2 Insight into Fungal Secondary Metabolism from Ten Years of LaeA Research

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CONTENTS

I. Introduction

Fungi are well known for their ability to produce copious numbers of bioactive small molecules known as natural products or secondary metabolites (SMs), the moniker used in this chapter. Since the discovery of penicillin in 1928 by Alexander Fleming, the number of partially or fully characterized fungal SMs has risen exponentially. The interest in fungal SMs lies primarily in their useful antibiotic and pharmaceutical activities, although several of these metabolites are also potent phytotoxins or mycotoxins, contributing adversely to plant, animal, and/or human health (Leitão and Enquita 2014). A literature survey of fungal metabolites, covering 1500 compounds that were isolated and characterized between 1993 and 2001, showed that more than half of the molecules had antibacterial, antifungal, or antitumor activity (Pelaez et al. 2005). In particular, certain members of the Ascomycetes and Basidiomycetes encode a large wealth of SMs that—as observed from genomes of sequenced fungi—remain largely untapped.

The first genetically characterized fungal SMs, the β-lactam antibiotics—penicillin and cephalosporins (Martin 1992) and the mycotoxins—aflatoxin and sterigmatocystin (Brown et al. 1996; Yu et al. 1995; Trail et al. 1995), revealed the near-universal clustered arrangement of genes involved in the production of a single SM. This clustering of fungal SM genes (reviewed in Hoffmeister and Keller 2007) has accelerated the ability to identify SM clusters in fungal genomes and led to the development of various bioinformatic algorithms, such as SMURF, antiSMASH, or MIDDAS-M (Khaldi et al. 2010; Medema et al. 2011; Umemura et al. 2013). While unable to predict intertwined superclusters containing genes for more than one SM (Wiemann et al. 2013) or account for genes outside of the cluster (Sanchez et al. 2011), these programs have greatly assisted in initial predictions of fungal SM gene clusters.

A major goal of studying SM is to understand how SM cluster genes are regulated. Some of the clusters contain cluster-specific transcription factors (e.g., AflR regulating expression of aflatoxin and sterigmatocystin clusters, Fernandes et al. 1998; Woloshuk et al. 1994) that, when activated naturally or through genetic manipulations, induce expression of other genes within the cluster (examples in Hoffmeister and Keller 2007; Brakhage 2013). Rarely, these types of in-cluster transcription

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factors have been reported to regulate another SM cluster such as AflR regulation of the asperthecin gene cluster (Yin et al. 2012). Thus, the discovery of LaeA, capable of regulating multiple SM clusters simultaneously, was remarkable and recognized early on as a useful tool in SM sleuthing (Bok and Keller 2004; Bok et al. 2006a). Here, we present an overview of LaeA function in SM production in fungi, and in so doing, compile a list of all SMs currently known to be regulated by this protein. For a more thorough review of LaeA impact on other aspects of fungal development, we refer the reader to Jain and Keller (2013).

II. LaeA Mechanism

LaeA was identified through a chemical mutagenesis of an *Aspergillus nidulans* norsolorinic acid-producing strain. This compound is a visible orange precursor of both sterigmatocystin and aflatoxin, and loss of its production is easy to screen (Butchko et al. 1999). Twenty-three single gene mutants were obtained with LaeA representing one of three mutants showing loss of a*flR* expression. Chemical characterization of *ΔlaeA* showed a decrease not only in sterigmatocystin production but also in multiple secondary metabolites (Bok and Keller 2004). The number and types of LaeA regulated SMs in *A. nidulans* and other fungi are described in the next section.

A. Methyltransferase

LaeA contains an *S*-adenosyl methionine (SAM)-binding site that when mutated yields a null-LaeA phenotype (Bok and Keller 2004), presumably indicative of methyltransferase activity. However, to date, other than demonstrating automethylation at a methionine residue near the SAM-binding site, a modification which is not required for in vivo function (Patananan et al. 2013), no substrate-specific methyltransferase activity has been found for LaeA. Interestingly, microarray analysis of the *A. fumigatus ΔlaeA* mutant shows it to be downregulated in the sulfur/methionine regulon (Perrin et al. 2007); however, no mechanistic connection between LaeA and this metabolic pathway has been established.

B. Epigenetics

Due to LaeA's similarities to methyltransferases, its localization in the nucleus, and its often precise regulation of SM clusters (Bouhired et al. 2007), it has been suggested that LaeA regulates transcription by protein lysine or protein arginine methyltransferase functions (Bok and Keller 2004; Bok et al. 2006b; Fox and Howlett 2008). Although no direct biochemical studies have demonstrated such a role, this protein has been linked to changes in chromatin structure in SM gene clusters where loss of LaeA leads to increased heterochromatin marks (Reyes-Dominguez et al. 2010). Several papers have indicated a role for LaeA in interactions with canonical histone-modifying enzymes, including HdaA, HstD, and CclA (Kawauchi et al. 2013; Bok et al. 2009; Shwab et al. 2007).

C. Velvet Complex Member

A clue to how LaeA works also came from the finding that it is a member of a nuclear complex known as the Velvet Complex composed of LaeA, VeA, and VelB (Bayram et al. 2008). Although first noted for its role in SM regulation, LaeA also has a profound effect on both asexual and sexual spore development, as do both VeA and VelB (Sarikaya Bayram et al. 2010; Bayram and Braus 2012). Thus, the Velvet Complex as a unit links morphological development with chemical development in all fungi examined so far (Wiemann et al. 2010; Lopez-Berges et al. 2013; Wu et al. 2012; Kosalková et al. 2009; Amaike and Keller 2009; Baba et al. 2012). When described, the phenotypes of deletants of these three genes are not equivalent but overlapping in some regulatory aspects of SM and morphological development.

III. Secondary Metabolites Regulated by LaeA

The initial characterization of LaeA in *A. nidulans* reported LaeA as positively regulating two well characterized endogenous SMs (sterigmatocystin and penicillin) as well as the heterologous lovastatin SM cluster genetically engineered into *A. nidulans* (Bok and Keller 2004). A second study of *A. nidulans* LaeA using microarray analysis identified additional uncharacterized SM clusters positively regulated by LaeA where one was characterized as producing terrequinone A (Bok et al. 2006a). Many more *A. nidulans* SMs have been discovered since these papers, and it is likely that LaeA regulates some, perhaps a majority, of these newly characterized SMs (Yaegashi et al. 2014). Microarray studies of at least four additional species (*A. fumigatus*, *A. flavus*, *Fusarium fujikuroi*, and *Trichoderma reesei*) show that many unknown and known SM clusters are regulated by LaeA; however, here we will only focus on those assigned to a metabolite (Bok et al. 2006a; Perrin et al. 2007; Georgianna et al. 2010; Karimi-Aghcheh et al. 2013; Wiemann et al. 2010 and Table 2.1). The reader should note that Table 2.1 represents only a small fraction of SMs regulated by LaeA, as many papers report an association of SM with LaeA without reporting what these metabolites are (Perrin et al. 2007; Georgianna et al. 2010; Karimi-Aghcheh et al. 2013; Wiemann et al. 2010; Rachmawati et al. 2013). Below, NRPS indicates a non-ribosomal peptide synthase derived SM, PKS a polyketide derived SM, and DMATS a dimethylallyl tryptophan synthase derived SM.

A. *Aspergillus* species

LaeA regulated SMs have been partially characterized in five *Aspergillus spp*., including *A. nidulans*, *A. fumigatus*, *A. flavus*, *A. oryzae*, *and A. carbonarius*. In *A. fumigatus*, LaeA regulated SMs include gliotoxin (NRPS, cluster size: 25 kb), fumitremorgin (NRPS, cluster size: 25 kb), pseurotin (PKS/NRPS hybrid, part of an intertwined cluster with fumagillin, cluster size: 50 kb), fumagillin (PKS/terpene hybrid), endocrocin (PKS, cluster size: 15 kb), festuclavine (DMATS), elymoclavine (DMATS), fumigaclavines (DMATS), helvolic acid (terpene, cluster size 17kbref), fumiquinazolines (NRPS cluster size: 15 kb), and hexadehydroastechrome (NRPS, cluster size: 25 kb). Several of these metabolites have been implicated as playing a role in virulence in this human pathogen (Abad et al. 2010).

LaeA in *A. flavus* regulates aflatoxin (PKS, cluster size: 80 kb), diastereomeric piperazines (two duplicated clusters encoding NRPS-like adenylating reductases, cluster sizes each: 13 and 20 kb), morpholine (NRPS), pyrazines (NRPS), cyclopiazonic acid (PKS/NRPS), 3-(p-hydroxyphenyl)-1,2-propanediol (NRPS), kojic acid (simple organic acid from glucose), aspergillic acid (NRPS), paspaline (DMATS), paspalinine (DMATS), aflatrem (DMATS, cluster size:10 kb), and aflavinines (DMATS). LaeA in *A. oryzae* regulates kojic acid and the heterologously expressed terrequinone A (NRPS, cluster size: 10 kb) and monacolin K (PKS/NRPS) clusters. LaeA in *A. carbonarius* regulates ochratoxin A (NRPS).

B. Other Genera

LaeA orthologs have been identified in other fungal genera. LaeA has been characterized in several *Fusarium* species including *F. oxysporum* where it regulates beauvericin (NRPS, cluster size: 10 kb), ferricrocin (NRPS), and triacetylfusarinine C (NRPS). Lae1 in *F. verticillioides* regulates bikaverin (PKS, cluster size: 12 kb), fumonisin (PKS, cluster size 43 kb), fusaric acid (PKS, cluster size: 13 kb), and fusarins (PKS/NRPS). FfLae1 in *F. fujikuroi* regulates gibberellin (terpene, cluster size: 15 kb), fumonisin (PKS, cluster size: 42 kb), fusarin C (PKS/NRPS, cluster size: 25 kb), and bikaverin (PKS, cluster size: 12 kb). FgLaeA in *F. graminearum* regulates trichothecenes (terpene, cluster size: 25 kb) and zearalenone (PKS, cluster size: 22 kb).

ChLae1 in *Cochliobolus heterostrophus* regulates T-toxin (PKS) and melanin (PKS). LaeA in *Monascus pilosus* regulates monacolin K

Table 2.1 LaeA linked secondary metabolite regulation in filamentous fungi

Gene name	Species	Secondary metabolites	References
LaeA	*Aspergillus nidulans*	Sterigmatocystin, penicillin, lovastatin	Bok and Keller (2004), Bok et al. (2006b)
		Hyphal pigments	Sarikaya Bayram et al. (2010)
		Terrequinone A	Bok et al. (2006a), Bouhired et al. (2007)
		Monodictyphenone, F9775A , F9775B	Bok et al. (2009)
LaeA	*A. fumigatus*	Gliotoxin	Bok et al. (2005), Bok and Keller (2004), Sugui et al. (2007), Ben-Ami et al. (2009), Perrin et al. (2007)
		Fumagillin	Dhingra et al. (2013)
		Fumitremorgin, pseurotin	Wiemann et al. (2013), Perrin et al. (2007)
		Endocrocin	Lim et al. 2012
		Festuclavine, elymoclavine, fumigaclavines	Perrin et al. (2007)
		Hexadehydroastechrome	Yin et al. (2013)
		Helvolbic acid	Lodeiro et al. (2009)
		Fumiquinazolines	Lim et al. (2014)
LaeA	*A. flavus*	Aflatoxin	Amaike and Keller (2011), Kale et al. (2008), Georgianna et al. (2010)
		Diastereomeric piperazines, morpholine, pyrazines, 3-(p-hydroxyphenyl)-1,2-propanediol	Forseth et al. (2013)
		Cyclopiazonic acid	Kale et al. (2008), Georgianna et al. (2010)
		Aspergillic acid, paspaline, paspalinine, aflatrem, aflavinines, kojic acid	Kale et al. (2008)
LaeA	*A. oryzae*	Kojic acid	Oda et al. (2011)
		Terrequinone A, monacolin K	Sakai et al. (2012)
LaeA	*A. carbonarius*	Ochratoxin A	Crespo-Sempere et al. (2013)
ChLae1	*Cochliobolus heterostrophus*	T-toxin, melanin	Wu et al. (2012)
LaeA	*Fusarium oxysporum*	Beauvericin	López-Berges et al. (2014)
		Triacetylfusarinine C , ferricrocin	Lopez-Berges et al. (2013)
Lae1	*F. verticillioides*	Bikaverin, fumonisins, fusaric acid, fusarins	Butchko et al. (2012)
FfLae1	*F. fujikuroi*	Gibberellin, fumonisins, fusarin C, bikaverin	Wiemann et al. (2010)
		Fusarin C	Niehaus et al. (2013)
FgLaeA	*F. graminearum*	Trichothecenes, zearalenone	Kim et al. (2013)
LaeA	*Monascus pilosus*	Monacolin K, pigments	Lee et al. (2013), Zhang and Miyake (2009)
LaeA	*Penicillium citrinum*	ML236B	Baba et al. (2012)
PcLaeA	*P. chrysogenum*	Penicillin	Kosalková et al. (2009), Kopke et al. (2013), Hoff et al. (2010), Martín et al. (2012), Veiga et al. (2012)
		Pigments	Kosalková et al. (2009)
Lae1	*Trichoderma reesei*	Sterigmatocystin, siderophore	Karimi-Aghcheh et al. (2013)

(PKS, cluster size: 42 kb) and various pigments. LaeA in *Penicillium citrinum* regulates ML236B (PKS/NRPS, cluster size: 20 kb). PcLaeA in *P. chrysogenum* regulates penicillin (NRPS, cluster size: 15 kb) and pigments. Lae1 in *Trichoderma reesei* controls siderophore (NRPS) and the heterologously expressed sterigmatocystin cluster (PKS, 60 kb).

IV. Processes Identified Through LaeA Microarrays

As mentioned above, several microarray studies have led to characterization of several SMs, including but not limited to terrequinone A (Bok et al. 2006b), piperazines (Forseth et al. 2013), pseurotin (Wiemann et al. 2013), fumagillin (Wiemann et al. 2013), endocrocin (Lim et al. 2012), fumiquinazoline (Lim et al. 2014), and hexadehydroastechrome (Yin et al. 2012). However, other non-SM genes regulated by LaeA also may impact SM production. Characterization of LaeA regulated transcription factors include the sporulation specific regulatory protein BrlA as mediating LaeA regulation of spore-specific SMs (Berthier et al. 2013; Lim et al. 2014), NosA as mediating germination defects of the *ΔlaeA* mutant (Soukup et al. 2012b), and MeaB, a bZIP protein, enhancing virulence in *A. flavus* (Amaike et al. 2013). Details of BrlA are discussed in Chap. 1.

Both BrlA and MeaB affect SM production. BrlA is required for transcription and production of several spore-specific SMs, including endocrocin, fumiquinazoline, fumigaclavines, trypacidin, and various uncharacterized SMs in *A. fumigatus* (Berthier et al. 2013; Lim et al. 2014; Twumasi-Boateng et al. 2009; Coyle et al. 2007; Gauthier et al. 2012). Currently, it is not known if LaeA regulation of spore SMs is also mediated by BrlA—or the appropriate sporulation transcription factor in non-Aspergilli—in other fungal spp. Although not reported to be through BrlA, one study suggested that LaeA regulation of aflatoxin in *A. flavus* might be mediated through alterations in conidial development (Chang et al. 2012), and it was noted that *laeA* loss also impacted hydrophobin content in *A. fumigatus* spores (Dagenais et al. 2010). MeaB had a regulatory impact on aflatoxin synthesis in *A. flavus* where loss of MeaB greatly reduced production of this mycotoxin (Amaike et al. 2013).

A microarray analysis of *Trichoderma reesei* showed that *lae1* loss in this species resulted in complete loss of enzymes (CAZymes) responsible for lignocellulose degradation. On the other hand, overexpression of *lae1* led to enhanced CAZyme gene transcription (Seiboth et al. 2012). Another study, this one in *P. chrysogenum*, resulted in the identification of 62 genes co-regulated by both PcVelA and PcLaeA. One gene positively regulated by both proteins was *PcchiB1* encoding a class V chitinase required for cell wall integrity and pellet formation in *P. chrysogenum* (Kamerewerd et al. 2011). These two studies did not examine if there was relationship between SM production and these enzymes.

V. Processes Identified Through LaeA Mutagenesis

A multicopy suppressor screen looking for restoration of secondary metabolism in an *A. nidulans ΔlaeA* background has resulted in the identification of several novel regulators of SM. RsmA (remediation of secondary metabolism A) is a bZIP protein that directly regulates the sterigmatocystin gene cluster by binding to the intergenic region of AflR and AflJ (Shaaban et al. 2010; Yin et al. 2012, 2013). Asperthecin was also regulated by RsmA, apparently through transactivation by AflR (Yin et al. 2012). Overexpression of RsmA partially restored sterigmatocystin synthesis but not sporulation defects in both *ΔlaeA* and *ΔveA* backgrounds. The RsmA ortholog in *A. fumigatus* positively regulates gliotoxin in that species (Sekonyela et al. 2013).

The same screen also found EsaA, a histone acetyltransferase, to be a global regulator of SM. Like RsmA, overexpression of EsaA partially restored sterigmatocystin synthesis (and again, not sporulation defects) in *ΔlaeA* (Soukup et al. 2012a). Moreover, EsaA was determined to increase transcript levels of multiple SM cluster genes; this increase was associated with an increase in total H4 acetylation and specifically H4K12 acetylation of SM gene promoters. As mentioned earlier, several histone-modifying enzymes have been found to be important in SM regulation, often in relation with LaeA functionality.

VI. Conclusion

Since its discovery in 2004, LaeA has provided the research community with a new paradigm of regulation of SM gene clusters in fungi. The global nature of SM regulation by LaeA, presumably as part of the Velvet Complex, suggests an evolved requirement for production of certain SM in concert with morphological development, possibly as part of a stress response in protecting fungi from both abiotic and biotic stresses (Hong et al. 2013). Although present in most Ascomycetes, LaeA and other members of the Velvet complex are conspicuously missing in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Recently, putative VeA and VelB orthologs have been found in the Basidiomycete *Ustilago maydis* (Karakkat et al. 2013), and it remains to be seen if LaeA also exists in this fungus.

Considering the large number of sequenced fungi and unknown SM clusters, LaeA is likely to continue to be a valuable tool in natural product studies, both as a means to activate endogenous SM clusters and also, increasingly, as a tool to activate heterologously expressed clusters. This was recently demonstrated where *laeA* overexpression in *A. oryzae* activated transcription of the monacolin K gene cluster from *M. pilosus* and the terrequinone A gene cluster from *A. nidulans* (Sakai et al. 2012). In another embodiment, *A. nidulans laeA* was overexpressed in *Cordyceps militaris* to awaken silent secondary metabolite clusters in that fungus (Rachmawati et al. 2013). An alternative approach in utilizing LaeA as a SM enhancer was recently demonstrated in *P. chrysogenum* where 1,3-diaminopropane and spermidine were found to enhance *laeA* transcript levels and, thus, increase penicillin production (Martín et al. 2012; Pfeifer and Khosla 2001).

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