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Secondary metabolism in fungi: does chromosomal location matter?

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Filamentous fungi produce a vast array of small molecules called secondary metabolites, which include toxins as well as antibiotics. Coregulated gene clusters are the hallmark of fungal secondary metabolism, and there is a growing body of evidence that suggests regulation is at least, in part, epigenetic. Chromatin-level control is involved in several silencing phenomena observed in fungi including mating type switching, telomere position effect (TPE), silencing of ribosomal DNA, regulation of genes involved in nutrient acquisition, and as presented here, secondary metabolite cluster expression. These phenomena are tied together by the underlying theme of chromosomal location, often near centromeres and telomeres, where facultative heterochromatin plays a role in transcription. Secondary metabolite gene clusters are often located subtelomerically and recently it has been shown that proteins involved in chromatin remodeling, such as LaeA, ClrD, CclA, and HepA mediate cluster regulation.

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Introduction

For many years it has been known that chromosomal location and histone modification have profound effects on gene transcription in a variety of organisms from yeast to humans. Filamentous fungi produce many bioactive small molecules, or secondary metabolites, that range from beneficial antibiotics to harmful toxins. Genes responsible for the production of these secondary metabolites are typically clustered and coregulated [1]. Interestingly, the order and location of biosynthetic genes within a cluster is important for their regulation. Additionally, secondary metabolite gene clusters have a tendency to be located near the ends of chromosomes in areas termed subtelomeric [2••,3•] — a region where chromatin modifiers impact transcription of these clustered genes. Here we review the importance of location, both specific locations of genes within a cluster, the chromosomal location of the entire cluster itself, and putative epigenetic forces on the genetic regulation of secondary metabolite gene clusters in fungi. We offer a view that secondary metabolite clusters are located in regions of facultative heterochromatin, which can be silenced and activated by both canonical and novel chromatin-mediated mechanisms.

Hallmarks of gene silencing in fungi

Eukaryotic organisms have evolved orchestrated mechanisms to regulate their large gene networks for proper development and appropriate environmental responses. In recent years, much interest has been focused on epigenetic and small RNA regulation of gene expression. Common to all eukaryotes, fungi possess several cellular devices important in gene silencing and activation. Early research in *Saccharomyces cerevisiae* identified the silent mating type loci (HML/HMR), which subsequently opened the door to an extensive body of work on positional effects in fungi as well as higher eukaryotes [4]. A key finding from the *S. cerevisiae* work was that exogenous genes were repressed when integrated at the silent mating type loci, thus indicating that repression was due to positional effects [5]. The mating type switching phenomenon has also been reported in fission yeast, *Schizosaccharomyces pombe*, where repetitive border elements facilitate the silencing effect [6].

An additional silencing mechanism is termed telomere position effect (TPE). This phenomenon was first reported in yeast and occurs when subtelomerically located genes are repressed [7]. In fungi, TPE has been demonstrated in *S. cerevisiae* [8], *Sc. pombe* [9], *Candida glabrata* [10], *Neurospora crassa* [11], and recently, *Aspergillus nidulans* ([12•], Palmer *et al.*, unpublished results). The extent of TPE is variable at the 32 yeast telomeres [13], but generally extends 20 kb indicating several hundred genes are regulated by TPE [14].

A commonality in the above instances of positional silencing of gene expression is the involvement of chromatin-level control, commonly termed the histone code, where residues on the histone tails are modified, which in turn results in alterations of chromatin structure [15]. Chromatin can exist in two states: euchromatin is transcriptionally active and characterized by low nucleosome density, while heterochromatin is transcriptionally silent

Table 1

Selected examples of chromatin-level control affecting aspects of fungal development.

Developmental aspect	Organism	Phenotypic description	Reference
Nitrate and proline utilization	*Aspergillus nidulans*	Nitrate and proline genes are clustered Inducing/repressive conditions alter nucleosome positioning in promoter and histone H3 acetylation patterns	[50,51]
Adhesion	*Candida glabrata*	Adhesins important pathogenicity factors in *C. glabrata* Adhesins are produced by telomerically located EPA genes EPA genes regulated by TPE and HDAC's	[52]
Growth and Reproduction Defects	*Neurospora crassa*	Severe growth defects in null mutants of the H3K9 methyltransferase and heterochromatin protein 1	[53,54]
	Aspergillus fumigatus	Null mutant of H3K9 methyltransferase shows impaired growth and delayed asexual development	[55]

and contains densely packed nucleosomes. Heterochromatin that can become activated under particular circumstances is sometimes referred to as facultative heterochromatin as illustrated by developmentally timed gene expression in *Drosophila* [16]. Histone tail residues that are hyperacetylated and methylated at lysine 4 of histone 3 (H3K4) are associated with gene transcription and euchromatin, while hypoacetylation and methylation of lysine 9 of histone 3 (H3K9) are associated with gene silencing and heterochromatin [17]. These generalities are not rigid, however, as H3K4 methylation is also associated with silencing in yeast subtelomeric and rDNA regions [18]. A few examples of chromatin-mediated control affecting aspects of development in fungi are listed in Table 1.

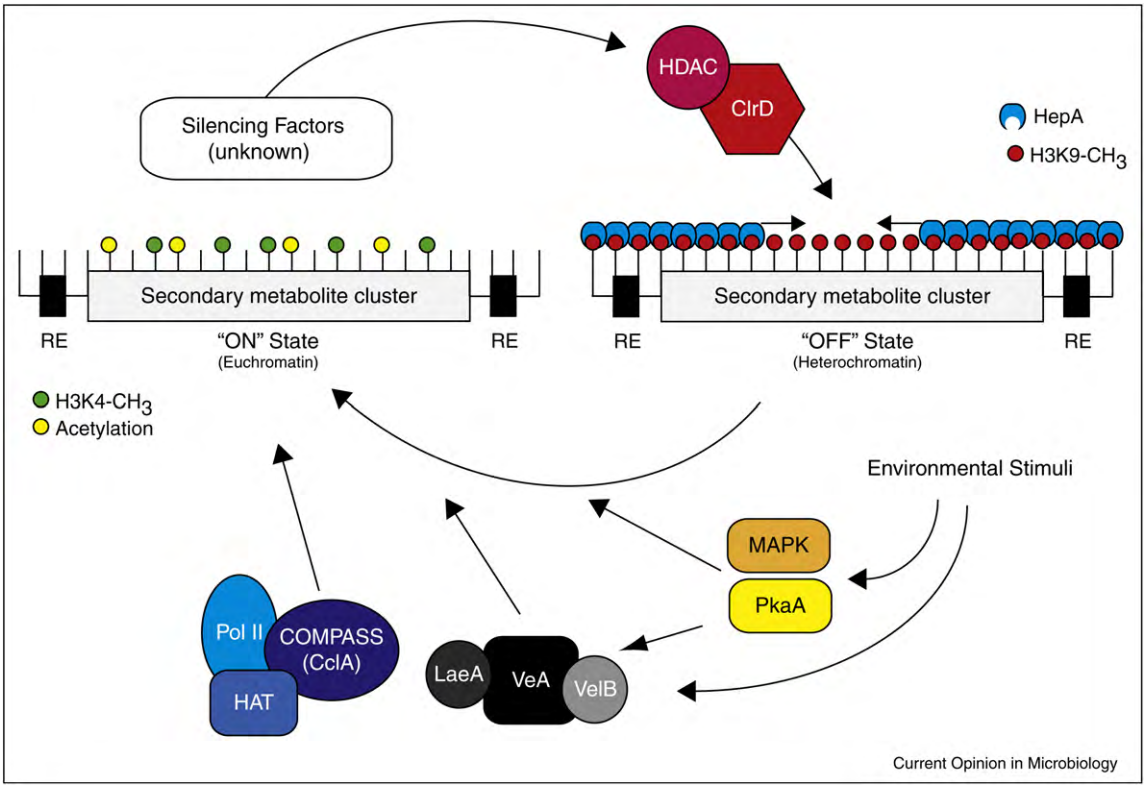
Regulation of secondary metabolite gene clusters in fungi

An unexpected finding upon inspection of several fungal genomes was the presence of vast numbers of secondary metabolite gene clusters [19]. Although most remain undefined, research on select gene clusters is quite robust and serves to illustrate several important points on the regulation of secondary metabolite gene clusters. The reader is directed to recent reviews detailing nonheterochromatic regulatory mechanisms employed to regulate these clusters [1,19–21]. Briefly, many clusters contain cluster specific transcription factors, often C6 zinc binuclear cluster proteins such as AflR for aflatoxin (AF)/sterigmatocystin (ST) biosynthesis in *Aspergillus* spp. [22] or Tri6 for trichothecene biosynthesis in *Fusarium* spp. [23] that function to activate biosynthetic genes in their respective cluster. Secondary metabolite clusters are also activated, and sometimes shut down, in response to a variety of environmental stimuli that include but are not limited to light, pH, carbon source, nitrogen source, ROS, and temperature (Figure 1) [24]. Environmental stimuli are translated to the nucleus through signal transduction cascades, such as the mitogen activating protein kinase (MAPK) cascade and the cAMP mediated PkaA cascade [25–29] and have been linked to activation of specific broad domain regulator factors including CreA (carbon metabolism), AreA (nitrogen metabolism) and PacC (pH sensor) [1].

The first hint that locality of secondary metabolite genes plays a role in their regulation came from characterization of one of the biosynthetic enzymes of the AF cluster in *Aspergillus parasiticus*, where localization of the *ver-1* gene outside of the AF cluster resulted in 500-fold lower expression than *ver-1* located inside the cluster [30]. Similarly, the AF biosynthetic enzyme *nor-1* was not expressed when located at two different positions outside of the AF cluster, which led to the conclusion that positional effects are important for expression of AF biosynthetic genes [31]. Insight into a mechanism controlling positional regulation of AF genes came with the discovery of LaeA, a global regulator of secondary metabolism in filamentous fungi ([32–35], Tudzynksi *et al.*, unpublished results). Recently, LaeA has been shown to be part of the *velvet complex*, consisting of LaeA–VeA–VelB, that functions to regulate development and secondary metabolism in response to light [36••]. LaeA regulation of gene clusters was found to be location dependent as placement of *aflR* outside of the ST cluster removes it from LaeA regulation, and conversely, placement of noncluster gene in the ST cluster puts it under LaeA control [37].

Although the precise function of LaeA remains enigmatic, several studies link LaeA activity with chromatin modifications. Recent data illustrate that mutations in *Aspergillus* histone modifying genes activate silent or poorly expressed gene clusters and, significantly, can partially remediate loss of secondary metabolite production in Δ*laeA* strains (Table 2). Three deletion mutants that produce increased levels of secondary metabolites target the H3K9 residue including HdaA, a histone deacetylase (HDAC) [38,39], HepA (heterochromatin protein 1) and ClrD (H3K9 methyltransferase) [40••]. The latter two mutations resulted in decreased H3K9 methylation inside ST cluster, which corresponded

Figure 1



A proposed model for chromatin-mediated control of secondary metabolite gene clusters. Secondary metabolite gene clusters are often flanked by repetitive elements (REs) and located in subtelomeric regions of the genome. The epigenetic marks of H3K4 methylation (H3K4-CH_3) and general histone acetylation have been shown to be associated with active gene transcription [17]. Thus, histone acetyltransferases (HAT) and the H3K4 methylation protein complex (COMPASS) are involved in initiation of transcription through RNA polymerase II (Pol II) [18]. Environmental stimuli are translated by signal transduction cascades, including but not limited to MAPK and PkaA, to trigger production of secondary metabolites [19]. These signals work independently and dependently through the LaeA containing velvet complex [25,26]. On the other hand, in several eukaryotic systems heterochromatin protein 1 has been shown to bind H3K9 methylation (H3K9-CH_3) and is associated with gene silencing. In *Aspergillus nidulans*, null mutants of the H3K9 methyltransferase (ClrD) and heterochromatin protein 1 (HepA) result in derepression of the ST gene cluster [40••]. Currently, the genetic components involved in initiation of heterochromatin at secondary metabolite gene clusters is unknown, RNAi-mediated heterochromatin formation could function this way as well as DNA binding repressors.

to increased ST production. In the same study, ChIP analysis showed that secondary metabolite deficient *ΔlaeA* strains contain increased H3K9 methylation in the ST cluster [40••]. Furthermore, HDAC inhibitors have been reported to increase secondary metabolite production in several fungi [38,41•]. Finally, again supporting a role for chromatin-level control, the order in which AF biosynthetic genes are transcribed mirrors

Table 2

Genes involved in chromatin-mediated control of secondary metabolism in *Aspergillus nidulans*.

Gene	Function	Secondary metabolism phenotype[a]	Reference
hepA	Heterochromatin protein 1	*ΔhepA* results in increased production of ST	[40••]
clrD	H3K9 methyltransferase	*ΔclrD* results in increased production of ST, partial remediation of ST in *ΔlaeA* background	[40••]
hdaA	Histone deacetylase	*ΔhdaA* results in increased production of ST and PN. Partial remediation of ST/PN in *ΔlaeA* background	[38]
cclA	H3K4 methyltransferase (part of COMPASS complex)	*ΔcclA* resulted in production of secondary metabolites from cryptic clusters	[43••]
laeA	Unknown	*ΔlaeA* results in loss of several secondary metabolites (ST, PN, TQ) and increased H3K9 methylation in the ST cluster	[37,40]

[a] ST = sterigmatocystin, PN = penicillin, and TQ = terrequinone A.

increased histone H4 acetylation patterns in the AF cluster [42•]. While these results confirm that histone modifications are directly linked with secondary metabolite cluster activation, it remains unclear if LaeA directly or indirectly modifies chromatin structure. It has long been speculated that LaeA could directly change chromatin structure through methylation of histones [32,37], however, a substrate for methylation by LaeA remains to be identified.

Chromosomal location of secondary metabolite gene clusters

As mentioned earlier, methylation of H3K9 is associated with heterochromatin, while methylation of H3K4 is more commonly associated with euchromatin and transcription. However, the COMPASS complex, which methylates H3K4 in yeast, is also associated with homothallic mating type silencing, ribosomal DNA silencing, and subtelomeric gene expression in this fungus [18]. Paralleling these observations, it was shown that a mutant defective in a component of the COMPASS complex activates silent secondary metabolite clusters in *A. nidulans* [43••]. These studies led to the discovery of the gene clusters responsible for producing emodin, F9775A/F9775B, and monodictyphenone in addition to shedding light on genome mining techniques leading to discovery of cryptic gene clusters [43••,44,45]. Moreover, these advances have led to ''chemical epigenetic mining'' where incorporation of exogenous acetylase/methylase inhibitors or activators have led to identification of novel fungal metabolites [41•,46,47••,48]. These data suggest that cryptic or silent secondary metabolite gene clusters are located in regions of facultative heterochromatin and can be turned on when chromatin structure is changed.

In the human pathogen *Aspergillus fumigatus*, null mutants of LaeA display reduced pathogenicity in murine models of invasive aspergillosis [35,49]. An interesting feature of the LaeA regulon was revealed by microarray analysis in *A. fumigatus*, which suggested there was a tendency for LaeA regulated secondary metabolite clusters to be located in subtelomeric regions [3•]. This observation was recently substantiated by expression profiling in *A. fumigatus*, which revealed subtelomeric regions, including toxin genes, were highly up regulated when exposed to the murine lung compared to normal laboratory growth [2••]. There is striking overlap between secondary metabolite clusters regulated by LaeA and the subtelomeric regions differentially regulated upon exposure to the murine model [2••]. Taken together, these data imply that subtelomeric location of secondary metabolite clusters may be important for their genetic regulation and biological function.

A conserved feature of subtelomeric DNA sequences, including secondary metabolite gene clusters, is the presence of repetitive elements (REs) composed of active transposable elements or transposon relics. Because active transposons have the potential to be disruptive in the genome, organisms employ complex regulatory mechanisms to limit their expression, such as RNAi-mediated heterochromatin formation [6]. A possible role for transposon regulation of a subtelomeric gene clusters was recently reported for the penicillin (PN) gene cluster [12•]. The PN cluster consists of only three genes and is located ~30 kb from the telomere of chromosome VI. Disruption of large areas of repetitive DNA sequences resulted in mutants producing significantly less PN. Characterization of one area, a 3.7 kb repeat termed PbIa (penicillin boundary element Ia) containing two transposons/transposon relics, showed its removal decreased PN production, whereas control strains harboring marker gene insertions to either side of PbIa had no effect on PN production. Subsequent *trans*-complementation experiments were unable to restore PN production. In contrast, deletion of the HDAC HdaA in the ΔPbIa background was able to restore production of PN, suggesting that a transposon mechanism of secondary metabolite cluster expression could involve localized chromatin modifications [12•].

Conclusions

This review highlights work suggestive of epigenetic regulation of secondary metabolite gene clusters in filamentous fungi. Recently there has been an increase in the number of examples of gene cluster regulation mediated by chromatin remodeling enzymes, including chemical epigenetic approaches. These studies reveal the importance of positional effects, both location effects within a cluster and chromosomal location effects on cluster regulation. Future studies are warranted to tease out the molecular mechanisms of epigenetic regulation. Interesting questions remain to be answered: which happens first — chromatin remodeling leading to transcription factor activation or transcription factor binding leading to chromatin remodeling? Does RNAi have a role in chromatin-mediated regulation of secondary metabolism? What role do repetitive elements that flank gene clusters have in regulation? Does LaeA directly or indirectly modify chromatin structure?

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