Identification of a 12-Gene Fusaric Acid Biosynthetic Gene Cluster in *Fusarium* Species Through Comparative and Functional Genomics

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In fungi, genes involved in biosynthesis of a secondary metabolite (SM) are often located adjacent to one another in the genome and are coordinately regulated. These SM biosynthetic gene clusters typically encode enzymes, one or more transcription factors, and a transport protein. Fusaric acid is a polyketide-derived SM produced by multiple species of the fungal genus *Fusarium*. This SM is of concern because it is toxic to animals and, therefore, is considered a mycotoxin and may contribute to plant pathogenesis. Preliminary descriptions of the fusaric acid (FA) biosynthetic gene (*FUB*) cluster have been reported in two *Fusarium* species, the maize pathogen *F. verticillioides* and the rice pathogen *F. fujikuroi*. The cluster consisted of five genes and did not include a transcription factor or transporter gene. Here, analysis of the *FUB* region in *F. verticillioides*, *F. fujikuroi*, and *F. oxysporum*, a plant pathogen with multiple hosts, indicates the *FUB* cluster consists of at least 12 genes (*FUB1* to *FUB12*). Deletion analysis confirmed that nine *FUB* genes, including two Zn(II)₂Cys₆ transcription factor genes, are required for production of wild-type levels of FA. Comparisons of *FUB* cluster homologs across multiple *Fusarium* isolates and species revealed insertion of non-*FUB* genes at one or two locations in some homologs. Although the ability to produce FA contributed to the phytotoxicity of *F. oxysporum* culture extracts, lack of production did not affect virulence of *F. oxysporum* on cactus or *F. verticillioides* on maize seedlings. These findings provide new insights into the genetic and biochemical processes required for FA production.

Collectively, species of the fungal genus *Fusarium* occur as pathogens or endophytes on a wide variety of economically important plants and can cause opportunistic infections in humans and other animals (Leslie and Summerell 2006). *Fu-*

sarium species also produce over 50 distinct families of secondary metabolites (SM) that vary tremendously in chemical structure and biological activity (Desjardins 2006; Vesonder and Golinski 1989). Some *Fusarium* SM are pigments, others are plant growth regulators, and some (i.e., fumonisins, trichothecenes, and zearalenone) are among the mycotoxins of greatest concern to food and feed safety. Although some *Fusarium* SM contribute to pathogenicity, the roles of most in the ecology of *Fusarium* spp. have not yet been elucidated. Fusaric acid (5-butylpicolinic acid [FA]) is a polyketide-derived SM produced by multiple *Fusarium* species, including *Fusarium oxysporum* and multiple members of the *F. fujikuroi* species complex (FFSC) (Bacon et al. 1996).

FA is of interest because it has long been associated with plant wilt symptoms caused by infection with some *Fusarium* species and it exhibits mild levels of toxicity to animals (Bacon et al. 1996; Bouizgarne et al. 2006; D'Alton and Etherton 1984). FA is phytotoxic to cotton (Stipanovic et al. 2011a), its production by *F. oxysporum* f. sp. *cubense* is correlated with pathogenicity on banana plants (Li et al. 2013), and it induces programmed cell death in plants (Jiao et al. 2013; Samadi and Behboodi 2006). Exposure to subtoxic doses of FA can also elicit plant defense responses (Bouizgarne et al. 2006). In addition to a mild toxicity to animals, FA exhibits acanthamoebicidal (Boonman et al. 2012), antioomycete (Son et al. 2008), and antimycobacterial activities (Pan et al. 2011).

Although fungal SM exhibit tremendous diversity in chemical structure and biological activity, their biogenesis exhibits several common themes. In a majority of cases, genes responsible for synthesis of the same SM are coordinately regulated and located adjacent to one another in the genome (Keller et al. 2005; Osbourn 2010). Such biosynthetic gene clusters generally include a key gene encoding an enzyme that catalyzes assembly of the SM chemical scaffold. Typically the enzyme is a polyketide synthase (PKS), non-ribosomal peptide synthase, terpene cyclase, or prenyltransferase. A majority of other cluster genes encode enzymes that catalyze modification of the chemical scaffold, e.g., oxidation, acylation, or cyclization. Most SM biosynthetic gene clusters also encode at least one transcription factor that regulates gene expression within the cluster and a protein that transports the SM out of the cell.

A five-gene FA biosynthetic gene (*FUB*) cluster was previously identified and characterized in two FFSC species, *F. ver-*

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verticillioides, a causal agent of ear and stalk rot of maize and a frequent endophyte of maize (Brown et al. 2012), and *F. fujikuroi*, the causal agent of “bakanae” disease of rice (Niehaus et al. 2014). Orthologs of the *FUB* cluster are also present in the other FFSC species *F. circinatum* and *F. mangiferae* as well as in *F. oxysporum*, which is closely related to but not a member of FFSC (Wiemann et al. 2013). The cluster genes were predicted to encode a PKS (*FUB1*), a hypothetical protein of unknown function (*FUB2*), an amino acid kinase (*FUB3*), a hydrolase (*FUB4*), and an acyltransferase (*FUB5*). Notably absent from the *FUB* cluster were genes encoding a transcription factor or a transporter. In *F. verticillioides* and *F. fujikuroi*, the five *FUB* genes were coregulated; i.e., they exhibited similar patterns of expression (Brown et al. 2012; Niehaus et al. 2014). Deletion of *FUB1* in both *F. verticillioides* and *F. fujikuroi* abolished FA production (Brown et al. 2012; Niehaus et al. 2014). In an analysis of *F. fujikuroi*, *FUB4* deletion abolished FA production, *FUB3* and *FUB5* deletion reduced production by 20 to 40%, and *FUB2* deletion had no effect on production (Niehaus et al. 2014). Based on predicted function of the five *FUB* genes and previous biochemical studies (Stipanovic et al. 2011b), a biosynthetic pathway for FA was proposed (Brown et al. 2012). The proposed pathway begins when the *FUB1*-encoded PKS catalyzes formation of a six-carbon chain and, then, the *FUB3*-encoded amino acid kinase catalyzes linkage of the polyketide to oxaloacetate and a nitrogen atom.

After the initial descriptions of the five-gene *FUB* cluster, we examined gene expression in genomic regions flanking the cluster in *F. verticillioides* and identified a region located 14.6 kb upstream of *FUB1* that included seven contiguous genes with patterns of expression similar to those of *FUB* genes (this study). Thus, the goal of the current study was to determine whether these seven genes as well as *FUB2* through *FUB5* are required for FA production in *F. verticillioides* and *F. oxysporum*. The results provide evidence that *FUB3* through *FUB5* as well as five genes within the seven-gene region are required for wild-type FA production. These results, combined with the presence of homologs of the seven-gene region near the *FUB* cluster in *F. fujikuroi*, indicate that the *Fusarium FUB* cluster consists of at least 12 genes rather than five genes, as originally described. The results also indicate that FA production is not required for wild-type levels of virulence in *F. oxysporum* and *F. verticillioides*. Together, the results of this study

provide further insight into genetic and biochemical pathways required for FA biosynthesis and will help guide further studies to understand the role of FA in the ecology of *Fusarium* species.

RESULTS

Identification of genes with *FUB*-like expression.

Microarray-based transcriptional analysis of *F. verticillioides* genes flanking the five-gene *FUB* cluster identified seven contiguous genes (FVEG_12528 to FVEG_12534), located in a region 14.6 kb upstream from *FUB1*, that exhibited patterns of expression similar to those of *FUB* genes (Fig. 1). Namely, expression levels for the *FUB* genes and FVEG_12528 through FVEG_12534 were low at 24 h, highest at 48 h, and progressively lower at 72 and 96 h. FVEG_12528 through FVEG_12534 were predicted to encode four enzymes (two dehydrogenases, an oxidase, and a sulfhydrylase), a major facilitator superfamily (MFS) transporter, and two Zn(II)₂Cys₆ transcription factors (Table 1). All other predicted genes in the 58-kb region 5′ of *FUB1* and the 16.6-kb region 3′ of *FUB5*, corresponding to the end of supercontig 20 in the *F. verticillioides* genome sequence database, exhibited very low levels of expression at all time points examined (Fig. 1). The four predicted genes, FVEG_12524 to FVEG_12527, in the 14.6-kb region between *FUB1* and FVEG_12528 also exhibited very low levels of expression at the time points examined (Fig. 1), i.e., their patterns of expression were not the same as those of *FUB1* through *FUB5* and FVEG_12528 through FVEG_12534.

Comparative genomic analysis.

Examination of publicly available *Fusarium* genome sequences revealed that homologs of genes FVEG_12528 through FVEG_12534 are located in the same genomic region as homologs of *FUB1* through *FUB5* in *F. fujikuroi* and *F. oxysporum* (Table 2). The homologous genes share 90 to 99% deduced amino acid sequence identity in *F. verticillioides*, *F. fujikuroi*, and *F. oxysporum* (Table 3). Because of their *FUB* gene-like expression patterns in *F. verticillioides*, as well as additional evidence described below, we will, hereafter, refer to FVEG_12528 through FVEG_12534 and their homologs in other species as *FUB6* through *FUB12*, respectively (Fig. 2). In addition, in the description of the *FUB* region immediately below, we use the gene models predicted for *F. oxysporum* f. sp. *lycopersici* Fol4287 as representative of *F. oxysporum*.

Fig. 1. Microarray-based analysis of gene expression in the *FUB* cluster region in *Fusarium verticillioides* grown for 24 to 96 h in GYAM medium (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K₂HPO₄, 2 mM MgSO₄, and 8.8 mM CaCl₂, pH 3.0).

The order and orientation of the *FUB6* through *FUB12* homologs are the same in all three *Fusarium* species; however, the position of the *FUB6* through *FUB12* segment relative to the *FUB1* through *FUB5* segment differs in *F. verticillioides* compared with the other species, i.e., in *F. verticillioides*, the

FUB6 through *FUB12* segment is located 5' of *FUB1*, whereas in *F. fujikuroi* and *F. oxysporum*, it is located 3' of *FUB5*, and the entire *FUB6* through *FUB12* segment is inverted relative to *F. verticillioides* (Fig. 2). The distance between the *FUB1* through *FUB5* and *FUB6* through *FUB12* segments differs in

Table 1. Predicted functions of genes in the *Fusarium verticillioides* *FUB* cluster region

FCD designation ^s	Gene name	Predicted function ^t	BLAST E value ^u	NCBI-CDD domain model	Delete/Comp. ^v
FVEG_12535		Methyltransferase	1.1×10^{-23}	COG4122	
FVEG_12534	<i>FUB12</i>	C6 transcription factor	1.5×10^{-10}	CD12148	x
FVEG_12533	<i>FUB11</i>	MFS ^w	2.3×10^{-15}	CD06174	x/c
FVEG_12532	<i>FUB10</i>	C6 transcription factor	4.3×10^{-4}	CD00067	x/c
FVEG_12531	<i>FUB9</i>	Oxidase	9.9×10^{-85}	CD02809	(x)
FVEG_12530	<i>FUB8^x</i>	Dehydrogenase	5.3×10^{-26}	CD05235	
FVEG_12529	<i>FUB7</i>	Sulphydrylase	4.2×10^{-154}	TIGR01326	x
FVEG_12528	<i>FUB6</i>	Dehydrogenase	9.4×10^{-74}	CD08295	
FVEG_12527		Aldolase	8.2×10^{-12}	COG0235	
FVEG_12526		Lactamase	7.6×10^{-8}	SMART00849	
FVEG_12525		Dehydrogenase	2.0×10^{-36}	CD00567	
FVEG_12524		Esterase	7.0×10^{-34}	PFAM00135	
FVEG_12523	<i>FUB1</i>	PKS ^y	1.2×10^{-133}	CD00833	x
FVEG_12522	<i>FUB2</i>	Unknown	na	na	x
FVEG_12521	<i>FUB3</i>	Amino acid kinase	1.9×10^{-102}	CD04247	x
FVEG_12520	<i>FUB4</i>	Hydrolase	1.5×10^{-9}	PFAM03959	x/c
FVEG_12519	<i>FUB5</i>	Acetyltransferase	1.3×10^{-79}	COG2021	x/c
Np ^z		Unknown	na	na	
FVEG_12518		Unknown (WD40)	5.6×10^{-48}	CD00200	

^s Designations from the *Fusarium* Comparative Database (FCD) at the Broad Institute. Gene designations in bold text are coexpressed during growth on GYAM (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K₂HPO₄, 2 mM MgSO₄, and 8.8 mM CaCl₂, pH 3.0).

^t Functions listed are based on the results of BLAST analysis.

^u BLAST E value refers to the probability score, generated by BLASTX of each gene against the National Center for Biotechnology Information (NCBI) database to protein domain models in the Conserved Domain Database (CDD).

^v The x indicates that the gene was deleted and c indicates that one of the gene deletion mutants was complemented with a wild-type copy of the gene.

^w MFS = major facilitator superfamily, a group of transporters that facilitate the transport of a variety of substrates across membranes.

^x The *FUB8* predicted protein includes three domains with similarity to domains in the CDD. In addition to the dehydrogenase domain, Fub8 has a phosphopantetheine attachment site (PFAM00550, 5.1×10^{-3}) and a fatty acid CoA ligase domain (CD05956, 7.0×10^{-12}).

^y PKS = polyketide synthase. The *FUB1* predicted protein includes five domains with similarity to domains in the CDD. CD00833 corresponds to the highly conserved and signature keto synthase domain associated with PKS.

^z Np = not predicted. The putative gene was predicted based on the presence of an open reading frame.

Fig. 2. Comparison of homologs of the *FUB* cluster region in *Fusarium verticillioides*, *F. fujikuroi*, 12 strains of *F. oxysporum* and *Trichoderma atroviride*. The genes considered to be part of the cluster are represented as blue arrows (*FUB2* through *FUB12* are labeled 2 through 12). The three green arrows (labeled A to C) and the red arrow represent genes with homologs in the three *Fusarium* species but that exhibit markedly different patterns of expression than *FUB* genes, according to expression analysis in *F. verticillioides*. In *F. verticillioides*, A is 450 bp and, thus, is truncated relative to homologs in *F. fujikuroi* and *F. oxysporum*, which are approximately 1,423 bp. Black arrows represent genes unique to each species. Gray shading indicates segments within the *FUB* cluster that exhibit conservation of gene order among species.

all three species: 4.9 kb in *F. oxysporum*, 10.5 kb in *F. fujikuroi*, and 14.6 kb in *F. verticillioides*. The 4.9-kb region in *F. oxysporum* includes two predicted genes, FOXG_21865 and FOXG_15242, that do not exhibit significant similarity to the genes between the *FUB1* through *FUB5* and *FUB6* through *FUB12* segments in *F. verticillioides*. However, in *F. verticillioides*, there is a truncated homolog of FOXG_21865 (gene labeled A in Figure 2) located immediately 3' to *FUB5*. This *F. verticillioides* FOXG_21865 homolog is predicted to encode a 152-amino acid protein, which is only about 42% the length of the FOXG_21865 predicted protein. In *F. fujikuroi*, the 10.5-kb region between the *FUB1* through *FUB5* and *FUB6* through *FUB12* segments includes four predicted genes. One of these genes (FFUJ_02112) is a homolog of

FOXG_15242 (labeled B in Figure 2), and a second is a homolog of FOXG_21865 but is not annotated in the Institute of Bioinformatics and Systems Biology (IBIS) *F. fujikuroi* genome database. The position of these two *F. fujikuroi* genes in relation to *FUB5* and *FUB6* are conserved in *F. fujikuroi* and *F. oxysporum* (Fig. 2). The other two predicted *F. fujikuroi* genes located between the *FUB1* to *FUB5* and *FUB6* to *FUB12* segments are FFUJ_02110 and FFUJ_02111 and are predicted to encode two different proteins of unknown function (Fig. 2). Neither of the latter two *F. fujikuroi* genes have homologs near the *FUB* cluster in *F. oxysporum* or *F. verticillioides*. The four predicted *F. verticillioides* genes located between the *FUB1* to *FUB5* and *FUB6* to *FUB12* segments do not have homologs in the corresponding region

Table 2. Predicted functions of genes in the *Fusarium oxysporum* *FUB* cluster region

FCD designation [†]	Gene name	Proteins (aa)	Predicted function [‡]	BLAST E-value [‡]	NCBI-CDD domain model	Deletion
FOXG 21868 ^w		534	Esterase	0.0	COG2272	x
FOXG 15253		515	Permease (MFS)	2.4×10^{-30}	TIGR02332	x
FOXG 15252		899	Rhamnosidase	1.2×10^{-170}	PFAM05592	x
FOXG 15251		1,012	Unknown	na	na	
FOXG 15250		750	Glucosidase	2.9×10^{-40}	PFAM00933	x
FOXG 15249		304	Epimerase	5.1×10^{-21}	CD05234	x
FOXG 15248	<i>FUB1</i>	2,410	Polyketide synthase	9.7×10^{-135}	CD00833	x
Np ^x	<i>FUB2</i>	104	Unknown	na	na	
FOXG 15247	<i>FUB3</i>	511	Amino acid kinase	5.0×10^{-96}	CD04247	x
Np		393	HET ^y	2.3×10^{-16}	PFAM06985	
FOXG 21866 ^z		729	Peptidase	4.6×10^{-3}	CD02619	x
FOXG 15244	<i>FUB4</i>	267	Hydrolase	7.8×10^{-10}	PFAM03959	x
FOXG 15243	<i>FUB5</i>	464	Acetyltransferase	2.2×10^{-77}	COG2021	x
FOXG 21865		371	Unknown	na	na	
FOXG 15242		378	Reductase	2.5×10^{-25}	CD08249	x
FOXG 15241	<i>FUB6</i>	358	Dehydrogenase	2.6×10^{-73}	CD08295	x
FOXG 15240	<i>FUB7</i>	434	Sulfhydrylase	1.0×10^{-154}	TIGR01326	x
FOXG 15239	<i>FUB8</i>	1,059	Dehydrogenase	1.2×10^{-6}	CD0523	x
FOXG 15238	<i>FUB9</i>	349	Oxidase	0.0	CD02809	x
FOXG 15237	<i>FUB10</i>	452	C6 transcription factor	9.8×10^{-4}	CD00067	x
FOXG 15236	<i>FUB11</i>	593	MFS ^z	0.0	CD06174	
FOXG 15235	<i>FUB12</i>	723	C6 transcription factor	2.3×10^{-10}	CD12148	
FOXG 15234			C6 transcription factor	1.1×10^{-6}	CD00067	

[†] Designations from the *Fusarium* Comparative Database (FCD) at the Broad Institute.

[‡] Putative functions listed are based on the results of BLAST analysis.

[‡] BLAST E value refers to the probability score, generated by BLASTX of each gene against the National Center for Biotechnology Information (NCBI) database to protein domain models in the Conserved Domain Database (CDD).

^w Formerly FOXG_15254 and FOXG_15255.

^x Np = not predicted. The putative gene was predicted based on the presence of an open reading frame and similarity to *FvFUB2*.

^y HET = a vegetative incompatibility protein; MFS = major facilitator superfamily, a group of transporters that facilitate the transport of a variety of substrates across membranes.

^z Formerly FOXG_15245 and FOXG_15246.

Table 3. Comparison of *FUB* homologs

Gene name	FVEG_# ^w	Proteins (aa)	FOXG_#	Proteins (aa)	Fv to Fo ^x	FFUJ_#	Proteins (aa)	Fv to Ff	<i>T. atroviride</i> JGI_#	Proteins (aa)	Fv to Ta
<i>FUB1</i>	12523	2,409	15248	2,410	94.7	02105	2,410	93.7	211357	2,422	55.6
<i>FUB2</i>	12522	109	Ng ^y	104	96.2	02106	104	98.1	Nh ^z	—	—
<i>FUB3</i>	12521	510	15247	511	96.3	02107	510	95.3	Nh	—	—
<i>FUB4</i>	12520	267	15244	267	97.0	02108	267	97.0	314350	271	59.2
<i>FUB5</i>	12519	463	15243	464	90.4	02109	427	92.6	260926	404	62.4
<i>FUB6</i>	12528	357	15241	358	96.6	02113	337	96.7	53082	356	72.2
<i>FUB7</i>	12529	433	15240	434	99.1	02114	433	98.9	93352	434	74.8
<i>FUB8</i>	12530	1,036	15239	1,059	92.3	02115	1,036	91.1	52932	1,067	54.6
<i>FUB9</i>	12531	387	15238	349	96.6	02116	358	97.2	314346	367	67.2
<i>FUB10</i>	12532	421	15237	453	97.8	02117	419	97.4	314352	378	39.8
<i>FUB11</i>	12533	593	15236	594	97.8	02118	574	96.3	53012	547	49.1
<i>FUB12</i>	12534	661	15235	662	96.1	02119	661	96.7	211358	672	46.2

^w Designations for the *FUB* genes at the *Fusarium* Comparative Database at the Broad Institute for *F. verticillioides* (Fv) and *F. oxysporum* (Fo), at the Munich Information Center for Protein Sequences for *F. fujikuroi* (Ff), and the Joint Genome Institute (JGI) for *Trichoderma atroviride* (Ta).

^x Fv to Fo, Fv to Ff, and Fv to Ta refers to the percent amino acid identity shared between the *F. verticillioides* predicted proteins and the *F. oxysporum*, *F. fujikuroi*, and *T. atroviride* homologous proteins respectively.

^y Ng = No gene model available.

^z Nh = No homolog in cluster.

in either *F. fujikuroi* or *F. oxysporum*. However, the gene (FVEG_12524) immediately 5' to *FUB1* in *F. verticillioide*s (illustrated in red in Figure 2) has a homolog (FFUJ_02103) immediately 5' to *FUB1* in *F. fujikuroi* (Fig. 2).

BLASTX analysis of the 12 *F. verticillioide*s *FUB* homologs against the National Center for Biotechnology Information (NCBI) NR database revealed homologs of *FUB1* and *FUB4* through *FUB12* in the genome sequence of *Trichoderma atroviride* (Fig. 2). The presence of a *FUB1* homolog in *T. atroviride* is consistent with a recent phylogenomic analysis of PKS-encoding genes in *Trichoderma* spp. (Baker et al. 2012). Six of the *T. atroviride* *FUB* homologs (*FUB4* through *FUB9*) are organized in the same order and orientation as in *F. fujikuroi* and *F. oxysporum*, albeit without additional genes between *FUB5* and *FUB6*. Predicted proteins encoded by *T. atroviride* *FUB1* and *FUB4* to *FUB12* exhibit 40 to 75% deduced amino acid sequence identity to their respective *Fusarium* homologs (Table 3). The genes flanking either side of the *T. atroviride* *FUB* regions exhibit no significant identity to the genes on either side of the *FUB* region in *Fusarium* spp. Although a homolog of *FUB3* is not present in the *T. atroviride* *FUB*

region, a predicted gene (TRIATDRAFT_129821) with 53.5% deduced amino acid sequence identity to *Fusarium FUB3* homologs is located 717 kb downstream of *FUB1* in the *T. atroviride* genome sequence. However, phylogenetic analysis using the deduced amino acid sequence of TRIATDRAFT_129821 suggests it did not originate in the *FUB* cluster, because the sequence is more closely related to aspartate kinase-like proteins in *Fusarium* and *Trichoderma* spp. that are not encoded in the *FUB* cluster than it is to *Fusarium FUB3* homologs (Supplementary Fig. 2; Supplementary Table 3). BLAST analysis did not identify a *FUB2* homolog in the *T. atroviride* genome nor any *FUB* homologs in the five other *Trichoderma* genome sequences in the Joint Genome Institute (JGI) and NCBI databases.

Functional analysis of *FUB* genes in *F. verticillioide*s.

For deletion analysis of *FUB2* through *FUB12* in *F. verticillioide*s, 1,111 hygromycin-resistant transformants were screened by polymerase chain reaction (PCR) to determine whether the coding regions of *FUB2* through *FUB12* had been deleted (Supplementary Table 4). No deletion mutants were recovered for

Table 4. Analysis of fusaric acid (FA) production in strains of *Fusarium verticillioide*s and *F. oxysporum* examined in this study

Genes	<i>Fusarium verticillioide</i> s		<i>Fusarium oxysporum</i>	
	Strains ^a	FA production (% wild type) ^y	Strains	FA production (% wild type) ^w
FOXG_21868	nh ^x		Δ21868	130
FOXG_15253	nh		Δ15253	nt
FOXG_15252	nh		Δ15252	68
FOXG_15251	nh		na ^y	
FOXG_15250	nh		Δ15250	56
FOXG_15249	nh		Δ15249	66
<i>FUB1</i>	ΔFvFub1.1	0	ΔFoFub1.5	5
	ΔFvFub1.5	0.7	na	
<i>FUB2</i>	ΔFvFub2.2	115	na	
	ΔFvFub2.3	85	na	
<i>FUB3</i>	ΔFvFub3.4	13	ΔFoFub3.9	0
	ΔFvFub3.11	7	ΔFoFub3.22	0
FOXG_21866	nh ^x		Δ21866	66
<i>FUB4</i>	ΔFvFub4.9	0	ΔFoFub4.1	nt
	ΔFvFub4.43	0	ΔFoFub4.6	nt
	ΔFvFub4.9AB8	163	na	
<i>FUB5</i>	ΔFvFub5.1	8	ΔFoFub5.9	nt
	ΔFvFub5.3	4	ΔFoFub5.12	nt
	ΔFvFub5.1AB8	88	na	
<i>FUB6</i>	nh		Δ15242	99
	na		ΔFoFub6.7	0
<i>FUB7</i>	na		ΔfoFub6.2	nt
	na		ΔFoFub7.7	56
<i>FUB8</i>	na		ΔFoFub7.5	nt
	na		ΔFoFub8.30	0
<i>FUB6</i> to <i>FUB9</i>	ΔFvFub69.2	0	ΔFoFub8.32	0
	ΔFvFub69.12	0	na	
<i>FUB9</i>	ΔFvFub9.29	80	na	
<i>FUB10</i>	ΔFvFub10.2	0	ΔFoFub9.9	nt
	ΔFvFub10.5	0	ΔFoFub10.1	0
	ΔFvFub10.5AB815	389 ^z	ΔFoFub10.3	0
<i>FUB11</i>	ΔFvFub11.6	0	na	
	ΔFvFub11.8	10	na	
	ΔFvFub11.8AB4	118	na	
<i>FUB12</i>	ΔFvFub12.54	18	na	
	ΔFvFub12.60	31	na	

^a The Δ symbol in a strain designation indicates that the strain has a deleted gene; AB indicates the deleted gene has been complemented.

^y FA production reported is relative to wild type, by percent. Not all mutants were examined for FA production at the same time. For mutants of *FUB1* to *FUB5* and *FUB10* to *FUB12*, the mean level of FA production for the wild type was 19.2 ± 5.8 μg/ml. For mutants of *FUB9* and the 4-*FUB* gene mutants, the mean level of FA production for the wild type was 30.1 ± 2.6 μg/ml. For mutants of *FUB10* and *FUB11*, the mean level of FA production for the wild type was 6.6 ± 0.5 μg/ml.

^w FA production reported is relative to wild type, by percent. All mutants were examined for FA production at the same time. The mean level of FA production for the wild type was 13.1 ± 3.1 μg/ml. nt = not tested.

^x nh = no homolog present

^y na = deletion mutant not available.

^z Three other independent *FUB10*-complemented strains, along with ΔFvFub10.5AB815, produced, on average, over 350% more FA than M-3125.

FUB6, *FUB7*, or *FUB8*, even though, collectively, 321 transformants were screened during attempts to individually delete these genes. In contrast, a screen of 140 transformants recovered following transformation with a DNA construct that simultaneously targeted *FUB6*, *FUB7*, *FUB8*, and *FUB9* identified two mutants (*fub6-9* mutants) in which the four genes were deleted. In total, 23 *FUB*-deletion mutants were identified. All transformants exhibited wild-type growth and conidiation on GYAM medium (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K₂HPO₄, 2 mM MgSO₄, and 8.8 mM CaCl₂, pH 3.0), maize kernels, V-8 juice agar, and yeast extract-peptone-glucose (YPG) agar. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of mutants indicated that FA production was abolished in *fub4*, *fub10*, and *fub6-9* mutants, was reduced by 76 to 95% relative to wild type in *fub3*, *fub5*, *fub11*, and *fub12* mutants, and was unaltered in *fub2* and *fub9* mutants (Table 4).

For complementation analysis of selected *fub4*, *fub5*, *fub10*, and *fub11* mutants, geneticin-resistant transformants were screened for the presence of the *FUB4*, *FUB5*, *FUB10*, and *FUB11* coding sequences, respectively. LC-MS/MS analysis of culture extracts revealed that the *FUB4*, *FUB5*, and *FUB11* complementation strains produced FA at levels that were 163, 88, and 118%, respectively, of those produced by the wild type (Table 4). In contrast, the *FUB10* complementation strain produced FA at levels that were over 350% of those produced by the wild type (Table 4).

Functional analysis of *FUB* genes in *F. oxysporum*.

For deletion analysis of genes in the *F. oxysporum* *FUB* cluster region, 10 to 30 transformants per transformation were arbitrarily selected for PCR analysis to determine if the target gene was replaced with the geneticin resistance gene (*GenR*) (data not shown). A total of 16 predicted genes were deleted by double homologous recombination. All 59 transformants, both deletion mutants and mutants in which the transforming DNA inserted ectopically in the genome, exhibited wild-type growth and conidiation on Czapek-Dox broth (CDB), minimal medium (MM), and YPG agar and liquid media. High performance liquid chromatography (HPLC) analysis of 3-week-old CDB culture extracts of mutants indicated no FA was present in extracts of the *fub3*, *fub6*, *fub8*, and *fub10* mutants, while minimal FA (5% of wild type) was observed in the *fub1* mutant. In contrast, significant levels of FA (56% of wild type) were observed in extracts of the *fub7* mutant. FA production by the *fub4*, *fub5*, and *fub9* mutants was not examined (Table 4). Deletion mutants of FOXG_15242 (located between *FUB5* and *FUB6*) and FOXG_21866 (located between *FUB3* and *FUB4*), and individual deletion mutants of the five genes immediately upstream of *FUB1* produced FA at levels ranging from 56 to 130% of wild-type levels.

Pathogenicity of *fub* mutants.

To assess the effect of FA production on pathogenicity, we examined severity of symptoms induced by *F. oxysporum* in an assay with the three-angled cactus and by *F. verticillioide*s in a maize seedling assay. In the cactus assay, the severity of symp-

toms induced by *F. oxysporum* *fub10* mutants was not consistently reduced compared with wild type and, in some cases, significant differences in severity were observed between the two mutants (Table 5; Fig. 3). In contrast, culture filtrates of the *fub10* mutants were significantly less phytotoxic than filtrates from the wild-type progenitor. These results indicate that FA production is not required for wild-type levels of virulence of *F. oxysporum* on three-angled cactus under the conditions examined but that the ability to produce FA can contribute to phytotoxicity of *F. oxysporum* culture filtrates. The disease symptoms induced by inoculation with living *F. oxysporum* *fub10* mutants suggest that *F. oxysporum* produces other, nonsecreted virulence factors that are sufficient to cause disease. In the maize seedling assay, disease severity induced by *fub1* and *fub10* mutants of *F. verticillioide*s did not differ significantly from their wild-type FA-producing progenitor (Supplementary Table 5).

FUB10 and gene expression.

The complete loss of FA production in the *F. verticillioide*s and *F. oxysporum* *fub10* mutants coupled with the similarity of the predicted *FUB10* protein (Fub10) to Zn(II)₂Cys₆ transcriptional regulators suggest that Fub10 regulates expression of other *FUB* genes. To examine the effect of *FUB10* on *FUB* gene expression, we performed quantitative (q)PCR analysis of multiple *F. verticillioide*s and *F. oxysporum* genes in the *fub10* deletion mutants and wild type.

In *F. verticillioide*s, expression of *FUB1*, *FUB3*, *FUB8*, and *FUB10* was assessed in *fub10* deletion mutants ΔFvFub10.2 and ΔFvFub10.5, the *FUB10* complementation strain ΔFvFub10.5AB815, and the wild-type progenitor strain (Table 6). In the wild-type strain, abundant transcripts of all four *FUB* genes were detected, a result that is consistent with microarray results (Fig. 1). By contrast, in the *fum10* deletion mutants, transcript levels for all four genes were minimal or undetectable. In the *FUB10* complementation strain, *FUB10* transcript levels were over threefold greater than in the wild type, and *FUB1*, *FUB3*, and *FUB8* transcript levels were approximately 23-fold greater than in the wild type (Table 6). These data are consistent with Fub10 being a transcription factor that positively regulates transcription of genes in the *FUB* cluster. In addition, the high levels of *FUB* gene transcripts in the *FUB10* complementation strain ΔFvFub10.5AB815 are consistent with the greater-than-wild-type levels of FA production for this and other *FUB10* complementation strains (Table 3).

The greater FA production and *FUB* gene transcription in the *F. verticillioide*s *FUB10* complementation strain compared with the wild type could be due to higher levels of transcription from a single copy of *FUB10* resulting from the presence of multiple copies of *FUB10* in complementation strain ΔFvFub10.5AB815 or from some other mechanism. qPCR analysis with *FUB10*-specific primers of genomic DNA from the wild-type progenitor, *fub10* deletion mutants, and the *FUB10* complementation strain revealed that the complementation strain contained 3.5-fold more *FUB10* DNA than the wild type (Table 7). No *FUB10* DNA was detected in the *fub10* deletion mutants. Thus, the high levels of FA production and of *FUB* transcripts in the *FUB10* complementation strain

Table 5. Pathogenicity of the selected *Fusarium oxysporum* transformants on cross sections of three-angled cactus shoots

Fungal strain	Symptom severity			
	Culture filtrate	Spore suspension (1 × 10 ⁴ /ml)	Spore suspension (1 × 10 ⁶ /ml)	Agar block
Foxy20 (wild type)	3.0 ± 0.0 a ^z	2.5 ± 0.5 a	4.5 ± 0.5 a	4.0 ± 0.0 a
ΔFoFub10.1	1.5 ± 0.5 c	1.5 ± 0.5 b	4.5 ± 0.5 a	3.2 ± 1.0 b
ΔFoFub1.1	2.5 ± 0.5 b	2.0 ± 0.6 ab	3.7 ± 0.8 b	4.0 ± 0.0 a
Control (CDB only)	0.0 ± 0.0 d			

^z The same letters in each column denote no significant difference at *P* < 0.05 by Duncan's multiple range test.

are likely due to the presence of multiple copies of *FUB10* in the genome of the complementation strain compared with the single copy of the gene in wild-type *F. verticillioides*.

In *F. oxysporum*, qPCR was conducted to assess expression of *FUB1*, *FUB3* through *FUB10*, FOXG_15242, FOXG_21866, FOXG_15249 through FOXG_15253, and FOXB_21868 in the *fub10* deletion mutant Δ15237 and the wild-type progenitor strain Foxy20 (Fig. 4). All nine *FUB* genes examined were

expressed more on CDB than in MM at all time points in the wild type, while none were expressed in the *fub10* deletion mutant (Fig. 4). These data are consistent with FA production by the wild type in CDB medium and no production in MM medium over the time course and further support the hypothesis that *FUB10* is a transcription factor that positively regulates transcription of *FUB* genes. In contrast, the expression of FOXG_15242 (located between *FUB5* and *FUB6*), FOXG_

Fig. 3. Pathogenicity of *Fusarium oxysporum* strains on three-angled cactus (*Hylocereus trigonus*). Three 0.5-cm-thick cross stem sections of the cactus were placed on moistened Whatman No. 2 filter papers in petri dishes. The central stem portions were treated with 0.1 ml of the Czapek-Dox broth (CDB) culture filtrate, 0.1 ml of spore suspensions with 10⁴ or 10⁶ spores per milliliter, or an agar block excised from a potato dextrose agar culture of the *fub1* mutant, *fub10* mutant, or their wild-type progenitor strain Foxy20, were incubated for 4 days and, then, were assessed for disease symptoms.

Table 6. *FUB* gene expression in *Fusarium verticillioides* *FUB10* deletion mutants and complementation strains by quantitative polymerase chain reaction

Strains ^w	Gene ^x			
	<i>FUB1</i>	<i>FUB3</i>	<i>FUB8</i>	<i>FUB10</i>
Wild type	608 a ^y	393 a	454 a	261 a
<i>FUB10</i> deletion ^z	0.6 c	1.1 c	0.5 c	0.0 c
<i>FUB10</i> complementation	12,980 b	11,610 b	8,260 b	831 b

^w Wild type refers to *F. verticillioides* strain M-3125, the parent strain of the *FvFUB10* deletion mutants. Deletion refers to *FUB10* deletion mutants ΔFvFub10.2 and ΔFvFub10.5 and complemented refers to *FUB10* complemented mutant ΔFvFub10.5AB815.

^x Quantity of transcript in culture per gene in nanograms per microliter.

^y Strain means for each gene (within a column) followed by different letters are significantly different based on differences of least squares means at *P* < 0.05.

^z The deletion strain was not included in the analysis for *FUB10* since the mean and variance were both zero and thus is automatically significantly lower than complemented mutant and wild type at *P* < 0.05.

21866 (located between *FUB3* and *FUB4*), and the six genes downstream of *FUB1* (FOXG_15249 through FOXG_15253 and FOXB_21868) were expressed both in CDB and MM in the wild type and in the *fub10* deletion mutant over time (data not shown). Although *FUB* gene expression did not generally increase over time, FA production was first detected at day six and increased linearly to day 21 (Supplementary Fig. 3). Consistent with transcript analysis, no FA was observed at any time point in the *fub10* deletion mutant.

DISCUSSION

Previous molecular genetic studies of *F. verticillioides* and *F. fujikuroi* provided evidence for a five-gene *FUB* cluster

(Brown et al. 2012; Niehaus et al. 2014). In the current study, analyses of cluster flanking regions provide evidence that the *Fusarium FUB* cluster consists of 12 genes, including the five previously described *FUB* genes. The evidence for this larger *FUB* cluster is that the 12 genes exhibit similar patterns of expression, their order and orientation is largely conserved among the three *Fusarium* species examined, and nine of them are required for wild-type FA production in one or both *F. oxysporum* and *F. verticillioides*. Furthermore, there is a 10-gene homolog of the *Fusarium FUB* cluster in the *T. atroviride* genome (Fig. 2).

The previously described five-gene *FUB* cluster did not include genes predicted to encode a transporter or transcription factor, two types of proteins frequently encoded by SM bio-

Table 7. Relative amount of *FUB10* coding DNA in *FUB10* complementation mutant genome by quantitative polymerase chain reaction (PCR)

Strains	Average Ct values ^u			Mean ^v	ΔCt^w	$\Delta\Delta\text{Ct}^x$	Fold difference ^y
	<i>FUB10</i>	<i>FUB8</i>	<i>CYP2</i>				
<i>FUB10</i> complementation ^z	14.95	17.11	16.2	16.65	−1.7	−1.81	3.5
Wild type	17.65	18.04	17.05	17.54	0.11		

^u Average threshold cycle (Ct) was calculated from three replica PCR reactions for each DNA.
^v Geometric mean of the two control genes *FUB8* and *CYP2* was calculated by multiplying the respective Ct values and taking the square root.
^w $\Delta\text{Ct} = \text{FUB10 Ct (per strain)} - \text{mean Ct}$.
^x $\Delta\Delta\text{Ct} = \Delta\text{Ct for FUB10 in } \Delta\text{FvFub10.5AB815} - \Delta\text{Ct for FUB10 in wild-type}$.
^y Fold difference (FD) more *FUB10* DNA in *FUB10* complementation mutant $\Delta\text{FvFub10.5AB815}$ as compared with the wild-type strain M-3125 was calculated using the formula $\text{FD} = 2^{-\Delta\Delta\text{Ct}}$.
^z *FUB10* complementation mutant = $\Delta\text{FvFub10.5AB815}$.

Fig. 4. Relative expression of nine *Fusarium oxysporum FUB* genes *FUB1*, *FUB3*, *FUB4*, *FUB5*, *FUB6*, *FUB7*, *FUB8*, *FUB9*, and *FUB10* under fusaric acid producing (Czapek-Dox broth [CDB]) and nonproducing conditions (minimal medium [MM]) over 18 days in wild-type strain Foxy20 and *fub10* deletion mutant $\Delta 15237$. The standard errors are indicated on top of each histogram bar.

synthetic gene clusters. The larger *FUB* cluster described in the current study includes a gene, *FUB11*, predicted to encode an MFS transporter and two genes, *FUB10* and *FUB12*, predicted to encode Zn(II)₂Cys₆ transcription factors. In addition to the Zn(II)₂Cys₆ binuclear cluster DNA-binding domain, *Fub12* is predicted to contain a fungal transcription factor regulatory domain. Thus, in addition to biosynthetic enzymes, the larger *FUB* cluster is predicted to encode proteins that could be involved in transport of FA across the cell membrane (*Fub11*) and regulation of *FUB* gene expression (*Fub10* and *Fub12*). The lack of FA production in *fub10* mutants and the reduced expression of multiple *FUB* genes in *fub10* mutants of both *F. oxysporum* and *F. verticillioides* suggest that *Fub10* is a FA pathway-specific transcriptional regulator. Although the deletion of the other *FUB* cluster transcription factor gene (*FUB12*) did not abolish FA production in *F. verticillioides*, the substantially reduced levels of FA in cultures of *fub12* mutants indicate that *FUB12* likely contributes to regulation of FA production.

The 12-gene, *Fusarium FUB* cluster is unusual in that it consists of segments that are separated from one another by a 4.9- to 14.6-kb region with putative non-*FUB* genes (Fig. 2). The evidence that these putative non-*FUB* genes do not contribute to FA production is i) they are not conserved in the three FA-producing fusaria examined, ii) they are absent in the *FUB* cluster homolog in *T. atroviride*, iii) in both *F. oxysporum* and *F. verticillioides*, the putative non-*FUB* genes that were examined exhibited patterns of expression that differed from those of *FUB* cluster genes, and iv) deletion of one of them (*FOXG_15242*) in *F. oxysporum* did not affect FA production. Because of the presence of the non-*FUB* genes within the *FUB* cluster, the *Fusarium* cluster could also be viewed as two or three smaller clusters located near one another.

The presence of two non-*FUB* genes in the *FUB3-FUB4* intergenic region of only some strains of *F. oxysporum* and their absence in the corresponding region of *F. fujikuroi* and *F. verticillioides* suggests that this non-*FUB* segment arose in *F. oxysporum* after it diverged from the FFSC. In contrast, the non-*FUB* segment in the *FUB5-FUB6* intergenic region is present in all strains of *F. oxysporum* examined and in *F. fujikuroi*, although in the latter species it includes two non-*FUB* genes that are not in *F. oxysporum*. This suggests that the non-*FUB* segment between *FUB5* and *FUB6* arose earlier than the non-*FUB* segment between *FUB3* and *FUB4*. We are currently examining the *FUB* cluster in a larger diversity of FA-producing fusaria to gain further insight into the origins of the non-*FUB* segments.

The organization of SM biosynthetic genes into multiple segments, rather than a cluster of contiguous genes dedicated to the same biosynthetic pathway, has been observed in other fungi. In *Neotyphodium lolii*, 10 *lol* genes involved in biosynthesis of the indole-diterpenes lolitrems are located in three segments separated from one another by 16- or 35-kb AT-rich retrotransposon relics (Young et al. 2006). The interruption of the *Fusarium FUB* cluster differs from the interruption of the *N. lolii lol* cluster in that the non-*FUB* sequences in *FUB*-gene intergenic regions have no apparent relationship to transposons but, instead, are predicted to encode metabolic or regulatory proteins (e.g., peptidase, dehydrogenase, HET protein). In some *Fusarium* species, trichothecene biosynthetic (*TRI*) genes are organized into three segments: a one-gene, a one- or two-gene, and a 12- or 14-gene segment. However, the *TRI*-gene segments differ from the *FUB*-gene segments in that they are distinct loci, and in *F. graminearum*, each of these *TRI* loci is on a different chromosome. Why the *Fusarium FUB* cluster is interrupted by one or two segments of non-*FUB* genes and whether this arrangement provides an advantage is not clear.

We are analyzing the *FUB* cluster in a phylogenetically wider range of fusaria and examining the role of FA production in the ecology of these fungi to address these issues.

In *F. verticillioides*, the *FUB1* to *FUB5* and *FUB6* to *FUB12* segments of the *FUB* cluster have undergone translocation and inversion relative to one another when compared with the homologous segments in *F. fujikuroi* and *F. oxysporum* (Fig. 2). A similar type of rearrangement of genes has also occurred in the *TRI* cluster during the evolutionary history of trichothecene-producing fusaria (Proctor et al. 2009). That is, in some species, a three-gene segment of the *TRI* cluster was inverted and translocated from one end of the cluster to the other. The relatively close phylogenetic relationship of *F. verticillioides* and *F. fujikuroi* (O'Donnell et al. 2013) indicates that the translocation within the *FUB* cluster occurred relatively recently.

Gene-function analyses done in *F. verticillioides* (current study) and *F. fujikuroi* (Neihaus et al. 2014) indicate that *FUB4* was essential for FA production, whereas *FUB3* and *FUB5* are not essential but were required for wild-type levels of FA production. In contrast, in a similar analysis done with *F. oxysporum*, *FUB3* was essential for production. The production of some FA by *F. verticillioides* and *F. fujikuroi fub3* or *fub5* mutants indicate that either the reaction catalyzed by these enzymes, a putative amino acid kinase and acetyltransferase, respectively, can occur spontaneously or that products of other genes can carry out the reaction, albeit at a lower efficiency. Why FA production was completely blocked in the *F. oxysporum fub3* mutants is unclear. This result suggests, however, that if *F. verticillioides* and *F. fujikuroi* have a gene located outside the *FUB* cluster that can compensate for *FUB3* deletion, a homolog of this noncluster gene is either absent or regulated differently in *F. oxysporum*.

In the current study, functional analysis of *FUB6* through *FUB12* in *F. verticillioides* and *F. oxysporum* revealed that five of these genes play a role in FA synthesis. Surprisingly, both putative Zn(II)₂Cys₆ transcription factor genes (*FUB10* and *FUB12*) appear to be involved; *FUB10* is essential for FA production, whereas *FUB12* is not essential but, instead, is required for wild-type levels of production (Table 4). qPCR analysis indicated that *FUB10* is required for transcription of other *FUB* genes and, thus, appears to be a FA pathway-specific regulator (Table 6; Fig. 4). How *FUB12* affects FA synthesis is not known, but our current hypothesis is that it modulates *FUB* gene transcription in response to levels of exogenous FA. Preliminary evidence in support of this hypothesis includes a rapid increase in expression of *FUB12* and the transporter gene, *FUB11*, in response to the addition of FA to cultures of wild-type *F. verticillioides* (D. W. Brown, unpublished) and the 95% reduction in FA production in *fub11* mutants (Table 4).

A specific role for *FUB3*, *FUB4*, *FUB5*, *FUB6*, and *FUB8* in synthesis of FA could not be assigned because no obvious FA intermediates were observed in culture extracts of the corresponding *fub* mutants (Fig. 5), and the biochemical mechanism by which FA is formed from a polyketide precursor remains unclear. Furthermore, the role of *FUB2*, *FUB7*, and *FUB9*, which encode a small protein of unknown function, a sulfhydrylase, and an oxidase, respectively, in FA synthesis is even less clear, because their deletion did not affect FA production. The presence of SM cluster genes that do not appear to be required for synthesis of the corresponding SM under laboratory conditions has been previously observed. For example, *TRI14* is a gene located in the trichothecene biosynthetic gene cluster of trichothecene-producing fusaria and is not required for trichothecene production in culture but appears to play a role in planta (Dyer et al. 2005). In addition, multiple genes in the aflatoxin biosynthetic gene cluster in *Aspergillus flavus* have not yet been assigned a role in synthesis (Yu 2012).

Although FA production has not been reported in *T. atroviride* to our knowledge, the presence and clustering of 10 *FUB* gene homologs in this fungus indicate that it has the genetic potential to produce FA or an FA-like metabolite. The largely syntenic organization of *FUB* genes in the *Fusarium* and *T. atroviride* clusters suggests that the separation of the *FUB* genes into two or three segments in the *Fusarium* cluster occurred after the *Fusarium* and *Trichoderma* clusters diverged from one another.

The knowledge that *FUB6*, *FUB8*, and *FUB10* are essential for FA production could aid in development of novel strategies to limit FA production. Although FA production is not required by *Fusarium* spp. for pathogenesis on maize seedling or cactus, production is required for phytotoxicity of *F. oxysporum* culture extracts on cactus (this study). In addition, FA is phytotoxic to cotton (Stipanovic et al. 2011a) and has been shown to negatively affect a variety of microorganisms (Boonman et al. 2012; Pan et al. 2011; Son et al. 2008). The identification of the *FUB* cluster and the creation of *FUB* gene deletion and complementation strains will facilitate future studies aimed at understanding the biochemical pathway required for FA formation as well as the impact of FA on plant-parasite interactions and plant-associated microbial communities. Our results highlight the contribution and limitations of an integrated approach of both functional (gene deletion, pathogenicity, and plant toxicity) and genomic (transcriptomic and comparative genetic) analyses to understanding the production and function of SM in fungi.

MATERIALS AND METHODS

Strains and media.

The wild-type strain of *F. verticillioides* used in this study was M-3125 (NRRL 13447) (Leslie et al. 1992). The wild-type strain of *F. oxysporum* was Foxy20 (KACC 47718) (Lee et al. 2013). Wild-type *F. verticillioides* RNA was prepared from cultures grown in GYAM medium for 24, 48, 72, or 96 h. RNA of *F. verticillioides* mutant strains was prepared from

cultures grown in GYAM for 48 h. RNA of *F. oxysporum* was prepared from cultures grown in CDB (MBCell, Seoul, Korea) and MM (1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of KCl, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g of sucrose, 2 g of NaNO_3 , and 0.2 ml of trace elements) (Leslie and Summerell 2006) for 3, 6, 9, 12, 15, and 18 days, in triplicate. FA 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 corn kernels and 1.2 ml of distilled water in a 4-dram glass vial. Each vial was inoculated with a single 3-mm² mycelial plug taken from a V-8 juice agar (Tuite 1969) culture of *F. verticillioides*. FA production by *F. oxysporum* was determined from CDB shake cultures (25 ml of CDB in 50-ml tubes incubated at 150 rpm at 25°C) inoculated with seven or eight 1-mm² mycelia plugs from a culture grown on potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD, U.S.A.) or CDB stationary cultures (20 ml of CDB in 90-mm petri dishes at 25°C) inoculated with 1×10^6 conidia per milliliter. Conidia were obtained by filtering a CMC (15 g of carboxymethylcellulose, 1 g of yeast extract, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of NH_4NO_3 , 1 g of KH_2PO_4) shake culture incubated at 150 rpm at 25°C for 3 days through sterile Miracloth (Calbiochem).

Gene identification.

Possible functions of proteins encoded by the predicted genes flanking the five-gene *FUB* cluster were assigned based on BLAST searches of the nonredundant sequence databases, using default parameters at the NCBI (Table 1) (Altschul et al. 1997). *F. verticillioides* and *F. oxysporum* nucleotide sequences were retrieved from the *Fusarium* Comparative database at the Broad Institute. *F. fujikuroi* nucleotide sequences were retrieved from the IBIS database (Wiemann et al. 2013). Transcript sequences were retrieved from the *F. verticillioides* Gene Index database at the Dana-Farber Cancer Institute (Brown et al. 2005). *T. atroviride* nucleotide sequences were retrieved from the JGI database. For each species, genomic DNA and coding region sequences recovered from a database were assembled with Sequencher (version 4.10.1; Gene Codes Corp., Ann Arbor, MI, U.S.A.) and DNASTAR Lasergene (version 7.1; DNASTAR, Madison, WI, U.S.A.) to confirm the positions of predicted start and stop codons as well as intron splice sites. These positions were modified manually in cases in which a gene model predicted by automatic annotation was deemed unlikely, based on cDNA sequences from the *F. verticillioides* Gene Index or from comparisons of sequences from different species.

DNA preparation and PCR amplification.

F. verticillioides genomic DNA used as template for PCR analysis was prepared using standard protocols (Sambrook et al. 2001) with minor modifications. Mycelia were scraped from one-week-old V-8 juice agar plates, were placed in 250 µl of DNA extraction buffer (200 mM Tris, pH 8.0, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid, 100 mg of 0.5-mm glass beads [Scientific Industries, Inc., Bohemia, NY, U.S.A.] and 0.5% sodium dodecyl sulfate), were vortexed for 5 min, and were incubated for 10 min at 65°C. Phenol/chloroform/isoamyl alcohol (25:24:1; 250 µl) was added 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, U.S.A.). *F. oxysporum* DNA was prepared as described previously (Leslie and Summerell 2006).

PCR was performed with a total reaction volume of 10 µl (5 µl of Promega GoTaq master mix [Madison, WI, U.S.A.], 1 µl of each primer [10 µM], and 50 pg of genomic DNA) in a MJ Research PTC-100 PCR thermocycler (Waltham, MA,

Fig. 5. Proposed biosynthetic pathway for fusaric acid biosynthesis in *Fusarium* species.

U.S.A.), 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.

Microarray analysis.

Microarrays were designed and produced by Roche NimbleGen (Madison, WI, U.S.A.) based on 12,414 gene models obtained from the Broad Institute and 875 unique expressed sequence tags from the *F. verticillioides* Gene Index. Each sequence was represented on the array by up to 12 unique 60-mer probes. 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, CA, U.S.A.). The GYAM experiment was conducted with three biological replicates for the wild-type strain after 24, 48, 72, and 96 h. The array platform and experimental data set is available at Gene Expression Omnibus under accession number GSE16900.

Gene deletion.

Inactivation of *F. verticillioides* genes was accomplished by deletion of the predicted coding region of each gene via the split-marker and protoplast transformation methods with the hygromycin-resistance gene (*HygB*) as the selectable marker (Catlett et al. 2002; Proctor et al. 1999), essentially as described previously (Brown et al. 2012). The split-marker deletion constructs were generated by first amplifying two approximately 800-bp fragments: X-upstream and X-downstream, which corresponded to the approximately 800-bp region immediately upstream and downstream, respectively, of the coding region of the targeted gene. For each fragment, “X” refers to one or more *FUB* gene designations, e.g., *FUB5*. The template for these PCR amplifications was genomic DNA from the wild-type strain of *F. verticillioides*. Next, two approximately 1,700-bp overlapping fragments, corresponding to the 5′ two thirds (*Hyg-5′*) and 3′ two thirds (*Hyg-3′*) of *HygB*, were amplified from plasmid pHygAsc (Brown et al. 2002). *Hyg-5′* was amplified with primers M13FL and HY, while *Hyg-3′* was amplified with primers M13RL and YG. Amplicons X-upstream and *Hyg-3′* were then fused by PCR, using nested primers XFN and HYN. Likewise, amplicons X-downstream and *Hyg-5′* were fused using nested primers XFN and YGN. Simultaneous deletion of *FUB6*, *FUB7*, *FUB8*, and *FUB9* was also accomplished by the split-marker method, by first generating amplicons *Fub6-9A* and *Fub6-9B*, which corresponded to the 800-bp region immediately downstream of the predicted coding region of *FUB6* and *FUB9*, respectively. All primers are listed in Supplementary Table 1.

Replacement of one or more of the target genes with *HygB* occurred by integration of the X-upstream/*Hyg-3′* and X-downstream/*Hyg-5′* fusion amplicons into the genome via three homologous recombination events (Catlett et al. 2002). Following the protoplast transformation protocol, hygromycin-resistant transformants were selected by overlaying the regeneration medium with 1% water agar containing 300 µg of hygromycin B per milliliter (Invitrogen, San Diego, CA, U.S.A.). Hygromycin-resistant transformants were subjected to three PCR analyses to determine whether targeted DNA had been deleted and replaced with *HygB* and whether the three predicted homologous recombination events had occurred. In the first PCR analysis, primers XLT and XRT, which were complementary to sequences just flanking the targeted gene, were expected to amplify a fragment corresponding to the coding region of the target gene from the wild type and a fragment corresponding to the *HygB* gene from deletion mutants. In the second PCR analysis, primers XLFN and Hygout1 were expected to amplify

an approximately 900-bp fragment from deletion mutants but no fragment from the wild type (Supplementary Fig. 1). In the third PCR analysis, primers XRFN and Hygout2 were expected to amplify an approximately 900-bp fragment from deletion mutants but no fragment from the wild type. Transformants that yielded the pattern of PCR bands predicted for deletion of the target gene and its replacement with *HygB*, referred to as ΔFvX (e.g., $\Delta FvFUB5$), were regrown from a single conidium prior to further analysis.

Inactivation of genes in *F. oxysporum* was accomplished by deletion of target coding regions via the split-marker approach and protoplast transformation, as described above for *F. verticillioides* but with some notable differences. *F. oxysporum* split-marker constructs were generated by first amplifying two 1,000-bp fragments, one corresponding to the 5′ and the other to the 3′ flanking regions (flanks) of the coding region of the target gene. Fusion PCR, as described by Yu and associates (2004), was then used to fuse these fragments to either end of *GenR*. For fusion PCR, the PCR-amplified fragments 5′ flank, *GenR*, and 3′ flank were mixed at a molar ratio of 1:3:1, respectively, under the following conditions: 95°C for 3 min, followed by 10 cycles of 94°C for 30 s, 58°C for 10 min, 72°C for 5 min, and a 10-min extension at 72°C. The resulting 5′flank-*GenR*-3′ flank fusion product was then used as a template in PCR to amplify split-marker fragments. Primers NF and gen-NR were used to amplify a split-marker fragment corresponding to the 5′flank fused to the 5′ approximate two-thirds of *GenR*, and primers NR and gen-NF were used to amplify a split-marker fragment corresponding to the 3′ approximate two-thirds of *GenR* fused to the 3′ flank. All PCR amplifications were performed using TaKaRa Ex Taq polymerase (TaKaRa Bio Inc., Shiga, Japan). Primers used in construction of split-marker fragments for *F. oxysporum* are listed in Supplementary Table 2. The *GenR* fragment used in fusion PCR was amplified from plasmid pII99 (Namiki et al. 2001) with primers gen-F and gen-R.

To generate *F. oxysporum* protoplasts, conidia were germinated overnight in YPG broth at 25°C. The resulting mycelia were collected by filtration (Whatman filter paper), washed with 1 M NH_4Cl and were then incubated in a Driselase (Sigma-Aldrich, St. Louis) solution with 1 M NH_4Cl (1.05 g to 70 ml) for 3 to 4 h at 30°C with agitation (50 rpm). The incubation was allowed to proceed until 10^6 protoplasts were formed per milliliter of solution. Protoplasts were harvested by centrifugation at 7,000 rpm for 5 min at 4°C.

Replacement of the target gene with *GenR* occurred by integration of the NF/gen-NR and NR/gen-NF fusion amplicons into the genome via homologous recombination (Yu et al. 2004). Following the protoplast transformation protocol, geneticin-resistant transformants were selected by overlaying the regeneration medium with 1% water agar containing 100 µg of geneticin per milliliter (Invitrogen). Geneticin-resistant transformants were screened initially by PCR to identify those missing the target gene. Selected PCR-positive deletion mutants of *FUB1*, *FUB3*, *FUB6*, *FUB8*, and *FUB10* were subjected to Southern analysis for verification. DNA probes for hybridization were labeled with [^{32}P] dCTP, using the Amer-sham Rediprime II labeling system (GE Healthcare, Buckinghamshire, U.K.). The probes were synthesized by PCR from genomic DNA of the wild-type *F. oxysporum* Foxy20 strain with one set of the internal primers per gene.

Mutant complementation.

One each of the *F. verticillioides* *fub4*, *fub5*, *fub10*, and *fub11* deletion mutants were complemented by transformation with vectors containing a wild-type copy of the target *FUB* gene plus approximately 700 bp upstream of the predicted start

codon and approximately 300 bp downstream of the predicted stop codon. The vectors also included *GenR* as a selectable marker (Marek et al. 1989). For example, the *fub4* complementation vector pFub4 was created by PCR amplification of a 1,794-bp fragment with primers Fub4AbF and Fub4AbR and wild-type *F. verticillioides* genomic DNA, followed by cloning the fragment into the pCR-XL-TOPO plasmid (Invitrogen). A fragment of DNA containing *GenR* was then cloned into the *NotI* restriction site present in the pCR-XL-TOPO element of pFub4. The resulting plasmid pFub4C was introduced into the genome of *fub4* deletion mutant Δ FvFub4.9 via protoplast-mediated transformation. Following the protoplast transformation protocol, geneticin-resistant transformants were selected by overlaying the regeneration medium with 1% water agar containing 300 μ g of geneticin per milliliter (Invitrogen). Geneticin-resistant transformants were subjected to a PCR screen to determine whether they carried the complementation construct. The screen employed vector-specific primers 1840 and 1841 which, for *FUB4*, *FUB5*, *FUB10*, and *FUB11*, amplify a DNA fragment larger than the targeted gene. Transformants that yielded the expected amplicon were reisolated from a single conidium and the resulting isolates were reanalyzed by PCR to confirm the presence of the complementation construct.

qPCR.

qPCR was carried out in *F. verticillioides* to examine differences in transcript levels for *FUB1*, *FUB3*, *FUB8*, and *FUB10* in the wild-type strain, *FUB10* mutants, and *FUB10* complementation strains after 48 h of growth in GYAM. single-stranded (ss)DNA was generated from RNA isolated from harvested mycelia using the Quantitect RT kit (Qiagen, Venlo, Netherlands). PCR reactions were carried out using the Rotor-Gene PCR machine (Qiagen), utilizing the Rotor-Gene SYBR Green kit (Qiagen) in 25 μ l. Reaction conditions followed the manufacturer's recommendations: 95°C for 5 min to activate the polymerase, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. A melting curve from 50 to 95°C with reads every 1°C was generated to ensure that individual amplicons were produced. Concentration of DNA measured in nanograms per microliter were determined by comparison to a standard curve generated for *CYP2* (FVEG_00403, encoding a putative cyclophilin) and *UBC* (FVEG_11477, encoding a putative ubiquitin conjugating enzyme) using primers 2193/2194 and 2195/2196, respectively. For the most part, means of three subsamples per flask were used so that three flasks per strain $\times$ time interaction were treated as the replication. Calculated concentrations (CALCONC) of transcripts of the same gene from the three *F. verticillioides* strains were analyzed by single-factor, mixed model analysis of variance (ANOVA) using a completely randomized design. Technical and biological replicates were combined. Levene's homogeneity of variance tests of all datasets indicated that transformation of data for *FUB1*, *FUB3*, and *FUB8* was required to stabilize variance for ANOVA; the transformations were $\text{Ln}\{(\text{CALCONC}-0.09)/21250\}$, $\text{Ln}\{(\text{CALCONC}-0.009)/16350\}$, and $\text{Ln}(\text{CALCONC}/12655)$ for *FUB1*, *FUB3*, and *FUB8*, respectively. ANOVA tests were conducted on transformed data when necessary but untransformed means are presented for ease of interpretation. When ANOVA indicated a significant ($P \leq 0.05$) F-test value, a least squares means analysis was used to determine which treatments differed from one another. SAS 9.2 software (SAS Institute Inc. Cary, NC, U.S.A.) was used for the statistical analyses.

qPCR was carried out with *F. verticillioides* genomic DNA to examine relative difference of the *FUB10* coding DNA in the *FUB10* complementation strain Δ FvFub10.5AB815 as compared with the wild-type. PCR reactions were carried out essentially as described above, except that approximately 2.5

ng of genomic DNA from each strain were used as template. Average threshold cycle (Ct) values were generated from three subsamples per genomic DNA. The calculated relative fold difference of the *FUB10* amplicon in complementation strain DNA as compared with wild-type DNA was normalized to the geometric mean of the two unmanipulated endogenous controls, *FUB8* and *CYP1* (FVEG_07816, encoding a Trm112-like protein; primers 2203 and 2204).

qPCR was carried out in *F. oxysporum* to examine differences in transcript levels for 17 genes in the wild-type and *FUB10* deletion mutant after growth on CDB, which induces FA production in *F. oxysporum*, and minimal medium, which does not induce FA production. Integrated DNA Technologies PrimerQuest software was used to design gene-specific PCR primers. Translation elongation factor 1 alpha (*EF1 α* , FOXG_03515) served as an endogenous control. Total RNA was extracted from harvested mycelia using the RNA extraction kit (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer's protocol. ssDNA was generated from RNA using the SuperScript III first-strand synthesis system (Invitrogen) as described previously (Lee et al. 2014). PCR reactions were performed in an iCycler iQ5 real time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). The PCR thermal cycling conditions were as follows: 95°C for 3 min, followed by 45 cycles at 95°C for 10 s, 60°C for 20 s, 72°C for 20 s. The PCR reaction contained 10 μ l of 2 $\times$ iQ SYBR green supermix (Bio-Rad), 0.5 μ l of each primer (10 pM), 1 μ l of template cDNA (12.5 ng), and water to a final volume of 20 μ l. Quantification values were automatically determined using the Bio-Rad CFX Manager version 1.6 and the Ct values were determined. In all experiments, negative control reactions containing no template DNA were subjected to the same procedure to detect DNA contamination. This experiment was done two times. In each experiment, three technical replicates were done for each treatment, and final Ct values are presented as the average of the replicates. Ct values were normalized with the Ct value of *EF1 α* and the normalized levels of each gene were compared with the level of the wild type on day 3 in MM.

Analysis of FA.

Ten-day-old cracked maize kernel cultures of *F. verticillioides* and culture filtrates from 21-day-old CDB cultures of *F. oxysporum* were extracted with acetonitrile/water (50% vol/vol) acidified to pH 2 with HCl. FA concentrations in *F. verticillioides* extracts were determined by HPLC coupled to a tandem mass spectrometer in a manner similar to that previously described (Brown et al. 2012). The LC-MS/MS apparatus included a DIONEX (ThermoFisher Scientific, Waltham, MA, U.S.A.) Ultimate 3000 HPLC coupled to a SCIEX (AB SCIEX, Framingham, MA, U.S.A.) QTRAP 3200. Briefly, 10 μ l of extract was injected into an 800 μ l/min gradient flow of water (solvent A) and acetonitrile (solvent B) through an XBridge (Waters, Milford, MA, U.S.A.) C18 chromatography column (4.6 mm diameter $\times$ 150 mm length, 3.5 μ m particle size). Solvents included 0.5% formic acid. A solvent program (0 min: 10% B, 1 min: 10% B, 8.5 min: 90% B, 10 min: 90% B, 12 min: 10% B, 13 min: 10% B) was used to effectively elute the FA for MS/MS detection. Approximately 10% of the LC eluent was directed to the electrospray ionization (ESI) source of the MS, while the remainder was directed to a waste container. Parameters for the MS/MS detection of the FA were empirically optimized to maximize sensitive detection. The ESI source was operated in positive mode at a voltage of 5,500 V and the collision energy for stimulation of ion fragmentation was set to 10. The MS was operated in multiple reaction monitoring mode to observe several distinctive ions produced upon fragmentation of the protonated $[\text{M}+\text{H}]^+$ FA ion. The parent

ion (m/z 180) and fragment ions (m/z 134, 152, 162) were monitored and quantitation was based upon integrated intensity of the m/z 134 ion. Operation of the LC-MS/MS instrument and interpretation of the acquired data were done utilizing the Analyst software provided by the MS instrument manufacturer.

F. oxysporum extracts were analyzed with an HPLC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) equipped with the diode-array detector set at 254 nm and Zobax C18 column (4.6×150 mm, 40°C). The mobile phase was acetonitrile and water containing 0.5% trifluoroacetic acid. The flow rate was 1.0 ml/min and the injection volume was 25 μl . The FA standard was purchased from Sigma-Aldrich.

Pathogenicity assays.

Pathogenicity of *F. oxysporum* and phytotoxicity of its culture filtrates were assessed with a cactus shoot assay, using the three-angled cactus *Hylocereus trigonus*, because the wild-type progenitor strain Foxy20 was originally isolated from cactus. Shoots of plants grown in the greenhouse for one year were cut into 0.5-cm-thick cross sections and were then placed on moistened Whatman No. 2 filter papers in an 8-cm petri dish. The central portion of each cross section was treated in one of the following ways: i) a 4-mm diameter block was excised from PDA cultures of *F. oxysporum* grown at 25°C for 7 days, ii) 0.1 ml of a suspension of 10^4 or 10^6 *F. oxysporum* conidia per milliliter, or iii) 0.1 ml of culture filtrate prepared by passing a 21-day-old CDB culture of *F. oxysporum* through a $0.2 \mu\text{m}$ filter. Each treatment was applied to three cross sections per dish with three replications. Symptom development was examined after 4 days of incubation at 25°C . Severity was assessed using a rating scale from 0 to 5 based on the percentage of tissue with visual symptoms in which 0 = no reaction, 1 = yellow or brown surface discoloration of the central portion of the cross section, 2 = central portion rotten and darkly discolored, 3 = central portion rotten but rot is less than 3 mm deep, 4 = central portion rotten more than 3 mm deep, 5 = whole cross section rotted (Choi et al. 2010). The experiment was repeated with essentially identical results.

Pathogenicity of *F. verticillioides* was assessed with a seedling assay using the hybrid white sweet corn Silver Queen (Johnny's Selected Seed) (Glenn et al. 2008; Larson et al. 2011). Fungicide-free seeds were surface-sterilized by soaking in 100% commercial bleach (6.25% sodium hypochlorite) for 10 min, were rinsed with sterile water four times, were allowed to soak for 4 h in sterile water, and were then heat-shocked at 60°C for 5 min. Thirty seeds were then incubated at 27°C overnight in 10 ml of water containing 1×10^6 *F. verticillioides* conidia per milliliter. Ten seeds were planted in 10-cm plastic pots containing growing mix (Sunshine Redi-earth, Bellevue, WA, U.S.A.) that was sterilized twice in an autoclave at 121°C for 20 minutes. Pots were placed in a growth chamber with the following conditions: 14 h of light at 30°C and 10 h of dark at 20°C . Disease symptoms were assessed after 16 days by counting the number of plants and measuring plant height.

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AUTHOR RECOMMENDED INTERNET RESOURCES

- Broad Institute *Fusarium* Comparative database:
www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html
- Gene Expression Omnibus: www.ncbi.nlm.nih.gov/geo
- Institute of Bioinformatics and Systems Biology (IBIS) website:
www.helmholtz-muenchen.de/en/ibis
- Joint Genome Institute (JGI) *Trichoderma atroviride* database:
genome.jgi-psf.org/Triat2/Triat2.home.html