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Identification of gene clusters associated with fusaric acid, fusarin, and perithecial pigment production in *Fusarium verticillioides*

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ABSTRACT

The genus *Fusarium* is of concern to agricultural production and food/feed safety because of its ability to cause crop disease and to produce mycotoxins. Understanding the genetic basis for production of mycotoxins and other secondary metabolites (SMs) has the potential to limit crop disease and mycotoxin contamination. In fungi, SM biosynthetic genes are typically located adjacent to one another in clusters of co-expressed genes. Such clusters typically include a core gene, responsible for synthesis of an initial chemical, and several genes responsible for chemical modifications, transport, and/or regulation. *Fusarium verticillioides* is one of the most common pathogens of maize and produces a variety of SMs of concern. Here, we employed whole genome expression analysis and utilized existing knowledge of polyketide synthase (PKS) genes, a common cluster core gene, to identify three novel clusters of co-expressed genes in *F. verticillioides*. Functional analysis of the PKS genes linked the clusters to production of three known *Fusarium* SMs, a violet pigment in sexual fruiting bodies (perithecia) and the mycotoxins fusarin C and fusaric acid. The results indicate that microarray analysis of RNA derived from culture conditions that induce differential gene expression can be an effective tool for identifying SM biosynthetic gene clusters.

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1. Introduction

Fungal secondary metabolite (SM) biosynthetic genes are typically located adjacent to each other (e.g. in a cluster) and tend to exhibit similar patterns of expression (Keller et al., 2005; Shwab and Keller, 2008). This tightly controlled regulation has been suggested as a main selective force for gene clustering (Khaldi and Wolfe, 2011). SM gene clusters usually include a core synthase gene responsible for synthesis of the SM parent chemical, genes responsible for chemical modifications (e.g. oxidoreduction, acyl or amino transfer, and dehydrogenation), one or more genes involved in transport of the SMs, and one or more genes that regulate cluster gene transcription. Most core synthase genes encode either a polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), or terpene cyclase (TC). Multiple methods have been used to identify SM biosynthetic gene clusters including over expression of transcriptional regulators located proximal to core genes, heterologous expression of the core synthase gene, altering cluster transcription by modifying chromatin structure (Bok et al., 2009; Chiang et al., 2009; Watanabe et al., 1999) and the bioinformatic (e.g. SMURF or Secondary Metabolite Unknown Regions Finder) identification of multiple SM genes located adjacent to a core gene by homology (Khaldi et al., 2010). The number of core synthase genes per fungal genome (Ma et al., 2010; Yoder and Turgeon, 2001) suggest that many fungal species can produce up to 10 fold more SMs than indicated by chemical analysis. In accordance with this genetic potential, fungi in the genus *Fusarium* have been found to produce a wide variety of SMs that are diverse in structure and biological activity. The most thoroughly studied *Fusarium* SMs are the mycotoxins, fumonisins, trichothecenes, and zearalenone. These SMs pose a threat to human and animal health because consumption of contaminated grain or grain-based food and feed has been associated with a variety of diseases (Glenn, 2007; Morgavi and Riley, 2007). The economic impact of these mycotoxins on health costs and effect on international trade is estimated to be in the 100 s of millions of dollars each year (Wu, 2007).

Fusarium verticillioides is one of the most common maize-associated fungi worldwide and can infect and colonize maize as an endophyte (i.e. without causing disease symptoms) or as a pathogen (i.e. causing symptoms). During growth on maize, the fungus can produce fumonisins and a number of other polyketide-derived SMs, including the toxins fusaric acid (5-butylpicolinic acid) and fusarins, the pigment bikaverin, and an uncharacterized violet pigment present in the walls of sexual fruiting bodies (perithecia). Production of these metabolites has been reported in other, but not all, *Fusarium* species as well as in other fungal genera. For example, fumonisin production has been reported in several *Fusarium* species within the *Gibberella fujikuroi* species complex (GFSC), in rare isolates of the closely related species

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Fusarium oxysporum (Proctor et al., 2004), and in *Aspergillus niger* (Frisvad et al., 2007) and *Tolypocladium* (Mogensen et al., 2011). In contrast, fusarin production occurs across a genetically broad range of *Fusarium* species including multiple GFSC and trichothecene-producing species (Desjardins and Proctor, 2001; Proctor et al., unpublished).

The role of these SMs in the ecology of *Fusarium* is not clear. However, they do exhibit varying levels of toxicity to plants and animals. For example, fumonisins affected maize sphingolipid synthesis and pathogenesis of *F. verticillioides* in some maize seedling assays (Glenn et al., 2008; Myung et al., 2011). However, they did not affect pathogenesis in other maize seedling assays or in an ear rot assay (Desjardins et al., 2007; Desjardins et al., 2002). Fumonisin consumption can cause a variety of diseases in animals and is epidemiologically associated with esophageal cancer and neural tube defects in humans (Gonzalez et al., 1999; Marasas et al., 2004). Fusaric acid is a broad-spectrum plant toxin and is thought to contribute to the severity of *F. oxysporum*-induced vascular wilt, damping-off and root rot diseases of numerous vegetable crops (Capasso et al., 1996). Fusaric acid is considered a mild toxin to mammals (Bacon et al., 1996). In contrast, fusarins have been reported to be mutagenic in the Ames *Salmonella* mutagenicity assay (Gelderblom et al., 1983; Gelderblom et al., 1984) but not carcinogenic in mouse and rat model systems (Gelderblom et al., 1986). Recently, it has been reported that fusarin C stimulates growth of a breast cancer cell suggesting that it acts as an estrogenic mimic (Sondergaard et al., 2011). Initial studies with fusarin-nonproducing *F. verticillioides* strains in maize seedling and ear rot assays indicate that they are not required for pathogenesis (Brown and Proctor, unpublished). *Fusarium* pigments (e.g. the naphthoquinones bikaverin and fusarubin) are best known for the protective role they provide fungi against environmental stresses such as bacteria and plants (Medentsev and Akimenko, 1998). Overall, fumonisins, fusarins, fusaric acid and bikaverin may contaminate maize and maize-based food and feed and as a result, pose a risk to human and livestock health.

Examination of the genetics of *Fusarium* SM biosynthesis is motivated by the need to limit mycotoxin contamination of agricultural products and to understand how SMs impact *Fusarium*-induced plant disease. Analysis of *F. verticillioides* genome sequence identified 16 genes predicted to encode PKSs (Kroken et al., 2003; Ma et al., 2010). The functions of two of these genes have been assigned based on studies in *F. verticillioides*: *FUM1* (*PKS11*) is required for fumonisin production (Proctor et al., 1999) and *PGL1* (*PKS3*) is required for production of the violet perithecial pigment (Proctor et al., 2007). The function of two more have been assigned based on studies in closely related *Fusarium* species: *PKS4* is required for bikaverin production (*Fusarium fujikuroi*) (Wiemann et al., 2009) and *PKS10* is required for fusarin C production (*Fusarium moniliformis* and *Fusarium venenatum*) (Song et al., 2004) and *Fusarium graminearum* (Gaffoor et al., 2005). To date, associated gene clusters have been identified for two of the PKS genes: *FUM1* (Proctor et al., 2003) and *BIK1* (Brown et al., 2008) based on gene expression and/or gene inactivation analysis.

The identification of multiple SMs that are likely derived from polyketides combined with the identification of the remaining PKS genes in the *F. verticillioides* genome (Desjardins and Proctor, 2007) provides an excellent opportunity to identify additional PKS gene-containing clusters and link them to production of specific SMs. The overall goal is to better understand the role of SMs in the life of *F. verticillioides*; in particular, with regards to the maize disease process. Thus, in the current study, we employed microarray analysis to identify clusters of co-expressed genes that include a PKS gene. The analysis confirmed the existence of the two previously described biosynthetic gene clusters and provided evidence for three heretofore uncharacterized clusters. In addition, results of functional analysis conducted here and previous studies linked the three novel clusters to production of the SMs fusarins, the violet perithecial pigment, and fusaric acid. The identification of these clusters will facilitate future analyses of whether the SMs have a role in plant pathogenesis.

2. Materials and methods

2.1. Strains and media

The wild-type strain of *F. verticillioides* used in this study was M-3125 (NRRL 13447) (Leslie et al., 1992). RNA was prepared from cultures grown in GYAM (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K_2HPO_4, 2 mM $MgSO_4$, and 8.8 mM $CaCl_2$, pH 3.0) medium for 12, 24, 48, 72, 96 or 120 h. Fusaric acid and fusarin production by *F. verticillioides* was determined from cracked maize kernel medium cultures grown for 10 days at 25 °C. The medium was prepared by autoclaving 2.5 g of cracked maize kernels and 1.2 mL of distilled water in a 4-dram glass vial. Each vial was inoculated with a single mycelial plug taken from a V-8 juice agar (Tuite, 1969) culture of *F. verticillioides*.

2.2. Gene identification

Tentative functions of proteins encoded by open reading frames (ORFs) flanking FVEG_11086 and FVEG_12523 were assigned based on BLAST searches of the non-redundant sequence databases, using the default parameters, at the National Center for Biotechnology Information (NCBI) (Tables 2 and 3) (Altschul et al., 1997). *F. verticillioides* and *F. graminearum* genomic nucleotide sequences were retrieved from the *Fusarium* Comparative database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). Transcript sequences were retrieved from the *F. verticillioides* Gene Index database at the Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=f_verticill) (Brown et al., 2005). *Fusarium solani* (teleomorph *Nectria haematococca*) genomic nucleotide sequences were retrieved from the Joint Genome Institute's fungal genome database (http://genome.jgi-psf.org/Necha2/Necha2.home.html). All sequences were assembled using Sequencher™ (version 4.10.1, Gene Codes Corp., Ann Arbor, MI) and examined manually.

2.3. DNA preparation and PCR amplification

DNA was prepared for PCR analysis as previously described (Butchko et al., 2003) with only minor modifications. Mycelia were scraped from one-week-old V-8 juice agar plates, placed in 250 µl of DNA extraction buffer (200 mM Tris, pH 8.0, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid, and 0.5% SDS), and incubated 10 min at 65 °C. Organic material was separated by the addition of 250 µl of phenol:chloroform:isoamyl alcohol (25:24:1) to the mixture, followed by vortexing and centrifugation. The aqueous phase was transferred to a new tube and genomic DNA was isolated using UltraBind following the manufacturer's directions (Mo Bio, Solana Beach, CA). PCR was performed with a total reaction volume of 10 µl (5 µl Promega GoTaq® master mix, 1 µl of each primer (10 µM), and 50 pg of genomic DNA) in a MJ Research PTC-100 PCR thermocycler (Waltham, MA) with the following program: 95 °C for 30 s followed by 57 °C for 30 s followed by 72 °C for 90 s for 30 cycles.

Table 1
Primers used in this study.

Primer name	Sequence 5'–3'
P6F	CCATTGCCTAGTTAGCTGATAG
P6LFI	GTCGTGACTGGGAAAACCTGGCGCGAGCCATTGGAGAGAGTCAT
P6RFI	TCCTGTGTGAAATTGTTATCCGCTGTCAAGCGTGAGACCAATAGCAC
P6RF	GAAGTCTCTTCGTCGGCAGG
P6FN	CTCTAAGCGACATATCTCTAC
P6RFN	CTACCTTGAAGCTCTTCAGC
P6T2F1	CACTAGCAACGGCCATGGAG
P6T2R1	CAGCTACAGAATGTCAGCTC
P6RFO1	GTGAGGTCAAGCTTCTCCTG
P6LFO1	CTGTGAGTTTCCTTACCAGC
P6TF	ATGACTCTCTCCAATGGCTC
P6TR	GTGCTATTGGTCTCACGCTTG
M13FL	CGCCAGGGTTTTCCCAGTCACGAC
M13RL	AGCGGATAACAATTTCACACAGGA
HY	GGATGCCTCCGCTCGAAGTA
YG	CGCCAGGGTTTTCCCAGTCACGAC
HYN	GCGCGTCTGCTGCTCCATAC
YGN	CCGAACTGCCCGCTGTTCTGC
Hygout1	CACGGTGCCTGACTGCGTTAGC
Hygout2	GGTGGGCCTTGACATGTGCAGC
719	GACGTTGTAAAACGACGGCCAGTG
878	GCAACGGCTCTTGCTTGAAGCTGT
879	CAGTGAAGAAGGCTTCGTGAATAGC
1098	ACCAAGCCTATGCCTACAGCATCC

2.4. Microarray analysis

Microarrays were designed and produced by Roche NimbleGen (Madison, WI) based on 12,414 gene models obtained from the Broad Institute in January, 2008 and 875 unique ESTs from the *F. verticillioides* Gene Index. Each sequence was represented on the array by up to 12 unique 60-mer probes. Total RNA was extracted using the RNeasy Mini kit (Qiagen Inc., Valencia, CA). DNA was removed by on-column DNase digestion with the RNase-Free DNase set (Qiagen Inc., Valencia, CA). Microarray hybridization, data acquisition and initial analysis were conducted by Roche NimbleGen, Iceland. Data were normalized by robust multiarray average (Bolstad et al., 2003; Irizarry et al., 2003) and were compared using the Acuity 4.0 microarray analysis software package (Molecular Devices Corp, Sunnyvale, USA). The GYAM experiment (Gene Expression Omnibus (GEO) accession no. GSE16900) was conducted with three biological replicas for the wild-type strain after 24, 48, 72 and 96 h.

2.5. Northern analysis

Total RNA was isolated from wild-type *F. verticillioides* via the TRIzol method (Gibco BRL, Gaithersburg, MD) and Northern blots were carried out as previously described (Proctor et al., 2003). DNA probes for hybridization were labeled with [^{32}P]dCTP with the Prime-A-Gene system (Promega, Madison, WI). DNA templates used to synthesize hybridization probes consisted of 700–1200-bp fragments amplified by PCR from *F. verticillioides* genomic DNA with specific primers. PCR Primers were designed based on predicted gene models FVEG_11075–FVEG_11092 from the Broad Institute's *F. verticillioides* genome sequence database.

Table 2
FUS cluster, flanking genes, and predicted function.

F. verticillioides FCD designation[a]	Gene name	Protein (AAs)	Predicted function[b]	BLAST[c] E value	Accession
FVEG_11077	–	415	Unknown[c]	–	–
FVEG_11078	*FUS9*	355	Methyltransferase	5×10^{-14}	ACG28379
FVEG_11079	*FUS8*	514	Monooxygenase	0.0	AAO73449
FVEG_11080	*FUS7*	461	Dehydrogenase	2×10^{-90}	ADK93993
FVEG_11081	*FUS6*	563	MFS transporter	0.0	EFY96024
FVEG_11082	*FUS5*	233	Oxidoreductase	3×10^{-25}	EDU39826
FVEG_11083	*FUS4*	439	Protease	1×10^{-5}	BAH20542
FVEG_11084	*FUS3*	220	Regulation	3×10^{-36}	AAH54190
FVEG_11085	*FUS2*	419	Hydrolase	2×10^{-176}	EFY96027
FVEG_11086	*FUS1*	3734	PKS-NRPS	0.0	AAT28740
FVEG_11087	*GHY1*	283	Glycosyl hydrolase	6×10^{-13}	CBN78991

[a] FCD refers to the *Fusarium* Comparative Database at the Broad Institute. Bolded genes are co-expressed during growth in GYAM. Non-bolded genes that flank the cluster do not have EST support.
[b] Predicted functions are based on results of BLAST analysis.
[c] BLAST E value refers to the probability score, generated by BLASTX of each gene against the NCBI database, to the predicted protein with the associated accession number.

Table 3
FUB cluster, flanking genes, and predicted function.

F. verticillioides FCD designation[a]	Gene name	Protein (AAs)	Predicted function[b]	BLAST E value[c]	Accession
FVEG_12524	*EST1*	507	Esterase	2×10^{-83}	ABK62698
FVEG_12523	*FUB1*	2409	PKS	0.0	AAR92213
FVEG_12522	*FUB2*	109	Unknown	–	–
FVEG_12521	*FUB3*	510	Aspartokinase	4×10^{-145}	XP_961274
FVEG_12520	*FUB4*	267	Hydrolase[d]	6×10^{-10}; 8×10^{-13}; $6.5 \times^{-17}$	XP_002909515; Pfam03959
FVEG_12519	*FUB5*	463	Acetyltransferase	4×10^{-130}	AAL09833
NP[e]	*FVX1*	152	Unknown	–	–
FVEG_12518	*HET1*	1253	HET[f]	2×10^{-115}	Q00808

[a] FCD refers to the *Fusarium* Comparative Database at the Broad Institute. Bolded genes are co-expressed during growth in GYAM.
[b] Functions listed are based on the results of BLAST analysis.
[c] BLAST E value refers to the probability score, generated by BLASTX of each gene against the NCBI database, to the protein with the associated accession number.
[d] The first E value refers to BLASTX analysis while the second and third E values refer to BLASTP to the accession and to the pfam group indicated respectively.
[e] NP = not predicted. The putative gene was predicted based on the presence of an ORF.
[f] HET = vegetative incompatibility protein HET or beta transducin-like protein.

2.6. FVEG_11086 inactivation

FVEG_11086 was inactivated with the integrative disruption strategy and the protoplast-mediated transformation method with the antibiotic hygromycin B (*HygB*) as the selectable marker (Proctor et al., 2007; Proctor et al., 1999; Turgeon et al., 1987). The disruption vector (pPKS10Δ/Hyg) was created by cloning a 652-bp fragment (nucleotides 339–990) of FVEG_11086, encoding a portion of the putative ketosynthase domain, into vector pUCH2-8, which contains the *HygB* resistance cassette (Alexander et al., 1998; Turgeon et al., 1987). A single, homologous recombination event between the FVEG_11086 coding region and the corresponding region of pPKS10Δ/Hyg would inactivate FVEG_11086 by generating two truncated copies of the coding region separated from one another by the disruption vector as previously described for *PGL1* disruption (Proctor et al., 2007). Hygromycin-resistant transformants were screened for integration of pPKS10Δ/Hyg into FVEG_11086 by PCR that employed primers that flanked the 652-bp fragment (primers 878 and 879) and were complementary to either the vector sequence (primer 719) or to the 3' end of *HygB* (primer 1098). In PCR, primer pair 878–879 was expected to yield a 780-bp amplicon from wild-type DNA and a > 6-kb amplicon from transformants in which pPKS10Δ/Hyg integrated into FVEG_11086 by a single homologous integration event. Primer pair 878–719 was expected to yield a 880-bp amplicon from FVEG_11086 disruption mutants but no amplicon from the wild type. Likewise, primer pair 879–1098 was expected to yield a 920-bp amplicon from FVEG_11086 mutants but no amplicon from the wild type (Supplemental Fig. 1). Disruption of FVEG_11086 was confirmed by Southern blot analysis using essentially the same strategy described previously for analysis of *PGL1* disruption (Proctor et al., 2007). Primers used in this study are listed in Table 1.

2.7. FVEG_12523 deletion

Mutation of FVEG_12523 was accomplished by deletion of about half of its predicted coding region via the split-marker and protoplast transformation method with *HygB* as the selectable marker (Catlett et al., 2002; Proctor et al., 1999). The disruption amplicons were created by first amplifying two ~800-bp fragments from wild-type *F. verticillioides* genomic DNA. The first fragment (Fub1-upstream) corresponded to an 800-bp region upstream of the FVEG_12523 start codon and was amplified with primer pair P6F-P6LFI. The second fragment (Fub1-downstream) corresponded to an 800-bp region that began 3.5 kb downstream of the FVEG_12523 start codon and was amplified with primer pair P6RFI-P6RF. Also, two fragments corresponding to overlapping regions of the *HygB* gene were amplified from plasmid pHygAsc (Brown et al., 2002). The first fragment (Hyg-5') included the 5' two thirds of the *HygB* gene and was amplified with primer pair M13FL-HY; and the second fragment (Hyg-3') included the 3' two thirds of *HygB* and was amplified with primer pair M13RL-YG. Fragments Fub1-upstream and Hyg-3' were fused by PCR using nested primers P6FN and HYN. Likewise, fragments Fub1-downstream and Hyg-5' were fused using nested primers P6RFN and YGN. Integration of the two PCR products into the genome via three homologous recombination events was expected to replace 3.5 kb of the FVEG_12523 coding region with *HygB* (Catlett et al., 2002). Hygromycin-resistant transformants were screened by PCR to determine whether targeted DNA had been deleted and replaced with *HygB* and then to determine whether homologous recombination events had occurred as predicted. In the first case, PCR with primers P6T2F1 and P6T2R1 was expected to yield a 500-bp amplicon from wild-type DNA and no amplicon from transformants in which the *HygB* cassette replaced a 3.5 kb portion of FVEG_12523. Second, PCR with primer pairs P6RFO-Hygout1 and P6LFO1-Hygout2 were expected to yield no amplicons from wild-type DNA and ~900 and ~940-bp amplicons respectively from FVEG_12523 disruption mutants (Supplemental Fig. 2).

2.8. Analysis of fusarin C and fusaric acid

For fusarins, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method similar to that described by Kleigrewe et al. (2011) and Maragos et al. (2008) was employed to monitor and quantify fusarin C, the fusarin analogue produced in greatest abundance by wild-type *F. verticillioides*. Care was taken to minimize exposure of samples to conditions previously shown to prompt isomerization. Fusarins were extracted from 2.5 g cracked maize cultures with 12 mL of acetonitrile/water (84% v/v). Cultures were allowed to steep for 2 h, with gentle shaking, and then filtered through a 0.45-μm-pore-size Gelman filter. The culture extracts were then analyzed for fusarin C by LC-MS/MS. The analytical method utilized a LC-MS/MS instrument consisting of a ThermoSpetraPhysics high performance liquid chromatography (HPLC) system and a ThermoFinnigan LCQ DECA ion trap mass spectrometer. The mass spectrometer was operated in positive mode utilizing an electrospray ionization (ESI) interface. Injections of 10 μl of analyte were eluted from a Metachem (Torrance, CA, USA) Inertsil C18 3×150 mm column with a 300 μl/min gradient flow of water/methanol (MeOH). The entire column flow was directed to the ESI interface of the mass spectrometer. HPLC solvents were acidified with 0.3% acetic acid. The gradient program consisted of the following steps: 0–10 min, 35% MeOH; 10–25 min, 35–95% MeOH; 25–26 min, 95% MeOH; 26–34 min, 95–35% MeOH; 34–35 min, 35% MeOH. ESI-MS/MS detection of fusarin C eluting from the column was accomplished by monitoring three characteristic fragment ions (*m/z* 364, 382, 396) of the *m/z* 432 $[M + H]^+$ ion of fusarin C in multiple reaction monitoring (MRM) mode. Quantitation of the fusarin C was done on the basis of the integrated intensity of the *m/z* 364 fragment compared to a calibration curve generated from fusarin C standard solutions. Signal for LC-MS/MS determination of fusarin C may contain a contribution from products of the decomposition of fusarin C during conduct of the LC-MS/ MS analysis (Kleigrewe et al., 2012).

Fusaric acid was extracted from cracked maize cultures with 12 mL of acetonitrile/water (50% v/v) acidified to pH 2 with HCl and analyzed by LC-MS/MS. Fusaric acid extract analysis was accomplished by use of a LC-MS/MS procedure developed for this study with the apparatus described above. Direct infusion (50 μl/min, 1 mg/mL fusaric acid (Sigma Chemical Co., St. Louis, MO, USA) in 1:1 water/methanol with 5% acetic acid) ESI-MS/MS analysis of fusaric acid produced a spectrum with dominant fragments of *m/z* 152 and 162. These dominant fragments were chosen for monitoring in LC-MS/MS experiments. HPLC solvents contained 0.3% trifluoroacetic acid as an ion pairing reagent. The HPLC gradient program (300 μl/min gradient flow) consisted of the following steps: 0–10 min, 15% MeOH; 10–25 min, 15–90% MeOH; 25–26 min, 90% MeOH; 26–34 min, 90–15% MeOH; 34–35 min, 15% MeOH. ESI-MS/MS detection of the fusaric acid ion eluting from the column was accomplished by monitoring the two fragment ions (*m/z* 152 and 162) and the parent ion *m/z* 180 $[M + H]^+$ ion. Quantitation of the fusaric acid was done on the basis of the integrated intensity of the *m/z* 162 fragment compared to that obtained from a calibration curve generated by analysis of fusaric acid concentration standards. Calibration curves generated for the LC-MS/MS analysis were linear over a range corresponding to 0.5–50 μg fusaric acid per gram cracked maize.

3. Results

3.1. Identification of clusters of co-expressed genes via microarray analysis

To identify SM biosynthetic gene clusters, we examined microarray data from wild-type *F. verticillioides* grown in liquid GYAM medium for 24, 48, 72 and 96 h. We focused the search for clusters by looking for patterns of co-expression of genes located within 50–80 kb of the 16 PKS genes previously identified in the *F. verticillioides* genome sequence (Kroken et al., 2003; Ma et al., 2010). Genes were considered members of a cluster if (1) they were adjacent to each other, (2) a majority were expressed to a similar degree and (3) they shared a similar pattern of expression. Some consideration was given for inclusion of a predicted gene in a putative cluster based on similarity to previously characterized SM genes. The analysis revealed the presence of five such clusters. Although genes within each cluster exhibited similar patterns of expression, the patterns were not the same between clusters. Two clusters correspond to the previously characterized *BIK* (FVEG_03379–FVEG_03384) and *FUM* (FVEG_00315–FVEG_00329) clusters (Fig. 1) (Brown et al., 2008; Proctor et al., 2003). Expression of most of the fourteen *FUM* cluster genes was readily observed at 48 h after which expression increased significantly through 96 h to a similar degree (Fig. 1A). In contrast, expression of three of the six *BIK* cluster genes increased steadily through the course of the experiment while the other three were only detected at 96 h (Fig. 1B). Genes flanking the clusters exhibited patterns of expression that were markedly different from those of genes within the clusters (Fig. 1A and B). Confirmation of co-expression of genes within the *BIK* and *FUM* clusters via microarray analysis in *F. verticillioides* indicates that the same approach may be useful for identification of novel SM biosynthetic gene clusters that include PKS genes.

Co-expression of genes in the three other regions with a PKS gene has not been described previously. The first of these regions included the PKS gene FVEG_11086, several homologs of which are required for production of fusarin mycotoxins in other fusaria (Gaffoor et al., 2005; Song et al., 2004). Within this region FVEG_11086 and FVEG_11078–FVEG_11085 exhibited significant expression at all four time points (Fig. 2A). In contrast, genes (e.g. FVEG_11071–FVEG_11077 and FVEG_11087–FVEG_11094) that flanked this region exhibited different patterns of expression. The second region included the previously characterized PKS gene *PGL1* (FVEG_03695), which is required for production of the violet perithecial pigment. Within this

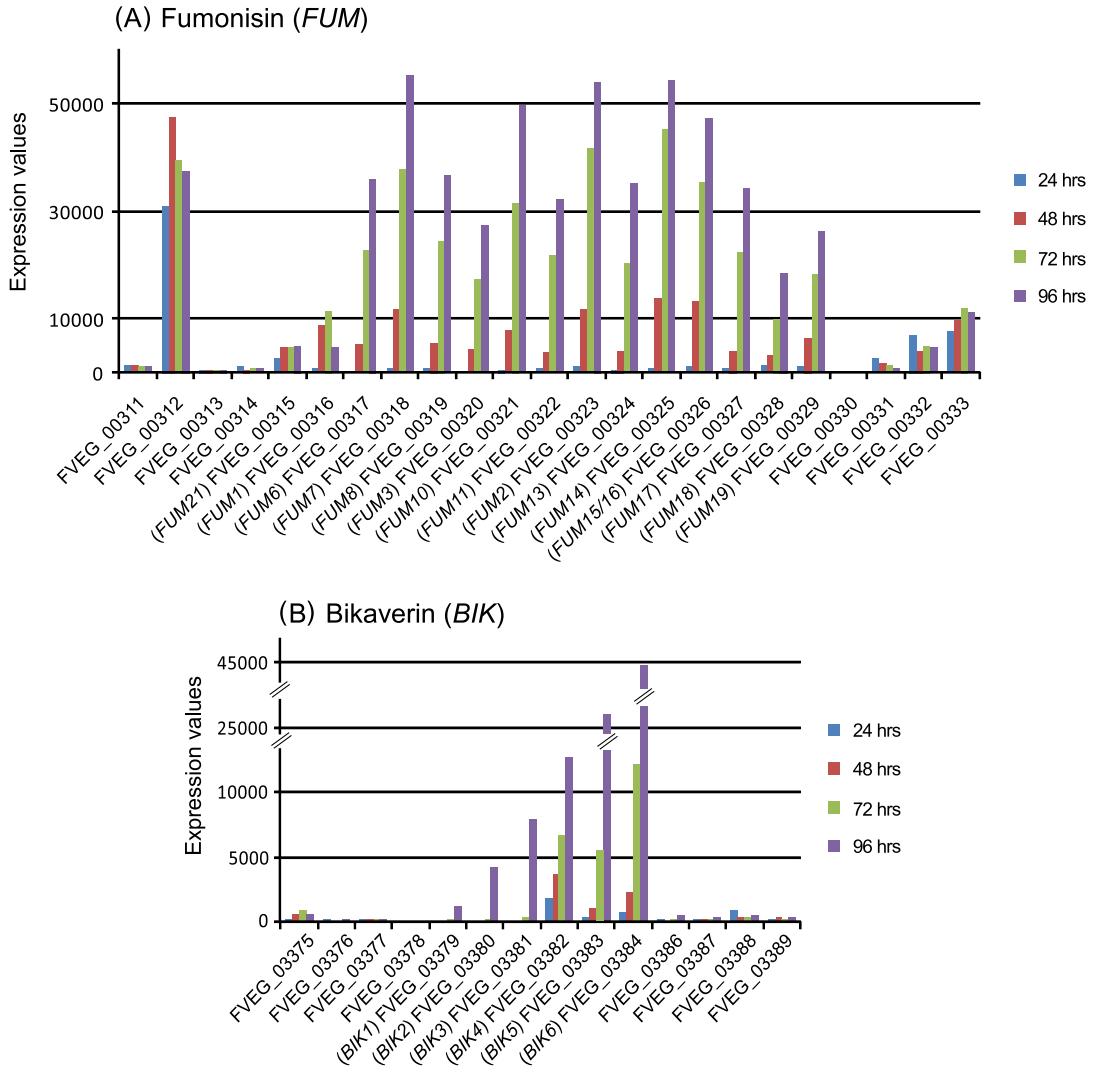

Fig. 1. Relative gene expression values (based on microarray analysis) following growth of *F. verticillioides* in GYAM medium for different lengths of time. (A) Fumonisin (*FUM*) gene cluster (B) Bikaverin (*BIK*) gene cluster.

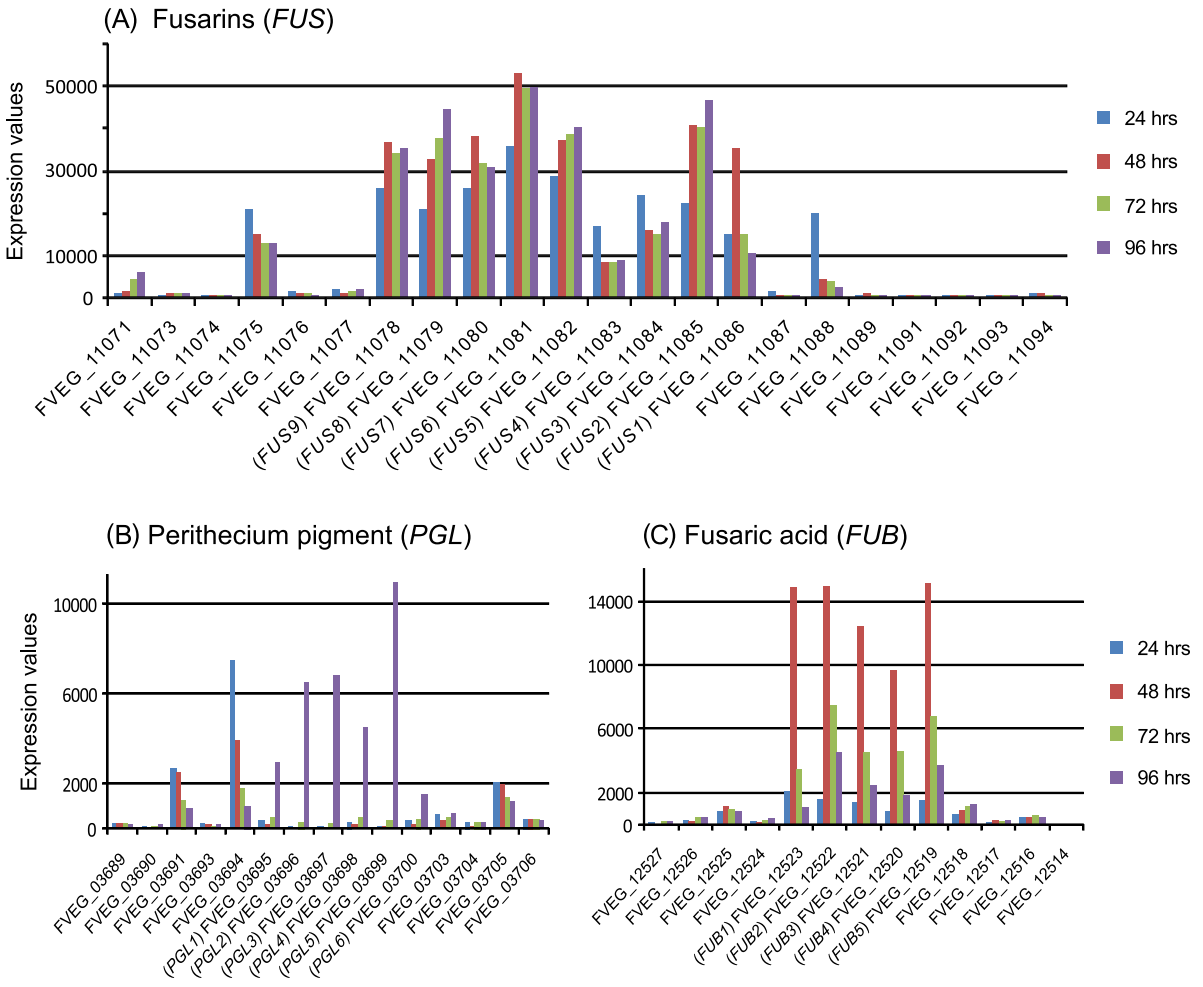

Fig. 2. Results of microarray analysis showing relative expression values for genes within three regions of the *F. verticillioides* genome after growth in GYAM medium for different lengths of time. (A) Nine genes (FVEG_11078–FVEG_11086 designated *FUS1–FUS9*) and thirteen flanking genes. (B) Six genes (FVEG_03695–FVEG_03700 designated *PGL1–PGL6*) and nine flanking genes. (C) Five genes (FVEG_12519–FVEG_12524 designated *FUB1–FUB5*) and eight flanking genes.

region, *PGL1* and genes FVEG_03696–FVEG_03700 did not exhibit significant expression until the 96-h time point (Fig. 2B). In contrast, genes flanking this region exhibited different patterns of expression. The third region included the uncharacterized PKS gene FVEG_12523. In this case, genes FVEG_12519–FVEG_12523 exhibited low levels of expression at 24 h, higher at 48 h, and then decreasing levels at 72 and 96 h (Fig. 2C). Again, genes flanking this region tended to exhibit different patterns of expression.

3.2. Gene models and predicted functions of co-expressed genes

The gene models for the nine co-expressed genes FVEG_11078–FVEG_11086 are supported by 406 Expressed Sequence Tags (ESTs) representing 336 different transcripts from the *F. verticillioides* Gene Index. The transcripts define 14 introns that have typical fungal GT/AG borders, including an atypically long, 546-bp intron in FVEG_11086, which was previously reported in the *Fusarium moniliforme* FVEG_1186 homolog *fusA* (Song et al. 2004). Excluding this intron, the average intron length is 53 (±7.2) bp. The predicted function of each gene, based on BLAST analysis is listed in Table 2. The gene models for the five co-expressed genes FVEG_12519–FVEG_12523 are supported by 44 ESTs representing 32 different transcripts. The transcripts define 10 of the 12 introns in the Broad gene models. All 12 introns have typical fungal GT/AG borders and an average length of 52 (±5.6) bp. The predicted function of each gene, based on BLAST analysis is listed in Table 3.

3.3. Northern analysis of FVEG_11086 and flanking genes

Expression of genes within a 55-kb region that included PKS gene FVEG_11086 was examined by Northern blot analysis of RNA prepared from GYAM cultures of wild-type *F. verticillioides* harvested at 12, 24, 48, 72 and 96 h of growth (Fig. 3). Although we were not able to detect a full-length FVEG_11086 transcript, estimated to be around 12 kb, we did observe significant hybridization signal between 1 and 3 kb at 24, 48, 72 and 96 h. The failure to detect a full-length transcript may be due to poor transfer of large RNAs onto the membrane while the detection of multiple, small RNAs may be due to the random mechanical breakage of large transcripts during the RNA extraction protocol (Diez et al., 1990). In contrast, bands of the expected sizes were observed for genes FVEG_11078–FVEG_11085 immediately upstream of FVEG_11086 as well as for FVEG_01187. Each gene exhibited little or no expression at 12 h but moderate to high levels of expression at 24 h that tended to decline after 72 h. In contrast, a majority of the genes in the region flanking FVEG_11078–FVEG_11087 tended to exhibit different patterns of expression (Fig. 3). Thus, in Northern analysis genes FVEG_11078–FVEG_11086 exhibited similar

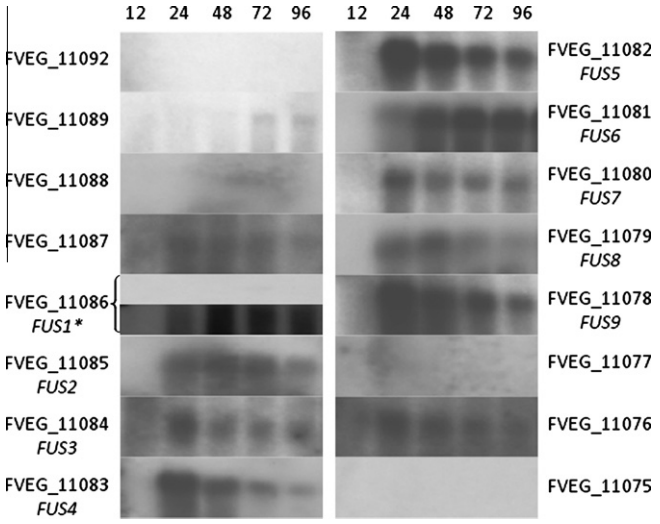

Fig. 3. Northern analysis of *FUS* gene cluster (FVEG_11078–FVEG_11086) and selected flanking genes after growth on GYAM medium. *The Northern analysis of FVEG_11086 is displayed in two sections. The top section is the portion of the blot corresponding to ~12 kb, the size expected for the FVEG_11086 transcript. The lower section is around 3 kb and includes a portion of the multiple hybridization signals observed.

patterns of expression when *F. verticillioides* was grown in GYAM medium.

3.4. Functional analysis of PKS genes FVEG_11086 and FVEG_12523 for fusarin C and fusaric acid biosynthesis

LC-MS/MS analysis of 10-day-old cracked maize culture extracts of hygromycin-resistant isolates recovered following transformation of wild-type *F. verticillioides* with the FVEG_11086 disruption vector (pPKS10Δ/Hyg) indicated that two transformants, designated GmT606 and GmT644, did not produce detectable levels of fusarin C (Fig. 4). In contrast, the wild-type progenitor strain and transformants in which pPKS10Δ/Hyg integrated ectopically (e.g. strain GmT601) produced 150–297 μg fusarin C per gram of cracked maize medium. PCR analysis indicated GmT644 carried a disrupted FVEG_11086 (Supplemental Fig. 1). Disruption of FVEG_11086 in GmT644 was confirmed by Southern blot analysis (data note shown). LC-MS/MS analysis of 10-day-old cracked maize culture extracts of hygromycin-resistant isolates recovered following transformation of wild-type *F. verticillioides* with the FVEG_12523 deletion amplicons indicated that two, designated GmTcm1 and GmTcm5, did not produce detectable levels of fusaric acid whereas a similar analysis of the wild-type progenitor indicated that it produced 18.4 μg fusaric acid per gram of cracked maize medium (Fig. 5). PCR analysis indicated that both GmTcm1 and GmTcm5 lacked half of the FVEG_12523 coding sequence (Supplemental Fig. 2). All four transformants exhibited wild-type growth and conidiation on a variety of growth media.

3.5. Comparative genomic analysis

Examination of available *Fusarium* genomic sequences revealed regions homologous to FVEG_11078–FVEG_11086 in *F. graminearum* and *F. solani* (Fig. 6, Table 3). *F. graminearum* has homologs of all nine *F. verticillioides* genes. The homologous genes share 78% deduced amino acid identity on average and are organized in the same order and orientation in the *F. graminearum* and *F. verticillioides* genomes. *F. solani* has homologs of eight of the nine

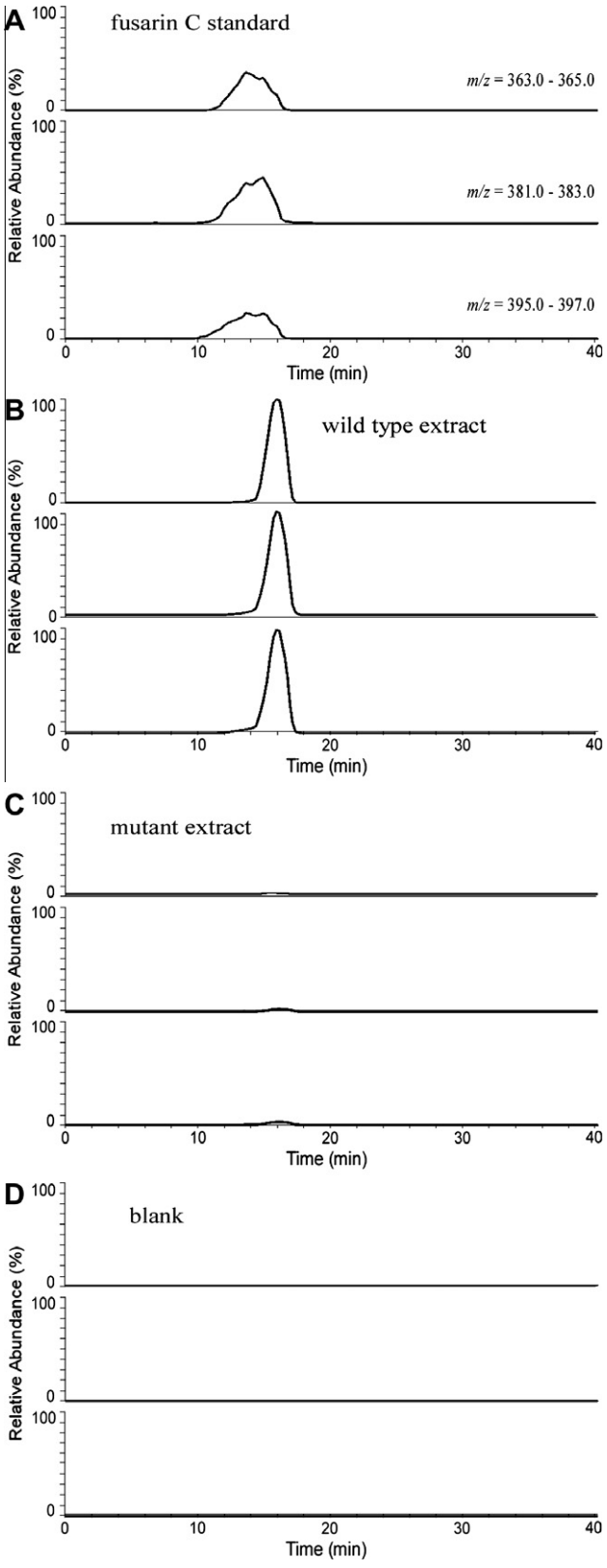

Fig. 4. Reconstructed LC-MS/MS (MRM) chromatograms for (A) a fusarin C standard (0.1 mg/mL), 10 day culture extracts of (B) NRRL 13447 and (C) GmT606 (Δ *FUS1*), and (D) a blank. The first panel in each set is the fragment ion mass range *m/z* 363.0–365.0, second panel is the fragment ion mass range *m/z* 381.0–383.0 and the third panel is the fragment ion mass range *m/z* 395.0–397.0 of fusarin C. The fusarin C parent ion is 432 $[M + H]^+$. The relative abundance has been normalized to the wild-type strain.

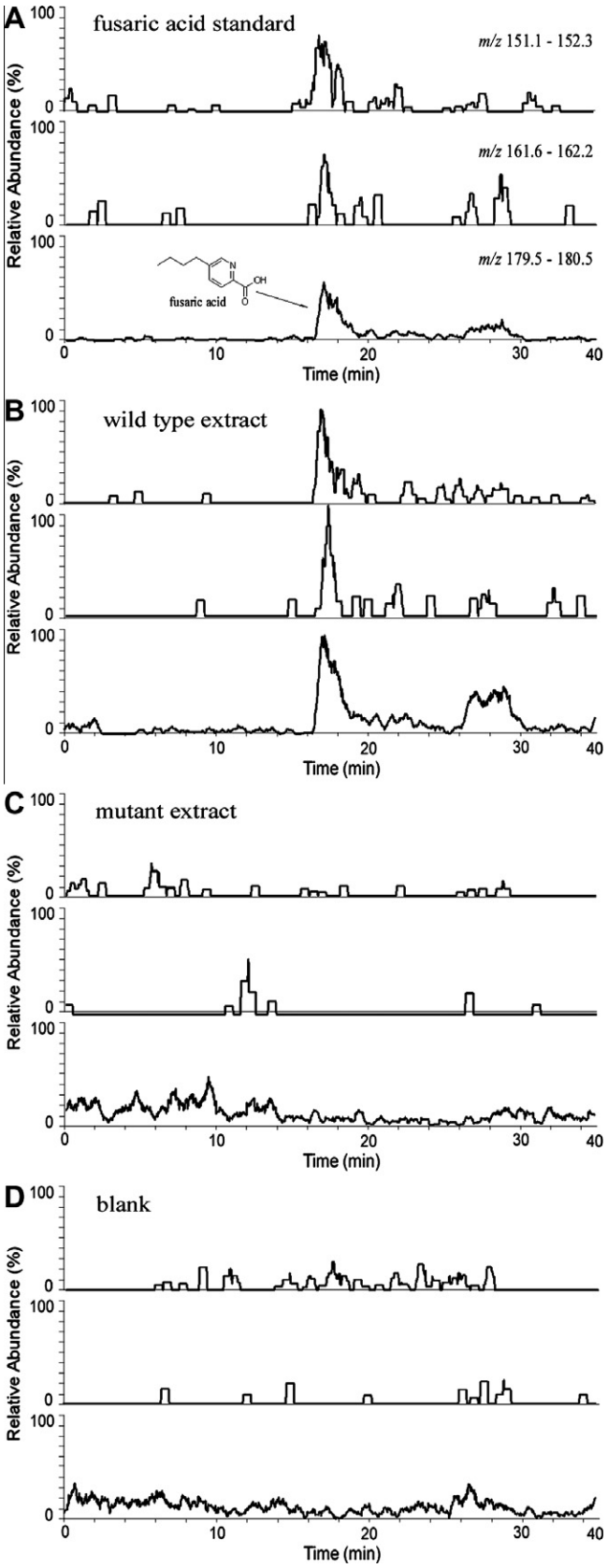

Fig. 5. Reconstructed LC-MS/MS (MRM) chromatograms for (A) a fusaric acid standard, 10 day culture extracts of (B) NRRL 13447 and (C) GmTcm1 (Δ *FUB1*), and (D) a blank. The first panel in each set is the fragment ion mass range *m/z* 151.1–152.3 while second panel in each set is the fragment ion mass range *m/z* 161.6–162.2. The third panel is the parent ion with a mass range of *m/z* 179.5–180.5. The relative abundance has been normalized to the wild type strain.

F. verticillioides genes; a homolog of FVEG_11082 is not present in *F. solani*. In addition, the homologous genes in *F. verticillioides* and *F. solani* share 68% deduced amino acid identity on average and exhibit marked differences in arrangement in the two genomes (Fig. 6, Table 4). Intergenic regions between homologous pairs of genes in all three fungi are of similar length (Fig. 6). The genes on either side of each gene cluster in each *Fusarium* do not share any similarity indicating that the genomic locations of each cluster are different (Fig. 6).

4. Discussion

Two hallmarks of genes involved in synthesis of a fungal SM are their co-expression and their clustering within a genome (Keller et al., 2005). Previous Northern and EST analyses established co-expression and clustering of fumonisin biosynthetic (*FUM*) genes in *F. verticillioides* (Brown et al., 2007; Proctor et al., 2003) and bikaverin biosynthetic (*BIK*) genes in *F. fujikuroi* (Wiemann et al., 2009). Functional analyses confirmed the roles of most genes within these clusters in the synthesis of fumonisins and bikaverin respectively. In the current study, analysis of microarray data from *F. verticillioides* grown in GYAM medium confirmed co-expression of genes within the *FUM* and *BIK* clusters (Fig. 1A and B) and indicated that it should be possible to use microarray data to identify additional SM biosynthetic gene clusters. Thus, co-expression of the three PKS genes (*PGL1*, FVEG_11086 and FVEG_12523) with their respective flanking genes provides evidence for three additional SM biosynthetic gene clusters. Furthermore, functional analyses of the PKS genes, done previously (Gaffoor et al., 2005; Proctor et al., 2007; Song et al., 2004) and during the course of the current study, link each of these clusters of co-expressed genes with production of an SM.

PGL1 homologs in *F. verticillioides* and *F. graminearum* were previously shown to be required for production of a violet perithecial pigment (Gaffoor et al., 2005; Proctor et al., 2007), a trait that can be used to distinguish between fusaria with a *Gibberella* teleomorph from those with a *Nectria* teleomorph. Co-expression of *PGL1* and the adjacent genes FVEG_03696–FVEG_03700 suggests that these six genes constitute a gene cluster that is responsible for synthesis of the perithecial pigment. Based on this and for consistency with the *PGL1* designation for the PKS gene, we propose that FVEG_03696–FVEG_03700 be designated *PGL2–PGL6* (Fig. 2B).

Initial evidence for the *PGL* gene cluster came from a study describing the conservation in position and orientation of the two genes immediately upstream of *PGL1* in *F. verticillioides*, *F. graminearum* and *F. solani*. The two genes are predicted to encode activities consistent with SM synthesis (Proctor et al., 2007). However, six additional genes upstream of *PGL1* are conserved in *F. verticillioides*, *F. graminearum*, and *F. oxysporum* but not *F. solani*. The most likely consequence of the lack of conservation of genes flanking *PGL1* in *F. solani* and *F. verticillioides* is production of different SM end products in the two fungi. As noted above, *F. verticillioides PGL1* is responsible for the synthesis of a violet pigment that accumulates in the walls of perithecia of some *Fusarium*/*Gibberella* species (Gaffoor et al., 2005; Proctor et al., 2007). In contrast, the pigment that accumulates in the perithecia of *F. solani* is red and results from the activity of a different PKS (encoded by *pksN*; JGI_101778) (Graziani et al., 2004). Although the SM end product of the *F. solani PGL1* (JGI_33672) homolog is unknown, this fungus has been reported to produce fusarubin (Fig. 1) (Kurobane et al., 1980), a metabolite that is not produced by *PGL1* mutants but is produced by wild-type *F. verticillioides* (M. Busman and R.H. Proctor, unpublished). *PGL1* is predicted to encode an non-reducing PKS (NR-PKS) which is consistent with the synthesis of the aromatic structure of fusarubin. Studies are in progress to determine

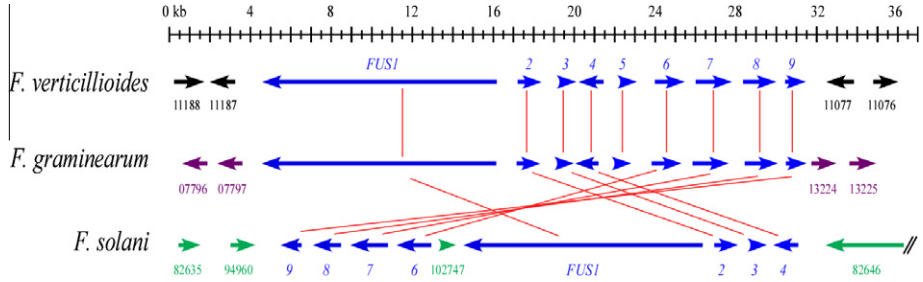

Fig. 6. Comparison of genomic organization of *FUS* gene clusters in *F. verticillioides* and *F. graminearum* and the *FUS*-like cluster in *F. solani*. Relative scale in kilo base pairs (kb) is indicated. The arrowheads indicate direction of transcription. The blue arrows represent cluster genes while the black arrows represent genes flanking the cluster. In contrast to the cluster genes, cluster-flanking genes differ in the three fungi. The green arrow represents a gene that is present within the *F. Solani FUS* cluster but that is absent in the cluster in the other two fungi. The numbers underneath the black arrows refer to database gene designations for each fungus respectively.

Table 4
Comparison of *Fusarium FUS* homologs.

Gene name	Fv (FVEG_#)[a]	Protein (AAs)	Fg (FGSG_#)	Protein (AAs)	Fg% to Fv[2]	Nh (JGI_#)	Protein (AAs)	Nh% to Fv
FUS9	11,078	355	07805	327	67.3	101,086	361	59.4
FUS8	11,079	514	07804	515	84.6	45,012	509	71.2
FUS7	11,080	461	07803	465	75.9	45,085	466	65.9
FUS6	11,081	563	07802	561	89.7	45,233	544	80.9
FUS5	11,082	233	07801	233	75.1	–	NA	NA
FUS4	11,083	439	07800	437	74.8	82,635	439	59.8
FUS3	11,084	220	13,223	220	74.3	62,410	219	69.1
FUS2	11,085	419	13,222	419	88.0	104,901	420	75.8
FUS1	11,086	3734	07798	3738	71.8	70,660	3742	64.3

[a] Designations for the *FUS* genes at the *Fusarium* Comparative Database at the Broad Institute for *F. verticillioides* (Fv) and *F. graminearum* (Fg) and the JGI website for *N. haematococca* (Nh).
[b] Fg% to Fv and Nh% to Fv refers to the percent amino acid identity shared between the *F. graminearum* and *N. haematococca* predicted proteins respectively to the *F. verticillioides* homologous proteins.

the relationship between fusarubin and the perithecial pigment. Fusarubin may be a monomer or building block of the perithecial pigment, which after polymerization or attachment to the perithecial wall, is recalcitrant to analysis due to its complex nature. It should be noted that the putative *F. graminearum PGL* cluster includes a gene, FGSG_09185 that is not present in the *F. verticillioides PGL* cluster. FGSG_09185 is located between the homologs of *PGL3* and *PGL4*. The most closely related FGSG_09185 homolog in *F. verticillioides* is FVEG_13758 (E value = 0.0), and it is located on a different chromosome. Recently, a homolog of the *PGL* cluster was identified in *F. fujikuroi* (Studt et al., 2012). Functional analysis of genes in the cluster demonstrated that the *F. fujikuroi* homologs of *PGL1*, *PGL2* and *PGL3* are required for production of fusarubins and that the *PGL1* homolog is required for perithecial pigmentation (Studt et al., 2012). Although the functional characterization of most of the cluster in *F. fujikuroi* provides strong evidence that fusarubins, which are red, are precursors of the perithecial pigment, which is violet, the structure of the perithecial pigment has yet to be determined.

Previously, putative homologs of FVEG_11086 were shown by gene inactivation analysis to be required for fusarin production in *F. graminearum* (*FUS1*) (Gaffoor et al., 2005), *F. moniliforme* (*fusA*) and *F. venenatum* (*fusA*) (Song et al., 2004). In addition, inactivation of a putative ortholog in *Metarhizium robertisii* (*NGS1*) blocked production of compound NG-391, which is structurally similar to fusarin C (Donzelli et al., 2010). Because *F. moniliforme* has been resolved into multiple morphologically similar species, including *F. verticillioides*, it is not clear whether the *F. moniliforme* strain examined by Song et al. was *F. verticillioides* or another species. Therefore, we inactivated *F. verticillioides FUS1* homolog, FVEG_11086, in strain FRC M-3125 (FGSC 7600, NRRL 20956), a standard fumonisin-producing, pathogenic, and mating type tester strain as well as the strain used to determine the *F. verticillioides* genome sequence and to generate the genetic linkage map of the fungus. The analysis confirmed that FVEG_11086 is required for fusarin production in *F. verticillioides* and, therefore, is a functional homolog of *FUS1/fusA*. Thus, FVEG_11086 will be referred to hereafter as *FUS1*.

Additional evidence that the nine *F. verticillioides FUS* genes constitute a gene cluster is that contiguous groupings of *FUS* homologs in the *F. graminearum* and *F. solani* genomes and that flanking genes on either side of the putative *FUS* cluster differ in each species (Fig. 3 and Table 3). This break in synteny, where homologous biosynthetic genes in related fungi are clustered but the clusters are located in different genomic locations, may be a common attribute in filamentous fungi (Proctor et al., 2009). In summary, based on the requirement of *FUS1* for fusarin production, the co-expression of *FUS1* and FVEG_11078–FVEG_11085, and the conservation of the cluster in *Fusarium*, we propose that these nine genes constitute a fusarin biosynthetic gene cluster. For consistency with the *FUS1* designation, we also propose that FVEG_11078–FVEG_11085 be designated *FUS9–FUS2* as shown in Fig. 3.

To our knowledge, fusarin production has not been reported in *F. solani*. However, the presence of a putative *FUS* gene cluster suggests that it has the genetic potential to produce fusarins or a fusarin-like metabolite(s). In contrast, the *F. oxysporum* genome does not contain a *FUS* gene cluster and this species has not been reported to produce fusarins.

Co-expression of PKS gene FVEG_12523 with adjacent genes FVEG_12519–FVEG_12522 suggests that this region could also be a SM biosynthetic gene cluster. Based on the co-expression and on the lack of production of fusaric acid in the FVEG_12523 deletion mutants, we propose that FVEG_12519–FVEG_12523 constitute a fusaric acid biosynthetic gene cluster (Fig. 7). Furthermore, we propose that the genes be designated as *FUB*, for fusaric acid biosynthetic genes. The PKS gene FVEG_12523 will be referred to

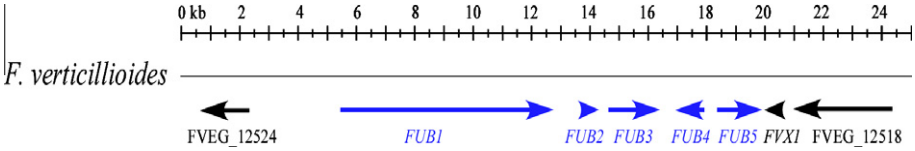

Fig. 7. Genomic organization of the putative *F. verticillioides* fusaric acid biosynthetic (*FUB*) gene cluster. Relative scale in kilo base pairs (kb) is indicated. The arrowheads indicate direction of transcription and the five sets of blue arrows represent the co-expressed cluster genes while the black arrows represent cluster-flanking genes. FVEG_12524 encodes a putative esterase, *FVX1* encodes a putative protein of unknown function and FVEG_12518 encodes a putative protein with similarity to vegetative incompatibility proteins or beta transducin-like proteins.

as *FUB1* and FVEG_12519–FVEG_12522 be referred to as *FUB5–FUB2*, as shown in Fig. 2C.

No gene involved in fusaric acid biosynthesis has yet been reported in the literature. Precursor feeding studies indicate that fusaric acid biosynthesis requires three acetate units (a triketide via a PKS) and aspartate (Dobson et al., 1967; Stipanovic et al., 2011b). Based on the observation that two of three other *Fusarium* SMs derived from acetate and an amino acid, fusarin C (Song et al., 2004), and equisetin (Sims et al., 2005), are synthesized from a PKS with a non-ribosomal peptide synthase module (PKS-NRPS), we expected that the fusaric acid PKS to have a NRPS module as well. In other PKSs with an NRPS module, the module serves to activate a specific amino acid and covalently attach it to the carbon chain generated by the PKS. In contrast, the amino acid in the third *Fusarium* SM (fumonisins) is linked to the polyketide carbon chain via an α-oxoamine synthase encoded by a gene, *FUM8*, in the *FUM* cluster (Proctor et al., 2008).

PKS-NRPS genes occur frequently in fungal genomes. In *F. verticillioides*, three PKSs include an NRPS module (Ma et al., 2010). Work in our laboratory demonstrated that two of these three were not involved in fusaric acid synthesis (R.H. Proctor and M. Busman, unpublished). The finding that the PKS gene *FUB1* is required for fusaric acid synthesis but does not have an NRPS module and the putative fusaric acid biosynthetic gene cluster does not include an α-oxoamine synthase gene indicates that fusaric acid synthesis involves a novel mechanism for the incorporation of an amino acid (or derivative). The function of the putative protein encoded by *FUB3*, an aspartokinase (i.e. amino acid kinase), its physical proximity to and co-expression with *FUB1* strongly suggest that *FUB3* is also involved in fusaric acid synthesis. Gene deletion studies are in progress to determine if this hypothesis is correct.

Based on the chemical structure of fusaric acid and previous published substrate feeding experiments (Dobson et al., 1967; Stipanovic et al., 2011b), we predict that the *FUB1*-encoded PKS (Fub1) catalyzes condensation of three acetate units to form a fully reduced 6-carbon polyketide chain (Fig. 8). The proposed function of *FUB3*, *FUB4* and *FUB5* in fusaric acid synthesis is speculative (Table 3 and Fig. 8). *FUB3*, encoding a putative amino acid kinase, likely plays a critical role in assimilating a nitrogen from glutamine or oxaloacetate to form fusaric acid. *FUB5* is predicted to encode an acetyltransferase, which suggests that it could be responsible for the addition of a methyl group to the carboxylic acid moiety of fusaric acid to yield methyl fusarate (Fig. 8, a metabolite that often occurs along with fusaric acid in *Fusarium* cultures (Amalfitano et al., 2002). *FUB4* is predicted to encode a hydrolase. A role for the hydrolase in fusaric acid synthesis is still more speculative. Nevertheless, based on the observation that methyl fusarate is more toxic than fusaric acid (Stipanovic et al., 2011a), the putative hydrolase may catalyze conversion of methyl fusarate to the less toxic fusaric acid by removing the methyl group (Fig. 8). The ability to readily interconvert these two materials may allow *Fusarium* to more precisely control relative concentrations of fusaric acid and methyl fusarate in order to mitigate their impact on host plants, bacteria, or other fungi. We are in the process of determining whether *FUB4* is involved in the proposed conversion of methyl

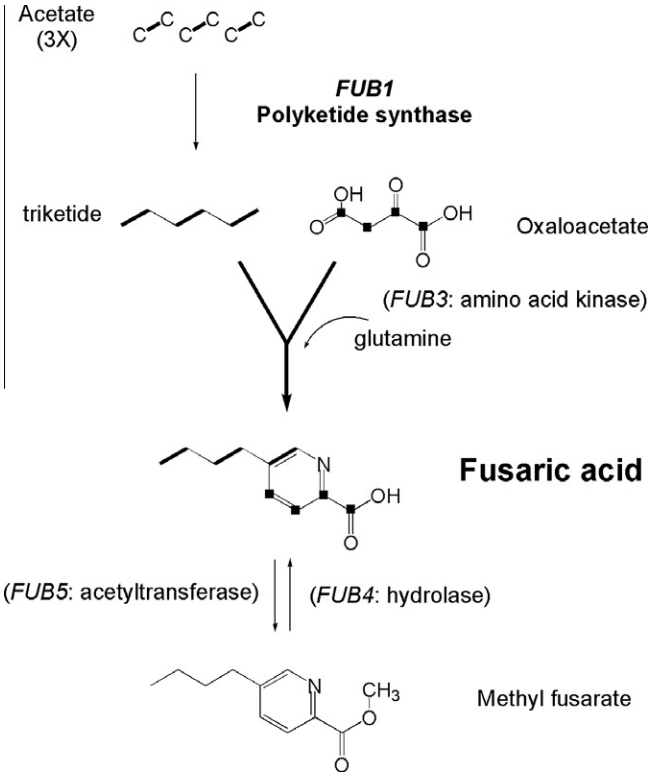

Fig. 8. A proposed biochemical pathway for fusaric acid biosynthesis in *F. verticillioides*. The assignment of *FUB1* is based on functional studies and the assigned roles for *FUB*3, *FUB4* and *FUB5* (in parenthesis) were based on BLAST analysis and biochemical considerations.

fusarate to fusaric acid and whether it serves a protective role *in planta* against the more harmful effects of methyl fusarate. In addition, the identification and inactivation of fusaric acid biosynthetic genes will allow rigorous examination of the role of fusaric acid in plant and mammalian diseases.

In early studies, researchers demonstrated the utility of microarray analysis for identifying SM biosynthetic gene clusters with mutant strains of *Aspergillus* in which production of the global regulatory protein LaeA was over expressed or was blocked by gene deletion (Bok et al., 2006; Bok and Keller, 2004). Gene clusters were identified by changes in expression in the mutants relative to the wild type. In the current study, microarray analysis of wild-type *F. verticillioides* confirmed co-expression of genes within the previously characterized *FUM* and *BIK* clusters and provided support for microarray-based identification of the *PGL*, *FUS* and *FUB* clusters by gene co-expression. The failure to observe expression of the 11 other PKSs under these conditions was not unexpected because 18 different culture conditions were required to observe expression of 14 of the 15 *F. graminearum* PKS genes. (Gaffoor et al., 2005). Additional evidence for the *PGL* cluster consists of the presence of cluster homologs in *F. verticillioides*,

F. oxysporum, and *F. graminearum*, whereas additional evidence for the *FUS* cluster consists of the presence of cluster homologs in *F. verticillioides*, *F. graminearum* and *F. solani* as well as Northern blot data showing co-expression of *FUS* genes in *F. verticillioides*. Thus, our results support the concept that SM biosynthetic gene clusters can be identified by whole genome-based microarray analysis of *Fusarium* grown in different conditions. Functional analysis by mutagenesis of all individual putative cluster genes is required to verify involvement and provide evidence for their roles in synthesis of the corresponding SMs. In the current study, we exploited differences in gene expression that occur over time in a semi-defined liquid medium that was designed to promote fumonisin production in *F. verticillioides* but also supports production of other secondary metabolites in the fungus. In future studies, whole-genome expression analysis of *Fusarium* grown under other culture conditions may provide evidence for additional SM biosynthetic gene clusters in this agriculturally important genus of filamentous fungi.

Acknowledgments

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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.2012.05.010.

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