, and future work is likely to dissect the nature of this cross-talk. However, it is not inconceivable that ergothioneine biosynthesis is increased to, in part, compensate for the abrogation of gliotoxin biosynthesis at a specific biosynthetic step.

NAPDH

Nicotinamide adenine dinucleotide phosphate requirement as a reductant in NRPS and PKS is well-established (Manavalan et al., 2010; Wilson et al., 2013) and recent work in mammalian cell lines (Fan et al., 2014) has demonstrated that both the oxidative pentose phosphate pathway and one-carbon metabolism are important cellular sources of NADPH. While it has been reported that metabolic engineering of the pentose phosphate pathway – consequent to increased abundance of 6-phosphogluconate dehydrogenase – can lead to enhanced cellular NADPH availability in *A. niger* (R Poulsen et al., 2005), to our knowledge little assessment of alternative sources of NADPH in fungi has been forthcoming. Thus, in future assessments of integrated fungal metabolism, it is possible that relevant interactions between NRPS, PKS, and availability of alternative reducing power require evaluation.

AMINO ACIDS

A link between amino acid biosynthesis and secondary metabolism has been described in the ascomycetous plant pathogen *Leptosphaeria maculans*. The NRP sirodesmin PL, an ETP, which is the causal agent of blackleg disease of *Brassica napus*, has been shown to be regulated by the cross pathway control gene *cpcA*, which in turn regulates amino acid biosynthesis. During amino acid starvation, amino acids are diverted from sirodesmin biosynthesis into amino acid biosynthesis, and a silenced *cpcA* mutant was shown to produce significantly higher levels of sirodesmin PL after prolonged amino acid starvation compared to the wild-type strain. However, it has yet to be established if this effect is mediated directly or indirectly through the sirodesmin pathway specific transcriptional factor, *sirZ* (Elliott et al., 2011). The rice pathogen *Fusarium fujikuroi* produces the NRP apicidin F under nitrogen-sufficient conditions through the global regulator AreB (Niehaus et al., 2014). Similarly, production of the *F. fujikuroi* PKS/NRPS-derived mycotoxin fusarin C is induced by an excess of nitrogen (Wiemann et al., 2010). Organic acids such as fumarate, anthranilic acid, 4-hydroxyphenylpyruvate, and indole-3-pyruvate also are incorporated into NRPs in fungi (Wackler et al., 2012; Steinchen et al., 2013; Walsh et al., 2013).

RIBOSOMAL PEPTIDE SYNTHETIC PATHWAY (RiPS)

The discovery of the biosynthetic mechanism, via a ribosomal peptide synthetic pathway (RiPS), for ustiloxin B in *A. flavus* by Umemura et al. (2014) can be thought to further contribute to the blurring distinction between primary and secondary metabolism. Ustiloxin B is a modified YAIG tetrapeptide, conjugated to norvaline via L-tyrosine. However, it appears that YAIG is derived from a proteinaceous precursor, followed by extensive enzymatic conversion, which is encoded by a discrete gene cluster, to ustiloxin B. Of special interest is the presence of a S atom in ustiloxin B, which is coincident with the presence of a GST, cysteine desulfurase and γ-glutamyl transpeptidase in the cognate gene cluster (Umemura et al., 2014). Although not noted by the authors, it is tempting to speculate that a similar biochemistry to that which has evolved to enable ETP biosynthesis in fungi, is also operational in the biosynthesis of a RiPS product, ustiloxin B, in an NRPS-independent manner. This work is the first example of RiPS in Ascomycetes.

METABOLITES AND NON-PROTEOGENIC AMINO ACIDS – THE ORNITHINE EXEMPLAR

Siderophores are NRPs secreted by fungi and bacteria that scavenge free ferric iron (Fe^{3+}) in the immediate environment to facilitate its transport into the cell, and this mechanism is deployed by both animal and plant pathogens (*Cochliobolus heterostrophus*) to acquire iron (Haas, 2012; Zhang et al., 2013). Because Fe^{3+} is required for primary metabolism, it is clear that these NRPs also act as a nexus between both primary and secondary functionalities within fungi. Since excess intracellular Fe^{3+} is deleterious, intracellular siderophores also exist to maintain intracellular Fe^{3+} homeostasis and prevent Fenton reaction oxidative damage (Eisendle et al., 2006). In *Aspergillus spp.* such as *A. nidulans* and *A. fumigatus*, triacetylfusarinine C (TAFC) and fusarinine C (FusC) are secreted to acquire Fe^{3+}, while FC is the key intracellular siderophore which effects Fe^{3+} storage. It is notable that in *A. niger*, TAFC is absent and coprogen B and ferrichrome are the main secreted siderophores, with the latter comprising the main intracellular siderophore (Franken et al., 2014). Biosynthesis of siderophores necessitates interaction between enzyme systems at multiple levels. Firstly, one of the key non-proteinaceous amino acids, L-ornithine, essential for siderophore biosynthesis, must be converted to *N*-hydroxy-L-ornithine via the action of ornithine monooxygenase, SidA

(Schrettl et al., 2004; Hissen et al., 2005). Interestingly, Beckmann et al. (2013) have demonstrated that mitochrondrially derived ornithine is primarily utilized for siderophore biosynthesis – indicating a localization-type cross-talk. These authors also demonstrated metabolite cross-talk between siderophore and polyamine formation, whereby ornithine acts a co-substrate for SidA and ornithine decarboxylase (ODC), the latter enzyme acting to provide biosynthetic precursors for polyamine biosynthesis. Secondly, mevalonate, a biosynthetic intermediate in ergosterol biosynthesis, is also required for siderophore biosynthesis and is enzymatically converted to anhydromevalonyl-CoA to facilitate incorporation into FusC and TAFC (Schrettl et al., 2007). Finally, in *A. fumigatus*, FusC is converted to TAFC by enzyme-mediated acetylation via the action of SidG in an acetyl CoA-dependent reaction. The cross-talk between ergosterol and siderophore biosynthesis has proven to be especially significant, as it has been demonstrated that inhibition of HMG CoA reductase, the enzyme responsible for mevalonate production (**Figure 1**), also attenuates siderophore biosynthesis, and virulence in a murine ocular keratitis model system, (Leal et al., 2013). Moreover, this inhibitory effect is enhanced by utilization of iron chelators to further inhibit iron uptake by fungi. Thus, interfering with metabolite cross-talk systems may bring therapeutic benefits.

METABOLITE CROSS-TALK AND OXIDATIVE STRESS

As aerobic organisms, fungi are subject to oxidative stress due to the production of reactive oxygen species (ROS) as side-products of metabolic pathways. Almost 90% of ROS in aerobic organisms is produced through the electron transport chain, while oxidases such as NADPH oxidase also contribute toward the ROS content of the cell (Takemoto et al., 2007; Starkov, 2008). Control of ROS levels is important as cellular components can be modified by ROS, particularly enzymes containing [Fe–S]-clusters and active thiol groups. Oxidative stress, whereby the ROS concentration rises above tolerable levels, thereby leading to deleterious modification of cellular components, can interfere with cellular metabolism and regulatory pathways (Lushchak, 2011). Enzymes such as catalases, glutathione peroxidases, and superoxide dismutases actively protect against oxidative stress, while fungi also produce antioxidant molecules such as α-tocopherols, ascorbic acid, carotene and GSH (Halliwell and Gutteridge, 2007). SM also interacts with ROS in many ways. Some SMs act as antioxidants (EGT, as described above) or otherwise protect against oxidative stress (Bello et al., 2012), while others use oxidative stress as a signal to initiate biosynthesis, while some SMs, like ETPs, enact toxic effects through ROS generation and redox reactions (Gardiner et al., 2005a).

Epipolythiodioxopiperazines are categorized by a signature disulfide bridge (Gardiner et al., 2005a) and gliotoxin is the best studied of the ETPs. It is produced by *A. fumigatus*, and its biosynthesis is mediated by the products of the *gli* gene cluster, with the NRP synthetase GliP starting the pathway through the fusion of L-phenylalanine and L-serine (Balibar and Walsh, 2006), while many of the other genes encode decorating enzymes. Gliotoxin toxicity is intrinsically linked to its disulfide bridge, and redox cycling between the disulfide and dithiol forms allows for the production of ROS. The ability of gliotoxin to form mixed disulfide bonds with proteins, resulting in their inactivation, is another example of the redox-dependant action of the disulfide bridge (Waring et al., 1995). *A. fumigatus* in part detoxifies gliotoxin by closing the disulfide bride via the oxidoreductase GliT, which ‘disarms’ the toxic effects of the molecule, although a cryptic reductase activity of GliT has also been noted (Scharf et al., 2010; Schrettl et al., 2010). Another ETP, sporidesmin, effects toxicity in a similar manner to gliotoxin (Middleton, 1974; Munday, 1982). The redox activity of gliotoxin does not appear to be limited to anti-microbial and virulence-related activity upon secretion and intracellular gliotoxin appears to play a role in maintaining the redox state of the cell. Owens et al. (2014) demonstrated that gliotoxin alleviates oxidative stress caused by H_2O_2 exposure in *A. fumigatus* which suggests that gliotoxin is not merely a toxin, but may play an important role in redox homeostasis in *A. fumigatus.* Thus, this SM appears to mediate cross-talk between ROS presence and the global response to ROS in fungi (**Figure 1**).

Reverberi et al. (2010) have also highlighted a link between production of SMs and oxidative stress. Interestingly, oxidative stress in *A. parasiticus* up-regulates aflatoxin biosynthesis (Jayashree and Subramanyam, 2000; Narasaiah et al., 2006; Reverberi et al., 2008). Conversely, aflatoxin biosynthesis is inhibited in response to antioxidant activity (Reverberi et al., 2005). The aflatoxin regulatory gene, *aflR*, encodes a $Zn(II)_2Cys_6$ DNA binding protein serves to activate gene expression encoding enzymes for the ST/aflatoxin biosynthetic pathway (Yu et al., 1996). The discovery of putative binding sites for the Yap1 ortholog, AP1, in the *aflR* promoter region further strengthens this suggested relationship (Reverberi et al., 2008). A similar relationship between oxidative stress and SM production can be seen in *Fusarium graminearum.* Addition of H_2O_2 to liquid cultures increased the accumulation of the type B trichothecenes, deoxynivalenol, and 15-acetyl-deoxynivalenol, while the addition of catalases down-regulated expression of the genes responsible for their biosynthesis (Ponts et al., 2006, 2007). Similar results showing antioxidants reducing SM biosynthesis have been encountered in *A. flavus* with aflatoxin (Chipley and Uraih, 1980), *F. verticillioides* with fumonisin B_1 (Beekrum et al., 2003), *A. ochraceus* with ochratoxin A (Fanelli et al., 2004) and *Penicillium expansum* with patulin (Sanzani et al., 2009; Tolaini et al., 2010). This demonstrates cross-talk between oxidative stress defense and SM biosynthesis across a range of fungi, producing several apparently unrelated SMs.

Siderophores, the iron-scavenging NRPs described above, have an essential role in oxidative defense. Excess intracellular iron can result in oxidative stress through Fenton reaction (Papanikolaou and Pantopoulos, 2005), while conversely, heme is essential for the function of many peroxidases (Conesa et al., 2002), an important family of enzymes in the detoxification of hydrogen peroxide. Thus it is important for fungi to manage iron levels carefully. Eisendle et al. (2006) demonstrated that oxidative stress causes an up-regulation of the intracellular siderophore ferricrocin in *A. nidulans.* Such an increase is also seen when exposed to iron excessive conditions, though it is unclear if this is due to the iron itself, or the resulting iron mediated oxidative stress on the cell.

Deletion of the siderophore synthetase *sidC* halts production of ferricrocin, resulting in multiple phenotypes, such as inefficient iron utilization, delayed germination in iron-replete conditions and elimination of cleistothecia formation in homothallic conditions. In addition to these phenotypes, the conidia of the *sidC* mutant display sensitivity to H_2O_2. A similar relationship role of siderophores in oxidative defense can be seen in *A. fumigatus*. Schrettl et al. (2007) investigated several siderophore biosynthetic gene deletions. The siderophore null mutant, Δ*sidA* demonstrated severe sensitivity to H_2O_2, while absence of either intracellular or extracellular siderophores separately resulted in a milder sensitivity. This suggests that both intra- and extra- cellular siderophores have a role in mediating oxidative damage and suggests redundancy in the system. Cross-talk between ROS detoxification and siderophores is not limited to the *Aspergillus* species. In *Alternaria alternata*, deletion of NPS6, a NRPS essential for extracellular siderophore biosynthesis, results in increases sensitivity to H_2O_2. In addition, expression of NPS6 in *A. alternata* is regulated by NOX, YAP1, and HOG1, genes involved in oxidative stress defense (Chen et al., 2014b). Deletion of NPS6 *in Cochliobolus miyabeanus, Fusarium graminearum*, and *Alternaria brassicicola* also resulted in increased sensitivity to oxidative stress (Oide et al., 2006).

Medentsev and Akimenko (1998) conducted a large scale investigation into naphthoquinones, redox active SMs produced by a large variety of filamentous fungi. The study demonstrated that many of these (fusarubin, anhydrofusarubin, javanicin, anhydrojavanicin, bostricoidin, norjavanicin, flaviolin, and 2-hydroxyjuglone) displayed activity against bacteria, yeast, fungi, and plant cells. The mode of action of these molecules was through interference with the host respiration system. By accepting the reducing equivalents from redox enzymes and transferring directly to oxygen, resulting in the respiratory chain being bypassed. In addition, naphthoquinones can act through generation of superoxide radicals and inhibition of glutathione reductases.

ENZYME AND CLUSTER CROSS-TALK

In addition to the clear interactions between primary and secondary metabolism described above, there are also several examples of cross-talk amongst gene clusters which are responsible for the synthesis of fungal SMs (**Figure 1**). Over-expression of the silent *inp* putative secondary metabolism cross-pathway regulator gene (*scpR*) in *A. nidulans* resulted in the overproduction of both the *inp* gene cluster and also production of the polyketide, asperfuranone. As no link had previously been described between these unrelated metabolites, this work highlighted some additional complexity of regulatory cross-talk in fungal secondary metabolism (Bergmann et al., 2010). Further recent findings are changing previously held views of distinct SM clusters encoding for single class of, or closely related, SMs. A FAD-dependent monooxygenase (FqzB) which is encoded by the fumiquinazoline biosynthetic pathway in *A. fumigatus* was also shown to catalyze spiro-carbon formation in the indole alkaloid spirotryprostatin A via an epoxidation route. The authors speculated that these interactions between unrelated fungal SM-encoding cluster genes may be a strategy of natural product producers to generate structural diversity (Tsunematsu et al., 2013).

FUMIGACLAVINES AND FUMIQUINAZOLINES

Fumigaclavine C, an ergot alkaloid, was demonstrated to aid in oxidative stress defense in *A. fumigatus* whereby deletion of either of the NRP synthetase genes *pesL* or *pes1* resulted in abrogation of the metabolite (O'Hanlon et al., 2012). Loss of fumigaclavine C in *A. fumigatus* resulted in H_2O_2 sensitivity, suggestive of its role in oxidative stress defense. It should be noted that, in contrast to *C. purpurea* (Haarmann et al., 2005), the role of an NRP synthetase in alkaloid synthesis has yet to be confirmed in *A. fumigatus*. Loss of fumigaclavine C was accompanied by elevated levels of another class of SM, the fumitremorgins. The increased production of fumitremorgins such as TR-2, fumitremorgin C, and verruculogen in response to fumigaclavine C diminution is indicative of cross-talk within the secondary metabolome. These authors have speculated that increased fumitremorgin levels are possibly due to increased isoprene availability due to decreased prenylation of fumigaclavine A due to PesL/Pes1 absence. This is supported by the findings of Wiemann et al. (2012), who saw an increase in the isoprene derived molecule gibberellin following loss of either PKS or NRPS activity due to deletion of *ppt1* in *Fusarium fujikuroi*. Interestingly, biosynthesis of fumiquinazolines A through F continues despite the loss of *pesL*, even though this NRP synthetase has been shown to mediate fumiquinazoline biosynthesis *in vitro* (Ames et al., 2010), an observation shared by Lim et al. (2014). This suggests that alternative NRP synthetases can be utilized for fumiquinazoline biosynthesis, which implies cross-talk between SM gene clusters.

REDOX CONTROL AND GENE CLUSTER MODULATION

O'Keeffe et al. (2014) have surprisingly demonstrated that an intact gliotoxin self-protection mechanism (Scharf et al., 2010; Schrettl et al., 2010), mediated by GliT, is essential to regulate the biosynthesis of apparently unrelated metabolites such as pseurotin A, fumagillin, and fumitremorgins. Specifically, using RNA-seq these authors have demonstrated that gene cluster expression encoding the biosynthesis of helvolic acid, pseurotin A, fumagillin and fumitremorgins is significantly attenuated in *A. fumigatus* Δ*gliT* upon gliotoxin exposure. Unexpectedly, alterations in the expression of genes involved in siderophore-mediated iron transport and siderophore biosynthesis was also observed, which was suggestive of a deficiency in iron-sensing. Along with alterations in multiple additional cellular systems, these data suggest that GliT-mediated gliotoxin formation is essential to ensure global SM biosynthesis in *A. fumigatus* can occur in a regulated manner, and that cellular redox control, amongst other cellular systems, may play an important role in regulating SM biosynthesis (**Figure 1**).

Further to the observations regarding fumitremorgin and fumigaclavine biosynthesis (O'Hanlon et al., 2012), overexpression of the *A. fumigatus* putative Zn(II)$_2$Cys$_6$ transcription factor *hasA* [which is part of the hexadehydro-astechrome (HAS) biosynthetic gene cluster] also resulted in the production of large amounts of fumitremorgins compared to the wild-type strain.

Deletion of the HAS NRPS in a background overexpressing *hasA* (OE*::hasA*Δ*hasD*) resulted in significantly increased fumitremorgin production. The authors hypothesized that the diversion of tryptophan and prenylation activity from HAS production toward fumitremorgin biosynthesis may occur in OE*::hasA*Δ*hasD* due to the inability of this mutant to utilize the pathway intermediates from HAS production. As fumitremorgins are potent tremorgenic mycotoxins this may account for the increased virulence of the OE*::hasA*Δ*hasD* strain compared to wild-type (Yin et al., 2013). This degree of metabolite cross-talk has one limiting affect, because it infers that caution must be exercised in interpreting the impact of metabolite-altering gene deletion experiments. In other words, if metabolite one disappears, and the level of an apparently unrelated metabolite two elevates – which change is altering phenotype?

Although HAS does not contribute to the uptake of extracellular siderophores or iron detoxification in *A. fumigatus*, an iron starvation phenotype and concomitant siderophore biosynthesis is induced by HAS or astechrome (Wiemann et al., 2014). It has now been demonstrated that iron levels regulate the biosynthesis of HAS, and other SM, which is dependent upon the bZIP protein, HapX and the GATA-type transcription factor, SreA. Wiemann et al. (2014) have also demonstrated that HAS not only influences iron acquisition but also the expression of multiple gene clusters involved in SM biosynthesis. They further speculate that intracellular levels of iron (and HAS) may trigger, at a systems level, primary metabolite availability to effect SM biosynthesis, in a redox-dependent manner. These contemporaneous observations are complementary to those of O'Keeffe et al. (2014) and suggest that we are beginning to piece together the systems interactions, via SM cross-talk, necessary to facilitate SM biosynthesis in this human opportunistic pathogenic fungus (**Figure 1**).

EFFLUX

The efflux of SMs is another aspect of secondary metabolism where evidence exists of the occurrence of cross-metabolite interactions. The rice pathogen *Magnaporthe grisea* produces the PKS–NRPS fusion natural product ACE1 from a 15-gene cluster. Although this cluster encodes a transporter for the MFS superfamily (MFS1), it has been shown that *MFS1* is not involved in the efflux of ACE1 as this gene has a deletion of a single base pair which results in an early stop codon. Therefore, it has been suggested that ACE1 must rely on another transporter which is encoded outside of the ACE1 gene cluster (Coleman and Mylonakis, 2009). Moreover, deletion of the sirodesmin ABC transporter gene *sirA* from *Leptosphaeria maculans* actually resulted in an increase (39%) of sirodesmin production and secretion compared to the wild-type strain. The production of deacetyl sirodesmin in the Δ*sirA* mutant also increased 27% compared to the wild-type. This seemingly contradictory result may be explained by the presence of alternate efflux mechanisms for these metabolites or a degree of redundancy across SM transporters. These alternate efflux mechanisms may be more effective than SirA, resulting in differential sirodesmin cluster feedback regulation which leads to the overexpression of this NRPS cluster (Gardiner et al., 2005b). In contrast, deletion of *gliA* from the gliotoxin gene cluster significantly decreased gliotoxin efflux, which indicates that some ETP-producing fungi may not have compensatory mechanisms to mediate natural product efflux (Wang et al., 2014). Evidence also exists to suggest that the MFS transporter DotC encoded within the dothistromin gene cluster of *Dothistroma septosporum* is not the only mechanism of toxin efflux in this organism (Bradshaw et al., 2009).

INTERTWINED GENE CLUSTERS

The *A. fumigatus* genes *psoF* (putative dual function methyltransferase and monooxygenase) or *psoG* (hypothetical protein) were predicted to be required for fumagillin biosynthesis due to their proximity to the characterized fumagillin encoding genes *fmaA* and *fmaB*. Surprisingly, deletion of *psoF* and *psoG* resulted in the abolition of pseurotin A biosynthesis. The Δ*psoF* strain accumulated a demethyl-deepoxy-synerazol (*m/z* 384.1447) compound instead of pseurotin A which is in agreement with the putative role of this enzyme (Wiemann et al., 2013). PsoF was recently characterized by Tsunematsu et al. (2014). This work highlighted an additional layer of complexity regarding the interactions between SMs and is undoubtedly one of the most dramatic examples of cross-talk between fungal metabolites, at the genetic level, which has been elucidated by Wiemann et al. (2013) in *A. fumigatus*. Here, the gene clusters encoding fumagillin and the NRP/polyketide hybrid, pseurotin A, are physically intertwined and co-regulated by LaeA via the $Zn(II)_2Cys_6$ Transcription Factor, FapR. In addition, fumitremorgin is also encoded by this supercluster; however, the genes encoding the biosynthesis of this metabolite are distinct from the intertwined region. Interestingly, although this supercluster is not present in completely intact form in related fungal species, there is sufficient co-localization of orthologs to allow Wiemann et al. (2013) to speculate that co-production of the aforementioned metabolites confers survival advantages on producing species. It is tempting to speculate that the products of this supercluster act synergistically or in a complementary manner, almost like subunits of a heteromeric enzyme, to effect survival of *A. fumigatus*, and related species in defined ecological niches.

It is also true to say that hybrid PKS-NRPS megasynthetases represent a unique concept in metabolite cross-talk, whereby PK and NRPs are reconstituted into unique molecular entities and this topic has been extensively reviewed elsewhere (Boettger and Hertweck, 2013).

BEYOND ENDOGENOUS: INTER-KINGDOM CROSS-TALK

Recent data have also described what may be cross-talk of natural products from different organisms. Culturing *A. fumigatus* MBC-F1-10 in the presence of *Streptomyces bullii* leads to the production of a diversity of *A. fumigatus* metabolites including ergosterol and seven diketopiperazine (DKP) class of alkaloids. Production of the antibiotic–antitumor metabolite glionitrin was induced in *A. fumigatus* following co-culture with a *Sphingomonas* isolate KMK-001 derived from mine-drainage system (Rateb et al., 2013). Moreover, physical interaction between the *S. rapamycinicus* and *A. fumigatus* resulting in the activation of a silent PKS gene cluster encoding fumicycline A (König et al., 2013). Bacterial metabolites have also been demonstrated to act as precursors of fungal metabolites, whereby phenazine metabolites from *Pseudomonas aeruginosa* were converted by *A. fumigatus* into new molecular species with enhanced toxicity, as well as additional, properties (Moree et al., 2012). It is likely that further examples of inter-kingdom interactions, which describe cross-talk leading to metabolite production, will be described as increasing attention is being focused on the impacts of co-culturing of microorganisms.

CONCLUDING REMARKS

Upon writing this manuscript, the authors could not help but be struck once again by two terms which continuously pervade work on fungal natural products – these are NRPS and *secondary* metabolism. In other words, we ask the question if research in and funding for, this exciting area of chemical biology is hindered by the use of the negative terminology of *non* and *secondary*? Perhaps it is time to describe the biosynthesis of these peptidyl entities in more positive ways, and not to refer to the processes of bioactive metabolite formation as a *secondary*, or somehow less important or optional, system. It is not – evolution generally sees to that! Perhaps CMPS (Cluster Mediated Peptide Synthesis) could replace NRPS, as it simultaneously (i) removes the negative terminology of *non*, (ii) increases the descriptive value of the acronym to suggest *gene cluster* involvement in the process, and (iii) offers the possibility of referring to NRPs as or CDPs (Cluster Peptides or Cluster Derived Peptides).

So, it is undoubtedly clear that significant cross-talk exists between (i) primary and secondary metabolism, (ii) different fungal metabolites, (iii) the enzymes involved in the synthesizing different metabolites, and (iv) various metabolite-encoding gene clusters (**Figure 1**). Indeed cross-talk even exists between kingdoms. As described herein, and elsewhere (Lim and Keller, 2014), the details, significance and potential of this exquisite orchestration of unforeseen molecular events are emerging at an ever-increasing rate. For the future, high-throughput analytical approaches, combined with new insights into, and revised conceptions of, fungal genetics and biochemistry, will yield further surprises which will continue to enthuse us to re-imagine our current perspective of distinct orders of cellular metabolic processes in these truly unique organisms.

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Nitrogen regulation of fungal secondary metabolism in fungi

Bettina Tudzynski*

Institute of Biology and Biotechnology of Plants, Westfaelische Wilhelms-University Muenster, Muenster, Germany

Edited by:
Nancy Keller, University of Wisconsin, USA

Reviewed by:
Patricia Coutinho Dos Santos, Wake Forest University, USA
Luyan Ma, Institute of Microbiology Chinese Academy of Sciences, China

****Correspondence:***
Bettina Tudzynski, Institute of Biology and Biotechnology of Plants, Westfaelische Wilhelms-University Muenster, Schlossplatz 8, 48143 Muenster, Germany
e-mail: tudzynsb@uni-muenster.de

Fungi occupy diverse environments where they are constantly challenged by stressors such as extreme pH, temperature, UV exposure, and nutrient deprivation. Nitrogen is an essential requirement for growth, and the ability to metabolize a wide variety of nitrogen sources enables fungi to colonize different environmental niches and survive nutrient limitations. Favored nitrogen sources, particularly ammonium and glutamine, are used preferentially, while the expression of genes required for the use of various secondary nitrogen sources is subject to a regulatory mechanism called *nitrogen metabolite repression*. Studies on gene regulation in response to nitrogen availability were carried out first in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa*. These studies revealed that fungi respond to changes in nitrogen availability with physiological and morphological alterations and activation of differentiation processes. In all fungal species studied, the major GATA transcription factor AreA and its co-repressor Nmr are central players of the nitrogen regulatory network. In addition to growth and development, the quality and quantity of nitrogen also affects the formation of a broad range of secondary metabolites (SMs). Recent studies, mainly on species of the genus *Fusarium*, revealed that AreA does not only regulate a large set of nitrogen catabolic genes, but can also be involved in regulating production of SMs. Furthermore, several other regulators, e.g., a second GATA transcription factor, AreB, that was proposed to negatively control nitrogen catabolic genes by competing with AreA for binding to GATA elements, was shown to act as activator of some nitrogen-repressed as well as nitrogen-induced SM gene clusters. This review highlights our latest understanding of canonical (AreA-dependent) and non-canonical nitrogen regulation mechanisms by which fungi may regulate biosynthesis of certain SMs in response to nitrogen availability.

Keywords: nitrogen regulation, secondary metabolites, AreA, AreB, MeaB, Nmr1, GS

INTRODUCTION

It is well known that secondary metabolites (SMs) are not required for viability but provide a competitive advantage to the microorganism producing them in various ways. They may improve nutrient availability (e.g., in the form of chelating agents such as siderophores), protect it against environmental stresses (e.g., pigments against UV irradiation), enhance its competitive interactions for nutrients with other microorganisms in ecological niches, decrease the fitness of their hosts, e.g., plants, animals, or humans, and act as a metabolic defense mechanism (Breitling et al., 2013). Many SMs act as pathogenicity factors, such as the host selective T-toxin from *Cochliobolus heterostrophus* (Turgeon and Baker, 2007), the cyclic peptide AM-toxin from *Alternaria alternata* (Markham and Hille, 2001) or the trichothecene deoxynivalenol (DON) produced by *Gibberella zeae* (Proctor et al., 1995; Jansen et al., 2005).

Secondary metabolites are only produced during specific conditions, and their biosynthesis is subject to diverse regulatory controls. As a consequence, many of the SM biosynthetic genes show little or no expression under typical laboratory conditions, and therefore the potential new SMs are either not produced, or are present at levels that are too low to be detected by standard methods (Brakhage, 2013). The increasing number of sequenced fungal genomes has revealed that fungi probably produce many more SMs than originally expected, though most of these new SMs are only predicted by bioinformatics analysis of putative SM gene clusters.

In the pre-genomics era, culture supernatants were screened to identify new SMs with activities of interest. These classical methods for activating SM genes involved the manipulation of culture conditions exemplified by the OSMAC (**o**ne **s**train, **ma**ny **c**ompounds) approach (Bode et al., 2002; Craney et al., 2013). This simple and inexpensive method led to the discovery of many new metabolites and gave first insight into the complex regulatory network which includes widely conserved general, as well as pathway-specific, regulation principles.

Also by limiting the rate of availability of a single nutrient, e.g., in continuous fermentations, normally silent gene clusters can be activated by controlling the specific growth rate. The strength of this approach has been demonstrated by growing *Aspergillus nidulans* in a chemostat under nitrogen limitation. By this approach, two novel polyketide metabolites, sanghaspirodins A and B, were discovered (Scherlach et al., 2011). Orsellinic acid biosynthesis is also induced under nitrogen limitation in continuous culture and is suggested to be a precursor for sanghaspirodins biosynthesis (Scherlach et al., 2011; **Table 1**). These studies revealed a significant impact of nutritional factors, such as carbon and nitrogen sources, on SM production and morphological differentiation (Keller et al., 1997; Calvo et al., 2002).

REGULATION OF SECONDARY METABOLISM BY NITROGEN AVAILABILITY

Of all environmental factors, the quality and quantity of the nitrogen source used in the growth media have a special effect not only on growth and differentiation, but also on the biosynthesis of many known fungal SMs, e.g., production of sterigmatocystin and aflatoxin in different *Aspergillus* species (Chang et al., 1995; Feng and Leonard, 1998; Calvo et al., 2002; Ehrlich and Cotty, 2002), gibberellin (GA; Jefferys, 1970; Giordano and Domenech, 1999), fusarubin (Studt et al., 2012), bikaverin (Wiemann et al., 2009), fusaric acid (Niehaus et al., 2014a), and fusarin (Díaz-Sánchez et al., 2012; Niehaus et al., 2013) in *Fusarium fujikuroi*, fumonisin in *F. verticillioides* (Kim and Woloshuk, 2008), or cephalosporin, penicillin, and patulin in *Acremonium chrysogenum*, *Penicillium chrysogenum*, and *P. urticae*, respectively (Rollins and Gaucher, 1994; Haas and Marzluf, 1995; Li et al., 2013). An overview of fungal SMs whose biosynthesis is affected by nitrogen availability is given in **Table 1**.

Recent genome-wide microarray experiments under nitrogen-sufficient and nitrogen-limiting conditions in *F. fujikuroi* revealed that the expression of 30 out of the 45 putative SM gene clusters depends on the quantity and quality of the nitrogen source. Among them are 13 clusters with a polyketide synthase (PKS) gene (e.g., those for fusaric acid, bikaverin, fusarubin, and fumonisin), the two with a diterpene cyclase (DTC) gene (GA and carotenoid clusters), two with a sesquiterpene cyclase (STC) gene, 11 with a non-ribosomal peptide synthetase (NRPS) gene (e.g., the apicidin F cluster), one with a dimethylallyl tryptophan synthase (DMATS) gene, and one with the only type III-PKS gene (Wiemann et al., 2013).

However, for most of the nitrogen-regulated SMs in fungi the molecular mechanism of the nitrogen dependency is not well understood. To better understand the complex nitrogen regulation network in general, the following paragraph provides an overview of fungal nitrogen regulators which ensure the preferential use of primary nitrogen sources and also confer the ability to use different secondary nitrogen sources when appropriate.

NITROGEN REGULATION IN FUNGI: THE GATA-TYPE TRANSCRIPTION FACTORS AreA AND AreB

Filamentous fungi are able to use many compounds as sole nitrogen sources, but preferentially use energetically favored nitrogen sources such as NH_4^+ and glutamine for as long as they are present in the medium. In the absence of these sources, less easily assimilated nitrogen sources such as nitrate, urea, uric acid, amines, amides, purines, and pyrimidines may also be used (Marzluf, 1997; Wong et al., 2008). The regulatory mechanism that enables preferential utilization of easily assimilated nitrogen sources in one circumstance, but selective utilization of these secondary nitrogen sources in another circumstance, is called *nitrogen metabolite repression*. This global regulatory circuit ensures the transcriptional activation of structural genes encoding enzymes and permeases required for scavenging and degradation of energetically less favored nitrogen sources (Wiame et al., 1985; Marzluf, 1997; Fraser et al., 2001; Magasanik and Kaiser, 2002). In ascomycetes, nitrogen metabolite repression is mediated by transcription factors belonging to the GATA family. The key regulatory genes *areA* in *A. nidulans* and *nit-2* in *N. crassa* were cloned more than 25 years ago (Fu and Marzluf, 1987; Caddick, 1992). The predicted amino acid sequences of AreA and NIT2 revealed a high level of similarity, mainly in the DNA-binding domain consisting of a Cys_2/Cys_2-type zinc finger motif and the adjacent basic region, while the N-terminal region is highly variable and dispensable for function in *A. nidulans* (Fu and Marzluf, 1990; Kudla et al., 1990; Caddick and Arst, 1998). Cross-genus complementation showed that *nit-2* and *areA* are functional orthologs (Davis and Hynes, 1987). NIT2/AreA were found to preferentially bind to at least two 5′HGATAR DNA motifs located within 30 bp of each other (Ravagnani et al., 1997).

The standard model of nitrogen metabolite repression is that AreA/NIT2 and their orthologs in several other ascomycetes (reviewed in Wiemann and Tudzynski, 2013) mediate derepression of many genes involved in utilization of secondary nitrogen sources in the absence of glutamine and ammonium. However, the fungus activates the transcription of catabolic genes only when their substrates are available. For this substrate-specific gene activation, additional pathway-specific transcription factors are involved which mediate induction of a set of genes in response to a specific inducer. The best characterized example is the nitrate assimilation system in *A. nidulans* where AreA and the pathway-specific transcription factor NirA act synergistically and physically interact to assure the use of nitrate as sole nitrogen source (Narendja et al., 2002; Muro-Pastor et al., 2004; Berger et al., 2008). Interpretation of recent data has unraveled the individual contributions of NirA and AreA in this complex activation/inactivation process. It was shown that AreA is required for histone H3 acetylation and concomitant chromatin restructuring in the bidirectional promoter of the nitrate and nitrite reductase genes, *niaD*, and *niiA* (Berger et al., 2008). NirA also participates in the chromatin-opening process during nitrate induction, but by an H3 acetylation-independent mechanism and only requires the presence of nitrate. In nitrogen-starved cells, when elevated AreA chromatin occupancy and histone H3 hyperacetylation have been obtained, the chromatin remodeling function of NirA is dispensable. However, continuous presence of high nitrate assimilation and subsequent accumulation of intracellular glutamine (Schinko et al., 2010) lead to lowered AreA activities. Under these conditions, the interaction between the nitrate-activated NirA and the NplA/KapK nuclear export complex seems to be disrupted and results in nuclear retention of NirA and partial compensation for reduced AreA occupancy at the promoter (Bernreiter et al., 2007; Berger et al., 2008).

Table 1 | Nitrogen-regulated secondary metabolites and regulators involved.

Secondary metabolite	Fungus	Nitrogen regulation	Nitrogen regulators involved	Reference
Aflatoxin	*Aspergillus parasiticus* *Aspergillus flavus*	NO_3 repression, NH_4 induction	AreA binds AflR promoter	Feng and Leonard (1998), Chang et al. (2000), Ehrlich and Cotty (2002)
Alternariol	*Alternaria alternata*	Nitrogen repression	No data	Brzonkalik et al. (2011)
Apicidin F	*F. fujikuroi*	Nitrogen induction	AreA-independent; AreB-dependent	Niehaus et al. (2014b)
Beauvericin	*F. oxysporum*	Nitrogen induction	AreA-dependent	López-Berges et al. (2014)
Bikaverin	*F. fujikuroi*	Nitrogen repression	AreA not essential; GS, MeaB, MepB, and TOR involved	Teichert et al. (2004, 2006, 2008), Wiemann et al. (2009), Wagner et al. (2010, 2013)
Cephalosporin	*Acremonium chrysogenum*	Nitrogen repression	AreA-dependent	Li et al. (2013)
Carotenoids	*F. fujikuroi*	Nitrogen repression	No data	Rodriguez-Ortiz et al. (2009)
Fumonisin	*F. verticillioides*	Nitrogen repression	AreA-dependent	Kim and Woloshuk (2008)
Fumonisin	*F. proliferatum*	Nitrogen repression	Nitrogen starvation stress regulated by HOG MAP kinase	Kohut et al. (2009)
Fusaric acid	*F. fujikuroi*	Nitrogen induction	AreA-independent; AreB-dependent	Niehaus et al. (2014a)
Fusarielin H	*F. graminearum*	Nitrogen repression	AreA-dependent	Giese et al. (2013)
Fusarin C	*F. fujikuroi*	Nitrogen induction	AreA and AreB-independent; GS-dependent	Díaz-Sánchez et al. (2012), Niehaus et al. (2013)
Fusarubins	*F. fujikuroi*	Nitrogen repression; alkaline pH	No data	Studt et al. (2012, 2013a)
Gibberellins	*F. fujikuroi*	Nitrogen repression	AreA-and AreB-dependent; MepB, GS, TOR, and MeaB involved	Mihlan et al. (2003), Teichert et al. (2004, 2006, 2008), Schönig et al. (2008), Wagner et al. (2010, 2013), Michielse et al. (2014)
Ochratoxin	*Aspergillus ochraceus*, *P. verrucosum*	Nitrogen-induced	No data	Abbas et al. (2009)
Orsellinic acid	*Aspergillus nidulans*	Nitrogen starvation-induced	No data	Scherlach et al. (2011)
Patulin	*P. urticae*	Nitrogen repression	No data	Rollins and Gaucher (1994)
Penicillin	*P. chrysogenum*	Nitrogen repression	Nre binds to the *ACV-IPN*-promoter	Haas and Marzluf (1995)
Spiroanthrones	*Aspergillus nidulans*	Nitrogen starvation-induced	No data	Scherlach et al. (2011)
Sterigmatocystin	*Aspergillus nidulans*	NH_4 repression NO_3 induces	No data	Feng and Leonard (1998)
Trichothecenes (DON)	*F. graminearum*	Nitrogen repression	AreA-dependent	Min et al. (2012), Giese et al. (2013)
Zearalenone	*F. graminearum*	Nitrogen repression	Contradictory results to AreA-dependency	Min et al. (2012), Giese et al. (2013)

The activity of AreA itself is regulated by several signaling processes that report the extracellular nitrogen availability and the intracellular nitrogen status (Caddick et al., 2006). Under nitrogen-limiting conditions, AreA activity is partially de-repressed due to increased levels of *areA* transcription and *areA* mRNA stability compared with nitrogen-sufficient conditions (Langdon et al., 1995; Platt et al., 1996; Morozov et al., 2000, 2001). Furthermore, AreA activity is controlled by nitrogen starvation-induced nuclear translocation of AreA and subsequent elevated AreA-dependent gene expression in *A. nidulans* and *F. fujikuroi* (Todd et al., 2005; Michielse et al., 2014). In *F. graminearum* AreA accumulates in the nucleus under both nitrogen-limiting conditions and with nitrate as a sole nitrogen source. GFP-AreA was still visible in the nuclei in rich complete medium though with much lower intensity of GFP fluorescence *(*Min et al., 2012).

In *A. nidulans*, AreA contains six conserved nuclear localization sites (NLSs), but only the simultaneous mutation of all of them prevented AreA nuclear accumulation (Hunter et al., 2014). In addition to nuclear translocation, the export from the nucleus also seems to be an additional mechanism for regulation of AreA activity: upon addition of a rich nitrogen source, AreA is exported from the nucleus within minutes and can no longer activate expression of its target genes (Todd et al., 2005).

In some plant pathogenic fungi, such as *F. oxysporum*, and the opportunistic human pathogen *P. marneffei*, the AreA orthologs have been found to be required for full virulence, probably due to the failure of mutants to fully adapt to nitrogen-poor conditions during infection (Divon et al., 2006; Bugeja et al., 2012). In *P. marneffei* it was assumed that AreA is likely to contribute to its pathogenicity by also regulating the expression of potential virulence factors such as extracellular proteases (Bugeja et al., 2012).

Another level of regulation of AreA activity involves its interaction with the co-repressor NmrA/Nmr1 in *A. nidulans*, *N. crassa*, and *F. fujikuroi* (Fu et al., 1988; Xiao et al., 1995; Andrianopoulos et al., 1998; Schönig et al., 2008). It has been proposed that AreA and NIT2 transcriptional activity is inhibited by interaction with NmrA and Nmr1, respectively. In *N. crassa* it has been shown that both the DNA-binding domain (residues 732–821) and the 12-amino-acid carboxy-terminal tail (residues 1006–1036) of NIT2 are essential for interaction with Nmr1. The level of de-repression achieved with the NIT2 proteins mutated in either the zinc finger or the C-terminus was similar to that observed in complete loss-of-function *nmr1* mutants (Pan et al., 1997). In *A. nidulans*, deletion of the 12 C-terminal residues of AreA or mutations in the zinc finger region also lead to a partially de-repressed phenotype similar to that of the Δ*nmrA* mutant (Platt et al., 1996).

Furthermore, proteolytic degradation of NmrA in *A. nidulans* occurred in an ordered manner, preferentially at the C-terminal site, thereby preventing the binding to the AreA zinc finger (Zhao et al., 2010). These data reveal a potential new layer of control of nitrogen metabolite repression by the ordered proteolytic cleavage of NmrA.

It has been reported that *nmrA* expression in *A. nidulans* is high under conditions of nitrogen-sufficiency, opposite to the pattern of *areA* expression (Wong et al., 2007). However, this is not the case in *F. fujikuroi* where *nmr1* is an AreA target gene whose expression is strictly repressed in the presence of adequate nitrogen (Schönig et al., 2008; Wagner et al., 2010). Despite this different expression pattern, Nmr1 in *F. fujikuroi* interacts with AreA as in *N. crassa* and *A. nidulans*, and deletion and over-expression of *nmr1* resulted in high sensitivity and resistance towards chlorate, respectively, indicating its regulating effect on nitrate reductase activity by determining AreA activity (Schönig et al., 2008).

In *A. nidulans*, full activation of some AreA-dependent genes, e.g., *gdhA*, requires also the function of a Zn(II)2Cys6 zinc binuclear cluster transcription factor, TamA, a homolog of the *S. cerevisiae* Dal81p protein (Davis et al., 1996). Recently it has been shown, that TamA has dual functions as a DNA-binding transcription factor and a non-DNA-binding co-activator of AreA which interacts with the C-terminal residues of AreA (Davis et al., 1996; Downes et al., 2013, 2014). At present the role of TamA has not been characterized in any other fungus.

In contrast to *S. cerevisiae*, which involves four GATA-type transcription factors in the regulation of nitrogen use, the positively acting Gln3p and Gat1p, and the negatively acting Dal80p (Uga43p) and Gzf3p (Nil2p; reviewed in Magasanik and Kaiser, 2002) filamentous ascomycetes have only two GATA-type nitrogen regulators, namely AreA and AreB. While AreA/NIT2 are responsible for activation of genes that allow utilization of energetically less-favored nitrogen sources in a similar manner to the yeast homologs Gln3p and Gat1p, AreB was suggested to repress the same set of genes as it has been shown for the yeast GATA factors Dal80p and Gzf3p (Wong et al., 2009). The first functional analyses of the second GATA transcription factor, AreB/NreB, in *A. nidulans* and *P. chrysogenum*, respectively, confirmed its proposed role as repressor of AreA target genes, probably through DNA-binding competition. Over-expression of AreB and NreB in these fungi resulted in loss of AreA/NreA-dependent gene expression indicating that this GATA factor negatively modulates AreA/NreA activity (Haas et al., 1997; Wong et al., 2009).

Recently it has been shown that in *F. fujikuroi* AreB can act both as repressor and activator of nitrogen-regulated gene expression. Thus, both GATA factors are essential for expression of the GA biosynthetic genes (see below). Some other AreA-dependent genes, such as *mepC* (Teichert et al., 2008) and *nmr1* (Schönig et al., 2008), showed significantly elevated transcription in the Δ*areB* deletion mutant indicating that AreA and AreB have common sets of target genes but can affect their expression in opposite ways. However, it is not yet known whether AreB directly binds to the promoters of AreA target genes, or if its effect is due to down-regulation of *areB* transcript levels in the Δ*areA* mutant (Michielse et al., 2014).

In *A. nidulans*, detailed analysis of *areA* and *areB* single and double mutants has shown that both GATA transcription factors negatively regulate the expression of arginine catabolism genes *agaA* and *otaA* under nitrogen-repressing conditions. AreA is necessary for the ammonium repression of *agaA* and *otaA* under conditions of carbon repression, while AreB is involved under carbon-limiting conditions (Macios et al., 2012).

Interestingly, no impact on the expression of nitrogen-regulated genes has been shown for the AreB ortholog in *N. crassa*, Asd4. This transcription factor plays a role in development of asci and ascospores (Feng et al., 2000).

In *A. nidulans* and *F. fujikuroi*, the *areB* gene encodes multiple products all containing the GATA zinc finger and a leucine zipper motif (Conlon et al., 2001; Michielse et al., 2014).

However, the biological role of these different proteins is not well understood. Recently, it has been shown for the first time by a bimolecular fluorescence complementation (BiFC) approach that one of the AreB isoforms in *F. fujikuroi* interacts with AreA in the nucleus when starved of nitrogen (Michielse et al., 2014). Furthermore, expression of both *areA* and *areB* is repressed by nitrogen and induced by nitrogen starvation. Interestingly, the *areB* transcription level is decreased 6-fold in the Δ*areA* mutant when nitrogen is scarce, whilst AreB does not affect *areA* expression.

OTHER REGULATORS INVOLVED IN NITROGEN REGULATION OF METABOLIC PROCESSES IN FILAMENTOUS FUNGI

How do fungi sense the extracellular nitrogen availability and their intracellular nitrogen status? Furthermore, it is not understood clearly whether fungi can sense different types of nitrogen sources, or whether the intracellular pool of *glutamine* alone provides the stimulus for expression or repression of nitrogen-responsive genes.

These processes have been intensely studied in *S. cerevisiae* where the "TOR" (target of rapamycin) kinase and the downstream TOR signaling pathway play a crucial role as global regulators of cell growth. Under nitrogen-sufficient conditions, TOR is able to sense amino acid-derived signals and to stimulate a set of anabolic processes, including translation, transcription, and ribosome biogenesis, whereas nitrogen depletion or inhibition of TOR by the TOR inhibitor rapamycin triggers G1 cell cycle arrest, protein synthesis inhibition, and autophagy (Rohde and Cardenas, 2004; Rubio-Texeira, 2007; Rohde et al., 2008; **Figure 1**).

However, the mechanisms by which signals of nutrient availability are sensed by TOR and transduced to transcription factors to elicit a response are not fully understood. In *S. cerevisiae*, TOR regulates two highly conserved phosphatases, Sit4, and PP2A, which in turn regulate cellular localization of the two positively acting GATA-type transcription factors, Gln3 and Gat1. In the presence of sufficient nitrogen, Gln3 and Gat1 are cytoplasmic, and NCR genes are not expressed. However, nitrogen starvation or treatment with rapamycin result in immediate nuclear translocation of both transcription factors and subsequent transcription of nitrogen-repressed genes (Tate et al., 2010).

Much less is known on the role of the TOR kinase in filamentous fungi. Despite the presence of putative homologs of the TOR kinase and the other components of the TOR cascade in the sequenced genomes of filamentous fungi (Fitzgibbon et al., 2005; Teichert et al., 2006), there is currently no experimental evidence that TOR signaling acts through regulating subcellular localization of the GATA factors AreA and AreB. Genome-wide transcriptome analysis in *F. fujikuroi* with RNA from rapamycin-treated mycelium revealed a partial de-repression of some AreA target genes. Like in yeast, genes involved in ribosome biogenesis, and translation initiation/elongation are down-regulated by rapamycin, whereas genes involved in autophagy and protection against oxidative stress are up-regulated by the TOR inhibitor (Teichert et al., 2006). In the tomato pathogen *F. oxysporum*, ammonium-mediated repression of cellophane penetration is reversed by rapamycin, indicating that this negative regulation of penetration by ammonium also requires an active TOR cascade (López-Berges et al., 2010). The nitrogen source and TOR also control other virulence-related functions, such as vegetative hyphal fusion and root adhesion in *F. oxysporum* (López-Berges et al., 2010).

Assimilation of different nitrogen sources, such as nitrate, nitrite, urea, or amino acids, results in formation of NH_4^+ which is subsequently converted first to glutamate via glutamate dehydrogenase (GdhA), and then to glutamine via *glutamine synthetase* (GS). It became the widely accepted view that glutamine – and not ammonium, nitrate or glutamate – is the key effector of nitrogen metabolite repression to ensure the preferential utilization of reduced nitrogen sources such as ammonium and glutamine when multiple N resources are available (Dunn-Coleman and Garrett, 1980; Dunn-Coleman et al., 1981; Wiame et al., 1985; Caddick et al., 1994; Magasanik and Kaiser, 2002).

Recent studies in *A. nidulans* showed a 50% drop in glutamine concentration within 5 min after transferring the mycelium into nitrogen-free medium, while the levels of most of the other amino acids were not significantly different from the levels measured in ammonium-grown cultures. These data suggest that glutamine is indeed the marker for the nitrogen status (Berger et al., 2008). The question to be asked is whether glutamine alone is the key co-repressor of many metabolic processes as proposed by Premakumar et al. (1979) and Margelis et al. (2001). It has been proposed for a long time that the GS has an important role not only in providing glutamine, but also as key regulator in the nitrogen regulatory network in yeast and filamentous fungi. Indeed, in *N. crassa* (Dunn-Coleman and Garrett, 1980; Dunn-Coleman et al., 1981; Mora, 1990) and *S. cerevisiae* (Crespo et al., 2002; Magasanik, 2005), treatment with methionine sulfoximine (MSX), a specific inhibitor of the GS, or loss-of-function mutations in the GS, lead to increased expression of nitrogen-regulated genes in cells grown on a preferred nitrogen source. In *F. oxysporum*, GS activity is strictly required for ammonium-mediated inhibition of Cellophane penetration, suggesting that glutamine, rather than ammonium, acts as a signal for nitrogen repression (López-Berges et al., 2010).

A regulatory role for GS has been also postulated for *F. fujikuroi*. A macroarray approach comparing transcriptional profiles of wild-type *F. fujikuroi* with those of the *gln1* deletion strain revealed a set of genes that are strongly up- or down-regulated in the mutant. For example, the gene for the cross pathway control, *CPC1*, and genes involved in translation control (*eEF1α*; *eIH5a*), stress response (*ddr48*; *cipC*), ribosome biogenesis, and histone modification (histone acetyltransferases) all indicate that GS plays a major role in regulating different processes in the fungal cells (Teichert et al., 2004; Wagner et al., 2013). Furthermore, point mutations in an ammonium binding domain of the *F. fujikuroi* GS led to de-repression of several nitrogen-repressed genes with ammonium as nitrogen source. These data indicate that the putative NH_4^+ binding

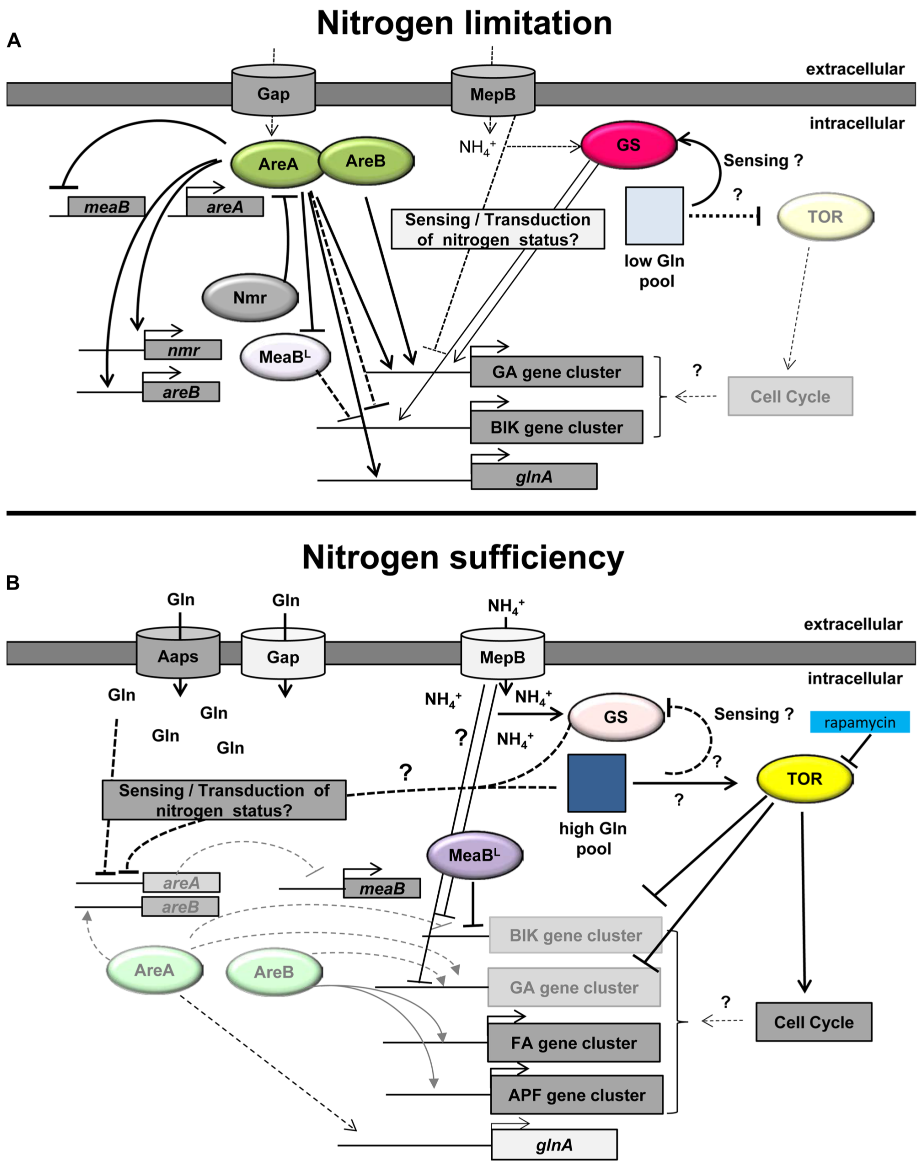

FIGURE 1 | Model of the nitrogen regulation of secondary metabolism in *Fusarium fujikuroi*. (A) Under nitrogen-limiting conditions, the intracellular glutamine (Gln) pool is low (light square). This nitrogen status of the cell is probably sensed by the GS and/or additional nitrogen sensors, e.g., the ammonium permease MepB. Under these conditions, the activity of TOR is low and *areA* is highly expressed. AreA subsequently activates the expression of *areB*. Both GATA transcription factors, AreA and AreB, are essential for the activation of the GA and probably also the fumonisin gene clusters. Active AreA represses the transcription of the negatively acting bZIP transcription factor MeaB^L resulting in high expression of the AreA-independent bikaverin gene cluster. AreA also induces the expression of a variety of genes including *nmr*, *glnA*, and *mepB*. Increasing levels of Nmr interact with AreA thereby putatively modifying its activity by a feed-back loop. **(B)** Under nitrogen sufficient conditions, TOR is active resulting in activation of the cell cycle. Rapamycin inhibits TOR activity resulting in partial derepression of nitrogen-repressed SM genes. The high intracellular Gln pool (dark blue square) is putatively sensed by the GS and/or other sensors and results in low *areA* mRNA levels. The AreA-dependent ammonium permease MepB and the general amino acid permease (Gap) are only slowly expressed, but other amino acid permeases (AAPs) and substrate-specific nitrogen transporters facilitate transport of nitrogen into the cell. The decreased level of active AreA leads to reduced GA (and fumonisin) gene expression and induction of *meaB*^{L} expression. MeaB^L itself is involved in repression of bikaverin (BIK) gene expression. The remaining levels of AreB directly or indirectly activate the expression of the nitrogen-induced fusaric acid (FA) and apicidin F (APF) gene clusters (modified after Wiemann and Tudzynski, 2013).

site D60/S62 causes *loss of* NH_4^+ *sensing* and subsequent loss of wiring of the repressing signal towards gene expression of nitrogen-regulated genes (Wagner et al., 2013; **Table 2**). Interestingly, deletion of the GS-encoding gene *gln1* resulted in significant up-regulation of several nitrogen regulatory genes, such as *areA*, *areB*, *mepB*, and *nmr*, strongly supporting the hypothesis that the GS itself plays a regulatory role (Wagner et al., 2013).

Besides the TOR pathway and the GS, one of the three ammonium permeases, "MepB," was shown to have transport and

Table 2 | Functions of regulators involved in nitrogen-dependent control of secondary metabolism.

Regulator	Function	Reference
AreA	GATA transcription factor; positive regulator of GA, fumonisin, DON, zearalenone, fusarielin H, beauvericin, and cephalosporin gene expression AreA is involved in chromatin accessibility and is essential for full virulence of some plant pathogens	Tudzynski et al. (1999), Berger et al. (2008), Kim and Woloshuk (2008), Min et al. (2012), Giese et al. (2013), Li et al. (2013), López-Berges et al. (2014)
AreB	GATA transcription factor, positive regulator of GA, fusaric acid and apicidin F biosynthesis AreA interacts with AreB under nitrogen-limiting conditions	Michielse et al. (2014), Niehaus et al. (2014a,b)
Nmr	Negative regulator of AreA activity as the deletion of *nmr1* results in increased chlorate sensitivity However, deletion of *nmr1* does not overrule the repression of GA, DON, zearalenone and fusarielin H genes under high nitrogen conditions in *Fusarium* spp.;	Schönig et al. (2008), Giese et al. (2013)
Tor	Inhibition of Tor by rapamycin led to up-regulation of GA and bikaverin biosynthesis genes under nitrogen-limiting conditions, but does not overrule their repression at high nitrogen	Teichert et al. (2006)
MepB	Strong de-repression of the GA and bikaverin biosynthetic genes in the *mepB* deletion mutant under nitrogen-sufficient conditions; sensing or regulatory role of MepB suggested	Teichert et al. (2008), Shnaiderman et al. (2013)
GS	Deletion of *gln1* resulted in loss of GA, bikaverin, fusarin and apicidin F gene expression and up-regulation of *areA*, *areB*, *mepB*, and *nmr1* genes; Postulated sensing and regulatory roles of the GS; affects energy metabolism	Teichert et al. (2004), Niehaus et al. (2013, 2014b), Wagner et al. (2013)
MeaB	bZIP transcription factor; affects expression of several nitrogen-regulated genes; elevated expression of GA and bikaverin biosynthesis genes in the deletion mutant under nitrogen-limiting conditions; overexpression of *A. nidulans meaB* led to impaired *in planta* aflatoxin B1 production	López-Berges et al. (2010), Wagner et al. (2010), Amaike et al. (2013)
Vel1/VeA	FfVel1 may partially overcome nitrogen repression of bikaverin genes *veA* mutant in *F. oxysporum* is impaired in growth on nitrate	Wiemann et al. (2010), López-Berges et al. (2014)

sensing functions and is probably part of the nitrogen regulation network (Teichert et al., 2008). Deletion of *mepB* resulted in impaired growth on media with low concentrations of ammonium as well as in de-repression of multiple genes (e.g., the genes *MTD1* and *AAP8* encoding a peptide transporter and an amino acid permease) that are normally repressed at high concentrations of ammonium (Teichert et al., 2008).

In *Colletotrichum gloeosporioides*, ammonia uptake by the germinating spores of the wild-type, but not of the Δ*mepB* strain with compromised ammonium transport, activated cAMP-mediated transcription of regulatory and catalytic PKA subunits (Shnaiderman et al., 2013). As a consequence, Δ*mepB* mutants showed 75% fewer appressoria and colonization than the wild-type demonstrating that MepB contributes to the virulence of this fungus, probably by activating the cAMP pathway similar to Mep2p, the sensing ammonium permease ("transceptor") in yeast (Van Nuland et al., 2006).

An additional central component of the nitrogen regulation network in fungi is the cross-pathway transcription factor "Cpc1" mediating the transcriptional up-regulation of genes involved in amino acid metabolism under conditions of amino acid imbalance (Barthelmess and Kolanus, 1990; Tian et al., 2007). In *F. fujikuroi* it has been shown that the expression of *cpc1* and Cpc1 target genes is not activated under nitrogen-limiting conditions. However, the *cpc1* gene as well as several genes involved in amino acid biosynthesis are significantly up-regulated in the *F. fujikuroi gln1* deletion mutant due to the significantly decreased glutamine pool and subsequent amino acid imbalance (Teichert et al., 2004; Schönig et al., 2009). Interestingly, in *A. nidulans*, a binding sequence motif for Cpc1 is present in the *prnB* promoter that is also regulated by AreA (Tazebay et al., 1997). Elucidation of the mechanisms by which AreA is interconnected with Cpc1 and GS should lead to a major advance in understanding the role of AreA in amino acid metabolism.

Beside these nitrogen regulators, the bZIP transcription factor "MeaB" has been shown to be involved in nitrogen repression of a set of genes. The *meaB* gene in *A. nidulans* was identified by mutations conferring resistance to toxic amino acid analogs and methylammonium ions, resulting in de-repression of nitrogen-regulated genes (Arst and Cove, 1973; Polley and Caddick, 1996; Wong et al., 2007). However, it is not clear whether there is a functional or physical interaction of MeaB with AreA. Contradictory reports exist about the role of MeaB in regulation of *nmrA* expression in *A. nidulans*. Whereas Wong et al. (2007) reported that under conditions of nitrogen sufficiency MeaB activates *nmrA* expression by binding to a conserved sequence in the *nmrA* promoter, only a slight or no impact of MeaB on *nmrA*/*nmr1* transcription was shown in comparative studies on *A. nidulans* and *F. fujikuroi*, respectively (Wagner et al., 2010). In addition, the postulated binding site for MeaB in the *A. nidulans nmrA* promoter is not conserved in the promoters of *nmrA* homologs in other genera of filamentous fungi (Wagner et al., 2010). Despite these different reports on the impact of MeaB on *nmrA*/*nmr1* expression, both MeaB, and NmrA/Nmr1 were regarded as repressive nitrogen regulatory proteins in *N. crassa*, *Fusarium* spp., and *Aspergillus* spp. (Pan et al., 1997; Wong et al., 2007; López-Berges et al., 2010; Wagner et al., 2010; Amaike et al., 2013). In *F. oxysporum*, MeaB and TOR were shown to be required for repression of the ability to penetrate Cellophane films overlaid on agar plates with high concentrations of ammonium. Both inhibition of TOR by its inhibitor rapamycin and deletion of *meaB* consistently enhanced the ability to penetrate Cellophane in the presence of ammonium (López-Berges et al., 2010). In *F. fujikuroi*, several AreA target genes, such as *mtd1* and *aap1* encoding a peptide permease and an amino acid permease, respectively, are partially up-regulated under starvation conditions in the Δ*meaB* mutant. However, these genes are not de-repressed under nitrogen sufficient conditions, when MeaB is translocated to the nucleus. Therefore, the de-repressing effect seems to be indirect (Wagner et al., 2010).

THE ROLE OF THE GATA FACTORS AreA AND AreB IN REGULATING SECONDARY METABOLISM

For a long time the major nitrogen regulator AreA and its orthologs were thought to be involved exclusively in regulating the use and metabolization of non-favored nitrogen sources (Arst and Cove, 1973; Marzluf, 1997). AreA orthologs have been identified and deleted in a number of filamentous ascomycetes (Wiemann and Tudzynski, 2013), but the impact of AreA inactivation on secondary metabolism is still not well understood.

The first indication that AreA ortologs may regulate SM biosynthesis came from studies on NreA in *P. chrysogenum* (Haas and Marzluf, 1995). The authors showed that this AreA ortholog binds not only to the intergenic promoter regions of the nitrate and nitrite reductase genes (*niiA-niaD*), but also to the intergenic region between *acvA* and *pcbC*, encoding the first two enzymes in penicillin biosynthesis. Unfortunately, the NreA-encoding gene has not been deleted due to very low homologous integration rates in this fungus. Therefore, the direct proof of an involvement of this GATA factor in regulation of penicillin biosynthesis has still not been achieved. Furthermore, it has been suggested that AreA may play a role in the regulation of the aflatoxin gene cluster in *Aspergillus parasiticus*, but this also remains to be proved (Chang et al., 2000).

In *F. fujikuroi*, it has been known for more than 50 years that fermentation of *F. fujikuroi* for GA production delivers highest yields under nitrogen-limiting conditions, and that the red pigment bikaverin, a by-product of GA fermentation, is induced under the same conditions (Borrow et al., 1964; Jefferys, 1970; Bu'lock et al., 1974). However, studies on the molecular mechanism of this nitrogen regulation were possible only after the identification of GA and bikaverin biosynthesis genes (Tudzynski and Hölter, 1998; Linnemannstöns et al., 2002; Wiemann et al., 2009). GAs were the first SMs for which the essential role of the GATA-type transcription factor AreA were proved unequivocally (**Table 1**; **Figure 1A**). Deletion of AreA almost fully abolished GA biosynthesis and expression of GA cluster genes (Tudzynski et al., 1999; Mihlan et al., 2003). Furthermore, AreA was shown to directly bind to the GATA/TATC sequence elements in the promoters of the GA genes by gel mobility shift assays. The binding of AreA was analyzed in more detail using the promoter of the *ent*-kaurene oxidase (*P450-4*) gene fused to the *Escherichia coli uidA* reporter gene (Mihlan et al., 2003). These findings were unexpected because GAs have no nitrogen in their structure and therefore cannot serve as nitrogen sources for the fungus.

As mentioned above, AreA activity was shown to be negatively regulated under nitrogen sufficient conditions by interacting with the repressor protein NmrA/Nmr1 in *A. nidulans* and *F. fujikuroi* (Lamb et al., 2003, 2004; Schönig et al., 2008). While the consequences of *nmrA* deletion on secondary metabolism have not been studied in *A. nidulans*, deletion of *nmr1* in *F. fujikuroi* did not result in the expected significant up-regulation of GA gene expression (Schönig et al., 2008). However, the Δ*nmr1* mutant is highly sensitive to chlorate indicating that Nmr1 specifically interacts with AreA to prevent nitrate utilization in the presence of glutamine, but does not act as a general repressor of AreA in all AreA-dependent pathways (Schönig et al., 2008).

More recently, some SMs from different *Fusarium* species were shown to be nitrogen repressed in an AreA-dependent manner. In *F. verticillioides*, the *areA* deletion strain was incapable of producing fumonisins on mature maize kernels and was compromised in its ability to grow under these *in planta* conditions unless ammonium was added. Furthermore, expression of *fum1, fum8,* and *fum12* was abolished under inducing *in vitro* conditions, while a mutant that constitutively expresses *areA* was able to produce fumonisins even under repressing conditions. These data indicate that AreA is essential for *FUM* gene expression (Kim and Woloshuk, 2008; Picot et al., 2010; **Tables 1** and **2**).

Recently, AreA was functionally characterized in *F. graminearum*, a pathogen of wheat and other cereals (Min et al., 2012; Giese et al., 2013). The virulence of Δ*areA* strains on wheat heads was significantly reduced compared with the wild-type strain. While Min et al. (2012) revealed loss of trichothecene biosynthesis, but no effect on zearalenone biosynthesis in the Δ*areA* strain, Giese et al. (2013) described AreA as a global regulator which is required for the production of DON, zearalenone, and fusarielin H regardless of the nutrient medium (**Tables 1** and **2**). However, the drastically decreased product levels and the presence of several putative tandem AreA binding sites, especially in the promoters of the zearalenone gene cluster, is not conclusive evidence for direct binding of AreA to the toxin gene promoters. The fusarin C and aurofusarin biosynthetic genes contained similar numbers of putative AreA binding sites, although these SMs are not affected in the Δ*areA* mutant.

Deletion of the putative AreA repressor gene *nmr* in *F. graminearum* had little effect on either growth or toxin production. Generally the *nmr* deletion mutant produced very low levels of DON, zearalenone, and fusarielin H under nitrogen sufficient conditions equivalent to the wild-type strain (Giese et al., 2013; **Table 2**). Similarly, GA production in *F. fujikuroi* was still repressed by high nitrogen in the *nmr1* deletion mutant although Nmr1 was shown to interact with AreA and to affect nitrate reductase activity (Schönig et al., 2008).

In F. oxysporum, AreA also contributes to chromatin accessibility and expression of a Velvet-regulated NRPS gene cluster, responsible for the biosynthesis of the mycotoxin beauvericin (López-Berges et al., 2014). Transcript levels of the key enzyme-encoding gene *BeaS* was significantly reduced in both the Δ*areA* and the Δ*veA* mutant. The authors suggest a combinatorial role for the Velvet complex and AreA in nitrogen use and secondary metabolism because the *F. oxysporum* Δ*veA* mutant was impaired in growth on nitrate (López-Berges et al., 2014).

Recently, the involvement of AreA in cephalosporin production has been demonstrated in *Acremonium chrysogenum* (Li et al., 2013). Consistent with the reduction of cephalosporin production, the transcription of *pcbAB*, *cefD2*, *cefEF*, and *cefG* encoding the enzymes for cephalosporin production was reduced in the Δ*AcareA* mutant. Band shift assays showed that AcAreA bound not only to the bidirectional promoter of the nitrate/nitrite reductase-encoding genes *niaD* and *niiA*, but also to the bidirectional promoter region of *pcbAB-pcbC*. Sequence analysis showed that all the AcAreA binding sites contain the consensus GATA elements. These results indicated that AcAreA plays an important role both in the regulation of nitrogen metabolism and cephalosporin production in *Acremonium chrysogenum* (Li et al., 2013; **Table 1**).

Surprisingly, AreA is not essential for the expression of bikaverin genes (*bik1*–*bik6*) in *F. fujikuroi* although they are co-regulated with GA genes under nitrogen-limiting conditions. Unexpectedly, *bik1* (former *pks4*) expression and bikaverin production were even stronger in the Δ*areA* mutant compared to the wild-type (Linnemannstöns et al., 2002). Therefore, a second non-canonical, AreA-independent mechanism of nitrogen metabolite repression must exist that mediates repression of *bik* genes at high nitrogen concentrations (Wiemann et al., 2009; Wiemann and Tudzynski, 2013). Furthermore, the loss of the VeA-encoding genes *Ffvel1* or *FfareA* affected nitrogen-mediated GA and bikaverin synthesis differently. Expression of the GA biosynthetic genes was significantly down-regulated in both the *Ffvel1* and *areA* deletion mutants compared with the wild-type, while the expression of the bikaverin cluster genes *bik1-3* were significantly stronger in the Δ*Ffvel1* mutant compared with the wild-type and the *areA* deletion strain and was still detected in the Δ*Ffvel1* mutant under normally repressing high nitrogen (60 mM glutamine) conditions (Wiemann et al., 2010). These data indicate that FfVel1 may partially overcome nitrogen repression of bikaverin genes.

Recently, a second family of PKS-derived red pigments has been identified in *F. fujikuroi*, the fusarubins (Studt et al., 2012). These pigments are responsible for the coloration of the perithecia. The fusarubin biosynthetic genes (*fsr1*–*fsr6*) are repressed by high nitrogen similarly to the *bik* genes. However, in contrast to the *bik* genes, which need both low nitrogen and acidic pH, the *fsr* genes are only expressed under low nitrogen conditions and alkaline pH (Studt et al., 2012). To keep alkalinity constant during the whole cultivation time in liquid media, nitrate has to be used, a nitrogen source which cannot be used by the Δ*areA* mutant. Therefore, a potential role of AreA in nitrogen-repressed fusarubin gene expression and biosynthesis cannot be excluded (Studt et al., 2012).

As for bikaverin and fusarubins, the expression of the structural genes of the carotenoid pathway, *carRA* and *carB*, is transiently increased upon nitrogen removal (**Table 1**). However, an involvement of AreA in this regulation has not been shown (Rodriguez-Ortiz et al., 2009).

A milestone in understanding the role of the two GATA transcription factors AreA and AreB were two important findings: (1) that both AreA and AreB can be involved in regulating expression of SM genes and (2) that AreB does not generally act as a negative counterpart of AreA (Michielse et al., 2014).

So far, a role of AreB as positive regulator of some SMs has been described only in *F. fujikuroi*. First of all, both AreA and AreB were shown to be essential for expression of the GA biosynthesis genes and concomitant GA production under conditions of nitrogen starvation. Both AreA and AreB co-localized and interact with each other in the nucleus under GA production conditions as shown by use of BiFC (Michielse et al., 2014; **Table 2**).

Interestingly, AreB is not only involved in regulating expression of nitrogen starvation-induced SMs. Recently it has been demonstrated that fusaric acid and apicidin F, both induced under nitrogen sufficient conditions, are not produced and the biosynthetic genes are almost not expressed in the Δ*areB* mutant (Niehaus et al., 2014a,b; **Table 1**; **Figure 1B**). However, AreB is not involved in regulation of all nitrogen-induced SMs as the formation of fusarin C is not affected in the Δ*areB* mutant of *F. fujikuroi* (Niehaus et al., 2013).

THE INVOLVEMENT OF OTHER NITROGEN REGULATORS IN SECONDARY METABOLISM

In *A. nidulans* and *N. crassa*, "NmrA/Nmr1" have been postulated to play a major role in regulating AreA activity. However, no data exist on its potential repressing effect on SM production. In *F. fujikuroi*, deletion of *nmr1* resulted in high sensitivity to chlorate indicating that Nmr1 affects the activity of nitrate reductase via its interaction with Are (Mihlan et al., 2003; Wagner et al., 2010). However, deletion of *nmr1* did not result in de-repression of the AreA-dependent GA biosynthesis genes in the presence of ammonium or glutamine, and over-expression of *nmr1* revealed only a slight repression (**Table 2**). The limited impact of *nmr1* deletion on the expression of AreA-dependent SM genes on the one hand, and the AreA-independent expression of other nitrogen-regulated SM genes, e.g., those for bikaverin, aurofusarin, and fusarin biosynthesis (Wiemann et al., 2009; Giese et al., 2013), suggest that additional factors must be involved in nitrogen regulation in general, and particularly in regulation of SM.

In *A. nidulans*, *F. fujikuroi* and *F. oxysporum*, the bZIP transcription factor "MeaB" affects expression of several nitrogen-regulated genes (López-Berges et al., 2010; Wagner et al., 2010; Amaike et al., 2013). However, not much is known about the role of MeaB in regulating SM. In *F. fujikuroi*, deletion of the gene resulted in increased transcription of the GA and bikaverin biosynthesis genes under inducing low nitrogen conditions, but did not overcome repression by glutamine. It is notable that full de-repression of the AreA-independent bikaverin biosynthesis genes was observed in the Δ*meaB*/Δ*areA* double mutant suggesting that both AreA and MeaB act as repressors of bikaverin biosynthesis in different pathways. Studies are still needed to test whether this strong de-repressing effect is due to direct binding of one or the other TF, or if both regulators act indirectly by affecting the activity of other regulators involved in bikaverin biosynthesis, e.g., the pathway-specific Zn(II)2Cys6 transcription factor Bik5 (Wagner et al., 2010).

In *A. nidulans*, loss of *meaB* did not significantly affect secondary metabolism. However, the *OE::meaB* strain had a much stronger phenotype. The mutant is impaired in seed colonization, lipase activity and *in planta* aflatoxin B1 production (Amaike et al., 2013). Further experiments will allow an unambiguous integration of MeaB into the established nitrogen regulation network in filamentous fungi.

Studies in *F. fujikuroi* and *Colletotrichum gloeosporioides* revealed that the ammonium permease "MepB" might be involved in sensing external nitrogen availability and the intracellular nitrogen status of the cell (Teichert et al., 2008; Shnaiderman et al., 2013). However, an impact of MepB on SM has been shown so far only for *F. fujikuroi.* The deletion of *mepB* in this fungus resulted in strong de-repression of the GA and bikaverin biosynthetic genes at early stages of growth when ammonium is still present at sufficient levels and secondary metabolism is not switched on in the wild-type strain (**Figure 1**). To be sure that this de-regulation of SM production is not due to reduced ammonium transport capacity, but to a sensing defect, the gene *mepC* encoding the second high capacity transporter MepC, was over-expressed in the Δ*mepB* background. While the strong growth defect was partially overcome, the SMs were still de-repressed at high ammonium concentrations, supporting the suggestion that MepB plays a sensing or regulatory role in addition to its function as a permease (Teichert et al., 2008). However, downstream signaling components, as in *C. gloeosporioides* (Shnaiderman et al., 2013), have not yet been identified in *Fusarium* spp..

As mentioned before, the "GS" seems to play an important role as key regulator in the nitrogen regulation network, mainly by regulating the intracellular glutamine levels (Dunn-Coleman and Garrett, 1980; Dunn-Coleman et al., 1981; Mora, 1990; Crespo et al., 2002; Magasanik, 2005). However, its potential function in regulating SM production has been studied only recently. In *F. fujikuroi*, deletion of the GS-encoding gene *gln1* resulted in surprising loss of GA and bikaverin production instead of the expected increased product yields due to the decreased glutamine pool in the mutant (Teichert et al., 2004). The GA and bikaverin biosynthetic genes are significantly down-regulated even under nitrogen-limiting conditions. The same results were obtained by inhibiting GS by MSX (Teichert et al., 2004). Recently, the biosynthesis of two nitrogen-induced metabolites, fusarin C (Niehaus et al., 2013) and apicidin F (Niehaus et al., 2014b) was also shown to be absolutely dependent on a functional GS. Therefore, the *gln1* mutant can neither produce GAs and bikaverin, nor fusarin and apicidin F (**Figure 1**).

Interestingly, the two GS-encoding genes of *N. crassa* (*Ncgln1* and *Ncgln2*) and even those of *Streptomyces coelicolor, glnA*, and *glnII,* fully restored not only the wild type-like growth, but also SM gene expression and product formation of the *F. fujikuroi gln1* deletion strain indicating the high level of functional conservation between prokaryotes and eukaryotes (Wagner et al., 2013).

Complementing the Δ*gln1* mutant with 14 different *F. fujikuroi gln1* copies with site-directed mutations in the 14 highly conserved GS domains resulted either in restoration of both glutamine synthesis and SM production, or of none of these activities. However, three site-directed mutations in the *gln1* gene partially restored secondary metabolism and GS-dependent gene expression, but not glutamine formation, demonstrating for the first time that the catalytic and regulatory roles of GS can be separated. One of these three mutants with a mutation in a postulated ammonium binding domain (mutant D60A/S62A) revealed a partial de-repression of the AreA-dependent GA and AreA-independent bikaverin gene expression when grown with NH_4^+ as sole nitrogen source (Wagner et al., 2013).

The reason for the strong impact of the GS on SM gene expression is unclear. However, the significantly reduced growth of the Δ*gln1* mutant (only 15% dry weight compared to the wild-type) in media with high glucose and low amounts of glutamine compared to the wild-type suggests a defect in efficient glucose use and ATP generation. All complemented *gln1* mutant strains with functional GS proteins revealed not only restored SM but also growth similar to the wild-type strain, while the glutamine auxotrophic strains accumulated significantly less biomass similar to the Δ*gln1* mutant. Surprisingly, the three deregulated mutants (D60A/S62A, G246A/G248A, and S72A/D73A) accumulated five- to sixfold more dry weight than the Δ*gln1* mutant and produce SMs. These data indicate that the down-regulation of secondary metabolism in the Δ*gln1* mutant is due to suboptimal energy balance and carbon source availability (Wagner et al., 2013).

In addition to transmembrane localized permeases which have transport and sensing functions ("transceptors"), and the GS which seems to be involved in ammonium sensing, additional internal nitrogen sensors must exist which are able to determine the nitrogen status of the cell and to affect expression of nitrogen-repressed SMs. One of them is the "TOR" protein kinase in *F. fujikuroi.* Genome-wide transcriptional analysis of the wild-type treated or not treated with rapamycin revealed a similar set of TOR-controlled genes, which are involved in anabolic processes, including translation, transcription, and ribosome biogenesis, similarly to the rapamycin-sensitive genes found in *S. cerevisiae* (Rohde and Cardenas, 2004; Teichert et al., 2006). In contrast to yeast which does not produce any SMs, inhibition of TOR by rapamycin resulted in partial deregulation of GA and bikaverin biosynthesis genes, but also of some other genes subject to nitrogen metabolite repression. However, this partial deregulation was obtained only with nitrogen concentrations no higher than 10 mM (Teichert et al., 2006). Rapamycin was insufficient to override the repressing effect of high nitrogen concentrations. Currently, it is not known whether inhibition of TOR by rapamycin results in nuclear translocations of the GATA transcription factors AreA and/or AreB, and if Sit4- and PP2A-like phosphatases are involved in dephosphorylation of AreA as it has been shown for the AreA homolog Gln3p in *S. cerevisiae* (Georis et al., 2008; Tate and Cooper, 2013). In *F. oxysporum* it has been shown that rapamycin treatment increased transcript levels of three AreA target genes (*niaD*, *niiA*, and *mepB*; López-Berges et al., 2010). The up-regulation of the sensing ammonium permease *mepB* might also contribute to the elevated expression of GA and bikaverin biosynthetic genes upon rapamycin treatment in *F. fujikuroi.*

Besides the TOR cascade, other signaling components seem to be involved in nitrogen sensing and subsequent regulation of secondary metabolism. In *F. proliferatum* it has been shown that the "HOG (high osmolarity glycerol)-type MAPK" pathway senses nitrogen starvation stress and regulates adaptation to these new conditions. Deletion of the HOG-encoding gene resulted in much stronger elevation of *FUM* gene expression upon transfer into a nitrogen-free medium compared to the wild-type but had no impact on FUM gene expression under nitrogen sufficient conditions (Kohut et al., 2009). A role of the stress-activated MAP kinase Sak1, a homolog of Hog1, in nitrogen sensing, and pigment production has also been shown in *Aspergillus fumigatus* (May et al., 2005).

Another example for the role signaling cascades may play in nitrogen regulation of secondary metabolism is the Gα protein/adenylate cyclase-mediated repression of the fusarubin production in *F. fujikuroi.* These PKS-derived perithecial pigments were only produced under nitrogen-limiting alkaline conditions (5 mM $NaNO_3$). Deletion of the Gα subunit FfG1 resulted in strong up-regulation of *fsr* gene expression and fusarubin production not only under low nitrate conditions but also under normally repressing high (60 mM) nitrate conditions (Studt et al., 2013a).

NITROGEN AVAILABILITY AFFECTS THE HISTONE MODIFICATION PATTERNS

Recent studies in several fungi showed that SM gene clusters in fungi can be regulated by chromatin-modifying enzymes, such as histone acetylases and deacetylases as well as histone methylases (Williams et al., 2008; Bok et al., 2009; Strauss and Reyes-Dominguez, 2011; Soukup et al., 2012). In general, gene expression has been associated with acetylation of histone H3 lysine 9 (H3K9ac) and dimethylation of histone H3 lysine 4 (H3K4me2), whereas gene silencing has been associated with trimethylation of histone H3 lysine 9 (H3K9me3; Bannister and Kouzarides, 2011; Strauss and Reyes-Dominguez, 2011).

In *F. fujikuroi*, many putative SM biosynthetic gene clusters were mapped to individual chromosomes, and most of them were shown to be located within subtelomeric regions. These regions are often subject to regulation by posttranslational modification of histones. To show whether microarray-based expression profiles and production levels for a certain SM fit with enrichment of a specific histone mark at the cluster region, genome-wide ChIP-seq analysis under nitrogen-limiting and nitrogen sufficient conditions were performed by using antibodies specific to two activating (H3K9ac and H3K4me2) and one silencing (H3K9me3) modifications. The presence of H3K9ac was correlated with gene expression across the GA, bikaverin, and fumonisin gene clusters at low concentrations of nitrogen, while H3K9ac was almost completely absent under repressing high-nitrogen conditions (Wiemann et al., 2013). These data support the hypothesis that histone acetylation is also associated with gene transcription in *F. fujikuroi.* However, this is not always the case. For example, the fusaric acid cluster showed little enrichment for H3K9ac under the inducing high nitrogen conditions (Wiemann et al., 2013). Therefore, other factors, including global or specific transcription factors, additional histone modifications, or other external signals such as pH or plant signals can also regulate expression of cluster genes.

A link between nitrogen regulation and histone modifications has been recently shown for the bikaverin biosynthesis in *F. fujikuroi.* While the *bik* genes are only expressed under nitrogen-limiting conditions in the wild-type strain, high *bik* gene expression levels under repressing (60 mM glutamine) and low expression levels under normally inducing conditions (6 mM glutamine) have been observed in the histone deacetylase deletion strain Δ*ffhda1* (Studt et al., 2013b). This contrasting expression pattern was confirmed by Northern blot and high-performance liquid chromatography with diode-array detection (HPLC-DAD).

CONCLUSION

Fungi are able to respond to quantitative and qualitative changes in nitroogen availability through complex regulatory mechanisms. Components of this regulatory network are nitrogen sensors, signaling cascades, e.g., the TOR cascade, transcription factors, and other regulatory proteins that might be able to interact. In contrast to *S. cerevisiae* with four GATA transcription factors involved in nitrogen regulation, filamentous fungi have only two, AreA and AreB. The former factor predominantly acts as positive regulator activating the expression of a set of nitrogen-regulated genes when preferred nitrogen sources, such as glutamine or ammonia, are limited. In the past years significant progress has been made in understanding how AreA functions. Besides its binding to the promoters of target genes, work in *A. nidulans* revealed an additional important role of AreA: the ability to mediate chromatin remodeling by increasing histone H3 acetylation. Much less is known about the role of AreB. Recent work in *F. fujikuroi* clearly showed that AreB can act both as positive and negative regulator and it regulates common target genes with AreA, but also AreB-specific target genes.

Beside genes involved in utilization and metabolization of different nitrogen sources, AreA is now well accepted as regulator of SMs. After the finding that AreA is essential for expression of GA biosynthetic genes in *F. fujikuroi*, several other SMs, mainly produced by species of the genus *Fusarium,* were also shown to depend on AreA. Recently it has been shown that both AreA and AreB are essential for GA gene expression, whereas only AreB positively affects apicidin F and fusaric acid biosynthesis.

Beside the two major transcription factors AreA and AreB, some other regulators have been shown to affect SM production, such as MeaB, Tor, and the GS. Also one of the three ammonium permeases, MepB, seems to play a role as sensor in addition to its transport function. A model of the function of individual components of the nitrogen regulation network in *F. fujikuroi* with emphasis on secondary metabolism is given in **Figure 1**.

However, despite the progress made in studying nitrogen regulation of secondary metabolism, the molecular mode of action, and possible interactions between and cross-talks with the regulators are not well understood. Because of the importance of nitrogen availability in regulating secondary metabolism, fundamental studies are needed to shed light on the functions of individual regulators, but also on the entire network, from sensing the nitrogen signal to the alteration of expression profiles.

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Fungal metabolic gene clusters—caravans traveling across genomes and environments

Jennifer H. Wisecaver* and *Antonis Rokas*

Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

Edited by:
Jonathan Palmer, US Forest Service, USA

Reviewed by:
David Fitzpatrick, Maynooth University, Ireland
Marina Marccet-Houben, Centre de Regulació de Genòmica, Spain

****Correspondence:***
Antonis Rokas, Department of Biological Sciences, Vanderbilt University, VU Station B 351364, Nashville, TN 37235, USA
e-mail: antonis.rokas@vanderbilt.edu

Metabolic gene clusters (MGCs), physically co-localized genes participating in the same metabolic pathway, are signature features of fungal genomes. MGCs are most often observed in specialized metabolism, having evolved in individual fungal lineages in response to specific ecological needs, such as the utilization of uncommon nutrients (e.g., galactose and allantoin) or the production of secondary metabolic antimicrobial compounds and virulence factors (e.g., aflatoxin and melanin). A flurry of recent studies has shown that several MGCs, whose functions are often associated with fungal virulence as well as with the evolutionary arms race between fungi and their competitors, have experienced horizontal gene transfer (HGT). In this review, after briefly introducing HGT as a source of gene innovation, we examine the evidence for HGT's involvement on the evolution of MGCs and, more generally of fungal metabolism, enumerate the molecular mechanisms that mediate such transfers and the ecological circumstances that favor them, as well as discuss the types of evidence required for inferring the presence of HGT in MGCs. The currently available examples indicate that transfers of entire MGCs have taken place between closely related fungal species as well as distant ones and that they sometimes involve large chromosomal segments. These results suggest that the HGT-mediated acquisition of novel metabolism is an ongoing and successful ecological strategy for many fungal species.

Keywords: metabolic gene cluster, gene innovation, horizontal gene transfer, microbial ecology, comparative genomics, secondary metabolism, specialized metabolism, physical linkage

GENETIC NOMADS—GENE INNOVATION THROUGH HORIZONTAL GENE TRANSFER

The ability to respond to dynamic ecological pressures, such as shifts in nutrient availability or biological interactions, is a defining characteristic of many successful species and often requires evolutionary innovation. Horizontal gene transfer or HGT—the transfer of genetic material from one organism to another through a process other than reproduction—is one source of innovation that can result in the rapid acquisition of genes that contribute to ecologically important traits (Gogarten and Townsend, 2005). In bacteria and archaea, HGT is a major contributor to gene innovation (Ochman et al., 2000; Boucher et al., 2003; Jain et al., 2003; Treangen and Rocha, 2011), with as much as 32% of genes in these organisms, depending on the species, having been recently acquired via HGT (Koonin et al., 2001), and with over 75% of prokaryotic genes having experienced at least one HGT event (Dagan et al., 2008; Kloesges et al., 2011).

Although once considered a process of limited effect outside of prokaryotes, we now know that HGT has occurred in all major eukaryotic lineages (reviewed by Huang, 2013), including multicellular plants (e.g., Yue et al., 2012; Li et al., 2014) and even animals, whose isolated germlines were once considered effectively inaccessible to foreign DNA (e.g., Dunning Hotopp et al., 2007; Graham et al., 2008; Danchin et al., 2010; Moran and Jarvik, 2010; Boschetti et al., 2011). Although the frequency of HGT is generally substantially lower in eukaryotic genomes compared to prokaryotic ones (Keeling and Palmer, 2008; Andersson, 2009), notable examples that invoke as well as showcase HGT's influence on eukaryotic evolution include the green plant radiation onto dry land (Yue et al., 2012), the repeated colonization of animal digestive tracts by microbial eukaryotes (Garcia-Vallve et al., 2000; Ricard et al., 2006), and even adaptation to life in boiling acid lakes in extremophile algae (Schönknecht et al., 2013). Elevated rates of HGT have also been coincident with the loss of typical eukaryotic traits such as sexual reproduction (Boschetti et al., 2012) and aerobic growth (Andersson et al., 2003; Loftus et al., 2005; Pombert et al., 2012).

Among eukaryotic lineages, the fungi are no exception and also show HGT-driven gene innovation, broadly resulting in expanded repertoires of secreted and transporter proteins and increased metabolic capacities (Richards et al., 2011). A survey of sixty fungal genomes detected hundreds of genes horizontally acquired from bacteria (Marcet-Houben and Gabaldon, 2010), and studies suggest that bacteria-derived genes serve various functions in diverse fungal lineages, from vitamin biosynthesis in yeast (Hall and Dietrich, 2007), to the production of secondary metabolites in filamentous fungi (van den Berg et al., 2008; Schmitt and Lumbsch, 2009; Lawrence et al., 2011). Other cases of HGT from bacteria have been implicated in the ability of some soil-dwelling fungi to utilize unusual carbon sources (Wenzl et al., 2005), host adaptation in fungal pathogens (Hu et al., 2014), and adaptation to anoxic environments in the model fermenter *Saccharomyces cerevisiae* (Gojkovic et al., 2004; Hall et al., 2005). Aside from bacteria, the list of donors of fungal genetic material that was acquired via HGT includes plants (Richards et al., 2009), microbial eukaryotes (Slot and Hibbett, 2007; Tiburcio et al., 2010), and, perhaps most frequently, other fungi (Wisecaver et al., 2014).

MOLECULAR TARIFFS—PATHWAY COMPLEXITY AS A BARRIER TO HGT

Phylogenomic surveys suggest that HGT has affected between 0.1–2.8% of genes on a typical fungal genome (Marcet-Houben and Gabaldon, 2010; Wisecaver et al., 2014). It may seem surprising that HGT in fungi is not more common, given the obvious advantage of acquiring a pre-adapted gene from the environment rather than *de novo* from non-coding sequence or through gene duplication and subsequent functional specialization. However, the structure of the eukaryotic cell, in which the genome is tightly packaged with chromatin and compartmentalized in the nucleus, as well as incompatibilities between potential donor and recipient organisms in their genome architecture or molecular machinery (e.g., differences in promoter regions, intron-splicing, and codon usage patterns), are all likely barriers to rampant HGT in eukaryotes (Keeling and Palmer, 2008; Richards et al., 2011). Fungal biology may also limit the opportunity of DNA exchange; many species of filamentous fungi grow through branching and fusion of hyphal mycelium and have evolved vegetative incompatibility mechanisms to limit cell fusion with genetically different individuals (Glass et al., 2000).

Studies of HGT in prokaryotes suggest that the propensity of a gene to undergo HGT is strongly associated with its biological function. For example, genes involved in replication, translation and transcription are transferred less often than genes participating in cellular metabolism (Jain et al., 1999; Nakamura et al., 2004). Cohen et al. (2011) argued that this trend is likely driven by the correlated factor of gene connectivity; genes whose products form many complex interactions are less likely to undergo successful HGT because they are less likely to be successfully integrated into a foreign system. In contrast, genes whose products function alone, without interacting with other proteins, are predicted to be the most amenable to HGT (Moran et al., 2012). Thus, the fact that eukaryotes have large genomes with complex gene interaction networks (Szklarczyk et al., 2015) may further explain partly why HGT is not as abundant in these organisms compared to prokaryotes.

METABOLIC CARAVANS—GENE CLUSTERING CAN FACILITATE THE TRANSFER OF ENTIRE PATHWAYS

The majority of high-throughput analyses in fungi and other organisms has examined and quantified the impact of HGT on a gene-by-gene basis, effectively assuming that each gene that has undergone HGT has done so independently of any other genes (e.g., Loftus et al., 2005; Dagan et al., 2008; Marcet-Houben and Gabaldon, 2010; Cohen et al., 2011; Kloesges et al., 2011; Boschetti et al., 2012; Yue et al., 2012; Schönknecht et al., 2013; Wisecaver et al., 2014). Yet, numerous discoveries in diverse fungal organisms have shown that multiple genes can be transferred together (Slot and Hibbett, 2007; Khaldi et al., 2008; Novo et al., 2009; Moran and Jarvik, 2010; Slot and Rokas, 2010, 2011; Khaldi and Wolfe, 2011; Campbell et al., 2012; Cheeseman et al., 2014; Greene et al., 2014). For example, the genome of a *S. cerevisiae* commercial wine strain contains a 17-kb DNA segment (including five protein coding genes with various functions) that was horizontally acquired from the yeast *Zygosaccharomyces bailii*, a common wine fermentation contaminant (Novo et al., 2009). More often, however, co-transferred genes are involved in the same function, effectively reducing the connectivity barrier to HGT. For example, two genes required for carotenoid biosynthesis in pea aphids were horizontally transferred in a single event from fungi and are responsible for a red-green color polymorphism in these insects that influences their susceptibility to predators (Moran and Jarvik, 2010).

An important prerequisite for the co-transfer of functionally associated genes is the genes' physical proximity in the donor genome. Although it was once assumed that the order of the genes in eukaryotic genomes was random, limiting opportunity for such co-transfers, a slew of studies suggests that this is not true (Lee, 2003; Hurst et al., 2004). In fungi, this non-randomness is perhaps best illustrated by metabolic gene clusters (MGCs), which typically consist of metabolic pathways whose constituent genes are physically linked in the genome. MGCs are common features of fungal genomes (Keller and Hohn, 1997; Hall and Dietrich, 2007; Wisecaver et al., 2014), and similar MGCs have also been discovered in several plant species (e.g., Frey et al., 1997; Qi et al., 2004; Field and Osbourn, 2008; Winzer et al., 2012; Itkin et al., 2013).

In fungi, MGCs most often code for metabolic pathways that are not required to sustain cellular life but instead confer accessory traits that allow organisms to better respond to ecological pressures. Fungal examples of such MGCs include those for resistance to xenobiotic arsenic (Bobrowicz et al., 1997) and degradation of plant defensive compounds (Greene et al., 2014) as well as utilization of uncommon or ecologically specialized forms of carbon (Hittinger et al., 2004), nitrogen (Jargeat et al., 2003; Wong and Wolfe, 2005) and other nutrients (Hull et al., 1989; Hall and Dietrich, 2007). Perhaps the most well-known fungal MGCs are those that code for the production of secondary metabolites (SMs) (Keller and Hohn, 1997; Keller et al., 2005)—the focus of this special Research Topic.

A growing number of studies shows that several fungal MGCs encoding diverse specialized metabolic pathways have undergone HGT (**Table 1**). Some of the transferred MGCs are involved in nutrient acquisition, including the *GAL* cluster for galactose utilization (Slot and Rokas, 2010) and the fHANT-AC cluster for nitrate assimilation (Slot and Hibbett, 2007), whereas others are found in plant-associated filamentous fungi and appear to have been acquired for degrading defensive SMs produced by plant hosts (Greene et al., 2014). However, most transferred MGCs are involved in the production of fungal SMs, including sterigmatocystin (Slot and Rokas, 2011), bikaverin (Campbell et al., 2012), fumonisin (Khaldi and Wolfe, 2011), and gliotoxin (Patron et al., 2007).

To quantify this association between fungal MGCs and HGT, a recent survey of metabolic genes from 208 fungal genomes and

Table 1 | Summary of published cases of HGT involving MGCs.

MGC	Donor[a]	Recipient[a]	No. genes[b]				Function	References
			D	R	T	C		
ACE1 biosynthesis	*Magnaporthe*	*Aspergillus*	15	6	5	6	SM production	Khaldi et al., 2008[c]
Bikaverin biosynthesis	*Fusarium*	*Botrytis*	6	6	6	na	SM production	Campbell et al., 2012, 2013
fHANT-AC for nitrate assimilation	Oomycetes	Dikarya	3	3[d]	3	2	Nutrient acquisition	Slot and Hibbett, 2007
fHANT-AC for nitrate assimilation	*Ustilago*	*Trichoderma*	3	3	3	2[e]	Nutrient acquisition	Slot and Hibbett, 2007
Fumonisin biosynthesis	*Fusarium*	*Aspergillus*	16	11	2	na	SM production	Khaldi and Wolfe, 2011
Fumonisin biosynthesis	Repeated transfer of 16–17-gene cluster between *Fusarium spp.*[f]						SM production	Proctor et al., 2013
GAL utilization	*Candida*	*Schizosaccharomyces*	5–6	3–4	4	4	Nutrient acquisition	Slot and Rokas, 2010
Gentisate catabolism	Between *Cochliobolus* and *Magnaporthe* grass pathogens[g]		6	6	6	na	Protection/defense	Greene et al., 2014
Gliotoxin and related ETP toxins	Multiple HGTs within Pezizomycotina		unknown[h]			18[i]	SM production	Patron et al., 2007[j]
Sterigmatocystin biosynthesis	*Aspergillus*	*Podospora*	23	24	23	23	SM production	Slot and Rokas, 2011
Tyrosine degredation	*Exophiala*	*Baudoinia*	5	8	4	na	Protection/defense	Greene et al., 2014

[a] *Donor and recipient taxonomic clade based on taxon sampling of each study.*

[b] *Number of MCG genes, where column D is the number of genes in the existing MGC in the donor lineage, R is the number of genes in the existing MGC in the recipient lineage, T is the number of gene trees supporting MGC-HGT reported by the original reference, and C is the number of gene trees supporting MGC-HGT confirmed by* Richards et al. (2011).

[c] *See also* Moore et al. (2014) *which argues that extensive gene duplication and loss could also explain the ACE1 gene phylogenies.*

[d] *Genes are not clustered in some fungal lineages.*

[e] *Reported phylogenies for nitrate reductase and the high affinity nitrate transporter.*

[f] *Transfers inferred based on phylogenetic incongruence between accepted species phylogeny and supermatrix tree of concatenated genes in fumonisin MGC.*

[g] *Insufficient phylogenetic evidence to infer the direction of HGT event.*

[h] Patron et al. (2007) *lists two possible patterns of MGC inheritance: one via HGT and the other via vertical inheritance involving multiple gene duplications and losses.*

[i] Richards et al. (2011) *does not reject the hypothesis of HGT, but states that the extensive differences between the gene histories and the species phylogeny make it difficult to differentiate between HGT over complex gene loss.*

[j] *See also* Ballester et al. (2014) *which describes phylogenetic patterns indicative of HGT in the ETP MCG in Penicillium expansum and Penicillium roqueforti.*

HGT showed that genes in MGCs were transferred 1.66 fold more often than their non-clustered counterparts (Wisecaver et al., 2014), with clustered genes involved in SM biosynthesis showing significantly higher rates of HGT than other clustered genes in filamentous fungi. Taken together, these studies suggest that the organization of metabolic pathways into discrete MGCs may facilitate their dispersal through HGT.

ECOLOGICAL CURRENCY—ADAPTATION MAY DRIVE THE GAIN AND LOSS OF METABOLIC GENE CLUSTERS

By circumscribing the available niches in which fungal species and their MGCs interact and evolve, ecology is a major determinant of HGT of fungal MGCs, even though deciphering the ecological "means and motive" associated with specific HGT events is not always straightforward. This is largely due to the fact that HGTs are inferences of ancient historical events. For example, the exact MGC donors and recipients can never be known with certainty, unless the HGT event is extremely recent; thus, in most cases putative donor and recipient species are instead approximated based on extant sequenced genomes, and the likely ecological circumstances associated with the event are deduced from what is known about their organisms' ecologies. Recurrent HGT events, as may be the case for the gliotoxin and related ETP toxin MGCs (Patron et al., 2007), as well as very ancient HGT events render such interpretations particularly difficult. For example, the fHANT-AC cluster for nitrate assimilation may have first evolved in oomycetes, fungal-like microorganisms actually related to brown algae and diatoms, before it was transferred to the ancestor of Dikarya fungi, making the task of inferring the ancient selective pressures that may have driven this HGT event challenging (Slot and Hibbett, 2007). Nevertheless, the suggested age of the fHANT-AC transfer (circa 500 Ma) is consistent with the estimated age of the plant colonization of dry land (Sanderson et al., 2004), leading Slot and Hibbett (2007) to speculate that the ability to utilize nitrate as a source of fixed nitrogen may have been a key innovation that facilitated the Dikarya to radiate alongside plants across terrestrial habitats.

Limited functional understanding of MGC constituent genes also confounds ecological interpretation of HGT. This is particularly challenging for MGCs involved in secondary metabolism, because they are often lineage-specific and their enzymatic activities are often poorly characterized. Inferences can be made based on homology, but there are limitations. For example, Khaldi et al. (2008) suggests that the ACE1 cluster may have been horizontally transferred from *Magnaporthe* to *Aspergillus*. In the rice blast fungus *Magnaporthe grisea*, the ACE1 cluster is expressed during fungal penetration of host leaves, suggesting its involvement in plant pathogenicity (Böhnert et al., 2004); however, *Aspergillus clavatus* is not a plant pathogen, indicating the MGC likely serves a different function in this species.

Despite the challenges of identifying and interpreting past HGT, examination of well-characterized HGT events involving fungal MGCs shows that they occur in a wide variety of ecological settings and may involve species with overlapping ecological niches. Putative examples include HGT between ubiquitous fungal saprobes (Khaldi et al., 2008; Khaldi and Wolfe, 2011; Slot and Rokas, 2011), natural fermenters (Slot and Rokas, 2010), and plant pathogens (Campbell et al., 2012; Greene et al., 2014). In one instance, a fungal MGC encoding the tyrosine degradation pathway shows evidence of HGT from *Exophiala* to *Baudoinia*, both of which are thermotolerant, as well as between *Cochliobolus* and *Magnaporthe*, both of which are grass pathogens (Greene et al., 2014).

EVOLUTIONARY OUTCOMES—METABOLIC GENE CLUSTERS FOLLOW DIFFERENT PATHS AFTER TRANSFER

Once integrated into a new genome, a horizontally transferred MGC may follow diverse evolutionary paths (**Figure 1**). In some cases, the original function of the MGC may be beneficial to the recipient organism, and purifying selection may act to preserve it. For instance, the MGC responsible for the biosynthesis of the SM sterigmatocystin, a highly carcinogenic mycotoxin, was likely horizontally transferred from *Aspergillus* to *Podospora* (Slot and Rokas, 2011). As the sterigmatocystin MGCs of *Podospora anserina* and *Aspergillus nidulans* are remarkably conserved in both synteny and sequence identity (**Figure 2**), one may infer strong selection for maintaining the MGC's structure and function in both the donor and the recipient. Similarly, strong purifying selection is the most likely explanation for the conservation of the nitrate reductase MGC across many fungal lineages for half a billion years (Slot and Hibbett, 2007).

The selection pressure may often differ across descendant lineages of the HGT recipient, and in some cases neutral evolution or even positive selection may result in partial or complete loss of a horizontally transferred MGC. For example, work by

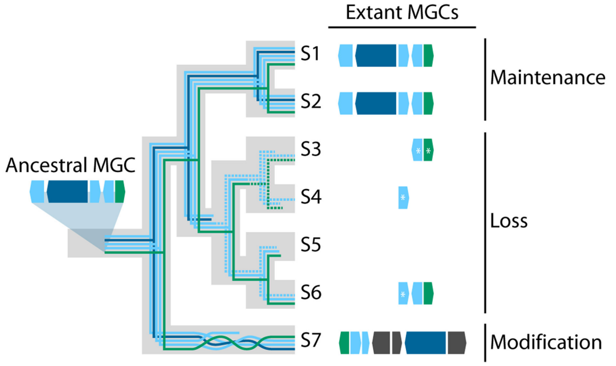


FIGURE 1 | Three evolutionary fates of transferred MGCs. In this hypothetical example, a five gene MGC is transferred into the common ancestor of seven species of fungi (taxa S1–S7). The large gray tree represents the underlying species phylogeny, and each colored line represents the evolutionary history of a gene within the MGC. Dashed lines indicate genes that have been pseudogenized and are no longer functional. Extant MGCs in taxa S1 and S2 have maintained the same number of genes in the same orientation as the ancestral MGC suggesting purifying selection has acted to preserve the MGC's original form and function. In contrast, taxa S3–S6 contain the MGC in varying stages of decay, indicative of neutral evolution or positive selection for its loss. Asterisks (*) indicate pseudogenes. Finally, the MGC in taxon S7 has been modified from its original form, having undergone gene rearrangement, gene loss, and recruited three additional genes (colored in dark gray), which suggests the MGC has experienced diversifying selection.

FIGURE 2 | The remarkable similarity of the sterigmatocystin MGC between *Aspergillus nidulans* and *Podospora anserina,* two organisms belonging to different fungal classes, is evidence of HGT. (A) Synteny conservation between the sterigmatocystin MGC from *Podospora anserina* and *Aspergillus nidulans.* Aligned regions were drawn using the progressive Mauve algorithm (Darling et al., 2010) and are shown in red. **(B)** Conservation of SM gene clusters between *Podospora anserina, Aspergillus nidulans* and *Aspergillus fumigatus.* Circular tracks were created using Circos (http://circos.ca); the outer black track shows the relative gene counts in each of the three species. However, to visualize the relative location of SM gene clusters, the width of genes in these clusters have been drawn at 20 times the width of unclustered genes. Average amino acid percent identities (%IDs) of all reciprocal best BLAST hits (RBBHs) between the three genomes are shown in gray. SM gene clusters were predicted using antiSMASH (Blin et al., 2013), and SM cluster type is indicated by the colored wedges of the inner track. SM clusters were considered homologous if greater than 50% of their genes were RBBHs. Black links and black numbers indicate homologous SM clusters between *Aspergillus nidulans* and *Aspergillus fumigatus* and the average %IDs of RBBHs of the clustered genes, respectively. The red link and red number indicate the only homologous SM cluster (sterigmatocystin) identified between *Aspergillus nidulans* and *Podospora anserina* and the average %ID of RBBHs of the clustered genes, respectively. There are no homologous SM clusters between *Aspergillus fumigatus* and *Podospora anserina.*

Campbell et al. (2012, 2013) suggests that the MGC for the biosynthesis of the SM bikaverin was horizontally transferred from *Fusarium* to *Botrytis.* Although a functional bikaverin MGC was identified in a rare *Botrytis cinerea* isolate (Schumacher et al., 2013), the MGC is more commonly present in various stages of decay in the genomes of several different *Botrytis* species (Campbell et al., 2013); for example, *Botrytis galanthina* and *Botrytis elliptica* retain only a few pseudogenes, whereas four other species appear to have lost the entire MGC altogether (Campbell et al., 2013).

Alternatively, once transferred into a new genome, fungal MGCs may experience diversifying selection that alters their functions. Although potentially the most interesting mode of selection, because of its ability to lead to the generation of novel pathways and metabolic products, diversifying selection is also the most challenging to identify and characterize because donor and recipient MGCs may appear very different following diversification. For example, Khaldi and Wolfe (2011) argued that the fumonisin MGC in *Aspergillus niger* was acquired via HGT from *Fusarium* but that the MGCs have since diverged in function, because the number and order of genes in the MGCs is no longer conserved. Similarly, diversifying selection following HGT may have also played a role in the discontinuous distribution of epipolythiodioxopiperazine (ETP) MGCs responsible for the fungal production of gliotoxin, sirodesmin and related mycotoxins. Patron et al. (2007) identified putative ETP gene clusters in diverse fungal species, but the MGCs varied dramatically in total gene length, suggesting they were responsible for the production of various SM products.

CELLULAR TRADE ROUTES—POSSIBLE MECHANISMS FOR THE ACQUISITION OF FOREIGN DNA

Ecological opportunity and motive aside, identifying the possible cellular mechanisms for the uptake and incorporation of foreign DNA is equally important when seeking to understand the effect of HGT on fungal genomes. Recent reviews by Richards et al. (2011) and Soanes and Richards (2014) list several observable mechanisms for fungal HGT, including conjugation, both natural and agrobacterial mediated transformation, and viral transduction. Both reviews also cite data supporting conidial and hyphal fusion as potential fungal-specific routes for DNA exchange, arguing that, although vegetative incompatibility mechanisms would act to limit fusion events, errors in such systems need only occur at a low frequency to represent a viable mechanism for fungal HGT. Once taken up by the recipient cell, incorporation of foreign DNA could occur via ectopic repair of double-stranded DNA breaks, which genomes are susceptible to during cellular stress. Moreover, MGCs are often located in dynamic, rapidly evolving regions of fungal chromosomes, such as subtelomeres (Keller et al., 2005) or near mobile genetic elements (Han et al., 2001), both of which have been associated with instances of HGT in other eukaryotes (Gladyshev et al., 2008; Keeling and Palmer, 2008).

Another viable route of HGT for fungal MGCs is whole chromosome transfer. Several fungal species contain accessory chromosomes (also known as supernumerary or conditionally dispensable chromosomes), which are not essential for normal growth but may carry genes for specialized functions that are beneficial in certain conditions (Covert, 1998). An extreme example comes from the genome of the plant pathogenic fungus, *Mycosphaerella graminicola*, in which eight of the 21 chromosomes can be lost with no visible effect on the fungus and may have originated via HGT from an unknown donor (Goodwin et al., 2011). Another example comes from *Fusarium oxysporum* f. sp. *lycopersici,* which contains four accessory chromosomes that account for over 25% of its genome (Ma et al., 2010).

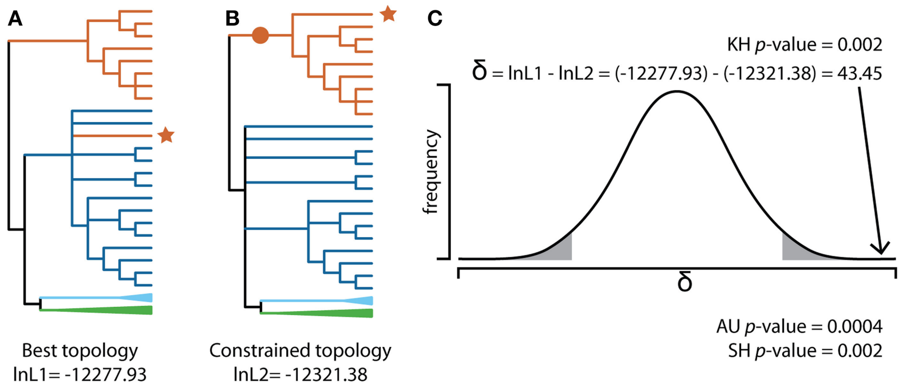
Importantly, accessory chromosomes have been shown to encode MGCs and can be transferred between fungal strains (He et al., 1998; Akagi et al., 2009; Ma et al., 2010; van der Does and Rep, 2012). The 1 Mb accessory chromosome in *Alternaria arborescens* contains 209 genes including ten putative MGCs (Hu et al., 2012). These 209 genes lack homology or have low sequence similarity to genes in other *Alternaria* spp. and have a different GC-content and codon bias compared to genes on essential chromosomes, leading Hu et al. (2012) to argue that the entire accessory chromosome, along with its ten MGCs, was obtained through HGT. Similarly, an accessory chromosome in some strains of *Nectria haematococca* contains a six gene pea pathogenicity (*PEP*) MGC required for causing disease on pea plants. Genes in the *PEP* cluster also have a different GC-content and codon bias compared to genes on essential chromosomes and may have been acquired via HGT (Temporini and Vanetten, 2004). Although this is an exciting and ongoing area of research in fungal biology, thus far horizontal chromosome transfer has only been documented between member of the same species or genus, so the relative impact of this process as a mechanism for fungal HGT is currently unknown.

EVOLUTIONIST'S TOOLKIT—METHODS FOR DETECTING HGT

Most cases of fungi-to-fungi HGT, including those involving MGCs, are first identified based on a sequence similarity search such as BLAST followed by a phylogenetic analysis, which demonstrates incongruence between gene trees and the established species phylogeny. Specifically, HGT is supported when genes suspected of being horizontally acquired have well-supported phylogenetic profiles that contradict accepted species relationships (Soanes and Richards, 2014). Whenever applicable, the hypothesis of HGT should be evaluated using a comparative topology test that examines whether the tree topology indicative of HGT is significantly better than other topologies that do not support the transfer event. Specifically, a comparative topology test compares the likelihood of the best topology to the likelihoods of one or more alternative topologies, given the sequence alignment, and computes the probability that the alternative topologies have a statistically lower fit to the data than the best topology (**Figure 3**, Shimodaira and Hasegawa, 2001). In the case of the sterigmatocystin MGC, comparative topology tests supported the placement of *Podospora* within or sister to the *Aspergillus* clade in nine out of the twenty three gene trees (Slot and Rokas, 2011).

Although many of the HGT events listed in **Table 1** are supported by multiple phylogenetic and genomic comparisons, in some cases the available evidence does not allow for robust inference of HGT or its directionality. In some instances a MGC is shared between distantly related fungal species, indicating HGT likely played a part in its current distribution, but limited taxon sampling of closely related strains and species makes it difficult to resolve the timing and directionality of the HGT event (Patron et al., 2007; Greene et al., 2014). Many MGCs, particularly those involved in the production of SMs, have diversified rapidly (Carbone and Ramirez-Prado, 2007). As a consequence, some genes in MGCs share little or no sequence similarity to any other known sequence making it challenging to even construct gene trees with strong phylogenetic signals, let alone identify cases of HGT.

In contrast to MGC genes that are lineage-specific, other MGC genes are members of large, multi-copy, multi-domain



Best topology
lnL1= -12277.93

Constrained topology
lnL2= -12321.38

FIGURE 3 | Evaluating HGT using a comparative topology test. (A) Maximum likelihood (ML) phylogeny of homologous sequences to stcI, a gene in the sterigmatocystin SM cluster, in *Podospora anserina* identified from a BLAST search of 161 Pezizomycotina genomes (JGI Mycocosm, download date 5 July 2014). Sequences were aligned and trimmed using MAFFT (Katoh and Standley, 2013) and trimAL (Capella-Gutierrez et al., 2009), respectively, and the phylogeny was created using RAxML (Stamatakis, 2014) using the PROTGAMMAAUTOF amino acid model of substitution and 100 bootstrap replicates. The resulting cladogram was midpoint rooted and branches supported by less than fifty bootstrap replicates were collapsed. This ML best tree depicts *Podospora anserina* (Sordariomycetes; red branch with red ⋆) grouping with Eurotiomycetes (dark blue branches). Other taxa in the phylogeny include additional Sordariomycetes (red branches), Leotiomycetes (light blue collapsed clade) and Dothideomycetes (green collapsed clade). **(B)** Best ML phylogeny using the same input data, but with the constraint imposed that *Podospora anserina* must group with other Sordariomycetes (red •). All other branches were resolved to obtain the maximum log-likelihood (-lnL), given the alignment using RAxML. **(C)** The difference in likelihood scores was evaluated to determine if the best topology represents a significantly better explanation of the data compared to the constraint topology. The Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989) assumes a normal distribution of log-likelihood differences (δ). In this example, $\delta = 43.45$ lies within the rejection region (gray area under curve) so one can reject the null hypothesis that the best topology is not statistically significantly better than the constraint topology (KH p-value = 0.002). Other tests, such as the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) and the Approximately Unbiased (AU) test (Shimodaira, 2002), improved upon the KH test by correcting for multiple comparisons as well as the fact that the ML best tree is known *a priori*. In this example, both the SH test and AU test also reject the null hypothesis. All comparative topology tests were run in CONSEL (Shimodaira and Hasegawa, 2001).

gene families (Kroken et al., 2003; Bushley and Turgeon, 2010). Membership in such large, dynamically evolving gene families often makes distinguishing orthologs from paralogs extremely challenging (Haggerty et al., 2014). The complex evolutionary history of gliotoxin, a SM produced by *Aspergillus fumigatus* and other disparate fungal species, is a powerful illustration of this challenge. Patron et al. (2007) identified gliotoxin and gliotoxin-like MGCs in 14 species of Ascomycota. The resulting gene trees were extremely hard to reconcile with the species phylogeny, suggesting a complex evolutionary history likely due to a combination of gene duplication, transfer, and loss (Patron et al., 2007). A second phylogenetic analysis with increased taxonomic coverage by Richards et al. (2011) re-analyzed the evidence for HGT for this MGC, but was still unable to conclusively differentiate between HGT and complex patterns of gene loss. Phylogenetic patterns indicative of HGT were also found for genes in the gliotoxin-like MCGs of *Penicillium expansum* and *Penicillium roqueforti* (Ballester et al., 2014).

A perhaps more general approach to evaluate the evidence for HGT takes advantage of gene tree-species phylogeny reconciliation algorithms (Abby et al., 2010; Stolzer et al., 2012). For example, the phylogenetic software Notung can assign costs to HGT, gene duplication, and gene loss and use those costs to determine the most parsimonious combination of these three events to explain a given gene tree topology given the consensus species phylogeny (Chen et al., 2000; Vernot et al., 2007; Stolzer et al., 2012). In such analyses, HGT events will be inferred when a gene tree topology that is contradictory to the species phylogeny cannot be more parsimoniously reconciled using a combination of gene duplications and losses. This general approach is heavily dependent on the relative costs assigned to the three evolutionary events under consideration, namely HGT, gene duplication, and gene loss. Thus, it is important to evaluate how the relative costs assigned to the different events influence HGT inference. As an example, we reconciled the gene tree of homologous sequences to stcI (a gene in the sterigmatocystin SM cluster) in *Aspergillus* and *Podospora anserina* to the consensus Pezizomycotina species phylogeny using different costs for HGT. Only when using a HGT cost over 11.33 times the duplication cost, does Notung no longer predict a HGT from *Aspergillus* to *Podospora anserina* (**Table 2**).

Because the phylogenetic signal in single gene phylogenies can be weak (Rokas et al., 2003) and a wide variety of other biological and methodological factors can also produce gene trees that differ from the species phylogeny (Salichos and Rokas, 2013), examination of the concordance of evolutionary histories of genes in MGCs and their joint difference from the species phylogeny can significantly strengthen inference of HGT, something that is obviously impossible to do in examinations of single gene HGT events. For example, further support for the HGT of the sterigmatocystin MGC comes from a pairwise comparison of the clusters in *Podospora anserina* and *Aspergillus nidulans*, which shows an extreme conservation of gene order as well as increased sequence similarity of the sterigmatocystin gene orthologs compared to the average sequence similarity of all gene orthologs (**Figure 2**,

Table 2 | Example gene tree-species phylogeny reconciliation analysis using Notung v2.8 (Vernot et al., 2007; Stolzer et al., 2012).

Notung run	Transfer cost	Duplication cost	Loss cost	No. equally parsimonious solutions	Reconciliation score	Recovers *Podospora* HGT
Figure S1C	-	1.5	1	1	123	no
Figure S1D	3	1.5	1	256	70	yes
Figure S1E	7.5	1.5	1	1	97.5	yes
Figure S1F	15	1.5	1	1	112.5	yes
Figure S1G	17	1.5	1	1	116.5	yes
Figure S1H	19	1.5	1	1	119.5	no
Figure S1I	30	1.5	1	1	123	no

Gene tree of homologous sequences to stcI (a gene in the sterigmatocystin SM cluster, Supplementary Figure S1A) was reconciled to the consensus species phylogeny (Supplementary Figure S1B) using different transfer costs. Only when using a transfer cost over 11.33 times the duplication cost, does Notung no longer predict a HGT from Aspergillus to Podospora anserina. See Supplementary Figure 1 for reconciled trees.

Slot and Rokas, 2011). Taken together, the topology tests, gene tree-species phylogeny reconciliation, and unusually high conservation in gene content, sequence, and microsynteny, all provide strong support for the HGT of this gene cluster. Moreover, Slot and Rokas (2011) also demonstrated conservation of a cis-regulating element between *Podospora anserina* and *Aspergillus nidulans* as well as evidence of expression of the sterigmatocystin MGC in *Podospora anserina*, which coupled with the fact that *Podospora* produces sterigmatocystin (Matasyoh et al., 2011), suggests that HGT resulted in the acquisition of a functional cluster in this species.

Detection of a rare genomic change shared between donor and recipient taxa can provide additional evidence for a putative HGT event (Moran and Jarvik, 2010; Li et al., 2014). In the case of the horizontally acquired *GAL* cluster in *Schizosaccharomyces,* the *GAL10* gene contains fused epimerase and mutarotase domains, a trait shared with putative MGC donors but absent in other fungal species (Slot and Rokas, 2010). Remarkably, *Schizosaccharomyces* species still retain a vertically inherited, unclustered *GAL10* paralog as well that contains only an unfused epimerase domain (Slot and Rokas, 2010).

NO MORE ANALOGIES—CONCLUSIONS AND PERSPECTIVE

Studies suggest that several fungal MGCs have been transferred and have followed diverse evolutionary paths across a wide range of fungi (**Table 1**) that vary in their ecological life history strategies, including opportunistic animal and plant pathogens, suggesting that HGT of MGCs is a source of gene innovation in fungal specialized metabolism. Given that most fungal genomes have yet to be fully explored, that many fungal MGCs—especially those that produce SMs—exhibit very narrow taxonomic distributions, and that circumscription of novel MGCs continues unabated, it seems likely that the current number of known cases of HGT of fungal MGCs is just the tip of the iceberg.

Although it is to be expected that many more cases of HGT involving MGCs are going to be discovered in the years to come, determining the rate of such events as well as the evolutionary fates of the transferred MGCs remains an ambitious goal. What is sorely needed are targeted, in-depth sequencing efforts of closely related strains and species that might capture the remarkably rapid turn-around of fungal MGCs, coupled with functional studies and improved algorithms that characterize the number and function of MGCs in those genomes.

The small number of reported HGT cases, most of which are very ancient, severely limits efforts to understand the ecological circumstances that underlie these exchanges and how they alter the structure of fungal communities. At present, we still lack any understanding as to whether particular ecological lifestyles are more prone to HGT events than others. *A priori*, one might expect that fungi that form intimate ecological associations such as mycoparasites, endophytes and lichens might be more prone to HGT than free-living fungi, but the relative dearth of genome representatives for most fungal lifestyles severely limits the scope of any such analysis.

Although several mechanisms for both the acquisition and assimilation of foreign DNA exist in fungi (Ma et al., 2010; Soanes and Richards, 2014), the relative contribution of these mechanisms to the observed patterns of HGT is unclear; moreover, the mechanisms for stages of HGT between cell entry and genome incorporation are completely uncharacterized (Scazzocchio, 2014). For example, how and how frequently does foreign DNA escape nucleases and how does foreign DNA enter the nucleus? Is the chance of successful integration associated with foreign DNA length and is there bias toward integration of smaller fragments? At what rate is foreign DNA acquired, and how does this rate vary across species and environments? Once integrated, what fraction of transfers have a measurable fitness effect, and what fraction of those are beneficial vs. deleterious? Do horizontally acquired MGCs become integrated into existing regulatory networks of metabolism or does the acquisition of MGCs precipitate the modification of existing metabolic and regulatory networks?

These outstanding questions on the prevalence and consequences of HGT of fungal MGCs, as well as the ecology and mechanisms driving transfer events, require systematic tests of hypotheses about the process that often require integrative data from diverse facets of fungal biology, from genomics to chemistry, and from ecological lifestyles to evolutionary history. Fortunately, the ever larger number of fungal genomes sequenced and the increasing importance given to the sampling of ecologically and taxonomically important groups, coupled with novel functional genomic technologies, is quickly bringing remarkable amounts of data to bear on these questions. For example, the 1000 fungal genome project (http://1000.fungalgenomes.org), an ongoing community sequencing initiative sponsored by the Joint Genome Institute, aims to sequence at least two genomes from each of the more than 500 fungal families, including genomes representative of all the major fungal lifestyles and morphologies; similarly, the yeast 1000 project, recently funded by the National Science Foundation (http://www.nsf.gov/awardsearch/showAward?AWD_ID=1442148), aims to decode the genomes of all ∼1000 yeast species belonging to the subphylum Saccharomycotina. Such projects promise to not only serve as comprehensive surveys of fungal metabolism but also provide unprecedented opportunities for evaluating and quantifying the impact that processes, such as HGT-mediated gene innovation, have had on the generation of fungal biodiversity.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00161/abstract

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Motif-independent *de novo* detection of secondary metabolite gene clusters—toward identification from filamentous fungi

Myco Umemura, Hideaki Koike and Masayuki Machida*

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba/Hokkaido, Japan

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Edited by:
Ozgur Bayram,
Maynooth University, National University of Ireland Maynooth, Ireland

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Correspondence:
Masayuki Machida,
Bioproduction Research Institute, Hokkaido Center, National Institute of Advanced Industrial Science and Technology, 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, Tsukuba/Hokkaido 062-8517, Japan
m.machida@aist.go.jp

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Secondary metabolites are produced mostly by clustered genes that are essential to their biosynthesis. The transcriptional expression of these genes is often cooperatively regulated by a transcription factor located inside or close to a cluster. Most of the secondary metabolism biosynthesis (SMB) gene clusters identified to date contain so-called core genes with distinctive sequence features, such as polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS). Recent efforts in sequencing fungal genomes have revealed far more SMB gene clusters than expected based on the number of core genes in the genomes. Several bioinformatics tools have been developed to survey SMB gene clusters using the sequence motif information of the core genes, including SMURF and antiSMASH. More recently, accompanied by the development of sequencing techniques allowing to obtain large-scale genomic and transcriptomic data, motif-independent prediction methods of SMB gene clusters, including MIDDAS-M, have been developed. Most these methods detect the clusters in which the genes are cooperatively regulated at transcriptional levels, thus allowing the identification of novel SMB gene clusters regardless of the presence of the core genes. Another type of the method, MIPS-CG, uses the characteristics of SMB genes, which are highly enriched in non-syntenic blocks (NSBs), enabling the prediction even without transcriptome data although the results have not been evaluated in detail. Considering that large portion of SMB gene clusters might be sufficiently expressed only in limited uncommon conditions, it seems that prediction of SMB gene clusters by bioinformatics and successive experimental validation is an only way to efficiently uncover hidden SMB gene clusters. Here, we describe and discuss possible novel approaches for the determination of SMB gene clusters that have not been identified using conventional methods.

Keywords: secondary metabolism, bioinformatics tool, filamentous fungi, gene expression, non-syntenic block

Introduction

Filamentous fungi produce a wide variety of secondary metabolites, some of which have industrial value, including potential for medical use. Recently developed large-scale screening technology using high-throughput robotics (High-Throughput Screening: HTS), typically by pharmaceutical companies, has dramatically accelerated the possibility of discovering novel compounds from microorganisms compared to the enormous efforts made before the introduction of HTS. Compared to chemically synthesized compounds, secondary metabolites or natural compounds have larger molecular weights, more complex structures, and often chirality. These characteristics are thought to result from the multiple enzymatic reactions involved in the synthesis of these compounds. A long history of studying secondary metabolism has revealed the involvement of enzymes with remarkable characteristics, such as polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS), which play major roles in synthesizing the backbone structure of metabolites. Enzymes such as cytochrome P450 monooxygenase, dehydrogenases, and methyltransferases are often involved in secondary metabolite biosynthesis (SMB) as well, although they are not specific to secondary metabolism. The genes encoding these enzymes have their own sequence characteristics and/or sequence motifs, which suggested that the genes are involved in secondary metabolism based on sequence similarity.

Revolutionary developments in sequencing technology have allowed us to sequence the genomes of fungal species in a relatively short period with reasonable cost and quality. Currently, the genome sequences of more than 1000 fungi have been deposited in the NCBI genome database as of March 2015 (NCBI genome database, http://www.ncbi.nlm.nih.gov/genome/browse/). The early days of fungal genomic analysis led to the discovery of genes containing motifs found in PKS, NRPS, and other enzymes known for SMB in the genomes of filamentous fungi such as *Neurospora crassa* (Galagan et al., 2003) and *Magnaporthe grisea* (Dean et al., 2005). The numbers of genes encoding PKS and NRPS in the individual genomes of eight *Aspergillus* species range from 17 to 35 and from 14 to 24, respectively (Rank et al., 2010), values much higher than expected before genome sequencing. Several bioinformatics tools have been developed to predict secondary metabolic genes. One major type of the tools first developed depends on the presence of domains typically existing in PKS, NRPS, and other known genes catalyzing the synthesis of secondary metabolites such as SMURF (Khaldi et al., 2010) and antiSMASH (Medema et al., 2011). In addition to these motif-dependent tools, motif-independent tools has been reported for detecting co-regulated gene clusters using transcriptome data. These tools take advantage of large-scale transcriptomic data, rapidly growing by the use of next generation sequencing technologies.

In this review, we describe the current development of bioinformatics tools for comprehensive detection of fungal SMB gene clusters, particularly those without the use of known motifs. Of them, we take a closer look at MIDDAS-M, which successfully discovered the ustiloxin B biosynthesis gene cluster encoding a novel pathway, Ribosomal Peptide Synthetic (RiPS) pathway in fungi, for the biosynthesis of a peptide compound in a manner different from NRPS.

Characteristic Localization of Secondary Metabolism Genes on Chromosomes

The sequencing of three *Aspergillus* species provided the first good opportunity for detailed genome comparison between closely related species of filamentous fungi (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005). Syntenic analysis between the genomes of *A. oryzae* and *Aspergillus nidulans* or *Aspergillus fumigatus*, revealed that non-syntenic blocks (NSBs) were distributed in a mosaic manner throughout the *A. oryzae* genome. In contrast to syntenic blocks (SBs), which harbored genes involved in basic cellular functions, NSBs, which occupy approximately 25% of the *A. oryzae* genome, harbored a large proportion of genes that were predicted to belong to secondary metabolism and secretory hydrolases.

Another remarkable feature of the NSB genes is that a significantly larger proportion of the genes are functionally unknown compared to the SB genes (Tamano et al., 2008; Umemura et al., 2012). **Figure 1** shows functional analyses of the *A. oryzae* genes localized to SBs and NSBs. Analysis using the Cluster of Orthologous Group (COG) and Eukaryotic Orthologous Group (KOG) (Tatusov et al., 2003) reveals that genes of unknown function comprise 67% of NSB genes, a significantly larger proportion than the 54% unknown function genes in SBs. In spite of a similar proportion of metabolism genes in SBs and NSBs, NSBs contain a larger proportion of genes involved in secondary metabolism and genes encoding secretory hydrolases and transporters than SBs. **Table 1** shows the localization of the PKSs and NRPSs identified in the *A. oryzae* genome. Although NSBs occupy only approximately 25% of the entire genome, 64% of PKS/NRPS genes are located in NSBs, a 2.5-fold enrichment in NSBs. Similar genomic islands were found in *A. fumigatus* (Perrin et al., 2007; Fedorova et al., 2008), *Magnaporthe oryzae* (Rehmeyer et al., 2006; Thon et al., 2006) suggesting common feature for genomics of filamentous fungi. Although most of the software tools to predict SMB gene clusters do not use the close relationship between SMB genes and NSBs described above, the information could play an important role for the development and evaluation of the software tools.

Detection Methods for SMB Gene Clusters Using Transcriptome Data

One conventional way to survey SMB genes from fungal genome sequences is to use known sequence motifs of enzymes involved in biosynthesis of a metabolic backbone such as PKS, NRPS, or dimethylallyl tryptophan synthases (DMATs) (Fedorova et al., 2012). The genes encoding these enzymes are called "core genes" or "backbone genes." SMURF (Khaldi et al., 2010), antiSMASH (Medema et al., 2011; Blin et al., 2013), ClustScan (Starcevic et al., 2008), and CLUSEAN (Weber et al., 2009) adopt this strategy and predict gene clusters typically composed of approximately 10–30 genes including the SMB core genes. The central algorithm used in these tools is hidden Markov model (HMM), in addition to

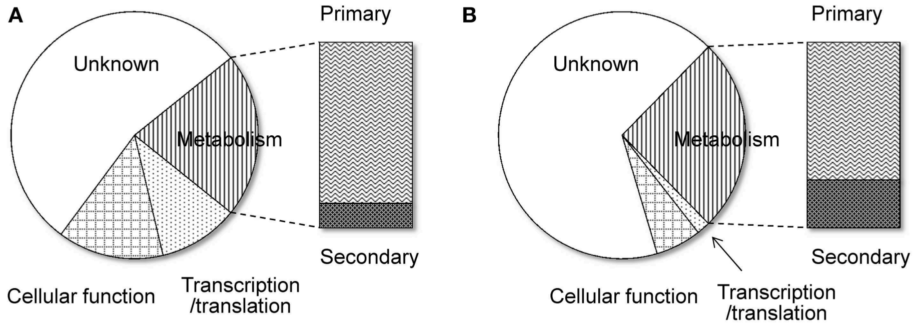

FIGURE 1 | Functional difference between the genes on SBs and NSBs. Genes in the *A. oryzae* genome were functionally classified by searching for them in the COG/KOG database (Tatusov et al., 2003) using BLASTP. **(A)** SBs, **(B)** NSBs.

TABLE 1 | Localization of the PKS/NRPS genes of *A. oryzae*.

Function	Gene ID[*1]	*E*-value	Top hit by BLASTP search[*2]
LOCATED ON SBs			
PKS	AO090026000001	1.00E4−161	Polyketide synthase-nonribosomal peptide synthetase [*Aspergillus oryzae*]
PKS	AO090206000074	0.00E+00	Polyketide synthase PKSL2 [*Aspergillus parasiticus*]
PKS	AO090701000530	0.00E+00	PKS-like enzyme, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090003001097	0.00E+00	L-aminoadipate-semialdehyde dehydrogenase large subunit [*Aspergillus terreus* NIH2624]
NRPS	AO090103000167	0.00E+00	Non-ribosomal siderophore peptide synthase Sid2 [*Aspergillus flavus* NRRL3357]
NRPS	AO090005001026	0.00E+00	AMP-dependent ligase, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090001000516	0.00E+00	NRPS-like enzyme, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090003000945	0.00E+00	NRPS-like enzyme, putative [*Aspergillus flavus* NRRL3357]
LOCATED ON NSBs			
PKS	AO090102000545	0.00E+00	Polyketide synthetase PksP [*Aspergillus flavus* NRRL3357]
PKS	AO090113000209	0.00E+00	Polyketide synthase, putative [*Aspergillus flavus* NRRL3357]
PKS	AO090010000048	0.00E+00	Polyketide synthase, putative [Aspergillus flavus NRRL3357]
PKS	AO090005000961	0.00E+00	Polyketide synthase, putative [*Aspergillus flavus* NRRL3357]
PKS	AO090010000404	0.00E+00	Polyketide synthase, putative [*Aspergillus flavus* NRRL3357]
PKS	AO090701000826	0.00E+00	Polyketide synthase, putative [*Aspergillus flavus* NRRL3357]
PKS	AO090009000131	0.00E+00	Polyketide synthase [*Botryotinia fuckeliana*]
NRPS	AO090103000223	0.00E+00	Non-ribosomal peptide synthase, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090026000585	1.00E−157	Non-ribosomal peptide synthase [*Aspergillus fumigatus* Af293]
NRPS	AO090026000378	0.00E+00	Non-ribosomal peptide synthase, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090102000338	0.00E+00	Non-ribosomal peptide synthase, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090010000498	0.00E+00	NRPS-like enzyme, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090020000240	0.00E+00	NRPS-like enzyme, putative [*Aspergillus flavus* NRRL3357]
NRPS	AO090003001545	0.00E+00	NRPS-like enzyme, putative [*Aspergillus flavus* NRRL3357]

[*1] *The list of PKS and NRPS genes was obtained from Umemura et al. (2013b).*

[*2] *The NCBI nr-aa database was used, but the top hits from the A. oryzae genome were removed.*

BLAST homology search algorithm (Altschul et al., 1990; Li et al., 2009), which depend certain sequence information of domains and genes. The methods are summarized in **Table 2**.

In addition to these tools using motif information of the core genes for the prediction of SMB gene clusters, a few tools are recently developed using transcriptome data to detect gene clusters in which the genes are cooperatively regulated. Andersen et al. defined the method to score the co-regulation of adjacent three genes. Using transcriptome data from 44 samples including four strains of *A. nidulans*, four different growth media, and five different carbon sources, they precisely determined the boundaries of 70 SMB gene clusters containing the core genes (Andersen et al., 2013). Gibbons et al. performed sliding window analysis to evaluate co-regulated gene clusters in *A. fumigatus*, and successfully detected 27 gene clusters upregulated under biofilm-like state, among which seven clusters are considered to

TABLE 2 | Detection methods for secondary metabolite biosynthetic gene clusters.

Type	Method	Algorithm[a]	References	Fungal strains tested in reference
Motif-dependent	SMURF	HMM	Khaldi et al., 2010	*A. flavus*, *A. oryzae*, *A. nidulans*, *A. niger*, *A. fumigatus*, *A. terreus*, *A. clavatus*, *N. fischeri,* et al.
	antiSMASH	profile HMM	Medema et al., 2011	*A. fumigatus*
	antiSMASH 2.0	profile HMM	Blin et al., 2013	–
	ClustScan	HMM	Starcevic et al., 2008	–
	CLUSEAN	Blast, HMM	Weber et al., 2009	–
	NP.searcher	Blast	Li et al., 2009	–
Motif-independent	Andersen's	Sliding window with coregulation coefficient	Andersen et al., 2013	*A. nidulans*
	Gibbons's	Sliding window with coregulation probability	Gibbons et al., 2012	*A. fumigatus*
	MIDDAS-M	Sliding window with deviation from standard distribution	Umemura et al., 2013a	*A. flavus*, *A. oryzae*, *F. verticillioides*
	MIPS-CG	Comparative genomics	Takeda et al., 2014	*A. flavus*, *A. oryzae*, *A. nidulans*, *A. fumigatus*, *A. terreus*, *F. verticillioides*, *F. graminearum*, *F. oxysporum*, *C. globosum*, *M. grisea*

[a] *HMM: hidden Markov model.*

be SMB ones as judged from existence of the core genes (Gibbons et al., 2012). Dhingra et al. adopted the method by Gibbons et al. to detect gene clusters regulated by the global transcription factor, *veA*, in *A. fumigatus* and successfully detected several potential SMB gene clusters (**Table 2**) (Dhingra et al., 2013). These methods are mainly used in combination with information of SMB core genes, to analyze fungal SMB gene clusters.

Transcriptomic analysis can be one of the best ways to identify the corresponding SMB genes of a fungus that produces a metabolite of interest. Secondary metabolism is "not essential to growth" and is activated only when necessary; transcriptional regulation plays a crucial role in this activation. Many SMB genes are activated after a logarithmic growth phase. In addition, various factors that affect the induction of secondary metabolism, such as temperature, carbon/nitrogen ratio, and medium composition, can potentially make an inducible condition very specific to each secondary metabolism (Brakhage, 2013). Therefore, analyzing the transcription profiles of producing and non-producing conditions and successively comparing these profiles should be an effective way to identify SMB gene clusters. Based on this idea, another motif-independent method, MIDDAS-M, was developed to detect gene clusters, whose component genes are co-regulated (Umemura et al., 2013a). This method uses induction ratios of whole genes under compound producing over non-producing conditions.

Most fungal SMB gene clusters already known to date include the core genes, but some gene clusters do not contain any of the SMB core genes. Recently, the genes responsible for kojic acid (KA) biosynthesis were identified from the *A. oryzae* genome (Terabayashi et al., 2010). The KA biosynthesis gene cluster consists of three genes, which are frequently found in SMB gene clusters, namely a Zn_2-Cys_6 (C6) fungal-type transcription factor, a major facilitator superfamily (MFS) transporter, and an oxidoreductase genes. However, none of the three genes are the core genes. This example clearly illustrates the importance of developing new methods for predicting genes involved in a novel SMB pathways.

Identification of a Kojic Acid Biosynthesis Gene Cluster Using the Transcriptome

A gene cluster for the biosynthesis of KA is one of the typical examples without the core genes. Small cluster size consisting of only three genes makes its detection more difficult as compared to most of the known SMB gene clusters consisting of 10–30 genes in general. KA was first identified in 1907 from a *koji*-culture, a solid-state culture of steamed rice inoculated with *A. oryzae* (Saito, 1907). However, despite the importance of KA, especially in the fermentation industry, the genes responsible for its production were only first identified in 2010 (Terabayashi et al., 2010). Biosynthesis pathway analysis of KA using an isotope tracer technique suggested the direct conversion of glucose to KA by no more than two or three enzymes (Arnstein and Bentley, 1953a,b,c), indicating that no SMB genes with typical motifs such as PKS or NRPS were involved.

To identify the genes responsible for KA biosynthesis, the KA-producing/KA-non-producing expression profiles of three pairs of conditions were selected for analysis: (i) without nitrate/with nitrate, (ii) 4th day/2nd day after cultivation, (iii) 7th day/4th day after cultivation (**Table 3**). Despite significant induction of KA production in all three pairs of conditions, no genes were found that were induced in more than two of the three pairs of conditions. Thus, the first two genes, AO090113000136 (*kojA*, oxidoreductase) and AO090113000138 (*kojT*, transporter), which were located very close to each other, were originally identified by one-by-one disruption approach referring to the transcriptome data. An additional gene, AO090113000137 (*kojR*, transcription factor) was found in between the two genes above.

TABLE 3 | Induction ratio rankings for the KA biosynthesis genes.

Producing/Non-producing	Index	AO090113000136 (*kojA*)	AO090113000137 (*kojR*)	AO090113000138 (*kojT*)	Cluster
7th day/4th day	*t*-value	5	22	2	1
	$m \times a$	1	14	2	1
	m-value	12	56	9	1
	a-value	51	1575	554	46
4th day/2nd day	*m*-value	1352	2216	1413	1126
Nitrate(−)/Nitrate(+)	*m*-value	2022	1185	1074	1103

In order to address most important factors for the detection of a co-regulated gene cluster only from gene expression profiles, several values obtained from simple calculation and statistics are compared using the KA gene cluster as a model case (**Table 3**). For the transcriptome data from the 7th day/4th day pair, the gene expression induction ratios (*m*-values) of *kojA*, *kojR*, and *kojT* rank relatively high: 12, 56, and 9, respectively. However, choosing the correct genes from these *m*-values is difficult, as the correct genes are not ranked first or very close to first. Calculating *t*-values using the statistical *t*-test significantly improved the selection power, as the *kojA*, *kojR*, and *kojT* rankings jump to 5, 22, and 2 from 12, 56, and 9, respectively. Another measuring index, $m \times a$, which is calculated by multiplying the *m*-value by the average expression level (*a*-value) based on high productivity of KA, is also effective for selecting the correct genes: the rankings of the three genes are 1, 14, and 2, respectively. However, the *m*-values and other indexes for the three genes rank from 1000 to 2000 for the 4th day/2nd day condition pair and the nitrate(−)/nitrate(+) pair. As KA production significantly increases for all three pairwise conditions, it is fairly difficult to select suitable conditions (in this case, the 7th day/4th day pair) to generate a useful transcriptome dataset for the identification of genes responsible for KA production.

To resolve this difficulty, an approach to select SMB genes based on the general characteristic of cooperative induction when they are producing secondary metabolites were developed. These genes, including genes related to well-known metabolites such as aflatoxin, sterigmatocystins, melanin, and trichothecenes, are clustered and co-regulated by a pathway-specific transcription factor (Keller and Hohn, 1997). In contrast, eukaryotic genes are generally not clustered in the genome, regardless of relationship of their functions. Therefore, averaging the *m*-values of the genes in a typical genomic region should yield a value close to zero, while the average *m*-value of the genes in an SMB gene cluster would yield a significantly larger value.

To test the above hypothesis, the average *m*-values are calculated for all possible virtual gene clusters containing three genes by moving a three-gene window function one gene at a time through the annotated genes of the *A. oryzae* genome. As expected, the average *t*-value, *m*-value and *a*-value for the KA-biosynthesis gene cluster are ranked 1st in the lists using the 7th day/4th day cultivation condition pair (**Table 3**). These results suggest that fluctuation noise in the gene expression values can be suppressed by averaging, particularly when the cluster contains a large number of genes. Noise suppression by averaging is the fundamental concept behind MIDDAS-M, which detects SMB gene clusters based on transcriptome data. However, the final form of the algorithm (described below) uses the summation, not the average, to evaluate cluster scores and uses other statistical tricks to improve detection sensitivity.

Mechanism of MIDDAS-M

Based on the idea of evaluating the expression value per gene cluster to detect functional SMB genes, we have developed a novel method to detect SMB genes that without using motif information from the SMB core genes. We named this method MIDDAS-M, which stands for motif-independent *de novo* detection algorithm for SMB gene clusters. As shown in **Figure 2**, the foundation of this method is the creation of a comprehensive list of virtual gene clusters (VCs) in a genome sequence, followed by the summation of the expression ratios of the genes in each VC. Using this method, the VCs containing genes that are co-expressed have large values, whereas other VCs have values close to the average. In other words, the gene induction ratios are distributed normally in a symmetric bell-shaped curve, but VCs that are candidate SMB clusters exhibit large absolute values that deviate from the symmetric normal distribution curve (**Figure 3**, middle). For a given cluster size *ncl*, this procedure can be described using the following equation:

$$M_{i,\,ncl} = \sum_{k=i}^{i+ncl-1} \frac{m_k - \overline{m}}{\sigma_m} \tag{1}$$

where m_k is the induction ratio or gene expression level of gene k, and $\overline{m}$ and σ_m are the mean and the standard deviation of all *m*-values, respectively. This equation normalizes the gene induction ratios via Z-score transformation, which makes the average zero and the standard deviation 1. After normalization, the top of the bell-shaped distribution centers on the y-axis ($x = 0$). Note that the M scores become largely negative when the expression value is the induction ratio of secondary metabolite non-producing vs. producing conditions.

The M scores for SMB gene cluster candidates can be distinguished from other VCs when the values are sufficiently large, but other VCs still yield many noisy peaks (**Figure 3**, right-middle). Therefore, we tested several equations to reduce the signal-to-noise ratio of the M scores for SMB cluster candidates, yielding the final form of the MIDDAS-M equation:

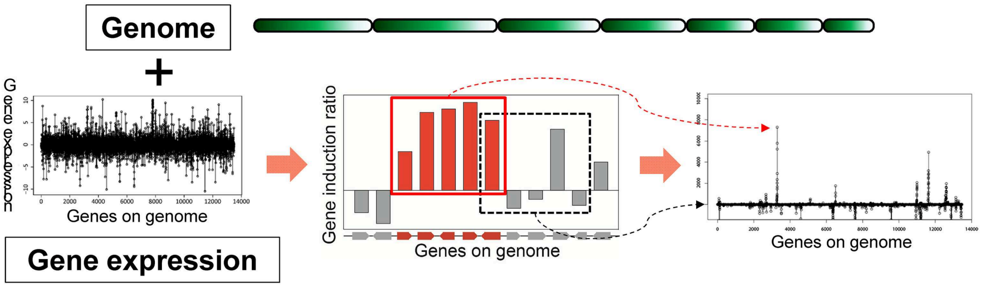

FIGURE 2 | Principles of MIDDAS-M. MIDDAS-M uses both genomic information, in which gene positions are annotated, and transcriptome data. When adjacent genes are co-expressed, as is the case with secondary metabolic genes, the sum of their induction ratios or expression values becomes large, whereas other summed values approach the average for all gene values.

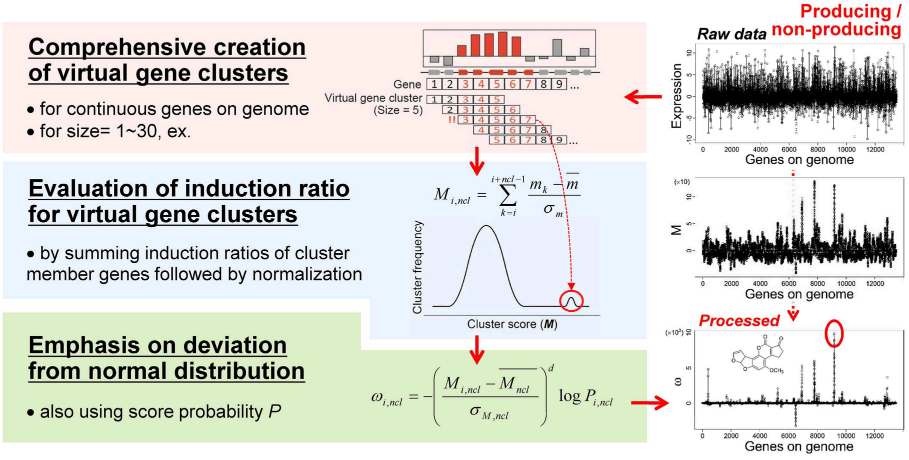

FIGURE 3 | The MIDDAS-M algorithm. After creating all possible virtual gene clusters for a genome sequence, the induction ratios (*M*) of the virtual gene clusters are evaluated by summing the induction ratios of the genes in each cluster. The SMB gene cluster candidates should have large *M*-values that deviate from the normal distribution, which is emphasized by the statistical evaluation.

$$\omega_{i,ncl} = -\left(\frac{M_{i,ncl} - \overline{M_{ncl}}}{\sigma_{M,ncl}}\right)^{d} \log P_{i,\,ncl} \tag{2}$$

where $\overline{M_{ncl}}$ and $\sigma_{M,ncl}$ are the mean and the standard deviation, respectively, of all M scores at a given cluster size ncl, d is a positive odd integer representing the order of the moment (set to 3 as default), and $P_{i,ncl}$ is the occurrence probability of $M_{i,ncl}$ in the distribution of all M scores at ncl. In Equation (2), two components result in the high sensitivity of MIDDAS-M detection: the moment and the rareness of the M scores (**Figure 4**). The moment, described as a Z-scored M-value to the dth power, indicates the degree of deviation from the normal distribution, whereas the rareness is described as the logarithm of the M score probability multiplied by -1, drastically emphasizing values with low probability, which will be closer to zero. The high sensitivity of MIDDAS-M is mainly derived from the moment, but the rareness also contributes to the sensitivity, especially when the template genome sequence is not well-defined. The moment can also be described as the degree of unbalance in a distribution. Gene induction ratios are normally distributed and symmetrical with respect to the y-axis, but the summed induction ratios, M, are unevenly distributed away from the normal distribution when the functional SMB gene cluster(s) are included in the analyzed transcriptome data. Most M values

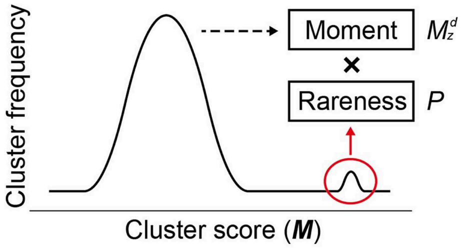

FIGURE 4 | Two factors for high sensitivity of MIDDAS-M. Based on the principle of MIDDAS-M, the algorithm emphasizes the *M*-values of the SMB gene cluster candidates using two factors: moment and rareness. The deviation of the *M*-values from the normal distribution is amplified by the moment, and the rareness of the *M*-values is expressed as a logarithm of the possibility.

in the bell-shaped distribution are near zero because of the normalization, whereas the *M* scores of possible SMB VCs become larger than 1; subsequently raising the *M* scores to the power of integer *d* yields quite large numbers.

Figure 5 shows the results of MIDDAS-M analysis using the same dataset as in **Table 3** to detect the KA biosynthesis gene cluster. A strong peak in the MIDDAS-M scores was obtained using only the data from the 7th day/4th day pair but not from the 4th day/2nd day pair or the nitrate(−)/nitrate(+) pair. These results are consistent with our previous results that the 7th day/4th day pair yielded more accurate gene rankings. However, statistical evaluation using MIDDAS-M correctly identified the KA biosynthesis genes even when datasets from the undesirable conditions were included.

Comparison of MIDDAS-M with SMURF and antiSMASH

The most valuable feature of MIDDAS-M analysis is that, unlike other tools for SMB gene cluster detection, it detects functional SMB gene clusters independent of the known motif sequences of the SMB core genes such as PKS, NPRS, and terpene cyclase (TC). **Table 4** summarizes the number of SMB gene cluster candidates in *Aspergillus flavus* and *Fusarium verticillioides* detected by MIDDAS-M compared to the number detected by SMURF (Khaldi et al., 2010) and antiSMASH (Medema et al., 2011; Blin et al., 2013), which are the major tools that use motif information from the SMB core genes. The total number of clusters detected by MIDDAS-M, SMURF, or antiSMASH differs between *A. flavus* and *F. verticillioides*; the number of clusters detected by MIDDAS-M or SMURF/antiSMASH is >5-fold or ∼2-fold higher, respectively, in *A. flavus* than in *F. verticillioides*. This difference may be a result of differences in the variety and sizes of the datasets used for the two species: for *A. flavus*, 28 datasets were obtained under various culture conditions, including liquid and maize solid media, whereas for *F. verticillioides*, only 4 datasets were obtained as time series using the same liquid medium. In *A. flavus*, 55 clusters were detected by SMURF, and 76 clusters were detected by antiSMASH; 49% and 46% of these clusters were also detected by MIDDAS-M, respectively. The number of clusters detected by MIDDAS-M are lower in *F. verticillioides*, but the percentage of SMURF (24%) and antiSMASH (27%) clusters detected by MIDDAS-M were similar, as in *A. flavus*. These data suggest that a certain percentage of the SMB gene clusters containing core genes are functional, whereas the rest might contain pseudo or silent genes that lost function or are inactive under most conditions. The percentage relationship between the genes detected by MIDDAS-M, SMURF and/or antiSMASH is presented as a Venn diagram for *A. flavus* (**Figure 6A**). Among the VCs detected by MIDDAS-M, 10% and 15% were also detected by SMURF and antiSMASH, respectively; the remaining 85% possess no known SMB core genes. Many of the VCs detected by MIDDAS-M may still be candidates for novel SMB gene clusters, as many contain "keystone" genes involved in secondary metabolism, such as P450, MFS transporters, and C6 transcription factors, and the occupancy increases according to the ω score (Umemura et al., 2013a).

The cluster sizes predicted by MIDDAS-M are often smaller than those by SMURF and/or antiSMASH (**Figure 6B**). Of the VCs detected by MIDDAS-M, those composed of three genes are frequently observed (>60% for *A. flavus*), whereas most clusters predicted by SMURF or antiSMASH harbored 10∼20 genes (Appendix S2) (Umemura et al., 2013a). The average cluster size detected by MIDDAS-M is 4.1, whereas those by SMURF and antiSMASH are 13.1 and 15.3, respectively, in *A. flavus* (**Figure 6B**). Frequency of the small gene clusters detected by MIDDAS-M is obviously higher than when assuming random distribution of the genes on the genome. Further, smaller size of the MIDDAS-M gene clusters does not depend on the absence of SMB core genes. **Figure 7** shows expression of genes in two clusters detected by all the three methods but with significantly

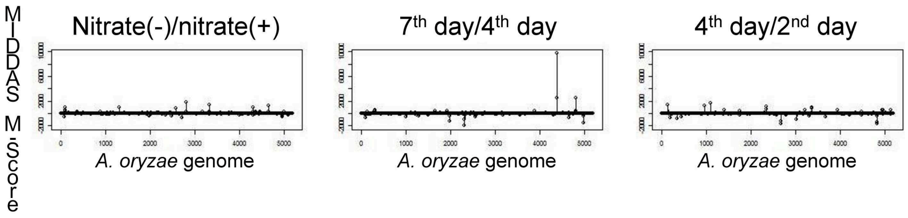

FIGURE 5 | Results of MIDDAS-M analysis of the KA biosynthesis gene cluster. MIDDAS-M scores were calculated for the entire *A. oryzae* genome using three pairs of conditions as in **Table 3**.

TABLE 4 | The number and characteristics of the SMB gene cluster candidates detected by MIDDAS-M.

Strain	MIDDAS-M[*1]	SMURF	By MIDDAS-M[*2]	%	antiSMASH	By MIDDAS-M[*2]	%
A. flavus	240	55	27	49	76	35	46
F. verticillioides	47	29	7	24	30	8	27

[*1] *The threshold is ≥1016.7 for A. flavus and ≥499.4 for F. verticillioides.*

[*2] *Counted when one or more genes in the cluster are detected by MIDDAS-M.*

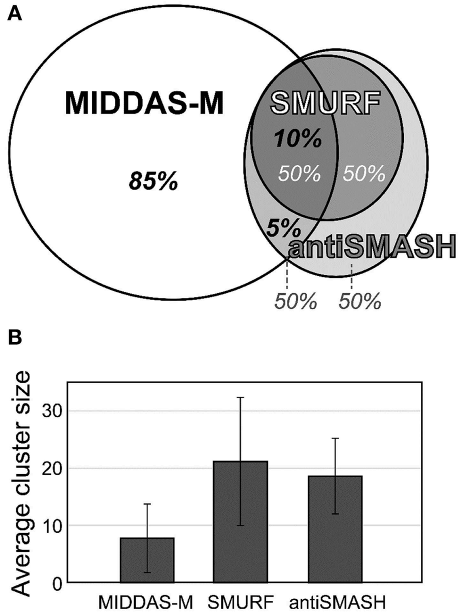

FIGURE 6 | Comparison of gene clusters in *A. flavus* detected by MIDDAS-M, SMURF, and antiSMASH. (A) Among the candidate SMB gene clusters detected by MIDDAS-M, 10% and 5% were also predicted by SMURF and antiSMASH, respectively. Conversely, approximately half the clusters predicted by SMURF or antiSMASH were detected by MIDDAS-M. **(B)** The average cluster size of VCs detected by MIDDAS-M SMURF and antiSMASH.

shorter length by MIDDAS-M than by the others as examples. MIDDAS-M predicted the aflatoxin biosynthesis gene cluster as two separate clusters (AFLA_139150–AFLA_139320 and AFLA_139370–AFLA_139410), which lacks five genes less than its actual number in total (**Figure 7A**) (Yu et al., 2004). The genes essential for the aflatoxin biosynthesis, AFLA_139140 (*aflYa*), AFLA_139340 (*aflS*), AFLA_139360 (*aflR*), AFLA_139420 (*aflT*), and AFLA_139440 (*aflF*) were excluded because their induction levels were significantly lower than those detected as the clusters. On the other hand, MIDDAS-M predicted the aflatoxin and cyclopiazonic acid (Chang et al., 2009) biosynthesis gene clusters separately, whereas SMURF and antiSMASH predicted the two clusters combined into a single cluster. Similarly, MIDDAS-M predicted another cluster, AFLA_023000–AFLA_023040, significantly shorter than in the case of SMURF and antiSMASH (**Figure 7B**). When judged directly from the expression profiles, AFLA_022810–AFLA_022990, which exhibit almost no expression differences in any conditions used in the analysis, can be apparently excluded. However, AFLA_023050–AFLA_023100, which seem to have induction/repression similar to but weaker than the genes in the cluster might be included in the cluster. The two examples above strongly suggest that MIDDAS-M can be fine-tuned (introduction of optional calculation for example) to make its prediction more accurate and practical by taking various experimentally validated results into consideration.

Table 5 shows the percentages of clusters containing a C6 transcription factor, an MFS transporter and a P450 enzyme in relation to the three detection methods. Of the gene clusters predicted by MIDDAS-M, SMURF and antiSMASH, 4%, 23%, and 17% contained a C6 transcription factor, respectively. The significantly smaller value for MIDDAS-M is thought to be due to smaller induction ratio of the transcription factor than that of the other genes in the clusters in general. The percentage of the C6 transcription factor further decreased to 2% when the clusters detected commonly by MIDDAS-M and SMURF/antiSMASH were used. However, because the value was calculated from only a small subset of the entire clusters (approximately 15% of the clusters detected by MIDDAS-M), it is not clear if the decrease might have any biological meaning. Smaller percentage of the clusters containing an MFS transporter detected by MIDDAS-M is thought to be due to smaller induction ratio of MFS transporters in general, similarly to the case of *aflT*, the transporter gene in the aflatoxin gene cluster (**Figure 7B**).

As described in the Introduction, genes belonging to category Q in the KOG classification, secondary metabolic genes, tend to localize to NSBs regardless of their distance from the telomeres or their expression levels (Umemura et al., 2012). SMB genes detected by MIDDAS-M also exhibit localization to NSBs: 72% of the identified genes are located in NSBs in *A. flavus*, even though NSBs comprise only 29% of the genome compared with the *A. nidulans* genome (Umemura et al., 2013a). **Figure 8** shows a projected view of all MIDDAS-M peaks obtained using transcriptome data from 28 culture conditions (previously presented in a 3D view) (Umemura et al., 2013a), with indicators for NSB regions, KOG Q-genes, and PKS/NRPS gene clusters predicted by SMURF. The localization of MIDDAS-M-detected clusters to NSBs is more obvious in the 3D view in our previous report (Umemura et al., 2013a).

There are several reports of global regulators for fungal secondary metabolism such as VeA and LaeA (Keller and Hohn, 1997; Reyes-Dominguez et al., 2010; Soukup et al., 2012; Bok et al., 2013). By comparing the *laeA* over-expression and its deletion mutants of *A. flavus*, 10 candidate clusters including those for aflatoxin and cyclopiazonic acid are detected by MIDDAS-M. This detection number is comparable to the number (17 clusters) obtained from comparison between *A. flavus* and *A. oryzae* under maize culture. Similarly, approximately 10 candidate clusters are detected by comparing transcriptomes from different temperatures such as 28°C/37°C under liquid A&M and solid maize cultures (Georgianna et al., 2010).

Toward Comprehensive Analysis of SMB Gene Clusters

Fungal secondary metabolites tend to be produced under limited culture conditions. Therefore, comprehensive analysis of transcriptome data obtained under various culture conditions is essential for the discovery of novel SMB gene clusters. MIDDAS-M is suitable for this purpose because it can concurrently process large-scale transcriptome datasets. For example, we used 28 transcriptome datasets obtained under various liquid and solid media culture conditions for our MIDDAS-M analysis of

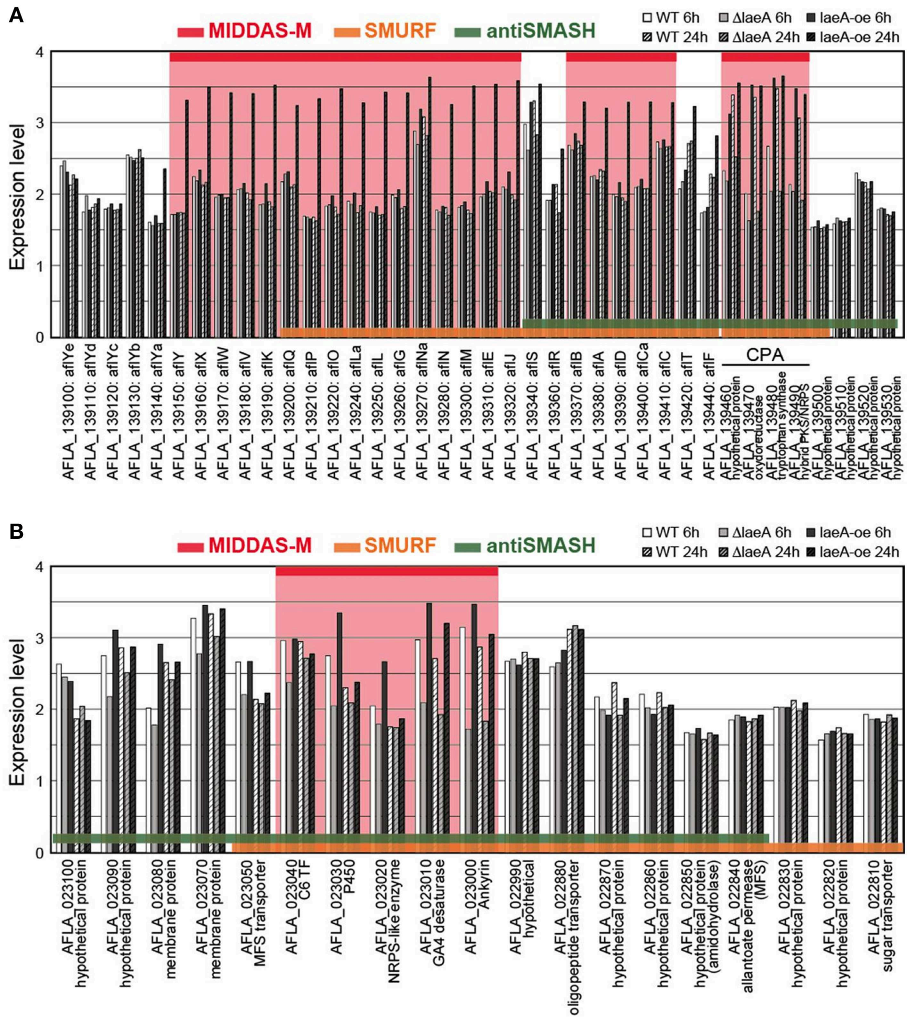

FIGURE 7 | Comparison of expression levels of gene clusters commonly detected by MIDDAS-M, SMURF, and antiSMASH. Transcriptional expression levels of the genes in the chromosomal regions containing **(A)** the aflatoxin and cyclopiazonic acid biosynthetic gene clusters and **(B)** another unknown gene cluster containing an SMB core gene AFLA_023020, which were commonly detected by MIDDAS-M, SMURF, and antiSMASH, are indicated in a logarithmic scale. The values were obtained from a control strain and a deletion and an over-expression mutants at 6 h and 24 h in the transcriptome dataset of GSE15435 in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). Gene IDs are indicated together with corresponding annotations from the NCBI database.

TABLE 5 | The percentage of clusters containing a C6 transcription factor, an MFS transporter, and a P450 in *A. flavus*.

Detection method	C6 (%)	MFS (%)	P450 (%)
MIDDAS-M	4	10	9
SMURF	23	36	36
antiSMASH	17	28	34
MIDDAS-M and SMURF/antiSMASH	2	7	30
MIDDAS-M only	5	10	5

A. flavus and several datasets for our analysis of *A. oryzae*, resulting in 378 sets of gene induction ratios (Umemura et al., 2013a). It is not difficult to perform MIDDAS-M analysis of 100 or more transcriptome datasets using recent high performance computers. **Figure 9** shows the relationship between the number and height of the detected MIDDAS-M peaks for each gene (**Figure 9A**) and for each culture condition combination (CCC) (**Figure 9B**). For each gene, the detected number of CCCs increases exponentially and strongly correlates with the peak value; that is, when a gene is detected as a member of a candidate VC in various CCCs, its maximum peak value tends to be strong (**Figure 9A**). SMB gene clusters that are rarely expressed tend to show weaker MIDDAS-M peaks; therefore, it is better to survey relatively small peaks to identify rare SMB gene clusters, which have a high probability of being novel.

Plotting the number of detected genes vs. CCCs after sorting by the number of detected genes yields an *S*-curve (**Figure 9B**, top), while the curve from plotting the number of detected genes per gene is very different (**Figure 9A**, top). One of the reasons is that the CCC data includes all possible combinations of two datasets from 28 conditions, so that one dataset appears 27 times, yielding a total of 378 CCCs. The number of detected peaks per CCC does not correlate with the maximum ω-value, but the highest peaks were observed for the cultivation condition pairs harboring a relatively small number of detected peaks. This effect may be partially derived from the principle of MIDDAS-M itself; that is, the ω-value has relation to the number of genes detected because the ω-value is evaluated as the degree of deviation from the normal distribution. When many VCs deviate from the normal distribution, the statistical center of the distribution may shift closer to the data points with large deviations, resulting in underestimated deviation values.

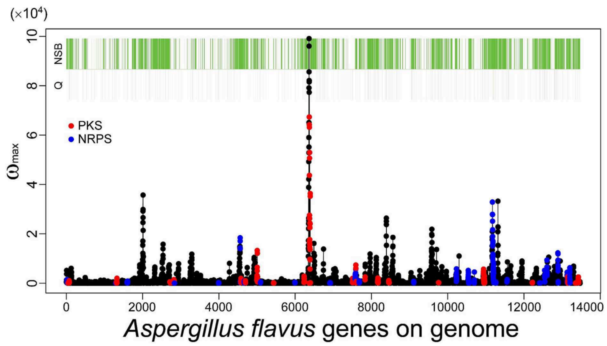

FIGURE 8 | MIDDAS-M peaks in *A. flavus* with indicators for the NSB regions, Q-genes, and PKS/NRPS gene clusters predicted by SMURF. The red and blue marks overlaid on the MIDDAS-M peaks indicate the genes predicted as PKS and NRPS, respectively, by SMURF. The green and gray bars at the top of the figure indicate the genes in NSBs identified from the comparison between the *A. flavus* and *A. nidulans* genomes and the Q-genes in the KOG database, respectively.

Many secondary metabolites are produced only under limited culture conditions. Several culture and media conditions are known to be suitable for the production of secondary metabolites, including solid maize culture, but the knowledge is empirical. This knowledge gap is one of the main reasons SMB genes have been difficult to identify. MIDDAS-M allows us to analyze a large number of culture conditions simultaneously to identify conditions in which rare SMB gene cluster(s) are expressed. As a case study, we examined a rare cluster that was observed in limited CCCs, as described in our previous report (Umemura et al., 2013a). The cluster, designated by a yellow circle in the middle of **Figure 10**, is detected only by comparing *A. oryzae* to *A. flavus* datasets, i.e., the cluster is expressed only in the former species. *A. oryzae*, a close relative of *A. flavus*, rarely produces secondary metabolites compared to *A. flavus*, despite the large number of orthologs found between the two species (Payne et al., 2006). *A. oryzae* is used in the food industry because of this low production of secondary metabolites. The functional annotations of the genes in this cluster from NCBI indicate that it contains a hydrolase, a dehydrogenase and two neighboring genes annotated as P450 and MFS transporters, which are often found in SMB gene clusters. In contrast, significantly more possible SMB gene clusters were detected in *A. flavus* using MIDDAS-M as expected. Considering the remarkable differences between the two species in terms of SMB gene expression, the corresponding gene cluster in *A. flavus* might be inactivated due to the high mutation frequency in NSBs. Alternatively, the product of this cluster may be beneficial to *A. oryzae*, yielding a good flavor during fermentation for example. This result demonstrates that MIDDAS-M analysis can be used to find rare SMB gene clusters due to its comprehensive analysis capabilities.

Recently, we have developed another software tool, MIPS-CG (motif-independent prediction without core genes), for the identification of completely novel SMB gene clusters with no

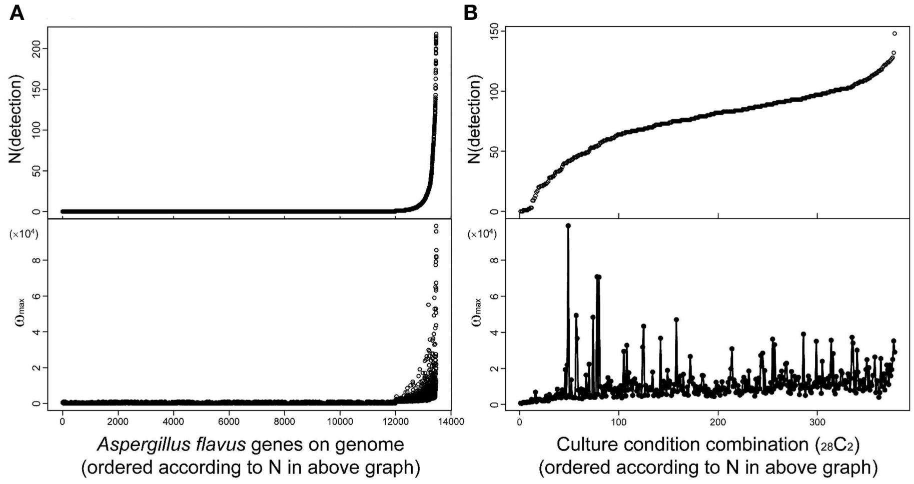

FIGURE 9 | Relationships between height and number of detected MIDDAS-M peaks in *A. flavus*. Top: The number of peaks detected by MIDDAS-M from 28 datasets of transcriptome data for *A. flavus*, according to the genes **(A)** and the culture condition combinations (CCC; $_{28}C_2 = 378$) **(B).** The numbers are sorted in ascending order. Bottom: The maximum ω score for each gene **(A)** and CCC **(B)**.

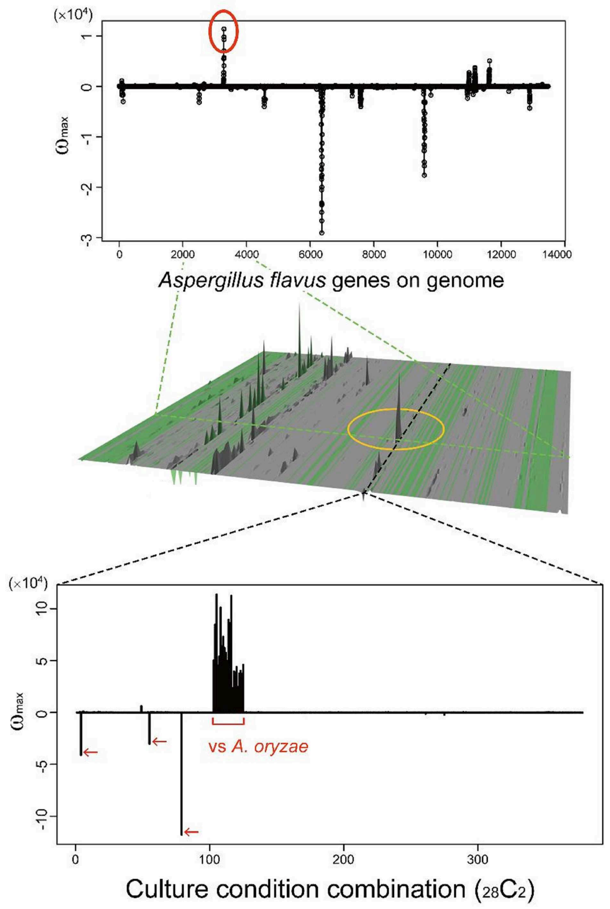

FIGURE 10 | A candidate SMB gene cluster rarely detected among various culture condition combinations. The peak in the middle of the 3D figure (Umemura et al., 2013a) is detected only in limited culture condition combinations (CCCs); the graph cut-outs show the position of a corresponding CCC (above) and gene (below).

known core genes (Takeda et al., 2014). MIPS-CG first detects gene clusters by searching for a pair of clustered genes with significant similarity between two genomes without using any known sequences or motifs. More importantly, MIPS-CG selects potential SMB gene clusters from the gene cluster candidates based on the discovery that SMB gene clusters are highly enriched on NSBs (Machida et al., 2005). The localization of SMB gene clusters on NSBs are clearly conserved in the results by the projected view of MIDDAS-M as described above. By applying appropriate values for several parameters, MIPS-CG successfully detected most of the known SMB gene clusters (21/24 gene clusters) from 10 filamentous fungal genomes without large differences from the experimentally determined positions of the cluster boundaries. As the two software tools, MIDDAS-M and MIPS-CG, use principally different algorithms to detect SMB gene clusters, combining the two software tools is expected to increase the probability and accuracy of detecting novel SMB gene clusters, especially ones without any known core genes.

Discovery of a Novel Type of Fungal Secondary Metabolic Pathway

It is thought that there is virtually little example of SMB gene clusters without known SMB core genes discovered using bioinformatics before experimental detection. Nonetheless, MIDDAS-M successively detected the ustiloxin biosynthetic gene cluster, which does not include PKS, NRPS, or any other known SMB core genes. Successive detailed analyses of the gene cluster revealed that ustiloxin biosynthesis is the first example of a ribosomal biosynthetic pathway in filamentous fungi (Umemura et al., 2013a, 2014). Ustiloxin B is a cyclic tetrapeptide, Tyr-Ala-Ile-Gly, whose tyrosine is modified with a non-protein coding amino acid, norvaline (**Figure 11**). The structure first indicated that the compound is synthesized by NRPS, but none of the NRPS-specific catalytic domains, A, C, PCP, and TE, were included in the gene cluster or within 10 genes adjacent to it. Instead, we found a gene whose translated amino acid sequence contains a 16× repeat of short peptides, including "YAIG," the exact sequence of the ustiloxin B cyclic moiety (**Figure 11**). Therefore, ustiloxin B is not synthesized by NRPS but is instead synthesized ribosomally as a precursor protein, UstA, followed by processing via peptidases (Umemura et al., 2014). Ribosomally synthesized natural compounds are designated as ribosomal peptides and have been reported in bacteria, plant, and cone snails, especially since the development of genome sequencing technologies in the twenty-first century (Velasquez and Van Der Donk, 2011; Schmidt, 2012; Arnison et al., 2013; Yang and Van Der Donk, 2013). Except one example, amanitin, produced by the *Amanita* mushroom (Hallen et al., 2007), the synthesis of ribosomal peptides in filamentous fungi had not been reported until the identification of the ustiloxin B biosynthetic gene cluster. The highly repeated structure of the precursor protein, UstA, has not been observed in bacteria, thus it might be a characteristic feature specific to filamentous fungi. By searching biosynthetic pathways similar to the ustiloxin one in filamentous fungi by analyzing their genome sequences and metabolite profiles, the world of fungal secondary metabolites will be widely extended.

Ustiloxin B, an inhibitor of microtubule assembly, was first discovered as a metabolite of the fungus *Ustilaginoidea virens*, which is a rice pathogen that causes false smut disease. As the compound was not known to be produced by *A. flavus* until the identification of the gene cluster by MIDDAS-M, the ustiloxin B biosynthetic gene cluster was discovered using a "gene to compound" strategy. This strategy includes certain inevitable difficulties. The production of secondary metabolites is generally unstable and often reduced in laboratory environments; thus, it is extremely difficult to identify target compounds among the thousands of other metabolites produced. Other experimental limitations include the detection sensitivity limit of the available instruments, the absence of a suitable extraction solvent, and the rapid degradation or volatile characteristics of the compound. We experienced all these situations while preparing fifty or more deletion mutants based on MIDDAS-M prediction, and we learned that at least the potential presence of a compound of interest should be indicated in the culture conditions. The

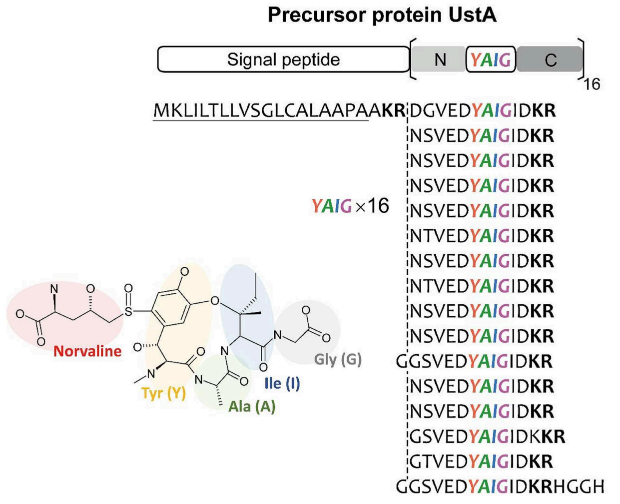

FIGURE 11 | The structure of ustiloxin B and its precursor protein, UstA. Ustiloxin B consists of a cyclic tetrapeptide, Tyr-Ala-Ile-Gly (YAIG), whose tyrosine is modified with the non-proteinogenic amino acid norvaline (Umemura et al., 2014). The cyclic YAIG amino acid sequence is directly encoded in the gene for the precursor protein UstA.

transcriptome-based methodology adopted by recent tools is the most straightforward and fastest approach for a "compound to gene" strategy because it detects only the expressed and potentially functional clusters in the culture conditions suited for the production of the compound.

Conclusion

Genome sequencing has shed light on comprehensive analysis of SMB gene clusters in filamentous fungi. In contrast to the importance of SMB gene clusters with the core genes such as PKS, NRPS, DMAT, and TC, lack of potential methodologies has made exploring SMB gene clusters without the core genes extremely difficult. MIDDAS-M is a powerful method for detecting novel SMB gene clusters, especially functionally active ones. Although the detected clusters were frequently located in NSBs, where SMB genes are highly enriched, only a few examples currently available confirm that the newly identified clusters actually produce secondary metabolites. Following points should be considered when using MIDDAS-M to search for SMB gene clusters: (i) the detected clusters may not be SMB gene clusters but instead have a currently unknown function; (ii) the clusters may not produce active polypeptide (enzymes, transporters, transcription factors, and so on); or iii) the products may be active yet remain inactive in the overall metabolism (possibly because part of the cluster is missing). Thus, detailed analyses of the novel clusters detected by MIDDAS-M are necessary to characterize the novel clusters and extend our knowledge of novel secondary metabolism.

The dramatic acceleration in sequencing fungal genomes due to the development of sequencing technologies after the twenty-first century (Chiang et al., 2009; Lim et al., 2012) allows the comprehensive analysis of SMB gene clusters from entire fugal species. Thus, the "gene to compound" strategy is quite attractive despite the difficulties described in the end of the previous section. This strategy is applied to recent attempts to awaken cryptic or silent SMB gene clusters in fungi by manipulating regulatory genes. In spite of dependence of MIDDAS-M on gene expression, MIPS-CG predicts SMB gene clusters only from genome sequence information. Combining these methods and motif-dependent methods such as SMURF and antiSMASH should significantly accelerate comprehensive analysis of secondary metabolism of filamentous fungi.

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Fungal natural products—the mushroom perspective

Marc Stadler[1]* and Dirk Hoffmeister[2]*

[1] *Department of Microbial Drugs, Helmholtz Centre for Infection Research, Braunschweig, Germany*
[2] *Department of Pharmaceutical Microbiology, Hans Knöll Institute, Friedrich Schiller Universität Jena, Germany*
**Correspondence: marc.stadler@helmholtz-hzi.de; dirk.hoffmeister@hki-jena.de*

Edited by:
Nancy Keller, University of Wisconsin-Madison, USA

Reviewed by:
Nancy Keller, University of Wisconsin-Madison, USA
Claudia Schmidt-Dannert, University of Minnesota, USA

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Among the first documented studies on the chemistry of fungal natural products were descriptions of quinoid pigments, i.e., the L-tyrosine- and L-phenylalanine-derived terphenylquinones atromentin and polyporic acid, respectively. The isolation of these compounds from mushroom fruiting bodies (basidiomes) was published around 1877 by Stahlschmidt and Thörner. Ever since, organic chemists embraced basidiomycetes as a prolific source of bioactive compounds and investigated these fungi with regard to compound isolation, structure elucidation, and synthesis (Gill and Steglich, 1987; Zhou and Liu, 2010; De Silva et al., 2013 and previous reviews referenced therein, Lorenzen and Anke, 1998; Richter et al., in press). Mushrooms seem to be particularly talented in producing unique terpenoids, and the molecular background behind the biosynthesis of some of those compounds has only recently been elucidated (Quin et al., 2014). Prominent examples of basidiomycete metabolites for lead structures in agrochemistry and drug research are, among others, the strobilurins, i.e., agriculturally used ß-methoxyacrylate fungicides from cultures of *Mycena*, *Oudemansiella*, *Strobilurus*, *Xerula* and several other basidiomycete genera (Sauter et al., 1999, Figure 1). Other examples are the pleuromutilins, the illudins, and the omphalotins (**Figure 1**). The pleuromutilins from cultures of species that are now placed in the genera *Clitopilus* and *Omphalina* served as scaffold for the development of the semisynthetic antibacterial antibiotic retapamulin (Kirst, 2013) which is clinically used for topical treatment of infections with *Staphylococcus aureus*. The illudins from *Lampteromyces* and *Omphalotus* species (Omphalotaceae) are sesquiterpenes featuring an unusual cyclopropane ring and are currently developed as anticancer drugs (Tanasova and Sturla, 2012). The omphalotins are cyclopeptides with pronounced nematicidal activites against root knot nematodes (Büchel et al., 1998), which are also exclusively found in the Omphalotaceae. Recently, the blazeispirols from *Agaricus subrufescens* were discovered as strong and selective agonists of the Liver X receptor (LXR alpha). Concurrently, relevant *in vivo* effects of blazeispirols in a mouse model were observed which might give rise to the development of a new antihypercholesterolemic agent from cultures of a medicinal mushroom (Grothe et al., 2011).

The above examples illustrate that basidiomycete secondary metabolomes merit further exploration. Perhaps fortunately for coming generations of Ph.D. students, the realm of basidiomycete metabolites is still underexplored, even after decades of intensive research to isolate and structurally elucidate compounds. This is also evident by the fact that toxic principles of mushrooms which repeatedly led to poisonings were identified only recently (**Figure 1**). Recent advances pertain to *Trogia venenata* fruiting bodies, in which the toxic 2*R*-amino-5-hexynoic acid and related compounds were found (Zhou et al., 2012). Cycloprop-2-ene carboxylic acid causing rhabdomyolysis was isolated from *Russula subnigricans*, a toxic mushroom native to East Asia (Matsuura et al., 2009). Furthermore, saponaceolide toxins with their unusual molecular skeleton were discovered in *Tricholoma terreum* (Yin et al., 2014).

For basidiomycetes, the genomic era set in later than for ascomycetes, and in numbers of genome projects the former are still lagging behind the latter. Still, the available genomic data impacted natural product research as it reveals a stimulating disparity: the number of natural product genes, best reflected by the number of genes for polyketide synthases and peptide synthetases exceeds the number of known compounds by far—even after decades of chemical research. The "house eater" fungus *Serpula lacrymans* encodes 21 PKS and NRPS genes (Eastwood et al., 2011), the average number of PKS genes per basidiomycete genome is four, according to a survey of 35 mostly saprotrophic species (Lackner et al., 2012).

The wealth of natural product biosynthesis genes in a given species contrasts the few compounds known from the same species. This situation is reminiscent of what was found for ascomycete genomes years ago, e.g., for the genera *Aspergillus*, *Penicillium*, *Fusarium*, and others (Keller et al., 2005; Desjardins and Proctor, 2007; Sanchez et al., 2012). However, the course research has taken (and will be taking) to make as much sense as possible out of the genomic data is quite different with basidiomycetes. This is due to a number of reasons that contrast the situation with ascomycetes.

(a) Basidiomycetes are mostly dikaryotic and hence little suitable for reverse genetics, although some species grow as monokarya *in vitro*.

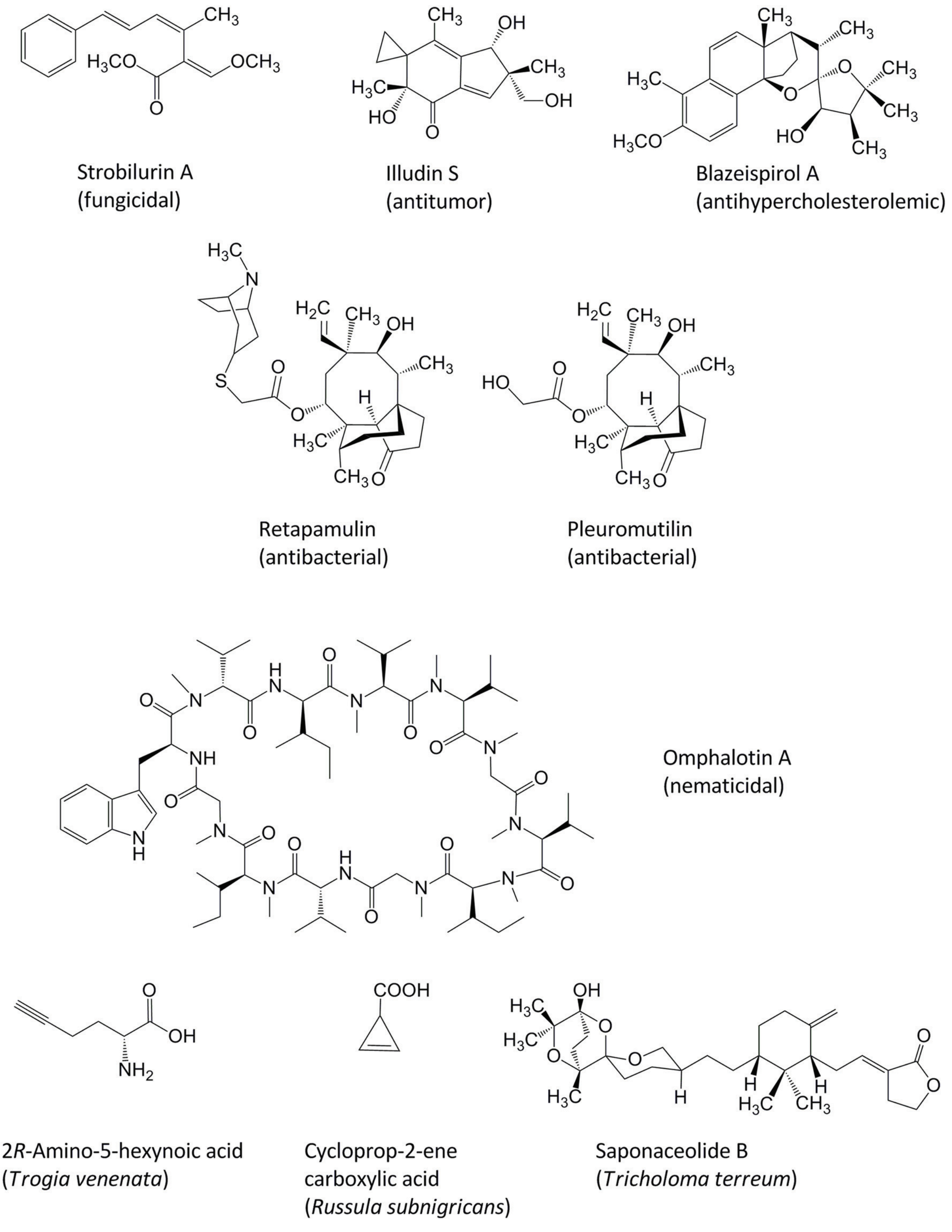

FIGURE 1 | Chemical structures of pharmacologically active basidiomycete natural products.

(b) Basidiomycetes are little amenable, if at all, to transformation and genetic manipulation, as only a very modest number of genetic tools and procedures are in place. Notable exceptions with regard to producers of pharmacologically active metabolites pertain to the honey mushroom *Armillaria mellea* (Baumgartner et al., 2010) which produces the melleolides, i.e., unusual sesquiterpene ester antibiotics (Bohnert et al., 2014). Also, a transformation procedure was established for the pleuromutilin producer

Clitopilus passeckerianus (Kilaru et al., 2009).

(c) The ecological preferences of numerous basidiomycetes have so far remained obscure and can only now be established by using modern methods of molecular ecology. For instance, some species that were hitherto regarded as saprotrophs, such as *Hygrocybe virginea*, are now suspected to possess hitherto unknown associations with plants, illustrating in-depth studies on their ecology might in future be rewarding (Tello et al., 2014).

(d) Many basidiomycetes, in particular the biotrophic pathogens, are difficult or virtually impossible to grow in axenic culture. This applies e.g., to the entire subdivision Pucciniomycotina, ("rust fungi") and to the obligate mycorrhizal taxa, comprising important families, such as the Russulaceae and Cortinariaceae, from whose basidiomes numerous unique secondary metabolites have already been obtained. Even in some other "saprotrophic" genera, such as *Pluteus*, the basidiospores do not readily germinate, and stable cultures can hardly be established from sterile mycelial plugs taken out of the basidome tissues using standard methodology. Protocols to culture rust fungi or mycorrhizal symbionts have been elaborated by competent mycologists several decades ago. We encourage the community to also emphasize teaching classical mycological techniques, to educate the coming generation of mycologists and prevent these valuable methods from slowly being forgotten. In fact, such techniques could be useful to facilitate work on the genomics and metabolomics of these organisms since stable cultures could be used for propagation of sufficient biomass and a number of other interesting tasks.

The typical approach to explore metabolic pathways includes gene inactivation, combined with chemical characterization of the resulting phenotype. Whereas for model species/genera such as *Aspergillus* and other ascomycetes, numerous procedures and protocols were in place for reverse genetics, to manipulate expression of silent natural product genes, and to harness -omics technologies, this is only modestly (if at all) the case for basidiomycetes. Hence, the above reasons add more complexity to research which aims at functionally characterizing individual genes and basidiomycete secondary metabolomes. Consequently, despite chemically intriguing and unique features of their natural products, and also for the lack of robust biotechnological expression systems, basidiomycetes have not become the objects of choice. As long as the respective genes, enzymes, and mechanisms are present elsewhere, e.g., in *Aspergillus* or *Fusarium* species, or in streptomycetes, these will be preferred organisms. Projects including these organisms will be sooner finished and sooner published. On the other hand, one new basidiomycete genome after the other is currently released and sequence data are made available at a much faster pace than biochemists and natural product chemists can keep up with. Hence, an increasing amount of (natural product gene) sequence data is produced and because verification by wet-bench work cannot keep pace the amount of hypothetical and misannotated natural produce genes is ever-increasing.

Despite all these challenges there are three encouraging reasons why basidiomycetes advance mycology and natural product chemistry. Firstly, unique structures, e.g., the ones mentioned in the introduction, deserve elucidation of the biochemically and mechanistically intriguing basis behind their biogenesis. Secondly, fungi as such are a widely unexplored source for novel biotechnological products in general (cf. Rambold et al., 2013), and this especially holds true for the basidiomycetes. These two reasons alone justify new genomes to be sequenced. Finally, basidiomycetes are of outstanding ecological significance and may be key to answer the question as to why natural products exist. Due to their ability to form mycorrhizae with conifers and deciduous trees, they are key elements of temperate and boreal climax vegetations. They efficiently degrade lignocellulose which makes them indispensable to keep the global carbon cycles going. The basidiomycetes and the existing genomes represent a good opportunity to follow a different approach, but still contributing substantially to natural product research: with perhaps a dozen of carefully chosen symbiotic, parasitic, and saprotrophic species and a concerted effort of mycologists, chemists, ecologists, biochemists and bioinformaticians we may come to a more profound understanding why these magnificent small molecules were evolved, beyond the established examples that they serve as defense agents and to compete with other microbes in their ecological niche.

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Silver linings: a personal memoir about Hurricane Katrina and fungal volatiles

Joan W. Bennett *

Department of Plant Biology and Plant Pathology, School of Environmental and Biological Sciences, Rutgers – The State University of New Jersey, New Brunswick, NJ, USA

In the aftermath of Hurricane Katrina, the levees protecting New Orleans, Louisiana failed. Because approximately 80% of the city was under sea level, widespread flooding ensued. As a resident of New Orleans who had evacuated before the storm and a life-long researcher on filamentous fungi, I had known what to expect. After the hurricane I traveled home with a suitcase full of Petri dishes and sampling equipment so as to study the fungi that were "eating my house." Not only were surfaces covered with fungal growth, the air itself was full of concentrated mold odor, a smell that was orders of magnitude more funky than any damp, musty basement I had ever encountered. The smell made me feel bad and I had to take regular breaks as I sampled. Being a mycotoxin expert, I knew a fair amount about "sick building syndrome" but believed that it was difficult to get enough respiratory exposure to toxins to cause the array of symptoms associated with the syndrome. So why was I feeling sick? Some Scandinavian experts had hypothesized that mold volatile organic compounds (VOCs) might be the fungal metabolites to blame for sick building syndrome and the time in my smelly, mold infested home made me think they might be right. After securing a new job and establishing a new laboratory, I endeavored to test the hypothesis that some volatile mold metabolites might be toxic. My laboratory at Rutgers University has interrogated the role of VOCs in possible interkingdom toxicity by developing controlled microcosms for exposing simple genetic model organisms to the vapor phase of growing fungi. Both *Arabidopsis thaliana* and *Drosophila melanogaster* exhibit a range of toxic symptoms that vary with the species of fungus, the duration of exposure, and other experimental parameters. Moreover, low concentrations of chemical standards of individual fungal VOCs such as 1-octen-3-ol also exhibit varying toxicity and cause neurotoxicity in a *Drosophila* model. Collectively, these data suggest that fungal VOCs may contribute to some of the adverse health effects reported by people exposed to damp indoor environments and that biogenic gas phase molecules deserve increased attention by the research community.

Keywords: volatile organic compounds (VOCs), sick building syndrome, 1-octen-3-ol (mushroom alcohol), double petri plate system, *Aspergillus*

Edited by:
Nancy Keller,
University of Wisconsin–Madison, USA

Reviewed by:
John Edgar Linz,
Michigan State University, USA
Deepak Bhatnagar,
United States Department of Agriculture, USA

****Correspondence:***
Joan W. Bennett,
Department of Plant Biology and Pathology, School of Environmental and Biological Sciences, Rutgers – The State University of New Jersey, New Brunswick, NJ 08901, USA
profmycogirl@yahoo.com

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Introduction

I am a fungal geneticist. For most of my professional career, I lived in New Orleans, Louisiana, USA where I was on the faculty at Tulane University and maintained a strong collaboration with scientists at the Southern Regional Research Laboratory, a branch of the U. S. Agricultural Research Service. My research concerned the genetics and physiology of fungal toxins (mycotoxins), especially the aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. I had plenty of experience studying filamentous fungi and was a self-proclaimed mycophile. Nevertheless, I never expected that fungi would change my life outside of the laboratory. Yet, that is pretty much what happened to me late in the summer of 2005, in the aftermath of Hurricane Katrina. Here is my story.

A Hurricane Memoir

In August 2005 I was about to start a sabbatical leave during which I planned to work on the annotation of the genome of *Aspergillus flavus*, an aflatoxigenic species that had just been sequenced with funding by the U. S. Department of Agriculture. My sabbatical plans were permanently altered on August 29, 2005 when Hurricane Katrina crossed the Gulf of Mexico. The day before the hurricane, my husband and I evacuated to a small town in eastern Louisiana so we were not in New Orleans when the hurricane hit and the levees failed. About 80% of the city flooded, including our house. The National Guard barred residents from returning home. Therefore, we drove to New Jersey, where my husband had friends, and found temporary housing. The weeks after Hurricane Katrina were not easy. At first, I watched a lot of TV, getting increasingly angry at the negative media slant put on New Orleans and its residents. A retired colleague, Dr. Gerhard Haas, asked me to give a lecture about my hurricane experiences at Fairleigh Dickinson University, so I used the internet connection at the Franklin Lakes, NJ, public library to obtain images, learn more about the devastation, and read up on categorizations used for hurricanes (1–5). I learned that although Katrina originally had been a powerful Category 5 storm, by the time it hit New Orleans it was "only a high 3." Nevertheless, storm surges caused by the hurricane breached the levees protecting the city and inundated areas below sea level. It took several weeks for the Army Corps of Engineers to pump the flood water out of the city. During that time, public health officials expressed concerns about infectious disease, water pollution, and heavy metal contamination. Almost no one paid any attention to mold. But I knew that the city – the drowned and dying vegetation, the water logged buildings, and their contents – were perfect substrates for filamentous fungi. New Orleans had become a paradise for molds and mildews.

My husband I did not return to New Orleans until early October. Good friends whose home had not flooded invited us to stay in their guest room. Before leaving New Jersey, I called a professional acquaintance, Dr. James White, Chair of the Plant Biology and Pathology, Department at Rutgers University and asked if I could come to his lab and make media. He readily agreed. So on October 4th, 2005 when my husband and I flew back to New Orleans for the first time since our evacuation, I traveled with a suitcase containing sleeves of sterile Petri plates containing fungal media, sampling equipment, disposable latex gloves, and face masks.

Before evacuating New Orleans, we had shut the windows, pulled the curtains, and locked all the doors to our house. We also had taken a last look at our beautiful green garden. By the time we returned to our neighborhood, the flood water was gone but most of the vegetation was dead from the prolonged anoxia. The lawns and our half-century-old azalea bushes were brown and shriveled. My husband and I did our best to brace ourselves. Nevertheless, when we opened the front door to our home, the reality of the devastation was hard to take (see **Figures 1A,B**).

There was no longer any standing water in the house but the carpets were still squishy; a car we had left behind in the garage was filled with muddy water. Several armchairs were lying on their side. We surmised that when the flood waters surged in they had knocked over the lighter pieces of furniture. As expected, mold was growing on almost every surface – carpets, curtains, upholstered furniture, and most heart breaking of all, some of my most beloved books. Our home had become a fungal utopia. What was unexpected was the intensity of the stench. Like everyone, I was familiar with the smell of damp basements, attics, and

FIGURE 1 | (A) The living room of the author's New Orleans home on October 6, 2005 in the aftermath of Hurricane Katrina. **(B)** Closer view of moldy books on flooded book shelf.

other spaces where microbial life grows in enclosed spaces. As an *Aspergillus* geneticist, I also was familiar with the characteristic scent that hits you when you open the door of an incubator full of growing fungi. But nothing had prepared me for the intensity of the odor in our flooded home. The stench was so strong that it didn't really smell like mold – it just smelled horribly funky and powerfully sickening.

For me, being able to slip into my role as a professional scientist helped with the psychological shock. I proceeded to sample the house. As usual, it was very hot in New Orleans and the lack of electric power meant there were no fans, much less any air conditioning. The combination of the heat, the emotional impact of seeing my mold-ravaged home, and the terrible smell of rot in the enclosed rooms made me feel sick. Since I was wearing a mask while I sampled, I didn't think that my physical reaction came from breathing mold spores. Nevertheless, my body was telling me, "Get out of here." So, I wiped off one of the wooden chairs and put it in the front yard. Over the next few hours I alternated my mold sampling and photography with sitting in the front yard, breathing the outdoor air, and letting my mind wander. Among other things, I thought about the bad smell and wondered where it came from. Were the odors caused by mold metabolism or were they incidental breakdown products of fungal degradative enzymes? I knew that fungi made odorant metabolites but I had never given them much attention. For example, I knew that some mushroom hunters use characteristic scents to help them identify delectable species. Chanterelles are known for their fruity, apricot-like odor, and the princess matsutake *Agaricus subrufescens* smells like almonds (Harper et al., 1968; Cronin and Ward, 1971). Further, mold volatiles have been used to detect contamination in stored grains (Schnürer et al., 1999). I also had read that workers in Scandinavia had postulated that mold volatile organic compounds (VOCs) might be responsible for some of the symptoms associated with "sick building syndrome," a poorly defined condition of unknown etiology that was associated with unhealthy indoor environments. With the benefit of hindsight, I wish that I had brought appropriate equipment for sampling VOCs but lacking such technological supports, I nevertheless started to form my own conjectures. Perhaps my own negative physical reaction was caused by the fungal odorants. Perhaps some mold VOCs were biologically active. Perhaps if I ever could re-establish my laboratory, I could devise a way of studying the toxigenic potential of fungal VOCs.

Starting Over

Now to flash forward and abbreviate several difficult months. My husband and I hired a crew of workers to gut our house and then we flew back to New Jersey. I brought my Katrina molds to Rutgers and subcultured them. Jim White suggested that I should meet with Robert Goodman, Dean of Cook College (now School of Environmental and Biological Sciences), the agricultural unit of Rutgers University. Dean Goodman invited me to complete my sabbatical at Rutgers. The following January we rented a house near the Rutgers campus and I started a new research project. I worked with a an undergraduate named Craig Pritch doing tentative taxonomic identifications of my Hurricane Katrina molds and reading the literature on sick building syndrome. At the end of the semester, Mr. Pritch submitted a short research paper with a humorous cover page showing a contaminated culture of *Trichoderma*, one of the most common molds found in my flooded home (see **Figure 2**). Despite the spelling errors, it remains my favorite-ever cover sheet for a student paper.

During my sabbatical time in the Department of Plant Biology and Pathology, Dr. Philip Furmanski, Executive Vice President of Rutgers University offered me a permanent job at the university and I accepted. The day after I received my tenure letter from Rutgers University I resigned from Tulane University. Although I was a senior scientist, I had decided that I would start a new research career, studying the possible physiological activity of fungal odorants. My husband I moved permanently to New Jersey in late August 2006, almost exactly 1 year after the hurricane that had changed our lives.

During my early months on the Rutgers faculty, I recruited my first new graduate student, Richard Hung, who had worked in my lab as an undergraduate at Tulane, and my first visiting scientist, Prakash Masurekar who had recently retired from Merck Corporation. They helped order supplies, equipment and set up the lab. We put the Katrina molds that I had collected into pure culture and then grew them individually on laboratory medium and gypsum board ("sheet rock") in controlled

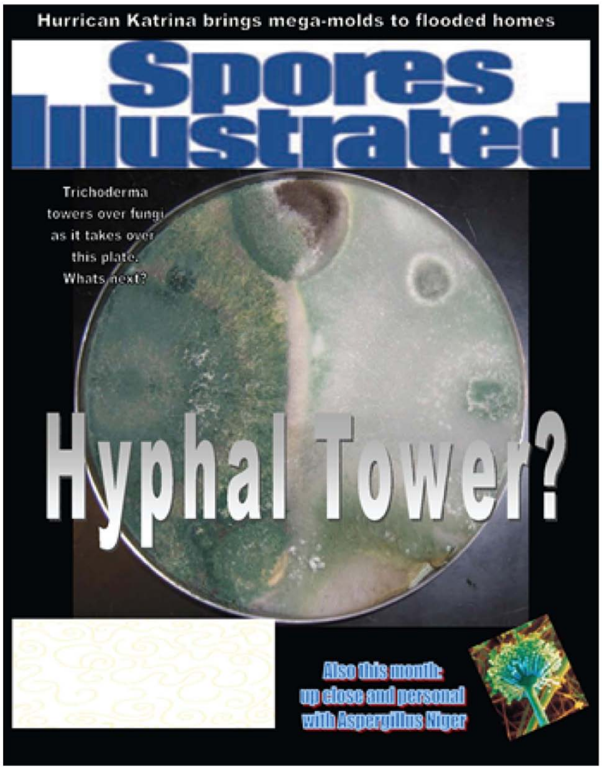

FIGURE 2 | Cover of undergraduate research paper about molds isolated after Hurricane Katrina, designed by Craig Pritch, May 2006.

microcosms. Richard Hung spent rather a lot of time working on developing appropriate collection methods (See **Figure 3**).

The fungal isolates were grown on gypsum board, VOCs from the head space collected, and using gas chromatography–mass spectrometry (GC–MS), profiles of VOCs were identified. The VOCs consisted of mixtures of different chemical classes: alcohols, aldehydes, hydrocarbons, aromatics, nitrogen-containing compounds, thiols, terpenoids, and their derivatives. Previous researchers had done a good job of cataloging the VOC profiles produced by "indoor molds" grown on different substrates and similar profiles of metabolites previously had been identified from contaminated building materials (Fiedler et al., 2001; Claeson et al., 2002; Matysik et al., 2008). Thus, we learned what others had discovered and published before us. Molds make many different VOCs. Different species of molds make different combinations of VOCs. They make them in different proportions and concentrations, depending on how long they are grown, and what they are grown on. These VOCs are produced as blends of complex mixtures that change dynamically with time.

Some Background on Sick Building Syndrome

For decades the international public health community has been concerned about the possible adverse health effects of molds in damp indoor environments. These adverse health effects are usually named "sick building syndrome" or less frequently "damp building syndrome" (Godish, 1995; Hodgson, 2000). Sick building syndrome is characterized by a group of non-specific symptoms that include fatigue, respiratory distress, skin problems, eye irritation, mental disturbances, and so forth (Hodgson, 2000, 2002; Burge, 2004). The "off gassing" of industrial solvents, air borne particulates, and exposure to mold toxins (mycotoxins) have been implicated as causes of this difficult-to-define syndrome (Godish, 1995; Straus, 2009) with molds considered one of the main risk factors (Li and Yang, 2004). Some experts question whether there is sufficient evidence to link molds and mycotoxins to the wide range of symptoms associated with this elusive health condition (Hardin et al., 2003; Kuhn and Ghannoum, 2003). Both the U. S. Institute of Medicine [IOM], 2004 and the World Health Organization [WHO], 2009 have published meta-reviews of the published literature in which it was concluded that certain respiratory health effects (e.g., asthma, allergy, and upper respiratory tract irritation) show an association with mold exposure but that there is no conclusive evidence linking mold exposure to the full set of symptoms attributed to sick building syndrome. Many data gaps exist. Available studies have shown a lack of standardized methods for measuring exposure to molds and difficulty in determining which of several disease-causing agents in damp indoor environments may be responsible for the adverse health effects (Institute of Medicine [IOM], 2004; World Health Organization [WHO], 2009). Moreover, although there is a large published literature on the adverse health effects of mycotoxins, far less is known about fungal VOCs. Danish workers were the first to postulate that VOCs caused or contributed to the adverse health effects associated with unhealthy indoor environments (Mølhave, 1992; Mølhave et al., 1993). Several laboratories have shown that certain industrial and biogenic VOCs are toxic in mammalian cell culture (Kreja and Seidel, 2002; Korpi et al., 2009). Furthermore, human volunteers exposed to vapors of mushroom alcohol (1-octen-3-ol) at a concentration of 1.9 ppm for 2 hours show an increase in inflammatory markers in their nasal secretions (Wålinder et al., 2008).

Obviously, people who complain of building-related ill health may have been exposed to numerous toxigenic and allergenic agents. These include interacting biological (e.g., mycotoxins), chemical (e.g., off-gassing of building materials), and physical (e.g., ventilation rates) factors that may interact synergistically to create "problem buildings." For detailed discussions of the complexities involved in the analysis of sick building syndrome, including psycho-social dimensions, see the monographs by Cone and Hodgson (1989), Godish (1995), and Straus (2009).

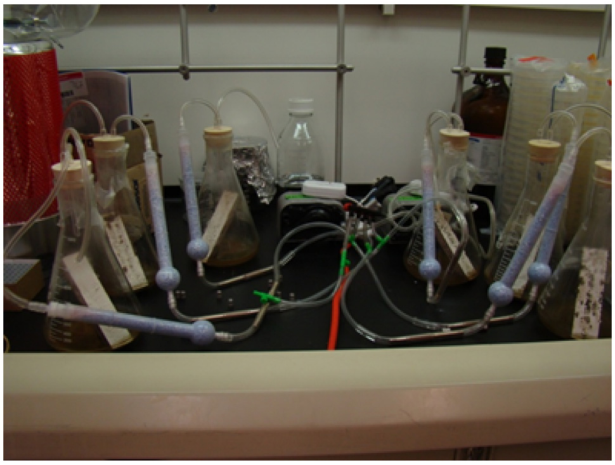

FIGURE 3 | Experimental set up for collecting volatiles emitted by molds growing on gypsum board (Image courtesy of Richard Hung).

Developing Model Systems

After cataloging the VOCs produced by our Katrina molds, we embarked on a new avenue of research that to our knowledge has not been done by other groups. We pioneered the use of genetic model systems to test the physiological activity of fungal VOCs in controlled environments. Our intent was to create simplified systems in which to isolate individual factors and to do so in model organisms that would allow us to investigate the mechanistic basis of any effects we uncovered. A long term goal was to create standardized toxicological assays for studying fungal volatile compounds in hopes of finding a way to link mold VOCs and negative health consequences.

Caenorhabditis elegans

Richard Hung obtained several strains of *Caenorhabditis elegans* and cultured them in the presence of growing molds or low concentrations of chemical standards of several of the VOCs we knew were components of the blends emitted by growing molds. We envisioned that we would record the behavior of wild type worms and then test mutants lacking certain known chemosensory genes. However, the experiments did not go well. After a short time, many worms would be found having crawled up the sides of the Petri plates. Most of the nematodes seemed to have vanished. We speculated that they crawled out of the plates and desiccated. Because these nematodes are tiny and translucent, we could not even find their dead bodies. We abandoned *C. elegans*.

Arabidopsis thaliana

Based on the observation that vegetative growth is suppressed in areas known to have truffles growing underground, Italian workers had hypothesized that plants may have the ability to detect the presence of fungi through their volatiles (Splivallo et al., 2007). The same workers determined that at high concentrations, the distinctive fungal VOC commonly called "mushroom alcohol" (1-octen-3-ol) inhibited root growth and lowered chlorophyll concentration in *Arabidopsis thaliana*. A Japanese group had shown that at lower doses, 1-octen-3-ol enhanced resistance of mature *A. thaliana* to *Botrytis cinerea*, and activated some of the same defense genes turned on by ethylene and jasmonic acid signaling (Kishimoto et al., 2007). Therefore, Richard switched his attention to using *A. thaliana* as a possible genetic model for assaying volatile toxicity. He determined that a low concentration (1 ppm) of racemic, (S)-(+)-1-ocen-3-ol or R–(-)-1-octen-3-ol had negative effects on *A. thaliana* seedling formation, biomass production, chlorophyll content, and electrolyte leakage of 2 week old plants (Hung et al., 2014a). Further, Richard joined with Samantha Lee, another graduate student in the lab, and evaluated the sensitivity of *A. thaliana* to the presence of eight biogenic and six anthropogenic VOCs using seed germination and plant growth assays. With the exception of ethanol, when compared to controls, all the VOCs we studied gave rise to significantly lower levels of chlorophyll in treated plants. Many showed severe discoloration and curling of leaves as well as localized cell death (Lee et al., 2014). Then, in separate experiments when testing the effect of exposing *A. thaliana* to VOCs from growing fungi, Richard unexpectedly discovered that the VOCs from a *Trichoderma* species *enhanced* plant growth (Hung et al., 2013, 2014b). *Trichoderma* is well known and widely used as a biocontrol species (Harman et al., 2004) but the contribution of its VOCs to plant vigor had not before been shown. Therefore, our laboratory has established a new research project that focuses on the plant growth enhancement effects of *Trichoderma* VOCs and shown that the effects are not limited to *A. thaliana* (unpublished data).

Drosophila melanogaster

About 2 years after the Hurricane, I hired a postdoctoral research associate named Arati Inamdar who had training in neurobiology. She developed a new bioassay using *Drosophila melanogaster*. We exposed both adult flies and third instar larvae. For the latter assay Arati devised double petri plate "sandwiches" consisting of two Petri dish bottoms separated by a lid into which a hole had been burned (see **Figure 4**). The hole was sealed carefully with a membrane that allowed VOCs to pass. On one side, we placed the fly larvae. In the other, we inoculated a growing culture of a mold isolated from my flooded home. We sealed the two plates together with Parafilm, placed them on a rotary shaker at constant temperature, and recorded the number of larvae, pupae, and adults that formed over a 2 week period.

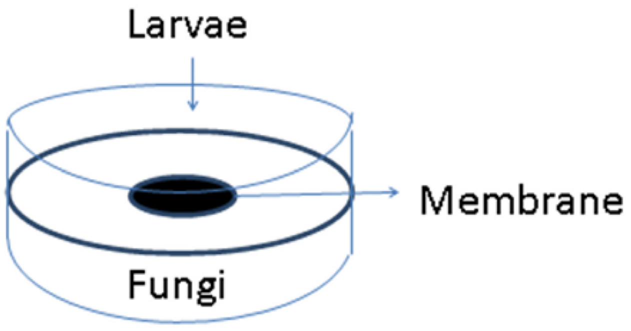

FIGURE 4 | Double Petri plate system to test volatile organic compounds (VOCs) emitted from a living culture of mold. *Drosophila* third instar larvae are placed on one plate and a growing mold culture on the other. A membrane permeable to VOCs separates the two plates (Image courtesy of Arati Inamdar).

The developmental stages of the flies were exposed to the VOC blends emitted by the growing fungi for the entire time. Isolates of *Aspergillus*, *Penicillium*, and *Trichoderma* from my flooded home were tested. Of these, the *Trichoderma* isolates were most toxic (Inamdar et al., 2012). Although different toxigenic effects were detected, it was impossible to know which specific compounds, or combination of compounds, were responsible for the negative effects we were seeing on fly metamorphosis and survival. The ever-changing profile of VOC production made it difficult to characterize biological effects. Which of the many VOCs was causing a given effect? Were multiple VOCs interacting in some synergist fashion? How much did substrate, and how much did genotype, dictate the kinds of VOCs produced?

After a long career as a geneticist, my mental preferences are reductionist. I don't like experiments where there are too many variables acting simultaneously. Therefore, we decided to come at our hypothesis from a different direction. Rather than exposing our model to the ever-changing mixture of VOCs emitted by the growing molds, we turned to using chemical standards of individual VOCs. In this way, we could study them one by one using carefully calibrated concentrations. We went to a chemical supply catalog and looked to see which common fungal VOCs were available for purchase. We bought a number of the cheapest ones and then, for each chemical, we tested a range of low concentrations against both third instar larvae and adult flies. For positive controls, we tested the vapor phase of toluene, benzene, formaldehyde, and xylene. After 15 days of exposure, benzene and toluene led to 50% mortality while mortality was under 20% for formaldehyde and xylene. The percent mortality for 1-butanol, 1-propanol, 1-hexanol, and 1-decanol were 2, 42 and 68%, respectively. The eight carbon compounds we tested were more toxic to adult and larval *Drosophila* than the non-C8 compounds. Of these, 1-octen-3-ol (mushroom alcohol) and 3-octanol are secondary alcohols while 3-octanone is a ketone. All these C8 compounds yielded 100% mortality within 24 h (Inamdar et al., 2012). Further, the flies exposed to 1-octen-3-ol demonstrated a number of movement disorders, similar to those seen in a fly model for Parkinson's disease (Inamdar et al., 2010). We went on and used the fly model, applying a combination of genetic, biochemical, and immunological approaches, to show that 1-octen-3-ol reduced dopamine levels and caused dopamine neuron degeneration in *D. melanogaster*. Furthermore, over-expression of the vesicular monoamine transporter (VMAT) rescued the dopamine toxicity and neurodegeneration. In contrast, mutations decreasing VMAT and tyrosine hydroxylase exacerbated toxicity. Uptake of dopamine was inhibited by vapor phase 1-octen-3-ol in human cell lines expressing the human plasma membrane dopamine transporter and human VMAT ortholog, VMAT2. Collectively, these data suggested that 1-octen-3-ol exerted toxicity via disruption of dopamine homeostasis and may represent a naturally occurring environmental agent involved in parkinsonism-like toxicity (Inamdar et al., 2013a). We also demonstrated that exposure of flies to 1-octen-3-ol stimulated the caspase-3 dependent apoptotic signaling pathway (Inamdar et al., 2013b). In addition, we showed that there was an induction of nitric oxide (NO) and its derivative, peroxynitrite by 0.5 ppm of 1-octen-3-ol an inflammatory response mediated via hemocytes, which are *Drosophila* innate immune cells. In other words, this ubiquitous fungal VOC, commonly associated with mold-contaminated damp indoor spaces, stimulated a NO mediated inflammatory response in nervous and respiratory tissues of *D. melanogaster* (Inamdar and Bennett, 2014). Together, these experiments with the *Drosophila* model may open a new avenue for mechanistic understanding of the possible human health effects of mold-emitted volatile chemicals.

Conclusion

The toxicity and biological potency of a number of industrial solvents such as formaldehyde, toluene, and benzene are well known, however, far less is known about the gas-phase biomolecules secreted by fungi, bacteria, and green plants. Some of the most penetrating studies have been conducted by entomologists who have shown that many VOCs serve as semiochemicals ("infochemicals"; Davis et al., 2013). In general, the literature on biogenic VOCs is scattered between food and flavor chemistry, entomology, chemotaxonomy, and a number of other subdisciplines that do not normally "talk to one another." Members of our laboratory, along with collaborators at Penn State in Seogchan Khan's group, have collaborated on writing several review articles in an attempt to bring together the literature on VOCs that has been developed in mycology, entomology, building science as well as the elegant chemical studies performed by food and flavor scientists (Morath et al., 2012; Bennett et al., 2013; Bitas et al., 2013). Another interesting research development is the use of volatiles as a non-invasive method for disease detection. For example, the "volatome" of *Aspergillus fumigatus* has been analyzed and may provide an early diagnostic tool for systemic aspergillosis infections (Heddergott et al., 2014).

We believe that in order to bring the research on fungal volatiles to its appropriate place in 21st century biology, we need better communication between fungal biologists, molecular biologists, entomologists, chemical ecologists, toxicologists, and all biologists interested in VOCs. The traditions of our respective disciplines do not make these associations easy to achieve, nor do the discipline-based approaches of the review committees associated with most funding agencies. The scientific community has a great deal to learn about the way in which VOCs influence ecosystem dynamics, especially the microbial ecosystems that function in indoor environments. The study of gas phase molecules produced by fungi increases our understanding of how fungi interact with their environment and with each other.

For me personally, the shift in my research focus has been intellectually stimulating. Sometimes bad experiences lead to good outcomes. That is what happened to me with Hurricane Katrina. The metaphoric black clouds of my Hurricane Katrina experience have provided a scientific silver lining and an entirely new research focus. The hurricane transformed my life. My flooded home, covered with mold growth, is what inspired my new research. I have changed jobs, the place where I live, and my perspectives about fungal metabolism. Like most biologists and biochemists, previously my experimental strategies were all based on "liquid phase biology." Even when I studied water-insoluble mycotoxins, I grew molds in liquid media and then partitioned target metabolites into liquid-phase, non-polar solvents. During the many decades when I worked on aflatoxin genetics and biosynthesis, I cultured thousands of plates and flasks of fungi in laboratory incubators. When I opened an incubator door and smelled that characteristic moldy smell, I ignored it. Now my "consciousness has been raised." I am alert to odors of all kinds – not just fungal odors – and have come to believe that volatile phase biology is a new scientific frontier. In nature, organisms do not live alone. They live in communities where interspecific communications frequently take place through gas-phase chemical signaling, especially in terrestrial environments. The possibilities for new discoveries are enormous. Scientists merely have to "open their noses," smell the world around them, and recognize that odorants have many undiscovered biological properties. We hope that our exploratory research will inspire others to work on volatile phase signaling in biology.

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Taxonomy, chemodiversity, and chemoconsistency of *Aspergillus*, *Penicillium*, and *Talaromyces* species

Jens C. Frisvad*

Section of Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark

Edited by:
Jonathan Palmer, United States Department of Agriculture Forest Service, USA

Reviewed by:
Antonello Santini, University of Naples Federico II, Italy
Rob Samson, CBS Fungal Biodiversity Centre, Netherlands

***Correspondence:**
Jens C. Frisvad, Section for Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark, Søltofts Plads B. 221, 2800 Kongens Lyngby, Denmark
e-mail: jcf@bio.dtu.dk

Aspergillus, *Penicillium*, and *Talaromyces* are among the most chemically inventive of all fungi, producing a wide array of secondary metabolites (exometabolites). The three genera are holophyletic in a cladistic sense and polythetic classes in an anagenetic or functional sense, and contain 344, 354, and 88 species, respectively. New developments in classification, cladification, and nomenclature have meant that the species, series, and sections suggested are natural groups that share many extrolites, including exometabolites, exoproteins, exocarbohydrates, and exolipids in addition to morphological features. The number of exometabolites reported from these species is very large, and genome sequencing projects have shown that a large number of additional exometabolites may be expressed, given the right conditions ("cryptic" gene clusters for exometabolites). The exometabolites are biosynthesized via shikimic acid, tricarboxylic acid cycle members, nucleotides, carbohydrates or as polyketides, non-ribosomal peptides, terpenes, or mixtures of those. The gene clusters coding for these compounds contain genes for the biosynthetic building blocks, the linking of these building blocks, tailoring enzymes, resistance for own products, and exporters. Species within a series or section in *Aspergillus*, *Penicillium*, and *Talaromyces* have many exometabolites in common, seemingly acquired by cladogenesis, but some the gene clusters for autapomorphic exometabolites may have been acquired by horizontal gene transfer. Despite genome sequencing efforts, and the many breakthroughs these will give, it is obvious that epigenetic factors play a large role in evolution and function of chemodiversity, and better methods for characterizing the epigenome are needed. Most of the individual species of the three genera produce a consistent and characteristic profile of exometabolites, but growth medium variations, stimulation by exometabolites from other species, and variations in abiotic intrinsic and extrinsic environmental factors such as pH, temperature, redox potential, and water activity will add significantly to the number of biosynthetic families expressed in anyone species. An example of the shared exometabolites in a natural group such as *Aspergillus* section *Circumdati* series *Circumdati* is that most, but not all species produce penicillic acids, aspyrones, neoaspergillic acids, xanthomegnins, melleins, aspergamides, circumdatins, and ochratoxins, in different combinations.

Keywords: ***Aspergillus, Penicillium, Talaromyces,*** **secondary metabolites, chemodiversity, chemoconsistency**

INTRODUCTION

The genera *Aspergillus sensu lato* and *Penicillium sensu lato* contain a high number of very diverse species. These species produce a large number of exometabolites, also known as secondary metabolites. Exometabolites are small molecules produced during morphological and chemical differentiation that are outward directed, i.e., secreted or deposited in or on the cell wall, and accumulated in contrast to endometabolites (primary metabolites), that are fluctuating in concentration (the fluxome), and either transformed into other endometabolites or feeding into exometabolites, exoproteins, exopolysaccharides, and morphological structures. While endometabolites can be found in almost all species of fungi (and most other kinds of organisms), exometabolites, exoproteins, and exopolysaccharides are taxonomically restricted, being produced in species-specific profiles. Some metabolites can occur both as endo- and exometabolites, for example citric acid. When citric acid is part of the mitochondrial fluxome, it should be regarded as an endometabolite, but when citric acid is secreted and accumulated (Goldberg et al., 2006; Andersen et al., 2011; Poulsen et al., 2012), as in *Aspergillus niger*, it must be regarded as an exometabolite. Accumulation of citric acid requires that there is a reductive pathway for it in the cytosol and that it can be secreted to the surroundings via an exporter. Thus the transport from the mitochondria to the cytosol, the cytosolic reduction, and the secretion requires a dedicated gene cluster. Such a gene cluster has been found in for example *A. terreus* that is coding for accumulating and secreting itaconic acid (van der Straat et al., 2014), but the gene cluster for citric acid accumulation has not been described yet. Some species related to *Aspergillus* and *Penicillium*, such as *Xeromyces bisporus*, are predominantly stress-selected (S-selected) and the lack of any competitors at very low water activities will have the consequence that *X. bisporus* produces no exometabolites (Leong et al., 2014). In *Aspergillus* most species produce a large number of exometabolites, but some stress selected species, such as *A. penicillioides* and *A. restrictus*, have only been reported to produce asperglaucide and cristatin A, and the related arestrictin A and B (Itabashi et al., 2006). However, the closely related xerotolerant/xerophilic species in the *Aspergillus* subgenus *Aspergillus* (formerly *Eurotium*) produce a high number of exometabolites in the ascomata, making them chemically very diverse (Slack et al., 2009).

Aspergillus AND *Penicillium* TAXONOMY AND NOMENCLATURE

Because of their importance, species of *Aspergillus* and *Penicillium* have been taxonomically treated several times, but the monographs by Raper and Thom (1949) for *Penicillium* and Raper and Fennell (1965) for *Aspergillus* are still regarded as cornerstones in the taxonomy of these fungi. In the period between these two monographs, however, several authors (Benjamin, 1955; Malloch and Cain, 1971) suggested to use names for the sexual state of the Aspergilli and Penicillia, whenever possible, to adhere to the Botanical Code in a nomenclatural sense. The use of *Penicillium* and *Aspergillus* for species that had not yet been found to produce a sexual state could keep their *Penicillium* and *Aspergillus* names, because of a special nomenclatural "exception" in the Botanical Code (Art. 59) that allowed to use two names for a specific fungal species, one for the asexual states (the "anamorph") and one for the sexual state (the "teleomorph"). Despite this it was recommended to use the sexual name for the whole fungus (the "holomorph"), whenever a sexual state had been found. For this reason many species in *Penicillium* were renamed *Eupenicillium* or *Talaromyces* (Pitt, 1980) and many *Aspergillus* species were renamed *Chaetosartorya*, *Emericella*, *Eurotium, Fennellia, Hemicarpenteles*, *Hemisartorya*, *Neocarpenteles*, *Neopetromyces*, *Neosartorya*, *Petromyces, Saitoa*, *Sclerocleista*, or *Warcupiella* (Rai and Chowdhery, 1975; Rajendran and Muthappa, 1980; Gams and Samson, 1986). To give one example of the name changes one can mention a fungus that was originally described as *A. fischeri.* Since this fungus was described including the sexual state, it could not be used for the asexual state anymore, so the correct name for the fungus according the nomenclatural rules before 2011 was *Neosartorya fischeri*, while *A. fischeri* had to be renamed *A. fischerianus* if one wanted only to refer to the asexual state. A full monographic revision of *Aspergillus* according to the Botanical Code has not been written, but lists of accepted *Aspergillus* and *Penicillium* species have been made (Pitt et al., 2000) and several revisions of the individual genera have been published.

In 2010 it was suggested to introduce a new nomenclatural system in which one fungus had only one name (Hawksworth et al., 2011; Hawksworth, 2012). This suggestion was adopted by the Botanical Congress in Melbourne (McNeill et al., 2012), and thus hereafter any species in fungi will only have one official name. The selection of those names is encouraged to take place by consensus among international experts in the group of fungi under consideration. The International Commission of *Penicillium* and *Aspergillus* (ICPA) has decided to use *Penicillium* for the monophyletic clade that includes *Penicillium* subgenera *Aspergilloides*, *Furcatum,* and *Penicillium sensu* Pitt (1980), *Eupenicillium*, *Chromocleista*, *Thysanophora* and *Eladia,* and *Talaromyces* for the monophyletic clade that includes *Talaromyces* itself and *Penicillium* subgenus *Biverticillium sensu* Pitt (1980). For *Aspergillus sensu lato,* a cladistic study using DNA sequence data, showed that most known *Aspergillus* species were included in a monophyletic clade (Houbraken and Samson, 2011), while a few rare species, such as *A. zonatus* and *A. clavatoflavus* were more closely related to other genera in the *Eurotiomycetes.* The nomenclatural consequence of this is to call all the species in the monophyletic clade *Aspergillus* (Houbraken et al., 2014; Samson et al., 2014) or retypify the genus *Aspergillus* with for example *A. niger*, and then subdivide *Aspergillus* into the genera *Aspergillus*, *Neosartorya*, *Emericella*, *Eurotium,* and *Chaetosartorya* (Pitt and Taylor, 2014). This would have the consequence that the name *Aspergillus* would only be used for a paraphyletic weakly supported clade representing subgenus *Aspergillus* and that the genus *Neosartorya* would be polyphyletic as it includes *Dichotomomyces.* Even though a majority of ICPA members voted for the *Aspergillus* solution, which includes mentioning the sexual state informally, for example *A. fischeri* (neosartorya-morph present), general consensus has not yet been reached. In this review *Aspergillus* names will be used, as suggested by Samson et al. (2014), as the name *Aspergillus* can be confidently used for the monophyletic clade that includes the genera listed above (Houbraken et al., 2014). All species formerly included in *Dichotomomyces*, *Cristaspora*, *Phialosimplex*, *Polypaecilum*, in addition to *Penicillium inflatum*, have been formally combined into *Aspergillus* (Samson et al., 2014), while *A. crystallinus*, *A. malororatus,* and *A. paradoxus* (*Hemicarpenteles paradoxus*) have been combined into *Penicillium*, as *P. crystallinum*, *P. malodoratum,* and *P. paradoxum* (Visagie et al., 2014b). This means that the presence of aspergilla in an isolate does not necessarily mean that the isolate belongs to *Aspergillus sensu stricto*, and the presence of penicilli in an isolates does not necessarily mean the species belong in *Penicillium sensu stricto*. However, in the majority of cases aspergilla or penicilli indicates that the species belong to *Aspergillus* and *Penicillium*, respectively.

Pitt and Taylor (2014) suggested to use *Aspergillus* for the paraphyletic subgenus *Circumdati* only (after potential re-typification of *Aspergillus* with *A. niger*), stating that this restricted use of the genus *Aspergillus* would make this genus phenotypically different from the closely related *Aspergillus* subgenus *Nidulantes* and therefore suggested the name *Emericella* for the latter monophyletic clade. However, there are many phenotypic traits in common between section *Circumdati* and *Nidulantes*, including the presence of hülle cells and the exometabolites kojic acid, aflatoxins, and sterigmatocystins in both subgenera (Raper and Fennell, 1965; Wiley and Simmons, 1973; Frisvad and Samson, 2004a; Frisvad et al., 2005; Zalar et al., 2008). Subgenus *Circumdati* includes species with both multiple cleistothecia in sclerotia (*Petromyces, Neopetromyces, Saitoa*; Udagawa et al., 1994; Yaguchi et al., 1994; Frisvad and Samson, 2000; Horn et al., 2013) and pseudoparenchymatous multiple ascomata in hyphal masses with or without hülle cells (*Fennellia* and the perfect state of *A. terreus*; Wiley and Simmons, 1973; Locquin-Linard, 1990; Yaguchi et al., 1994; Samson et al., 2011a; Arabatsis and Velegraki, 2013), while several species in *Aspergillus* section *Nidulantes* produce pseudoparenchymatous single ascomata and hülle cells.

Assistance in choosing between *Aspergillus* (Samson et al., 2014) versus the genera *Eurotium, Emericella, Neosarotrya* and *Chaetosartorya, Phialosimplex, Polypaecilum, Dichotomomyces,* and *Cristaspora* (Pitt and Taylor, 2014) can be sought from scientific databases. It is very clear that while *Aspergillus* has been used in 56178 publications the other genera, when all added, have only been used in 1093 publications (approximately 2%; **Table 1**).

A classification of isolates into sections and series in *Penicillium, Talaromyces,* and *Aspergillus* based on phenotypic characters will show that these supraspecific taxa are natural polythetic classes (Beckner, 1959) in exometabolite, ecophysiological, and morphological characters. For example in *Aspergillus* subgenus *Circumdati* section *Circumdati* (the former *A. ochraceus* group) most species, but not all, produce aspyrones, penicillic acids, xanthomegnins, ochratoxins, melleins, circumdatins, neoaspergillic acids, and aspergamides/stephacidins (Frisvad et al., 2004a,b; Finefield et al., 2012; Visagie et al., 2014a). In addition individual species produces exometabolites that are only accumulated by few species in the section. *A. westerdijkiae* and *A. ochraceus* can both produce all the exometabolites listed above, but in addition *A. westerdijkiae* produces preussin and mellamide, not produced by *A. ochraceus*.

Table 1 | References to *Aspergillus, Penicillium, Talaromyces,* and associated genera (Web of Science, as of 18 October, 2014).

Genus	No hits in web of science
Aspergillus	56178
Penicillium	18011
Talaromyces	645
Eurotium	421
Emericella	379
Neosartorya	278
Eupenicillium	187
Thysanophora	35
Petromyces	31
Dichotomomyces	12
Fennellia	10
Basipetospora	9
Polypaecilum	7
Chaetosartorya	6
Eladia	5
Hemicarpenteles	5
Phialosimplex	5
Warcupiella	4
Neopetromyces	4
Chromocleista	4
Sclerocleista	3
Saitoa	1
Neocarpenteles	1
Cristaspora	1
Hemisartorya	0

At present *Aspergillus* comprises 344 species (Samson et al., 2014), *Penicillium* 354 species (Visagie et al., 2014b), and *Talaromyces* 88 species (Yilmaz et al., 2014). These genera include species that have been reported to produce large numbers of exometabolites (**Table 2**).

CHEMODIVERSITY

Species of *Aspergillus*, *Penicillium,* and *Talaromyces* are extraordinarily productive concerning exometabolites. A comparison with other genera shows that most exometabolites have been

Table 2 | Individual exometabolites produced by important genera of filamentous fungi ranked according to highest number of exometabolites reported (according to AntiBase).

Genus	Number of exometabolites reported
Aspergillus	1984
Penicillium	1338
Fusarium	507
Trichoderma	438
Talaromyces	316
Phoma	263
Drechslera, Curvularia, Bipolaris, Cochliobolus	258
Alternaria and *Ulocladium*	231 + 7
Chaetomium	230
Acremonium	187
Phomopsis	186
Xylaria	143
Stachybotrys	138
Pestalotiopsis	133
Claviceps	130
Cladosporium	113
Botrytis	102
Byssochlamys/Paecilomyces sensu stricto	94
Hypoxylon	88
Cordyceps	77
Clonostachys	72
Arthrinium	26
Nigrospora	25
Septoria and Stagonospora and *Parastagonospora*	22
Stemphylium	17
Trichophyton	10

Species of Penicillium listed were revised to Talaromyces if they belonged there (Yilmaz et al., 2014).

reported from *Aspergillus* (1984), next-most from Penicillium (1338), and fifth-most by *Talaromyces*, (316), with only *Fusarium* (507) and *Trichoderma* (438) producing more exometabolites *in toto* (**Table 1**). The number of exometabolites pr species is 5.77 for *Aspergillus*, 3.77 for *Penicillium*, and 3.58 for *Talaromyces*. These number per species are clearly underestimates as some exometabolites are produced by more than one species in a genus, in addition to the fact that many species have not been examined and that some exometabolites are only expressed under unique circumstances and thus may remain undetected (Sanchez et al., 2012; Brakhage, 2013; Scherlach et al., 2013; Takahashi et al., 2013; Bertrand et al., 2014; Marmann et al., 2014). Light, pH, redox potential, temperature, water activity, carbon sources, nitrogen sources, iron starvation, and exometabolites from other species can all have a regulatory effect on the regulatory proteins for exometabolite expression in a fungus (Brakhage, 2013). A majority of the exometabolites produced by *Penicillium* and *Aspergillus* are only found sporadically in other genera, but a large number of exometabolites are in common between *Aspergillus* and *Penicillium*. On the other hand exometabolites from *Talaromyces* are nearly all unique to that genus (Samson et al., 2011b), or only shared with few other species.

The same exometabolite may be produced by widely different species. For example aflatoxin is produced by the species listed in *Aspergillus* section *Flavi* (15 spp.), *Aspergillus* section *Nidulantes* (3 spp.), *Aspergillus* section *Ochraceorosei* (2 spp.), and *Aschersonia* (2 spp.; Frisvad and Samson, 2004a; Frisvad et al., 2005; Zalar et al., 2008; Varga et al., 2009, 2011; Kornsakulkarn et al., 2012, 2013; Massi et al., 2014). Three species in *Aspergillus* section *Flavi* and all the seven species outside section *Flavi* listed above only produce aflatoxins of the B type. It is surprising that aflatoxins have never been found in *Penicillium*, but they have been found in the unrelated scale insect fungi *Aschersonia coffea* and *Aschersonia marginata* (Kornsakulkarn et al., 2012, 2013). However, the precursor sterigmatocystin, although end-product for some species, has been found in a large number of unrelated genera (Rank et al., 2011), suggesting that this complicated gene cluster has been horizontally transfered between species in widely different genera, as shown by Slot and Rokas (2011) for *Podospora anserina* and *A. nidulans*. Fungal species are specifically associated to certain habitats or few plant, animal, or other kind of organisms (Filtenborg et al., 1996), and will therefore produce exometabolites in reponse to the challenges in the particular habitat. For example *P. herquei* was thought to be a soil fungus saprophyte (Kwaśna, 2004), but recent studies have shown tha the leaf-rolling weevil (*Euops chinensis*) have developed mycangia to inoculates leaves with *P. herquei* conidia to protect the weevil eggs (Li et al., 2012). *P. herquei* produce a species specific profile of exometabolites, of which several are antibiotically active (Petit et al., 2009; Tansakul et al., 2014). Thus the specificity in both association of fungal species to other species and the profile of exometabolites are factors that have boosted the evolution of so many exometabolites.

Dichotomomyces cejpii was transferred to *A. cejpii* by Samson et al. (2014), and this new combination is supported by chemotaxonomic evidence. *D. cejpii* was reported to produce gliotoxin, xanthocillin X monomethylether, tryptoquivalones, JBIR-03, emindole SB, emindole SB beta-mannoside, and 27-*O*-methylasporyzin (Varga et al., 2007; Harms et al., 2014). While gliotoxin, tryptoquivalones, and xanthocillins (Frisvad et al., 2009; Zuck et al., 2011) indicates a relationship to *A. fumigatus* and tryptoquivalones a close relationship to *A. clavatus*, as supported by DNA sequences (Varga et al., 2007), production of emindole SB indicates a relationship to *Aspergillus* section *Nidulantes*. The report of emindole SB, emeniveol, asporyzin A-C, and JBIR-03 from a marine-derived *A. oryzae* (Qiao et al., 2010), indicates what they identified as "*A. oryzae*" is a fungus related to *A. cejpii* or a species in *Nidulantes* rather than *A. oryzae*, however.

CHEMOCONSISTENCY AND OSMAC

The abbreviation OSMAC (one strain many compounds) was introduced by Bode et al. (2002) where the authors showed, among several examples, that a strain of *A. westerdijkiae* produced a series of exometabolites that could be ordered into different biosynthetic families. Furthermore, by using several media a more full profile of these exometabolites could be revealed. The idea that one strain can produce several exometabolites was already introduced by Frisvad (1981) and Frisvad and Filtenborg (1983, 1989). These authors showed that terverticillate penicillia produced a unique profile of different exometabolites and also that certain media, such as Czapek yeast autolysate (CYA) agar and yeast extract sucrose (YES) agar were very efficient for production of a large number of different exometabolites, while further media may increase the number of exometabolites expressed (Bills et al., 2008; Nielsen et al., 2011; Frisvad, 2012). Furthermore they showed that these profiles of exometabolites were species specific and consistent from isolate to isolate, i.e., the isolates in anyone fungal species were chemo consistent (Larsen et al., 2005). One of the original terms for exometabolites or secondary metabolites was idiolites, the latter indicating that production of exometabolites was strain specific, however, exometabolite profiles are clearly species specific (Frisvad et al., 2008). However, a single mutation in a gene in an exometabolite gene-cluster will often be sufficient for loss of phenotypic expression (Susca et al., 2014), and this may be the reason some authors call the production of certain exometabolites "strain-specific" (i.e., Engel et al., 1982). The ability to produce mycophenolic acid in *P. roqueforti* is retained in most strains, however, (Frisvad and Filtenborg, 1989; Geisen et al., 2001; Frisvad and Samson, 2004b), but is not as consistent as in *P. brevicompactum*, where a non-producing strain has never been found (Frisvad and Filtenborg, 1989; Frisvad and Samson, 2004b). Reasons for observing "unusual " or "unexpected" exometabolites in a species may be horizontal gene transfer of a gene cluster in only one or few strains, hybridization (which is not common in filamentous fungi), or epigenetic priming. Raper and Thom (1949) mentioned a strain of *P. citrinum* (NRRL 822, their group III in a subdivision of *P. citrinum* "transitional toward *P. chrysogenum* series") produced both citrinin, known from this species, and penicillin, known from *P. chrysogenum*. They also mentioned that their strain had the cultural appearance of both *P. citrinum* and *P. chrysogenum*. We have re-examined this strain, and indeed it had characters of both species, and appeared to be a (rare) hybrid. However, exometabolites from co-occurring species from the same habitat may stimulate the epigenome by acting as inhibitors of histone acetylation or methylation, and this exometabolite stimulation will be one of many ways of having silent exometabolite gene clusters in filamentous fungi expressed (Bertrand et al., 2014). It was recently shown that *A. niger* could produce sclerotia with many hitherto not expressed aflavinins in them (Frisvad et al., 2014) simply by stimulating *A. niger* with whole fruits or rice. Whether this stimulation is caused by extrolites from those whole fruits or rice or from a physical stimulation is not yet known. Furthermore variations in the growth medium and ecophysiological factors such as pH, temperature, and water activity will obviously also stimulate expression of gene clusters of exometabolites that were initially thought to be silent.

In conclusion *Aspergillus, Penicillium,* and *Talaromyces* contain species that produce a very large number of species-specific exometabolites with a high degree of chemoconsistency. The chemodiversity of the many species in these three genera is extremely high and many more bioactive compounds from the species will be found in the future. Both ecological and genetic/molecular approaches are needed to fully explore this treasure-trove of natural products.

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Recent advances in genome mining of secondary metabolites in *Aspergillus terreus*

Chun-Jun Guo*[1] *and Clay C. C. Wang*[1,2]

[1] *Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA, USA*
[2] *Department of Chemistry, College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA, USA*

Edited by:
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Reviewed by:
Axel A. Brakhage, Friedrich Schiller University Jena, Germany
Sean Doyle, National University of Ireland Maynooth, Ireland

****Correspondence:***
Clay C. C. Wang, Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Ave PSC 406A, Los Angeles, CA, USA
e-mail: clayw@usc.edu

Filamentous fungi are rich resources of secondary metabolites (SMs) with a variety of interesting biological activities. Recent advances in genome sequencing and techniques in genetic manipulation have enabled researchers to study the biosynthetic genes of these SMs. *Aspergillus terreus* is the well-known producer of lovastatin, a cholesterol-lowering drug. This fungus also produces other SMs, including acetylaranotin, butyrolactones, and territram, with interesting bioactivities. This review will cover recent progress in genome mining of SMs identified in this fungus. The identification and characterization of the gene cluster for these SMs, as well as the proposed biosynthetic pathways, will be discussed in depth.

Keywords: genome mining, *Aspergillus terreus*, secondary metabolites, natural products

INTRODUCTION

Filamentous fungi, such as species found within the genus *Aspergillus*, are known to produce a wide variety of natural products displaying a broad spectrum of biological activities. Genome sequencing of members in the genus *Aspergillus* revealed that the number of secondary metabolite (SM) genes or gene clusters greatly exceeds the number of SMs identified so far. This suggests that more types of SMs still remain to be discovered. Efficient and high-throughput genome sequencing techniques have now greatly facilitated the genome mining of SMs identified in *Aspergillus* species. More and more studies have shown that most fungal SM genes are clustered, often separated from each other by less than 2 kb within the chromosome (Keller et al., 2005). Striking examples of fungal SM clusters include those responsible for the biosynthesis of fumonisin (Proctor et al., 2003) and sterigmatocystin (Brown et al., 1996). The clustering of these SM genes is fortuitous for researchers since upon discovering one responsible gene within a cluster, other genes that are within close proximity of the identified genes may be involved in the biosynthesis of the same natural product.

Analysis of the fungal genome makes it feasible to characterize these SM gene clusters via genetic manipulations. Verification of function of the relevant genes can be achieved via several approaches. These approaches include targeted deletion or over-expression of the relevant genes in the native organism, heterologous expression in an alternative host, or *in vitro* biochemical characterization. Recent advances in the genome editing of *Aspergillus nidulans* have greatly expedited the SM genome mining of this fungal species (Sanchez et al., 2012). One advance is the development of a fusion PCR technique that allows quick synthesis of linear PCR fragments that are used in the transformation of filamentous fungi (Yu et al., 2004; Szewczyk et al., 2006). Another advance concerns the establishment of an efficient gene targeting system in the fungus *A. nidulans*. This is accomplished via targeted deletion of the *A. nidulans* homolog (*nkuA*) of the human KU70 gene which is essential for non-homologous end joining of DNA in DNA double-strand breaks (Nayak et al., 2006; Szewczyk et al., 2006). Later, this *ku70* deletion toolbox was expanded to other *Aspergillus* species and boosted the gene targeting efficiency in these species (Kuck and Hoff, 2010).

The fungus *Aspergillus terreus* is known to produce lovastatin, which became the first cholesterol-lowering drug of its class approved for human use in the United States. Besides lovastatin, *A. terreus* produces a number of biologically relevant compounds such as sulochrin, terretonin, asterriquinone, and butyrolactone. The strain *A. terreus* NIH 2624, a patient isolate, was sequenced by the Broad Institute as part of the Broad Fungal Genome Initiative. A 10 × coverage genome sequence has been completed, and the data are publicly available through the Broad Institute website. Analysis by Secondary Metabolite Unique Regions Finder (SMURF) showed that *A. terreus* NIH2624 contains 28 polyketide synthase (PKS) genes, 22 non-ribosomal peptide synthetase (NRPS) genes, one hybrid PKS/NRPS gene, two PKS-like genes, and 15 NRPS-like genes (Khaldi et al., 2010). This review will cover the most current knowledge of the genome mining of SMs produced by *A. terreus*. The review consists of three major sections: genome mining of PKS-derived natural products, genome mining of NRPS-derived natural products, and genome mining of hybrid PKS-NRPS derived natural products by *A. terreus*.

GENOME MINING OF PKS-DERIVED NATURAL PRODUCTS IN *A. TERREUS*

BIOSYNTHESIS OF LOVASTATIN IN *A. TERREUS*

Perhaps the most well-known molecule produced by *A. terreus* is lovastatin. Lovastatin is an inhibitor of the enzyme (3*S*)-hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase which reduces HMG-CoA to mevalonate. The biosynthesis of lovastain has been extensively reviewed in literature reports (Hill, 2006; Cox, 2007; Campbell and Vederas, 2010; Chiang et al., 2010; Chooi and Tang, 2012). Two highly reducing PKSs (HR-PKSs), LovB (lovastatin nonaketide synthase) and LovF, play critical roles in the formation of the lovastatin core structure (**Figure 1**). In the pathway, the HR-PKS LovB, together with the trans-acting enoyl reductase (ER) protein LovC, are responsible for the production of dihydromonacolin L acid (**1**). The release of **1** from LovB is catalyzed by a thioesterase LovG (Xu et al., 2013a). The oxidative conversion of **1** to monacolin J acid (**2**) involves LovA. The other HR-PKS LovF synthesizes the 2-methylbutyrate moiety of lovastatin (**3**) and the covalent attachment of this moiety to **2** is catalyzed by a transesterase, LovD (**Figure 2**). Recent efforts

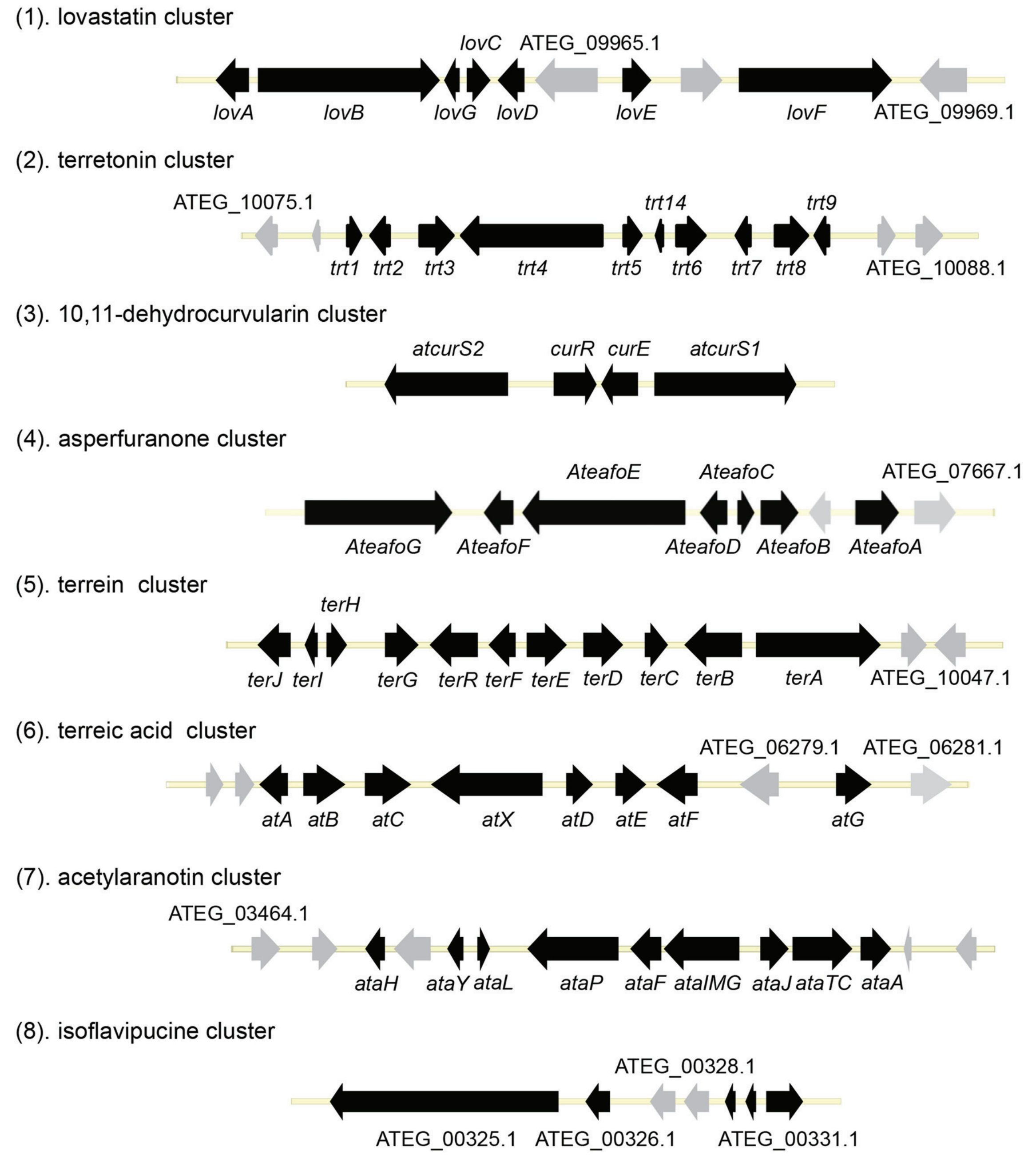


FIGURE 1 | Organization of the secondary metabolite gene clusters identified in *A. terreus*.

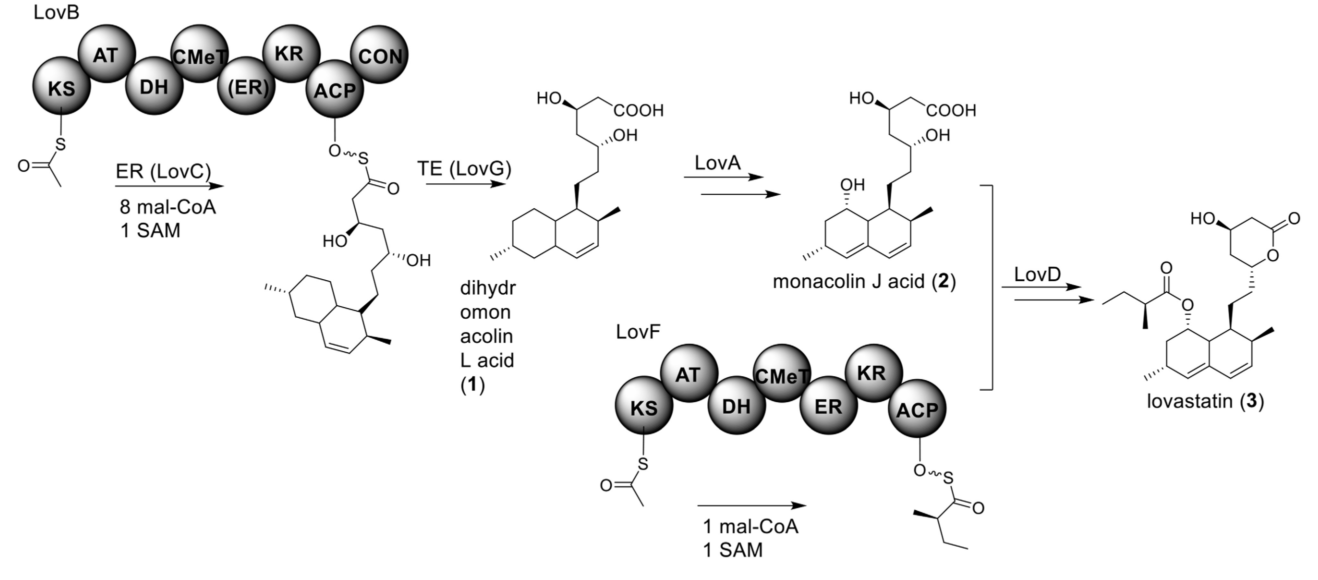


FIGURE 2 | Biosynthesis of lovastain in *A. terreus*.

in engineering LovD using a directed evolution strategy generated a mutant LovD containing 29 point mutations that greatly improves (~1000 fold) the protein's efficiency in the synthesis of the drug simvastatin (Jimenez-Oses et al., 2014), representing an excellent example of how the combination of synthetic biology and protein chemistry may facilitate drug discovery and production.

BIOSYNTHESIS OF TERRETONIN IN *A. TERREUS*

Terretonin is one type of meroterpenoids with mixed origins (**Figure 3**). Previous studies by Simpson and Vederas in the 1980's using isotopic labeled precursors have shown that terretonin originates from both polyketide and terpenoid pathways (McIntyre et al., 1982, 1989). Recent genome mining studies revealed that the terretonin cluster contains a total of 10 genes (Guo et al., 2012). The cluster includes a non-reducing PKS (NR-PKS) gene, *trt4* (ATEG_10080.1), and a prenyltransferase gene, *trt2* (**Figure 1**). Trt4 is responsible for the biosynthesis of 3,5-dimethylorsellinic acid (**4**) and Trt2 catalyzes the alkylation of compound **4** to give **5** (**Figures 1**, **3**) (Guo et al., 2012; Itoh et al., 2012). The following methylation (**5** to **6**), epoxidation (**6** to **7**) and cyclization (**7** to **8**) are proposed to be catalyzed by proteins encoded by *trt5*, *trt8*, and *trt1*, respectively (Guo et al., 2012; Matsuda et al., 2012). Interestingly, the Abe group discovered that the methylation step catalyzed by Trt5 is an essential step for cyclization (Matsuda et al., 2012). Upon formation of the tetracyclic precursor, Trt9 is proposed to oxidize compound **9** to **10** and Trt3 hydroxylases **10** to give **11** (**Figure 3**). Next, the putative protein Trt6 catalyzes the intra-lactonization reaction to give **12**, while the conversion of **12** to terretonin (**13**) might involve other enzymes that are not identified by Guo et al. (2012).

BIOSYNTHESIS OF 10,11-DEHYDROCURVULARIN IN *A. TERREUS*

The polyketide origin of the compound 10,11-dehydrocurvularin (**14**) was established by incorporation of stable isotope labeled precursors (Arai et al., 1989). The biosynthetic cluster for 10,11-dehydrocurvularin was identified in *A. terreus* AH-02-30-F7. The cluster is proposed to contain four genes, an HR-PKS gene (*Atcurs1*), an NR-PKS gene (*Atcurs2*), a GAL4-like transcription regulatory gene (*AtcursR*) and a major facilitator superfamily gene (*AtcursE*) (**Figure 1**) (Xu et al., 2013b). The biosynthesis of 10,11-dehydrocurvularin in *A. terreus* was characterized by heterologous expressing the HR-PKS and NR-PKS pair in *Saccharomyces cerevisiae* (Xu et al., 2013b). In the pathway, the HR-PKS AtCURS1 is proposed to synthesize the tetraketide 7(*S*)-hydroxyotc-2(*E*)-enoic acid (**Figure 4**). This tetraketide is then loaded onto the starter unit ACP transacylase domain (SAT) of AtCURS2, followed by four chain extension cycles to release the final product **14**. In the cluster, the gene *AtcursR* is predicted to encode for a fungal transcription regulator and *AtcursE* may code for an exporter involved in the transporting of 10,11-dehydrocurvularin (**14**) (Xu et al., 2013b). Further investigation of the product template (PT) domain of AtCURS2, which catalyzes the ring cyclization and aromatization, revealed that the cyclization mode of this domain could be reshaped from the bacterial folding mode, as shown in the biosynthesis of compound **14**, to the fungal mode by three selected point mutations (Xu et al., 2013c). Such rational control of fungal polyketide ring cyclization should facilitate the engineering of natural products with novel chemical scaffolds.

BIOSYNTHESIS OF ASPERFURANONE IN *A. TERREUS*

The azaphilone asperfuranone (**20**) was first isolated from *A. nidulans* after activating two PKSs encoded by *afoE* and *afoG* (Chiang et al., 2009). Later a highly homologous cluster was identified in the *A. terreus* genome (**Figure 1**) (Chiang et al., 2013). In this case, the individual genes from *A. terreus* were heterologously expressed in *A. nidulans* and the whole asperfuranone pathway was reconstituted. The asperfuranone cluster in *A. terreus* contains a total of seven genes including two PKS genes

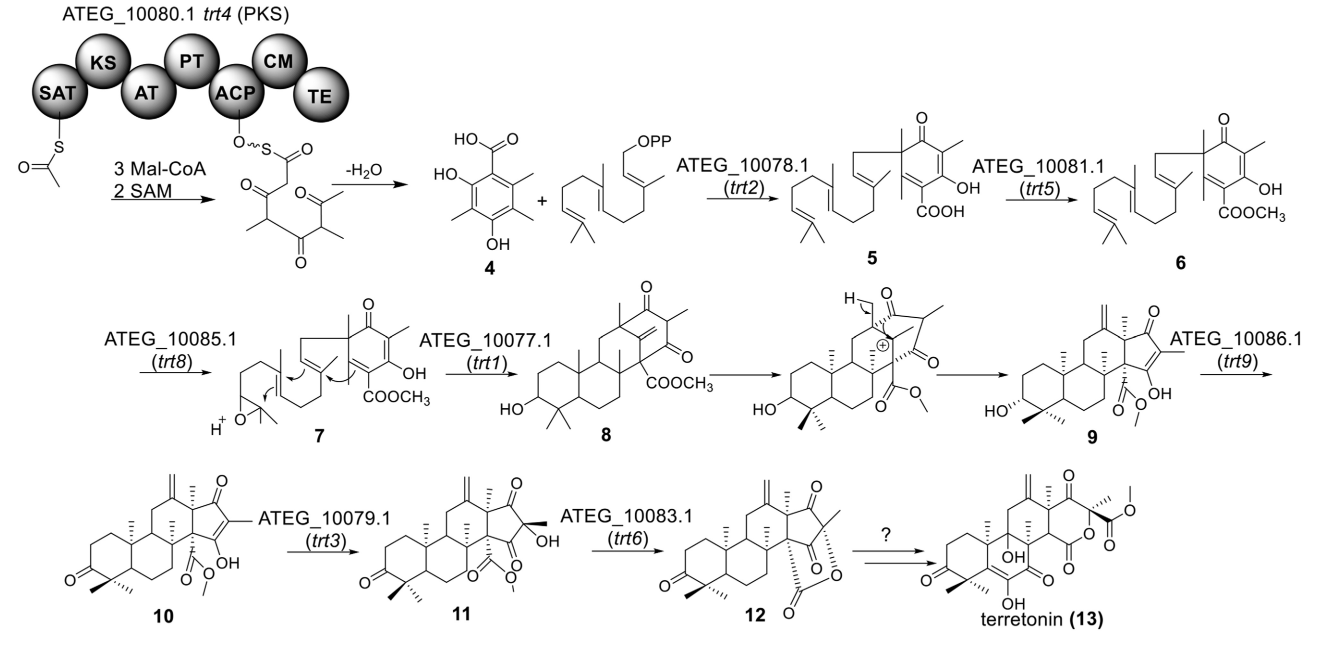


FIGURE 3 | Biosynthesis of terretonin in *A. terreus*.

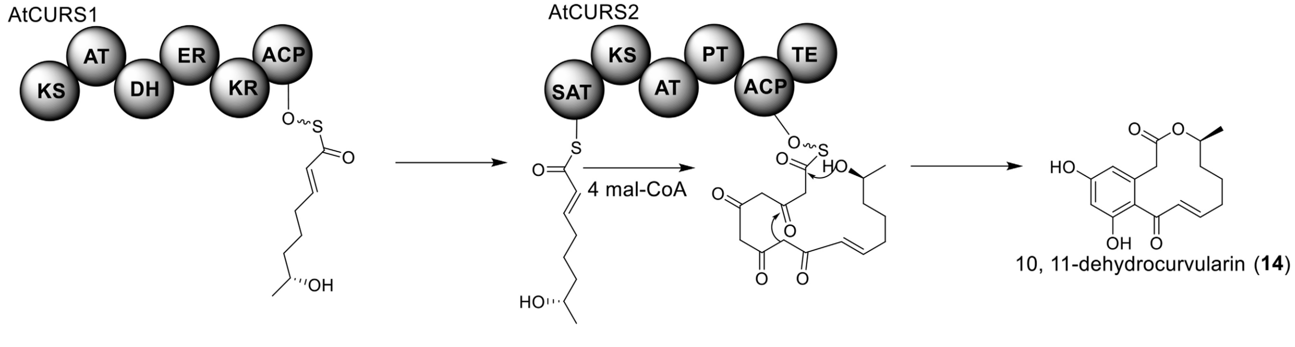


FIGURE 4 | Biosynthesis of 10,11-dehydrocurvularin in *A. terreus*.

ateafoE and *ateafoG* (**Figure 1**). In the pathway, the gene *AteafoG* encodes the HR-PKS that is responsible for the production of the PK intermediate **15.** The enzyme AteafoC might facilitate the transfer of the side chain **15** from AteafoG to AteafoE. Next, AteafoD catalyzes the dearomatization of the precursor **16** to give intermediate **17**, followed by hydroxylation by AteafoF to **18.** Last, the authors proposed the existence of an endogenous reductase in *A. nidulans* that catalyzes the conversion of **19** to asperfuranone (**20**) (**Figure 5**).

BIOSYNTHESIS OF TERREIN IN *A. TERREUS*

The terrein (**25**) cluster was serendipitously identified while the Brock group were initially looking for secondary metabolites that are involved in the biosynthesis of conidia pigment in *A. terreus* (Zaehle et al., 2014). Previous isotope-labeling studies revealed that the biosynthesis of terrein originates from a polyketide-based pathway (Birch et al., 1965) and involves the contraction of a six-membered ring precursor to give the five-membered ring terrein (**25**) (Hill et al., 1981). The terrein cluster contains a total of 11 genes, including an NR-PKS gene *terA* (ATEG_00145.1) and a transcription factor gene, *terR* (**Figure 1**). In the terrein biosynthetic pathway as shown in **Figure 6**, the NR-PKS encoded by *terA* condensates one acetyl-CoA and four malonyl-CoA to synthesize the precursor 2,3-dehydro-6-hydroxymellein (**23**). Heterologous expression of *terA* in *A. niger* revealed that the NR-PKS TerA is also capable of producing compounds **21** and **22** by incorporating different number of malonyl-CoAs as substrates. The reduction of compound **23** to 6-hydroxymellein (6-HM) (**24**) is probably catalyzed by TerB, but it is also possible that other unspecific ketoreductases may perform this reduction. The authors proposed that the conversion of 6-HM (**24**) to the final product terrein (**25**) might involve four genes, *terC*, *terD*, *terE*, and *terF* while the specific roles of these genes await further verification. Interestingly, the study also revealed that

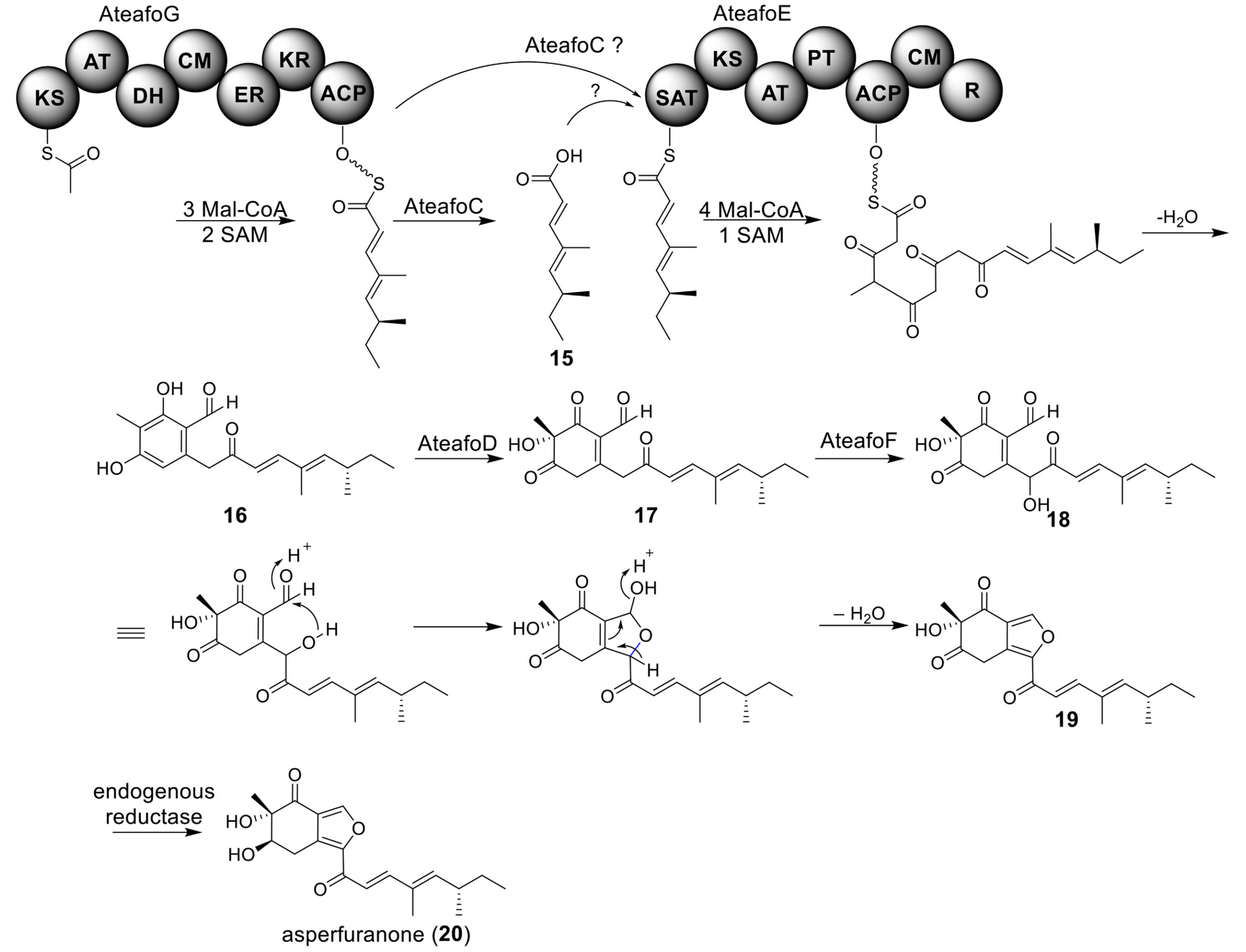


FIGURE 5 | Biosynthesis of asperfuranone in *A. terreus*.

terrein (**25**) has phytotoxic activity. In the two experiments, terrein (**25**), rather than the intermediates or shunt products, inhibited root elongation and caused lesions on fruit surface (Zaehle et al., 2014), representing a prime example of how fungal secondary metabolites might play a role in fungus-plant interactions.

BIOSYNTHESIS OF TERREIC ACID IN *A. TERREUS*

Terreic acid (**30**) is a natural product isolated from *A. terreus* with anti-bacterial activity (Yamamoto et al., 1980). A later study recognized its inhibitory effect against Bruton's tyrosine kinase (Btk), and this compound has been used as a chemical probe to examine the function of Btk (Kawakami et al., 1999). Previous research using isotope-labeled precursors has shown that the biosynthesis of terreic acid originated from one polyketide, 6-methylsalicylic acid (6-MSA, **26**) (Read and Vining, 1968; Read et al., 1969). The PKS gene *atX* was cloned and identified as a 6-MSA (**26**) synthase (Fujii et al., 1996). A recent bioinformatic analysis study predicts that the PKS gene *atX* and its surrounding genes encodes the biosynthetic cluster which is responsible for terreic acid biosynthesis (Boruta and Bizukojc, 2014). The cluster was experimentally characterized using a targeted gene deletion method (Guo et al., 2014). The cluster contains a total of eight genes, including a partially reducing PKS (PRPKS) gene, *atX*, and a transcription factor gene *atF* (**Figure 1**). The pathway starts with the formation of 6-MSA (**26**) by AtX using one acetyl-CoA and three malonyl-CoA units as its substrates, followed by a decarboxylative hydroxylation catalyzed by AtA to give **27**. Compound **27** could be degraded to a shunt product, (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid. The degradation of **27** may be catalyzed by a putative catechol 1,2-dioxygenase in *A. terreus*, but the coding gene for this enzyme is not identified in the terreic acid cluster (Guo et al., 2014). The hydroxylation of **27** to give **28** is possibly catalyzed by AtE. The oxidative conversion of **28** to **29** might involve AtG, but this proposal is not confirmed in the study. Last, AtC catalyzes the oxidation of compound **29** to give the final product terreic acid (**30**) (**Figure 7**).

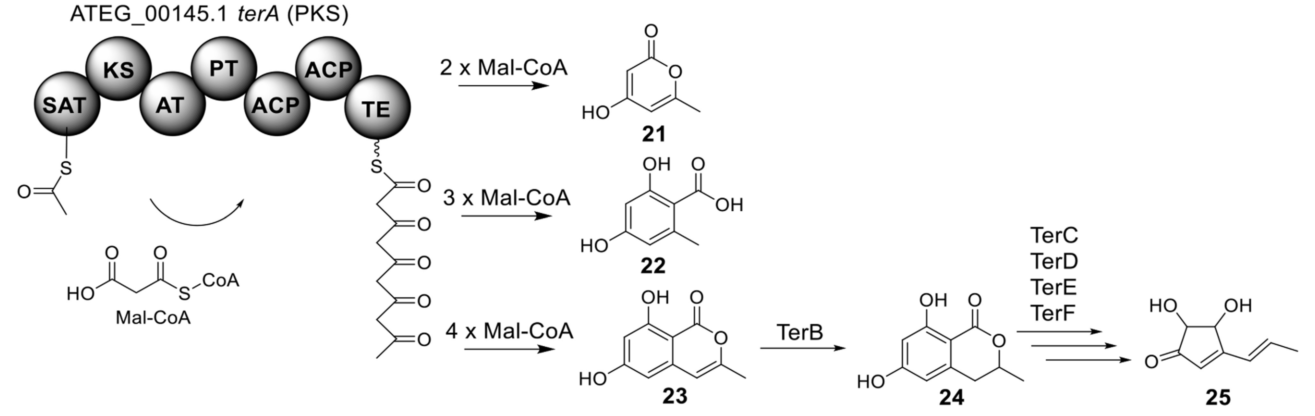


FIGURE 6 | Biosynthesis of terrein in *A. terreus*.

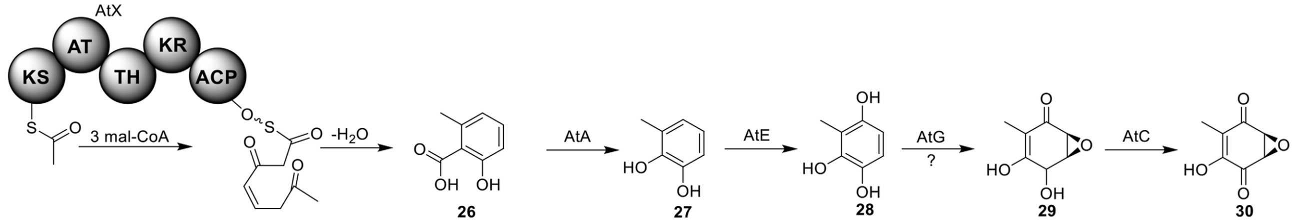


FIGURE 7 | Biosynthesis of terreic acid in *A. terreus*.

IDENTIFICATION OF THE PRODUCTS OF SIX NR-PKS GENES IN *A. TERREUS*

The previous studies successfully linked several PKS-derived SMs with their biosynthetic clusters in *A. terreus*. Using a heterologous expression strategy, researchers also identified the products of six NR-PKS genes in *A. terreus* (Chiang et al., 2013). The products of these NR-PKSs are shown in **Table 1**. The NR-PKS encoded by ATEG_00145.1 synthesizes a mixture of compounds, **21**, **22**, **23**, and **31**. The plasticity of ATEG_00145.1 is also demonstrated in the study of terrein biosynthesis (Zaehle et al., 2014). The products of the NR-PKS, encoded by ATEG_03432.1, are compounds **32** and **33**, and the product of the ATEG_03629.1 encoded NR-PKS is **34**. The NR-PKS atrochrysone carboxylic acid synthase (ACAS) encoded by ATEG_08451.1 produces atrochrysone **35** and its derivative emodin **36** with the aid of a β-lactamase encoded by ATEG_08450.1. This result matches the previous finding reported by Awakawa et al. (2009) As expected, ATEG_10080.1 (*trt4*) synthesizes and releases the PKS product 3,5-dimethylorsellinic acid (**4**), which is presumed to be the first precursor in the biosynthesis of terretonin (**Figure 3**) (Guo et al., 2012; Itoh et al., 2012). As aforementioned, the products of the NR-PKS AteafoE and its adjacent HR-PKS AteafoG incorporate into the asperfuranone pathway as shown in **Figure 5** (**Table 1**).

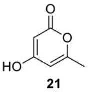
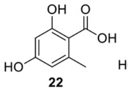
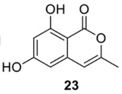
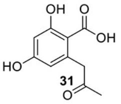
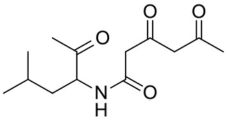
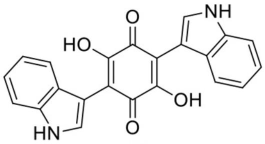
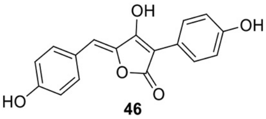
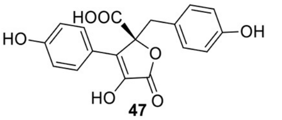
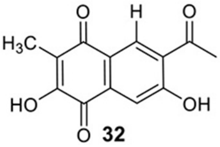
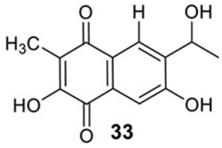
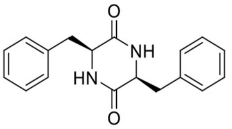
BIOSYNTHESIS OF NRPS-DERIVED NATURAL PRODUCTS IN *A. TERREUS*

BIOSYNTHESIS OF ACETYLARANOTIN IN *A. TERREUS*

Acetylaranotin belongs to one group of SM toxins named epipolythiodioxopiperizine (ETP). This type of natural products is usually featured by the presence of unique di- or poly-sulfide bridges. The di- or poly-sulfide bridges are presumed to mediate the molecule's cytotoxicity, either by cross-linking vital proteins via cysteine bonds, or by generating reactive oxygen species via redox cycling (Scharf et al., 2011a). An early study using stable isotope-labeled precursors showed that the diketopiperazine (DKP) precursor, compound **37**, could be incorporated intact into acetylaranotin (**44**) (Boente et al., 1981). This indicates that the acetylaranotin pathway may include an NRPS that catalyzes the condensation of two L-phenylalanines to give **37**. Bioinformatic analysis of the ETP clusters dispersed in filamentous ascomycetes uncovered two putative clusters in *A. terreus* that might encode the biosynthetic pathway for acetylaranotin (Patron et al., 2007).

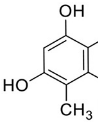
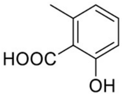
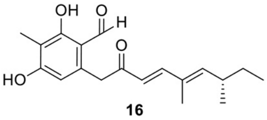
Using the gene targeted deletion strategy, one of the two clusters, named *ata* cluster, was found to be responsible for acetylaranotin production in *A. terreus* (Guo et al., 2013b). The *ata* cluster contains a total of nine genes, including one NRPS gene, *ataP* (**Figure 1**). In the pathway, the NRPS AtaP catalyzes the condensation of two phenylalanines to give compound **37**. Next, the C domain of the protein AtaTC is proposed to catalyze the dual hydroxylation of compound **37** to **38**. A series of studies have fully elucidated the functions of the enzymes that are involved in the installation of the disulfide moiety in gliotoxin (Scharf et al., 2010, 2011b, 2012, 2013; Schrettl et al., 2010; Davis et al., 2011; Gallagher et al., 2012). Similar to their homologs in the gliotoxin cluster, AtaG, AtaJ, AtaI are involved in the conversion of the dual hydroxyl groups in **38** to thiol groups in **39**, followed by the AtaT-mediated

Table 1 | The core synthesis genes and their products identified in *A. terreus*.

Genes	Name	Type	Putative domain architecture	Released product	Downstream metabolites
ATEG_00145.1	*terA*	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	 21  22  23  31	Terrein
ATEG_00228.1		NRPS	Multidomains		
ATEG_00282.1		HR-PKS	KS-AT-DH-KR-ACP-C		
ATEG_00325.1		PKS-NRPS hybrid	KS-AT-(DH)-(CMeT)-(KR)-ACP-C-A-T-R/C	 49	Isoflavipucine
ATEG_00700.1	*atqA*	NRPS-like	A-T-TE	 45	Asterriquinones
ATEG_00881.1		NRPS	A-T-C		
ATEG_00913.1		Undefined	KS-AT		
ATEG_01002.1		NRPS	A-T-C-C-A-T-C-A-T-C-A-T-R		
ATEG_01052.1		NRPS-like	A-T-R		
ATEG_01894.1		HR-PKS	KS-AT-DH-ER-KR-ACP		
ATEG_02004.1	*apvA*	NRPS-like	A-T-TE	 46	Aspulvinones
ATEG_02403.1		NRPS-like	A-T-R-KR		
ATEG_02434.1		HR-PKS	KS-AT-DH-ACP		
ATEG_02815.1	*btyA*	NRPS-like	A-T-TE	 47	Butyrolactones
ATEG_02831.1		NRPS	A-T-C		
ATEG_02944.1		NRPS	C-A-C-A-T-C		
ATEG_03090.1		NRPS-like	A-T		
ATEG_03432.1		NR-PKS	SAT-KS-AT-PT-ACP-CMeT-R	 32  33	
ATEG_03446.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP		
ATEG_03470.1	*ataP*	NRPS	T-C-A-T-C	 37	Acetylaranotin

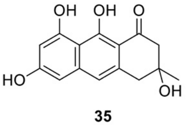
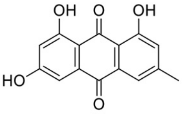
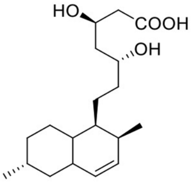
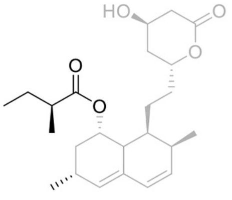
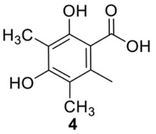
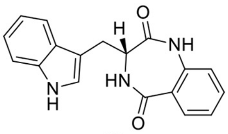
(Continued)

Table 1 | Continued

Genes	Name	Type	Putative domain architecture	Released product	Downstream metabolites
ATEG_03528.1		NRPS	A-T-C-A-T-C		
ATEG_03576.1		NRPS	C-A-T-C-A-T-C		
ATEG_03629.1		NR-PKS	SAT-KS-AT-PT-ACP-ACP-CMeT-TE	 **34**	
ATEG_03630.1		NRPS-like	A-T-R	Reduce **34** to its aldehyde form	
ATEG_04322.1		NRPS	C-A-T-C		
ATEG_04323.1		NRPS	A-T-C-C-A		
ATEG_04718.1		HR-PKS	KS-AT-DH-KR-ER-KR-ACP		
ATEG_04975.1		NRPS-like	A-T-R		
ATEG_05073.1		NRPS	A-T-C-A-T-C-T-C-T-C		
ATEG_05795.1		NRPS-lke	A-T-R		
ATEG_06056.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP		
ATEG_06113.1		NRPS	A-T-C-C-A-T-C		
ATEG_06206.1		Undefined	KS-AT		
ATEG_06275.1	*atX*	PRPKS	KS-AT-TH-KR-ACP	 **26**	Terreic acid
ATEG_06680.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP		
ATEG_06998.1		NRPS-like	A-T-R		
ATEG_07067.1		HR-PKS	KS-AT-DH-ER-KR-ACP		
ATEG_07279.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP		
ATEG_07282.1		HR-PKS	KS-AT-DH-CMeT-KR-ER-KR-ACP		
ATEG_07358.1		NRPS	A-T-C-A-T-C-T		
ATEG_07379.1		HR-PKS	KS-AT-DH-ACP-ACP-TE		
ATEG_07380.1		NRPS-like	A-T-R		
ATEG_07488.1		NRPS	A-T-C-A-T-C		
ATEG_07500.1		HR-PKS	KS-AT-DH-ACP-ACP-TE		
ATEG_07659.1	*AteafoG*	HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP	 **16**	Asperfuranone
ATEG_07661.1	*AteafoE*	NR-PKS	SAT-KS-AT-PT-ACP-CMeT-R		Asperfuranone
ATEG_07894.1		NRPS-like	A-T-R		
ATEG_08172.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP		
ATEG_08427.1		NRPS	A-C-A-T-C-T		

(Continued)

Table 1 | Continued

Genes	Name	Type	Putative domain architecture	Released product	Downstream metabolites
ATEG_08451.1		NR-PKS	SAT-KS-AT-PT-ACP	 **35**  **36**	
ATEG_08662.1		NR-PKS	SAT-KS-AT-ACP-CMeT-R		
ATEG_08678.1		NRPS-like	A-T-R		
ATEG_08899.1		NRPS-like	A-T-TE		
ATEG_09019.1		NRPS	C-A-T-C-C-T-A-T-C-A-T-C-A-T-C		
ATEG_09033.1		NRPS-like	A-T-R		
ATEG_09064.1		NRPS	A-T-C-A-T-C		
ATEG_09068.1		NRPS	C-A-T-C-A-T-R		
ATEG_09088.1		HR-PKS	KS-AT-DH-CMeT-ER-KR		
ATEG_09100.1		HR-PKS	KS-AT-DH-CMeT-ER-KR-R		
ATEG_09142.1		NRPS-like	A-T-R		
ATEG_09617.1		HR-PKS	KS-AT-DH-CMeT-KR-ACP		
ATEG_09961.1	*lovB*	HR-PKS	KS-AT-DH-CMeT-(ER)-KR-ACP-CON	 **1**	lovastatin
ATEG_09968.1	*lovF*	HR-PKS	KS-AT-DH-CMeT-ER-KR-ACP	 lovastatin (**3**)	lovastatin
ATEG_10080.1	*trt4*	NR-PKS	SAT-KS-AT-PT-ACP-CMeT-TE	 **4**	Terretonin
ATEG_10305.1	*anaPS*	NRPS	A-T-C-A-T-C	 **48**	Epi-aszonalenins

Domains shown in bracket are supposed to be non-functional.

oxidation to form the transannular disulfide bridge as shown in intermediate **40**. Next, AtaF catalyzes the dual epoxidation of **40** followed by the spontaneous nucleophilic attack of the amide nitrogens to yield intermediate **41**. The acetylation of **41** to **42** is catalyzed by AtaH, and the oxepine ring formation involves the protein AtaY to yield acetylaranotin **44** (**Figure 8**). The proteins AtaH and AtaY can function independently, but the detailed catalytic mechanism of AtaY still awaits further investigation. Two recent studies unveiled that the *S*-alkylation of gliotoxin was catalyzed by a SAM dependent methyltransferase encoded by a gene *tmtA/gtmA* outside the gliotoxin cluster (Dolan et al., 2014; Scharf et al., 2014). These studies suggest that the *S*-methylation of acetylaranotin to give bisdethiobis(methylthio)acetylaranotin is likely to be catalyzed by a methyltransferase encoded by a gene outside the acetylaranotin cluster.

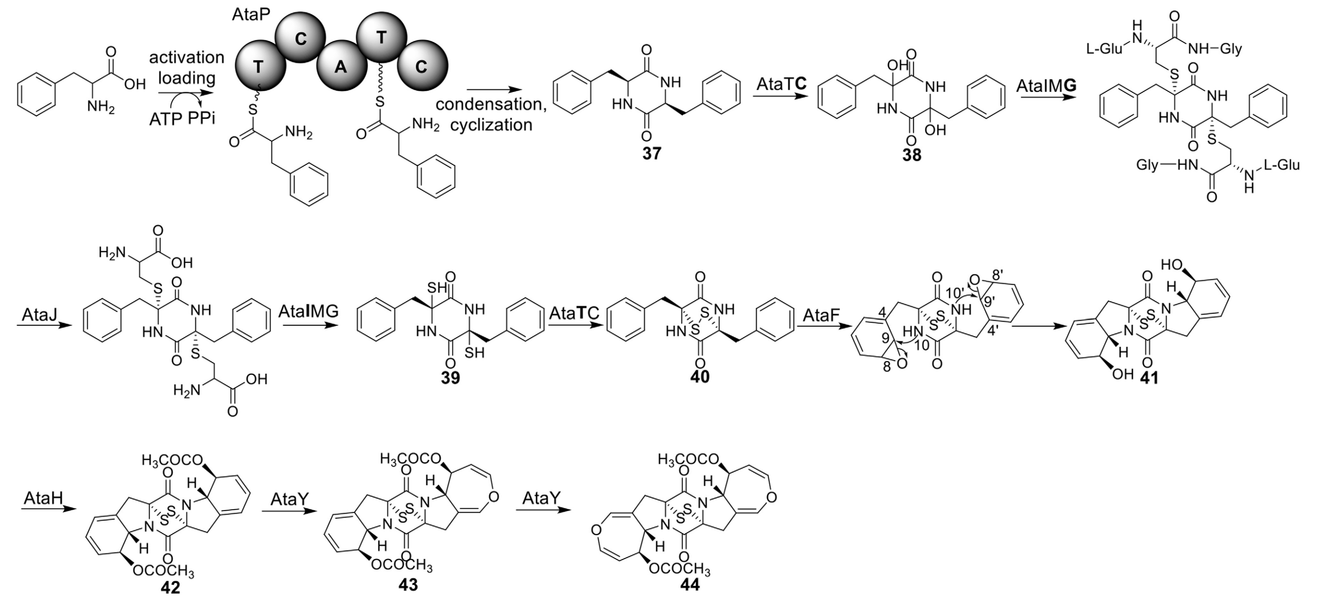


FIGURE 8 | Biosynthesis of acetylaranotin in *A. terreus*.

IDENTIFICATION OF THE PRODUCTS OF FOUR NRPS OR NRPS-LIKE GENES IN *A. TERREUS*

Screening of the genome-sequenced strain *A. terreus* NIH 2624 showed that this fungus is able to biosynthesize four main types of secondary metabolites including aspulvinones, butyrolactones, asterriquinones, and aszonalenins (Guo et al., 2013a). The NRPS-origin of these four types of natural products was revealed in previous studies (Kiriyama et al., 1977; Nitta et al., 1983; Balibar et al., 2007; Yin et al., 2009). To link these molecules to their core synthesis NRPS genes, a mutant library was created by individually deleting a total of 21 NRPS or NRPS-like genes in *A. terreus*. Screening of the mutants' SM profiles, in comparison with that of the wild type, showed that three NRPS-like genes *atqA* (ATEG_00700.1), *apvA* (ATEG_02004.1), and *btyA* (ATEG_02815.1), are responsible for the synthesis of the core structures of asterriquinones, aspulvinones, and butyrolactones, respectively (**Table 1**) (Balibar et al., 2007; Guo et al., 2013a). The NRPS gene *anaPS* encoded by ATEG_10305.1 is proposed to biosynthesize the (R)-benzodiazepinedione core (**Table 1**) (Yin et al., 2009; Guo et al., 2013a). Serendipitously, deletion of one NRPS-like gene *atmelA* (ATEG_03563.1) generates an albino mutant that abolishes the brown conidia melanin in *A. terreus*. It is possible that AtmelA synthesizes the first precursor that incorporates into the conidia pigment produced by *A. terreus*. Since all the identified conidial melanins in other *Aspergillus* species are derived from a PKS pathway, another possibility is that this NRPS-like gene might be involved, but not directly related in the melanin biosynthesis of *A. terreus* (Guo et al., 2013a).

AN NRPS-LIKE GENE IS INVOLVED IN ACTIVATING AND REDUCING OF AN ARYL ACID RELEASED FROM AN NR-PKS

Zhao group recently reported that an NRPS-like protein encoded by ATEG_03630.1 could catalyze the reduction of compound **34** to an aryl aldehyde (Wang et al., 2014). Compound **34** is released by its adjacent NR-PKS ATEG_03629.1, The putative domain architecture of the NRPS-like protein is A-T-R. The aryl acid product **34**, upon its release from the TE domain of the NRPKS, is then loaded onto the A domain. And the R domain is responsible for the reduction of the aryl acid **34** to its aldehyde form (Wang et al., 2014). Interestingly, a subsequent study revealed that the substrate of the A domain of this NRPS-like enzyme could be engineered to anthranilate via bioinformatic analysis and mutagenesis (Wang and Zhao, 2014).

BIOSYNTHESIS OF HYBRID PKS-NRPS NATURAL PRODUCTS IN *A. TERREUS*

BIOSYNTHESIS OF ISOFLAVIPUCINE IN *A. TERREUS*

Only one hybrid PKS-NRPS gene, ATEG_00325.1, can be identified in the genome of *A. terreus* NIH 2624. The Brock group identified that the PKS-NRPS encoded by this hybrid gene is responsible for the core synthesis of isoflavipucine and dihydroisoflavipucine (Gressler et al., 2011). RT–PCR analysis of expression of the hybrid gene and its surrounding genes, under isoflavipucine producing condition, suggested that the cluster for isoflavipucine biosynthesis might contain five genes, ATEG_00325.1, ATEG_00326.1 (transcription factor gene), ATEG_00329.1, ATEG_00330.1, and ATEG_00331.1 (**Figure 1**) (Gressler et al., 2011). In the pathway, the hybrid PKS-NRS synthesizes and releases the linear precursor (**49**). The conversion of the precursor **49** to final product includes heterocyclization (**49** to **50**), oxidation (**50** to **51**), transamination and ring arrangement (**51** to **52**), and epoxidation to give flavipucine (**53**) (**Figure 9**). However, the enzymes that are responsible for the conversion of the linear precursor **45** to isoflavipucine (**54**) are not identified in this study and needs further investigation.

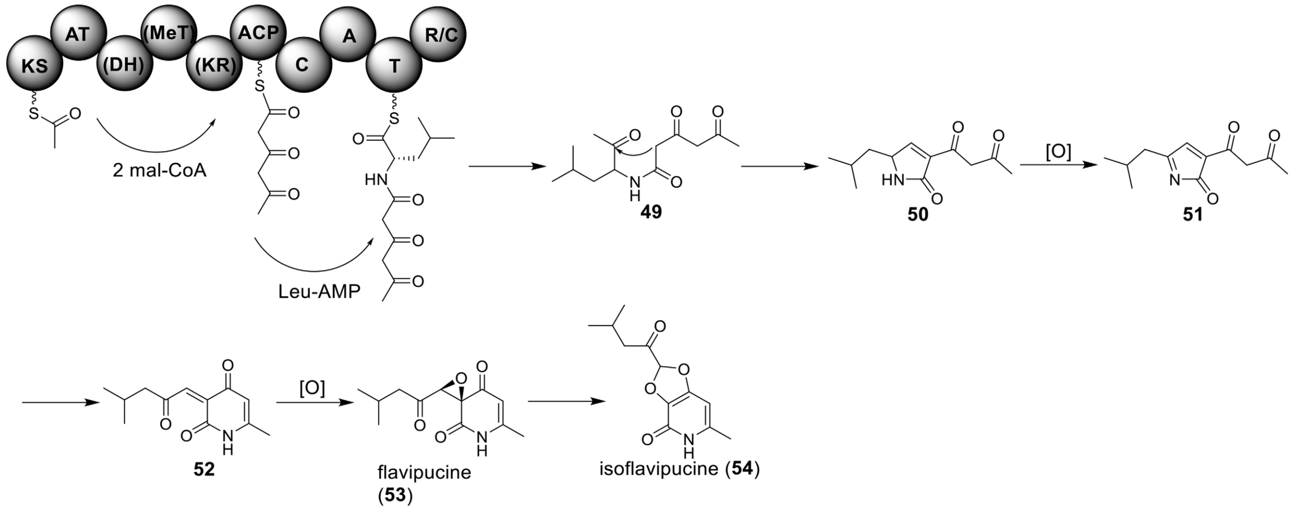


FIGURE 9 | Biosynthesis of flavipucine in *A. terreus*.

CONCLUSION

In the fungus *A. terreus*, most of the genome mining experiments are carried out in the strain NIH 2624 because it is the only *A. terreus* strain in which the genome sequence is publicly available. A combination of bioinformatic analysis and experimental verification have enabled researchers to elucidate the functions of genes and proteins that are involved in the biosynthesis of several SMs produced in *A. terreus*. However, as shown in **Table 1**, there are still many other SM genes or gene clusters in *A. terreus* whose products still remain elusive. With the advance of genome sequencing and manipulation techniques, an increasing amount of effort will focus on deciphering the products of these silent or cryptic genes or gene clusters, as well as engineering the characterized pathways to produce second generation molecules with improved bioactivities.

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Fungal secondary metabolite dynamics in fungus–grazer interactions: novel insights and unanswered questions

Marko Rohlfs*

Johann-Friedrich-Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Göttingen, Germany

Edited by:
Ozgur Bayram, National University of Ireland Maynooth, Ireland

Reviewed by:
Jeffrey William Cary, United States Department of Agriculture – Agricultural Research Service, USA
Frank Kempken, Christian-Albrecht Kiel University, Germany

****Correspondence:***
Marko Rohlfs, Johann-Friedrich-Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Berliner Strasse 28, 37073 Göttingen, Germany
e-mail: mrohlfs@gwdg.de

In response to fungivore grazing fungi are assumed to have evolved secondary metabolite-based defense mechanisms that harm and repel grazers, and hence provide a benefit to the metabolite producer. However, since research into the ecological meaning of highly diverse fungal secondary metabolites is still in its infancy, many central questions still remain. Which components of the enormous metabolite diversity of fungi act as direct chemical defense mechanisms against grazers? Is the proposed chemical defense of fungi induced by grazer attack? Which role do volatile compounds play in communicating noxiousness to grazers? What is the relative impact of grazers and that of interactions with competing microbes on the evolution of fungal secondary metabolism? Here, I briefly summarize and discuss the results of the very few studies that have tried to tackle some of these questions by (i) using secondary metabolite mutant fungi in controlled experiments with grazers, and by (ii) investigating fungal secondary metabolism as a flexible means to adapt to grazer-rich niches.

Keywords: *Aspergillus*, chemical defense regulation, fungivory, inducible resistance, secondary metabolites

INTRODUCTION

Fungal metabolism is an important source of an apparently endless diversity of organic compounds which are not obviously required for the producer's normal growth and metabolism – one reason why they are called secondary metabolites (SMs). This chemical diversity raises a pertinent yet hardly addressed basic research question in fungal biology: why are SMs produced in such variety, especially since the SM biosynthesis is encoded in clusters of genes whose transcriptional activation is embedded in a tightly regulated network comprising the activity of signaling pathways, epigenetic regulators, fungal hormones, and cellular secretion processes (Tsitsigiannis and Keller, 2007; Roze et al., 2011; Brakhage, 2013)?

Because many fungal SMs are toxic to other organisms, in particular animals, one line of arguments follows the idea that fungi produce specific SMs as direct defense compounds to achieve protection from grazers foraging for nutrient-rich food sources (e.g., Janzen, 1977; Martin, 1979; Camazine et al., 1983; Vining, 1990; Gloer, 1995; Spiteller, 2008). Putative direct defense compounds have emerged primarily due to pharmacological evidence for their deleterious effects on grazers. This evidence stems from studies that report relationships between fungivore mortality, decreased fecundity, and feeding on purified compounds mixed into artificial diets or on fungi expressing the proposed chemical defense trait constitutively (e.g., Wright et al., 1980; Paterson et al., 1987; Panigrahi, 1993; Gloer, 1995; Rohlfs and Obmann, 2009). Such studies provide at best weak correlative evidence as they do not evaluate whether these compounds increase fungal fitness under grazing pressure, which is the defining criterion of a chemical compounds-based defense.

In a series of recent studies (see below), the use of genetically modified fungi manipulated in the expression of candidate defense gene products has corroborated the relationship between SM regulatory mechanisms and resistance against grazers, yet manipulation of pathway-specific defense products have revealed conflicting results. Moreover, first studies have demonstrated complex chemical changes in fungi after fungivore attack, which could in part be related to changes in the capacity to resist grazing. The present review intends to provide a critical discussion of these recent findings and stimulate more research into the ecological causes and consequences of fungal chemical diversity.

INVESTIGATING FUNGAL RESISTANCE WITH GENETICALLY MANIPULATED FUNGI

Since more than a decade, functional genetic approaches have provided unparalleled insights into how fungi regulate their chemical diversity. Despite the availability of an increasing number of well–controlled mutant fungi, functional evaluations have largely been restricted to biochemical and medical aspects of fungal biology. Very few studies have used genetically modified fungi to conduct functional analyses of SM genes in the context of interactions with grazers.

GLOBAL SM REGULATION

Six of these studies focused on *Aspergillus* spp. *laeA* loss-of-function mutants in interactions with different soil arthropods, Collembola, (Rohlfs et al., 2007; Janssens et al., 2010; Staaden et al., 2011; Stötefeld et al., 2012) or facultative fungivorous *Drosophila melanogaster* larvae (Trienens et al., 2010; Caballero Ortiz et al., 2013). The methyltransferase-domain protein LaeA is essential for both appropriate developmental processes coupled with the biosynthesis of various SMs, including mycotoxins, such as sterigmatocystin (Bok and Keller, 2004; Sarikaya Bayram et al., 2010). Chemical deficient Δ*laeA Aspergillus* sp. mutants generally are less detrimental to grazer fitness than wild type fungi (reduced mortality, enhanced reproductive output) and, when given the choice, fungivores prefer grazing on the Δ*laeA* mutants. The latter appears to be mediated by a combined effect of differences in both the non-volatile SM and the volatile chemical profile (Staaden et al., 2011; Stötefeld et al., 2012). The LaeA-dependent capacity to harm insect grazers seems to be fungal species-specific though: while Δ*laeA Aspergillus nidulans* almost entirely lost the potential to kill fruit fly *D. melanogaster* larvae, Δ*laeA A. flavus* and Δ*laeA A. fumigatus* were still fatal to the insects, yet the onset of larval mortality was significantly delayed (Trienens et al., 2010). Importantly, compared to wild type, the lack of expression of *laeA* in all fungi tested increased their susceptibility to grazer damage (Rohlfs et al., 2007; Trienens et al., 2010; Stötefeld et al., 2012).

Loss of the capacity to kill fruit fly larvae was also evident for *A. nidulans* impaired in the expression of VeA (Trienens and Rohlfs, 2012), which indicates that an intact velvet (VelB-VeA-LaeA) complex (Bayram et al., 2008) is required for building resistance against grazers. Therefore, global regulatory mechanisms controlling both SM diversity and development have a distinct function in mediating protection from fungivore attack, and I would predict a similar role of these mechanisms in fungi beyond *Aspergillus*.

PATHWAY-SPECIFIC SM REGULATION

A review of the literature indicates that there are only five published studies that have used pathway-specific mutant strains to test for the influence of single SMs on the outcome of interactions with fungivores (Scheu and Simmerling, 2004; Staaden et al., 2010; Trienens and Rohlfs, 2012; Yin et al., 2012; Cary et al., 2014). As demonstrated by the use of knock-out mutants in feeding assays with Collembola, *Folsomia candida* and *Protaphorura armata*, and the nitidulid beetle *Carpophilus freemani*, polyketide synthase-driven formation of pigments dihydroxynaphthalene melanin in *A. fumigatus* conidia and asparasone A in sclerotia of *A. flavus* proved to impair or deter grazers, respectively, (Scheu and Simmerling, 2004; Cary et al., 2014). The latter is of particular interest because it is the first demonstration of a grazer-deterring compound whose biosynthesis is confined to fungal tissue that has a special function in ensuring survival under unfavorable conditions. However, how such pigments contribute to directly repelling grazers is unknown.

The polyketide sterigmatocystin is a characteristic mycotoxin of *A. nidulans*, which has insecticidal properties (Chinnici et al., 1983; Rohlfs and Obmann, 2009). In accordance with the proposed role of sterigmatocystin in mediating resistance against grazers, Collembola avoided feeding on a mutant *A. nidulans* strain over-expressing the bZIP transcription factor gene, *rsmA*, which results in a great increase of sterigmatocystin (Yin et al., 2012). RsmA activates the C6 transcription factor AflR, the ST pathway-specific regulatory factor required for transcriptional activation of ST biosynthetic genes. One would expect a benefit to grazers when exposed to *A. nidulans* deficient in the formation of sterigmatocystin. Interestingly, this hypothesis was not supported by Trienens and Rohlfs (2012). Compared with mortality in the presence of sterigmatocystin-producing wild type *A. nidulans*, *Drosophila* larvae did not demonstrate increased survival when confronted with 1-day old *A. nidulans* mutants (Δ*aflR*, Δ*stcJ*, Δ*stcE*, Δ*stcU*) incapable of producing sterigmatocystin. To our surprise, when the insects were exposed to initially 2-days old colonies, larvae suffered even higher mortality on substrate infested with the sterigmatocystin deficient mutant strains (Trienens and Rohlfs, 2012). A similar grazer response was observed when *F. candida* was offered a sterigmatocystin deficient Δ*aflR A. nidulans* mutant: relative to the wild type strains (Rohlfs et al., 2007), the Collembola suffered unusually high mortality and did not reproduce whatsoever (Albert, 2007). Thus, even though an artificial increase in sterigmatocystin biosynthesis enhances the capacity of *A. nidulans* to resist grazing, the loss of this compound does not reduce resistance as one would expect.

In conclusion, sterigmatocystin does not seem to be the major anti-grazer compound in wild type *A. nidulans*. In contrast, sterigmatocystin biosynthesis appears to hamper the ability of *A. nidulans* to develop even better protection against grazers. In search for an explanation of this phenomenon, one could argue that there are only a few key intermediates of the basic metabolic pathways that provide the starting points for the SM pathways. For example, acetyl-CoA is the precursor molecule for compound biosynthesis from the polyketide and the isoprenoid pathway. Possibly, artificial inactivation of the sterigmatocystin pathway leads to higher amounts of acetyl-CoA available for shunting into other pathways which produce more efficient anti-grazer compounds. If this turns out to be an adequate explanation for sterigmatocystin deficient *A. nidulans* being more detrimental to grazers than the wild type, fungi may suffer ecological costs of (high) SM diversity. And perhaps global, e.g., LaeA-dependent, regulatory mechanisms might prove to constrain fungi in activating the optimal combination of pathways for conquering grazers with a less diverse but more effective blend of SMs.

RESPONSES OF FUNGI TO GRAZERS

It has been well appreciated that fungal chemical phenotypes can vary with abiotic conditions such as light, water, temperature or the availability of nutrients (Schmidt-Heydt et al., 2008; Atoui et al., 2010; Schmidt-Heydt et al., 2010; Nielsen et al., 2011). Also, interactions with bacteria have been established as critical determinants of fungal SM composition (Brakhage and Schroeckh, 2011). Some recent analyses of the chemical responses to animal grazers provide first evidence of an inducible chemical compound-based defense response in fungi, which comprises the biosynthesis of so-called cryptic metabolites.

EVIDENCE FROM *Aspergillus nidulans*

Grazing by Collembola, *F. candida*, was found to induce an increase in the formation of sterigmatocystin, some meroterpenoids (mixed polyketide/terpenoid orgin) and emericellamides (mixed polyketide/peptide origin) in *A. nidulans* (Döll et al., 2013). In choice experiments, Collembola preferred un-grazed colonies to grazed, probably due to changes in the volatiles released by damaged colonies (Staaden et al., 2011), and when forced to feed

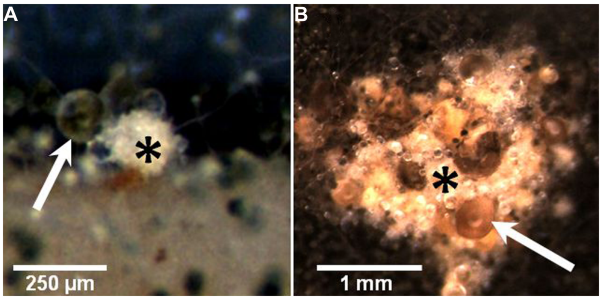

FIGURE 1 | Images depicting the localized formation of guttation droplets on the sexual fruiting bodies (cleistothecia) of *A. nidulans,* which are possibly involved in mediating protection from grazing by Collembola (see Döll et al., 2013). (A) To the left of the initial stage (primodium) of a cleistothecium (*asterisk*) a strikingly large droplet (*arrow*) is formed in addition to some smaller ones. The whitish appearance of the primordium is due to layers of so-called Hülle cells which are assumed to nurse and protect the developing ascospores within the cleistothecium. It is not obvious whether the guttation droplets are produced by the Hülle cells or aerial hyphae surrounding the fruiting bodies. **(B)** A cluster of larger cleistothecia (*asterisk*), surrounded by a dark mat of conidia-producing tissue. The cluster is covered by voluminous droplets of light-brown color (*arrow*). Numerous smaller and apparently colorless droplets attached to single aerial hyphae are also visible.

on un-grazed or grazed mold the animals grew slower on previously attacked colonies. Moreover, grazer-challenged *A. nidulans* colonies intensified significantly the formation of sexual fruiting bodies (cleistothecia), which appeared to be the only fungal tissue that was not consumed after prolonged grazing (Döll et al., 2013). Possibly, the positive correlation of the appearance of cleistothecia and the intensive formation of guttation droplets on the sexual fruiting bodies (**Figure 1**) is a means of protecting this valuable tissue from grazers. For example, guttation droplets produced by *Penicillium* and *Stachybotrys* are known to contain high amounts of toxic SMs (Gareis and Gareis, 2007; Gareis and Gottschalk, 2014), yet, no report exists on the SM content in *A. nidulans* guttation droplets exists (and whether it changes under grazer pressure). Nonetheless, at least for *A. nidulans*, combined investment in SM formation and sexual development seems to be a strategy to maintain high fitness in grazer-rich niches (**Figure 2**).

These findings are supported by an experiment that examined the effect of larval *Drosophila* grazing on the expression of *A. nidulans* genes. In the presence of the insect larvae, *A. nidulans* exhibited shifts in the transcriptional activity of many genes, including those involved in signal transduction, hormonal signaling, and SM biosynthesis (Caballero Ortiz et al., 2013); interestingly, *laeA* ranked highest among those genes that were found up-regulated in response to the insects. Comparable with the results of the Collembola-*A. nidulans* experiment described above, grazed wild type colonies were found to kill *Drosophila* fly larvae more rapidly than unchallenged ones. An intriguing finding here was that, in a subsequent experiment, *D. melanogaster* larvae were able to use a Δ*laeA* mutant of otherwise fatal *A. nidulans* as the only available food source that promoted development into adult flies (Caballero Ortiz et al., 2013). That is LaeA-mediated activation of

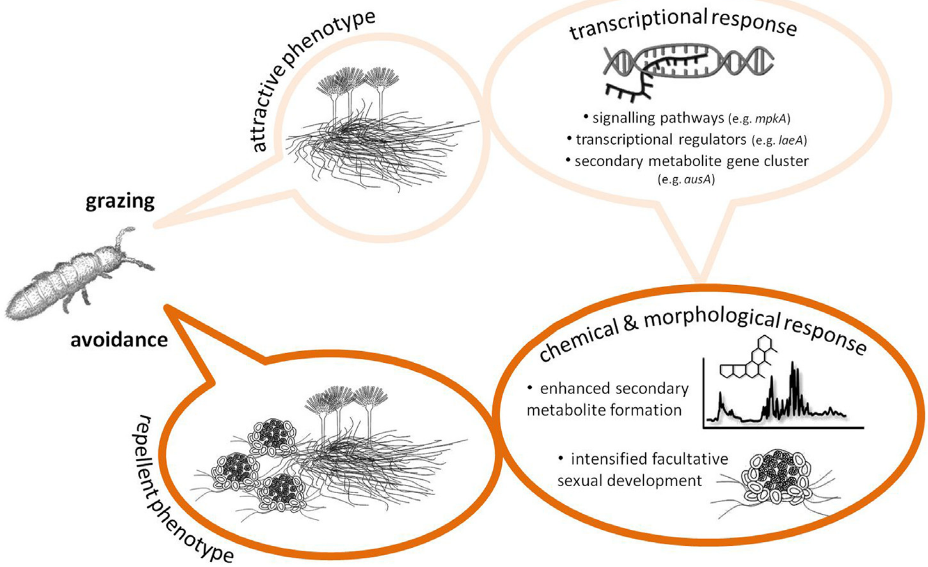

FIGURE 2 | Scheme summarizing the results of some recent studies demonstrating a grazer-induced defense response in the model fungus *Aspergillus nidulans* [see text and publications by Döll et al. (2013) and Caballero Ortiz et al. (2013) for details]. This scheme may serve as a modifiable blueprint for future studies providing evidence for or against an inducible chemical defense in fungi, add more specific information from other fungal systems, or contribute to general, system-independent properties of fungal chemical responses to grazers, e.g., hormone and pheromone signaling. Although not indicated in detail, it should also be specified how both putative defense compounds affect fungivore behavior and physiology, to be better able to determine the ecological consequences of fungal secondary metabolites (SM) biosynthesis and hence the selective forces that may have, at least in part, shaped fungal SM diversity.

SM formation in response to grazing to a large extent prevents the fungus from being consumed and used as a suitable diet by these facultative fungivores.

Finally, terpenoid compounds that function as hormones in arthropods, the so-called juvenile hormones (JH), have recently been found to be synthesized by *A. nidulans*, in particular under larval fruit fly grazing pressure (Nielsen et al., 2013). And when confronted with a JH over-expression mutant, fly larvae were significantly smaller. Possibly, the anti-grazer defense mechanisms of *A. nidulans* comprise the activity of insect hormone analogs that derange grazer endocrine processes.

Taken together, these findings illustrate the extent to which fungal morphological and chemical properties are affected by dynamic interactions with grazers and thereby reveal a number of new candidate defense pathways that may mediate direct resistance. Considering this chemical diversity of compounds we still lack convincing evidence for the identity of SMs used by fungi to repel and/or harm grazers. Even in this single *A. nidulans* system, it seems possible that more than one master defense compound exists, which are likely to interact with grazers via an array of perhaps equally diverse (patho-) physiological and behavioral mechanisms in the animals.

EVIDENCE FROM OTHER FUNGI

Using model fungal systems has many obvious advantages, yet there is a strong need to investigate the idea of inducible chemical compound-based resistance in other fungi. Outside the *A. nidulans* system, to the best of my knowledge, there is no good evidence of inducible chemical compound-based resistance in fungi. The study by Bleuler-Martínez et al. (2011) shows the induction of fruiting body lectins (carbohydrate-binding proteins) by nematode worms piercing hyphae and sucking in the cytoplasmatic content of *Coprinopsis cinerea*. In spite of this clear demonstration of an induced fungal response, it remains to be tested whether lectins provide an effective protection against the nematode or other grazers, since lectin toxicity has only been tested against non-fungivores (Bleuler-Martínez et al., 2011; Schubert et al., 2012; Žurga et al., 2014). Relevant to this discussion is the finding by Balogh et al. (2003), who did not find a positive influence of a lectin-deficient *Arthrobotrys oligospora* mutant on grazing by *F. candida*.

These very first studies illustrate the significance of grazing in determining the chemical profile of fungi but also the complexities and ambiguities involved in establishing a conceptually sound and direct connection between SM formation and fungal fitness. Therefore, separating functionally relevant from irrelevant chemical responses of fungi to grazing will be of utmost importance for the correct annotation of putative fungal defense traits that mediate protection from grazers. For example, genome-wide gene expression analyses should follow to reveal fungal responses to grazing the abovementioned studies may have overlooked.

CONCLUSION

We are only beginning to appreciate fungal chemical dynamics under varying ecological conditions, and we should investigate deeper the full repertoire, kinetics, and sites of secondary metabolite biosynthesis in response to grazers to be able to update and rearrange the lists of putative defense compounds (in *A. nidulans* and other fungi) on the basis of inducible reactions. Carefully conducted experiments with model grazers (e.g., Collembola, *Drosophila* larvae, nematodes, etc.) exposed to purified compounds will shed light on the mode of action in fungivores, e.g., whether fungal chemicals decrease or suppress the feeding response, act as acute toxins that interfere with intermediary metabolism or cellular functions, or cause chronic tissue and organ malfunctions which ultimately lead to impaired development and reduced fecundity. Finally, a large fraction of fungal SMs likely have evolved to allow effective control of and/or communication with other microbes in their environment, and we should thus think about the possibility that it is not the grazer itself that is directly affected by specific metabolites, but the endogenous microorganisms fungivores require for the control of their immune system, food breakdown, and detoxification. Thus, in order to fully appreciate the complete anti-grazer potential of SM biosynthesis in fungi, we need to combine their inducible dynamics with the influence of these compounds on grazer behavior, physiology, and multi-species interactions in their environment.

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Heterologous production of fungal secondary metabolites in Aspergilli

Diana Chinyere Anyaogu and Uffe Hasbro Mortensen*

Section for Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark

Edited by:
Nancy Keller, University of Wisconsin–Madison, USA

Reviewed by:
Clay Chia Chun Wang, University of Southern California, USA
Wei Xu, University of California, Los Angeles, USA

****Correspondence:***
Uffe Hasbro Mortensen, Section for Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark, Soltofts Plads, Building 223, 2800 Kongens Lyngby, Denmark
e-mail: um@bio.dtu.dk

Fungal natural products comprise a wide range of compounds. Some are medically attractive as drugs and drug leads, some are used as food additives, while others are harmful mycotoxins. In recent years the genome sequence of several fungi has become available providing genetic information of a large number of putative biosynthetic pathways. However, compound discovery is difficult as the genes required for the production of the compounds often are silent or barely expressed under laboratory conditions. Furthermore, the lack of available tools for genetic manipulation of most fungal species hinders pathway discovery. Heterologous expression of the biosynthetic pathway in model systems or cell factories facilitates product discovery, elucidation, and production. This review summarizes the recent strategies for heterologous expression of fungal biosynthetic pathways in Aspergilli.

Keywords: secondary metabolite, *Aspergillus*, gene clusters, fungi, heterologous expression, polyketide synthase

INTRODUCTION

Filamentous fungi produce a plethora of secondary metabolites, SMs, like polyketides (PK), terpenes, and non-ribosomal peptides (NRP). Several fungal SMs dramatically impact human life either because they are harmful mycotoxins, like carcinogenic aflatoxin (Eaton and Gallagher, 1994) and fumonisin (Voss and Riley, 2013), or because they are used to efficiently combat human disease, e.g., penicillin and lovastatin (Campbell and Vederas, 2010). Importantly, analyses of fully sequenced fungi show that the number of SMs known to be produced by these fungi is too low to account for the number of genes and gene clusters that potentially may lead to production of SMs (Szewczyk et al., 2008). This strongly suggests that the chemical diversity of the metabolomes produced by filamentous fungi is much larger than what is currently known, and it is therefore very likely that new harmful mycotoxins and new blockbuster drugs await discovery.

The rapid accumulation of fully sequenced genomes has accelerated the discovery of novel SMs dramatically. However, this sequence resource cannot be directly translated into chemical structures of new compounds despite that genes and gene clusters are often readily identified by bioinformatics tools (Khaldi et al., 2010; Andersen et al., 2013; Blin et al., 2013). For example, the exact structures of products released by fungal type I polyketide synthases are difficult to predict due to the iterative use of the different catalytic domains in these enzymes. Similarly, subsequent decorations performed by tailoring enzymes encoded by other genes in the cluster toward formation of the mature end product(s) are complex and not easy to predict. Another challenge is that many SMs are not readily produced under laboratory conditions although several approaches have been successfully employed to activate silent clusters (for reviews, see Brakhage and Schroeckh, 2011; Chiang et al., 2011; Klejnstrup et al., 2012; Wiemann and Keller, 2014; Yaegashi et al., 2014). To link novel SMs to genes, and to map novel biosynthetic pathways, extensive genetic manipulations of the strains are typically required. Since, most new gene clusters uncovered by sequencing projects will be situated in fungi with no available genetic tools, this type of analysis may not be straight forward. Moreover, it may be difficult to purify sufficient amounts of a desired compound from these fungi to allow for thorough characterization of its bioactivity. An alternative approach is to transfer genes and gene clusters to hosts with strong genetic toolboxes thereby facilitating product discovery, production, and characterization. Here we review SM production in Aspergilli based cell factories. Considerations and strategies concerning central steps toward fungal SM production are presented for inspiration: choice of host, how to produce the first intermediate in the pathway, and how to establish the remaining part of the pathway.

HOST CHOICE FOR HETEROLOGOUS EXPRESSION OF FUNGAL SECONDARY METABOLITES

Heterologous expression of SM genes has mainly been performed in baker's yeast *Saccharomyces cerevisiae* (Tsunematsu et al., 2013) and in the filamentous fungi *Aspergillus oryzae* and *A. nidulans*. Each of these model organisms offers specific advantages. For *S. cerevisiae* a superior genetic toolbox for strain construction has been developed and novel genes can easily be engineered into a wealth of single- and multi-copy expression plasmids or into chromosomes. For example, gene targeting and fusion of DNA fragments by homologous recombination (HR) is highly efficient in *S. cerevisiae*. Moreover, *S. cerevisiae* contains an insignificant endogenous secondary metabolism (Siddiqui et al., 2012). This fact simplifies the analysis of strains equipped with new pathways as they are not complicated by the presence of a multitude of other SMs; and the risk of undesirable side reactions due to cross chemistry between the novel and endogenous pathways is minimized. However, lack of secondary metabolism also means that yeast is not naturally geared for SM production and may contain limiting amounts of, or even lack, relevant building blocks (Kealey et al., 1998; Mutka et al., 2006). Moreover, localization of relevant enzymes for aflatoxin production into specialized vesicles in *A. parasiticus* indicate that fungi may possess specialized compartments for SM production, which yeast may not contain (Roze et al., 2011); and as introns are few in *S. cerevisiae* (Spingola et al., 1999) and differ from those in filamentous fungi (Kupfer et al., 2004), mRNA splicing could be problematic. For these reasons filamentous fungi may often be more appropriate for heterologous SM production. *A. oryzae* is often used for this purpose because it possesses a limited endogenous secondary metabolism and *A. nidulans* because a strong genetic toolbox has been developed for this fungus (for review see, Meyer, 2008; Meyer et al., 2011). Importantly, the recent development of efficient tools for gene targeting in filamentous fungi, including strains where random integration is minimized due to mutation of genes required for non-homologous end-joining (Ninomiya et al., 2004; Nayak et al., 2006; Takahashi et al., 2006), has further stimulated the use of these organisms as hosts for SM pathway reconstitution experiments.

HETEROLOGOUS EXPRESSION OF POLYKETIDE SYNTHASES

The fact that the product(s) released by fungal type I PK synthases (PKSs) cannot easily be predicted from their primary sequence has sparked a major interest in expressing PKS genes in model fungi with the aim of identifying these products. In yeast, two 2 μ based multi-copy plasmids harboring the 6-methylsalicylic acid (6-MSA) synthase gene from *Penicillium patulum* and the PKS activating PPTase gene from *Bacillus subtilis* were successfully used to produce 6-MSA (Kealey et al., 1998). Similarly production of green pigment has been achieved in a *wA*Δ *yA*Δ (white) *A. nidulans* (Holm, 2013) via co-expression of the PKS gene *wA* and laccase gene *yA* harbored on two AMA1 (Aleksenko and Clutterbuck, 1997) based plasmid. However, if multiple plasmids are needed to form a complex end-product, these vectors may have limited value since sufficient markers may not be available, and since 2 μ and AMA1 plasmids segregate unevenly during mitosis (Albertsen et al., 2011; Holm, 2013; Jensen et al., 2014).

More stable expression has been achieved by integrating PKS genes randomly into the genome of a model filamentous fungus via the non-homologous end-joining pathway. Using this concept, Fujii et al. (1996) successfully linked 6-MSA production to the PKS gene *atX* from *A. terreus* by expressing *atX* host *A. nidulans.* Considering that foreign SMs may be toxic in the new host, it is advisable to employ an expression strategy that minimizes this risk. For production of 6-MSA and enniatins in *A. nidulans* and *A. niger*, this was achieved by fusing the PKS and NRPS genes to the inducible promoters, amyB (Fujii et al., 1996) and Tet-on (Richter et al., 2014), respectively. Over the years, a number of other PKS genes have been linked to products using this strategy in *A. nidulans* and *A. oryzae* including the PKS genes for production of 1,3,6,8-tetrahydroxynaphthalene, alternapyrone, and 3-methylorcinaldehyde by (Fujii et al., 1999, 2005; Bailey et al., 2007).

Random integration may trigger unpredictable pleiotropic effects that alter the expression of neighboring genes, hence, complicating subsequent analyses (Verdoes et al., 1995; Palmer and Keller, 2010). Moreover, since multiple copies of the gene often integrate simultaneously into the same site, strains may suffer genetic instability and lose expression over time. Taking advantage of the development of strains and techniques for efficient gene targeting, these problems can be eliminated by inserting genes into a defined locus. This facilitates not only subsequent strain characterization, but also sets the stage for experiments analyzing mutant varieties of the gene where equal expression levels of the alleles are important to fairly judge the impact of individual mutations. Using this approach, Hansen et al. (2011) demonstrated that *mpaC* from *P. brevicompactum* encodes a PKS producing 5-methylorsellinic acid. In this case, *mpaC* was introduced into a defined site, *IS1*, on chromosome I of *A. nidulans*, which supports expression of non-toxic genes in a variety of tissues without affecting fitness. Moreover, to simplify the integration of genes into *IS1*, a set of vectors pre-equipped with targeting sequences, genetic markers, promoters and terminators and a USER-cloning cassette (Nour-Eldin et al., 2006) allowing for seamless ligation free insertion of relevant genes into the vector was developed. Using this technology, *ausA*, from *A. nidulans*, and *yanA*, from *A. niger*, have been shown to encode PKSs producing 3-,5-dimethyl orsellinic acid and 6-MSA, respectively (Nielsen et al., 2011; Holm et al., 2014). In a variation of this approach, Chiang et al. (2013) used fusion Polymerase chain reaction (PCR) to merge an *alcA* promoter and PKS genes followed by integration into the *wA* locus of *A. nidulans.* Correctly targeted transformants could therefore easily be identified as white colonies. The authors expressed nine non-reducing (NR) PKS genes from *A. terreus* in this manner and identified six products. Heterologous production of PKs is complicated by the fact that not all synthases possess a domain providing a product release mechanism (Awakawa et al., 2009; Du and Lou, 2010) and by the fact that some PKSs require a starter unit different from Ac-CoA (Hoffmeister and Keller, 2007). In the study by Chiang et al. (2013), two of the nine NR-PKSs analyzed did not contain such a domain and for one, a product was achieved by co-expressing a gene encoding a thioesterase activity. In addition, two NR-PKS were predicted to employ unusual starter units. For one NR-PKS, production of this starter unit was successfully delivered by co-expressing a gene encoding a highly reducing PKS and the collaborative effort of the two enzymes resulted in production of an intermediate for production of asperfuranone (Chiang et al., 2013).

TRANSFER OF GENE CLUSTERS TO HETEROLOGOUS HOSTS

Reconstitution of most SM pathways depends on the expression of multiple genes since the SM scaffold delivered by the synthase is further decorated by tailoring enzymes. Moreover, genes providing transcriptions factors, transporters and/or a resistance mechanism may also be required. Construction of strains for heterologous end-product production is therefore a major challenge as it requires not only transfer, but also activation, of large gene clusters. Two principles are generally employed for constructing DNA fragments that allow transfer of gene clusters into another fungal host. Firstly, DNA fragments harboring entire, or a large part of, gene clusters have been identified in cosmid/fosmid libraries and transferred into vectors with a selectable fungal marker (**Figure 1A**). Secondly, PCR fragments covering the gene cluster have been stitched together using a variety of methods including USER Fusion, Gateway cloning and yeast recombination to create suitable transformation vectors (**Figure 1B**). When gene clusters have been transformed into the host, activation has been achieved by three different methods. Firstly, in cases where the native gene cluster harbors a TF gene, it has been possible to activate the genes in the cluster by equipping the TF gene with a constitutive or inducible promoter known to work in the host. Secondly, in gene clusters without a TF gene, activation has been achieved either by overexpressing the global regulator LaeA or by individually swapping cluster gene promoters for constitutive or inducible promoters. Like for integration of PKS genes, and for the same reasons, integration strategies based on random or directed integration have been used (**Figure 1C**). In many cases these strategies have been combined and successful examples are provided below.

Cosmids harboring the entire penicillin biosynthetic pathway from *P. chrysogenum* were introduced to *Neurospora crassa* and *A. niger*, resulting in the production of penicillin (Smith et al., 1990). Similary, cosmids harboring the citrinin biosynthetic pathway from *Monascus purpureus* and the monacolin K gene cluster from *Monascus pilosus* were individually integrated into random positions in the genome of *A. oryzae.* In the case of citrinin, the transformant directly produced citrinin, but in small amounts. However, as the cluster contains a TF gene, additional copies of the activator gene (*ctnA*) controlled by the *A. nidulans trpC* promoter were subsequently introduced in the strain to boost production. Impressively, this resulted in a 400 fold increase of citrinin production (Sakai et al., 2008). The monacolin K gene cluster does not contain a TF gene. In this case, the cluster was activated by overexpressing the gene encoding the global activator LaeA (Sakai et al., 2012). A limitation of this strategy may be difficulties in isolating cosmids containing a fragment that harbors the entire gene cluster, especially if clusters are large. For example, the reconstruction of the terrequinone A gene cluster in *A. oryzae* was based on a fosmid containing an incomplete gene cluster. The remaining part of the cluster was subsequently obtained by PCR, cloned into a vector and transformed into the *A. oryzae* strain harboring the partial terrequinone A gene cluster (Sakai et al., 2012).

Several PCR based strategies have been used for transferring gene clusters from the natural producer to a model fungus. For clusters harboring a TF gene, PCR fragments covering the entire gene cluster have been amplified, fused, and inserted via a single cloning step into vectors predestined for site specific integration in the genome of the host by HR. Multiple PCR fragments can be orderly assembled by different strategies. For example, PCR fragments of the geodin and neosartoricin B clusters were physically linked by *E. coli* based USER fusion and by yeast based HR, respectively (Nielsen et al., 2013; Yin et al., 2013). Importantly, in both cases the promoter controlling expression of the TF gene was swapped for a strong constitutive promoter during the cluster re-assembly process. Large inserts (>15 kb) may not be propagated stably in a cloning vector and large clusters need to be subdivided into smaller fragment cassettes, which together represent the entire cluster. Multiple subsequent integrations depend on marker recycling, which can be achieved by using *pyrG* as a selectable/counterselectable marker. A faster method employs a two marker system for cluster transfer (Nielsen et al., 2013). During one transformation cycle, one of the markers is used to select for integration of the first cluster cassette and the other marker for the next cassette. By ensuring that integration of one cassette eliminates the marker contained by the preceding cassette, numerous cluster cassettes can be integrated sequentially by alternating the use of the two markers. Advantageously, when the gene clusters is inserted in a controlled manner it can be subjected to further genetic dissection to clarify the biochemical pathway toward end product. With the geodin cluster this was exploited to demonstrate that *gedL* encodes a halogenase using sulochrin as substrate (Nielsen et al., 2013).

Polymerase chain reaction based reconstruction of clusters that do not contain an activating TF gene requires more elaborate genetic engineering as all cluster genes need to be equipped with new promoters and terminators. In one strategy, cluster ORFs were inserted either individually or in pairs into expression cassettes in plasmids carrying different selection markers. Using this approach several small gene clusters containing four to five genes have been, fully or partially, reconstituted by randomly introducing the genes into the genome of *A.* oryzae. Several SMs have been achieved by this method including tennelin, pyripyropene, aphidicolin, terretonin, and andrastin A (Heneghan et al., 2010; Itoh et al., 2010; Fujii et al., 2011; Matsuda et al., 2012, 2013). Construction of larger clusters in *A. oryzae* has been limited by the number of available markers. To bypass this problem, Tagami et al. (2013, 2014) used the high co-transformation frequency with *A. oryzae* to integrate two vectors in one round of transformation using selection for only one marker. This allowed for reconstituting clusters with six and seven genes for production of paxilline and aflatrem, respectively (Tagami et al., 2013, 2014). Addressing the same problem, Gateway cloning was used to construct expression vectors containing up to four genes (Pahirulzaman et al., 2012; Lazarus et al., 2014). Utilizing this approach Wasil et al. (2013) expressed different combinations of the synthase and tailoring genes from the aspyridone pathway from *A. nidulans* in *A. oryzae.* An alternative approach to save markers is to generate synthetic polycistronic genes where all genes in the construct are under the control of a single promoter and where all ORFs are separated by a sequence encoding the viral 2A peptide that results in co-translational cleavage, hence, resulting in the formation of independent enzymes (Kim et al., 2011). Using this concept Unkles et al. (2014) reconstituted the penicillin gene cluster from *P. chrysogenum* as a single three ORF polystronic gene by yeast mediated HR. Random genomic integration of this construct resulted in penicillin production in *A. nidulans.*

A strategy for gene cluster activation based on promoter/terminator swapping has also been implemented in gene cluster transfer methods where genes are inserted into defined integration sites (Hansen et al., 2012; Mikkelsen et al., 2012;

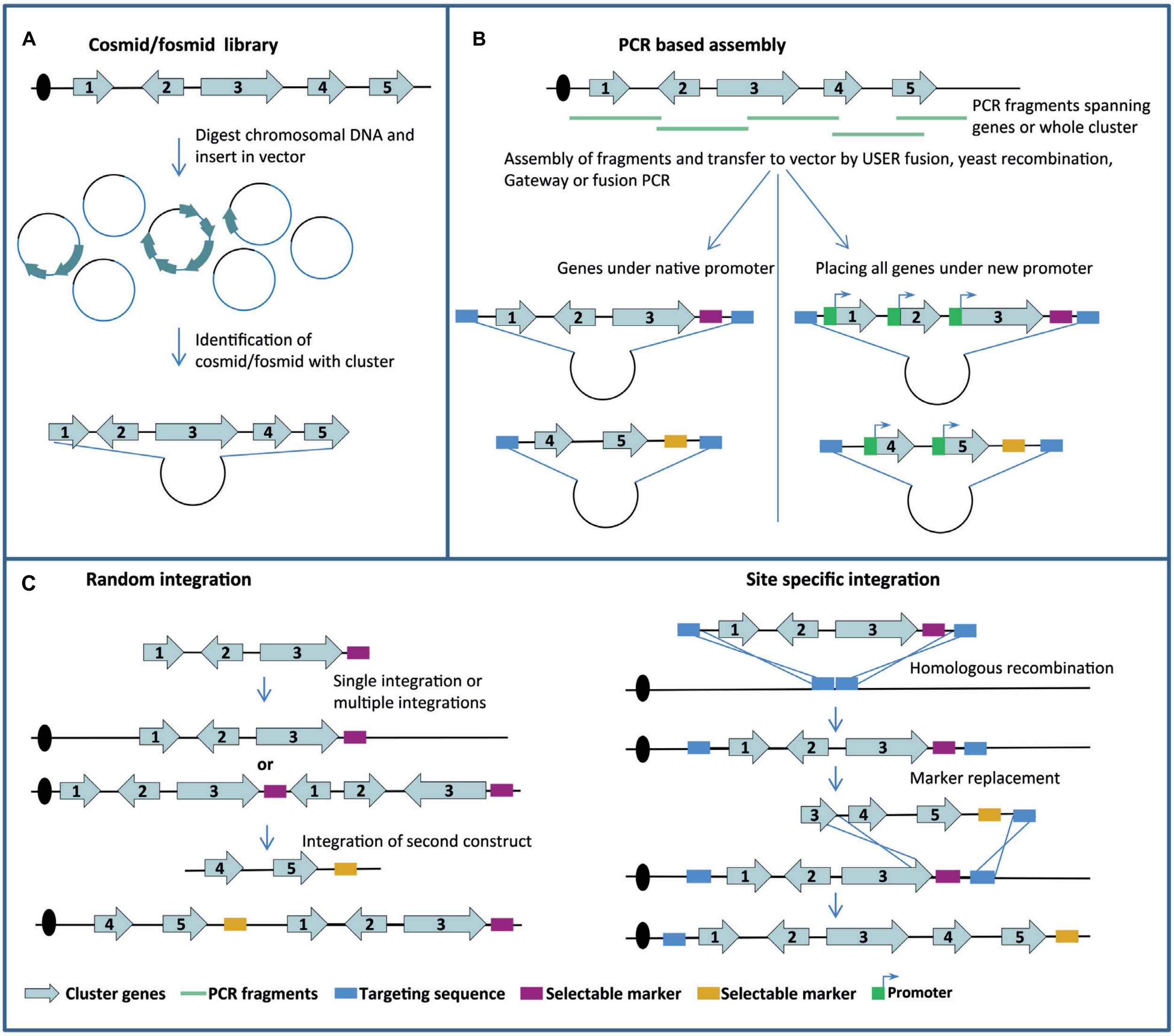

FIGURE 1 | Overview of principles employed for constructing DNA fragments (A,B) and for integration in host genomes (C).

Chiang et al., 2013). Specifically, expression plasmids containing one to two cluster genes were constructed by USER cloning or by fusion PCR and integrated into the expression sites in *S. cerevisiae* and *A. nidulans* to allow for production of the pigment precursor rubrofusarin in yeast (Rugbjerg et al., 2013) and for partial and fully reconstitution of the pathways for mycophenolic acid and asperfuranone production, respectively, in *A. nidulans* (Hansen et al., 2012; Chiang et al., 2013).

PERSPECTIVES

The rapid development of molecular tools for cluster transfer and re-engineering in heterologous hosts is now at a stage where high-throughput experiments can be performed, and we therefore predict that novel SMs, genes, pathways and enzymes routinely will be discovered using this approach. For now most efforts have been proof of principle cases analyzing genes and gene clusters from genetically well-characterized organisms, but the next wave of breakthroughs will likely concern SMs originating from genetically exotic fungi. In addition, the natural reservoir of SMs will likely expand dramatically as synthetic biology based approaches using bio-bricks of promoters, terminators and SM genes are combined in intelligent or in random ways in model fungi to deliver compounds that nature never invented. Together, we envision that heterologous production will serve as a major driver for SM discovery and development delivering compounds that can be used in the food and pharma industries. Accordingly, physiologically well-characterized fungal cell factories should preferentially be employed as platforms for novel SMs discovery and development. These fungi display superior fermentation properties and extensive metabolic engineering toolboxes, hence, shortening the way toward large scale production.

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Next-generation sequencing approach for connecting secondary metabolites to biosynthetic gene clusters in fungi

Ralph A. Cacho[1], Yi Tang[1,2] and Yit-Heng Chooi[3] *

[1] Chemical and Biomolecular Engineering Department, University of California, Los Angeles, Los Angeles, CA, USA
[2] Chemistry and Biochemistry Department, University of California, Los Angeles, Los Angeles, CA, USA
[3] Plant Sciences Division, Research School of Biology, The Australian National University, Canberra, ACT, Australia

Edited by:
Jonathan Palmer, United States Department of Agriculture – Forest Service, USA

Reviewed by:
Philipp Wiemann, University of Wisconsin–Madison, USA
Liliana Losada, J. Craig Venter Institute, USA

***Correspondence:**
Yit-Heng Chooi, Plant Sciences Division, Research School of Biology, The Australian National University, Linnaeus Way, Canberra, Acton, ACT 2601, Australia
e-mail: yh.chooi@anu.edu.au

Genomics has revolutionized the research on fungal secondary metabolite (SM) biosynthesis. To elucidate the molecular and enzymatic mechanisms underlying the biosynthesis of a specific SM compound, the important first step is often to find the genes that responsible for its synthesis. The accessibility to fungal genome sequences allows the bypass of the cumbersome traditional library construction and screening approach. The advance in next-generation sequencing (NGS) technologies have further improved the speed and reduced the cost of microbial genome sequencing in the past few years, which has accelerated the research in this field. Here, we will present an example work flow for identifying the gene cluster encoding the biosynthesis of SMs of interest using an NGS approach. We will also review the different strategies that can be employed to pinpoint the targeted gene clusters rapidly by giving several examples stemming from our work.

Keywords: filamentous fungi, secondary metabolites, gene clusters, next generation sequencing, genome mining

INTRODUCTION

Human health has been benefited from the secondary metabolites (SMs) produced by fungi. These small molecules, also known as natural products, include important clinical drugs like the antibiotic penicillins (Kardos and Demain, 2011), the cholesterol-lowering statins (Endo, 2010), the immunosuppressive cyclosporins (Britton and Palacios, 1982) and the antifungal echinocandins (Balkovec et al., 2014). Microbial SMs, including those from bacteria and fungi, continue to serve as important sources of molecules for drug discovery. For many decades, the fascinating and diverse structures of microbial SMs have inspired the organic chemists to embark on a quest to elucidate their biosynthetic pathways. Many basic insights into SM pathways were obtained by organic chemists using isotopic tracers during the 1950s (Bentley, 1999). The research shifted to the molecular biology of SM biosynthesis with the availability of tools for DNA cloning and sequencing. This is marked by several landmark papers, which described the molecular cloning of whole SM biosynthetic pathway on a contiguous stretch of DNA from actinomycete bacteria (Malpartida and Hopwood, 1984; Cortes et al., 1990; Donadio et al., 1991). Around the same period, the first fungal SM gene cluster, the penicillin biosynthetic gene cluster with the core non-ribosomal peptide synthetase (NRPS) gene encoding L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase had been discovered in the fungus *Penicillium chrysogenum* (Díez et al., 1990). This is followed by the discovery of the terpenoid gene cluster encoding trichothecenes (Hohn et al., 1993), and polyketide gene clusters encoding aflatoxin/sterigmatocystin biosynthesis in *Aspergillus* sp. (Brown et al., 1996; Yu et al., 2004) and lovastatin biosynthesis in *A. terreus* (Kennedy et al., 1999). This hallmark trait of gene clustering was then observed in almost all other classes of fungal SM pathways including indole alkaloids and terpenoids (Keller et al., 2005). The tendency for the biosynthetic genes in microbial SM pathways to cluster on a chromosomal locus greatly accelerated the elucidation of enzymatic steps involved in biosynthesis of individual SM compounds using molecular biology approaches. Consequently, identification of the gene cluster that encodes the production of a given SM is now becoming the common first step toward elucidating the molecular and enzymatic basis for the biosynthesis of a given SM. Subsequent verification of the predicted gene cluster is often achieved via targeted deletion and/or heterologous expression of key biosynthetic genes. Further characterization of the biosynthetic pathway can be done by deletion of the individual biosynthetic genes in the cluster or reconstruction of the whole pathway in heterologous systems.

Targeted SM gene cluster discovery in fungi in the pre-genomic era is a tedious and time-consuming process. Traditionally, this was done by either complementation of blocked mutants by cosmid libraries (e.g. Mayorga and Timberlake, 1990; Hendrickson et al., 1999), or insertional mutagenesis followed by plasmid rescue from the blocked mutant (e.g., Yang et al., 1996; Chung et al., 2003). Both of these aforementioned methods rely on screening of blocked/complementation mutants, which work well for pigment compounds but can be cumbersome if the phenotype, cannot be easily observed or assayed. For example, in the pioneering work to identify lovastatin gene cluster, 6000 mutants were screened for restored lovastatin production by HPLC/TLC after transformation of *A. terreus* with a cosmid library (Hendrickson et al., 1999). Other methods for identifying key biosynthetic gene include antibody screening of cDNA expression library (e.g., Beck et al., 1990), differential display reverse transcriptase-PCR (e.g., Linnemannstons et al., 2002), suppression subtractive hybridization-PCR (O'Callaghan et al., 2003). However, these methods often lead to isolation of a single gene or partial gene cluster. Further cosmid library walking is required to obtain the whole gene cluster. Due to the relatively large genome size, genome scanning method, such as that demonstrated for discovery of enediyne antitumor antibiotic pathways in actinomycete bacteria (Zazopoulos et al., 2003), is less feasible for fungi.

Biosynthesis of polyketide SMs has been subjected to more intensive studies among other classes of SM pathways in fungi (Chooi and Tang, 2012). One of the earlier major advances in identification of fungal polyketide SM gene clusters is the development of degenerate primer PCR based on conserved ketosynthase (KS) domain of polyketide synthases (PKSs). The KS domain DNA fragments are then use as probes to identify the cosmid library clone carrying the whole or partial gene cluster. This method has been employed to localize the aflatoxin and fumonisin PKS genes (Feng and Leonard, 1995; Proctor et al., 1999). The subsequent primer sets developed to target KS domains of a non-reducing (NR-), partial-reducing (PR-), and highly-reducing polyketide synthases (HR-PKSs) are especially useful for localizing specific PKS gene clusters in cosmid libraries (Bingle et al., 1999; Nicholson et al., 2001). Pioneering work by Kroken et al. (2003) which performed a phylogenomic analysis of the PKS genes in the genomes of *Gibberella*, *Neurospora*, *Cochliobolus*, and *Botrytis* species revealed that fungal PKSs are likely to be derived from eight major lineages (Kroken et al., 2003). Nevertheless, despite the degeneracy of the primers and subdivision of fungal PKS genes in to different subclasses, some PKS genes in the genome can be still be missed by the degenerate primer PCR approach. Furthermore, it has to be bear in mind that, although most SM pathways are clustered on chromosome in fungi some of them can be split into two or three smaller subclusters, such as the pathways for dothistromin in *Dothistroma septosporum* (Chettri et al., 2013), tryptoquivaline in *A. clavatus* (Gao et al., 2011), echinocandin in *Emericella rugulosa* (Cacho et al., 2012), and prenylated xanthones in *A. nidulans* (Sanchez et al., 2011). There are also instances where the gene clusters of multiple SM pathways are intertwined together, such as the fumitremorgin, fumagillin, and pseurotin supercluster (Wiemann et al., 2013a). In such cases, the absence of whole genome sequence information can complicate the identification of the complete gene set for the target SM pathway.

Whole genome sequencing (WGS) of the target SM-producing fungus bypasses the need for the cumbersome library construction, screening, and chromosome walking. More importantly, the genome sequence can reveal the inventory of all the SM gene clusters in the producing fungus. Even though each fungus can harbor 30–50 SM gene cluster, the number is still finite and one of them must encode the SM of interest. For example, WGS of *G. zeae* with the Sanger sequencing method has allowed the systematic deletion of all 15 PKS genes in the fungal genome, which lead to identification of the gene cluster for zearalenone, aurofusarin, fusarin C and an unidentified black perithecial pigment (Gaffoor et al., 2005). This opens up the opportunities for detailed characterization of these pathways for zearalenone (Kim et al., 2005; Zhou et al., 2010; Lee et al., 2011), aurofusarin (Frandsen et al., 2006; Frandsen et al., 2011), and fusarin C (Niehaus et al., 2013). The development of next-generation sequencing (NGS) technologies in the last decade has dramatically lowered the cost for DNA sequencing and put the power of microbial WGS in the hand of individual laboratories (van Dijk et al., 2014). This technology revolution has energized the natural product research field and sparked some exciting NGS-based targeted SM gene cluster discovery projects in fungi (**Table 1**). Our work that used such NGS approach includes the discovery of SM clusters encoding viridicatumtoxin and griseofulvin (Chooi et al., 2010), tryptoquialanine (Gao et al., 2011), echinocandin (Cacho et al., 2012),

Table 1 | Examples of biosynthetic gene clusters assigned to their respective compounds using next-generation sequencing technology.

Species	Sequencing Method	Characterized SM biosynthetic gene clusters	Reference
Penicillium aethiopicum	454	Griseofulvin	Chooi et al. (2010)
		Viridicatumtoxin	Chooi et al. (2010)
		Tryptoquialanine	Gao et al. (2011)
Emericella rugulosa	Illumina	Echinocandin B	Cacho et al. (2012)
Aspergillus alliaceus	Illumina	Asperlicin	Haynes et al. (2012)
Aspergillus sp. MF 297-2	Illumina	(−)-notoamide A	Li et al. (2012)
A. versicolor NRRL 35600	Illumina	(+)-notamide A	Li et al. (2012)
P. fellutanum ATCC 20841	Illumina	Paraherquamide A	Li et al. (2012)
Malbranchea aurantiaca RRC1813	Illumina	Malbrancheamide	Li et al. (2012)
Glarea lozoyensis	Illumina	Pneumocandin	Chen et al. (2013)
A. fischeri	Illumina	Ardeemin	Haynes et al. (2013)
Fusarium fujikuroi	454	Apicidin F	Niehaus et al. (2014)
Eupenicillium brefeldianum	454/Illumina	Brefeldin	Zabala et al. (2014)
F. heterosporum	Illumina	Equisetin and fusaridione A	Kakule et al. (2013)

asperlicin (Haynes et al., 2012), ardeemin (Haynes et al., 2013), and brefeldin (Zabala et al., 2014). Other studies that have taken the advantage of NGS have identified the SM clusters for fungal bicyclo[2.2.2]diazaoctane indole alkaloids (Li et al., 2012), equisetin (Kakule et al., 2013), and pneumocandin (Chen et al., 2013).

Contemporary natural product research programs are now increasingly based on the understanding of the relationship between the SM molecules and the biosynthetic genes (Walsh and Fischbach, 2010). Although such studies are primarily motivated by the desire to understand the molecular and enzymatic basis of SM biosynthesis, the potential benefits and implications derived from such work are manifold. Firstly, the new chemical insights obtained in elucidating the metabolic pathway can be used to design more efficient total synthesis routes for complex natural products. Secondly, knowledge about the gene cluster can facilitate the metabolic engineering effort to increase the yield of useful SMs for commercial production (Pickens et al., 2011). The knowledge also forms the basis for generation of new SM analogs by mutasynthesis and combinatorial biosynthesis. A recent example is the combinatorial biosynthesis of benzenediol lactones (Xu et al., 2014b), which built on previous studies (Reeves et al., 2008; Wang et al., 2008; Zhou et al., 2010; Xu et al., 2013b). Novel biocatalysts useful for green chemistry and chemoenzymatic process development may also be discovered from SM pathways. For example, the characterization of the acyltransferase LovD in lovastatin pathway led to the development of green chemistry process for the semisynthetic cholesterol-lowering drug simvastatin (Xie et al., 2006, 2009; Gao et al., 2009; Xu et al., 2013a). Furthermore, the gene cluster information will be useful for knowledge-based genome mining for structurally-related compound in other fungi, e.g., the discovery of the immunosuppressive neosartoricin based on viridicatumtoxin biosynthesis genes (Chooi et al., 2012, 2013a). Importantly, these established links between genes and SMs are valuable knowledge that contributes to the overarching aims for (1) accurate prediction of SM structures based on DNA sequences and (2) rational design of biosynthetic pathways for synthesis of organic molecules. Finally, bridging the gaps between genes and molecules may facilitate our understanding of the natural functions of SMs using comparative genomics and transcriptomics tools (Chooi and Solomon, 2014).

It is important to note that a SM/natural product-motivated fungal genome sequencing project has different aims compared to the conventional WGS project coordinated by an international consortium of researchers associated with large sequencing centers. The major goal is to obtain the whole SM gene cluster that encode the targeted SM on a contiguous stretch of DNA sequence contig or scaffold. That means the completeness of the genome sequence coverage and whole-genome annotation is of lower priorities. The key question is how to rapidly narrow down and accurately pinpoint the correct SM cluster in the genome that encodes the production of a target SM. Getting an accurate initial prediction will significantly reduce the time spent on gene cluster verification. Below, we will provide the general work flow and guidelines for researchers who are considering adopting NGS technologies for targeted SM gene cluster discovery based on our own experience. We will also use several examples stemming from our work, two PKS pathways and two NRPS pathways, to illustrate the concepts and strategies.

NEXT-GENERATION *DE NOVO* FUNGAL GENOME SEQUENCING AND ASSEMBLY

The standard NGS-based targeted SM gene cluster discovery work flow used in our laboratory is presented in **Figure 1**. The fungal strain acquired from culture collections or her sources are first verified for production of the targeted compound before its genomic DNA was sent for sequencing. A variety of culture media and conditions can also be tested to optimize the production of the target compound. Gene deletion or disruption followed by the detection of loss of target compound production is still the most common method for initial gene cluster verification. Alternatively, if genetic transformation is proven to be difficult on the fungus, expression of the backbone biosynthetic enzymes [e.g., PKS, NRPS, terpene synthase, or dimethylallyltryptophan synthase (DMATS)] in the candidate SM gene cluster in a heterologous system may be another way to verify the gene cluster.

The next thing to consider will be the choice of sequencing method. The choice will be mainly based on the cost, sequence quality, sequence read length, speed and project throughput (the number of strains to be sequenced together). There are currently several NGS technologies on the market suitable for *de novo* fungal WGS, with Illumina now dominating the market

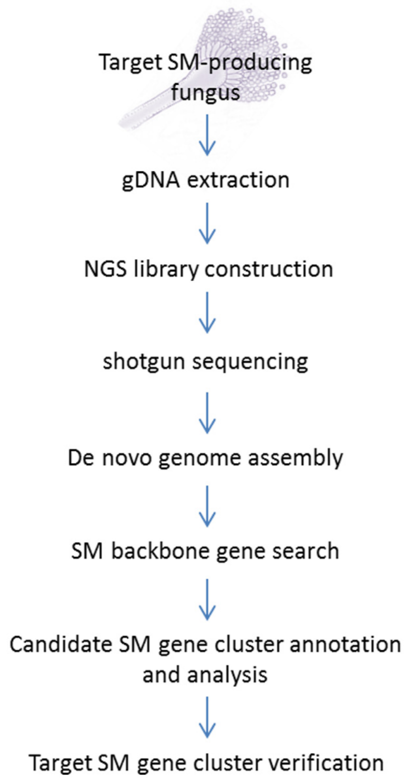

FIGURE 1 | Next-generation sequencing (NGS) workflow for targeted secondary metabolite (SM) gene cluster discovery.

(Nowrousian, 2010; van Dijk et al., 2014). We have experience in both Roche/454 FLX Titanium and Illumina HiSeq2000, but other DNA sequencing platforms may work for this purpose as well. For an overview of the different latest NGS technologies, the readers are referred to van Dijk et al. (2014). The *de novo* sequencing of *P. aethiopicum* (synonym *P. lanosocoeruleum*) IBT5753 genome is one of the earliest examples of SM-motivated fungal WGS undertaken by individual laboratories, which used the Roche/454 FLX Titanium platform (Chooi et al., 2010).

The introduction of Illumina HiSeq2000 (now superseded by HiSeq2500) with ∼100 bp paired end (PE) reads dramatically reduced the cost of sequencing and increased the sequencing output. The shorter read length of HiSeq2000 is compensated by the deeper coverage and the paired-end nature of the Illumina reads. The Illumina PE reads allowed the assembly of longer scaffolds with gaps, which can be filled in later using routine PCR and Sanger sequencing if any of the scaffold harbor interesting SM gene cluster. Longer PE information can be obtained by generating mate pair libraries with larger insert size (e.g., 3 kbp or 5 kbp). Indeed, it has been recently demonstrated that good fungal genome assembly can be obtained on platform with shorter sequence reads (50 bp) like SOLiD using mate pair libraries (Umemura et al., 2013b).

We first used HiSeq2000 for sequencing of the brefeldin-producing *Eupenicillium brefeldianum* ATCC 58665 and the echinocandin-producing *E. rugulosa* (Cacho et al., 2012; Zabala et al., 2014). The amount of sequence data acquired for assembly of the ∼32 Mbp *E. rugulosa* genome is more than sufficient despite the shorter read length (N50 = 235 kbp). In fact, we can get equally good quality assembly using half the amount of data. Now, we routinely perform HiSeq2000 sequencing multiplexed for four fungal genomic samples per lane (∼200X depth of coverage each genome) and can routinely obtain good quality assembly for the purpose of SM gene cluster discovery. Using this arrangement, the cost of sequencing per fungal strain can be lower than the cost required for cosmid/fosmid library construction, screening, and chromosome walking, not to mention the significant time savings. With the use of mate pair libraries, more fungal genomic samples can likely be included into HiSeq2000 per lane yet still yield fine assembly. One recent study shows that the optimum sequencing depth for small bacteria to medium eukaryotic genomes with 2X 100 bp PE Illumina reads is 50-100X (Desai et al., 2013).

There is more than a dozen of software available for assembly of short reads generated by NGS, some are more memory intensive than the others, and there is differences in assembly speed as well. The popular ones including Velvet (Zerbino and Birney, 2008), SOAPdenovo (Li et al., 2010a), AllPATHS (Butler et al., 2008), and ABySS (Simpson et al., 2009). With optimization, some software may allow some small-medium size genomes to be assembled on a standalone workstation (Kleftogiannis et al., 2013). There are several studies that compare the efficiency and assembly quality of different assembly software (Lin et al., 2011; Zhang et al., 2011). We have experience mostly in using the SOAPdenovo assembler developed by BGI (Li et al., 2010a). Our SOAPdenovo assemblies were run on the UCLA Hoffman2 computer cluster. The assemblies can usually be completed with 32–128 GB memory requested from the cluster, depending on the genome size and the amount of input data. For the standard 2X 100 bp PE reads, we often use a *k*-mer size of 63 or 79 for the SOAPdenovo assembly and were able to obtain good results. The latest version, SOAPdenovo2, promised to improve memory efficiency and assembly quality, and more optimized for the longer Illumina reads (rather than the 35–50 bp reads from older Illumina platforms; Luo et al., 2012). Many NGS providers also provide service for sequence assembly with a fee.

LOCATING THE TARGET SECONDARY METABOLITE GENE CLUSTER

The first task after obtaining the scaffolds generated from the assembly is to narrow down the scaffolds containing the candidate SM gene clusters. Despite the enormous structural diversity, most fungal SMs can be divided into four major classes, polyketides, non-ribosomal peptides, terpenes, and indole alkaloids, based on the limited classes of carbon building blocks they derived from (Keller et al., 2005). Thus, an efficient way of narrowing down the scaffolds that may contain the target gene cluster is to search for genes encoding backbone biosynthetic enzymes that synthesized the specific class of compounds correspond to the target SM. This can be most easily achieved by performing a TBLASTN search against a database generated from the assembled fungal genome scaffolds using the "makeblastdb" command in the NCBI stand-alone BLAST application[1]. Depending on the class of SM, an arbitrary chosen conserved domain of the corresponding backbone enzymes can be used as a TBLASTN query. For example, a protein query sequence of a KS domain of PKS for polyketide SMs, an adenylation (A) domain of NRPS for non-ribosomal peptide SMs, terpene synthase for terpenoid SMs and DMATS for prenylated indole alkaloids. The TBLASTN will generate a list of scaffolds containing the SM gene clusters belongs the corresponding SM classes ranked based on homology to the query sequence. Alternatively, commercial bioinformatics software programs with intuitive graphic user interface (GUI) that support BLAST on local database are also available, e.g., CLC Genomics Workbench[2] (CLC Bio) and Geneious[3] (Biomatters). After locating the scaffolds containing gene clusters of the target SM classes, the number of candidate scaffolds can be further narrowed down with comparative genomics analysis (See Comparative Genomics Approach for Target SM Gene Cluster Prediction) followed by gene predictions and more in-depth knowledge-based bioinformatics analysis (see **Figure 2** and Section "Pinpointing Target SM Gene Cluster with Knowledge-Based Analysis – Retrobiosynthesis").

For fungal gene predictions (the locations and exon–intron structures of genes on individual scaffolds) we routinely use FGENESH (Softberry; Solovyev et al., 2006), which can yield relatively accurate predictions for fungi. The web server allows direct submission of FASTA nucleotide sequences with gene-finding parameters trained using datasets from several fungal species[4].

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[1] http://www.ncbi.nlm.nih.gov/guide/howto/run-blast-local/

[2] http://www.clcbio.com/

[3] http://www.geneious.com/

[4] http://www.softberry.com/berry.phtml?topic=index&group=programs&subgroup=gfind

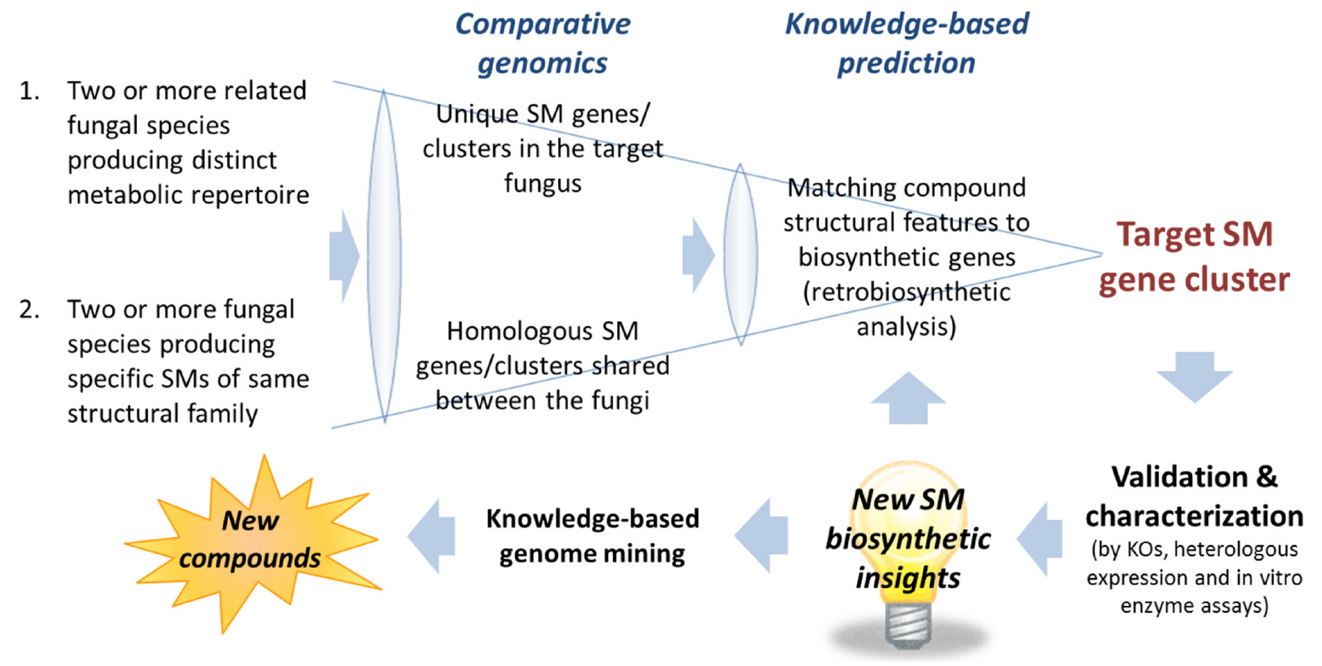

FIGURE 2 | Comparative genomics and knowledge-based prediction strategies for targeted SM gene cluster discovery. New biosynthetic knowledge and insights into gene-to-molecular structure relationship would be useful for target SM gene cluster predictions and knowledge-based genome mining for discovery of novel SM compounds.

Alternatively, AUGUSTUS also provides relatively accurate fungal gene prediction[5] based on training sets from various fungal species (Stanke et al., 2004). Individual protein sequences predicted in a candidate scaffold are then submitted to NCBI BLASTP server for detailed conserved domain analysis (using the integrated NCBI Conserved Domain Search feature) and homologous sequence comparison. The EBI Interproscan also offers similar conserved domain prediction and protein functional analysis. As each gene cluster usually contain 3–15 genes (>20 genes in some cases), it is often not too time consuming when the number of candidate scaffolds has been narrowed down significantly. Several SM gene cluster prediction software programs have also been developed, which can aid this process considerably (see Pinpointing Target SM Gene Cluster with Knowledge-Based Analysis – Retrobiosynthesis).

COMPARATIVE GENOMICS APPROACH FOR TARGET SM GENE CLUSTER PREDICTION

Comparative genomics can be a useful approach for filtering of candidate gene clusters (**Figure 2**). The increasing number of sequenced fungal genomes in public databases, due in part to the lower sequencing cost enabled by NGS technologies, allows one to find a suitable genome of related sequenced organism for comparison with the organism of interest. Since the genomes of different organisms, even those belonging to the same genus, would encode different array of SMs, one can compare the SM backbone biosynthesis gene inventory of one organism with a closely related organism to rapidly narrow down the unique SM gene cluster. This approach is especially useful when there is pre-existing knowledge about the SM repertoire of the reference organism used for comparison. For example, in the search of SM gene clusters responsible for the biosynthesis of viridicatumtoxin and griseofulvin in *P. aethiopicum* (Chooi et al., 2010), we compared the PKS inventory of *P. aethiopicum* with that of *P. chrysogenum* based on prior knowledge from previous chemotaxonomy studies (Frisvad and Samson, 2004). Since it is known that *P. chrysogenum* produces neither viridicatumtoxin nor griseofulvin, we can first filter out the orthologous PKS genes shared between the two fungi (**Figure 3A**; Section Case Study A: Griseofulvin and Case Study B: Viridicatumtoxin). Similar the genome-wide comparison of NRPS genes in *A. nidulans* and *E. rugulosa* was used to narrow down the candidate echinocandin gene cluster (Cacho et al., 2012). It has to be bear in mind that the absence of report about the presence of a target SM compound in a specific species does not always correlate with the absence of the gene cluster in the genome as there can be strain-strain variation and many SM gene clusters could be transcriptionally silent. For example, TAN-1612, reported in another *A. niger* strain as BMS-192548 (Kodukula et al., 1995; Shu et al., 1995), is not detected in the sequenced *A. niger* ATCC 1015. However, the corresponding gene cluster can be identified and activated by transcriptional regulator overexpression (Li et al., 2011). The abilities to produce fumitremorgin by *A. fumigatus* (Kato et al., 2013), and gibberellin and beauvericin by *G. fujikuroi* (Wiemann et al., 2013b), also vary from strain to strain. Nonetheless, such a

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[5] http://bioinf.uni-greifswald.de/webaugustus/predictiontutorial.gsp#example data

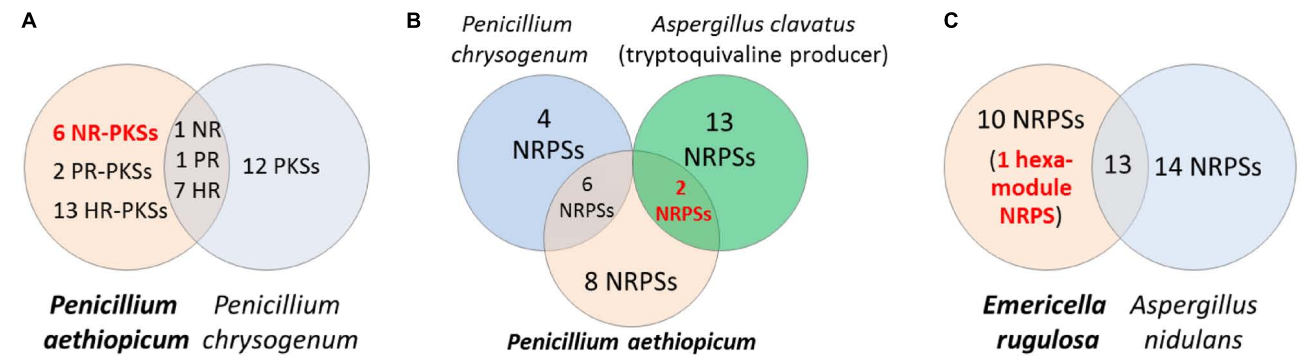

FIGURE 3 | Comparative genomics approach for discovery of SM biosynthetic gene cluster. Comparison of the PKS genes in *Penicillium aethiopicum* and *P. chrysogenum* was utilized in searching for the griseofulvin and viridicatumtoxin gene cluster **(A)**. In order to narrow down possible candidates for the tryptoquialanine gene cluster in *P. aethiopicum*, NRPS genes that are non-orthologous to *P. chrysogenum* NRPS genes but are orthologous to the NRPS genes of the tryptoquivaline producer *A. clavatus* were found **(B)**. The echinocandin NRPS gene was found in *Emericella rugulosa* was found by searching for a hexamodule NRPS gene in *E. rugulosa* that is non-orthologous to *A. nidulans* NRPS genes (**C**).

comparative genomics approach had served as a useful first-pass filter in our hands, especially for fungal species that have been characterized chemotaxonomically, such as those species in the *Aspergillus* and *Penicillium* genera (Frisvad and Samson, 2004; Frisvad et al., 2007).

Conversely, comparative genomics can also be utilized for identifying the biosynthetic gene clusters of a target group of SMs bearing structural similarities but made by less-related fungal species (**Figure 2**). In this case, orthologous gene clusters in two organisms that are capable of synthesizing the same family of compounds are targeted. An example of the latter strategy is demonstrated in our work on tryptoquialanine/tryptoquivaline gene cluster identification in *P. aethiopicum*/*A. clavatus* (Gao et al., 2011; **Figure 3B**). The strategy is also well-demonstrated in the work by the Sherman group in investigating the biosynthesis of fungal bicyclo[2.2.2]diazaoctane indole alkaloids (−)- and (+)-notoamide A, paraherquamide A, and malbrancheamide A by *Aspergillus* sp. *MF297-2, A. versicolor* NRRL35600, *P. fellutanum* ATCC20841, and *Malbranchea aurantiaca* RRC1813, respectively (Li et al., 2012). More recently, NGS-driven comparative genomics of four *Stachybotrys* strains from two chemotypes producing either atranones or stratoxins have revealed two unique gene clusters, which possibly encode the biosynthesis of the two respective terpenoid-derived SMs (Semeiks et al., 2014). However, the identities of the two gene clusters are yet to be verified experimentally. Such comparative genomics approach is highly compatible with the high-throughput nature of Illumina sequencing as genomic samples from multiple fungal strains can be multiplexed on a single lane of an Illumina sequencer flow cell. In the case where the target SM gene cluster is split into more than one scaffold in one of the fungal genome assemblies, either due to disruption of assembly by repetitive sequence or the pathway is encoded by multiple loci, comparative genomics of the different strains can help localize the complete biosynthetic gene sets. This approach has facilitated the identification of genes for biosynthesis of tryptoquivaline in *A. clavatus*, which was separated into three genomic loci, by comparing with the tryptoquialanine-producing *P. aethiopicum* genome (Gao et al., 2011).

Lastly, one can use comparative genomics to make an educated guess on where the boundaries of the SM gene cluster are located. Oftentimes, SM gene clusters are flanked by syntenic blocks containing highly-conserved core genes (Machida et al., 2005; Tamano et al., 2008). Similarly, we have observed this trend in our studies toward the discovery of gene clusters encoding griseofulvin, viridicatumtoxin, cytochalasin, and tryptoquialanine. All four putative SM gene clusters are flanked by syntenic block of genes with high shared identity (>85%) in closely related ascomycetes species (Chooi et al., 2010; Gao et al., 2011; Qiao et al., 2011). In fact, a study reported a motif-independent bioinformatics approach for detection of SM gene cluster based on non-syntenic blocks in fungal genomes (Umemura et al., 2013a). One of the software that is useful for identifying and visualizing syntenic regions across multiple genomes is Mauve (Darling et al., 2004). While it does not guarantee that genes that are not highly conserved to a related organism are part of the cluster, this strategy is helpful toward minimizing the number of genes subjected to further functional analysis.

PINPOINTING TARGET SM GENE CLUSTER WITH KNOWLEDGE-BASED ANALYSIS – RETROBIOSYNTHESIS

As mentioned above, gene predictions of individual scaffolds can be performed using software like FGENESH or AUGUSTUS after narrowing down the number of candidate SM gene clusters to a handful of scaffolds. Further detailed bioinformatics analysis of the individual SM gene clusters is then needed to identify the target gene cluster. Some software programs have been developed to aid SM gene cluster predictions. Two of the popular software programs available for prediction of SM gene clusters are antiSMASH (Medema et al., 2011; Blin et al., 2013) and SMURF (Khaldi et al., 2010). SMURF is specific for predicting SM gene clusters in fungal genomes, while antiSMASH can be used for both bacteria and fungi. Guidelines and detailed protocols for using these two and other related SM biosynthetic gene prediction programs can be found in Fedorova et al. (2012). SMURF requires a protein FASTA file and a gene coordinate file as input data. On the other hand, antiSMASH can accepts single nucleotide FASTA file and can be a very useful tool for getting an initial idea of the composition of SM gene cluster on each candidate scaffold along with gene annotation suggestions. Besides providing the predicted domain architecture of multi-domain backbone enzymes (i.e., PKSs and NRPSs), the smCOG (SM Cluster of Orthologous Groups) analysis module in antiSMASH also predicts the function of probable tailoring enzymes encoded in the cluster based on conserved domain analysis. Unfortunately, the substrate prediction function of antiSMASH is yet to be as useful for fungal SM clusters compared to bacterial ones. Moreover, intron prediction using antiSMASH is not as accurate compared to FGENESH, which can potentially hamper efforts toward heterologous expression of fungal SM biosynthetic enzymes in *E. coli* or yeast. With increasing number of established connections between fungal SM gene clusters and molecular structures, this feature is likely to improve in the future.

Nonetheless, a good understanding of the biochemistry of SM biosynthesis and knowledge about the relationship between biosynthetic genes and molecular structures is often needed to accurately pinpoint the SM gene cluster of interest. An excellent introduction to the common building blocks, enzymes, and biochemical reactions involve in SM biosynthesis is available (Dewick, 2009). The specific question that will be asked by the researcher is "what kind (and combination) of enzymes and precursors are likely to be involved in the biosynthesis of the target SM compound?" Such analytic-deductive approach is sometimes referred as "retro-biosynthetic analysis" where the SM structure are taken apart into simpler intermediates and precursors to help determine the enzymes and biological building blocks required for the target SM biosynthesis. Here, we will focus on the general strategies that can be adopted for pinpointing target PKS and NRPS gene clusters.

Polyketides

A great majority of fungal PKSs belong to the iterative type I PKSs whereupon the different PKS catalytic domains are juxtaposed on a single large polypeptide and the single set of PKS domains performs all the necessary catalytic activity during the biosynthesis of the polyketide. Fungal iterative type I PKSs are further classified into three major classes based on the degree of β-keto reduction performed by the PKS; namely the NR-PKSs, the PR-PKSs, and the HR-PKSs. The enzymology and classification of fungal iterative type I PKSs have been reviewed extensively (Cox, 2007; Crawford and Townsend, 2010; Chooi and Tang, 2012). As a general rule, aromatic polyketide compounds are synthesized by NR-PKSs, while HR-PKSs produce aliphatic compounds. PR-PKSs, on the other hand, has been shown to produce compounds lacking a phenolic hydroxyl group at the aromatic ring on the position where a β-keto group has been reduced to alcohol on the polyketide chain before cyclization, such as 6-methylsalicylic acid (Beck et al., 1990; Fujii et al., 1996) and (*R*)-mellein (Chooi et al., 2015). Thus, depending on the nature of the target polyketide compound, the number of candidate scaffolds can be further narrowed down by targeting NR-, PR-, or HR-PKS using corresponding KS domain. Querying the local BLAST database consist of the assembled genomic scaffolds with a corresponding KS domain will resulted in scaffolds containing the corresponding group of PKS genes appearing on top of the TBLASTN hit list. The specific group of PKSs encoded in the candidate scaffolds can be subjected to further scrutiny to pinpoint the PKS gene responsible for biosynthesis of the target SM. Type III PKSs, which present mainly in plants but can be found in some fungi as well, but are usually very limited in number (one or two) in most fungal genomes and are known to synthesize resorcylic acid-type compounds (Hashimoto et al., 2014).

In addition to the minimal PKS domains, a typical NR-PKS also contains the starter unit:ACP transacylase (SAT; Crawford et al., 2006); the product template (PT; Crawford et al., 2008, 2009). An NR-PKS could also contain a thioesterase/Claisen-like cyclase (TE/CLC; Fujii et al., 2001; Korman et al., 2010) or a terminal reductive domain (Bailey et al., 2007) for product release. Although examples of NR-PKSs utilize an in-*trans* releasing domain (Awakawa et al., 2009; Li et al., 2011) and NR-PKS that do not require any releasing domain or enzyme (Cacho et al., 2013) have been characterized as well. Of these aforementioned NR-PKS domains, the PT domain, which controls the first ring cyclization of the incipient reactive polyketide backbone to form the aromatic product, was shown to be useful in predicting the product of the NR-PKS. In the work by Li et al. (2010b), sequences of PT domains from characterized NR-PKS were subjected to phylogenetic analysis resulting in the grouping of the PT domains in accordance to their respective cyclization regiospecificity. The authors validated the model by demonstrating the previously uncharacterized PT domain of *An03g05440* from *A. niger*, predicted to catalyzed a C2-C7-type cyclization, does indeed perform the expected cyclization mode in a chimeric PKS *in vitro* system. Ahuja et al. (2012) has made similar observation for NR-PKSs in *A. nidulans.* Such PT domain analysis has facilitated the identification of viridicatumtoxin and TAN-1612 gene cluster (Chooi et al., 2010; Li et al., 2011).

Unlike NR-PKSs, HR-PKSs utilize the triad of the β-keto reductive domains ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains to introduce complexity in the incipient polyketide during each extension cycle. However, in contrast to NR-PKSs, there is a lack of in-depth bioinformatic studies to predict the products of uncharacterized HR-PKS. While more studies in deciphering the biosynthetic rules programmed within these tailoring domains is needed to construct an accurate model of predicting HR-PKS product, important clues can be garnered about the possible product of the HR-PKS using rudimentary bioinformatic analysis. For instance, conserved domain prediction analysis tools can help reveal the presence of in-*cis* polyketide tailoring domains in the HR-PKS protein sequence such as a *C*-methyltransferase domain that would indicate α-methylation during one or more polyketide extension cycle. This feature has been exploited for identification of the PKS gene encoding the biosynthesis of the tetraketide side chain of squalestatin in a genomic FNA library using *C*-methyltransferase domain sequence probe (Cox et al., 2004). High sequence similarity and proximity of phylogenetic relationship with a characterized HR-PKS could, to a limited extend, suggest that the unknown PKS produce similar chain length and structure (Kroken et al., 2003).

Fungi use division of labor between NR-PKS and HR-PKS to generate compounds with an aromatic portion and highly-reduced aliphatic portion respectively (Chooi and Tang, 2012). The linear aliphatic chains generated by HR-PKSs are often used as starter units for NR-PKSs. Compounds generated by such NR/HR two-PKS systems include the resorcylic acid family of compounds (Zhou et al., 2010; Xu et al., 2013b) and asperfuranone (Chiang et al., 2009). The linear aliphatic acyl chain may also be attached to an aromatic polyketide portion as an ester, such as in the case of azanigerone (Zabala et al., 2012) and chaetoviridin (Winter et al., 2012). Thus, such structural features, if present in the target SM, will be a useful tell-tale for predicting the target SM gene cluster as there is limited (one or two) such two-PKS gene cluster(s) in filamentous fungal genomes surveyed to date. SM gene clusters encoding hybrid polyketide-nonribosomal peptide compounds (Boettger and Hertweck, 2013), such as cytochalasins (Qiao et al., 2011; Ishiuchi et al., 2013), equisetin (Kakule et al., 2013), pseurotin (Maiya et al., 2007), and fusarin C (Niehaus et al., 2013) can be identified easily in a genome as well as there is usually one or two PKS-NRPS hybrid gene(s) in most ascomycete fungal genomes. Further analysis of the tailoring enzymes encoding in the vicinity of these PKS genes can help pinpointing the exact target SM gene cluster (see Tailoring Enzymes).

Non-ribosomal peptides

Unlike iterative fungal PKSs, most fungal NRPSs are modular. Like the bacterial counterparts, fungal NRPSs are assembly-line-like protein complexes arranged in functional units known as modules (Finking and Marahiel, 2004; Strieker et al., 2010). Each NRPS module is minimally comprise of three domains: an adenylation (A) domain that selects and activates the amino acid (aa) substrate of the module, a thiolation (T) or peptidyl carrier protein (PCP) domain that serves as a covalent tether for the aa substrate or the growing peptide chain and a condensation (C) domain that catalyzes the peptide bond formation. One notable characteristic of NRPS enzymology is the ability of NRPSs to incorporate non-proteinogenic aa into the NRPS product (Walsh et al., 2013). In addition, NRPSs typically follow the collinearity rule such that the substrate specificity, the number and the linear arrangement of the module within the assembly determines the composition of the NRPS product. Thus, in most cases, the candidate NRPS-encoding genomic scaffolds can be narrowed down easily by first matching the number of modules in the NRPSs to the number of aa residues linked by peptide bonds in the target non-ribosomal peptide product. The presence of the in-line tailoring domain in the NRPS assembly line such as an epimerization (E) or N-methylation (M) domains can further indicate that the NRPS product undergoes respective modification.

There are some exceptions to the co-linearity rule for some fungal NRPSs, in which certain domains or modules on these NRPSs are used iteratively. Notable examples are the fungal siderophore NRPSs, which involved the iterative use of one of the A domains (activating an identical aa residue more than once in a complete NRPS catalytic cycle). These NRPSs harbor additional T–C partial modules that extend the non-ribosomal peptide products beyond the number of complete A–T–C modules in the NRPSs (Schwecke et al., 2006; Johnson, 2008). There are also other non-canonical NRPSs, such as those that synthesize fungal cyclooligomer depsipetides (Glinski et al., 2002; Xu et al., 2008; Sussmuth et al., 2011) and the recently identified fungisporin NRPS from *P. chrysogenum* (Ali et al., 2014).

Pioneering studies by the Marahiel group led to the development of the A domain 10 aa code, which aided in prediction of the substrate specificities of adenylation of uncharacterized bacterial NRPSs via sequence alignment (Conti et al., 1997; Stachelhaus et al., 1999). Other algorithms that predict adenylation domain substrate specificity and online servers that utilized these algorithms were subsequently developed and are now in widespread use for preliminary bioinformatic characterization of NRPS genes (Challis et al., 2000; Röttig et al., 2011). However, the utility of the A domain 10 aa code and NRPS analysis software programs for prediction of fungal NRPS domain is still limited for fungi compared to bacteria, as there remains a lack of sequence-substrate specificity relationship for fungal NRPSs. Nonetheless, the new NRPSpredictor2 software incorporates A domain substrate specificity prediction for fungal NRPSs and can be a useful starting point (Röttig et al., 2011). Some progress have been made in this area for the A domain 10 aa codes for anthranilate (Ames and Walsh, 2010), α-keto acids (Wackler et al., 2012), and L-tryptophan (Xu et al., 2014a). Phylogenetic and structural analysis of fungal A domains may also yield some insights into the possible substrate of the A domain and the structural class of the final product (Ames and Walsh, 2010; Bushley and Turgeon, 2010). For instance, characterization of the anthranilate-activating fungal A domain in fumiquinazoline F biosynthesis (Ames and Walsh, 2010) led to the discovery of the gene clusters of fungal anthranilic acid-containing non-ribosomal peptides (Gao et al., 2011; Haynes et al., 2012, 2013).

Tailoring enzymes

Since genes for the biosynthesis of SMs are typically clustered together, the types of tailoring reaction the polyketide or non-ribosomal backbone undergoes can be surmised based on the type of functional domains encoded in vicinity of the PKS or NRPS gene, respectively. Essentially, if the backbone biosynthetic gene analysis narrowed down the target SM gene cluster to a couple of possibilities, the combination of tailoring enzymes that matches the SM structure would allow one to confidently pinpoint the right cluster. Tailoring enzymes involved in functional-group transfer such as methyltransferases, acyltransferases, prenyltransferases, and halogenases are especially helpful in correlating a gene cluster to its corresponding compound since there is little ambiguity on what type of reaction these enzymes catalyzes. As described in Section 4 below, the co-localization of multiple methyltransferase genes with an NR-PKS gene was critical toward the discovery of the griseofulvin gene cluster, while the presence of a prenyltransferase gene and a methyltransferase gene flanking an NR-PKS gene pinpointed the correct viridicatumtoxin gene cluster (Chooi et al., 2010). Genes encoding redox enzymes, such as flavoenzymes, cytochrome P450s, and non-heme iron oxygenases, can suggest that the product of the cluster undergoes oxidative transformation. However, their role in biosynthesis can be ambiguous as these enzymes can catalyze diverse redox reactions including hydroxylation, epoxidation, oxidative cleavage, and rearrangement. This is illustrated by our work in functional elucidation of the redox enzymes in viridicatumtoxin, tryptoquialanine, and echinocandin B pathways. As described below, some of the functions of these redox enzymes turned out to be quite surprising. Thus, caution is advised on inferring the function of genes for redox enzymes based on conserved domain analysis, especially in cases where there is no known characterized enzyme that share high sequence similarity with the enzyme of interest.

EXAMPLES ILLUSTRATING THE STRATEGIES FOR TARGETED SM GENE CLUSTER DISCOVERY

CASE STUDY A: GRISEOFULVIN

Griseofulvin is an antifungal polyketide made by several different *Penicillium* species (Frisvad and Samson, 2004). To identify the griseofulvin gene cluster, a curation of the PKS genes in *P. aethiopicum* was performed. Using a local BLAST search of the genome using an arbitrary KS as a query, 30 putative intact PKS genes were found within the genome of *P. aethiopicum*. Comparison of the PKS genes in *P. aethiopicum* and the closely related species *P. chrysogenum* revealed that the former contains six NR-PKS genes that are not orthologous to *P. chrysogenum* NR-PKS genes (van den Berg et al., 2008; Chooi et al., 2010). Since *P. chrysogenum* was not known to produce griseofulvin, it was inferred that one of the six non-orthologous NR-PKS genes in *P. aethiopicum* was responsible for the biosynthesis of griseofulvin (**Figure 3A**).

Based on the distinct structural features of griseofulvin, the presence of multiple methyltransferase genes as well as a chlorinase gene in the gene cluster encoding griseofulvin is expected (**Figure 4**). Using these search criteria, the *gsf* gene cluster, the candidate gene cluster for the biosynthesis of the compound, was found. Along with the expected three *S*-adenosyl-methionine (SAM) methyltransferase genes (*gsfB-D*) and a flavin-dependent halogenase gene (*gsfI*) flanking the NR-PKS gene *gsfA*, genes for the two redox enzymes GsfE and GsfF were also found within the cluster (**Figure 4**). Deletion of the *gsfA* gene led to loss of production of griseofulvin in *P. aethiopicum* and thus confirming the role of the *gsf* cluster in the biosynthesis of the compound (Chooi et al., 2010). A subsequent paper by our group revealed the function of the genes in the cluster as well as the regioselectivity of the *O*-methyltransferases GsfB, C, and D (Cacho et al., 2013). A combination of gene deletion studies and reconstitution of the enzymatic reaction in the same study also revealed the role of GsfF and GsfE in the grisan ring formation and cyclohexadienone reduction, respectively.

CASE STUDY B: VIRIDICATUMTOXIN

Viridicatumtoxin is another aromatic polyketide synthesized by *P. aethiopicum*. It contained a naphthacenedione core reminiscent of the core of the bacterial tetracyclines. Moreover, viridicatumtoxin also contained a cyclized geranyl moiety (**Figure 5**), indicative of the possible presence of a prenyltransferase gene within the cluster. As in the case of griseofulvin, viridicatumtoxin was not known to be produced by *P. chrysogenum* and likewise, one of the non-orthologous NR-PKS genes in *P. aethiopicum* was presumably responsible for the biosynthesis of the compound (**Figure 3A**). In addition to the aforementioned cyclized terpene group, viridicatumtoxin also contained an *O*-methyl group similar to what was found in griseofulvin. Using these two structural features as "landmarks," the viridicatumtoxin biosynthetic (*vrt*) gene cluster is localized on a scaffold (Chooi et al., 2010). Flanking the NR-PKS gene *vrtA* are genes encoding a farnesyl diphosphate synthase analog (geranyl diphosphate synthase), a prenyltransferase gene *vrtC* and a SAM-dependent *O*-methyltransferase gene *vrtF*; all three are in agreement with the two distinctive chemical features found in the compound. Deletion of the NR-PKS gene *vrtA* confirmed the role of the cluster in the biogenesis of the compound (Chooi et al., 2010).

Subsequent characterization of the prenyltransferase VrtC opened the doors toward the discovery of new fungal monoterpenoid biosynthetic gene cluster through genome mining (Chooi et al., 2012). In addition to the aforementioned genes, other genes encode five redox enzymes (*vrtE*, *vrtG*, *vrtH*, *vrtI*, and *vrtK*), a PLP-dependent threonine aldolase gene *vrtJ* and an acyl-CoA ligase gene *vrtB* were also found in the *vrt* gene cluster (Chooi et al., 2010). It was later found that VrtG and other related dimanganese-dependent thioesterase catalyze the Claisen-type cyclization in viridicatumtoxin and other fungal naphthacenediones (Li et al., 2011). Surprisingly, the cytochrome P450 VrtK, instead of a

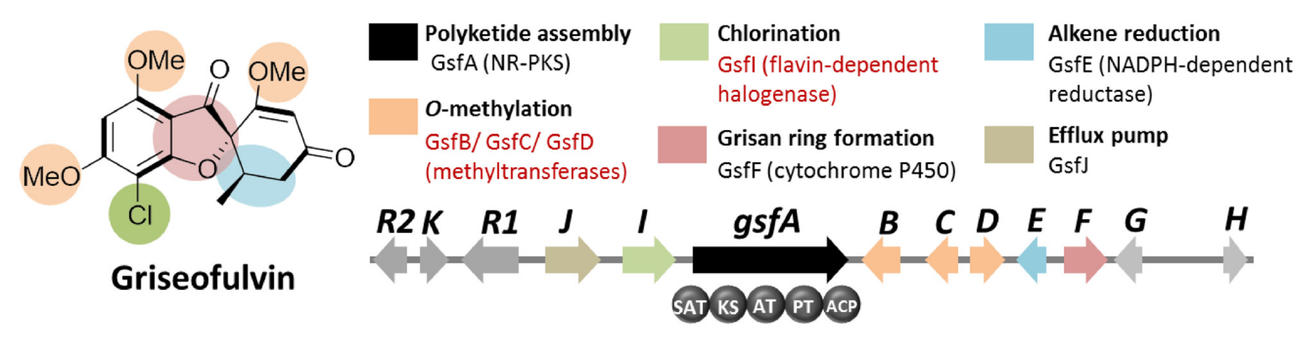

FIGURE 4 | Griseofulvin biosynthetic gene clusterin *P. aethiopicum*. Shown are the roles of the genes in the biogenesis of the compound. The non-reducing polyketide synthase (NR-PKS) gene *gsfA* is found via comparison of PKS genes in *P. aethiopicum* and *P. chrysogenum*. Two structural features, *O*-methylation and chlorination (in red text), were used as criteria to find the candidate gene clusters.

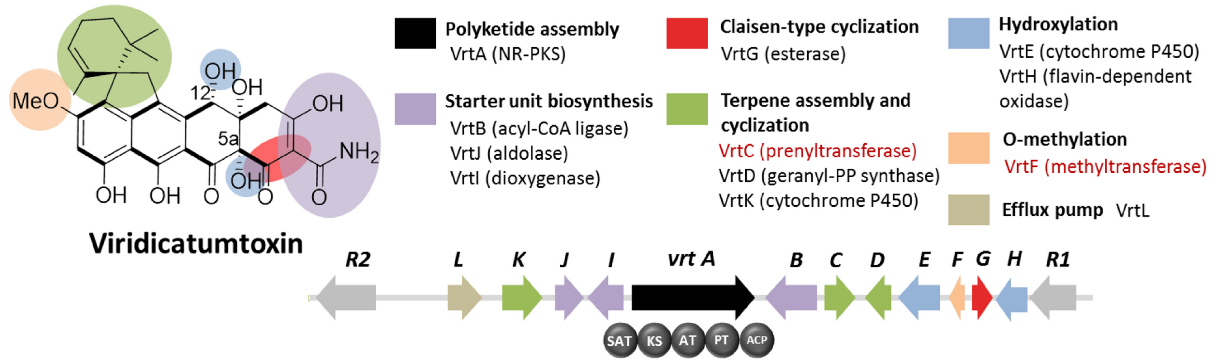

FIGURE 5 | Viridicatumtoxin biosynthetic gene cluster in *P. aethiopicum*. As with the case for the griseofulvin, the NR-PKS gene *vrtA* is non-orthologous to PKS genes in *P. chrysogenum*. The presence of the prenyltransferase gene *vrtC* and the *O*-methyltransferase gene *vrtF* (in red text) led to the identification of the gene cluster.

terpene cyclase, was shown to mediate the cyclization of the geranyl moiety found in viridicatumtoxin to afford a spirobicyclic structure fused to the tetracyclic core (Chooi et al., 2013b). Additionally, later studies revealed the role of VrtE and VrtH in the hydroxylation in the 5 and 12a positions in viridicatumtoxin, respectively (Chooi et al., 2013a,b).

CASE STUDY C: TRYPTOQUIALANINE

Tryptoquialanine (**Figure 6**) is a quinazoline-containing indole alkaloid produced by *P. aethiopicum*. It is structurally similar to a known tremorgenic mycotoxin from *A. clavatus* tryptoquivaline. Due to the presence of the non-proteinogenic aa anthranilic acid in the scaffold of the peptide, it was inferred that the compound is assembled by a NRPS (Ames and Walsh, 2010). Comparative bioinformatic analysis of the NRPS genes between *P. aethiopicum* and *P. chrysogenum* revealed the presence of 10 NRPS genes in *P. aethiopicum* that are non-orthologous to the NRPS genes in *P. chrysogenum* and were therefore candidates for tryptoquialanine biosynthesis (Gao et al., 2011). On the other hand, comparative bioinformatic analysis of the NRPS genes in *P. aethiopicum* with NRPS genes with the tryptoquivaline-producing *A. clavatus*, led to the identification of two NRPS genes in *P. aethiopicum* that are orthologous with *A. clavatus* NRPS genes but not with *P. chrysogenum* NRPS genes (**Figure 3B**). The two candidate NRPS genes, found on two separated scaffolds, encode a tri-module NRPS annotated as *tqaA* and a single-module NRPS annotated as *tqaB*. Primer walking and fosmid sequencing later revealed that both NRPS genes are co-localized in one segment of the genome. This demonstrates that cosmid library can be used to complement NGS-based targeted SM gene cluster discovery. Furthermore, the discovery of *tqa* cluster facilitated the identification of the tryptoquivaline biosynthetic genes, which are distributed on three separated genomic loci in *A. clavatus* (Gao et al., 2011).

Sequence analysis of TqaA revealed high overall shared identity with Af12080, which was previously implicated to be involved in the biosynthesis of fumiquinazoline A, a related quinazoline-containing alkaloid from *A. fumigatus* (Ames and Walsh, 2010*)*. Subsequent in-depth characterization of TqaA revealed its role in assembling fumiquinazoline F from anthranilate, L-tryptophan and L-alanine (Gao et al., 2012). The study also demonstrated the function of the terminal condensation domain in cyclization of the NRPS product and facilitated future genome mining of fungal cyclic non-ribosomal peptides. On the other hand, the

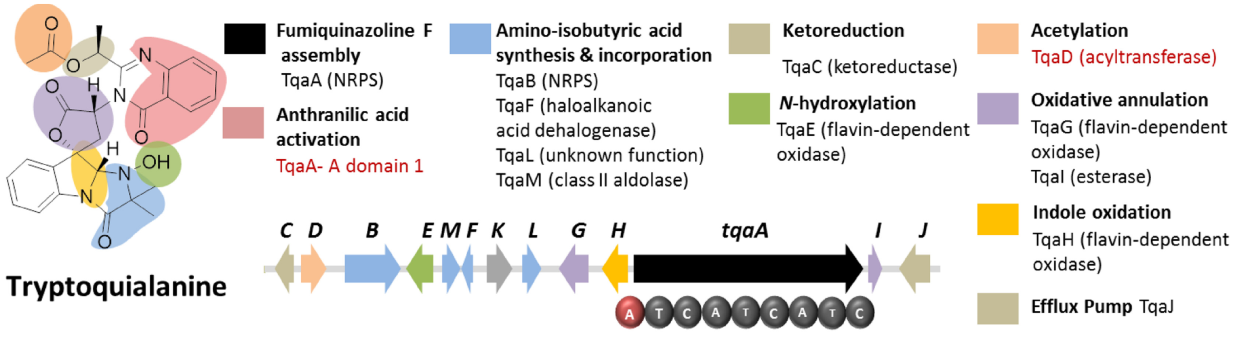

FIGURE 6 | Tryptoquialanine biosynthetic gene cluster in *P. aethiopicum*. The presence of acyltransferase gene *tqaD* and the anthranilic acid-activating A domain of TqaA (in red text) were clues to identification of the cluster.

second NRPS TqaB, based on the preliminary retrobiosynthetic analysis, presumably installs the 2-amino-isobutyric acid portion of the imidazolindolone pendant group (Gao et al., 2011). Interestingly, this reflects the arrangement of the aa building blocks, where anthranilic acid, L-tryptophan and L-alanine make up the main peptide chain while the fourth building block 2-amino-isobutyric acid is added to the modified L-tryptophan side chain.

Figure 6 shows the other genes involved in the biosynthesis of tryptoquialanine (Gao et al., 2011). Based solely on the conserved domains of the enzymes encoded by the *tqaA* genes, only the function of TqaD in the installation of the acetyl group in the tryptoquialanine can be confidently assigned. The function of the remaining genes in the cluster could only be assigned by targeted deletion of individual genes (Gao et al., 2011). This revealed that *tqaE* and *tqaH* are responsible for *N*-hydroxylation and the 2,3-epoxidation of the pendant indole ring of tryptoquialanine, respectively. *tqaC* encodes a short-chain dehydrogenase that reduces the ketone in tryptoquialanone. Meanwhile, *tqaG* and *tqaI* were demonstrated to encode enzymes that mediate the formation of *N*-deoxytryptoquialanone. Finally, knockout of *tqaL* and *tqaM* demonstrated that both genes are required for the biosynthesis of the 2-amino-isobutyric acid.

CASE STUDY D: ECHINOCANDIN B

Echinocandin B is a cyclic lipopeptide made by the ascomycetes *E. rugulosa.* Due to their efficacy against a broad range of *Candida* species, semisynthetic derivatives of natural echinocandins such as anidulafungin, micafungin, and caspofungin are currently in use as frontline treatment against invasive candidiasis (Kett et al., 2011). Structurally, echinocandin B is consist of six aa: (4*R*, 5*R*)-4,5-dihydroxy-L-ornithine, two units of L-threonine, (3*R*)-3-hydroxy-L-proline, (3*S*, 4*S*)-3,4-dihydroxy-L-homotyrosine, and (3*S*, 4*S*)-3-methyl-4-hydroxy-L-proline. In addition, a linoleic acid is appended to the cyclic peptide comprising of the six-aa (**Figure 7**). As with the examples given above, genome mining for the echinocandin biosynthetic gene cluster is initiated with comparative genomic analysis; in this case, between the NRPS genes in *E. rugulosa* and *A. nidulans* A4 (von Dohren, 2009; Cacho et al., 2012). Since *A. nidulans* A4 was not known to produce echinocandin B, NRPS genes orthologous to both species can be eliminated as candidates for echinocandin synthetase. Annotation of the unique NRPS genes in *E. rugulosa* revealed that only one of the ten non-orthologous NRPS genes encode for a six-module NRPS (one NRPS module is minimally consist of a C, A and T domain), which was the required number for the assembly of echinocandin B based on the collinearity rule (**Figure 3C**). In addition, the six-module NRPS, annotated as EcdA, contained an additional C-terminal condensation domain that is expected to catalyze the cyclization of the full-length peptide product (Gao et al., 2012), in accordance with the cyclic nature of the compound.

In addition to the NRPS gene *ecdA*, other genes found in the cluster in agreement to the chemical features of the compound were also found in the cluster such as the acyl-CoA ligase gene *ecdI* and the redox enzymes *ecdG, H* and *K* (**Figure 7**; Cacho et al., 2012). The presence of *ecdI*, in conjunction with its co-localization with the NRPS gene *ecdA*, was highly indicative that the product of the *ecd* gene cluster is a lipopeptide since EcdI, based on its predicted conserved domain, belongs to a family of enzyme that can convert a carboxylic acid to the more reactive acyl-CoA. Enzymatic characterization of EcdI verified its role in the activation and transfer of linoleic acid to the N-terminal thiolation domain of EcdA.

While the presence of the multiple genes for redox enzymes in the locus suggested that the product of the *ecd* gene cluster undergoes a plethora of oxidative modification steps, the low shared identity of the protein sequences of EcdG, H and K with of then characterized protein in sequence databases hindered the deciphering of the exact roles of the enzymes solely by bioinformatic analysis. Later gene knockout and enzymatic reconstitution study revealed the regioselectivity of EcdG toward the C3

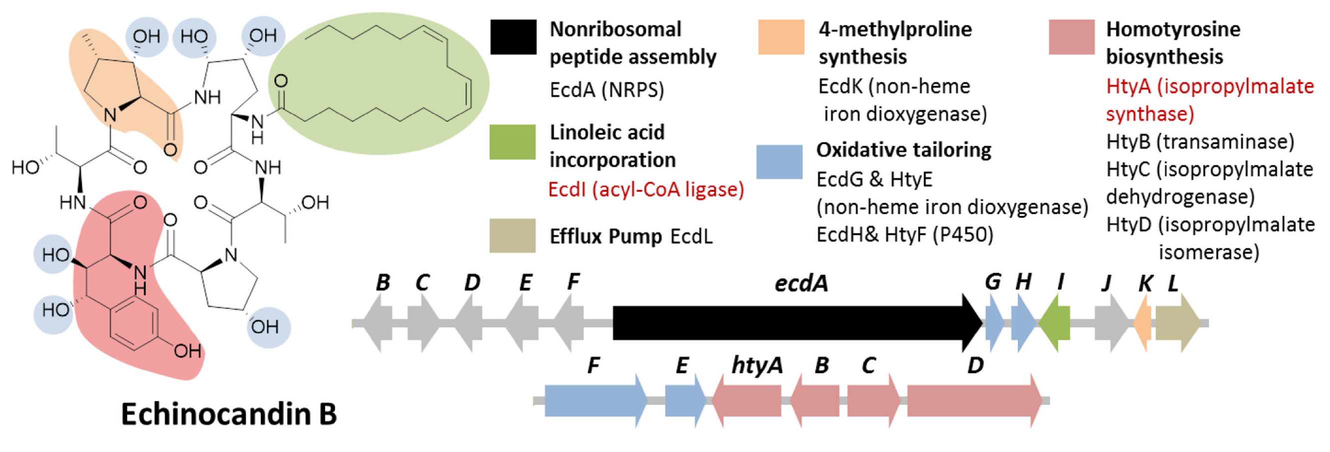

FIGURE 7 | Echinocandin B biosynthetic gene cluster in *E. rugulosa*. The gene cluster was found in two loci (*ecd* and *hty*). The *ecd* cluster contained a six module NRPS gene *ecdA* and an acyl-CoA ligase gene *ecdI* (in red text), indicating that the gene cluster is involved in the biosynthesis of a lipo-hexapeptide SM. The separate homotyrosine biosynthesis *hty* gene cluster at a different locus was located by searching for an isopropylmalate synthase (IPMS) homolog (in red text).

of L-homotyrosine and regioselectivity of EcdH toward C4 and C5 of L-ornithine in echinocandin B biosynthesis (Jiang et al., 2013). On the other hand, EcdK was revealed to perform the two-step oxidation of L-leucine to afford 5-hydroxy-L-leucine and γ-methyl-glutamic acid-γ-semialdehyde en route to the biosynthesis of (4*R*)-*R*-methyl-L-proline (Jiang et al., 2013).

Notably missing in the *ecd* gene cluster, however, were the genes required for the biosynthesis of L-homotyrosine, another non-proteinogenic aa building block of echinocandin B. Based on previous labeling studies on L-homotyrosine biosynthesis demonstrated that the latter originated from acetate and L-tyrosine (Adefarati et al., 1991). It was also proposed that the pathway is analogous to that of L-leucine biosynthesis, the first step of which is catalyzed by isopropylmalate synthase (IPMS). Thus, we predicted that a homolog of IPMS was involved in the first step of the biosynthesis of L-homotyrosine and found by genome mining the IPMS-like gene *htyA* in the *E. rugulosa* genome that is non-orthologous to the IPMS gene in *A. nidulans* A4 genome. Subsequent deletion of *htyA* implicated its role in the biosynthesis of L-homotyrosine (Cacho et al., 2012). Flanking the *htyA* gene were additional genes presumably involved in the biosynthesis of L-homotyrosine (*htyB*, *C*, and *D*) as well as two additional oxidase genes *htyE* and *htyF*.

Shortly after the studies on echinocandin B biosynthesis were reported, the biosynthetic gene cluster for the structurally-related pneumocandin B was described (Chen et al., 2013). The pneumocandin biosynthetic gene clusters contained gene homologs echinocandin gene cluster include *ecdA*, *ecdI*, cytochrome P450 genes (e*cdH* and *htyF*), *ecdG*, *htyE*, and *ecdK* and the L-homotyrosine biosynthetic genes. Interestingly, whereas the echinocandin B biosynthetic genes are located at multiple loci, the pneumocandin biosynthetic genes are situated in a single gene cluster. In addition, the pneumocandin biosynthetic gene cluster also contained a HR-PKS gene for the biosynthesis of the 10,12-dimethyl-myristic acid and an additional 2-oxoglutarate, non-heme iron-dependent oxygenase gene (GLAREA10042) which presumably is involved in the hydroxylation of L-glutamine at C3; both corresponding to the structural features of pneumocandin B but not of echinocandin B. The recent availability of several fungal genomes that encode production of the echinocandin family of lipopeptides has allowed bioinformatics comparison of these gene clusters and enables functional prediction of the unique pathway genes in correspondence to the unique structural features of individual echinocandin analogs (Bills et al., 2014).

CONCLUSION AND OUTLOOK

Next-generation sequencing technologies have significantly accelerated the process of targeted SM gene cluster discovery. The whole process from extraction of genomic DNA of the producing-fungus, sequencing, to initial identification of the target SM gene cluster can occur in one to three months. Using the described strategies, we can often identify the target SM gene cluster in the genome, within 2 weeks from receiving the sequencing data, with high accuracy. Compared to the traditional genomic library screening approach, which can take years beginning from the process of identifying the first biosynthetic gene to finally obtaining the complete gene cluster, such pace of discovery is unimaginable as recent as half a decade ago. The more arduous task ahead is the subsequent gene cluster verification and characterization as fungal genetic transformation protocols need to be developed for individual organisms. The development of heterologous expression systems and *in vitro* recombinant enzyme characterization methods greatly complement the genetic approach and can provide biosynthetic insights unattainable by traditional knockout approach. More and more fungal natural product research labs are expected to take advantage of NGS technology for identifying the gene cluster encoding the biosynthesis of SMs of interest. The increasing throughput and lowering cost for NGS would also encourage the simultaneous sequencing of multiple strains that produce a same family of compounds, which enables the use of powerful comparative genomics tools in the gene cluster identification and promotes combinatorial biosynthesis of fungal natural products.

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The importance of mass spectrometric dereplication in fungal secondary metabolite analysis

Kristian F. Nielsen and Thomas O. Larsen*

Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark

Edited by:
Nancy Keller, University of Wisconsin–Madison, USA

Reviewed by:
Tim S. Bugni, University of Wisconsin–Madison, USA
Nicholas Oberlies, University of North Carolina at Greensboro, USA

Correspondence:
Thomas O. Larsen, Department of Systems Biology, Technical University of Denmark, Soeltofts Plads B221, Kongens Lyngby DK-2800, Denmark
e-mail: tol@bio.dtu.dk

Having entered the *Genomic Era*, it is now evident that the biosynthetic potential of filamentous fungi is much larger than was thought even a decade ago. Fungi harbor many cryptic gene clusters encoding for the biosynthesis of polyketides, non-ribosomal peptides, and terpenoids – which can all undergo extensive modifications by tailoring enzymes – thus potentially providing a large array of products from a single pathway. Elucidating the full chemical profile of a fungal species is a challenging exercise, even with elemental composition provided by high-resolution mass spectrometry (HRMS) used in combination with chemical databases (e.g., AntiBase) to dereplicate known compounds. This has led to a continuous effort to improve chromatographic separation in conjunction with improvement in HRMS detection. Major improvements have also occurred with 2D chromatography, ion-mobility, MS/MS and MS^3, stable isotope labeling feeding experiments, classic UV/Vis, and especially automated data-mining and metabolomics software approaches as the sheer amount of data generated is now the major challenge. This review will focus on the development and implementation of dereplication strategies and will highlight the importance of each stage of the process from sample preparation to chromatographic separation and finally toward both manual and more targeted methods for automated dereplication of fungal natural products using state-of-the art MS instrumentation.

Keywords: liquid chromatography, metabolomics, mass spectrometry, diode array detection, dereplication

INTRODUCTION

Filamentous fungi are prolific producers of secondary metabolites (SM) of importance to humankind. Useful fungal metabolites include drugs, food colorants, feed additives, industrial chemicals, and biofuels (Bills, 1995; Bode et al., 2002; Firn and Jones, 2003; Butler, 2004). Fungi are also known for their negative consequences as contaminants of food and feed due to the production of mycotoxins which can be cytotoxic, immunotoxic, estrogenic, or carcinogenic (Miller, 2008; Shephard, 2008). Fungi can also cause invasive human infections, especially in immuno-compromised individuals (Larsen et al., 2007).

Having entered the *Genomic Era* it is now clear that the biosynthetic and metabolic diversity potential of filamentous fungi, is still vast, due to the presence of many cryptic SM encoding gene clusters. This is particularly true for non-model organisms. The abundance of cryptic gene clusters has resulted in the use of many strategies to stimulate gene expression aimed toward the discovery of novel bioactive compounds and characterization of their biosynthetic pathways (Bode et al., 2002; Bergmann et al., 2007; Bok et al., 2009; Schroeckh et al., 2009; Sarkara et al., 2012; Sørensen et al., 2012; Droce et al., 2013; Sorensen and Sondergaard, 2014). Uncovering the full chemical potential of any micro-organism is a challenging exercise. Firstly, expression of metabolites related to a given gene cluster is highly regulated and may only be expressed under special condition, and furthermore each cluster may be responsible for more than 10 end products and a similar number of stable intermediates also present in detectable concentrations (Degenkolb et al., 2006; Chiang et al., 2010; Nielsen et al., 2011b; Ali et al., 2014; Holm et al., 2014; Petersen et al., 2015). In addition, crosstalk between biosynthetic pathways can result in compounds that are products of more than one gene cluster (Nielsen et al., 2011b; Tsunematsu et al., 2013). Overall this results in an extremely complex pool of diverse small organic molecules to identify by chemical analysis, especially when considering a single species of *Aspergillus* and *Fusarium* contains 50–80 gene clusters (Sanchez et al., 2012; Lysoe et al., 2014).

It is common for natural products to have identical elemental composition (up to 130 for some terpenes) making unambiguous identification very challenging. As such, without access to authentic standards, elemental composition alone – obtained by high resolution mass spectrometry (HRMS) –is not enough to unambiguously identify compounds (Nielsen et al., 2011a; El-Elimat et al., 2013). Consequently, fast identification of previously described compounds without reference standards – known as dereplication – is a very challenging task. As reference standards of most secondary metabolite standards are not available, fast dereplication is vital for progress in both drug discovery and pathway elucidation projects (Cordell and Shin, 1999; Dinan, 2005; Zhang, 2005; Bitzer et al., 2007; Feng and Siegel, 2007; Stadler et al., 2009; Zengler et al., 2009; Nielsen et al., 2011a). Dereplication is most often done by Ultra high performance liquid chromatography (UHPLC) coupled to diode array detection (DAD) and HRMS, in combination with database searching. Proper dereplication strategies ensures that time consuming and costly efforts with isolation and subsequent NMR based structure elucidation can be focused solely on novel compounds (Cordell and Shin, 1999) or that re-isolation of known compounds can be done in an efficient way based on functional groups and thus fewer steps (Månsson et al., 2010). This paper will highlight important issues and recent approaches toward fast and reliable dereplication of fungal NPs primarily based on UHPLC-DAD-HRMS techniques.

THE IMPORTANCE OF SAMPLE PREPARATION

The chemical diversity which is found both between and within the many classes of secondary metabolites (e.g., polyketides (PKs), non-ribosomal peptides (NRPs) and terpenoids), makes it impossible to quantitatively extract all secondary metabolites from a given fungus using a single procedure. Consequently, sample preparation is an important aspect of secondary metabolite profiling and will, no matter the choice of method, lead to bias toward certain types of compounds. To avoid extracting too many polar media components, primary metabolites and sugars an organic extraction is needed using one or more water-immiscible solvents such as ethyl acetate (EtOAc; Nielsen et al., 2011a; Stadler et al., 2014), dichloromethane (DCM; Abdel-Mawgoud et al., 2008), or 1-butanol (Yokota et al., 2012). The latter is efficient for the extraction of lipopeptides but has a high boiling point (118°C), thus requiring both N_2 and heating for evaporation. The pH is vital for an organic extraction, as ionizable molecules will be extracted into the organic phase to a much higher degree in their neutral form than when in a charged state. As almost 50% of all described fungal NPs (Månsson et al., 2010) contains an acidic moiety, a low pH extraction is necessary in most cases but can be supplemented with a neutral extraction (e.g., for stability reasons). Solvents such as ethers (highly flammable and able to form explosive peroxides) as well the carcinogenic and environmental damaging chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) are being phased out. Methanol and ethanol are also efficacious but, due to their high polarity, also results in the extraction of large quantities of salts and polar interfering substances which can quickly clog analytical HPLC columns. On the other hand, methanol/ethanol extracts can also contain highly non-polar waxes, sterols and triglycerides, however, these can usually be flushed out of the column at 80°C with a mixture of acetonitrile-isopropanol for 1 h. We have experienced that injection of 1 μl of crude methanol extracts from marine media clogged an LC-MS electrospray source after analyzing only four extracts.

For extraction with EtOAc an essential process is the centrifugation of the 2-phase system as hard as possible then leaving behind the common interfacial agglomeration containing cells. Drying EtOAc extracts with anhydrous Na_2SO_4 can also result in cleaner extracts as EtOAc can contain up to 8% water. A major pitfall in sample preparation is the possibility of unwanted chemical reactions taking place. For example, alcohols can form esters with carboxylic acids and lactones (e.g., in homoserine lactones, rubratoxins, statins) under acidic conditions and can also catalyze various intramolecular changes (Rundberget et al., 2004). Acid catalyzed reactions can be especially problematic when evaporating organic extracts, since the lower volatility of acids such as formic and acetic acids will result in upconcentration when using volatile solvents like EtOAc and DCM, especially in the presence of residual water. Examples we have observed include; up to 50% loss of patulin during evaporation from EtOAc (Boonzaaijer et al., 2005); loss of trichothecenes prior to derivatization for GC-MS analysis (Langseth and Rundberget, 1998); up to 80% loss of fumonisins due to binding to the silanol groups of non-derivatized glass with up to 80% lost (Nielsen et al., 2015). We have used a fast extraction procedure to minimize the risk of studying artifact peaks, this procedure involves extraction into acetonitrile-water (1:1; in a ultrasonication bath or a beadbeater for circa 10 min), followed by centrifugation and subsequent transfer of the centrifugate directly to an auto-sampler compatible vial for analysis (Mogensen et al., 2011).

In some cases samples need to be purified on small solid phase extraction (SPE) columns to remove chromatography impairing lipids and phospholipids (Degenkolb et al., 2006; Pucci et al., 2009) or abundant but common secondary metabolites (e.g., as is *Stachybotrys*; Hinkley and Jarvis, 2000; Andersen et al., 2002). But SPE can also be used to simplify very complex extracts into several fractions. For example, ion exchange SPE can be used to separate an extract into acidic, neutral and basic analytes (Månsson et al., 2010), which may resolve new peaks and simplify the mass spectra. For an orthogonal separation to the common reversed phase analytical separation, we prefer diol or amino-propyl normal phase SPE (Bladt et al., 2013; Petersen et al., 2015) as we have found them to better separate compound classes compared to, e.g., pure silica.

ANALYTICAL SEPARATION

When grown on rich growth media fungi often produce in excess of 100 SMs (Stadler et al., 2007, 2014; Bertrand et al., 2013; Kildgaard et al., 2014; Klitgaard et al., 2014; Wolfender et al., 2015). With such complex mixtures it is practically impossible to acquire enough separation power to provide complete resolution of all individual metabolites and media components and allow for subsequent spectroscopic analysis. On balance, by far the best choice for separation is reverse phase (RP) chromatography, since it's polarity is well suited to most SMs, especially with the emergence of more polar phases such as pentafluoro phenyl, biphenyl, phenyl (Kildgaard et al., 2014; Wolfender et al., 2015) and also columns with various embedded groups (Euerby and Petersson, 2005). Many vendors have introduced improved the solid core particles of their stationary phase, ensuring no diffusion through the particle center (e.g., Poroshell, Kinetex, Ascentis, and Cortecs). Furthermore, hybrid chemistry particles (e.g., Waters BEH) provide sharper peaks as well as reduced tailing due to secondary interactions at a wide pH range. Performing LC at low pH is statistically preferably as 50% of all secondary metabolites contain an acidic moiety, while ca. 10% have a basic moiety (Månsson et al., 2010). Since sharper peaks are obtained from the non-charged state of SMs, low pH is preferable in RP chromatography. Undoubtedly the most prevalent buffers/acidifiers used are formic and acetic acid (Varga et al., 2013). Although triflouroacetic acid (TFA) is commonly utilized to lower the pH, this has the consequence of significantly suppressing negative ionization MS detection. We have chosen formic acid as, in addition to its lower pK_a, it suppresses microbial growth in solvent reservoirs to a greater extent than acetic acid. We have found that, in general, formic acid appears to give sharper peaks at lower concentrations than acetic acid, and thus lower suppression of the UV signal. Although the use formic acid to adjust the pH is generally satisfactory, for some compounds it results in poor ionization, for example type B trichothecenes (Sulyok et al., 2006). Formic acid may also not be optimal if the majority of analysis is performed in negative mode – in which case acetic acid performs better – or if the analysis is performed under non-acidic conditions (Sulyok et al., 2006; Zhang et al., 2012).

Comprehensive 2D LC (Dugo et al., 2009; Chen et al., 2012) have recently been introduced by several of the UHPLC manufactures and introduced some interesting possibilities for orthogonal separation through the use of a long high-polarity HPLC column (20–30 min run) in the first dimension and a short highly retentive small particle C_{18} in the second dimension (20–30 s runs).

Whereas RP chromatography is usually a suitable choice for the separation of the wide range of middle-polarity secondary metabolites mainly produced by fungi, smaller very polar compounds are usually poorly retained. As a result of the lack of retention this chemical window has been exploited to a much lesser degree due to difficulties with analysis. To better investigate compounds in this high polar and/or ionic window (compounds with LogD < -3) techniques such as HILIC (Sørensen et al., 2007; Creek et al., 2011), high-pH ion-chromatography (IC; van Dam et al., 2002; Kvitvang et al., 2014) or ion-pair chromatography (Lebrun et al., 1989; Werner, 1993; Magdenoska et al., 2013) are needed to sufficiently retain the analytes. Common to all these techniques is that the window of separation is much narrower than for the extremely versatile RP, thus requiring more optimization. A major benefit of these techniques can be found in that many NP chemists only examine organic extractable compounds thereby overlooking many interesting high polar compounds (Cannall, 1998; Dufresne, 1998; Shimizu, 1998; Månsson et al., 2010). Other alternative choices for retention of polar compounds include anion or cation mixed mode columns (Apfelthaler et al., 2008). Examples such as the Dionex Trinety or the PrimeSep columns can retain anionic and/or cationic analytes strongly while also performing conventional RP chromatography (Lammerhofer et al., 2008).

Though LC is the dominant technique for the separation of compounds from complex mixtures there are several alternatives. For volatile compounds such as terpenes or compounds (e.g., acids) that can be derivatized to heat stable analytes GC-MS can be an efficient choice. GC-MS can be beneficial both due to the superior separation (10-100 higher peak capacity) but also due to the reproducible EI^+ ionization that can be employed. Furthermore, technological improvements such as comprehensive GC × GC are becoming more affordable and dedicated TOFMS detectors for GC are being developed delivering accurate mass (<5 ppm accuracy) as well as GC-APCI interfaces for molecular mass identification.

Most recently, significant technological advances have occurred in the previously neglected field of supercritical fluid chromatography (SFC). Improvements have been made to SFC with better columns and much enhanced reproducibility by both Waters and Agilent. Together with novel column systems, many for enantiomeric separation, we foresee that this technique will become very valuable for analysis of SMs from the mid-polar region to the extremely non-polar (e.g., waxes and sterol esters). The technology can now be used with UV detection and is especially well suited for APCI.

DIODE ARRAY DETECTION

Ultra high performance liquid chromatography MS is often combined with the relative inexpensive DAD as UV/Vis spectra provide information on chromophores which can be used for database searching (Hansen et al., 2005; Larsen et al., 2005; Bitzer et al., 2007; de la Cruz et al., 2012). More often DAD is used as second criteria after a MS based search, as neither AntiBase nor Dictionary of Natural Products provides a direct UV/Vis search from UV/Vis max data nor do they provide whole spectra. Many secondary metabolites, such as non-reduced PKs and NRPs contain conjugated chromophore systems. As such many metabolites have distinct UV-spectra (**Figure 1**) that are, in some cases, indicative of the basic skeleton of the given compound class, for example, quinazolines and γ-naphtopyrones produced by many *Aspergilli* and *Penicillia* (Larsen et al., 1999; Nielsen et al., 2009). Consequently, UV/Vis spectral chromophores can provide a means to differentiate compounds with the same elemental composition and can be highly valuable in dereplication for exclusion or confirmation of candidates during a database search. Though often useful, many compounds lack or contain few conjugated double bonds and only giving rise to one absorption band. Examples of compounds without diagnostic UV/Vis spectra include trichodermin, patulin, and deoxynivalenol (**Figure 1**). In cases such as these the UV/Vis spectrum is of little or no use. Other compounds, such as mycophenolic acid, contain a highly diagnostic chromophore (**Figure 1**), with three absorption maxima. Despite this informative finger print, compounds like mycophenolic acid also require MS verification due to the existence of at least 55 known compounds containing the 5,7-dihydroxy-methylphtalide core of mycophenolic acid and four with the cichorine core, as revealed by a substructure search in AntiBase 2012.

Analysis of UV/Vis spectra can also be very powerful for biosynthetic pathway elucidation studies, since compounds which belong to the same pathway often contain the same chemical scaffold and thereby chromophore system. This phenomenon was recently demonstrated during elucidation of the yanuthone biosynthetic pathway (Holm et al., 2014). Furthermore, DAD can be very useful for detection and discovery of compounds of the same type across species and genera, especially in cases where a certain class of compound has shown great promise as a new potential drug lead. Hansen et al. (2005) have developed an algorithm X-hitting for automated comparison of full UV spectra from LC-DAD analysis against a UV-library of standards as well as spectra across samples. This allows for both the identification of known compounds as well as new compounds with UV spectra similar to known compounds (Hansen et al., 2005; Larsen et al., 2005). Altogether DAD is a cheap and complementary spectroscopic technique easily performed in combination with full scan MS. Interestingly a recent software made in the

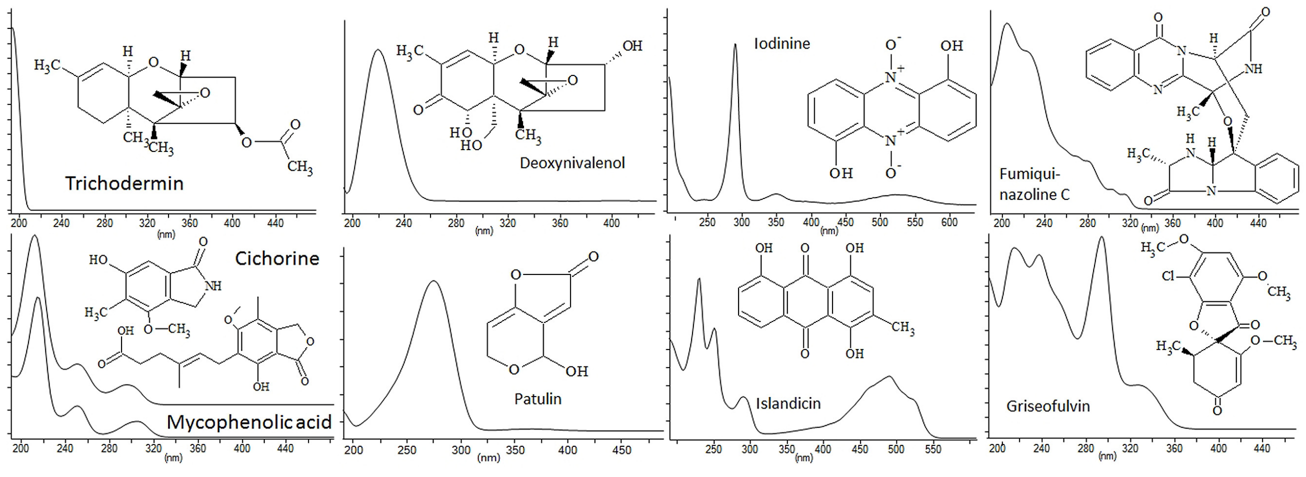

FIGURE 1 | UV/Vis spectra (pH 3) and structures of some common fungal natural products illustration the diversity in absorption range depending on the number of conjugated double bonds present in the molecules.

same R environment[1] as the popular XC-MS package (*vide infra*) for LC-MS, is now also available for LC-DAD (Wehrens et al., 2014).

Other (non-MS) detectors which are often used in combination with LC are evaporative light scattering detectors (ELSD; Bitzer et al., 2007; Yang et al., 2014) and the Corona cad detectors. These detectors give more quantitative information about the amount of compounds eluting from the LC unit, in contrast to MS detectors which are highly biased.

MASS SPECTROMETRY

Today all MS instrumentations coupled to LC rely on atmospheric pressure ionization (API) techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photo ionization (APPI). These are all soft ionization techniques, as such only limited fragmentation fungal SMs takes place at standard ionization conditions compared to what is seen when using hard ionization such as electron impact (EI) which is used for gas chromatography. Although limited fragmentation is usually observed, the default ion-source settings from many of the LC-MS manufactures, especially for ESI, cause quite extensive fragmentation to occur for very low molecular mass SMs (<300 Da) on most LC-MS systems (Nielsen et al., 2011a; Klitgaard et al., 2014; Rasmussen et al., 2014). Thus can the task of identifying the molecular mass in an API generated spectra become quite troublesome as a mix of fragment ions adducts and dimeric and double charged ions may be generated (**Figure 2**; Nielsen et al., 2011a; Kildgaard et al., 2014; Klitgaard et al., 2014). Consequently, the task of identifying the molecular mass in API generated spectra comes down to interpretation of the adduct patterns that can be seen for the different types of secondary metabolites (**Figure 2**).

Correct assessment of the molecular mass (M) is often not trivial as in-source fragmentation and adduct formation can lead to incorrect assignment of ions such as $[M+Na]^+$, $[M+NH_4]^+$ or $[M\text{-}H_2O+H]^+$ as $[M+H]^+$ (Nielsen et al., 2011a) or due to the low abundance of the $[M+H]^+$ ion due to poor ionization under the selected conditions. A combination of both ESI^+ and ESI^- ionization will assign the mass unambiguously, while just one polarity will only assign the mass unambiguously, when several adducts in a given mass spectrum all points toward the same molecular mass (Nielsen and Smedsgaard, 2003; Nielsen et al., 2011a). When the mass, or even better elemental composition, has been determined, this information can be searched against the literature to determine whether the compound is likely known or novel. *AntiBase* is currently the most suitable database for dereplication of fungal secondary metabolites as it only contains microbial metabolites. The 2014 version contains 43000 recorded compounds, including ~18000 fungal and 21000 bacterial compounds. *Dictionary of Natural Products* additionally contains a large number of plant metabolites (some of which are produced by endophytic fungi and not the plant themselves), thus is this database a valuable addition to AntiBase. The databases Scifinder/CAS and RSC/Chemspider are highly biased toward synthetic compounds, and of less value. Furthermore hits in these databases cannot be sorted by organism type (Lang et al., 2008; Nielsen et al., 2011a). For Marine derived fungi MarineLit (Blunt et al., 2008) may be of interest and now available via RSC/Chemspider[2].

In recent years data handling software has become the most crucial component of successful dereplication. Modern UHPLC-MS instruments can provide 50–200 data files per day with MS and tandem MS data, this can take many hours if not days per data file to manually handle, thus mandating more automated dereplication approaches. As with manual dereplication, the indisputable first step in automated dereplication is determination of the elemental composition of the individual compounds in the sample. For this accurate mass is vital. Depending on the mass range and

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[1] http://www.r-project.org/

[2] http://pubs.rsc.org/marinlit/

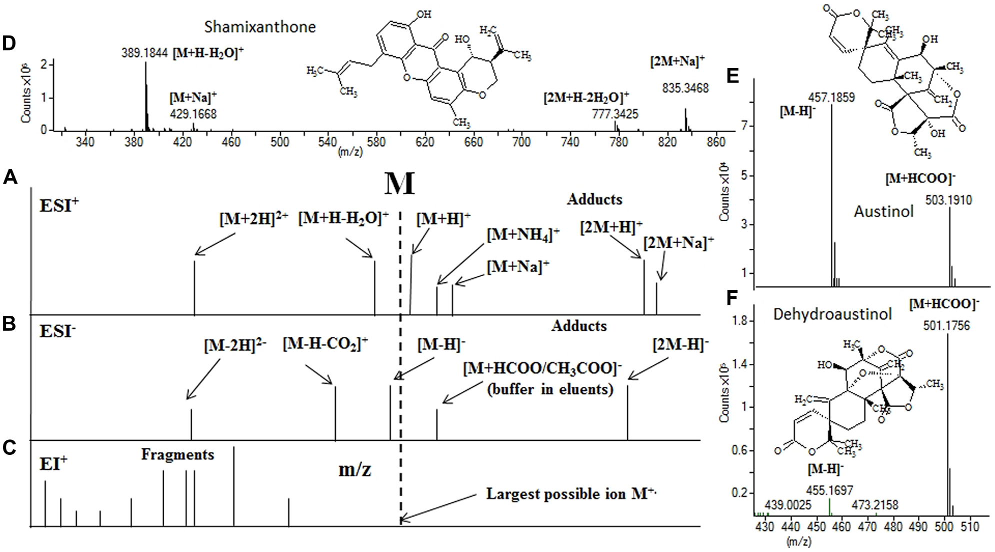


FIGURE 2 | Position of ions relative to the monoisotopic mass, of ESI$^+$ (A), ESI$^-$ (B) and EI$^+$ (C) along with adduct ions, and 3 example ESI spectra. In shamixanthone **(D)** the $[M+H]^+$ ion is absent but the molecular mass can easily be assigned using the Δ40 (39.9925) between the $[M+Na]^+$ and the $[M+H-H_2O]^+$. In **(E,F)** the big difference of formate adduct intensity on the austinols (same run, elutes 0.4 min in-between) is illustrated. For instrument settings see Kildgaard et al. (2014).

the instrumental accuracy the elemental composition can often be determined unambiguously up to 300 Da at an accuracy of 5 ppm, while 1–2 ppm is required for compounds with masses up to 5–600 Da (Kind and Fiehn, 2007; Nielsen et al., 2011a). When accurate isotope ratio assessment is also reliable, it is possible to eliminate close lying elemental composition candidates with different numbers of carbon atoms (Bitzer et al., 2007; Lehner et al., 2011; Nielsen et al., 2011a; Bueschl et al., 2012; Sleno, 2012; Xian et al., 2012; Nagao et al., 2014).

Today the market is dominated by time of flight (TOF), Orbitrap, and Fourier transform ion cyclotron (FT-ICR) based mass spectrometers. TOF instruments deliver both superior isotopic ratios as well as an unmatched scan speed providing ample time for concurrent MS/MS experiments or the possibility of ultra-fast separation runs. A serious drawback of TOF based instruments is overloading of the detector leading to poor mass accuracy, which was very pronounced on the earlier TDC type detectors. Even for the newest 4 GHz ADC detectors overloading is still a problem and TOF related software needs to limit analysis to scans from the non-overloaded parts of the chromatographic peak (Kildgaard et al., 2014). Overall this results in poorer mass resolution than the two other common types of HRMS instruments. The FT-ICR technology provides an unsurpassed mass accuracy and resolution, for example an FT-ICR can easily resolve the A+2 ion of sulfur containing ions into the $^{12}C_x{}^{13}C_2{}^{32}S$ and $^{12}C_{x+2}{}^{34}S$ isotopomers. Unfortunately FT-ICR instruments suffer from high running costs for magnets, high acquisition price, as well as slow scan capability. The practical limitations of FT-ICR instruments have made Orbitrap type instruments much more popular as they do not require the expensive cooling of the superconducting magnets. In contrast to TOF based instruments Orbitraps do not suffer from the detector overload and needs less frequent mass calibration, though they deliver significantly slower scan speeds. The linear ion-trap (LIT)-orbitrap types can provide MS^n for fragmentation trees due to a dual detector providing both high and low resolution spectra, while Quadrupole (Q)-Exactive provides MS/MS as a QTOF and better quantitative performance than the Lit-Orbitrap, as well as the possibility of positive/negative switching.

Some of the earlier Orbitrap types used an ion-trap for fragmentation and helium as collision gas, which does not provide the same fragment ions as heavier gasses. Furthermore, ion-trapping usually has a limited m/z trapping window, a limitation that have been overcome by adding an additional collision cell on newer models (Perry et al., 2008). With respect to MS/MS spectra obtained from QqQ and QTOF instruments, we find the Q-Orbitrap to be superior. A class of lower cost instruments, which can perform MS^n, are ion-trap (IT) spectrometers, albeit with low mass accuracy. This is very useful for peptide identification, and for other polymeric molecules made up of known units. In regard to dereplication, the common occurrence of chromatographic peaks indicating an unknown compound renders IT instruments inferior to accurate mass instruments. Triple quadrupole instrumentations have very poor full-scan sensitivity and are cost wise a poor choice for dereplication applications, but highly valuable for environmental analyses where sensitivity is the most important parameter (Sulyok et al., 2006; Sørensen et al., 2009).

For a number of years ion-mobility has been used in a range of configurations, for example, in the Waters Synapt instrument used for differentiating drift times of the fragment ions after MS/MS, however this has limited applications in SM analysis. Recently (May et al., 2014), a much higher resolution ion-mobility interface (positioned between the API source and a QTOF) has been disclosed and appears extremely promising to resolve co-eluting compounds which often have very different masses and cross sectional areas. Deconvolution of chromatographically co-eluting compounds can occur as the pseudo molecular ions and simple fragment ions (e.g., $[M+H-H_2O]^+$) have almost the same cross-section and thus same drift times. In contrast the co-eluting compounds are likely to have different cross sectional area and different drift times so can then be deconvoluted in the drift time dimension. This will provide a cleaner MS spectrum and more reliable interpretation of the molecular mass. Theoretically, a three dimensional deconvolution using both retention time and drift time can be used, but has to our knowledge not been developed.

AUTOMATED TARGETED ANALYSIS

An alternate dereplication strategy is to search LC-MS data files for the masses (preferable accurate masses or elemental compositions) of a list of possible known compounds. This strategy, named aggressive dereplication, has been shown to work well for lists up to 3000 compounds (Kildgaard et al., 2014; Klitgaard et al., 2014), using the software from several manufactures. A major challenge is fragile compounds that easily fragment in the ion-source and thus appear as another elemental composition, leading to erroneous compound identification. The technology can be improved tremendously by introducing pseudo MS/MS in a second scan trace, so the instrument alternates between high and low fragmentation during a run (MS-All, MS-E, All-Ions; Ojanpera et al., 2006; Broecker et al., 2011; Guthals et al., 2012; El-Elimat et al., 2013) to obtain compound specific fragment ions known from our own standards, literature data, or from *in-silico* fragmentation (Hill and Mortishire-Smith, 2005; Kangas et al., 2012; Hufsky et al., 2014). The MS-All, MS-E, All-Ions strategy also has the advantage that the $[M+Na]^+$ is often enhanced in the fragmentation trace and an accurate confirmation of $[M+Na]^+$ to $[M+H]^+$ or $[M+H-H_2O]^+$ cuts down on the number of false positives resulting from misidentification of in-source fragment ions (e.g., $[M+H-H_2O]^+$ being identified as $[M+H]^+$ of another compound). Here we have observed that using an algorithm locking the pair like Bruker Target 1.3, Agilent Quant (*vide infra*), or ACD/Spectrus Processor provides 2–10 fold less false positives than Agilent Qual (Kildgaard et al., 2014) or Bruker Target 1.2 (Klitgaard et al., 2014) where one cannot require pairs of $[M+Na]^+$ and $[M+H]^+$ or $[M+H-H_2O]^+$.

CONSTRUCTION OF COMPOUND DATABASE

An important part of targeted analysis is construction of the compound database to be used for searching. Kildgaard et al. (2014) and Klitgaard et al. (2014) used ACD Chemfolder (Advanced Chemistry Development, Toronto, ON, Canada) for construction of a database including: (i) a number of in-house reference standards; (ii) a selection of common fungal compounds from AntiBase; (iii) common impurity compounds known from blank samples or as common media components, and finally iv) a number of tentatively identified compounds. Compound-database handlers like Chemfolder or Chemfinder have the advantage of the ability to perform substructure searches, which is why these are preferred for a master database. For each compound, major adducts (known or predicted) were registered in the database [41]. If known and/or predicted fragment ions from an alternating broad band fragmentation (MS-E, All-Ions) are included the specificity is greatly increased.

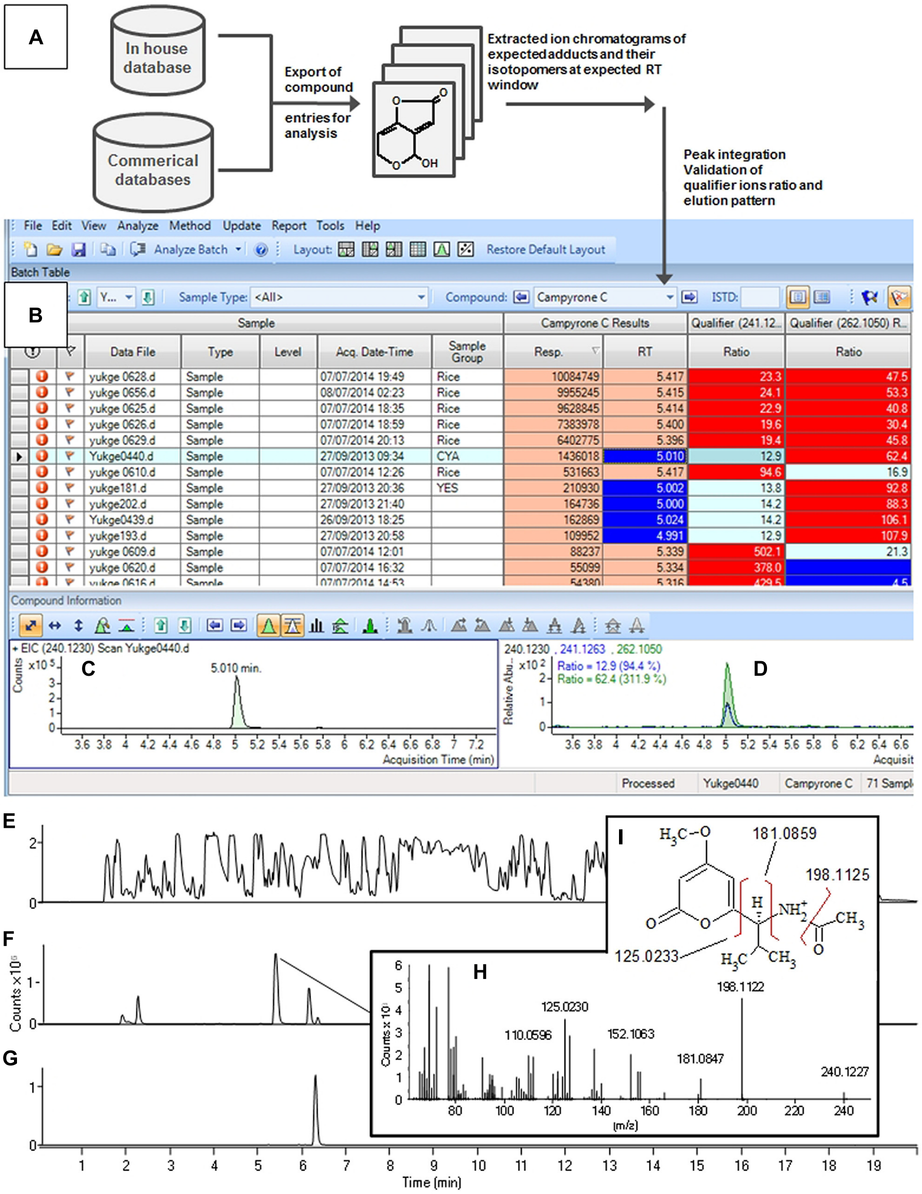
The next step is to create a taxonomically relevant search list (if chemotaxonomic data from properly identified fungal identifications are available). If a species specific search list is not available data from the whole genus may be used depending on the number of compounds described. However, a balance must be found between the number of false positives from compounds with the same elemental composition and with compounds failing to be identified and requiring subsequent manual deconvolution.

Minor in-house programming (e.g., Excel) is required to transfer from a Chemfolder/Chemfinder database to a ready search list for the MS vendor software (Bruker Target analysis (Klitgaard et al., 2014), Agilent Masshunter-find by formula (Kildgaard et al., 2014), or Waters ChromaLynx etc. Different settings for retention time, mass accuracy of peaks, area cut-off, among others can be used to process data-files depending on the nature of the samples.

In some concurrent large screening studies of black aspergilli, we have used a multi-sample screening tool, Agilent MassHunter Quant (similar packages available from Waters and Thermo), developed for LC-QqQ targeted quantification of pesticides, for example. Despite being outside the original design the software can also handle high-resolution data. Thus a target list can be imported via an XML file and one can convert a Chemfolder/Chemfinder database (e.g., $[M+H]^+$, $[^{13}C_1M+H]^+$ $[M+Na]+$, $[^{13}C_1M+Na]^+$, with RT window and fragment ions) to an XML file and import into MassHunter Quant (**Figures 3B–D**).

The advantage of the multi-sample screening approach is that whole batches of anywhere from 10 to 100s of samples including blanks, fungal strains grown on multiple media can be screened simultaneously. This not only gives fast processing, but a high chance of identifying a compound not previously detected while concurrently identifying the most prodigious producer of the batch. In many cases several peaks of interest are identified but with a proper selection of qualifier ion and comparisons between species and media and blanks usually only 2–3 peaks in the whole batch require further manual inspection. Here the sample with the most intense peak can be selected automatically as it is most likely to provide the best MS/HRMS and possible UV/Vis data. This is illustrated in **Figure 3** where a part of the batch of samples is shown, here screening for campyrone C, using $[M+H]^+$ (240.1230 ± 20 ppm, **Figure 3C**). **Figure 3D** shows the qualifier chromatograms, including $[M+Na]^+$, showing that the compound has the right elemental composition. The compound was then manually verified (**Figures 3E–H**) through conclusive MS/HRMS spectra and a correct elution order of the A or B isomers.

Altogether, the target analysis approach makes it possible to easily identify chromatographic peaks which are both likely to represent already known compounds and, even more importantly, also peaks that do not correspond to known compounds. Thus the target approach can quickly support prioritization in relation to which compounds might need to be produced in larger scale for



	Sample				Campyrone C Results		Qualifier (241.12...	Qualifier (262.1050) R...
Data File	Type	Level	Acq. Date-Time	Sample Group	Resp.	RT	Ratio	Ratio
yukge 0628.d	Sample		07/07/2014 19:49	Rice	10084749	5.417	23.3	47.5
yukge 0656.d	Sample		08/07/2014 02:23	Rice	9955245	5.415	24.1	53.3
yukge 0625.d	Sample		07/07/2014 18:35	Rice	9628845	5.414	22.9	40.8
yukge 0626.d	Sample		07/07/2014 18:59	Rice	7383978	5.400	19.6	30.4
yukge 0629.d	Sample		07/07/2014 20:13	Rice	6402775	5.396	19.4	45.8
Yukge0440.d	Sample		27/09/2013 09:34	CYA	1436018	5.010	12.9	62.4
yukge 0610.d	Sample		07/07/2014 12:26	Rice	531663	5.417	94.6	16.9
yukge181.d	Sample		27/09/2013 20:36	YES	210930	5.002	13.8	92.8
yukge202.d	Sample		27/09/2013 21:40		164736	5.000	14.2	88.3
Yukge0439.d	Sample		26/09/2013 18:25		162869	5.024	14.2	106.1
yukge193.d	Sample		27/09/2013 20:58		109952	4.991	12.9	107.9
yukge 0609.d	Sample		07/07/2014 12:01		88237	5.339	502.1	21.3
yukge 0620.d	Sample		07/07/2014 16:32		55099	5.334	378.0	
yukge 0616.d	Sample		07/07/2014 14:53		54380	5.316	429.5	4.5

FIGURE 3 | Aggressive (A–C) batch processing of 150 samples (12 shown), here sorted by peak area (Response column) of mass of [M+H]$^+$ of campyrone C (240.1230 ± 20 ppm) shown in (C). While **(D)** shows the overlaid extracted ion chromatograms of the same as well as $^{13}C_1{}^{12}C_{12}$ [M+H]$^+$ isotopomer and the [M+Na]$^+$ ion. **(F–I)** Manual verification of data file with highest peak intensity of campyrone C. **(E)** Shows the highly complex Base Peak chromatogram, while, the extracted ion chromatograms of [M+H]$^+$ of campyrone C and A/B respectively. **(G)** Shows the MS/MS spectrum at 10 eV of the tentatively identified campyrone C with **(H)** Showing the structure of campyrone C along with **(I)** theoretical masses of 3 likely fragmentation of the molecule. For instrument settings see Kildgaard et al. (2014).

semi-preparative isolation and possibly full structural characterization based on isolation and NMR spectroscopy. In our view this approach is very well suited to fungi that has already been studied significantly and when, for example, a new species related to a well characterized species is to be investigated.

PEAK-FINDING

A more classical approach to investigation of a full scan UHPLC-HRMS file is to use a peak finding algorithm based on molecular features. This approach uses deconvolution of the time profiles and adds peaks together and possibly resolves the adduct pattern (Kuhl et al., 2012; Kildgaard et al., 2014). Most vendor software as well as the open source software XC-MS (Smith et al., 2006) with the Camera package (Kuhl et al., 2012) offers this feature although not all can search Chemfolder/Chemfinder Structure Data Format (SDF). However they can all search ChemSpider and similar public databases. This approach is probably best suited to extracts from species where the taxonomy is not well known and can be used in a true unbiased metabolomics workflow (Cox et al., 2014; Macintyre et al., 2014; Wolfender et al., 2015), and can be especially useful when combined with bioactivity or gene knock-outs.

A major obstacle for this method is that *all* samples to be compared need to be processed at the same time, as changes in media batches, impurities in solvents, filters, plastic and glassware (often strongly ionizing impurities) strongly influences data analysis. In addition to this there are problems associated with the likelihood of changes in secondary metabolism which are often seen for many organisms, even though great efforts are made to standardize cultivation conditions. Finally analytical separation, cleanliness of ion-sources, changes in LC-MS solvents and many other things also may cause changes in data. One can standardize data between batches but this demands an enormous amount of quality assurance work as it needs to include at least all the variable things mentioned above and is likely to still result in large uncertainties.

Thus, if using a metabolomics approach to find small peaks in extracts, a very strong experimental design, with 4–6 replicates per condition is needed, and ideally all samples need to be processed and analyzed in a single batch. If this strict control is not implemented the peak picking algorithm and unbiased data analysis will likely find the sample preparation day as the most significant parameter, especially when lowering the intensity threshold. In such cases 5,000–30,000 chemical features (Cox et al., 2014; Macintyre et al., 2014; Wolfender et al., 2015) may be detected and statistically some will always be correlated to a given hypothesis. Despite these shortcomings, for extracts with very high media background and very low signal intensity from the secondary metabolites this approach is the most effective. In our view this approach is highly time-consuming and if searching for a bioactive compound, an assay guided approach may be far less time consuming.

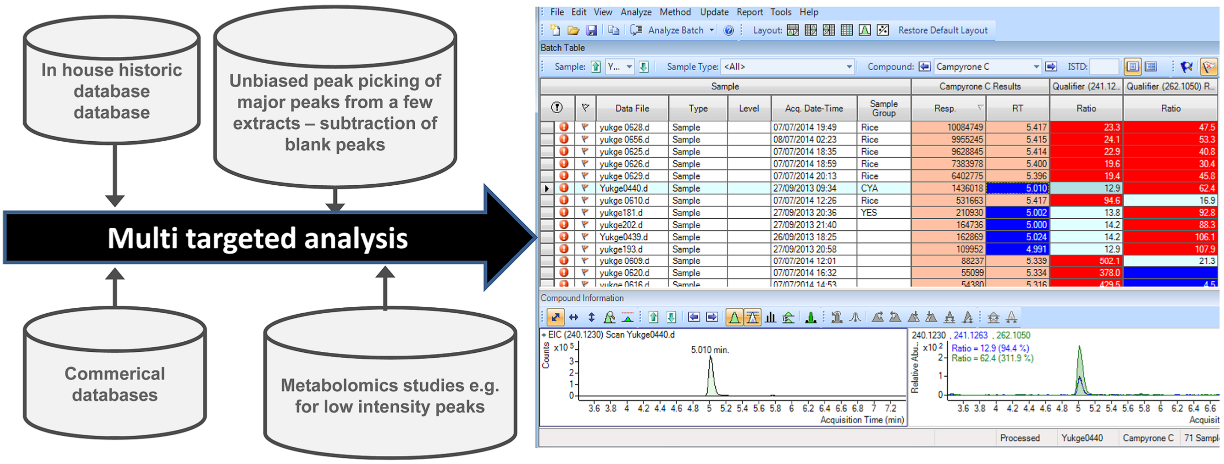
The metabolomics workflow is often not easily implementable with the workflow in a natural product laboratory as different species and strains often need substantial optimization in terms of growth and analysis (**Figure 4**). However, for gene-to-product linking type of projects, it can be the only way to search for low intensity peaks in defined studies, as one cannot add samples from later studies.

API MS/MS LIBRARIES

As mentioned above, UHPLC-HRMS is often not enough for a positive identification and a library based identification – as is possible for EI^+ fragmentation – would be highly desirable. If matching LC-MS generated MS spectra to a library they must be reproducible to give reliable matches. This is not possible with full scan MS due to the differences in in-source fragmentation settings and adduct formation and spectra of the same compound can be very different even on the same instrument. However MS/MS spectra of the pseudomolecular ions, in general, produces the same fragment ions, although the fragmentation energy to provide the same spectrum may be very different and also will vary between the harder argon toward the softer nitrogen (Baumann et al., 2000; Fredenhagen et al., 2005; Lee et al., 2005; Pavlic et al., 2006; Oberacher et al., 2012; El-Elimat et al., 2013; Kildgaard et al., 2014).

Thus an additional scan type, MS/MS, can be used simultaneously with full-scan, when using a QTOF, IT-Orbi-Trap, Q-Orbi-Trap, as these can perform auto-MS/MS, or data dependent MS/MS. While TOF and Orbi-trap (Excactive) instruments cannot isolate an ion for MS/MS, they can perform broad band excitation (All-Ions or MS-E) fragmentation at some point in the ion-optics. A corollary is that fragmentation in an ion-trap often provides very different fragments than a collision cell as fragment ions that are outside the excitation window in the ion-trap are no longer accelerated and thus fragmented. To remedy this shortcoming some Orbi-Trap type spectrometers come with an additional hexa- or octapole collision cell.

Compared to forensic science and toxicology (Broecker et al., 2011) only few in-house MS/MS libraries with fungal metabolite



Sample							Campyrone C Results		Qualifier (241.12...	Qualifier (262.1050) R...
ⓘ	▿	Data File	Type	Level	Acq. Date-Time	Sample Group	Resp.	RT	Ratio	Ratio
●	▿	yukge 0628.d	Sample		07/07/2014 19:49	Rice	10084749	5.417	23.3	47.5
●	▿	yukge 0656.d	Sample		08/07/2014 02:23	Rice	9955245	5.415	24.1	53.3
●	▿	yukge 0625.d	Sample		07/07/2014 18:35	Rice	9628845	5.414	22.9	40.8
●	▿	yukge 0626.d	Sample		07/07/2014 18:59	Rice	7383978	5.400	19.6	30.4
●	▿	yukge 0629.d	Sample		07/07/2014 20:13	Rice	6402775	5.396	19.4	45.8
●	▿	Yukge0440.d	Sample		27/09/2013 09:34	CYA	1436018	5.010	12.9	62.4
●	▿	yukge 0610.d	Sample		07/07/2014 12:26	Rice	531663	5.417	94.6	16.9
●	▿	yukge181.d	Sample		27/09/2013 20:36	YES	210930	5.002	13.8	92.8
●	▿	yukge202.d	Sample		27/09/2013 21:40		164736	5.000	14.2	88.3
●	▿	Yukge0439.d	Sample		26/09/2013 18:25		162869	5.024	14.2	106.1
●	▿	yukge193.d	Sample		27/09/2013 20:58		109952	4.991	12.9	107.9
●	▿	yukge 0609.d	Sample		07/07/2014 12:01		88237	5.339	502.1	21.3
●	▿	yukge 0620.d	Sample		07/07/2014 16:32		55099	5.334	378.0	

FIGURE 4 | Suggested workflow in fungal natural products research depending on the problem, number of strains, species and of peaks are minor or major, as well as if it is as well-studied or new organism, the different data-parts may differ significantly.

spectroscopic data are available thus far (Kildgaard et al., 2014). The major reasons are firstly; the lack of requirement to publish MS/HRMS spectra and secondly; the lack of standardization of fragmentation energies between instrument manufactures. Although only containing relatively few microbial natural products, the two libraries that can be helpful, to identify lipids, medium polar primary metabolites, vitamins, and other coumpunds are Massbank (Horai et al., 2010) and Metlin (Smith et al., 2005; ~10,000 compounds with spectra).

In addition it is unreliable to use *in silico* predictors for prediction of fragmentation of NPs, since they often represent highly condensed and complex ring structures, only leaving room for verification of some fragments from a structure in a spectrum (Hill and Mortishire-Smith, 2005; Hufsky et al., 2014). Despite of these challenges we anticipate that the use of MS/HRMS spectral libraries will become much more pronounced in the near future, especially since the different vendors can see a huge advantage in being able to supply their customers with dedicated spectral libraries.

PRECURSOR SELECTION

The use of MS/MS on one of the pseudomolecular ions does indeed already provide very reproducible MS/MS spectra within one brand of instrument (Fredenhagen et al., 2005) as recently demonstrated in our laboratory (Fredenhagen et al., 2005; Kildgaard et al., 2014). Thus, good reproducibility can be achieved if the collision energy is calibrated and the nature of metal ion adducts are known, since $[M+Na]^+$ and $[M+K]^+$ fragment far less and with different mechanisms than $[M+H]^+$, $[M+NH_4]^+$ (Oberacher et al., 2009, 2011; Wurtinger and Oberacher, 2012; Kildgaard et al., 2014). For robustness, many of the known adduct ions should be included as it increases the identification confidence especially between related compounds (Kildgaard et al., 2014).

FRAGMENTATION

Importantly, different energies for MS/MS are needed as the stability of compounds varies significantly (Kildgaard et al., 2014). This can be acquired in two ways: (i) as several distinct energies (Smith et al., 2005), or ii) by ramping the collision energy, thus acquiring an average spectrum. For fungal metabolites we have found the spectral quality (Kildgaard et al., 2014) to be much higher when using distinct energies, especially when using accurate mass of the fragment ions. In such cases only 3–7 fragment ions are needed, as long as they are distributed equally over the mass range (neither in the very low range, with common fragments shared with many other compounds, nor close to the molecular ion where the losses may not differentiate from related compounds). Agilent Technologies and Metlin have chosen to acquire spectra at the three different fragmentation energies 10, 20, and 40 eV (Kildgaard et al., 2014), which we also found efficient for microbial metabolites, except for large peptides where 60 eV had to be included, and we suggest a possibility to change the window for these when going to larger masses where much more energy is generally needed.

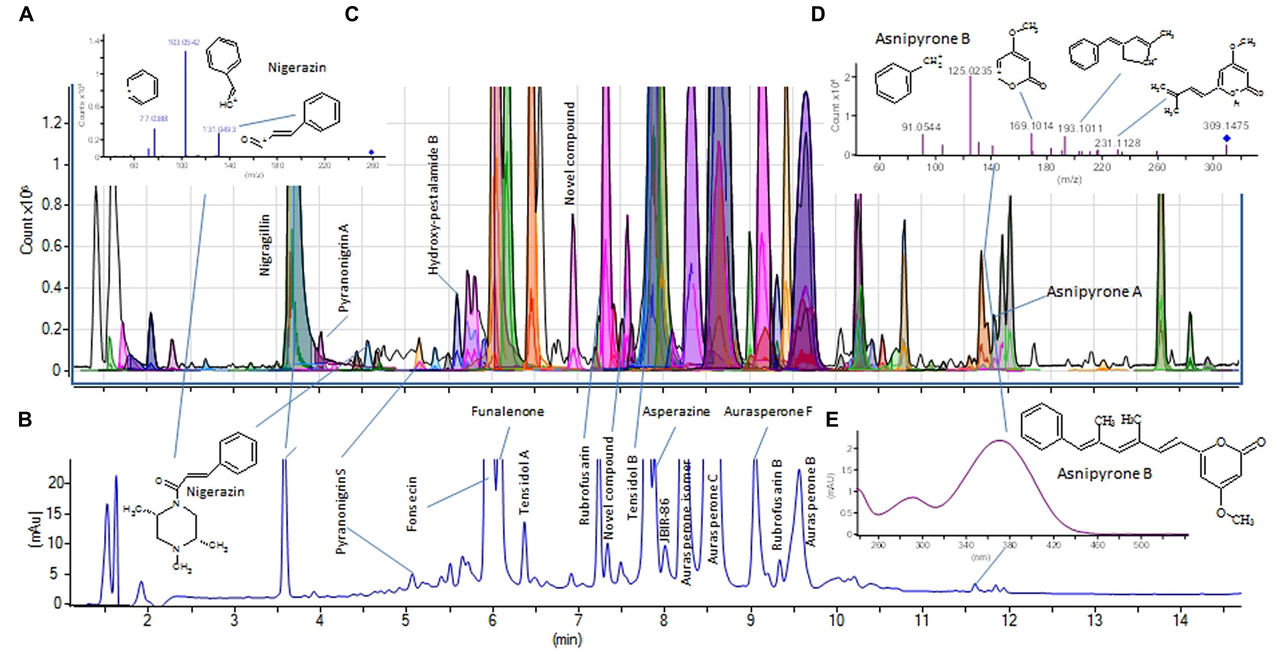


FIGURE 5 | UHPLC-DAD-HRMS-MS/HRMS analysis of an *Aspergillus tubingensis* extract. (A) shows the BasePeak chromatogram. Peaks automatically assigned from Auto-MS/MS data searched in the MS/HRMS database are colored. **(B)** shows the UV/Vis chromatogram (200–640 nm), while **(C)** shows the MS/HRMS spectrum of nigerazin A (20 eV) or B used to tentatively ID this peak. **(D)** and **(E)** shows the MS/HRMS (20 eV) and UV/Vis spectra of asnipyrone B, respectively, with the UV/Vis spectrum matching literature data perfectly, and also eluting before asnipyrone A (having an extra methyl group). For instrument settings see Kildgaard et al. (2014).

ALGORITHMS AND LIBRARY SCORING

Different algorithms have been used to search experimental MS/MS spectra contained in small in-house MS/MS libraries for tentative identification of fungal SMs. For example the NIST (National Institute of Standards and Technology) algorithm, developed for full scan EI$^+$ spectra and the Mass Frontier software for MS^n spectra were compared by Fredenhagen et al. (2005) to search low resolution MS/MS data, with the latter found to be superior. Similarly El-Elimat et al. (2013) used ACD-IntelliXtract, which allows inclusion of accurate masses of the fragments, but does not use the parent ion data as search entry [64]. We use the Agilent search algorithm that is an integral part of the Agilent MassHunter software for fast and automated search in our in-house MS/HRMS library of more than 1300 compounds for unambiguous identification of especially fungal metabolites belonging species in the genera *Aspergillus*, *Penicillium*, and *Fusarium* (**Figure 5**).

The software allows background subtraction and merging of spectra over chromatographic peaks into a single spectrum prior to automatic searching against the library. Importantly, searching of MS/HRMS spectra against a given in-house library in MassHunter allows for both forward and reverse scoring using the parent mass for matching of peaks in the unknown spectrum against the library spectra or vice versa. Often both forward and reverse scoring are needed for correct identification as shown in our work for the identification of patulin (Kildgaard et al., 2014). Part of our library (277 microbial compounds) can be downloaded as PCDL format from the homepage of the Technical University of Denmark (Nielsen and Frisvad, 2014).

Manual interpretation of MS/HRMS spectra might even be used to predict the structure of unknown compounds based on careful inspection of the obtained fragmentation patterns. This approach is especially applicable for NRPs due to their sequential composition of amino acids (**Figure 6**).

For ion-traps users (especially IT-Orbi trap) the so called MS-trees offers an additional dimension to identification of a substructure of an analyte molecule by MS/MS/MS (MS^3; Ridder et al., 2012; Rojas-Cherto et al., 2012), as fragment ions from

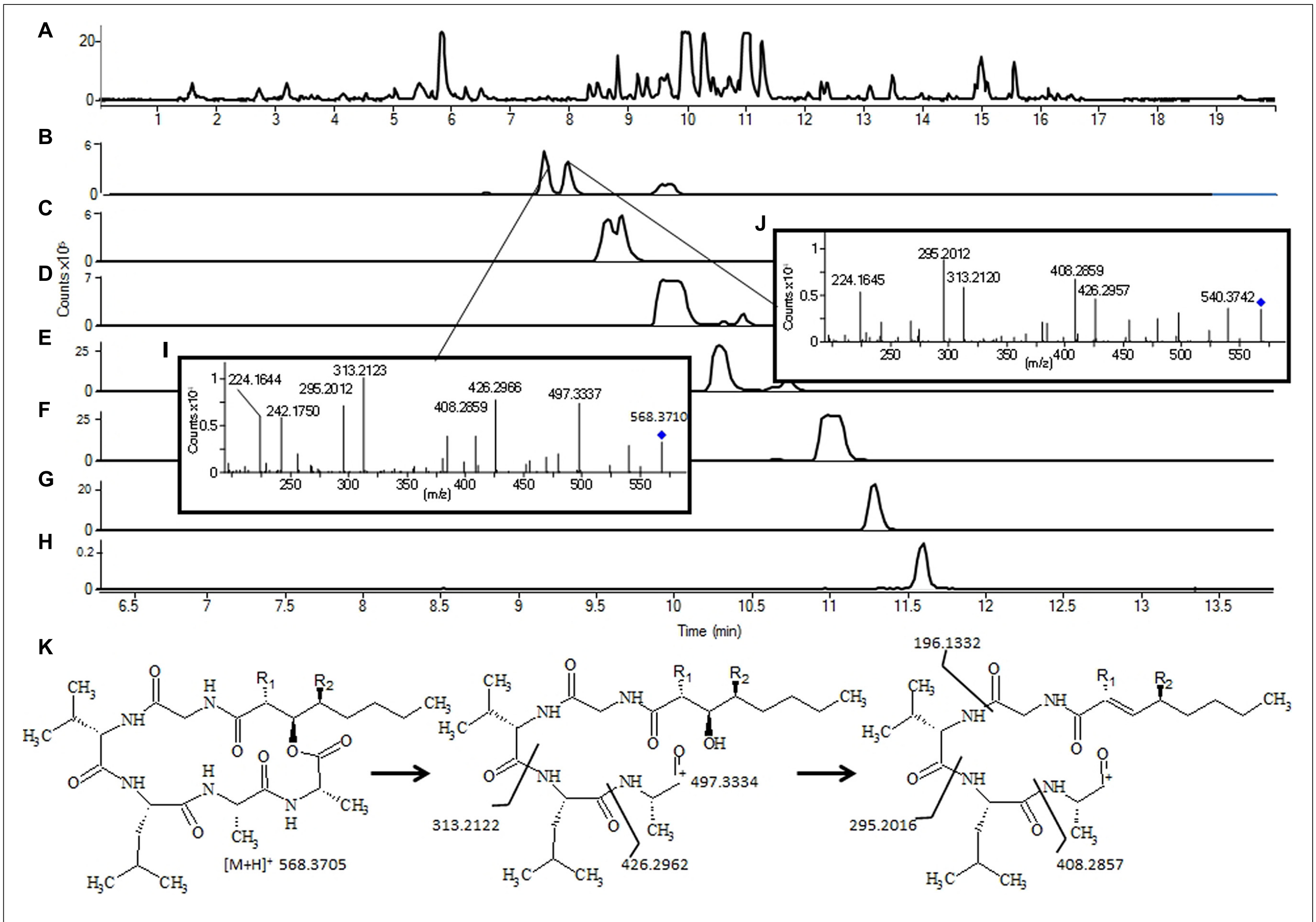


FIGURE 6 | Tentative identification of emericellamides in an extract of *Aspergillus nidulans* grown for 7 days on yeast extract sucrose agar: (A) Base Peak chromatogram; (B) novel emericellamides where MS/HRMS and retention time suggest the structure shown in (K) with (I) RT 9.17 (R_1 = CH_3, R_2 = H) and (J) RT 9.33 (R_1 = H, R_2 = CH_3). Water loss from the fatty acid chain is more favored when R_1 = H as a conjugation with the carbon can occur. **(C)** shows other novel emericellamides G (R_1 = H, R_2 = H) presumably with the fatty chain 2 × CH_2 longer. **(H)**, emericellamide B; **(G)**, emericellamide H (emericellamide E, with R_1 = CH_3, R_2 = CH_3); **(F)**, emericellamide F; **(E)**, emericellamide A; and **(D)**, emericellamide C. For instrument settings see Kildgaard et al. (2014).

the first MS/MS event subsequently can be further fragmented and matched in a MS/MS library for assigning parts of a given molecular structure.

MS NETWORKING

In recent years the Dorrestein and Bandeira labs (Guthals et al., 2012; Watrous et al., 2012) have been key drivers toward development of new networking MS/MS approaches. Here MS/MS spectra of compounds in a given sample are compared pairwise and structurally related compounds are clustered based on the presence of similar fragments and neutral loses. In this way several compounds belonging to the same pathways have very convincingly been linked together including compounds which belong to both known and novel biosynthetic pathways (Watrous et al., 2012; Yang et al., 2013). A major current drawback is the lack of back-integration of raw data for evaluation of the corresponding full scan data and retention time, making detailed analysis extremely time consuming. Also source code is not available in an open-source format as, e.g., XC-MS and it is thus impossible to troubleshoot data in cases where the analysis fails.

ISOTOPE LABELING FOR STRUCTURE AND BIOSYNTHESIS INVESTIGATION

The availability of LC-MS instruments and increased availability of stable isotope (^{13}C, ^{2}H, ^{15}N, ^{34}S) labeled substrates has made more reliable determination of the elemental composition of a given compound more robust through determination of the number of carbon (Bueschl et al., 2012), nitrogen (Bode et al., 2012) or sulfur atoms in the molecule (Brock et al., 2014). However, the technology is also promising for incorporation of precursors involved in PK and NRP biosynthetic studies (McIntyre et al., 1982, 1989; Townsend and Christensen, 1983; Steyn et al., 1984; Simpson, 1998; Baran et al., 2010; Bode et al., 2012; Fuchs et al., 2012; Proschak et al., 2013; Brock et al., 2014; Huang et al., 2014), as recently exploited for proving that yanuthone production in *A. niger* is produced via 6-methylsalisylic acid and not shikimic acid (Holm et al., 2014; Petersen et al., 2015).

SURFACE TECHNIQUES

During the last 7 years surface desorption techniques have been introduced for secondary metabolite screening. These techniques can both ionize and detect surface excreted compounds and compounds in agar, for example, the identification of compounds present between colonies of different organisms for studying interactions. Current methods can be separated into two different sub families: desorption electrospray ionization mass spectrometry (DESI), direct analysis in real time (DART) and variants of them, and they are all soft ionization techniques (Watrous et al., 2012; Hsu et al., 2013; Bouslimani et al., 2014). DESI (Esquenazi et al., 2009) is based on charged droplets of organic solvent and a gas jet hitting the surface and then sampling ions close by (Gurdak et al., 2014), whereas DART uses a gas ionized by a plasma. Both have a poor spatial resolution (mm range) and can only be applied for major structures as DESI imprint of exudate droplets (Figueroa et al., 2014). Another major field of surface desorption techniques are the matrix assisted laser desorption techniques (MALDI). MALDI has a much higher spatial resolution (down to several 100 μm), but still not low enough to provide cellular resolution of fungi (Watrous and Dorrestein, 2011; Watrous et al., 2012; Bouslimani et al., 2014).

CONCLUSION AND PERSPECTIVES

The recent advancements in separation sciences, high resolution mass spectrometry, as well as data mining tools have dramatically improved our possibilities for dereplication of fungal natural products in complex mixtures. At the same time these advances call for more automated methods for analysis since the amounts of data that can be generated on a single instrument in a short time is truly immense. Among other techniques, dereplication based on auto-MS/MS has proven to be very robust and effective on a given instrument. However, the use of MS/MS settings between manufactures and scoring algorithms are calling for standardized methods for both generation and comparison of MS/MS patterns.

The ultimate solution for future dereplication would be the construction of an open natural product database, where all relevant chemical and taxonomic information have been merged together. In particular this should include chromatographic and spectroscopic data (HR MS/MS, UV/Vis, NMR) as well as valid taxonomic information about the source organism, in many cases maybe even including linking biosynthetic origin of a given natural product to the related gene cluster.

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A chemical ecogenomics approach to understand the roles of secondary metabolites in fungal cereal pathogens

Yit-Heng Chooi* and Peter S. Solomon

Plant Sciences Division, Research School of Biology, The Australian National University, Canberra, ACT, Australia

Edited by:
Jonathan Mark Palmer, USDA Forest Service, USA

Reviewed by:
Armen Trchounian, Yerevan State University, Armenia
Gelhaye Eric, Université de Lorraine, France

****Correspondence:***
Yit-Heng Chooi, Plant Sciences Division, Research School of Biology, The Australian National University, Building 134, Linnaeus Way, Canberra, Acton, ACT 2601, Australia
e-mail: yh.chooi@anu.edu.au

Secondary metabolites (SMs) are known to play important roles in the virulence and lifestyle of fungal plant pathogens. The increasing availability of fungal pathogen genome sequences and next-generation genomic tools have allowed us to survey the SM gene cluster inventory in individual fungi. Thus, there is immense opportunity for SM discovery in these plant pathogens. Comparative genomics and transcriptomics have been employed to obtain insights on the genetic features that enable fungal pathogens to adapt in individual ecological niches and to adopt the different pathogenic lifestyles. Here, we will discuss how we can use these tools to search for ecologically important SM gene clusters in fungi, using cereal pathogens as models. This ecological genomics approach, combined with genome mining and chemical ecology tools, is likely to advance our understanding of the natural functions of SMs and accelerate bioactive molecule discovery.

Keywords: fungal secondary metabolites, ecological genomics, chemical ecology, genome mining, plant pathogen

INTRODUCTION

The interactions of fungal plant pathogens with their hosts are highly complex and involve both secondary metabolites (SMs) and small secreted proteins as pathogenicity factors (often defined as effectors). The role of SMs in mediating the virulence of fungal plant pathogens is increasingly being recognized (Mobius and Hertweck, 2009; Collemare and Lebrun, 2011). These fungal SMs facilitate infection by altering host cell structure or function via diverse mode of actions. Some of these SMs are host-selective toxins (HSTs) while others are non-host-selective general phytotoxins. Some classic examples of small molecule HSTs are found amongst the phytopathogens in the Dothideomycete class (Stergiopoulos et al., 2013; Muria-Gonzalez et al., 2014), including victorin, T-toxin, and HC-toxins. Other well-known examples of non-host selective phytotoxins include cercosporin, tentoxin, beticolin, depudecin, AAL-toxin, deoxynivalenol (DON) etc., each with different mode of actions.

The increasing number of phytopathogen genome sequences has revealed a large number of uncharacterized SM gene clusters in these fungi, particularly within the Dothideomycete but also other ascomycete pathogens such as *Magnaporthe* and *Fusarium* species. This indicates that we have merely scratched the surface of the SM repertoire in phytopathogens. Many SM gene clusters that encode the production of small molecules that have a role in host interactions are likely waiting to be discovered from these genomes. These phytopathogens SMs are equally likely to act as suppressors of other competing microbes that shared the ecological niches as well as deterrents of herbivores and fungivores (insects and other animals).

From a natural product discovery perspective, the flood of phytopathogen genome sequences presents an exciting opportunity for genome mining of bioactive molecules. Since eukaryotes, from fungi, plants to human beings, share many core biochemical pathways, many SMs that are targeted at plants and other eukaryotic microorganisms are likely to interact with macromolecules in human as well. Indeed, many molecules with human biological targets have been discovered from plant-associated fungi. Notable examples include, squalene synthase inhibitors zaragozic acids (Bergstrom et al., 1995), actin polymerization inhibitors cytochalasans (Scherlach et al., 2010), calmodulin inhibitors ophiobolins (Au et al., 2000), and various histone deacetylase inhibitors including depudecin, apicidin, and HC-toxin (Walton, 2006). These molecules have high clinical relevance and often serve as drug leads in drug discovery programs. Phytopathogens, which have to compete with other microbes in the environment, are also potential source of antimicrobial compounds. For example, aspergillomarasmine A, which was recently shown to be a promising metallo-β-lactamase inhibitor to combat antibiotic resistant bacteria (King et al., 2014), was originally isolated from the cereal pathogen *Pyrenophora teres* (Haenni et al., 1965). Despite that the biological targets of some of these bioactive SMs are known, the biological roles and ecological functions of these SMs often remain enigmatic.

It has been recognized that the chemical ecology research can advance the discovery of bioactive molecules (Caporale, 1995). With the new next-generation genomic tools now at our disposal, we believe it is time to explore the synergy of ecological genomics and chemical ecology for advancing the understanding of the SM functions in phytopathogens. We termed this emerging integrated approach chemical ecogenomics. Combined with the increasingly mature genome mining tools and heterologous systems for expression of fungal SMs, this approach is likely to greatly accelerate the discovery of SM virulence factors and bioactive molecules. Similar strategies have been proposed for the study of insect pheromones (Tittiger, 2004), the role of fungal SMs in interactions with animals (Kempken and Rohlfs, 2010; Rohlfs and Churchill, 2011), and for natural product discovery in endophytic fungi (Kusari et al., 2012), coprophilous fungi (Bills et al., 2013) and Gammaproteobacteria (Vizcaino et al., 2014). Here, we would like to take some of these ideas one step further and to propose a basic chemical ecological framework for the study of SMs in phytopathogens, in particular, in cereal pathogens (**Figure 1**).

CHEMICAL ECOGENOMICS – THE NEXUS OF CHEMICAL ECOLOGY AND ECOLOGICAL GENOMICS

Ecological genomics seeks to understand the function of genes and genome in biotic and abiotic interactions among organisms and their natural environments (Ungerer et al., 2008). Functional genomics tools, including comparative genomics, transcriptomics, and proteomics, are used to study the genome, transcriptome, and proteome dynamics in an ecological context. These studies often identify candidate genes that are important to a given ecological interactions or environmental niche. The identified genes are subjected to further functional verification. Similar studies focusing specifically on host–pathogen interactions in both human and plant pathogenic fungi are also often referred as pathogenomics (Pompe et al., 2005; Schmidt and Panstruga, 2011).

On the other hand, chemical ecology is the study of small molecules that mediate the biotic and abiotic interactions of organisms (Caporale, 1995). The small molecule metabolites that provide the organisms with adaptive advantages in specific ecological niches are often synonymous to SMs. A typical chemical ecology study often involves the isolation and structural characterization of the putative molecules involved in a given ecological interactions. The molecules are then used to test out the proposed function. The advantage of studying the function of genes and molecules in an ecological context is that they often provide important clues to their natural functions.

The SM biosynthetic genes that encode the production of SMs that mediate ecological interactions are essentially a subset of ecologically important genetic traits. Hence, studies in chemical ecology and ecological genomics have significant overlap and can be highly synergistic. Indeed, molecular genetics and genomics are also transforming the microbial SM (natural product) field, shifting the focus towards understanding biosynthesis and genes-to-molecules relationship (Walsh and Fischbach, 2010) and genomics-guided natural product discovery (Challis, 2008). Bringing these different, but related, fields together will help us bridge the gaps between genes, molecules, and functions.

The cereal pathosystems are perfect test beds for this multipronged chemical ecogenomics approach. Besides the large number of uncharacterized SM gene clusters in the genome, many of them are amenable to genetic manipulation and have established virulence assays (e.g., whole plant and detached leaf assays). An outline of how this emerging approach can be employed to study the SMs in cereal pathogens is presented in **Figure 1**. We will use some recent ecogenomics/pathogenomics studies in plant pathogens to illustrate the prospect of employing this strategy for understanding the SM functions in these pathogens and for bioactive molecule discovery.

GENOME EVOLUTIONARY DYNAMICS OF SM BIOSYNTHETIC GENES IN FUNGAL PHYTOPATHOGENS

Secondary metabolite gene clusters are often not essential for the growth and survival of fungi under ideal conditions, but they confer selective advantages on the organisms by producing SMs that may act as defense molecules, signals, siderophores, or

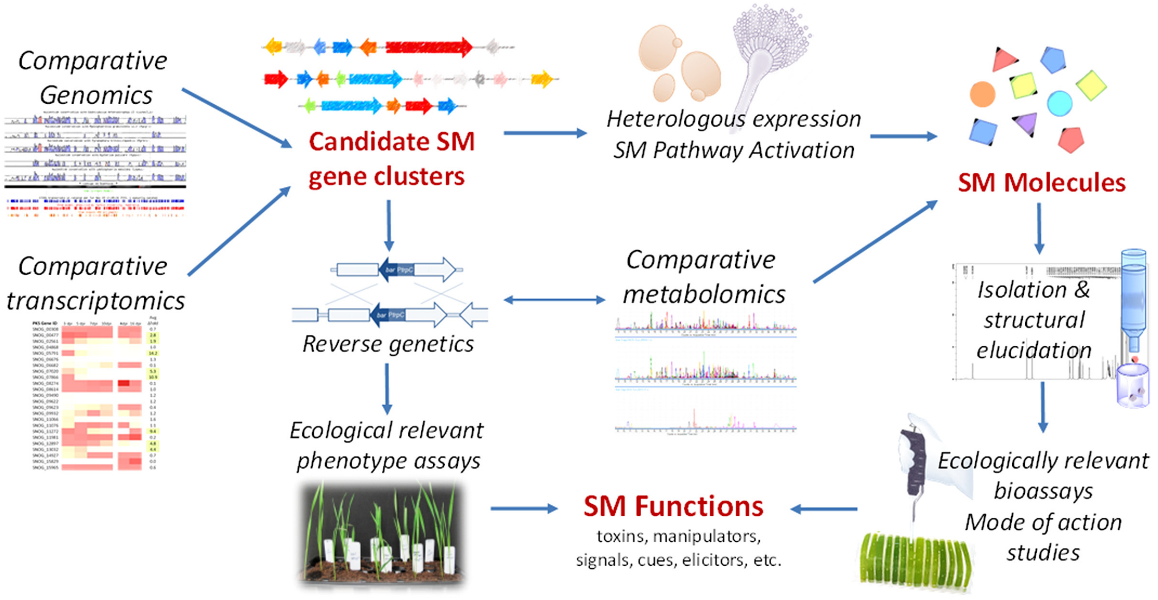


FIGURE 1 | An integrated chemical ecogenomic approach for understanding the functions of secondary metabolites (SMs) and bioactive molecule discovery. The strategy incorporates the common tools in ecological genomics, genome mining and chemical ecology.

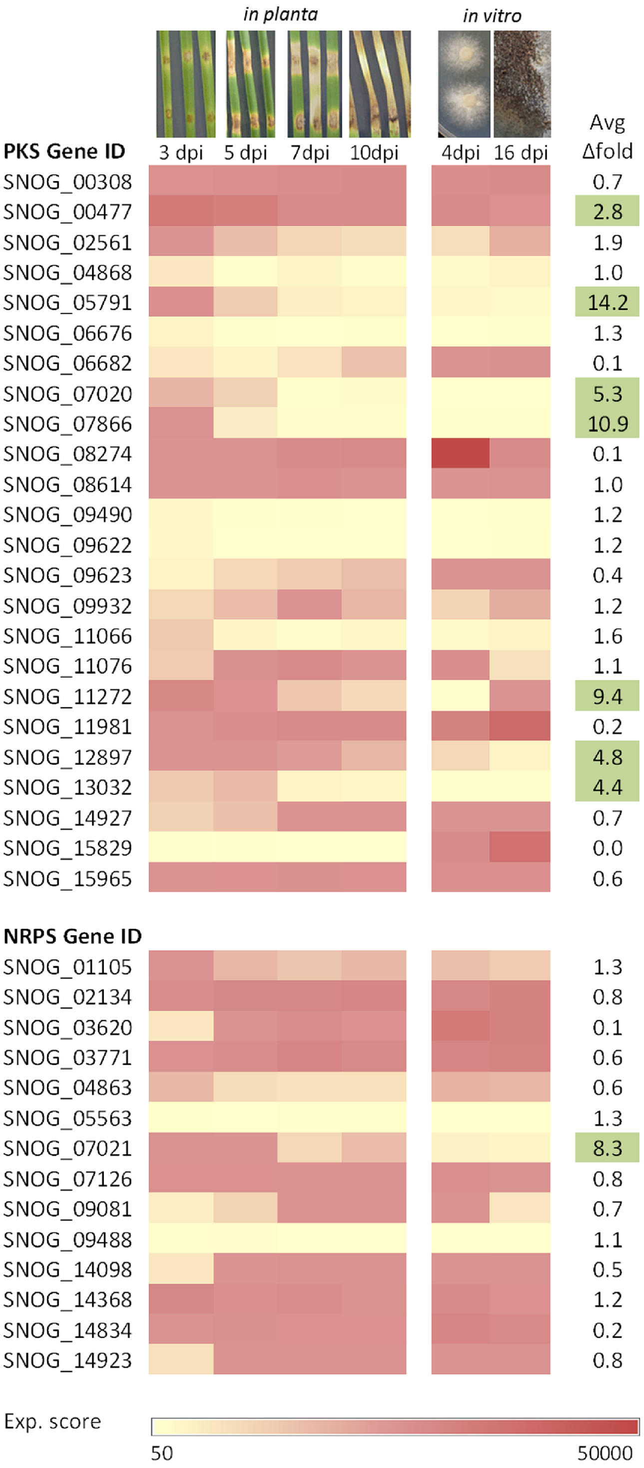
modulators in parasitic/endophytic interactions (Fox and Howlett, 2008; Collemare and Lebrun, 2011; Kusari et al., 2012). Thus, they are often subjected to adaptive evolution via a combination of genetic drifts and natural selection. Evolution mechanisms by gene loss, genetic mutation, gene duplication and divergence, genome rearrangement, fragment recombination and horizontal gene transfer (HGT) are commonly observed among SM gene clusters (Carbone et al., 2007; Patron et al., 2007; Fischbach et al., 2008; Proctor et al., 2009). The evolutionary dynamics of these SM gene clusters combined with the bio-ecological knowledge of the organisms can thus be used to infer the importance of individual SM gene clusters in environmental adaptations. Similar approaches have been recently employed to identify candidate effectors in plant pathogens (Stukenbrock et al., 2010; Gardiner et al., 2012; Ohm et al., 2012; Condon et al., 2013; Manning et al., 2013; Syme et al., 2013).

INTRASPECIFIC COMPARATIVE GENOMICS – SPOT THE DIFFERENCES

With ready accesses to microbial genome sequencing enabled by next-generation sequencing technologies, genome sampling and re-sequencing is becoming a routine. In specific reference to cereal and other crop pathogens, comparative genomics and phylogenomics analyses on different strains of the same species with different host range or virulence profiles may reveal SM gene clusters that are important to virulence or pathogenicity. Using this approach, Brandon et al. have identified candidate SM gene clusters that may play a role in the virulence of *Cochliobolus* spp. (Condon et al., 2013). In one of the examples from the study, phylogenomic comparison of non-ribosomal peptide synthetase (NRPS) genes between pathotype 2 (isolate ND90Pr) and pathotype 0 (isolate ND93-1) of the barley pathogen *Cochliobolus sativus* revealed a NRPS gene cluster that is unique to pathotype 2. Deletion of one of the two NRPS genes in the gene cluster significantly reduced the virulence of *Cochliobolus sativus* pathotype 2 on barley cultivar Bowman. However, the SM molecule(s) encoded by this gene cluster remains to be identified. As a proof of concept, the authors further demonstrated the utility of this comparative phylogenomic approach to pull out the two polyketide synthase (PKS) genes previously identified to be involved in the host-specific T-toxin biosynthesis in *Cochliobolus heterostrophus* race T strains (Baker et al., 2006). As expected, the two PKS genes are present in all race T strains but absent in all race O strains (Condon et al., 2013). Comparative genomics of the wheat pathogen *Parastagonospora nodorum* SN15 strain with virulent and avirulent strains have also identified a significant number of strain-specific genes and genomic regions (Syme et al., 2013). Among those is a SM gene cluster that is absent in the genome of the avirulent SN79 strain but present in the two other virulent strains. Based on microarray data from a previous study (Ipcho et al., 2012), the gene cluster appeared to be up-regulated exclusively *in planta* during wheat leaf infection (**Figure 2**). Although it remains to be confirmed, it is tempting to speculate the gene cluster may encode a SM that plays a role in virulence.

INTERSPECIFIC COMPARATIVE GENOMICS – SPOT THE SIMILARITIES

Horizontal gene transfer has now been recognized to be a common phenomenon among fungi (Fitzpatrick, 2012). In crop



PKS Gene ID	Avg Δfold
SNOG_00308	0.7
SNOG_00477	2.8
SNOG_02561	1.9
SNOG_04868	1.0
SNOG_05791	14.2
SNOG_06676	1.3
SNOG_06682	0.1
SNOG_07020	5.3
SNOG_07866	10.9
SNOG_08274	0.1
SNOG_08614	1.0
SNOG_09490	1.2
SNOG_09622	1.2
SNOG_09623	0.4
SNOG_09932	1.2
SNOG_11066	1.6
SNOG_11076	1.1
SNOG_11272	9.4
SNOG_11981	0.2
SNOG_12897	4.8
SNOG_13032	4.4
SNOG_14927	0.7
SNOG_15829	0.0
SNOG_15965	0.6

NRPS Gene ID	Avg Δfold
SNOG_01105	1.3
SNOG_02134	0.8
SNOG_03620	0.1
SNOG_03771	0.6
SNOG_04863	0.6
SNOG_05563	1.3
SNOG_07021	8.3
SNOG_07126	0.8
SNOG_09081	0.7
SNOG_09488	1.1
SNOG_14098	0.5
SNOG_14368	1.2
SNOG_14834	0.2
SNOG_14923	0.8

FIGURE 2 | Transcriptomic profiles of PKS and NRPS genes in *P. nodorum* on detached leaf assays (*in planta*) and during growth on minimal medium (*in vitro*). The microarray data is based on Ipcho et al. (2012). Expression scores were normalized: <500 essentially indicative of no expression, >50000 indicates massive expression (out of dynamic range). Average fold difference is calculated by dividing the average expression score *in planta* over the average *in vitro* (highlighted in green indicates > two-fold difference).

pathogens, HGT is thought to be a major evolutionary force that drives the emergence of new fungal crop diseases (Oliver and Solomon, 2008; Mehrabi et al., 2011). A well-known example is the interspecific horizontal transfer of the ToxA effector gene from *P. nodorum* to *Pyrenophora tritici-repentis*, resulting in the emergence of the tan-spot disease on wheat (Friesen et al., 2006). HGT of whole or partial SM gene clusters have also been proposed (Patron et al., 2007; Slot and Rokas, 2011; Wight et al., 2013). In a more extreme example, horizontal transfer of whole supernumerary chromosomes containing HST biosynthetic gene clusters has been proposed to confer pathogenicity to different pathotypes of *Alternaria alternata* (Akagi et al., 2009; Mehrabi et al., 2011). In fact, horizontal transfer has been proposed to be a principal driving force behind the evolution of clustering of SM biosynthetic genes (Walton, 2000). On the other hand, in some cases, the absence/presence of some SM gene clusters between closely related species can be explained by the loss of the gene clusters via genetic drifts (Chooi et al., 2010).

Given that the survival of a SM gene cluster is relying on its ability to confer advantages to the organism, we can expect that the conservation of a SM cluster across multiple species that share similar ecological niches may play similar bio-ecological roles. A good example is the recent discovery of immunosuppressive compounds from human pathogenic fungi (Chooi et al., 2013). Homology searches and comparative genomics identified a homologous gene cluster that is present among *Aspergillus fumigatus*, *Neosartorya fischeri,* and six dermatophytic fungi. The conserved gene clusters were demonstrated to produce the immunosuppressive compound neosartoricin (Chooi et al., 2013; Yin et al., 2013).

An example from the cereal pathogens is the SM gene cluster from *Cochliobolus carbonum* that encodes production of the host-selective HC-toxin required for pathogenicity to *hmhm* maize. Interspecific comparative genomic analysis identified HC-toxin gene cluster is present in another maize pathogen *Setosphaeria turcica* (Condon et al., 2013). Interestingly, the HC-toxin gene cluster is also present in another plant-associated fungus, *Alternaria jesenskae*, which does not appear to be pathogenic to most plants (Wight et al., 2013). Comparative phylogenomic analysis of NRPS genes among plant pathogens has also revealed that some NRPS genes have undergone recombination and modular rearrangement (Bushley and Turgeon, 2010). For example, modules 1 of ChNPS1 and ChNPS3 from *Cochliobolus heterostrophus* share high similarity to the modules in the AM-toxin synthetase of *Alternaria alternata* (Johnson et al., 2000), but other modules of ChNPS1 and ChNPS3 group with other cyclic peptide NRPSs and mono/bi-modular NRPSs in the phylogenetic analysis (Bushley and Turgeon, 2010). In such cases, care has to be taken in interpreting NRPS homology across different species as such NRPS module rearrangements will resulted in different SM products.

In *P. nodorum*, our detailed analysis of the SM gene clusters revealed several genes encoding PKSs and NRPSs that are highly conserved ($\geq$70% protein identity) among several dothideomycete cereal pathogens, such as *Cochliobolus* spp., *Leptosphaeria maculans,* and *Pyrenophora tritici-repentis* (Chooi et al., 2014b). One common PKS gene among *P. nodorum, P. tritici-repentis,* and *L. maculans* has been shown to be responsible for the production of an antifungal compound phomenoic acid in *L. maculans* (Elliott et al., 2013). Phomenoic acid was proposed to be an antifungal substance used by the pathogens to outcompete other fungi in their environment.

TRANSCRIPTOME DYNAMICS OF SM BIOSYNTHETIC GENES IN ECOLOGICAL INTERACTIONS

The production of SMs in fungi is highly regulated and often in response to specific biotic interactions and environmental perturbations (Keller et al., 2005; Brakhage, 2013). Therefore, the temporal and spatial expression of SM gene clusters may provide clues to the natural function of the encoded SM molecules in fungi. Traditionally, reverse transcriptase-PCR (RT-PCR) is used to monitor the expression of multiple backbone biosynthetic genes (e.g., PKS and NRPS genes) in fungi, while microarray allows the profiling of global transcriptome dynamics. Recent availability of next-generation RNA-Seq technologies has revolutionized transcriptomic profiling. Unlike microarray, RNA-Seq is not dependent on gene annotations and can provide information about transcript splicing as well. RNA-Seq also allows the simultaneous quantification of transcripts from more than one organism and is thus perfectly suited for the study of organismal interactions. For plant pathogens, these transcriptomic tools can be used to probe the expression of SM gene clusters during various stages of infection. Similar approaches can also be employed to probe fungal–fungal and fungal–bacterial interactions.

A classic example of SM gene cluster that is specifically expressed during host–fungus interactions is the ACE1 PKS-NRPS hybrid gene cluster from the rice pathogen *Magnaporthe grisea* (Collemare et al., 2008). The expression of ACE1 gene in *M. grisea* is highly up-regulated during the penetration into the host plant and the protein was localized to appressoria specifically. The gene *ace1* confers avirulence toward rice cultivar *Pi33* carrying a corresponding resistance (*R*) gene (Bohnert et al., 2004). ACE1 is likely to play a role in infection or manipulation of the host cell. Nonetheless, the identity of the SM product of ACE1 gene cluster and its function in *M. grisea* remain to be identified.

Recent global transcriptomic studies of plant pathogens have revealed several SM gene clusters that were expressed during infection. A remarkable example that highlighted the possible roles of fungal pathogen SMs *in planta* can be found in a recent RNA-Seq-based transcriptome study of *Colletotrichum higginsianum* (O'Connell et al., 2012). As many as 12 SM gene clusters (out of 39) were up-regulated before appressorial penetration and during biotrophic phase, but down-regulated during the necrotrophic stage. Similar observations were made on *Colletotrichum orbiculare* (Gan et al., 2013) and *Magnaporthe oryzae* (Soanes et al., 2012). Since the plant host remains healthy and asymptomatic during the biotrophic phase, the authors reasoned that the encoded SMs are unlikely to function as phytotoxins but perhaps as small molecule effectors that manipulate the host cells in ways that benefit the fungus or facilitate infection. Fungal SMs are often being screened for phytotoxic activities but their roles in biotrophy of fungi are largely unexplored and warrant further investigation.

To gain some insights in to the expression pattern of SM gene clusters in necrotrophic pathogens, here, we extracted the previous microarray data of *P. nodorum* during wheat leaf infection (Ipcho et al., 2012). During *in planta* stage, eight PKS genes and one NRPS genes, out of 24 and 14, respectively, were on average up-regulated twofold or more (**Figure 2**). There are also several genes that are up-regulated at the end of the necrotrophic phase (7 day post inoculation, dpi) before switching to saprotrophy. Close homologs of some of these genes can be found in other plant pathogens. For example, SNOG_05791, which was highly up-regulated at 3 dpi *in planta*, exhibits 82% head-to-tail protein identity to the alternapyrone synthase PKSN in *A. alternata* (Fujii et al., 2005). The final SM product and function of the PKSN gene cluster is yet to be characterized in *A. alternata* and it would be interesting to determine if the encoded metabolites play a role in the virulence of *P. nodorum* and *A. alternata*. We are in the process of teasing out the SMs encoded by these candidate gene clusters.

BRIDGING THE GAPS BETWEEN GENES, MOLECULES AND FUNCTIONS

Ecological genomics is a powerful approach for inferring functionality and narrowing down ecologically relevant SM gene clusters. However, to obtain deeper insights into the bio-ecological functions of these SM gene clusters, the encoded SM molecules have to be first identified. Traditional chemical ecology studies often involved fractionation of an active crude extract guided by ecologically relevant bioassays (e.g., growth inhibition or behavioral response of an interacting organism). However, this method is not suitable if the compounds are only produced in response to specific biotic interactions. Modern metabolomic techniques have enabled the measurement of the metabolome dynamics of organisms during interactions (Prince and Pohnert, 2010). Nonetheless, in systems involved intimate physical interactions between two organisms (e.g., plant–fungal interactions), it is often difficult to track the origin of the metabolites. Furthermore, the amount of compounds that can be obtained through such interaction studies is often limited, which hinders further molecular characterization and mode of action studies.

Genome mining is increasingly becoming a popular approach for natural product discovery in recent years (Challis, 2008; Wiemann and Keller, 2014). To obtain the SMs from a candidate gene cluster, the SM pathway can either be expressed in various heterologous hosts after reconstructing the pathway with host-compatible regulatory elements or in the native producer via consecutive promoter replacements (Ahuja et al., 2012; Lim et al., 2012; Tsunematsu et al., 2013; Lazarus et al., 2014; Yaegashi et al., 2014). Alternatively, the expression of a silent SM gene cluster can be activated by overexpression of pathway-specific transcriptional regulator, if such regulator is present in the candidate SM gene cluster (Bergmann et al., 2007; Brakhage and Schroeckh, 2011). These methods are capable of producing the desired SM compounds in significant quantities and at the same time establish the important link between genes and molecules. The ready access to genome sequencing means that the availability of DNA sequences is no longer a bottleneck. However, translating SM gene clusters to small molecule products, via the above mentioned methods, remains a time-consuming endeavor. So far, genome mining efforts are focusing on (1) identifying the SM gene cluster of a known compound, (2) discovering analogous SMs from gene clusters that share homology to characterized ones, and (3) untargeted genome mining motivated by the quest to understand gene-to-molecule relationships. The application of ecological genomic tools adds a new dimension to genome mining and will help us navigate the vast genomic information in search for bioactive molecules (**Figure 1**).

Traditional reverse genetics and mutant phenotyping (i.e., virulence of mutants against the plant host) will still play important roles in casting light on the potential function of the SMs (**Figure 1**). For example, the presence and absence of specific compound in the wild type and mutant during inter-organism interactions can be verified by metabolic profiling. On the other hand, the information obtained from comparative phenotype assays between mutant and wild type will facilitate the development of specific bioassays to test the hypothetical function of the compounds obtained by ecogenomics-guided SM gene cluster mining. More recently, using this multi-pronged strategy, we have identified the SNOG_00477 PKS gene that is strongly expressed *in planta* to encode (*R*)-mellein production in *P. nodorum* by gene disruption and heterologous expression in yeast (Chooi et al., 2014a). We further demonstrated that mellein is a strong inhibitor of wheat seed germination. In conclusion, we believe this integrated chemical ecogenomic approach will accelerate the bridging of gaps between genes, molecules and functions, effectively linking genotype-to-phenotype. This multi-pronged approach is also applicable to other microbial ecological systems and will propel the next frontiers in fungal SM research.

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