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Lae1 regulates expression of multiple secondary metabolite gene clusters in *Fusarium verticillioides*

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ABSTRACT

The filamentous fungus *Fusarium verticillioides* can cause disease of maize and is capable of producing fumonisins, a family of toxic secondary metabolites linked to esophageal cancer and neural tube defects in humans and lung edema in swine and leukoencephalomalacia in equines. The expression of fumonisin biosynthetic genes is influenced by broad-domain transcription factors (global regulators) and Fum21, a pathway-specific transcription factor. LaeA is a global regulator that in *Aspergillus nidulans*, affects the expression of multiple secondary metabolite gene clusters by modifying heterochromatin structure. Here, we employed gene deletion analysis to assess the effect of loss of a *F. verticillioides laeA* orthologue, *LAE1*, on genome-wide gene expression and secondary metabolite production. Loss of Lae1 resulted in reduced expression of gene clusters responsible for synthesis of the secondary metabolites bikaverin, fumonisins, fusaric acid and fusarins as well as two clusters for which the corresponding secondary metabolite is unknown. Analysis of secondary metabolites revealed that, in contrast to a previously described *Fusarium fujikuroi lae1* mutant, bikaverin production is reduced. Fumonisin production is unchanged in the *F. verticillioides lae1* mutant. Complementation of the *F. verticillioides* mutant resulted in increased fumonisin production.

1. Introduction

The filamentous fungal genus *Fusarium* includes many species that cause plant disease and produce secondary metabolites (SMs); that is, metabolites that are not essential for growth but that can provide an ecological advantage on some substrates or in some environments. *Fusarium* SMs also include toxins such as fusaric acid, fusarins and trichothecenes; pigments such as aurofusarin, bikaverin and bostrycoidin; and the plant growth regulators gibberellins. *Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland), commonly associated with maize worldwide, is a causal agent of ear and stalk rot of maize, and can produce a family of toxic SMs, fumonisins, that can contaminate maize kernels destined for food and feed (Marasas, 1996). Fumonisins have been implicated as a cause of human esophageal cancer and neural tube defects (Doko et al., 1995; Franceschi et al., 1990; Rheeder et al., 1992; Suarez et al., 2000; Sydenham et al., 1990; Yoshizawa et al., 1994). They are also responsible for multiple animal diseases including leukoencephalomalacia in equines and lung edema in swine (Colvin et al., 1993; Harrison et al., 1990; Oswiler et al., 1992). Reduction or elimination of fumonisin contamination in food and feed produced from maize is critical to food safety. One strategy to accomplish this goal is to limit fumonisin production in maize by reducing the expression of *F. verticillioides* genes responsible for the synthesis of the mycotoxins.

In fungi, genes directly involved in the synthesis of a given SM or family of structurally similar SMs are typically located adjacent to one another in gene clusters. SM biosynthetic gene clusters generally encode proteins that can be divided into four classes based on their function in SM biosynthesis: polyketide synthases (PKSs), nonribosomal peptide synthatases (NRPSs) and terpene synthases (TSs), that catalyze synthesis of the core structure of SMs; cytochrome P450s and flavin-dependent monooxygenases, dehydrogenases, methyltransferases and acyltransferases that catalyze modification of the core structure; transporters; and transcriptional regulators. In general and regardless of function, genes within SM biosynthetic gene clusters often exhibit similar patterns of expression. This co-regulation within SM biosynthetic gene clusters is influenced by narrow and broad-domain transcription factors (Yu and Keller, 2005). Both types of transcription factors bind to promoter regions of the clustered biosynthetic genes and either activate or inhibit gene expression. Narrow-domain transcription factors are generally encoded by genes located within an SM biosynthetic gene cluster and tend to bind only to promoter

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regions of genes in the cluster. Thus, such transcription factors are considered to be pathway specific in that they directly affect genes in only one SM biosynthetic pathway. In contrast, broad-domain transcription factors are encoded by genes located elsewhere in the genome and can bind to promoter regions in multiple SM clusters as well as to genes involved in other physiological processes. Thus, broad-domain factors are not pathway specific.

The fumonisin biosynthetic gene (*FUM*) cluster consists of 17 genes and is located on *F. verticillioides* chromosome 1 (Butchko et al., 2003, 2006; Proctor et al., 1999a,b, 2003; Seo et al., 2001; Zaleta-Rivera et al., 2006). Fum21 is a fumonisin pathway-specific regulator; it is a Zn(II)2Cys6 zinc binuclear DNA binding protein and activates transcription of other *FUM* cluster genes (Brown et al., 2007). The nitrogen regulator AreA, the pH regulator PacC, and the sugar uptake regulator Zfr1 are broad-domain regulators; they are encoded by genes located outside the *FUM* cluster but affect *FUM* gene expression, presumably by binding to *FUM* gene promoter regions but they also affect other physiological processes (Flaherty et al., 2003; Flaherty and Woloshuk, 2004; Kim and Woloshuk, 2008).

In addition to the *FUM* cluster, four other biosynthetic gene clusters for four other polyketide derived metabolites (bikaverin, fusaric acid, fusarin, perithecial pigment) have been identified in *F. verticillioides*. The bikaverin biosynthetic gene (*BIK*) cluster consists of six genes and has been characterized most extensively in *Fusarium fujikuroi*, a species that is closely related to *F. verticillioides* (Linnemannstons et al., 2002; Brown et al., 2008; Wiemann et al., 2009). The fusaric acid biosynthetic gene (*FUB*) cluster consists of five genes, the fusarin biosynthetic gene cluster (*FUS*) consists of nine genes and the perithecial pigment cluster (*PGL*) consists of seven genes and have recently been described in *F. verticillioides* (Brown et al., 2012b). Each cluster has been characterized by gene expression analysis and in some cases by comparative analysis of genomic DNA sequences. The gene encoding the polyketide synthase required for fusarin production has been characterized in *F. verticillioides* and *Fusarium venenatum* (Song et al., 2004) and the polyketide synthase required for the perithecial pigment has been described in *Fusarium graminearum* and *Fusarium solani* (Gaffor et al., 2005 and Graziani et al., 2004).

In *Aspergillus*, transcriptional regulation of multiple SM gene clusters can also occur through changes in heterochromatin structure induced by the multi-protein velvet complex, which includes VeA, VelB and LaeA (reviewed in Bayram and Braus, 2011). A key component of this complex, the LaeA protein, is encoded by *laeA* and was initially identified in a screen of *Aspergillus nidulans* to identify genes required for synthesis of the mycotoxin sterigmatocystin (Butchko et al., 1999). Subsequent work revealed that LaeA is critical for expression, either positive or negative, of multiple SM gene clusters in *Aspergillus* as well as in other fungi (Bok and Keller, 2004; Bok et al., 2006a,b; Bouhired et al., 2007; Reyes-Dominguez et al., 2010; Hoff et al., 2010; Wiemann et al., 2010). In *Aspergillus*, VeA is required for ascospore production as well as for the production of the SM sterigmatocystin (Bayram and Braus, 2011; reviewed in Calvo, 2008). The VeA homolog in *F. verticillioides*, FvVe1, has been shown to regulate both SM production and development. Loss of FvVe1 results in alteration of the ratio of microconidia and macroconidia (reviewed in Calvo (2008) and Li et al. (2006)) as well as the loss of fumonisin and fusarin production (Myung et al., 2009).

Recently, Wiemann et al. (2010) demonstrated that loss of the VeA component of the velvet-like complex in *F. fujikuroi* affects secondary metabolism, development and virulence on rice plants. Microarray analysis of a *Ffvel1* mutant led to the identification of a *laeA* homolog in *F. fujikuroi* (Wiemann et al., 2010). Analysis of *F. fujikuroi lae1* mutants found that they were similar to the *Ffvel1* mutants with respect to reduced gibberellins production and virulence on rice plants, but differ in their effect on bikaverin production (Wiemann et al., 2010).

The objective of this study was to examine the effect of Lae1 on gene expression in *F. verticillioides*. Microarray analysis of a *lae1* deletion mutant revealed that Lae1 can positively or negatively affect transcription of multiple SM gene clusters and hundreds of SM-like genes across the *F. verticillioides* genome. In some but not all cases, changes in SM gene expression were accompanied by changes in SM production. To our knowledge, this is the first report of a genome-wide analysis of the effects of the Lae1 on gene expression and SM production in *Fusarium*.

2. Materials and methods

2.1. Fungal strains and culture conditions

Fungal strains used are listed in Table 1. Wild-type strain M-3125 (NRRL 20960) (Leslie et al., 1992) was the parent strain to the *LAE1* deletion strains T21.1 and T22.1. Strains were cultured on V8 juice agar medium for the production of conidia and in liquid GYEP (5% glucose, 0.1% yeast extract, 0.1% peptone) for production of mycelia for genomic DNA preparation. Secondary metabolites were assayed from cracked corn medium at 11 days or liquid GYAM (Seo et al., 2001) for 2, 4 and 6 days shaking at 250 rpm at 27 °C (Desjardins et al., 1996; Seo et al., 2001).

2.2. Nucleic acid manipulations and fungal transformation

DNA was isolated using standard protocols (Sambrook et al., 1989). The deletion of the *F. verticillioides LAE1* was accomplished using a construct described by Wiemann et al. (2010) to delete the *F. fujikuroi LAE1*. Southern analysis was used to characterize the deletion strains and the complemented strain. Genomic DNA was digested with *EcoRI*, separated on agarose gel by electrophoresis, and blotted to nylon membrane. Three different probes were used to characterize the strains. Primer pair rb438 and rb442 was used to amplify a 484 base pair fragment of the *LAE1* coding region. Primer pair rb432 and rb435 was used to amplify a 673 base pair fragment corresponding to the region upstream of the *LAE1* coding region. Primer pair rb694 and rb697 was used to amplify an 844 base pair fragment corresponding to the region downstream of the *LAE1* coding region. DNA fragments were labeled with ^{32}P utilizing Ready-To-Go DNA labeling beads (GE Healthcare, Buckinghamshire, UK). Membranes were hybridized using ULTRAhyb solution (Life Technologies, Grand Island, NY) and washed following manufactures directions. The *LAE1* complementation plasmid, Pl-98, was constructed by first amplifying a 2700 bp DNA fragment from genomic DNA of the wild-type strain corresponding to approximately 1 kb upstream of the predicted Lae1 translational start site and the entire predicted coding sequence using primers rb424 and rb428. The fragment was subcloned into a plasmid containing a gene conferring resistance to geneticin (Marek et al., 1989). The *LAE1* fragment was sequenced to determine that no mutations were introduced through PCR amplification. Transformation of *LAE1* deletion strain T22.1 was as previously described (Proctor et al., 1999b). Characterization of the add-back strains was accomplished by amplifying PCR products with primers specific to *F. verticillioides LAE1* coding region.

2.3. DNA microarrays and RT-PCR

DNA microarrays (Roche-NimbleGen Systems (Madison, WI)) consisted of approximately 336,000 60-base pair probes, with each sequence in the *F. verticillioides* gene index represented by a set of 6 probes. Each probe is represented twice on each microarray. Total

Table 1
Fungal strains used in this study.

Strain name	Genotype	Antibiotic resistance gene	Reference
M3125	Wild-type	None	Leslie et al. (1992)
T21.1	*LAE1* deletion	Hygromycin	This study
T22.1	*LAE1* deletion	Hygromycin	This study
GMTcp-263	*LAE1* deletion, *LAE1* add-back	Hygromycin, geneticin	This study
GMTcp-264	*LAE1* deletion, *LAE1* add-back	Hygromycin, geneticin	This study

RNA was isolated from 2, 4 and 6 day liquid GYAM cultures using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) and DNA was removed using the RNase-Free DNase kit (Qiagen Inc., Valencia, CA) Nucleotide labeling, hybridization and data acquisition were done by NimbleGen Systems. Raw intensity values were normalized to internal controls on each chip and between chips using NimbleScan Bioanalyzer software, utilizing Robust Multichip Analysis (Irizarry et al., 2003). Normalized data was analyzed using Acuity 4.0 from Molecular Devices Corporation (Sunnyvale, CA) and P values were generated by a Student's *T*-test using the same software. Differences in gene expression are considered significant at a *P* value less than 0.05. Samples of RNA were extracted from three biological replicates of *F. verticillioides* strains cultured in liquid GYAM. End-point reverse transcription PCR (RT-PCR, 30 cycles) was used to assay gene expression. Stratagene (Santa Clara, CA) Easy-A One-tube RT-PCR kits were used following manufactures protocol. Primers are listed in Table 2.

2.4. Metabolite analysis

Fumonisin B1, B2 and B3 (Plattner et al., 1996), fusarin C (Maragos et al., 2008) and bikaverin (Vesonder et al., 1989) standards were prepared as previously described. To assess toxin production, strains were grown in triplicate for 6 days in liquid GYAM medium or on cracked corn medium for 11 days. Cracked corn cultures were extracted with acetonitrile:water (1:1) as previously described (Proctor et al., 1999b). Care was taken to utilize conditions that would minimize decomposition of fusarin C in all samples (Maragos et al., 2008; Kleigrewe et al., 2011). The fumonisin, fusarin C and bikaverin content of liquid culture media or cracked corn extracts was determined by liquid chromatography–mass spectroscopy (LC–MS) or liquid chromatography–tandem mass spectroscopy (LC–MS/MS). Analyses were conducted with a LC–MS/MS system composed of a ThermoSpectraPhysics high performance liquid chromatography system, consisting of a P4000 pump and AS3000 autosampler, coupled to a ThermoFinnigan DECA ion trap MS operated in positive electrospray ionization mode. Filtered liquid culture medium or cracked corn extract samples were injected into a linear gradient flow on a Metachem (Torrance, CA, USA) Inertsil C18 (150×3 mm) column. For all analyses a 300 μL/min water/methanol (MeOH) gradient program (0 min: 35% MeOH, 5 min: 35% MeOH, 15 min: 95% MeOH, 20 min: 95% MeOH, 25 min: 35% MeOH) with 1% acetic acid was used. LC–MS and LC–MS/MS quantitations were made by monitoring a 2 Thomson (Cooks and Rockwood, 1991) window around the *m/z* of the ion of interest. LC–MS determination of fumonisins was achieved by monitoring *m/z* 722 (FB1) and 706 (FB2 and FB3) protonated ([M + H]+) ions as previously described (Plattner et al., 1996). Quantitation was done by comparison of the FB1, 2 and 3 chromatographic peak areas to those for fumonisin concentration standards. LC–MS/MS determination of fusarin C was done by monitoring several distinctive fragments (*m/z* 364, 382, 394) of the [M + H]+ ion (*m/z* 432) in manner similar to that previously described (Maragos et al., 2008; Kleigrewe et al., 2011). Quantitation was based on comparison of the chromatographic peak area for the *m/z* 364 ion to those for fusarin C concentration standards. LC–MS/MS determination of bikaverin was done by monitoring several distinctive fragments (*m/z* 337, 355, 365) of the [M + H]+ ion (*m/z* 383) (Busman et al., submitted for publication). Quantitation was based on comparison of the chromatographic peak area for the *m/z* 355 ion to those for bikaverin concentration standards. Quantitative assessments of the LC–MS and LC–MS/MS experiments were made using the Xcalibur software supplied by the manufacturer of the MS instrument. Microsoft Excel (Redmond, WA) was used for statistical analysis and P values were generated using a Student's *T*-test.

Table 2
Primers used in this study.

Primer name	Gene	Sequence
rb291	TEF1	ATGGGTAAGGAGGACAAGAC
rb292	TEF1	GGAAGTACCAGTGATCATGTT
rb371	FUM21	GCCAAACGTGCCCTTTGGTTCTTA
rb374	FUM21	TGCATCTTGCCCTACTCACAGC
rb375	FUM1	TTTGTCGAAATTGGCCCTCACAGC
rb378	FUM1	ACAACGTCATTGAGGACCTGGTGA
rb405	FvVE1	CTTTGATCTGGAACCTGCCG
rb456	FvVE1	GCTGGTCTATCTTGAGAGCC
rb414	BIK1	AGTGTTGGCAGACATCACC
rb416	BIK1	CGCTTTGGCAATTTACCGAG
rb436	LAE1	GGATTCTGGCAACATGGCCG
rb440	LAE1	AAGTCCGTTTGAATACTGCC
rb424	LAE1	AACCCAAGGACAATTGTGCTG
rb428	LAE1	CACCAGGCCGATTTGGATTT
rb473	LAE1	CTTCATTGGGACATTTGGATTT
rb445	HYGB	CCAAGGACTATGACACTGGC
rb464	HYGB	TAGCAGCACGCCATAGTGA
rb432	lae1	CAAATATCGGCTTAAACGCC
rb435	lae1	CGGCCATGTTGCCAGAATCC
rb438	LAE1	GCCAAAACAATATGACATTG
rb442	LAE1	CCAGACTATGAGACAGCCCG
rb694	lae1	CAACGCAAGGAACACGAA
rb697	lae1	TGTCCACCACCTTCTGCTT

3. Results

3.1. Identification and deletion of F. verticillioides LAE1

In the *Fusarium* Comparative Database (Brown et al., 2005), *LAE1* is designated as FVEG_00539 and is located on chromosome 1. The predicted amino acid sequence of the *F. verticillioides LAE1*-encoded protein (Lae1) is 70% identical to *F. fujikuroi* Lae1 and 32% identical to *A. nidulans* LaeA. Two *lae1* deletion mutants were generated by transformation of wild-type *F. verticillioides* strain M-3125 with a vector engineered for deletion of the *F. fujikuroi LAE1* homolog (Wiemann et al., 2010) and are designated T21.2 and T22.1 (Fig. 1).

3.2. Genome-wide gene expression in lae1 mutant

We employed microarray analysis to compare genome-wide gene expression in *lae1* mutant strain GMTcp-258 and wild-type

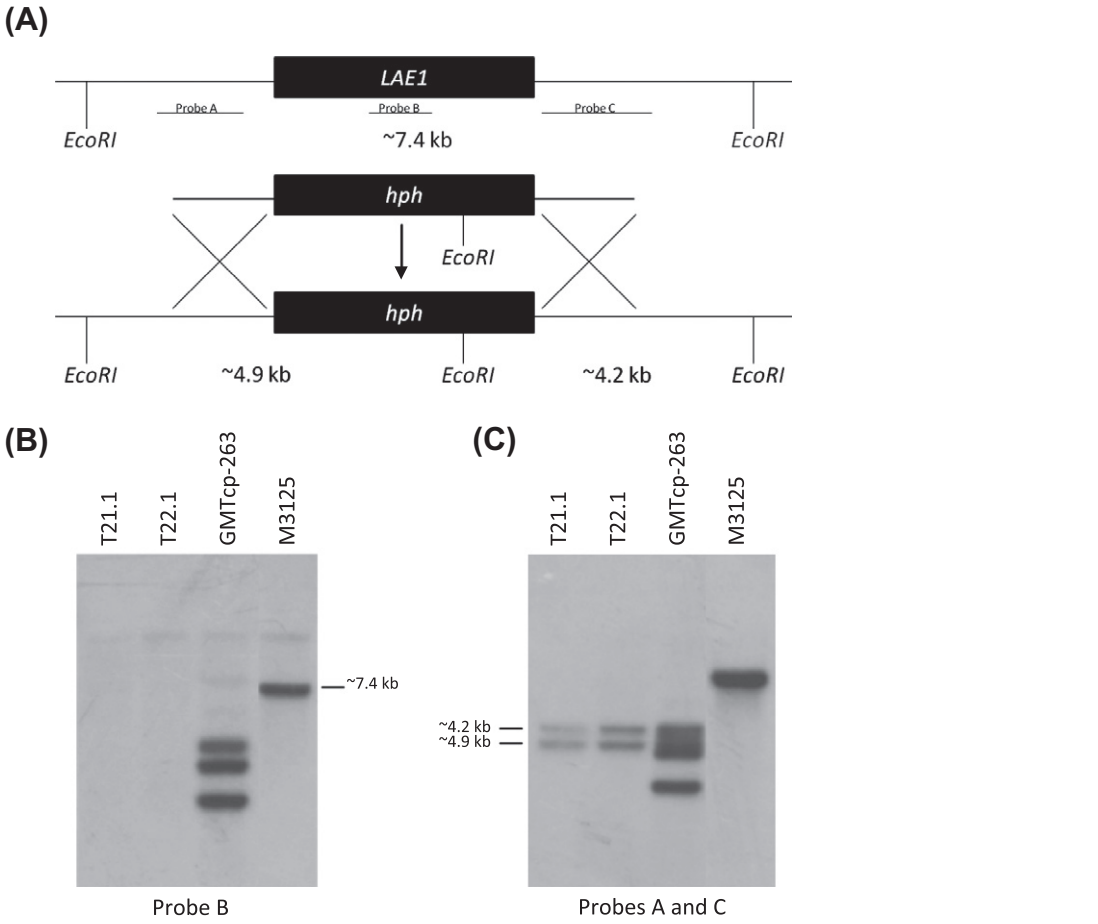

Fig. 1. Southern analysis of *LAE1* deletion strains and *LAE1* complemented strain. Genomic DNA was digested with *EcoRI* and hybridized with one fragment corresponding to the coding region and two fragments corresponding to the flanking regions. PCR primers used to generate these fragments are listed in Table 2. (A) Map of the *LAE1* locus, marker gene and resulting integration. (B) Southern analysis using probe corresponding to the coding region (Probe B) illustrating the lack of bands in the deletion strains (T21.1 and T22.1), the presence of bands in the complemented strain (GMTcp-261) and the presence of the ~7.4 kilo base pair band in the wild-type strain (M-3125). (C) Southern analysis using probes corresponding to the regions flanking *LAE1* (Probe A and Probe c). The integration of the marker gene results in the addition of an *EcoRI* site. Corresponding bands (~4.2 and ~4.9 kilo base pairs) are present in the *LAE1* deletion strains as expected.

F. verticillioides after 6 days of growth in a liquid medium, GYAM, that is favorable for production of fumonisins and some other secondary metabolites. Microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE32608 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32608). A total of 7910 genes of the 14,196 genes represented on the microarray exhibited expression that was significantly ($P < 0.05$) different in the *lae1* deletion mutant compared to its wild-type progenitor strain. Of these genes, 4800 exhibited reduced expression and 3110 exhibited increased expression in the *lae1* mutant. Blastp analysis with the National Center for Biotechnology Information (NCBI) database revealed that many of these genes in the subsets described below have homology to genes that are likely involved in SM biosynthesis, including genes encoding monooxygenases, dehydrogenases, reductases, and Zn2(II)Cys6 zinc binuclear DNA binding transcription factors.

Among the 4800 genes that exhibited reduced expression, 201 exhibited at least fourfold reduction in expression (Supplemental Table 1). These 201 genes included 10 sets of two adjacent genes and 10 sets of three or more adjacent genes. Three of the latter sets corresponded to the bikaverin (*BIK*), fusaric acid (*FUB*), and fusarin (*FUS*) biosynthetic gene clusters (Fig. 2). Another set includes four genes without significant homology to genes in the NCBI database, and a fifth set located 15 kb from the *FUB* gene cluster includes seven genes with significant homology to genes likely to be involved in SM biosynthesis (Fig. 2D).

Within the group of 3110 genes that exhibited increased expression in the *lae1* mutant compared to wild-type *F. verticillioides*, 74 exhibited at least fourfold greater expression and included seven sets of two or more adjacent genes (Supplemental Table 2). One group included a PKS gene (FVEG_00079) for which the corresponding polyketide-derived metabolite is not known. Forty-two of the 74 genes share significant similarity to genes of unknown function (e.g., hypothetical proteins) by BLASTP while 18 share similarity to genes that are or are predicted to be involved in secondary metabolism (e.g. dehydrogenases, methyltransferases, transporters, and monooxygenases). Interestingly, three of the four genes with homology to methyltransferases also shared homology to *laeA*-like genes in *Aspergillus*. An alignment of the deduced amino acid sequence of LAE1 and the four other *F. verticillioides* methyltransferases genes revealed that they all include the putative HemK domain that is characteristic of the S-adenosyl-L-methionine-dependent methyltransferase superfamily (Fig. 3).

3.3. Expression of PKS genes and their flanking genes

The *F. verticillioides* genome contains 16 polyketide synthase genes, including the PKS genes required for synthesis of bikaverin (*BIK1*), fumonisin (*FUM1*), fusaric acid (*FUB1*), and fusarins (*FUS1*)

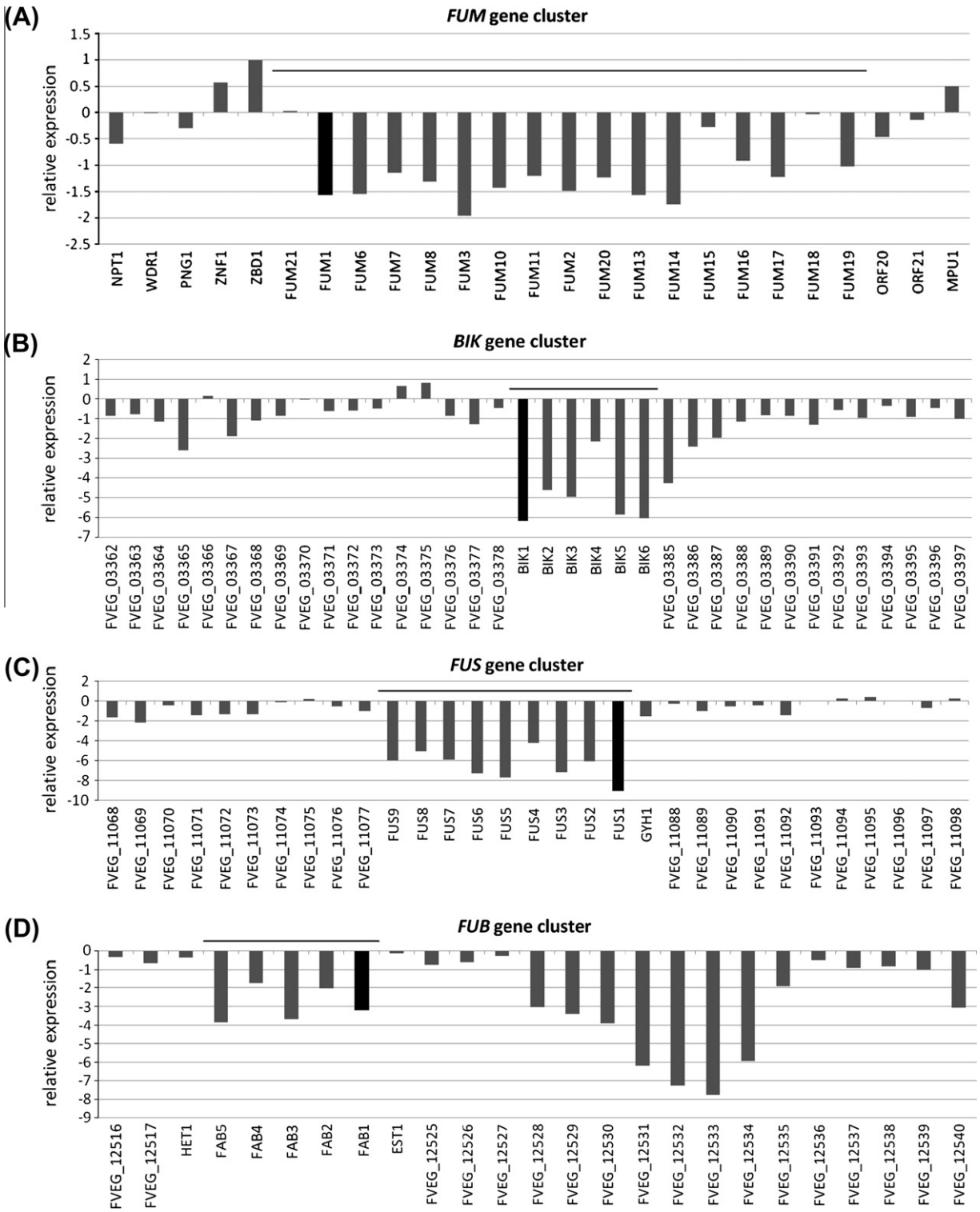

Fig. 2. Decreased expression of the *FUM*, *BIK*, *FUS* and *FUB* clusters in the *F. verticillioides lae1* deletion mutant strain T22.1. Data were generated by DNA microarray analysis of RNA isolated following six days of growth in GYAM medium. Bars represent fold change in the *lae1* mutant compared to the wild-type on a $\log_2$ scale such that a difference of 2 represents a fourfold change in expression. (A) The expression of the *FUM* cluster, (B) expression of the *BIK* cluster, (C) expression of the *FUS* clusters and (D) expression of the *FUB* cluster. Horizontal lines represent the extent of the gene cluster. In each case, the PKS gene is shaded black.

(Ma et al., 2010; Brown et al., 2012b). In order to identify novel SM gene clusters responsible for synthesis of polyketides, we analyzed expression of the PKS gene and surrounding genomic regions in the *lae1* mutant compared to the wild-type progenitor strain M-3125. The genomic regions examined included all predicted genes within 40 kilobase pairs (kb) of either side of the PKS gene. In many cases, the predicted function of flanking genes are unknown; in the Broad Institute's *Fusarium* Comparative Database these genes with unknown functions are annotated as "predicted proteins" or "conserved hypothetical proteins", and in blast analyses against the

```
LAE1          ------------------------------------MVVMPPQ-----------------N
FVEG_10732    --------------------------MAEPT-INVEPDLVPDEQSEEEHDSSIGSSLA--
FVEG_12679    MDKTDAVSQTNPRFPTEILPESTPPGMSSQGGQDLVAAQPLEENEEDDDDSALGQDFA--
FVEG_13182    --------------------------MAQEA-NEHESPLQADDAASLSHDSALAASLESG
                                                       :

LAE1          SVNESEGRYLQDGFWQHGRFYGSWK-PGKYLFPIDSEELNRLDIFHKVFLLARDNKPFLA
FVEG_10732    STSESLRSSILDYRKENGRTYHRYK-DGKYNLPNDDVEKERLDLQHNLFILMLDNKLGLA
FVEG_12679    SSTASLTSSILEYRKFQGRTFNSDKYEIEYFAPNDERQKESIDISHHYLMLLLDGKLSLA
FVEG_13182    SYLTSLNSSVLNYKYENGRRYHAFR-EGVYLVPNDDEEQDRMDLGHHIYRLLLGGDLFLA
              *   *    : :    .** :   :    *  * *. : : :*: *.   *    .  **

LAE1          PIR---RTSPRIMDIGTGTGIWAINVAEECLSDAQIMAVDLNQIQPALIPPGFMPKQYDI
FVEG_10732    PPNNPNSKAKRVLDVGTGTGIWAIDYADEH-PEAKVIGVDLSPIQPDFVPPNVEFFIDDI
FVEG_12679    PSP---EDFEKVLDIGTGTGIWAIDFADQY-PNAEVVGTDLSPIQPDWVPPNVRFELEDA
FVEG_13182    PIG---DKVKRVLDLGTGTGIWAMDFADEY-PQAEVLGTDLSPIQPPWTPPNCVFEVDDF
              *         :::*:********:: *::   :*::::..**. ***   **       *

LAE1          EEPSWGPLLADCDLIHMRMLLGSIQTDLWPQVYHNAFEHLAPGIGFLEHIEVDWIPRCDD
FVEG_10732    E-EPWNFS-EPFDYVHSRMMTFSIK--SWPNLASSIYDNLVPG-GYVELLEIDLFAHSDD
FVEG_12679    T-SNWTWSNDTFDFVHMRYLIGAIA--DWGALFKDAFRCCKPG-GFVESVEVNPTFFSDD
FVEG_13182    E-SDWLFR-QPFDFIHARELEGCIS--NNAQFFTRALASLAPG-GYLEMQAVHSEFKSDD
                  *       * :* * :  .*       .          ** *::*   :.    .**

LAE1          DERPANSAFVKWAELFLDGMDRFNRRVRVTPQEHRQMLEATGFTDIRQEVIKAYVCPWSA
FVEG_10732    KTLTEDHHLSKLIHLLDEASTKIGRPFQDNKKN-KDILRDAGFVDIVETVFKWPTNRWPL
FVEG_12679    GTASDVTAVQTWNKLFREASKAFGRSFCEIEGD-AELLAAAGFVDLQVTDFKVPVGGWAK
FVEG_13182    NTKEKAKNALLWMKNMVEGSSKFGKPLNVAPGW-KKEMEDAGFVDVEQKILKVPIGSWPR
                           . : :.   : : .        . :  :**.*:    :*     *

LAE1          DRNEREIARWFNIGLSH--SLEAMSLKPLIEKLGFEAEEVRELCERAKRETCVLRYHTYC
FVEG_10732    DKRYKEIGEWNNLNMDSFKGLEALSMAALTRVLGWTQEEVTVFLAKVRRDLNDRTIHAYW
FVEG_12679    DSKLRQVGEFLRATMEN--DLEGYTLLAWQQILGWPKDEYQLFLMDMRKALRDKKVHSYI
FVEG_13182    DPKLKEIGKFQSVQEAQ--VITSYTPGIFSRVLGWSDEEIQVFMAKVKKDLSNPSIHLYL
              * . :::..:          : . :     . **:  :*   :    ::       * *

LAE1          NM*---------
FVEG_10732    PVYSTYGRRPLD
FVEG_12679    RVRFLNAHKP--
FVEG_13182    PVYFIWGRKPE-
               :
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Fig. 3. Alignment of Lae1 with three other putative methyltransferases encoded in the *F. verticillioides* genome and that are more highly expressed in the *lae1* deletion mutant. The HemK domain is shaded and an * (asterisk) indicates identical amino acid residues while a : (colon) indicates strongly conserved amino acid residues and a . (period) indicates weakly conserved residues (http://www.ebi.ac.uk/Tools/msa/clustalw2/). FVEG_10732, FVEG_12679 and FVEG_13182 share 34%, 29% and 30% identity with *F. verticillioides* LAE1 respectively and exhibit 36%, 28% and 32% identity to *A. nidulans* LaeA respectively.

NCBI database, done as part of the current study, these genes do not exhibit significant identity to genes with known functions.

We found that 14 PKS genes exhibited significantly decreased expression while two exhibited significantly increased expression in the *lae1* mutant relative to wild-type (Supplemental Table 3). Four of the PKS genes with significantly decreased expression are *BIK1*, *FUB1*, *FUM1* and *FUS1*. Analysis of the 40-kb regions on either side of these four genes revealed that numerous genes in the flanking regions also exhibited reduced expression, including genes corresponding to the *BIK*, *FUB*, *FUM* and *FUS* clusters (Fig. 2). Co-expression of genes in the *BIK*, *FUB*, *FUM* and *FUS* clusters observed for the wild-type progenitor strain in the current study is consistent with previously reported co-expression of the genes (Proctor et al., 1999b; Brown et al., 2012b). In the current experiment, the expression of all *FUM* genes, except the regulatory gene *FUM21*, was approximately threefold lower in the *lae1* mutant (Fig. 2A). The expression of the six *BIK* genes, the nine *FUS* genes and the five *FUB* genes were 16 to >500 fold less in the *lae1* mutant (Fig. 2B–D). Although expression of the other 10 PKSs were between 2 and 6-fold less in the *lae1* mutant, genes flanking these other PKS genes did not exhibit patterns of changed expression indicative of gene clusters as is the case for the *BIK*, *FUB*, *FUM* and *FUS* clusters.

The two PKS genes that exhibited increased expression, a 2-fold increase, in the *lae1* mutant compared to wild-type are designated FVEG_00079 and FVEG_10535 in the *F. verticillioides* genome database (Fig. 4). Three genes flanking FVEG_00079 also exhibited a >2 to 6-fold increase in expression in the *lae1* mutant. These flanking genes, FVEG_00076, FVEG_00077 and FVEG_00078, are predicted

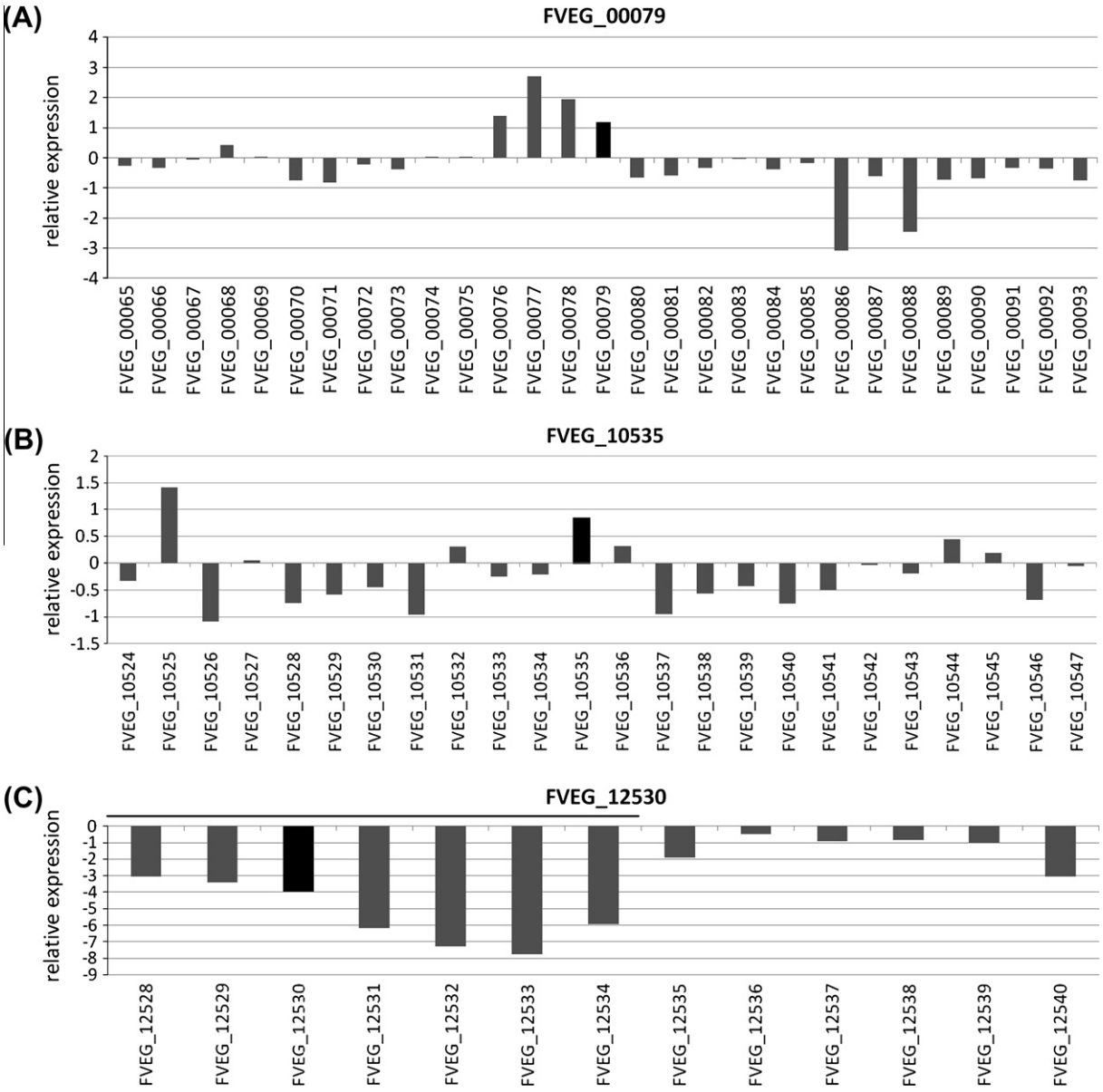

Fig. 4. Microarray analysis of expression of previously undescribed gene clusters that are likely involved in SM biosynthesis based on the predicted functions of the genes. In each case, the PKS or NRPS gene is shaded black.

to encode a protein of unknown function, an acetyltransferase, and an integral membrane protein respectively. The co-expression of these flanking genes combined with the close proximity of a PKS gene and the acetyltransferase are consistent with these genes constituting a polyketide-derived SM biosynthetic gene cluster. In contrast, the lack of co-expression of the genes flanking the PKS gene FVEG_10535 (Fig. 4B) provides no support for this gene being part of an SM biosynthetic gene cluster.

3.4. Identification of putative gene cluster with an NRPS-like gene

The genome-wide expression analysis also revealed that seven genes located 15 kb upstream of *FUB1* exhibited more than 4-fold decreased expression in the *lae1* mutant compared to wild-type *F. verticillioides* (Fig. 4C). In the *Fusarium* Comparative Database, these genes are designated FVEG_12528 through FVEG_12534 and exhibit sequence homology to genes involved in secondary metabolism. FVEG_12532 and FVEG_12534 are predicted to encode Zn2(II)Cys6 binuclear DNA binding domain proteins; FVEG_12528 and FVEG_12531 are predicted to encode dehydrogenases; FVEG_12529 is predicted to encode a lyase; FVEG_12530 is predicted to encode a NRPS-like protein; and FVEG_12533 is predicted to encode a major facilitator (MFS) transporter. The co-expression of these SM-like genes suggests that they may constitute a SM biosynthetic gene cluster.

3.5. Complementation of LAE1 deletion strain

The *F. verticillioides lae1* deletion mutants were genetically complemented with a wild-type copy of *F. verticillioides LAE1*. Two independent *LAE1* add-back strains were identified by PCR with primers specific to *LAE1* coding sequence and are designated GMTcp-263 and GMTcp-264. Expression of *LAE1*, *FUM1*, *FUM21*, *BIK1*, *Ve1* and *TEF1* (transcription elongation factor) in the *lae1* mutant were compared to the add-back transformant GMTcp-263 by end point reverse transcription PCR (RT-PCR) over time.

As expected, expression of the bikaverin and fumonisin PKSs (*BIK1* and *FUM1* respectively) were restored in the *LAE1* add-back strain (Fig. 5). Expression of *Ve1* was not altered in the *lae1* mutant strains and *LAE1* expression was restored in the add-back strain. No morphological differences with regard to growth rate or conidiophore production on solid media between the *lae1* mutants or the complemented mutants and the wilt-type strain were observed.

3.6. Secondary metabolite analysis

Analysis of production of SMs in the two *lae1* mutant strains revealed no statistically significant reduction in fumonisin production while bikaverin and fusarin production were both reduced compared to wild-type (Fig. 6A). In liquid culture, no fusarins were detectable. On cracked corn, bikaverin production was reduced by 70% ($P < 0.01$). Complementation of the lae1 mutant resulted in a 50% increase ($P < 0.05$) in fumonisin production compared to wild-type *F. verticillioides*. In contrast, bikaverin production was restored only to approximately 40% ($P < 0.05$) of wild-type levels while fusarin C was restored to approximately 10% ($P < 0.05$) in the *lae1* mutant complemented strain (Fig. 6B and C). Fig. 7 shows representative chromatograms from LC–MS or LC–MS/MS measurements of fumonisin (A), bikaverin (B) and fusarin C (C) production.

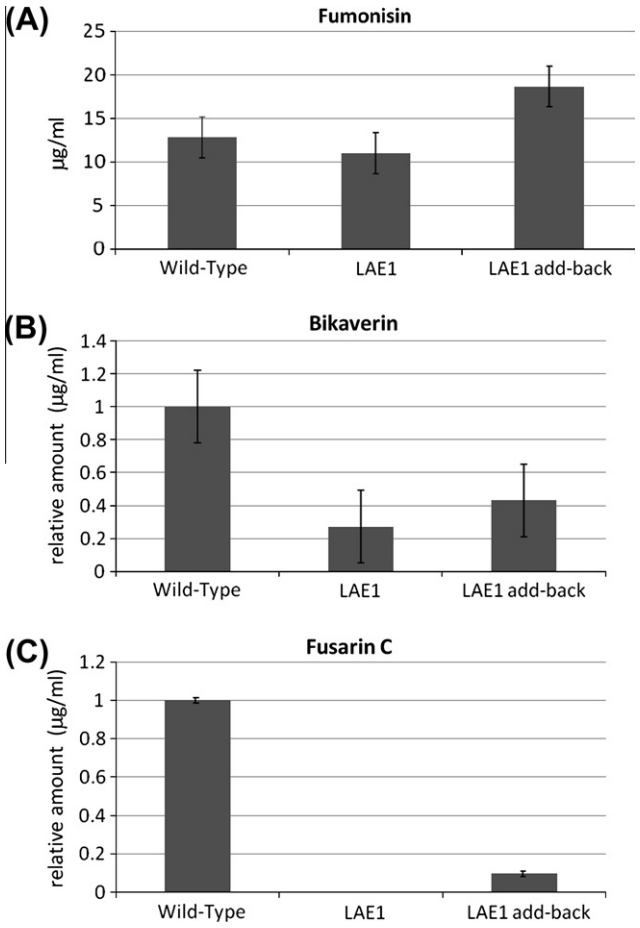

Fig. 6. Secondary metabolite analysis. Fumonisin production (A), bikaverin production (B), and fusarin C production (C) in wild-type (M-3125) LAE1 deletion strain T22.1 (LAE1) and LAE1 complementation strain GMTcp-263 (LAE1 Addback).

4. Discussion

In *Aspergillus* species, the velvet complex (composed of the proteins VeA, VelB and LaeA) is involved with the regulation of secondary metabolism and development by affecting gene expression at the chromatin level (Bayram et al., 2008; Bok and Keller, 2004; Bok et al., 2006a,b; Reyes-Dominguez et al., 2010). A recent review catalogs the prevalence of the velvet complex across the fungal kingdom and is reported to be present in Basidiomycetes, Hemiascomycetes, Zygomycete and Chytridiomycetes (Bayram and Braus, 2011). One goal of studies on the velvet complex in diverse fungi is to discover new strategies to control SM synthesis in order to limit mycotoxin contamination of crops and to discover new fungal natural products with novel biological activities. It is clear that although the general function of the velvet complex is conserved, its relative impact on the synthesis of different SMs is species specific within a genus. Here, we describe the effects of loss of Lae1 in *F. verticillioides* by analysis of deletion mutants. Loss of Lae1 resulted in the loss of expression of the *FUM*, *BIK*, *FUS* and *FUB* gene clusters and one previously undescribed gene cluster containing an NRPS-like gene (FVEG_12530) and the increase in expression of another previously undescribed

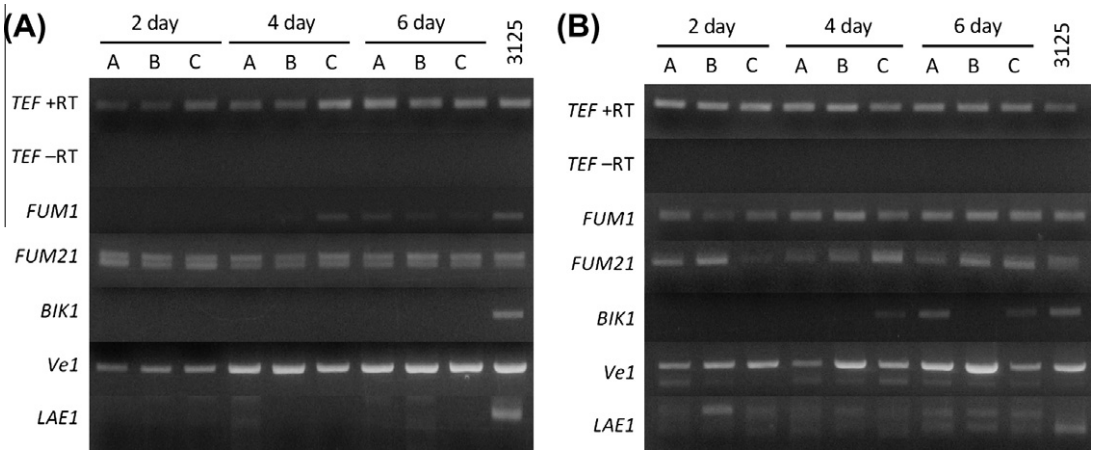

Fig. 5. Expression analysis of secondary metabolite genes in the *lae1* deletion mutant T22.1 (A) and *LAE1* complemented GMTcp-263 (B) strains. Headings A, B and C for each time point indicate biological replications. Expression of the transcription elongation factor gene (*TEF1*) was used as a control, both with and without reverse transcriptase (RT) to illustrate the absence of genomic DNA contamination in the RNA samples. Two bands generated by *FUM21* and the *LAE1*-specific primers represent different alternative splice forms. Total RNA from a 6 day culture of wild-type strain M-3125 was used as a positive control.

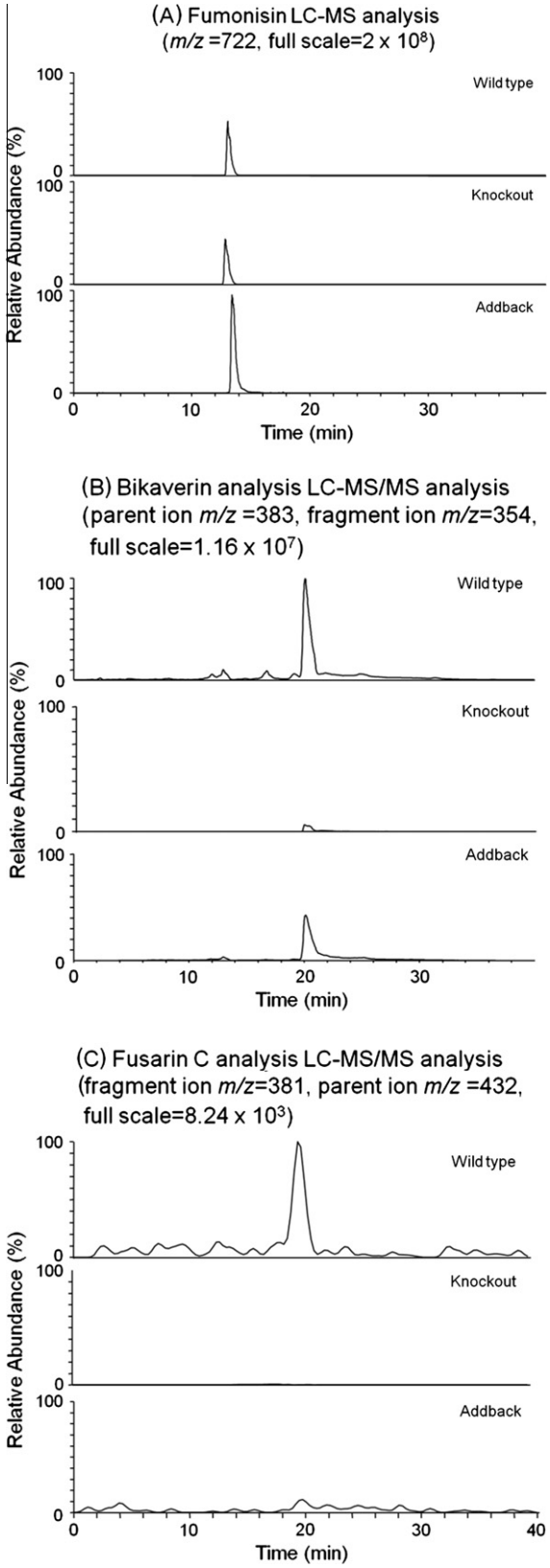

Fig. 7. Representative chromatograms for analysis after *F. verticillioides* growth in liquid GYAM (A and C) or on cracked corn (B). Wild type isolate is M-3125, knockout out is T22.1, addback is GMTcp-263. (A) LC–MS fumonisin analysis from 6 day liquid GYAM cultures, $m/z = 722$, full scale = 2×10^8. (B) LC–MS/MS bikaverin analysis from 11 day crack corn cultures, parent ion $m/z = 383$, fragment ion $m/z = 354$, full scale = 1.16×10^7. (C) LC–MS/MS fusarin C analysis from 6 day liquid GYAM cultures, fragment ion $m/z = 381$, parent ion $m/z = 432$, full scale = 8.24×10^3.

gene cluster containing a PKS (FVEG_00079). In the case of the only other PKS containing gene cluster whose product has been determined, the PGL gene cluster required for perithecial pigment production (Gaffor et al., 2005; Graziani et al., 2004; Proctor et al., 2007), the expression of the genes in this cluster were uncoordinated in the *lae1* mutant, that is, two of the genes including the PKS were expressed 2-fold less, two genes had no change in expression while two others were expressed more than 4-fold higher than wild-type (data not shown). The *PGL* gene cluster likely contains six genes based on coordinate expression in the wild-type strain M-3125 when cultured on liquid GYAM media (Brown et al., 2012b).

Here, we have shown that deletion of the *F. verticillioides LAE1* homolog results in altered expression of multiple SM gene clusters, both positive and negative, and a reduction in production of the SMs bikaverin and fusarin C. Despite decreased expression of *FUM* cluster genes, fumonisin production in the *lae1* mutant strain was not significantly reduced. The observation that the *LAE1* complemented strain produced 50% more fumonisins than wild-type indicates that *LAE1* affects fumonisin production in addition to *FUM* gene transcription. The differential effect on fumonisin production by *lae1* mutants as compared to *FvVe1* mutants in *F. verticillioides* is similar to the differential effect on bikaverin production by respective *F. fujikuroi* mutants (Wiemann et al., 2010). In contrast, we found that the effect on bikaverin production in the *lae1* mutants differed from *F. fujikuroi LAE1* mutants. In the *F. verticillioides lae1* mutant, *BIK* gene expression and bikaverin production was greatly reduced while in the *F. fujikuroi LAE1* mutant, *BIK* gene expression and bikaverin production were only slightly reduced (Wiemann et al., 2010). A comparison of these studies in *F. fujikuroi* and *F. verticillioides* demonstrate that the velvet complex, although conserved among fungi, can have different effects on production of the same SM in closely related species. Previously, the *VeA* homolog *FvVe1* in *F. verticillioides* was shown to positively affect production of two SMs, fumonisins and fusarins (Myung et al., 2009). In *F. fujikuroi*, all three components of the velvet complex have been described to affect SM synthesis as well as sexual and asexual development and pathogenicity on rice plants. FfVel1 and FfVel2 positively affect the production of gibberellic acids, fumonisins and fusarin C and negatively affect the production of bikaverin. FfLae1 has a positive effect on gibberellins and bikaverin production (Wiemann et al., 2010). A *Fusarium* homolog of *VelB* was identified and described by Wiemann et al. (2010) in *F. fujikuroi* as *FfVel2*. Nucleotide sequence similarity search by BLAST of the *F. verticillioides* genome database with *FfVel2* revealed the homolog as FVEG_01498. Expression of this gene in the *F. verticillioides lae1* mutant was not different than in the wild-type strain.

Microarray analysis of gene expression in the *lae1* mutant and wild-type *F. verticillioides* revealed differential expression of two novel gene clusters. Although the SMs that are synthesized by the enzymes encoded by the genes in these clusters are not known, the identification of the genes in a putative cluster can be a critical first step in identification of such metabolites. Work with the *laeA* deletion strain in *Aspergillus* species led to the identification of multiple new SM gene clusters as well as the corresponding metabolites (Bok et al., 2006b; Bouhired et al., 2007; Perrin et al., 2007). Further exploration of the expression patterns in the *lae1* mutant under different growth conditions in *F. verticillioides* could yield insight into previously undiscovered gene clusters and potential metabolites. Similarly, overexpression of *LAE1* in *F. verticillioides* might also aid in the identification of novel SM gene clusters. We have recently described the phylogenomics of the *F. verticillioides* PKSs and discussed domain analysis as it can be applied to predicting the types of natural products expected from these gene clusters (Brown et al., 2012a).

The regulation of expression of SM biosynthetic gene clusters is proving to be complex. Visentin et al. (2012) have recently

described the epigenetic regulation of a *FUM1* promoter:green fluorescent protein gene fusion in *F. verticillioides*. They conclude that histone modification of the promoter region of *FUM* genes is involved in transcriptional regulation. The role of *A. nidulans LaeA* in regulation histone acetylation has been described (Reyes-Dominguez et al., 2010). Furthermore, many environmental and nutritional factors affect gene expression via broad-domain transcription factors. In *F. verticillioides*, nitrogen regulatory elements, pH regulatory elements as well as sugar transporters affect *FUM* gene cluster expression (Bluhm and Woloshuk, 2006; Bluhm et al., 2008; Flaherty et al., 2003; Kim and Woloshuk, 2008). In *Aspergillus*, genes that regulate conidia and ascospore production also affect secondary metabolite pathways (Bayram and Braus, 2011 and references therein). The discovery of the velvet complex has revealed yet another level of regulation of gene expression: i.e. regulation via changes in heterochromatin structure.

It is unlikely that all fungal SMs have been identified. The number of putative SM gene clusters in both *Aspergillus* and *Fusarium* exceed the number of SMs characterized in each of these fungi (Amaike and Keller, 2011; Ma et al., 2010). One highly successful strategy to determine the function of some of these cryptic SM gene clusters has been to force their expression by deleting or overexpressing different components of the velvet complex (Bok et al., 2006b; Bouhired et al., 2007). The effects of either strategy are unpredictable but consistent; some gene clusters require *LAE1* for expression, while others are expressed in its absence (Bok et al., 2006b and the present work). Understanding the effects of the velvet complex on SM biosynthesis by *Fusarium* will allow a more complete understanding of how Lae1 and associated proteins regulate production of known metabolites and aid in the identification of new metabolites produced by the numerous other gene clusters present in *Fusarium* genomes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.06.003.

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