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Secondary metabolic gene cluster silencing in *Aspergillus nidulans*

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Summary

In contrast to most primary metabolism genes, the genes involved in secondary metabolism and certain nutrient utilization pathways are clustered in fungi. Recently a nuclear protein, LaeA, was found to be required for the transcription of several secondary metabolite gene clusters in *Aspergillus nidulans*. Here we show that LaeA regulation does not extend to nutrient utilization or the spoC1 sporulation clusters. One of the secondary metabolite clusters regulated by LaeA contains the positive regulatory (i.e. *aflR*) and biosynthetic genes required for biosynthesis of sterigmatocystin (ST), a carcinogenic toxin. Analysis of ST gene cluster expression indicates LaeA regulation of the cluster is location specific as transcription of genes bordering the ST cluster are unaffected in a Δ*laeA* mutant and placement of a primary metabolic gene, *argB*, in the ST cluster resulted in *argB* silencing in the Δ*laeA* background. ST cluster gene expression was remediated when an additional copy of *aflR* was placed outside of the cluster but not when placed in the cluster. Site-specific mutation of an s-adenosyl methionine (AdoMet) binding site in LaeA generated a Δ*laeA* phenotype suggesting the protein to be a methyltransferase.

Introduction

Filamentous fungi display many unique characteristics that render them of great interest to the research community. Among these characteristics is the production of natural products, or secondary metabolites (Bennett, 1987). These compounds often have obscure or unknown functions in the producing organism but have tremendous importance to humankind. Secondary metabolites display a broad range of useful antibiotic and immunosuppressant activities as well as less desirable phyto- and mycotoxic activities.

These biological properties of natural products have spurred efforts towards identifying genes involved in their synthesis. Accumulating data from these studies dispelled an original premise that fungal metabolic genes would be scattered throughout the genome. In fact, the hallmark of secondary metabolite genes, in contrast to most genes involved in primary metabolism, is that they are clustered in fungal genomes (Keller and Hohn, 1997). Examples of secondary metabolite gene clusters are well exemplified in the genus *Aspergillus* and include those synthesizing antibiotics (penicillin, PN), pharmaceuticals (lovastatin) and toxins (aflatoxin, AF) and sterigmatocystin (ST) (reviewed in Zhang *et al*., 2004).

One model of the clustering phenomenon predicts a need for physical linkage due to a regulatory mechanism(s) (reviewed in Zhang *et al*., 2004). Several transcription factors, typically $Zn(II)_2Cys_6$ binuclear zinc cluster and Cys_2His_2 zinc finger proteins, have been found to regulate secondary metabolite genes. These include metabolite-specific factors such as AflR regulating AF and ST biosynthetic genes (Woloshuk *et al*., 1994; Fernandes *et al*., 1998; Chang *et al*., 1999; Ehrlich *et al*., 1999) and also global transcriptional regulators including AreA (nitrogen regulation; Marzluf, 1997; Mihlan *et al*., 2003), PacC (pH regulation; Tilburn *et al*., 1995) and CreA (carbon catabolite repressor; Dowzer and Kelly, 1991; Espeso and Peñalva, 1992). However, a need for physical grouping of genes cannot be explained from these studies.

Recently, we identified a nuclear transcriptional regulator, LaeA, of secondary metabolite synthesis through complementation of a ST biosynthesis mutant in *Aspergillus nidulans* (Bok and Keller, 2004). Sequence analysis of LaeA showed no homology to known transcription factors but rather indicated some similarity to protein methyltransferases. Loss of LaeA silenced ST and PN production in *A. nidulans*, lovastatin production in *Aspergillus terreus* and gliotoxin production in *Aspergillus fumigatus* leading to the hypothesis that LaeA was involved in global regulation of secondary metabolite gene clusters. A role for global regulation of secondary metabolite gene clusters was further substantiated by recent microarray analysis of *A. nidulans laeA* mutants

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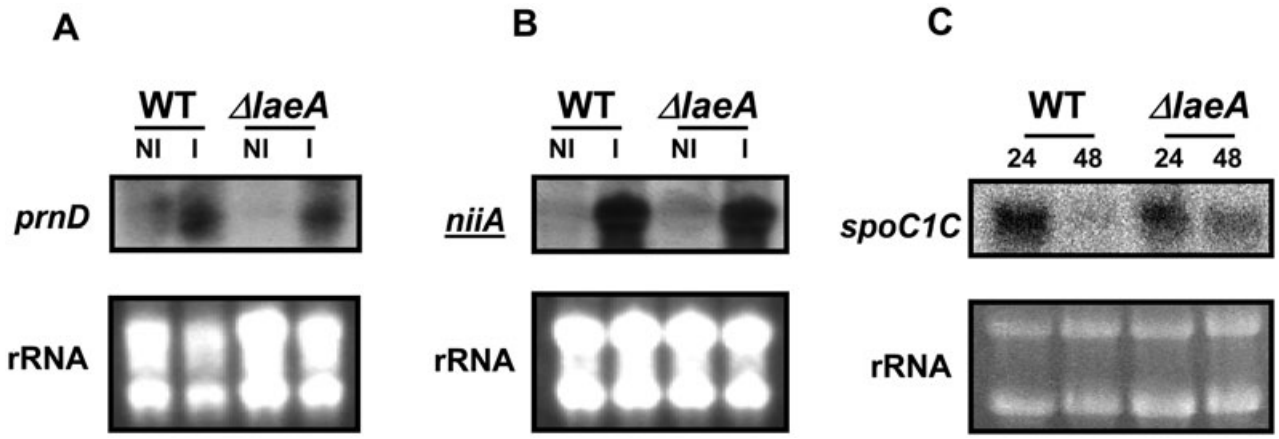

Fig. 1. Gene expression analysis of *prnD* (A), *niiA* (B) and *spoC1C* (C) in WT (RDIT2.3) and Δ*laeA* (RJW46.4) strains. *A. nidulans* WT and Δ*laeA* strains were cultured in *prnD*, *niaD* and *spoC1C* induction media as described in *Experimental procedures*. Blots were hybridized with *prnD*, a gene in the proline gene cluster, *niiA*, a gene in the nitrate gene cluster and *spoC1C*, a gene in the *spoC1* sporulation cluster. NI, not induced; I, induced. 24 and 48 represent hours of growth after switching to GMM solid media. Ethidium bromide-stained rRNA is indicated for loading.

which highlighted the potential of LaeA to identify novel clusters and their metabolites (Bok *et al*., 2006).

Here we present several lines of evidence demonstrating that LaeA regulation of gene clusters appears specific to secondary metabolite clusters and, furthermore, is chromosome location specific. Placement of a primary metabolic gene, *argB*, in the ST cluster results in downregulation of *argB* expression in the Δ*laeA* background. Additionally, an extra copy of *aflR* remediates ST biosynthesis in a Δ*laeA* background when placed outside of the ST cluster but not inside the cluster.

Results

Nutrient utilization clusters are not regulated by LaeA

In addition to gene clusters involved in secondary metabolism, fungi also possess catabolic pathways for the utilization of low-molecular-weight nutrients whose genes are often arranged in clusters. To examine the possibility that *laeA* might also regulate nutrient utilization cluster genes in *A. nidulans*, we examined transcripts from the proline (Garcia *et al*., 2004) and nitrate (Narendja *et al*., 2002) gene clusters in conditions known to induce or repress these genes in the wild-type (WT) strain (Muro-Pastor *et al*., 1999; Garcia *et al*., 2004). As shown in Fig. 1, representative transcripts were unaffected in the Δ*laeA* background in both non-induced and induced conditions for *prnD* (Fig. 1A) or *niiA* (Fig. 1B) gene expression. Additionally, a representative transcript from the conidiation-specific SpoC1 gene cluster (Gwynne *et al*., 1984) was also examined and found not to be regulated by LaeA at 24 h although there appeared to be a slight increase of expression at 48 h (Fig. 1C).

Genes bordering the ST cluster are not regulated by LaeA

Gene expression data of the *A. nidulans* Δ*laeA* strain compared with WT showed that selected genes in the ST cluster (defined by Brown *et al*., 1996) were downregulated (Bok and Keller, 2004). These findings are extended by recent microarray work illustrating a similar pattern of regulation of secondary metabolite gene clusters where all or nearly all genes within a cluster are down- or upregulated in a Δ*laeA* or overexpression *laeA* strain, respectively, with little effect on genes outside of the cluster (Fig. 2A and Bok *et al*., 2006). Here we present supportive data of these microarray results by assessing a transcriptional profile of the entire 60 kb ST gene cluster and flanking regions by Northern analysis in a Δ*laeA* strain compared with WT (Fig. 2B). Virtually every gene in the cluster is downregulated in the Δ*laeA* strain compared with WT. This is contrast to two flanking transcripts that show little if any difference in regulation in the two strains. Thus, LaeA regulation is specific to the cluster region and not border genes.

Remediation of ST gene expression in ΔlaeA *is dependent on location of an extra copy of* aflR

The gene encoding the ST pathway-specific transcription factor AflR is located in the ST cluster (Brown *et al*., 1996; Fernandes *et al*., 1998). An extra copy of *aflR*, whether in the ST cluster or placed at the *trpC* locus, increases ST cluster gene expression and subsequent ST production (Fig. 3 and data not shown). We investigated whether an extra copy of *aflR* could remediate Δ*laeA* silencing. As shown in Fig. 3, *aflR* and *stcU* (a ST biosynthetic gene formerly called *verA* and regulated by

Fig. 2. A. Microarray analysis identifies gene clusters regulated by LaeA. Sterigmatocystin (ST) gene cluster. Shown are expression ratios (Δ*laeA* to WT) for genes on chromosome IV in the region AN7800.2–AN7830.2. Asterisks indicate the *stcW* (AN7804.2) and *stcA* (AN7825.2) genes of the cluster, relative to the genome sequence annotation. Penicillin gene cluster. Shown are expression ratios (Δ*laeA* to WT) for genes on chromosome II in the region AN2621.2–AN2623.2. Asterisks indicate the first (AN2621.2, *acvA*) and last (AN7823.2, *aatA*) genes of the cluster, relative to the genome sequence annotation. Terrequinone A gene cluster. Shown are expression ratios (Δ*laeA* to WT) for genes on chromosome V in the region AN8513.2–AN8526.2. Asterisks indicate the first (AN8513.2, *tdiA*) and last (AN8517.2, *tdiE*) genes of the cluster, relative to the genome sequence annotation. Reprinted with permission from Elsevier from Bok *et al.* (2006).
B. Transcript analysis of the ST gene cluster and genes immediately upstream and downstream of the gene cluster. *A. nidulans* WT (RDIT2.3) and Δ*laeA* (RJW46.4) strains were grown in liquid shaking GMM for 12 h, 24 h, 48 h and 72 h at 37°C, 300 r.p.m. *stcA* and *stcU* are two characterized ST biosynthetic genes near either end of the ST gene cluster (Brown *et al.*, 1996). Blots were hybridized with *stcA*, *stcU*, pL11C09 (a cosmid covering AN7825.2–AN7803.2), a *stcA*-flanking PCR product (AN7826.2) and *stcX*-flanking PCR product (AN7801.2). Sequence is found at http://www.broad.mit.edu/annotation/fungi/aspergillus/. Ethidium bromide-stained rRNA is indicated for loading.
C. Schematic explanation of relative locations of biosynthetic genes in ST gene cluster. Solid arrows indicate genes in ST gene cluster, and hatched arrows indicate flanking transcripts.

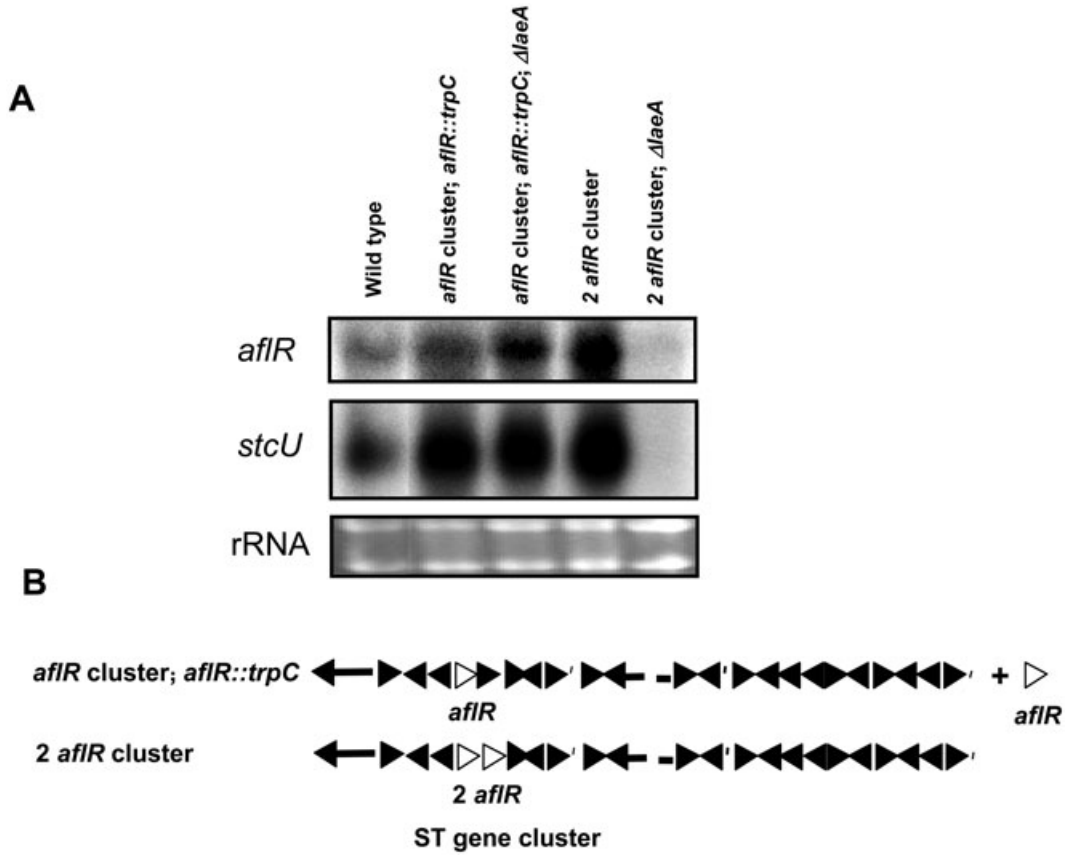

Fig. 3. A. An extra copy of *aflR* placed outside of the ST cluster can remediate ST gene expression in the Δ*laeA* background. WT (RDIT2.3);*aflR*; *trpC::aflR* (RDN01.55); *aflR*; *trpC::aflR*; Δ*laeA* (RJW54.8), two *aflR* in the cluster (RDN04.8) and two *aflR* in the cluster; Δ*laeA* (RDN05.2) were grown in liquid shaking GMM for 48 h at 37°C, 300 r.p.m. Blots were hybridized with *aflR* and *stcU*. Ethidium bromide-stained rRNA is indicated for loading.
B. Schematic explanation of placement of *aflR* genes (clear arrows) in the ST gene cluster or at the *trpC* locus.

aflR; Keller *et al*., 1994) were expressed in a Δ*laeA* background when the extra copy of *aflR* was placed at the *trpC* locus but not when placed in the ST cluster.

A primary metabolism gene is regulated by LaeA when placed in the ST cluster

To further investigate the possibility that gene regulation by LaeA was location dependent, we identified a gene involved in arginine metabolism, *argB* encoding ornithine carbamoyltransferase (Berse *et al*., 1983), that was not regulated by LaeA when *argB* was located at its native locus (Fig. 4, lanes 3 and 4, and Table 1). Strains were created where *argB* was removed from its native locus and placed in the ST cluster using its own promoter. Using these strains, a comparison of *argB* mRNA levels in WT and the Δ*laeA* mutant at 10 h, a time period when *stc* genes would not be expressed but optimal for *argB* expression, clearly shows *argB* transcription to be downregulated in the Δ*laeA* mutant (Fig. 4, lanes 7 and 8). Quantification of *argB* showed an approximately 1/3-fold lower expression level in the cluster in the Δ*laeA* background (Table 1). *argB* expression remained depressed in the Δ*laeA* strain at later, ST-inducing time points (e.g. 48 and 72 h, data not shown). This downregulation of *argB* expression was reminiscent, although not as severe, to the silencing of ST gene expression in a Δ*laeA* background (Figs 2 and 3 and Bok and Keller, 2004).

LaeA is a putative methyltransferase

The sequence of LaeA shows it contains conserved motifs commonly found in protein methyltransferases (Bok and Keller, 2004). Following procedures used to identify putative methyltransferase function, the AdoMet binding motif – LDL**G**C**G**TG – was mutated by replacing the two underscored glycines with alanines based on a modified Hamahata *et al*. (1996) method. Similarly to the Δ*laeA* strain, the strain carrying the mutation in the AdoMet binding site abolished the

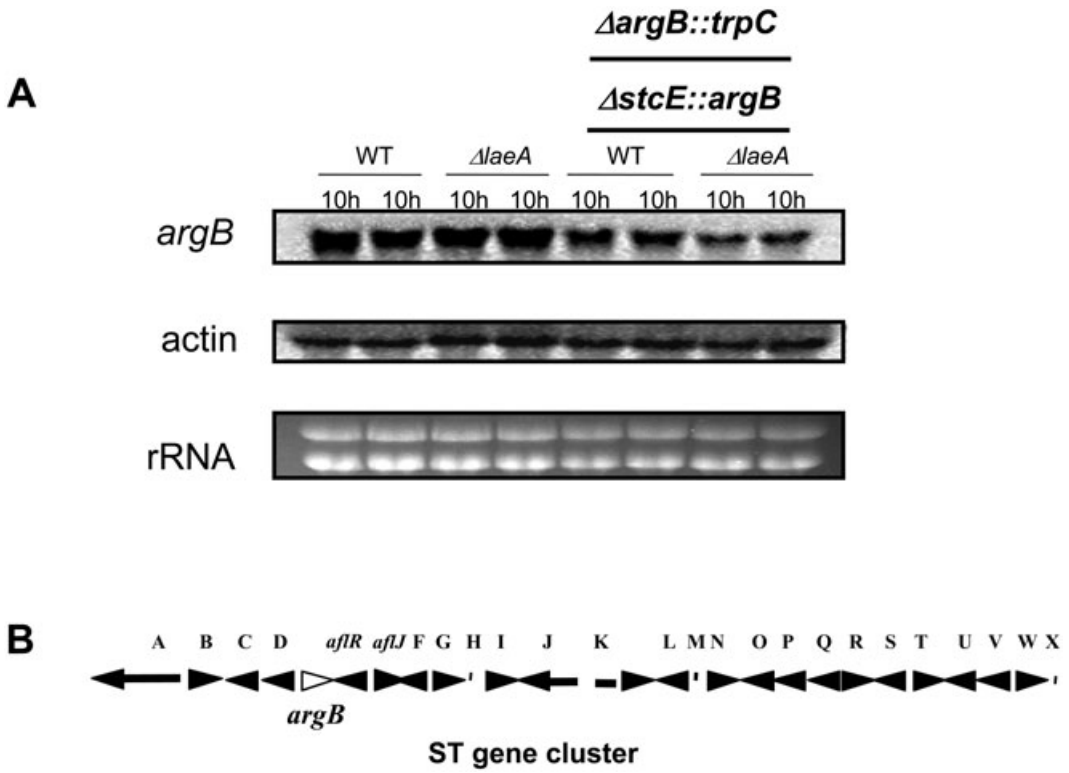

Fig. 4. A. *argB* expression is repressed in Δ*laeA* when placed in the ST cluster. WT (RDIT2.3), Δ*laeA* (RJW46.4), Δ*laeA*; Δ*stcE::argB*; Δ*argB::trpC* (RDN14.2) and Δ*stcE::argB*; Δ*argB::trpC* (RDN13.1) were grown in liquid shaking GMM for 10 h at 37°C, 300 r.p.m. Experiments were duplicated. Blots were hybridized with *argB* and actin. Ethidium bromide-stained rRNA is indicated for loading.
B. Schematic location of *argB* gene (clear arrow) in ST gene cluster.

expression of *aflR* and *stcU* required for ST production (Fig. 5). This result supports LaeA as a likely methyltransferase.

Discussion

The cluster arrangement of fungal genes involved in a unifying metabolic or developmental process has elicited much discussion on origination and maintenance of such clustering (reviewed in Zhang *et al*., 2004). No one model can explain the finding of such varied gene clusters as nutrient utilization, mating type, pathogenicity islands and secondary metabolism in the fungal genome. However, the identification of LaeA, a nuclear protein shown to transcriptionally activate several secondary metabolite gene clusters in *Aspergillus* spp. (Bok and Keller, 2004), suggested a possible global mechanism involved in cluster gene regulation. Here we present evidence indicating that LaeA regulation does not extend to nutrient utilization clusters nor the SpoC1 conidiation-specific cluster. Furthermore, the mechanism of LaeA regulation of gene expression appears coupled to chromosome location, as moving genes in or out of the ST cluster (a cluster known to be regulated by LaeA) directly correlated to gain or loss of transcriptional regulation mediated by LaeA.

Table 1. Quantification of *argB* expression in Fig. 4.

Lanes	*argB* (area volume)	Actin (area volume)	*argB*/actin
WT	112683	49124	2.195
WT	100108	52504	1.907
Δ*laeA*	105590	54865	1.925
Δ*laeA*	110929	57719	1.922
Δ*argB::trpC*; Δ*stcE::argB*	85274	51562	1.654
Δ*argB::trpC*; Δ*stcE::argB*	83532	53891	1.550
Δ*argB::trpC*; Δ*stcE::argB*; Δ*laeA*	55440	52164	1.063
Δ*argB::trpC*; Δ*stcE::argB*; Δ*laeA*	60682	54713	1.109

Transcripts were calibrated by ImageQuantTLv 2005 (Amersham Bioscience).

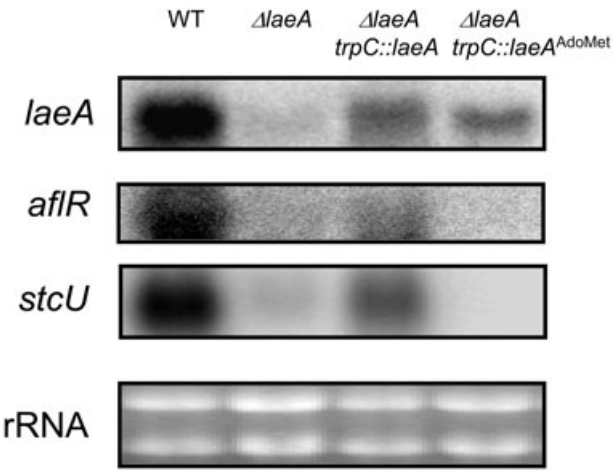

Fig. 5. Mutation of the AdoMet-binding motif in LaeA presents a Δ*laeA* transcription phenotype. WT (FGSC26), Δ*laeA* (RJW40.4), Δ*laeA*; *trpC::laeA* (TJW63.56) and Δ*laeA*; *trpC::laeA*^{AdoMet} (TJW60) were grown in liquid shaking GMM for 48 h at 37°C, 300 r.p.m. Mutations were introduced in the s-adenosyl methionine (AdoMet) binding site of LaeA (DLGCGTG → DLACATG) based on previously publication (Hamahata *et al*., 1996) to inactivate methyltransferase activity. Blots were hybridized with *laeA*, *aflR* and *stcU*. Ethidium bromide-stained rRNA is indicated for loading.

To address our hypothesis that *laeA* is specific to secondary metabolite gene cluster regulation, we examined the effects of loss of *laeA* on expression of a representative gene from two well-characterized primary metabolite gene clusters, the proline and nitrate utilization gene clusters. Our methods were based on well-established conditions that result in inductive or non-inductive conditions for these two clusters as described in Garcia *et al*. (2004) and Muro-Pastor *et al*. (1999) respectively. Loss of *laeA* appeared to have neither an effect on suppressing expression in induced conditions nor activating expression in the non-induced conditions (Fig. 1A and B). We also examined *spoC1C* expression in Δ*laeA* as compared with WT following conditions described for spoC1 cluster expression (Law and Timberlake, 1980; Gwynne *et al*., 1984). The spoC1 cluster contains genes expressed during early conidiation events, a time frame (24 h) when most secondary metabolites are not expressed. As show in Fig. 1C, *spoC1C* expression was similar in both strains at 24 h although there did appear to be a slight increase in *spoC1C* expression in Δ*laeA* as compared with WT at 48 h. Together, these results indicate that *laeA* has little impact on gene expression in these non-secondary metabolite gene clusters. This conclusion is supported by recent microarray analysis of *laeA* mutants compared with WT (Bok *et al*., 2006).

To further investigate factors affecting LaeA regulation of secondary metabolite gene clusters, we focused on gene regulation in the ST cluster. This approximately 60 kb cluster is bound on one side by *stcA*, encoding a polyketide synthase required for generating the ST carbon backbone (Yu and Leonard, 1995), and *stcW*, encoding a flavin-requiring monooxygenase (Keller *et al*., 2000), as well as the uncharacterized *stcX* (Brown *et al*., 1996). The sixth gene in the cluster is *aflR*, encoding a positive acting $Zn(II)_2Cys_6$ transcription factor required for the transcription of the biosynthetic *stc* genes (Fernandes *et al*., 1998). An examination of the expression of most genes in the ST cluster along with two uncharacterized border genes on either side of *stcA* and *stcX*, respectively, showed LaeA regulation was limited to the characterized ST cluster (Fig. 2 and Bok *et al*., 2006). This expression profile was similar to that of an *aflR* disruption strain (Yu *et al*., 1996). Thus, theoretically, LaeA regulation of the ST cluster could be entirely mediated by *aflR* suppression. Our finding that an extra copy of *aflR* could remediate the Δ*laeA* phenotype, but only when placed outside of the ST cluster (Fig. 3), suggests that cluster location of *aflR* may play the most important role in mediating LaeA regulation for ST cluster expression. This finding also raises questions on LaeA function. If our assumption is that LaeA activity is required for accessibility to ST cluster chromatin, it is not intuitive that a *trpC*-located *aflR* should rescue ST gene expression in a Δ*laeA* background. However, unlike the loss of *aflR*, loss of *laeA* does not result in a complete null ST phenotype. Depending on what media or temperature the fungus is grown, there is some ST production in the *laeA* mutant (data not shown). We speculate that the ST cluster in the Δ*laeA* strain is not entirely closed and, should there be sufficient *aflR* expression (such as at the *trpC* locus), the resulting gene product would be able to activate ST gene expression in the Δ*laeA* mutant. Our results are reminiscent to findings in *Saccharomyces cerevisiae* where it was shown that overexpression of the transcription factor PPR1 can suppress the silencing of an *ura3* gene placed at the telomere (Aparicio and Gottschling, 1994). However, we currently do not know whether the mechanism underlying these results is related in these two fungi.

In earlier studies we found both the ST and PN clusters were regulated by LaeA (Bok and Keller, 2004; Bok *et al*., 2006; Fig. 2A). A significant difference in these clusters, one pertinent to our observations with AflR and ST regulation, is that PENR1, the HAP-like transcriptional complex regulating PN biosynthesis, is not located in the PN cluster. It is also involved in expression of numerous non-PN genes (Litzka *et al*., 1998; Brakhage *et al*., 1999). Interestingly *penR1* was not regulated by LaeA based on our microarray analysis (Bok *et al*., 2006). Therefore, neither presence of a cluster-located transcription factor nor transcriptional regulation of such a factor is a requirement for LaeA control of secondary metabolite gene cluster expression. We suggest that LaeA exerts another layer of regulation on biosynthetic genes within a cluster

in addition to the regulation by the pathway-specific regulator. For example, a study by Liang *et al*. (1997) showed that the expression of *ver-1*, an orthologue of *A. nidulans stcU* and involved in AF biosynthesis in *Aspergillus parasiticus*, varied depending on location of this gene in the genome. Variation in *ver-1* expression as described by Liang *et al*. (1997) may be in part due to loss of regulation by LaeA.

The importance of chromosome location in LaeA regulation of gene expression was also supported by the *argB* expression profile. Figure 4 clearly shows *argB* expression is LaeA mediated when placed in the ST cluster. Although not silenced to the same degree as *stc* genes, it is nevertheless downregulated in the Δ*laeA* background when placed in the ST cluster. We found this downregulation both at an early time point, 10 h, when ST genes are not expressed and at late, ST-compatible time points (48 h; data not shown). This experiment also supports a non-AflR mechanism involved in LaeA control of gene expression in the ST cluster region of the chromosome as *argB* is not regulated by AflR, nor is *aflR* expressed at 10 h.

Previous results showed LaeA to be a nuclear-located protein with motifs most similar to those of protein methyltransferases (Hamahata *et al*., 1996; Bok and Keller, 2004), proteins involved in gene regulation through modification of chromatin structure (Peterson and Laniel, 2004). Here we show that site-specific mutation of the conserved AdoMet-binding motif in LaeA generated a strain with an identical phenotype to the loss of function allele (Fig. 5), thus supporting LaeA as a methyltransferase. Our current efforts are directed towards the possibility that LaeA could be involved in methylation changes of histone proteins and/or their associated activating complexes, and by these means affects transcription of select secondary metabolite gene clusters.

Experimental procedures

Plasmid construction

Plasmids were constructed using standard techniques. Turbo (Stratagene) was used for PCR reactions. Primers for PCR and probes are listed in Table 2. Plasmid pDN02 was constructed by ligation of a 2.5 kb ApaI fragment, containing the *aflR* gene and 400 bp of the native promoter, into pPK1, a pBluescript SK-based plasmid containing a 1.9 kb blunt end ligated SspI fragment containing *argB*. Plasmid pJW20 was constructed by ligation of the 2.5 kb ApaI fragment containing *aflR* into pSH96, a pBluescript SK-based plasmid containing a 1.8 kb blunt end-ligated SacI–EcoRI fragment containing the 5′-end of the *trpC* gene. pJW63.4 was constructed using double-joint PCR reactions (Yu *et al*., 2004) to introduce mutations in the s-adenosyl methionine (AdoMet) binding site of LaeA (DLGCGTG → DLACATG) based on a previous publication (Hamahata *et al*., 1996) to inactivate methyltransferase activity. To introduce mutations in the AdoMet binding site, a 1.8 kb 5′ fragment of *laeA* was amplified by using two primers, LAE1 (Bok and Keller, 2004) and Rmet. Another two primers, Fmet and LAE2 (Bok and Keller, 2004), created a 2 kb 3′ PCR product of *laeA*. These two purified fragments were mixed and a 3 kb fragment was amplified by two nested primers, Mt1 and OER (Bok and Keller, 2004), containing HindIII restriction enzyme sites at both ends, to yield a final 3 kb fragment containing the entire modified *laeA* gene. This 3 kb fragment was subcloned into the HindIII site of pSH96 (Wieser and Adams, 1995) to created pJW63.4.

Table 2. Primers.

Primer	Sequence
ActF	5′-TCTCGTTATCGACAATGGTTG-3′
ActR	5′-GAGAACGGCCTGAATAGAGA-3′
Fmet	5′-CCTCGATCGCCCAGATACCAGTGGCACAGGC TAGGTCCAGAAACCGGCCG-3′
Rmet	5′-AAGGCCGGTTTCTGGACCTAGCCTGTGCCAC TGGTATCTGGGCGATCGAG-3′
NiiF	5′-CGTGTAGGTCTGTCGTCGTA-3′
NiiR	5′-CCATCTCAATACCAGGAGCAATG-3′
PrnF	5′-TCTTTCCTCCGTTCTCCGCT-3′
PrnR	5′-CGTCGCAACTCAAGCAATAGA-3′
SpoC1CF	5′-ACCTAAACAATAAGCCGACTACAG-3′
SpoC1CR	5′-CACACTCAATCTCATCACCAGAC-3′
5′flankingF	5′-TACGGGTTCTCGAAGCAGCGC-3′
5′flankingR	5′-ACCAGTTCAAAGGTCTGTAAGCC-3′
3′flankingF	5′-AGCATCGCGATGAACGAGCCC-3′
3′flankingR	5′-AATTGACCAAACAATCGCCAGGG-3′
stcAF	5′-ATGGCCAGTCACGCTGAGCCA-3′
stcAR	5′-ATGTTGTCAATCTGCGCAGGCTT-3′

Nucleic acid analysis

DNA extractions from fungal and bacterial strains, restriction enzyme digestion, gel electrophoresis, blotting, hybridization and probe preparation were performed according to standard methods (Sambrook *et al*., 1989; Shimizu and Keller, 2001). Total RNA was isolated from lyophilized mycelia using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. RNA blots were hybridized with a 1.3 kb EcoRV–XhoI *aflR* fragment from pJW20, a 2 kb PCR product by primers stcAF and stcAR, a 0.7 kb SacII–KpnI fragment from pRB7 containing the *stcU* coding region (Bok and Keller, 2004), a 1.0 kb SacI–SphI *argB* fragment from pDN02, a 3 kb *laeA* fragment from pJW45.4 (Bok and Keller, 2004), a 0.9 kb PCR product by primers NiiF and NiiR for *niiA*, a 0.4 kb PCR product by primers PrnF and PrnR for prnD, a 1 kb PCR product by primers SpoC1CF and SpoC1CR for spoC1 and a 0.4kb PCR product by primers ActF and ActR for the actin gene. Also *A. nidulans* cosmid pL11C09, which contains most of the ST gene cluster (Brown et al., 1996), was used as a probe for mRNA expression. Flanking transcripts of the ST gene cluster were probed with a 2 kb PCR product amplified by primers 5′flankingF and 5′flankingR for a transcript upstream of *stcA* (http://www.broad.mit.edu/annotation/fungi/aspergillus/, contig1.132, 243110–245278 bp), and with a 3 kb PCR product amplified by primers 3′flankingF and 3′flankingR for a transcript down-

Table 3. *Aspergillus nidulans* strains used for this study.

Strain	Genotype	Source
FGSC26	*biA1, veA1*	FGSC
RAMB38	*biA1; methG1; veA1; trpC801, ΔaflR::argB*	Bergh
RDIT2.3	*veA1*	Tsitsigiannis
RDIT30.23	*argB2; methG1; veA1; trpC801*	Tsitsigiannis
RDIT30.34	*methG1; pyrG89; veA1; trpC801*	Tsitsigiannis
RDIT44.35	*biA1; pabaA4; pyrG89; veA1; trpC801*	Tsitsigiannis
RDN01.55	*veA1; trpC::aflR*	This study
RDN02.21	*argB2; veA1; ΔaflR::argB; trpC::aflR*	This study
RDN04.8	*aflR::argB::aflR; ΔargB::trpC; trpC801; veA1*	This study
RDN05.2	*methG1; ΔargB::trpC; veA1; aflR::argB::aflR; ΔlaeA::methG, trpC801*	This study
RDN13.1	*ΔargB::trpC; veA1; ΔstcE::argB; trpC801*	This study
RDN14.2	*methG1; ΔargB::trpC; veA1; ΔstcE::argB; ΔlaeA::methG, trpC801*	This study
RJH256	*biA1; argB2; veA1; ΔaflR::argB; trpC801*	Hicks
RJW3	*pyrG89, wA3; pyroA4; ΔstcE::argB; veA1; trpC801*	This study
RJW31	*biA1; wA3; argB2; ΔstcE::argB, veA1*	This study
RJW33.2	*methG1; wA3; pyroA4; argB2; veA1; ΔstcE::argB, trpC801, ΔlaeA::methG*	Bok and Keller (2004)
RJW40.4	*biA; methG1; veA1; ΔlaeA::methG*	Bok and Keller (2004)
RJW46.4	*methG1; veA1; ΔlaeA::methG*	Bok and Keller (2004)
RJW54.8	*methG1; veA1; ΔlaeA::methG, trpC::aflR*	This study
RJW55.8	*methG1; argB2; veA1; ΔaflR::argB; ΔlaeA::methG, trpC::aflR*	This study
RMS011	*pabaA1, yA2; veA1; ΔargB::trpC; trpC801*	Stringer *et al*. (1991)
TDN02.3	*pabaA1, yA2; veA1; ΔargB::trpC; aflR::argB::aflR; trpC801*	This study
TJW35.5	*biA1, methG1; wA3; argB2; veA1; ΔstcE::argB; ΔlaeA::methG*	Bok and Keller (2004)
TJW57.9	*methG1, wA3; pyroA4; argB2; veA1; ΔstcE::argB; ΔlaeA::methG, trpC::aflR*	This study
TJW60	*biA1; wA3; methG1; argB2; veA1; ΔstcE::argB, ΔlaeA::methG; trpC::laeA*^{AdoMet}	This study
TJW63.56	*biA1; wA3; methG1; argB2; veA1; ΔstcE::argB, ΔlaeA::methG; trpC::laeA*	This study

FGSC, Fungal Genetic Stock Center.

stream of *stcX* (http://www.broad.mit.edu/annotation/fungi/aspergillus/, contig1.132, 180034–182950 bp).

Fungal strains and culture conditions

The fungal strains used in these experiments are shown in Table 3. All strains were maintained as glycerol stocks and were grown at 37°C on solid minimal media plates or in minimal media liquid shake cultures both containing 1% glucose (GMM) as the sole carbon source (Shimizu and Keller, 2001). For the expression of *argB*, 50 ml of GMM was inoculated with 10^7 conidia per ml and incubated for 10 h at 37°C, 300 r.p.m. for each fungal strain analysed. For the expression of *spoC1C*, the method of Law and Timberlake (1980) was followed for induction of conidiophore formation. Briefly, 50 ml of GMM was inoculated with 10^6 conidia ml^{-1} of WT or Δ*laeA* and incubated for 20 h. Mycelia were harvested by filtration. To induce conidiation, the unwashed mycelia were placed on 9 cm Whatman no. 1 paper on top of solid GMM and incubated at 37°C. Conidia and mycelium were harvested at 24 h and 48 h for mRNA extraction. For *niiA* expression, the method of Muro-Pastor *et al*. (1999) was followed. Briefly, 100 ml GMM, substituting 5 mM urea (a non-induced, derepressed condition for *niiA* expression) for nitrate, was inoculated with 10^6 conidia per ml of WT or Δ*laeA*. To obtain enough mycelia, four flasks of WT and four flasks of Δ*laeA* were grown. After incubating for 7 h at 37°C at 300 r.p.m., the mycelia were harvested and washed and incubated in GMM-no nitrogen source medium for 20 min. The mycelia were then transferred to flasks containing either GMM + 5 mM ammonium D-(+) tartarate (non-induced, repressed conditions) or GMM + 10 mM $NaNO_3$ (induced, derepressed conditions). The flasks were incubated for another 2 h in these media after which the mycelia were filtered, washed and used for RNA isolation. A method by Garcia *et al*. (2004) was followed for *prnD* experiments. Here 10^6 conidia per ml of WT and Δ*laeA* were inoculated in 100 ml of FMM (minimal medium containing 0.1% fructose instead of glucose) containing 5 mM urea as the nitrogen source. To obtain enough mycelia, four flasks of WT and four flasks of Δ*laeA* were grown. After incubating for 8 h at 37°C at 300 r.p.m., the mycelia were harvested, washed and transferred to flasks containing either MMG + 20 mM ammonium D-(+) tartarate (non-induced, repressed conditions) or MMF + 5 mM urea + 20 mM L-proline (induced, derepressed conditions). The flasks were incubated for another 2 h in these media after which the mycelia were filtered, washed and used for RNA isolation. For other expression experiments, we followed our previously published culture method (Shimizu and Keller, 2001). Sexual crosses were performed according to Pontecorvo *et al*. (1953).

Recombinant DNA techniques

Fungal transformations were performed accordingly to standard techniques (Miller *et al*., 1985), with some minor modifications where protoplasts were embedded in minimal media top agar instead of spread by a glass rod on solid media. To examine location-dependent *argB* expression, strain TDN02.3 was constructed by transformation of strain RMS011 with plasmid pDN02, and strain TJW57.9 was constructed by transformation of strain RJW33.2 with plasmid

pJW20. To identify a putative methyltransferase function of *laeA*, strain TJW60 was constructed by transformation of strain RJW32.2 with plasmid pJW63.4. To examine location effect of *aflR*, RDN01.55 and RJW54.8 were created by sexual cross between TJW57.9 and RDIT44.35. RDN04.8 and RDN05.2 were created by sexual crosses between TDN02.3 and RJW33.2, and between TDN02.3 and RDIT30.23 respectively. Genetic backgrounds of created strains were confirmed by Southern blot analyses and/or PCR analyses.

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References

Aparicio, O.M., and Gottschling, D.E. (1994) Overcoming telomeric silencing: a *trans*-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev* **15:** 1133–1146.

Bennett, J. (1987) Mycotoxins, mycotoxicoses, mycotoxicology and mycopathologia. *Mycopathologia* **100:** 3–5.

Berse, B., Dmochowska, A., Skrzypek, M., Wegleaski, P., Bates, M.A., and Weiss, R.L. (1983) Cloning and characterization of the ornithine carbamoyltransferase gene from *Aspergillus nidulans*. *Gene* **25:** 109–117.

Bok, J.W., and Keller, N.P. (2004) Global regulation of secondary metabolic gene clusters. *Eukaryot Cell* **3:** 527–535.

Bok, J.W., Hoffmeister, D., Maggio-Hall, L., Murillo, R., Glasner, J., and Keller, N.P. (2006) Genomic mining for *Aspergillus* natural products. *Chem Biol* **13:** 31–37.

Brakhage, A.A., Andrianopoulos, A., Kato, M., Steidl, S., Davis, M.A., Tsukagoshi, N., and Hynes, M.J. (1999) HAP-like CCAAT-binding complexes in filamentous fungi: implications for biotechnology. *Fungal Genet Biol* **27:** 243–252.

Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., *et al.* (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc Natl Acad Sci USA* **93:** 1418–1422.

Chang, P.K., Yu, J., Bhatnagar, D., and Cleveland, T.E. (1999) Repressor–AFLR interaction modulates aflatoxin biosynthesis in *Aspergillus parasiticus*. *Mycopathologia* **147:** 105–112.

Dowzer, C.E., and Kelly, J.M. (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Mol Cell Biol* **11:** 5701–5709.

Ehrlich, K.C., Montalbano, B.G., and Cary, J.W. (1999) Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene* **230:** 249–257.

Espeso, E.A., and Peñalva, M.A. (1992) Carbon catabolite repression can account for the temporal pattern of expression of a penicillin biosynthetic gene in *Aspergillus nidulans*. *Mol Microbiol* **6:** 1457–1465.

Fernandes, M., Keller, N.P., and Adams, T.H. (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol Microbiol* **28:** 1355–1365.

Garcia, I., Gonzalez, R., Gomez, D., and Scazzocchio, C. (2004) Chromatin rearrangements in the *prnD-prnB* bidirectional promoter: dependence on transcription factors. *Eukaryot Cell* **3:** 144–156.

Gwynne, D.I., Miller, B.L., Miller, K.Y., and Timberlake, W.E. (1984) Structure and regulated expression of the SpoC1 gene cluster from *Aspergillus nidulans*. *J Mol Biol* **180:** 91–109.

Hamahata, A., Takata, Y., Gomi, T., and Fujioka, M. (1996) Probing the S-adenosylmethionine-binding site of rat guanidinoacetate methyltransferase. Effect of site-directed mutagenesis of residues that are conserved across mammalian non-nucleic acid methyltransferases. *Biochem J* **317:** 141–145.

Keller, N.P., and Hohn, T.M. (1997) Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet Biol* **21:** 17–29.

Keller, N.P., Kantz, N.J., and Adams, T.H. (1994) *Aspergillus nidulans verA* is required for production of the mycotoxin sterigmatocystin. *Appl Environ Microbiol* **60:** 1444–1450.

Keller, N.P., Watanabe, C., Kelkar, H., Adams, T.H., and Townsend, C. (2000) Requirement of monooxygenase-mediated steps for sterigmatocystin biosynthesis by *Aspergillus nidulans*. *Appl Environ Microbiol* **66:** 359–362.

Law, D.J., and Timberlake, W.E. (1980) Developmental regulation of laccase levels in *Aspergillus nidulans*. *J Bacteriol* **144:** 509–517.

Liang, S.-H., Wu, T.-S., Lee, R., Chu, F.S., and Linz, J.E. (1997) Analysis of metabolisms regulating expression of the *ver-1* gene, involved in aflatoxin biosynthesis. *Appl Environ Microbiol* **63:** 1058–1065.

Litzka, O., Papagiannopolous, P., Davis, M.A., Hynes, M.J., and Brakhage, A.A. (1998) The penicillin regulator PENR1 of *Aspergillus nidulans* is a HAP-like transcriptional complex. *Eur J Biochem* **251:** 758–767.

Marzluf, G.A. (1997) Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61:** 17–32.

Mihlan, M., Homann, V., Liu, T.W., and Tudzynski, B. (2003) AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. *Mol Microbiol* **47:** 975–991.

Miller, B.L., Miller, K.Y., and Timberlake, W.E. (1985) Direct and indirect gene replacements in *Aspergillus nidulans*. *Mol Cell Biol* **5:** 1714–1721.

Muro-Pastor, M.I., Gonzalez, R., Strauss, J., Narendja, F., and Scazzocchio, C. (1999) The GATA factor AreA is essential for chromatin remodelling in a eukaryotic bidirectional promoter. *EMBO J* **18:** 1584–1597.

Narendja, F., Goller, S.P., Wolschek, M., and Strauss, J. (2002) Nitrate and the GATA factor AreA are necessary for *in vivo* binding of NirA, the pathway-specific transcriptional activator of *Aspergillus nidulans*. *Mol Microbiol* **44:** 573–583.

Peterson, C.L., and Laniel, M.A. (2004) Histones and histone modifications. *Curr Biol* **14:** 546–551.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., Macdonald, K.P., and Bufton, A.W.J. (1953) The genetics of *Aspergillus nidulans*. *Adv Genet* **5:** 141–238.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Shimizu, K., and Keller, N.P. (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157:** 591–600.

Stringer, M.A., Dean, R.A., Sewall, T.C., and Timberlake, W.E. (1991) Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Dev* **5:** 1161–1171.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., *et al.* (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *EMBO J* **14:** 779–790.

Wieser, J., and Adams, T.H. (1995) *flbD* encodes a Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. *Genes Dev* **9:** 491–502.

Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E., and Payne, G.A. (1994) Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. *Appl Environ Microbiol* **60:** 2408–2414.

Yu, J.H., and Leonard, T.J. (1995) Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. *J Bacteriol* **177:** 4792–4800.

Yu, J.H., Butchko, R.A.E., Fernandes, M., Keller, N.P., Leonard, T., and Adams, T.H. (1996) Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr Genet* **29:** 549–555.

Yu, J.H., Hamari, Z., Han, K.H., Seo, J.A., Reyes-Dominguez, Y., and Scazzocchio, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet Biol* **41:** 973–981.

Zhang, Y.-Q., Wilkinson, H., Keller, N.P., and Tsitsigiannis, D.I. (2004) Secondary metabolite gene clusters. In *Handbook of Industrial Microbiology*. An, Z. (ed.). New York: Marcel Dekker, pp. 355–386.

