***Fusarium verticillioides* SGE1 Is Required for Full Virulence and Regulates Expression of Protein Effector and Secondary Metabolite Biosynthetic Genes**

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The transition from one lifestyle to another in some fungi is initiated by a single orthologous gene, *SGE1*, that regulates markedly different genes in different fungi. Despite these differences, many of the regulated genes encode effector proteins or proteins involved in the synthesis of secondary metabolites (SM), both of which can contribute to pathogenicity. *Fusarium verticillioides* is both an endophyte and a pathogen of maize and can grow as a saprophyte on dead plant material. During growth on live maize plants, the fungus can synthesize a number of toxic SM, including fumonisins, fusarins, and fusaric acid, that can contaminate kernels and kernel-based food and feed. In this study, the role of *F. verticillioides* *SGE1* in pathogenicity and secondary metabolism was examined by gene deletion analysis and transcriptomics. *SGE1* is not required for vegetative growth or conidiation but is required for wild-type pathogenicity and affects synthesis of multiple SM, including fumonisins and fusarins. Induced expression of *SGE1* enhanced or reduced expression of hundreds of genes, including numerous putative effector genes that could contribute to growth in planta; genes encoding cell surface proteins; gene clusters required for synthesis of fusarins, bikaverin, and an unknown metabolite; as well as the gene encoding the fumonisin cluster transcriptional activator. Together, our results indicate that *SGE1* has a role in global regulation of transcription in *F. verticillioides* that impacts but is not absolutely required for secondary metabolism and pathogenicity on maize.

Fungi are an ecologically diverse group of organisms that can live on a variety of substrates in different environments and can exhibit vastly different lifestyles. Animal- and plant-

pathogenic fungi often exist among the normal microflora of their hosts without causing disease symptoms. Wor1/Ryp1 is a transcriptional regulator governing transition between different lifestyles that also requires morphological transition and was first characterized in the human pathogens *Candida albicans* and *Histoplasma capsulatum* (Huang et al. 2006; Nguyen and Sil 2008). In *C. albicans*, Wor1 controls transition between white and opaque cell types, which grow preferentially at different host infection sites, by affecting the expression of several hundred genes (Huang et al. 2006; Zordan et al. 2007). In *H. capsulatum*, the Wor1 homolog, Ryp1, controls transition from saprophytic filamentous growth to pathogenic yeast-like growth at 37°C (Nguyen and Sil 2008).

Wor1/Rhyp1 orthologs have been identified in the plant-pathogenic fungi *Fusarium graminearum* (Fgp1) (Jonkers et al. 2012), *F. oxysporum* (Sge1) (Michielse et al. 2009b), *Botrytis cinerea* (Reg1) (Michielse et al. 2011), and *Verticillium dahliae* (Sge1) (Santhanam and Thomma 2013). Inactivation of genes encoding these homologous proteins (collectively referred to hereafter as Sge1) results in some phenotypes common to all four of these plant-pathogenic fungi as well as some phenotypes that are unique to one or two species. In all species examined, *SGE1*-inactivation mutants exhibit reduced levels of pathogenicity on their respective hosts. However, in the different fungi, the reduction may result from loss of different virulence factors such as effector proteins or phytotoxic secondary metabolites (SM) that require Sge1 for their production. In *F. oxysporum*, reduced virulence was attributed to reduced expression of effectors (Michielse et al. 2009b). Effectors are secreted proteins and include small cysteine-rich peptides, cell-wall-degrading enzymes, and necrosis-inducing proteins (de Jonge et al. 2011). Effectors and SM can alter host physiology, likely by suppressing defenses, and thereby allow pathogens to grow in the host (de Jonge et al. 2011; Rafiqi et al. 2012). The *F. oxysporum* Sge1 homolog controls expression of numerous effectors including secreted-in-xylem (SIX) proteins, some of which are required for pathogenicity on tomato (Houterman et al. 2007, 2009; Michielse et al. 2009b; Takken and Rep 2010; van der Does et al. 2008). Although the *V. dahliae* Sge1 homolog was shown to positively regulate some effectors and negatively regulate others, a direct role in disease has not yet been established (Santhanam and Thomma 2013). In *F. graminearum* and *B. cinerea*, reduced virulence was attributed to loss of production of phytotoxic sesquiterpenoids (Jonkers et al. 2012; Michielse et al. 2011). The *F. graminearum* Sge1 homolog, Fgp1, affects the expression of a gene cluster responsible for

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synthesis of trichothecene toxins, which contribute to the ability of this fungus to cause wheat head blight (Jonkers et al. 2012). As with *Wor1/Ryp1* in the human pathogens, *Sge1* homologs in plant-pathogenic fungi play a critical role in pathogenicity, perhaps by enabling fungi to grow in planta (Michiels et al. 2009b).

F. verticillioides is one of the most common maize-associated fungi in most regions of the world where this crop is grown. The fungus can be readily isolated from maize and maize products, as well as from dead plant material in the soil (Burgess 1981; Nelson 1992). Although it occurs most frequently as an endophyte, causing no obvious detrimental effects, *F. verticillioides* can also occur in maize as a pathogen, causing seed, root, stalk, and ear rot as well as seedling blight (Bacon et al. 2001; Murillo et al. 1999; Oren et al. 2003). The biological, environmental, and physical factors that affect transition of *F. verticillioides* between its saprophytic, endophytic, and pathogenic growth phases are poorly understood.

During growth in maize, *F. verticillioides* can produce SM, including some that are toxic to animals (e.g., mycotoxins), that can contaminate maize and maize-based products. Fumonisin mycotoxins are a particular concern, because their consumption is epidemiologically associated with esophageal cancer and neural tube defects in humans and has been demonstrated to cause diseases in horses, swine, and experimental rodents (Glenn 2007; Marasas et al. 2004; Morgavi and Riley 2007). The contamination of grain and grain products with mycotoxins has a significant impact on health costs and international trade estimated to be in the hundreds of millions of dollars each year (Wu 2007). The role of fumonisins in the ecology of *F. verticillioides* is not clear. In some studies, fumonisin production contributed to the pathogenicity of *F. verticillioides* on maize seedlings (Glenn et al. 2008; Myung et al. 2011) but, in others, it did not (Desjardins et al. 2007). In addition, results from two studies provided no evidence for a role for fumonisin production in the ability of *F. verticillioides* to cause maize ear rot (Desjardins and Proctor 2007; Desjardins et al. 2002). However, because most SM exhibit some level of toxicity or other biological activities, they have the potential to play a role in saprophytism by suppressing growth of competing microorganisms or in endophytism by suppressing host defenses or growth of other endophytes. It is also possible that toxic SM could contribute to pathogenicity of a microorganism by suppressing growth of competing pathogens.

Based on the previously characterized critical roles of *Sge1* homologs in SM production and virulence in plant-pathogenic fungi, we sought to determine whether *Sge1* functions in SM production, pathogenicity, and gene regulation in *F. verticillioides*. Our results show that *Sge1* is required for wild-type levels of virulence on maize seedlings and wild-type fumonisin production in culture. Transcriptomic analysis indicates that *Sge1* affects the transcription of hundreds of genes, including genes associated with the synthesis of at least four SM families and numerous potential effector genes.

RESULTS

In silico characterization of *F. verticillioides* *SGE1*.

Previously, Jonkers and associates (2012) noted that the *F. verticillioides* gene model FVEG_09150 in the Broad Institute's Comparative *Fusarium* Database is predicted to encode an *Sge1*-like protein. Here, further characterization of FVEG_09150 indicated that it consists of a 987-bp, intronless coding region predicted to encode a 328-amino-acid (aa) protein. Like other *Sge1* homologs (Jonkers et al. 2012), the predicted FVEG_09150 protein consists of a conserved amino-terminal region of approximately 220 aa and a variable carboxy termi-

nal region of approximately 120 aa. The conserved region of FVEG_09150 shares 80 and 82% amino acid sequence identity with the corresponding region of the *Sge1* homologs in *F. oxysporum* (Fo*Sge1*) and *F. fujikuroi* (Ff*Sge1*; FFUJ_07864) respectively, while the conserved regions of Fo*Sge1* and Ff*Sge1* share 89% identity. In contrast, the FVEG_09150 variable region shares 42 and 58% amino acid sequence identity with the corresponding region in Fo*Sge1* and Ff*Sge1*, respectively, while the variable regions in Fo*Sge1* and Ff*Sge1* share 30% identity. FVEG_09150 is represented by accession TC33724 in the *F. verticillioides* Gene Index, an expressed sequence tag (EST) database at the Dana Farber Cancer Institute (Brown et al. 2005). TC33724 comprises 20 EST derived from six cDNA libraries, each generated from a different growth condition. Seventeen of the EST were derived from *F. verticillioides* grown in a liquid medium for different periods of time, two were from growth on cracked maize kernel medium, and one was from germinating conidia (Brown et al. 2005). No FVEG_09150/TC33724 EST were recovered from growth of the fungus on excised maize seedling roots and shoots. Analysis of transcriptomic data (Gene Expression Omnibus [GEO] accession number GSE16900) derived from *F. verticillioides* grown for 24, 48, 72, and 96 h in a liquid fumonisin production medium revealed significant expression of FVEG_09150 at all timepoints. The high levels of sequence identity of the predicted FVEG_09150 protein and *Sge1* homologs in other fusaria indicate that the predicted FVEG_09150 protein is the *F. verticillioides* *Sge1* homolog. The EST and microarray data indicate that the corresponding gene, *SGE1*, is expressed under multiple growth conditions.

SGE1 deletion, complementation, and inducible expression.

In order to examine its function, the *F. verticillioides* *SGE1* was deleted, complemented, and subjected to inducible expression. For deletion, we used the split-marker gene deletion strategy combined with protoplast-mediated transformation (Catlett et al. 2002; Turgeon et al. 1987) to replace the *SGE1* coding region with the hygromycin B resistance gene, *HygB* (Catlett et al. 2002; Turgeon et al. 1987) (Fig. 1A). For complementation, a fragment that included the *SGE1* promoter, coding, and 3' flanking regions was cloned adjacent to the geneticin resistance gene *GenR* to create plasmid p*SGE1C* (Bluhm et al. 2008). For inducible expression, we first generated plasmid pXLN2P::SGE1, in which the *SGE1* coding and 3' flanking regions were fused to the promoter region of the *F. verticillioides* xylanase gene *XLN2* (FVEG_12502) (Fig. 2). Protoplast-mediated co-transformation was then used to introduce pXLN2P::SGE1 and plasmid pKS-Gen, which carries *GenR* into the *sge1* mutant Δ*SGE1*.12.

A polymerase chain reaction (PCR) screen identified four independent transformants that lacked the *SGE1* coding region, a result that was confirmed by Southern analysis in two of the transformants: Δ*SGE1*.6 and Δ*SGE1*.12 (Fig. 1B and C). Following transformation of the *sge1* mutant Δ*SGE1*.12 with the complementation vector p*SGE1C*, complemented mutants were identified by a PCR assay that amplified the entire *SGE1* coding region using primers specific to the *SGE1* promoter and terminator regions (data not shown). Three complemented mutants (strains Δ*SGE1*.12AB1, Δ*SGE1*.12AB15, and Δ*SGE1*.12AB19) identified in this analysis were selected for further study. Following transformation of the *sge1* mutant Δ*SGE1*.12 with the inducible expression vector pXLN2P::SGE1, transformants with the *XLN2* promoter-*SGE1* fusion construct were identified by PCR using primers specific to the *XLN2* promoter and *SGE1* coding sequence (data not shown). In transformants carrying the fusion construct, expression of *SGE1* should be induced by xylose. We confirmed xylose-induced expression

in one transformant, strain GMTcp-272, by growing it in SYP medium (100 mM sorbitol, 0.25% peptone, and 0.075% yeast extract) and adding 50 mM xylose after 18 h of incubation. The SYP medium was chosen to help minimize general transcription; thus allowing for easier visualization of new transcripts induced by the appearance of *Sge1*. Using reverse-transcriptase (RT)-PCR with *SGE1*-specific primers 1836 and 1838, we observed *SGE1* expression in strain GMTcp-272 at 5, 15, and 30 min after the addition of xylose (Fig. 2B). In contrast, no *SGE1* expression was observed in the *sge1* progenitor strain Δ SGE1.12 (Fig. 2B) or in the wild-type progenitor of the *sge1* mutant (data not shown). Thus, addition of xylose to cultures of the *SGE1* inducible-expression strain GMTcp-272 generated high levels of expression of *SGE1* within 5 min.

We also assessed whether addition of xylose to cultures of GMTcp-272 resulted in expression of a gene that might be regulated by *SGE1*. In *F. oxysporum*, the gene *SIX2* is regulated by *SGE1* (Michielse et al. 2009b; Rep et al. 2002). The gene model FVEG_03307 in the *Fusarium* Comparative Database is likely the *F. verticillioide*s *SIX2* ortholog because it shares 68.2% deduced amino acid identity with *F. oxysporum* *SIX2*. RT-PCR analysis with the FVEG_03307-specific primers 1881 and 1882 revealed expression of FVEG_03307 30 min after addition of xylose to cultures of GMTcp-272 (Fig. 2C). In contrast, no expression of FVEG_03307 was observed following addition of

xylose to cultures of the *sge1* mutant or wild type. These results provide evidence that *SGE1* functions similarly in *F. verticillioide*s and *F. oxysporum* with respect to regulation of *SIX2* and, therefore, potentially other genes as well.

Phenotypic characterization of mutants and inducible-expression strain.

Vegetative growth and conidiation of two independent *sge1* mutants (strains Δ SGE1.6 and Δ SGE1.12) did not differ significantly from the wild type in two liquid media (GYP [2% glucose, 1% peptone, and 0.3% yeast extract] and CMC [15 g of carboxymethylcellulose], 1 g of NH_4NO_3 , 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g of yeast extract per liter]) and on two solid media (V8 and sGYP [GYP plus 1.5% agar]) (Supplementary Table S1; Supplementary Fig. S1).

The effect of the deletion of *SGE1* on pathogenicity of *F. verticillioide*s was examined using a standard maize seedling assay (Glenn et al. 2008; Larson et al. 2011) with 'Silver Queen' and plant height as a measure of disease severity (Fig. 3). In the assay, the virulence of both *sge1* mutants was significantly reduced compared with the wild type (Table 1). Complementation of the *sge1* mutant Δ SGE1.12 restored virulence to wild-type levels (Table 2; Fig. 3). Although virulence of the *SGE1* inducible-expression strain was not restored to wild-type levels, in the presence of supplemental xylose, it exhib-

Fig. 1. *SGE1* deletion in *Fusarium verticillioide*s. **A**, Split-marker approach used for deletion, showing the three homologous recombination events required for replacement of the *SGE1* coding region (white arrow) with the hygromycin resistance gene, *HygB* (gray arrow). Small black arrows labeled as 1382, 1385, 1388, 1389, Hygout1, or Hygout2 indicate positions of primers used for polymerase chain reaction (PCR) analysis of transformants. **B**, PCR strategy used to determine whether *SGE1* was deleted, showing results from a representative transformant, strain Δ SGE1.12. In PCR, primer combinations 1382-Hygout2 and 1385-Hygout1 yielded approximately 800-bp amplicons that confirm integration of transforming DNA into the *SGE1* locus. These primer combinations do not yield an amplicon from the wild type or transformants with an intact *SGE1*. In addition, primers 1388 and 1389, which are complementary to the region immediately outside of the targeted deletion, confirmed the presence of *HygB* by amplifying a 2,500-bp product from *sge1* mutants but a 1,100-bp product from the wild type or transformants with an intact *SGE1*. Lane 1, DNA ladder; lanes 2 and 3, primer 1388-1389 amplicons; lane 4, primer 1382-Hygout2 amplicon; lane 5, primer 1385-Hygout1 amplicon. Sizes of amplicons are indicated in base pairs to right of gel image. **C**, Southern analysis of Δ SGE1.12. Genomic DNA was digested with *AseI* and probed with a fragment of DNA downstream from the predicted *SGE1* start codon (e.g., the left flank). *SGE1* sequence includes an *AseI* restriction site; thus, transformants digested with this enzyme, lacking *SGE1*, and probed would display a DNA band larger (7.1 kb) than the band observed in the wild type (4.3 kb).

ited significantly higher levels of virulence than the *sgel* mutant (Δ SGE1.12) from which it was derived (Supplementary Table S2). The addition of xylose had no effect on virulence of the wild type or any effect on median height of uninoculated plants based on Dunnett's test at $P \leq 0.05$.

Pathogenicity of Δ SGE1.12 and three complemented *sgel* mutants was also examined using a modified maize seedling assay in which pieces of maize stalk colonized by a single *F. verticillioides* strain were placed into the soil 24 h prior to sowing. Visual inspection did not reveal any differences in colonization of the stalk pieces by the *sgel* mutant and complemented mutant strains. In this experiment, two of the three complemented strains exhibited significantly higher levels of virulence than the *sgel* mutant progenitor strain Δ SGE1.12 (Table 2). The lack of discernible differences in growth in and on laboratory media and in maize stalks of *sgel* mutants and the wild type or complemented mutants indicates that a functional *SGE1* is not essential for the saprophytic lifestyle of *F. verticillioides*. In contrast, the reduction in maize seedling disease caused by the mutant relative to the wild type and complemented mutants indicates that *SGE1* contributes to the pathogenic lifestyle of the fungus.

We also examined the effect of *SGE1* deletion on production of three *F. verticillioides* SM (fumonisins, fusaric acid, and fusarins), using a previously described cracked maize kernel medium assay and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (Brown et al. 2012). Both *sgel* mutant strains (Δ SGE1.6 and Δ SGE1.12) exhibited reduced production of fumonisins and increased production of fusarins. On average, the levels of fumonisins and fusarins produced by the mutants were 39 and 140%, respectively, of the levels produced by the wild-type progenitor strain (Table 3). Fusaric acid production was variable in *sgel* mutants, ranging from 48 to 109% of wild-type levels. Fumonisin production increased 4.4-fold in two *SGE1*-complemented strains and twofold in the third strain relative to the *sgel* mutant progenitor (Table 3). Fumonisin production also increased 4.8-fold in the *SGE1* inducible-expression strain in the absence of exogenous xylose (Table 3). This result was not unexpected because the maize pericarp contains hemicellulose which includes xylan, a polymer of xylose (Nghiem et al. 2011). The failure of fumonisin production to be restored to wild-type levels in the three complemented mutants could result from integration of the complementation construct into a genomic region that did not

A Construction of *SGE1* expression vector

B Xylose induced expression of *SGE1*

C *SGE1* induced *SIX2* expression

Fig. 2. Induced expression of *SGE1* and putative effector gene *FvSIX2*. **A**, Construction of *SGE1* inducible-expression vector pXLN2P::SGE1 by ligating the *SGE1* coding region (white arrow) and its 3' flanking region immediately downstream of the *XLN2* promoter region in plasmid pXLN2P. **B**, Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of induced expression of *SGE1*. *sgel* mutant strain Δ SGE1.12 and *SGE1* inducible-expression strain GMTep-272 were grown in liquid SYP medium; after 18 h of incubation, cultures were induced by addition of 50 mM xylose; and mycelia were harvested for RNA preparation 5, 15, and 30 min later. RT-PCR with *SGE1*-specific primers 1836 and 1838 yielded the predicted 436-bp amplicon at each time point from the *SGE1* inducible-expression strain. No PCR product was amplified from the *sgel* mutant (Δ SGE1.12). **C**, Induction of the *FvSIX2* expression in *SGE1* inducible-expression strain. *FvSIX2*-specific primers 1881 and 1882 amplified the predicted 374-bp product from only the *SGE1* inducible-expression strain. Gel lanes: lane L, DNA ladder; lanes 5, 15, and 30 indicate time (in minutes) after induction by addition of xylose. Sizes of amplicons are indicated in base pairs to the right of gel image.

allow *SGE1* to function as effectively as the native gene in the wild-type strain.

***SGE1* positively and negatively regulates multiple genes.**

To begin to elucidate the genetic pathways through which *SGE1* affects pathogenicity and secondary metabolism, we conducted a transcriptomic analysis of the *sge1* mutant, *SGE1* inducible-expression strain, and their wild-type progenitor. Each strain was grown in liquid SYP medium for 18 h, at which time 50 mM xylose was added. RNA was extracted from cultures 30 min later and subjected to microarray analysis. In pairwise comparisons of the three strains, 142 to 1,388 genes exhibited a greater than twofold change in expression, either an increase or a decrease, as a result of *SGE1* deletion or induced expression (Supplementary Tables S3, S4, S5, and S6; Table 4). In some cases, changes in expression were greater than eightfold. The data also indicate that *SGE1* affects expression of more genes in positive manner (i.e. increases expression) than in a negative manner (i.e. decreases expression). For example, 168 genes were greater than fourfold more highly expressed in the wild type than in the *sge1* mutant, whereas only 34 genes were greater than fourfold more highly expressed in the mutant than the wild type (Table 4). Likewise, 435 genes were greater than fourfold more highly expressed in the *SGE1* inducible-expression strain than in the *sge1* mutant, whereas only 117 genes were greater than fourfold more highly ex-

pressed in the mutant relative to the inducible-expression strain (Table 4).

There was considerable overlap between the groups of genes that exhibited higher or lower levels of expression in the wild type and *sge1* mutant compared with the *SGE1* inducible-expression mutant. Of the 135 genes that were >8-fold more highly expressed in the *SGE1* inducible-expression strain relative to the wild-type, 130 of them were also >8-fold more highly expressed in the *SGE1* inducible-expression strain relative to the *sge1* mutant, while the remaining five genes were, on average, 5.6-fold more highly expressed.

Table 1. Pathogenicity of *sge1* mutants of *Fusarium verticillioides* in maize seedlings assay

Strain	Genotype	Number of plants ^a	Mean height (cm) ^b
None	...	28	36.4 ± 6.7**
M-3125	Wild type	26	24.2 ± 6.6
Δ <i>SGE1</i> .6	<i>sge1</i> Mutant	22	31.7 ± 6.8**
Δ <i>SGE1</i> .12	<i>sge1</i> Mutant	26	34.8 ± 5.3**

^a At the time disease was assessed, the number of living maize seedlings above 10 cm that resulted from 30 seeds sown in three pots (10 seeds/pot).

^b Height of each seedling was determined by measuring distance from the soil surface to the distal end of the longest leaf. Maize seedlings infected with M-3125 had significantly lower mean height than strains followed by ** based on Dunnett's test at $P \leq 0.05$.

Table 2. Pathogenicity of complemented *sge1* mutants of *Fusarium verticillioides* in a maize seedlings assay

Strain ^a	Genotype	Number of plants ^b	Mean height (cm) ^c
Experiment A			
None	...	30	36.7 ± 6.1
M-3125	Wild type	28	27.3 ± 6.1 ^d
Δ <i>SGE1</i> .12	<i>sge1</i> mutant	26	33.1 ± 7.4
Δ <i>SGE1</i> .12AB1	Complemented <i>sge1</i> mutant	26	25.9 ± 7.2 ^e
Δ <i>SGE1</i> .12AB15	Complemented <i>sge1</i> mutant	30	27.1 ± 8.5 ^e
Δ <i>SGE1</i> .12AB19	Complemented <i>sge1</i> mutant	28	29.8 ± 7.9 ^d
Experiment B			
None	...	30	41.1 ± 9.0
Δ <i>SGE1</i> .12	<i>sge1</i> mutant	29	36.1 ± 6.6
Δ <i>SGE1</i> .12AB1	Complemented <i>sge1</i> mutant	30	37.1 ± 6.5
Δ <i>SGE1</i> .12AB15	Complemented <i>sge1</i> mutant	29	29.4 ± 8.1 ^f
Δ <i>SGE1</i> .12AB19	Complemented <i>sge1</i> mutant	30	33.4 ± 7.2 ^f

^a In experiment A, maize seed were inoculated prior to sowing in 10 ml of water with 1×10^6 spores and incubated overnight. Plant heights were assessed after 16 days. In experiment B, corn stalk pieces (approximately 3 by 1 cm) were inoculated with a spore suspension and allowed to incubate for 5 days. Five corn stalk pieces were then placed into the soil in each pot 24 h prior to sowing seed. Plant heights were assessed after 21 days.

^b At the time disease was assessed, the number of living maize seedlings above 10 cm in height that resulted from 30 seeds sown in three pots (10 seeds/pot).

^c Height of each seedling was determined by measuring distance from the soil surface to the distal end of the longest leaf.

^d Maize seedlings infected with M-3125 had a significantly lower mean height than uninoculated seedlings based on Dunnett's test at $P \leq 0.05$.

^e Maize seedlings infected with all three complemented *sge1* mutants had significantly lower mean height than seedlings infected with the parent *sge1* mutant based on Dunnett's test at $P \leq 0.05$.

^f Maize seedlings infected with two of the three complemented *sge1* mutants had a significantly lower mean height than seedlings infected with the parent *sge1* mutant based on Student's *t* test at $P \leq 0.05$.

Fig. 3. Results of a seedling blight assay in which conidia of wild-type *Fusarium verticillioides* (strain M-3125), an *sge1* mutant (strain Δ*SGE1*.12), and a complemented *sge1* mutant (strain Δ*SGE1*.12AB1) were applied to seeds of 'Silver Queen' maize. Seed were then sown, and seedling height was evaluated 16 days after sowing. The *sge1* mutant had a small but not significant effect on seedling growth, whereas the wild type and complemented mutant significantly reduced growth.

Expression of effector genes.

The *F. verticillioides* genome includes multiple genes that are predicted to encode putative effectors, including predicted cell-wall-degrading enzymes (e.g., glycoside hydrolase [GH]), necrosis-inducing proteins (NEPs), and small, cysteine-rich proteins (de Jonge et al. 2011). Some of these genes are among those affected by *SGE1* deletion and induced expression. Of approximately 107 genes predicted to encode small, secreted, cysteine-rich proteins (Ma et al. 2010), 36 exhibited greater than twofold higher expression and only two exhibited greater than twofold lower expression in the *SGE1* inducible-expression strain than the *sge1* mutant (Supplementary Table S7). In contrast, 14 exhibited greater than twofold higher expression and none exhibited greater than twofold higher expression in the wild type compared with the *sge1* mutant. Thus, of the genes encoding small, cysteine-rich proteins that were affected by *SGE1*, almost all were positively affected.

SGE1 also had a positive effect on expression of GH genes more often than it had a negative effect. Of the 297 putative GH genes in the *F. verticillioides* genome (Ma et al. 2010), 54 exhibited greater than twofold higher expression and 17 exhibited greater than twofold lower expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant (comparison group 1). Likewise, 30 exhibited greater than twofold higher expression and four exhibited greater than twofold lower expression in the wild type compared with the *sge1* mutant (comparison group 2) (Supplementary Table S8). As expected, there is considerable overlap between these two groups. The existence of 12 GH genes more highly expressed in the wild type compared with the *sge1* mutant suggests that

loss of *SGE1* affects some genes more than induced expression of *SGE1*. Taken together, these results provide further evidence that *SGE1* can serve to positively and negatively regulate transcription, even of the same gene, depending on context.

The *F. verticillioides* genome includes three genes (FVEG_04647, FVEG_10941, and FVEG_13350) that are predicted to encode NEPs. Expression of FVEG_10941 was greater than fivefold higher in the *SGE1* inducible-expression strain compared with the *sge1* mutant, and greater than twofold higher in the wild type compared with the *sge1* mutant. In contrast, expression of FVEG_04647 and FVEG_13350 did not differ significantly in the *sge1* mutant compared with either the *SGE1* inducible-expression strain or the wild type.

Orthologs of multiple effector genes exist in both *F. verticillioides* and *F. graminearum*. However, few of these genes that were affected by *SGE1* in *F. verticillioides* appear to be affected in the same manner by the *SGE1* ortholog, *FGP1*, in *F. graminearum* (Jonkers et al. 2012). Of the 14 *F. verticillioides* cysteine-rich protein-encoding genes with lower expression in the *sge1* mutant relative to the wild type, five have orthologs in *F. graminearum*. In a similar manner, of the 30 *F. verticillioides* GH effector genes with significantly reduced expression (greater than twofold reduction) in the *sge1* mutant relative to the wild type, 20 have orthologs in *F. graminearum*. *F. graminearum* has orthologs of all three putative *F. verticillioides* NEP genes but none were reported to be transcriptionally affected by *FGP1* (Jonkers et al. 2012).

Expression of SM biosynthetic genes and gene clusters.

Because of the reduced production of fumonisins and increased production of fusarins in *sge1* mutants, we also assessed the effect of deletion and induced expression of *SGE1* on expression of genes required for synthesis of these and other SM. For this assessment, we utilized the microarray data from the experiment described above (i.e., SYP medium cultures; addition of xylose at 18 h; and sampling 5, 30, and 360 min later). A potential limitation of the experiment for such an analysis is that, in laboratory culture media, expression of SM biosynthetic genes is typically low at and before 24 h of overall growth. Nevertheless, differences in expression of some genes were observed. Expression of the fumonisin and fusarin polyketide synthase (PKS) genes (*FUM1* and *FUS1*, respectively) was low and essentially unchanged in the *sge1* mutant relative to the wild type. In contrast, *FUM1* expression was 1.7-fold higher and *FUS1* expression was 13-fold higher in the *SGE1* inducible-expression strain compared with the *sge1* mutant (Table 5).

Differences in expression indicate that five other *F. verticillioides* PKS genes may also be regulated by *SGE1*. A comparison of the wild type versus the *sge1* mutant expression levels revealed that one gene, FVEG_00079, was >14-fold more highly expressed in the wild type (Table 5). In contrast, four PKS genes (bikaverin PKS [*BIK1*], FVEG_00079, FVEG_10497, and FVEG_08425) exhibited greater than twofold higher

Table 3. Secondary metabolite production in *sge1* deletion mutants (Δ SGE1 strains), complemented *sge1* mutants (Δ SGE1.12AB strains), and wild-type (strain M-3125) *Fusarium verticillioides*

Strain	Secondary metabolite	
	Fumonisin ^a	Fusarin ^b
Experiment A		
M-3125 ^c	350 ($\pm$ 136)	250 ($\pm$ 27)
Δ SGE1.6	130 ($\pm$ 63)	352 ($\pm$ 48)
Δ SGE1.12	146 ($\pm$ 47)	348 ($\pm$ 18)
GMTcp-272	296 ($\pm$ 15)	226 ($\pm$ 83)
Experiment B		
M-3125 ^d	137 ($\pm$ 158)	NA
Δ SGE1.12	22 ($\pm$ 25)	NA
Δ SGE1.12AB1	94 ($\pm$ 18)	NA
Δ SGE1.12AB15	43 ($\pm$ 16)	NA
Δ SGE1.12AB19	99 ($\pm$ 119)	NA
GMTcp-272	105 ($\pm$ 21)	NA

^a Total fumonisins was determined by combining the amount of FB₁, FB₂, and FB₃ detected.

^b NA = not available.

^c Levels (ng/ μ l) were determined in extracts of three cultures per strain.

^d Levels (ng/ μ l) were determined in extracts of two cultures per strain.

Table 4. Numbers of genes exhibiting increased or decreased expression as a result of deletion or induced expression of *FvSGE1*^a

Fold change	Wild type (<i>SGE1</i>) vs. <i>sge1</i> mutant (<i>sge1</i>)		Wild type (<i>SGE1</i>) vs. inducible-expression strain (IESGE1)		<i>sge1</i> mutant (<i>sge1</i>) vs. inducible-expression strain (IESGE1)	
	<i>SGE1</i> > <i>sge1</i>	<i>sge1</i> > <i>SGE1</i>	<i>SGE1</i> > IESGE1	IESGE1 > <i>SGE1</i>	<i>sge1</i> > IESGE1	IESGE1 > <i>sge1</i>
1–2	1,461	1,103	2,104	1,526	2,720	1,879
2–4	544	108	829	566	505	952
4–8	110	25	148	145	93	212
>8	58	9	53	135	24	223
Total ^b	2,173	1,245	3,134	2,372	3,342	3,266

^a Strains used in this experiment were wild-type strain M-3125, *sge1* mutant strain Δ SGE1.12, and *SGE1* inducible-expression strain GMTcp-272. Values are numbers of genes exhibiting increase or decrease in expression.

^b Fold change in expression. Total = total number of genes that exhibited increased or decreased expression in strain comparisons indicated ($P < 0.01$).

expression and one PKS gene, FVEG_10535, exhibited two-fold lower expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant (Table 5).

The microarray data indicate that the expression of genes flanking some of the PKS genes exhibited changes in expression in the *sge1* mutant relative to the wild type or *SGE1* inducible-expression strains in a manner similar to changes exhibited by the PKS genes. The coregulation of genes within the *FUM*, *FUB*, *FUS*, and *BIK* gene clusters has been previously described (Brown et al. 2012). The nine genes in the *FUS* cluster exhibited similar changes in expression in the mutant relative to both the wild type and the *SGE1* inducible-expression strain (Fig. 4), whereas genes within other clusters did not always exhibit similar changes in expression. For example, only four of 17 *FUM* genes and three of six *BIK* genes were more highly expressed in the *SGE1* inducible-expression strain than the *sge1* mutant. The lack of similar patterns of expression for genes within the *FUM* and *BIK* clusters likely reflects low transcript levels for the genes at 18.5 h of incubation, the timepoint at which the transcriptome was examined here, rather than dissolution of coregulation of the clusters in the *sge1* mutant or *SGE1* inducible-expression strain over longer time frames.

Similar to expression patterns of the *FUS* cluster, three PKS genes and one or two genes adjacent to them exhibited similar changes in expression in response to *SGE1* deletion or induced

expression. First, PKS gene FVEG_08425 and the putative dehydrogenase gene, FVEG_08426, adjacent to it both exhibited significantly higher (twofold) levels of expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant. Second, PKS gene FVEG_10535 and the putative methyltransferase gene, FVEG_10536, adjacent to it both exhibited significantly lower levels of expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant. Finally, PKS gene FVEG_00079 and two genes, FVEG_00078 and FVEG_00077, contiguous with it all exhibited much higher (23-fold average) levels of expression in the wild type than the *sge1* mutant. This three-gene cluster has been described previously (Ma et al. 2010) and is conserved in the genome sequences of *F. verticillioides*, *F. fujikuroi*, *F. pseudograminearum*, *F. graminearum*, and some strains of *F. oxysporum*. In *F. graminearum*, two of the three genes in the cluster exhibit greater than twofold higher levels of expression in the wild type than the *fgp1* (= *sge1*) mutant (Jonkers et al. 2012). In addition to the PKS, the cluster encodes a putative G-protein coupled receptor (GPCR) of the Pth11 family (FVEG_00078) (Ma et al. 2010) and a putative acyltransferase (FVEG_00077). *PTH11* was originally described in *Magnaporthe* spp. and is required for host surface recognition prior to appressorium development (DeZwaan et al. 1999). The SM products of this cluster have not yet been determined for any species of *Fusarium*.

Table 5. Effect of *SGE1* on expression of *Fusarium verticillioides* PKS genes^a

PKS	WT	<i>sge1</i>	IESGE1	Fold difference in expression	
				WT vs. <i>sge1</i>	IESGE1 vs. <i>Sge1</i>
<i>FUM1</i> ^b	5.4	4.9	5.7	nd	1.7
<i>FUS1</i> ^b	8.5	8.2	11.9	nd	13.0
<i>FUB1</i> ^b	6.2	6.1	6.2	nd	nd
FVEG_00079	8.5	4.7	9.5	14.0	27.9
<i>BIK1</i>	5.9	5.3	6.8	nd	2.8
FVEG_10497	4.8	4.2	5.7	nd	2.8
FVEG_08425	4.8	3.9	5.1	nd	2.3
FVEG_10535	13.8	13.5	12.5	nd	-2.0
<i>PGL1</i>	6.0	6.0	6.9	nd	nd
FVEG_12610	5.3	4.6	5.4	nd	nd
FVEG_09961	5.2	4.5	5.4	nd	nd
FVEG_01914	9.6	9.2	9.0	nd	nd
FVEG_11932	8.3	8.6	9.5	nd	nd
FVEG_13715	5.7	5.0	5.6	nd	nd
FVEG_05537	7.0	7.3	6.9	nd	nd
FVEG_01736	4.9	4.5	5.1	nd	nd

^a Wild type (WT), *sge1* deletion mutant (*sge1*), and *SGE1* inducible-expression strain (IESGE1) expression values are indicated in log2 scale. The change in expression for each gene was calculated by determining the differences in log2 scale value determined for each gene and converting it to a linear value; nd = no significant difference.

^b Genes corresponding to secondary metabolites for which production levels were determined.

Fig. 4. Differences in expression of nine *FUS* cluster genes in the wild type (strain M-3125) versus an *sge1* mutant (strain Δ SGE1.12) and in *sge1* mutant versus *SGE1* inducible-expression strain (GMTcp-272) of *Fusarium verticillioides*.

Fig. 5. Results of quantitative polymerase chain reaction (qPCR) analysis of four likely secondary metabolite biosynthetic genes in wild-type strain M-3125, *sge1* mutant strain Δ SGE1.12, and *SGE1* inducible-expression strain GMTcp-272 (*SGE1* Exp). After 18 h of incubation, cultures were induced by the addition of 50 mM xylose, and mycelia were harvested for RNA preparation 5, 30, and 360 min later. The genes analyzed were *FUS1*, *FUS8*, *FVEG_00078*, and *FVEG_00079*. Columns with the same letters in the same graph are not significantly different.

Validation of gene expression in *FUS* and *FVEG_00079* clusters.

To validate the microarray results, we performed quantitative PCR (qPCR) analysis with four genes that appeared to be regulated by *SGE1*. These genes were also chosen because they are likely to be involved in secondary metabolism, which is a major focus of this study. *FUS1* and *FUS8* are part of the *FUS* gene cluster and encode a putative PKS and monooxygenase, while *FVEG_00079* and *FVEG_00078* encode a putative PKS and GPCR, respectively. qPCR was carried out in triplicate for three biological replicates for each gene at 5, 30, and 360 min after induction with xylose. Analysis of variance (ANOVA) of data for each gene showed significant differences ($P < 0.05$) in expression between the strain–time interactions. In agreement with the microarray results, *FUS1* expression values were significantly different in the *SGE1* inducible-expression strain compared with the wild type and the *sge1* mutant at 30 min (Fig. 5A). Although *FUS8* expression was not significantly different in the three strains after 30 min, it was significantly different after 5 min (Fig. 5B). Also in agreement with the microarray results, both *FVEG_00078* and *FVEG_00079* expression levels were lower in the *sge1* mutant at the 30-min timepoint compared with both the *SGE1* inducible-expression strain and the wild type (Fig. 5C and D). We also found a significant change in expression for all four genes in the wild type between 5 and 30 min, possibly an effect of the inducing agent xylose. In contrast, no significant change was observed for either *FVEG_00078* or *FVEG_00079* in the *sge1* mutant between 5 and 30 min or for all four genes between 30 and 360 min. This lack of change could reflect interactions or an overlap between the effects of xylose and *SGE1* on the transcriptome. Overall, the data indicate that *SGE1* can regulate some SM biosynthetic genes, but additional analyses are necessary to further elucidate the mechanism by which this regulation occurs.

Expression of genes associated with conidiation.

Although *SGE1* deletion did not have a noticeable effect on conidiation in *F. verticillioides*, it does affect conidiation in other plant-pathogenic fungi that have been examined (Jonkers et al. 2012; Michielse et al. 2009b, 2011; Santhanam and Thomma 2013). Therefore, we examined the effect of *SGE1* deletion and induced expression on the expression of potential conidiation genes in *F. verticillioides*. To do this, we utilized microarray data to assess expression of five genes previously shown to be involved in conidiation in *F. verticillioides* as well as *F. verticillioides* homologs of five genes that affect conidiation in other fungi (Supplementary Table S9). Expression of the *F. verticillioides* homologs of the *REN1*, *ABA1*, *FLBC*, *FLBA1*, *FLBA2*, *STUA*, and *HYD2* genes did not differ significantly in the *sge1* mutant or *SGE1* inducible-expression strain compared with the wild type. In contrast, expression of the *F. verticillioides* homolog of *NPS2*, which is involved in spore cell wall development and hydrophobicity in *Alternaria brassicicola* and *Cochliobolus heterostrophus* (Kim et al. 2007), was greater than fourfold lower in the *sge1* mutant relative to the wild type and 2.5-fold higher in the *SGE1* inducible-expression strain relative to the *sge1* mutant. Expression of *HYD2*, which encodes a putative hydrophobin required for conidiation in *F. verticillioides* (Fuchs et al. 2004), did not differ in the *sge1* mutant relative to the wild type but was 3.5-fold lower in the *SGE1* inducible-expression strain relative to the *sge1* mutant. Although we did not observe any obvious effect of the loss of *SGE1* on conidiation in the *sge1* mutants, the changes in gene expression for some genes involved in conidiation in other fungi in the *sge1* mutant and in the *SGE1* induced-expression strain suggests that additional studies are warranted.

Expression of membrane proteins.

Cell surface structures are likely a critical aspect of how fungi transition between phases of their lifecycles because such structures contribute to how fungi present themselves to their environment and how they sense extracellular molecules in the environment. If *SGE1* contributes to the ability of fungi to manage this transition, the expression of genes encoding cell surface proteins would likely be effected by the appearance or loss of *SGE1*. Two large groups of cell surface proteins are GPCRs and secreted anchored (SA) proteins. GPCRs are a large family of transmembrane domain proteins that bind ligands outside the cell and activate signal transduction pathways inside the cell. Of the 102 putative GPCRs identified in *F. verticillioides* (Ma et al. 2010), 29 exhibited greater than twofold lower expression and two exhibited greater than twofold higher expression in the *sge1* mutant compared the wild type. In contrast, 20 exhibited greater than twofold higher expression and 12 exhibited greater than twofold lower expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant (Supplementary Table S10).

SA proteins contain a putative secretion signal peptide and can be further divided into two groups based on whether they are predicted to be glycosylphosphatidylinositol (GPI) anchored to a membrane or whether they contain a unique pentapeptide [GS][PAVNKR]C[IRKHN]P (referred to as GPCR proteins) after the signal peptide (Ma et al. 2010). Of the 128 predicted SA genes, six exhibited greater than twofold lower expression and 25 exhibited greater than twofold higher expression in the *sge1* mutant compared with the wild type. In contrast, 18 exhibited greater than twofold higher expression and 26 exhibited greater than twofold lower expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant (Supplementary Table S11). Seventeen of the SA genes appear to be negatively regulated by *SGE1* in that they exhibited significantly (greater than twofold) higher expression in the *sge1* mutant than in the wild type and significantly lower expression in the *SGE1* inducible-expression strain than in the wild type. The predicted SA proteins encoded by these 17 genes include the GPCR peptide.

Included in the SA-encoding genes negatively regulated by *SGE1* are six that share significant similarity to one another (Supplementary Table S12). Two other genes encode secreted proteins that are not anchored and also share significant similarity to each other and the six GPCR proteins. Transcripts for this family of eight genes in the *sge1* mutant range from 4.1-fold to >23-fold more than the wild type (Supplementary Table S13). Expression of all eight genes was significantly less in the wild type compared with the *sge1* mutant (mean 8.8-fold difference) but significantly greater in the *SGE1* inducible-expression strain compared with the *sge1* mutant (mean 5.5-fold difference). To what extent these genes are regulated by *SGE1* and whether and how they contribute to the ability of *F. verticillioides* to live as a saprophyte, endophyte, or pathogen remain to be determined.

Differing effects of *SGE1* among *Fusarium* spp.

Previously, Jonkers and associates (2012) carried out a microarray-based transcriptomic analysis of an *fgp1* (= *sge1*) mutant and wild-type strain of *F. graminearum*. Here, we compared these *F. graminearum* data with data generated in the current study for equivalent strains of *F. verticillioides* in order to compare the types of genes regulated by *SGE1* orthologs in different species of *Fusarium*. The data sets are not exactly equivalent because the cultures used to prepare RNA for each species employed different growth media and incubation times. Nevertheless, comparisons of the two data sets were informative. Approximately 64% of the genes in the *F. verticil-*

lioides and *F. graminearum* genomes are orthologous (Ma et al. 2010). Of the 774 *F. verticillioides* genes that exhibited greater than twofold lower expression in the *F. verticillioides* *sge1* mutant versus the wild type, 372 have orthologs in *F. graminearum*. Of these 372 orthologs, 57 exhibited greater than twofold lower expression and 33 exhibited greater than twofold higher expression in the *F. graminearum* *fgp1* mutant compared with its wild-type progenitor. Of the 156 *F. verticillioides* genes with greater than twofold higher expression in the *F. verticillioides* *sge1* mutant versus the wild-type, 100 had orthologs in *F. graminearum*. Of these 100 orthologs, six exhibited greater than twofold higher expression and six others exhibited greater than twofold lower expression in the *fgp1* mutant compared with the wild type. In a similar manner, we compared our data with preliminary transcriptome data for the wild type and an *sge1* mutant of *F. fujikuroi* grown under a third set of growth conditions (C. Michielse and B. Tudzynski, personal communication). Approximately 71% of the genes in the *F. verticillioides* and *F. fujikuroi* are orthologous (Wiemann et al. 2013). Of the 774 *F. verticillioides* genes with greater than twofold lower expression in the *sge1* mutant versus the wild type, 421 have orthologs in *F. fujikuroi*. Of these 421 genes, 36 exhibited greater than twofold lower expression and 21 exhibited greater than twofold higher expression in *F. fujikuroi* *sge1* mutant compared with its wild-type progenitor. Of the 156 *F. verticillioides* genes with greater than twofold higher expression in the *sge1* mutant versus the wild type, 85 have orthologs in *F. fujikuroi*. Five of these orthologs exhibited greater than twofold lower expression and six exhibited greater than twofold higher expression in the *F. fujikuroi* *sge1* mutant compared with the wild type. These comparisons indicate that *SGE1* regulates markedly different sets of genes in different species of *Fusarium*, a finding that is consistent with a comparative analysis of *sge1/fgp1* mutants of *F. graminearum* and *F. oxysporum* (Jonkers et al. 2012).

Despite this general observation, we identified seven orthologs with greater than twofold lower expression in *sge1* mutants of *F. verticillioides*, *F. fujikuroi*, and *F. graminearum*. Two of the seven genes are adjacent to each other in all three organisms; one of these, FVEG_07524, is predicted to encode a sugar transporter and the other, FVEG_07525, a glycoside hydrolase. Three of the seven genes are predicted to encode secreted proteins: a lipase (FVEG_09233), a chitinase (FVEG_08470), and a pectinesterase (FVEG_10795). The final two genes are predicted to encode an amino acid lyase (FVEG_10108) and a protein involved in synthesis of amino-sugars (FVEG_02064). Although the similar regulation of these genes by *SGE1* implies a similar function, whether it be virulence, secondary metabolism, or other phenotype in the three *Fusarium* spp., how they contribute in *F. verticillioides* remains to be determined.

SGE1 regulated genes that are not required for disease on maize.

We used gene deletion analysis to assess the role in pathogenicity of seven other *F. verticillioides* genes that are positively regulated by *SGE1*. Also, some of these genes are homologs of genes that have been shown to play a role in disease in other fungi. For example, FVEG_02173 has a homolog (FOXG_03305) in *F. oxysporum* f. sp. *lycopersici* that contributes to pathogenicity on tomato (Michielse et al. 2009a). FVEG_02431 has a homolog in *Metarhizium anisopliae* that is linked to virulence on insects (Wang and St Leger 2007). However, *F. verticillioides* mutants in which either FVEG_02173 or FVEG_02431 were deleted exhibited wild-type levels of virulence in the maize seedling assay (Supplementary Table S14).

Killer protein 4 (KP4) is a small, cysteine-rich viral toxin that inhibits *Fusarium* and plant root growth and is secreted by

the maize smut fungus *Ustilago maydis* (Allen et al. 2008; Gage et al. 2002). Previously, we identified six KPL-like (killer protein-like [KPL]) genes in the genome of *F. verticillioides*. Five of the six KPL genes encode small, secreted, cysteine-rich proteins and, thus, could serve as effectors (Brown 2011). Microarray analysis of the five putative effectors revealed that expression of *KPL1*, *KPL3*, and *KPL5* was 2.4- to 5.1-fold (average 3.7-fold) lower in the *sge1* mutant compared with the wild type. Only *KPL5* was greater than twofold more highly expressed in the *SGE1* inducible-expression strain compared with the *sge1* mutant. *F. verticillioides* mutants lacking *KPL1*, *KPL2*, and *KPL3* (a triple gene mutant) or strains lacking either *KPL5* or *KPL6* caused levels of disease similar to the wild type. Again, despite being regulated by *SGE1*, *KPL* genes did not contribute significantly to pathogenicity of *F. verticillioides* on maize seedlings under the conditions examined.

DISCUSSION

Fungi can live and compete in widely diverse habits, including as saprophytes on virtually any carbon source or in commensal, mutualistic, or parasitic relationships with plants, animals, insects, or other fungi. How plant-associated fungi interact with their host can change with time and environment. For example, *F. verticillioides* can exist as a saprophyte, endophyte, and parasite over a single growing season. The ability of fungi to alter the physiology of plant hosts in order to live endophytically or parasitically has been attributed to production as well as nonproduction of effectors, including small, cysteine-rich proteins, cell-wall-degrading enzymes, necrosis-inducing proteins, and SM (de Jonge et al. 2011; Rafiqi et al. 2012). Because of its importance in pathogenicity and its ability to regulate effector-encoding genes in other fusaria, we characterized *F. verticillioides* *SGE1* to begin to elucidate genetic regulation of effectors and their relationship to endophytism and parasitism in this maize-associated fungus. We found that *SGE1* did not affect saprophytic growth on maize stalks and a variety of media, whereas pathogenicity on seedlings was affected. One possible explanation is that *SGE1* plays a role in the transition of the fungus from a saprophyte on maize stalks to growth within the plant as an endophyte or parasite. Although *Sge1* orthologs in other plant-pathogenic fungi have been found to be important for disease, this is the first characterization of a role in lifestyle transition, similar to what was first noted in yeast.

Included in our approaches to examine the role of *SGE1* in regulation of gene expression was the generation of the *SGE1* inducible-expression strain of *F. verticillioides*, in which the *SGE1* coding region was fused to the promoter region of a gene encoding a putative xylanase (*XLN2*), such that expression of *SGE1* could be induced by addition of xylose to culture media. This approach to controlled gene expression has been utilized successfully in *Penicillium* and *Aspergillus* spp. (Wong et al. 2009; Zadra et al. 2000). The major advantage is that it can reveal genes first induced by the rapid appearance of *Sge1*: those more likely directly regulated. Our results demonstrate that this inducible-expression system resulted in rapid and high levels of expression of *SGE1*, within 5 min of induction, and can lead to expression of *SGE1*-regulated genes within 30 min (Fig. 2). A limitation of this system is that xylose induced transcriptional changes over time for three of the four genes we examined by qPCR (Fig. 5). In our microarray experiment, this limitation was controlled for by comparing transcriptional differences between similarly treated strains at a single timepoint. Our results indicate that the *XLN2* promoter system could be a useful tool for characterization of other regulatory or structural genes in *F. verticillioides*.

The results reported here indicate that the *F. verticillioides* *SGE1* affects a lifestyle transition (saprophyte to pathogen), secondary metabolism, and the expression of hundreds of genes but does not affect vegetative growth or conidiation under the laboratory conditions examined. Our results also indicate that the effect of *SGE1* on pathogenicity in the genus *Fusarium* differs among species. In *F. graminearum* and *F. oxysporum*, *SGE1* deletion almost completely abolished pathogenicity in wheat head blight and tomato seedling wilt assays, respectively. In *F. graminearum*, pathogenicity and SM synthesis are connected; trichothecene production is required for spread of the fungus between wheat spikelets (Bai et al. 2002; Seong et al. 2009). *F. graminearum* *fgp1* deletion mutants are unable to synthesize trichothecenes and cause only minimal disease in the inoculated spikelet (Jonkers et al. 2012). This effect on virulence may be related to general growth developmental defects in the mutants or to a direct role of *SGE1* in the initial infection. In contrast, *SGE1* deletion in *F. verticillioides* resulted in reduced virulence compared with the wild type but it did not abolish pathogenicity. In addition, *SGE1* deletion did not completely block production of any of the *F. verticillioides* SM examined; fumonisin production was reduced, fusaric acid production was variable, and fusarin production was enhanced in *sge1* mutants. Although no clear role for an *F. verticillioides* SM in pathogenicity has yet been established, the impact of *SGE1* on expression of some, unknown PKSs (e.g., FVEG_00079) but not others over the time frame of the experiment are intriguing and will be examined further.

The mechanism by which *SGE1* reduces fumonisin and increases fusarin production is not clear. Transcriptomic analysis over a longer time frame may help to determine whether the respective changes in production are due to changes in *FUM* and *FUS* gene expression. Expression of most genes shown previously to affect fumonisin production (*FCC1*, *FCK1*, *LAE1*, *VE1*, *AREA*, *PAC1*, *HXK1*, *ZFR1*, and *FST1*) (Woloshuk and Shim 2013) were not significantly different in all three strains. The only gene previously shown to impact fumonisin synthesis found to differ significantly was *FUM21*, the fumonisin pathway-specific regulatory gene, which exhibited twofold lower expression in the *sge1* mutant compared with the wild type and 3.6-fold higher expression in the *SGE1* inducible-expression strain compared with the *sge1* mutant. A twofold reduction in *FUM21* expression could lead to the observed 50% reduction in fumonisin production over 10 days. Alternatively, carbon or nitrogen depletion or changes in culture pH could have affected fumonisin synthesis over the incubation period without an early impact on *AREA*, *PAC1*, or any of the other genes mentioned above.

In contrast to the *FUM* cluster, the response of the entire *FUS* cluster to *SGE1* (Fig. 4) after 30 min suggests that either *Sge1* directly affects transcription by binding to *FUS* gene promoters or it induces transcription of another gene that then activates expression. The *FUS* gene cluster is unusual among SM gene clusters in that it does not include a transcription factor gene (Niehaus et al. 2013). An ability of *Sge1* to bind *FUS* gene promoter sequence could be examined by promoter binding or gel shift assays (Fernandes et al. 1998). The *SGE1* inducible-expression strain could be used to help identify a possible intermediate transcriptional regulator by accessing the transcriptome at earlier timepoints after induction.

Membrane proteins play a host of critical functions ranging from allowing cells to communicate with their environment to controlling a variety of important metabolic processes. The effect of *SGE1* on the transcription of numerous GPCRs and SA proteins suggests a role for these two major groups of membrane proteins in pathogenicity or lifestyle change. A decrease in GPCRs can occur after stimulation and is one mecha-

nism to control their responsiveness (Claing et al. 2002). The appearance of *SGE1* transcripts potentially bypassed the role of one or more of the GPCRs apparently negatively regulated by *SGE1*, precipitating their downregulation. We are currently examining the roles that some of these GPCRs play in pathogenicity by gene deletion. In contrast, the unknown function of the vast majority (48 of 53) of SA genes precludes much speculation as to their role in virulence or lifestyle change. These effects on GPCR and SA gene expression indicate that *SGE1* likely impacts the content of GPCR and SA proteins in the cell membrane of *F. verticillioides* and, thereby, contributes to the ability of the fungus to sense and adapt to environmental changes.

In summary, similar to the *SGE1* orthologs described in other plant-pathogenic fungi, *F. verticillioides* *SGE1* is required for wild-type levels of virulence and affects secondary metabolism. A comparison of the transcriptomes of the wild type, the *sge1* mutant, and the *SGE1* inducible-expression strain provided significant insight into the genes regulated by *SGE1*. The number of different genes upregulated by *SGE1* in the inducible-expression strain compared with those downregulated in the *sge1* deletion mutant was significant. The observation by Jonkers and associates (2012) and in the current study that few orthologous genes are regulated by *SGE1* in the three *Fusarium* spp. thus far examined may reflect the predominant use of *sge1* deletion mutants in these studies and when the transcriptome was sampled. Perhaps more orthologous genes could be found by comparing genes induced in different *Fusarium* spp. following artificial induction of *SGE1*, as was done in the current study. Although previous experiments indicated that fusarin is not required for disease, the rapid expression of the entire *FUS* gene cluster after the induction of *SGE1* suggests that it may play a role in fungus-plant interactions. Experiments are underway to better understand the role of this metabolite as well as to determine the chemical product resulting from the PKS encoded by FVEG_00079 and coregulated genes and determine whether they are required for disease or other aspects of the maize-*F. verticillioides* interaction.

MATERIALS AND METHODS

Strains and media.

The wild-type *F. verticillioides* strain used in this study was M-3125 (NRRL 13447) (Leslie et al. 1992). Strains Δ SGE1.6, Δ SGE1.12, and Δ SGE1.28 were derived from M-3125 by deletion of the *SGE1* gene. The *SGE1* inducible-expression strain GMTcp-272 and the complemented *sge1* mutants Δ SGE1.12AB1, Δ SGE1.12AB15, and Δ SGE1.12AB19 were derived from strain Δ SGE1.12. All strains were routinely maintained on V8 juice agar (Tuite 1969). Conidial production was assayed by growth of strains in liquid CMC medium (15 g of carboxymethylcellulose [Sigma-Aldrich, St. Louis], 1 g of NH_4NO_3 , 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g of yeast extract [Becton, Dickinson and Company, Franklin Lakes NJ, U.S.A.] per liter), GYP medium (2% glucose, 1% peptone, and 0.3% yeast extract), solid GYP (GYP plus 1.5% agar), and V8 juice agar plates. RNA was prepared from cultures grown in SYP medium (100 mM sorbitol, 0.25% peptone, and 0.075% yeast extract) at 28°C and 200 rpm. For fumonisin, fusarin C, and fusaric acid production, strains were grown in cracked maize kernel medium for 10 days at 25°C in the dark. 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 mycelial plug taken from a V8 juice agar culture of *F. verticillioides*.

Nucleic acid sequence analysis.

Nucleotide sequences of *F. oxysporum* gene *SGE1* (gene model FOXG_10510) and the *F. verticillioides* genes *SGE1* (FVEG_09150) and *SIX2* (FVEG_03307) were retrieved from the Broad Institute's Fusarium Comparative Database. The nucleotide sequence of the *F. oxysporum* gene *SIX2* (FOXG_16416) was retrieved from the National Center for Biotechnology Information (NCBI), accession CAE55868. EST sequences corresponding to FVEG_09150 and FVEG_03307 were retrieved from the *F. verticillioides* Gene Index at the Dana-Farber Cancer Institute-Harvard School of Public Health. Preparation of the *F. verticillioides* EST libraries has been described previously (Brown et al. 2007). Nucleotide sequence data were assembled using Sequencher (version 5.0; Gene Codes Corp., Ann Arbor, MI, U.S.A.). Alignments of amino acid and nucleotides were carried out using the DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada). Sequence similarity analyses were also done by BLASTX and BLASTP searches against the NCBI nonredundant databases (Altschul et al. 1997; Zhang et al. 1998).

F. verticillioides genomic DNA used in PCR analyses was prepared as previously described (Butchko et al. 2003) but with modifications. Mycelia were scraped from 1-week-old V8 juice agar cultures, placed in 250 μl of DNA extraction buffer (200 mM Tris [pH 8.0], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate), and incubated for 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, which was then vortexed for 30 s and centrifuged at 15,000 rpm for 5 min. The aqueous phase was transferred to a new tube, and genomic DNA was isolated using the Ultra-Clean DNA Purification Kit (Mo Bio, Solana Beach, CA, U.S.A.) following the manufacturer's directions. The resulting genomic DNA (50 pg) was used in PCR with 5 μl of Promega GoTaq master mix and 10 μM of each primer in a total reaction volume of 10 μl . PCR was carried out in a PTC-100 PCR thermal cycler (MJ Research, Waltham, MA, U.S.A.) using 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.

Southern analysis was used to characterize the deletion strains. Genomic DNA was digested with *AseI*, separated by agarose gel electrophoresis, and blotted to nylon membrane. The hybridization probe for this analysis was generated by PCR from genomic DNA with primer pair 1383 and 1384, and corresponded to a 718-bp region upstream of the *SGE1* coding region. The amplification product was labeled with ^{32}P utilizing Ready-To-Go DNA labeling beads (GE Healthcare, Buckinghamshire, U.K.). Membranes were hybridized using ULTRA-hyb solution (Life Technologies, Grand Island, NY, U.S.A.) and washed following the manufacturer's directions.

Gene deletion of *SGE1*.

The *SGE1* (FVEG_09150) coding region was deleted via the split-marker approach (Catlett et al. 2002; Turgeon et al. 1987) and protoplast transformation method with the selectable marker gene *HygB*, which confers resistance to the antibiotic hygromycin B (Catlett et al. 2002; Turgeon et al. 1987). Primers used for amplification of the split-marker construct are listed in Supplementary Table S15. To begin preparation of the split-marker constructs, four DNA fragments were amplified by PCR. The first fragment (5'-Flank) corresponded to the 764 bp immediately upstream of the predicted *SGE1* start codon and was amplified with primer pair 1382/1384 from genomic DNA of the wild-type strain of *F. verticillioides*. The second fragment (3'-Flank) corresponded to the 779 bp immediately downstream of the predicted *SGE1* stop codon and was amplified with primer pair 1385/1387 from wild-type genomic DNA.

The third fragment (HY) consisted of the 5' two-thirds of the *HygB* gene and was amplified with primer pair M13RL/HY from plasmid pHygAsc (Brown et al. 2002). The fourth fragment (YG) corresponded to the 3' two-thirds of *HygB* and was amplified with primer pair M13FL/YG from pHygAsc. Two split-marker constructs were then generated by fusion of fragments 5'-Flank and HY and fusion of fragments 3'-Flank and YG (Catlett et al. 2002; Turgeon et al. 1987). Fusion of 5'-Flank and HY employed a final nested PCR step with primers 1383 and HYN, while fusion of 3'-Flank and YG employed a final nested PCR step with primers 1386 and YGN.

To delete the *SGE1* coding region, approximately 2 µg of both split-marker constructs was introduced into the wild-type *F. verticillioides* strain via protoplast-mediated transformation. Deletion was predicted to result from homologous recombination between DNA sequences of the *SGE1* 5' and 3' flanking regions in *F. verticillioides* genome and in the split-marker constructs and between the two partial copies of *HygB* in the split-marker constructs (Fig. 1). These three homologous recombination events were predicted to result in replacement of the 1,242-bp *SGE1* coding region with the 2,500-bp *HygB* resistance cassette. Following the transformation protocol, genomic DNA of hygromycin-resistant transformants, isolated after growth on V8 agar containing hygromycin B (Sigma) at 300 µg/ml, was subjected to three PCR screens to determine whether the *SGE1* coding region had been deleted and replaced by *HygB*. In the first screen, primers 1382 and Hygout2 were predicted to amplify an 800-bp fragment if the *SGE1* coding region had been deleted but no fragment if *SGE1* was intact (Fig. 1A). In the second screen, primers 1385 and Hygout1 were predicted to amplify an 800-bp fragment if the *SGE1* coding region had been deleted and no fragment if the gene was intact (Fig. 1A). In the third screen, primers 1388 and 1389 were predicted to yield a 2.5-kb amplicon if *SGE1* was deleted and replaced by *HygB* and a 1.2-kb amplicon if *SGE1* was intact (Fig. 1B). All transformants that yielded amplicons consistent with *SGE1* deletion were reisolated from a single conidium and then reanalyzed by PCR with all three primer pairs.

Complementation of the *sge1* mutant.

The *SGE1* complementation plasmid pSGE1C was constructed by first cloning a 2,446-bp PCR fragment that spanned from 1,101 bp upstream of the predicted start codon of *F. verticillioides* *SGE1* to 359 bp downstream of the predicted *SGE1* stop codon. The fragment was amplified from genomic DNA of the wild-type strain with primers 2145 and 2146, then cloned into the pT7-Blue-3 cloning vector (pT7Blue perfectly blunt cloning kit, EMD Chemicals, Darmstadt, Germany). A fragment containing the geneticin resistance gene *GenR* was then cloned into the *NotI* site, creating the complementation plasmid pSGE1C (Bluhm et al. 2008). pSGE1C was introduced into the genome of *sge1* mutant strain Δ*SGE1*.12 via protoplast-mediated transformation. Transformants were identified by their ability to grow in the presence of geneticin (Gibco) at 300 µg/ml, and by PCR analysis to determine whether they contained *SGE1* with primers 2145 and 2146. 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 pSGE1C.

Generation of *SGE1* inducible-expression strain.

Generation of a xylose-inducible *SGE1* expression vector (pXLN2P::SGE1) employed a previously constructed plasmid, pXLN2P (D. W. Brown, unpublished), that includes 900 bp of DNA corresponding to the region immediately upstream of the *F. verticillioides* xylanase gene *XLN2* (FVEG_12502) coding

region (i.e., the *XLN2* promoter). pXLN2P also includes *AscI* sites, such that coding regions of genes can be cloned into it immediately downstream of the *XLN2* promoter via *AscI* digestion and ligation. A 1,276-bp fragment corresponding to the *SGE1* coding region and terminator sequence was amplified from wild-type *F. verticillioides* genomic DNA with primers 1496 and 1497 and cloned into the pT7-Blue-3 cloning vector. Both primer sequences included an *AscI* site that facilitated cloning of the DNA fragment downstream of the *XLN2* promoter in pXLN2P (Fig. 2). The resulting plasmid, pXLN2P::SGE1, was introduced into the genome of strain Δ*SGE1*.12 via protoplast-mediated transformation. To do this, plasmid pXLN2P::SGE1 was co-transformed into protoplasts along with plasmid pKS-Gen, which carries the geneticin resistance gene *GenR*, so that transformants could be selected by their ability to grow in the presence of geneticin (Gibco) at 300 µg/ml.

Geneticin-resistant transformants were subjected to two PCR screens to determine whether they carried the *GenR* gene and the chimeric *XLN2* promoter::SGE1 (*SGE1* inducible-expression) construct pXLN2P::SGE1. The first screen employed primers 649rb and 650rb, which amplify a 634-bp fragment from *GenR*. The second PCR screen employed primers 1531 and 574rb, which amplify a 1,291-bp amplicon that spans the *XLN2* promoter and *SGE1* elements of pXLN2P::SGE1 (Fig. 2). Transformants that yielded the expected amplicons in both PCR screens were reisolated from a single conidium, and the resulting isolates were reanalyzed by PCR to confirm the presence of *GenR* and the inducible-expression construct.

Deletion of *SGE1* regulated genes.

Mutation of seven *F. verticillioides* genes positively regulated by *SGE1* was accomplished by deletion of their predicted open reading frames via the split-marker, as described above for *SGE1*. For FVEG_02173, FVEG_02431, *KPL5*, and *KPL6*, the deletion amplicons corresponded to an approximately 800-bp region upstream of the respective start codon and an approximately 800-bp region downstream of the respective stop codon. The simultaneous deletion of *KPL1*, *KPL2*, and *KPL3*, corresponding to 3.2 kb of genomic sequence, was accomplished in a similar manner. In the latter case, the first amplicon corresponded to an approximately 800-bp region downstream from the *KPL1* stop codon, while the second amplicon corresponded to an approximately 800-bp region downstream from the *KPL3* stop codon. Hygromycin-resistant transformants, isolated after growth on V8 agar containing hygromycin B (Sigma-Aldrich) at 300 µg/ml, were subjected to three PCR screens to determine whether the targeted genes' coding region had been deleted and replaced by *HygB*, as described above.

Maize seedling assays.

Seedling assays were carried out essentially as described previously (Glenn et al. 2008; Larson et al. 2011) with the hybrid white corn Silver Queen. Fungicide-free seed were obtained from Johnny's Selected Seeds, Winslow, ME, U.S.A. Seed were surface sterilized by soaking in 100% commercial bleach (6.25% sodium hypochlorite) for 10 min, rinsed with sterile water four times, allowed to soak for 4 h in sterile water, and then heat shocked at 60°C for 5 min. Thirty seeds were then incubated at 27°C overnight in 10 ml of water containing *F. verticillioides* at 1×10^6 conidia/ml, with or without 50 mM D-xylose (Sigma). Ten seeds were then sown in autoclaved growing mix (Sunshine Redi-earth, Bellevue, WA, U.S.A.) contained in 10-cm plastic pots. Pots were placed in a growth chamber with the following conditions: 14 h of light at 30°C

and 10 h of darkness at 20°C. Disease symptoms were assessed after 16 days by counting the number of plants and measuring height of each plant from soil to distal end of longest leaf. The inoculum for the modified maize seedling assay was prepared by first cutting dried maize stalks lengthwise and then into approximately 3-cm pieces. The stalks were autoclaved twice for an hour each time prior to inoculation. The stalks were incubated at 28°C for 6 days to allow ample growth. Five stalk pieces were placed into the top approximately 6 cm of autoclaved growing mix per pot. After 24 h, 10 washed seeds were sown per pot around the stalks.

Expression analysis.

For gene expression analyses, strains of *F. verticillioides* were grown in 25 ml of SYP in 125-ml flasks at 28°C and 200 rpm. Cultures were initiated by adding conidia to the SYP medium at final concentration of 1×10^6 conidia/ml. The inducer, 50 mM D-xylose, was added 18 h after culture initiation. Mycelia were harvested by vacuum filtration and flash frozen in liquid nitrogen. RNA samples for RT-PCR analysis were prepared from cultures harvested 5, 15, and 30 min after addition of D-xylose, while RNA samples for microarray and qPCR analysis were prepared from cultures harvested 5, 30, and 360 min after addition of D-xylose. Total RNA was extracted from mycelia using the RNeasy plant mini kit (Qiagen Inc., Valencia, CA, U.S.A.). RNA quality was checked using the 2100 Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, U.S.A.) and quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Prior to RT-PCR, DNA was removed from 25- μ l samples of RNA by digestion with DNase (Thermo Fisher Scientific Turbo DNA-Free kit). Approximately 80 ng of RNA served as a template per PCR reaction using the Agilent Easy-A 1-Tube RT-PCR kit (Agilent Technologies, Santa Clara, CA, U.S.A.).

Microarrays were designed and produced by Roche NimbleGen (Madison, WI, U.S.A.) based on 14,383 *F. verticillioides* gene models obtained from the *Fusarium* Comparative Database and unique EST from the *F. verticillioides* Gene Index. Each sequence was represented on the array by up to six unique 60-mer probes. The probe set was represented twice on each array at randomly assigned positions to provide technical replication. Microarray hybridization, data acquisition, and initial analyses were conducted by Roche NimbleGen, Reykjavik, Iceland. Data were normalized by robust multiarray average (Bolstad et al. 2003; Irizarry et al. 2003) and were compared using Acuity 4.0 microarray analysis software (Molecular Devices Corp, Sunnyvale, CA, U.S.A.). The *SGE1* experiment (GEO accession number GSE56664) was conducted with RNA extracted from three biological replicates for the wild-type strain M-3125, the *sgel* mutant strain Δ SGE1.12, and *SGE1* induced-expression strain GMTcp-272 30 min after the addition of xylose.

qPCR was carried out in triplicate for three biological replicates for each gene. Single-stranded DNA was generated from RNA isolated from mycelia harvested after 5, 30, and 360 min after the addition of xylose to the medium using the Quantitect RT kit (Qiagen Inc.). PCR reactions were carried out using the Rotor-Gene PCR machine (Qiagen Inc.) utilizing the Rotor-Gene SYBR Green kit (Qiagen Inc.) in 25 μ l. Reaction conditions followed the manufacturer's recommendations: 95°C for 5 min to activate the HotStarTaqDNA 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 products were produced. Concentration of DNA in nanograms per microliter were determined by comparison to a standard curve generated for the β -tubulin gene (FVEG_04081) using primers 490rp and 491rp. The means of three subsamples per flask were used so that three flasks per

strain-time interaction were treated as the replication. A completely randomized design mixed-model, two-factor ANOVA was performed comparing calculated concentration (nanograms per microliter) between three strains (the wild type, the *sgel* mutant, and the *SGE1* inducible-expression strain) at 5, 30, and 360 min separately for four genes (*FUS1*, *FUS8*, FVEG_00078, and FVEG_00079). Levene's homogeneity of variance tests were performed to determine whether data transformation was necessary prior to ANOVA. All analyses were performed on transformed data where necessary. Treatment comparisons were made using differences of least squares means with a Bonferroni adjustment when significant *F* test values from the ANOVA were obtained. SAS 9.2 (TS2M3) software was used for all analyses. Outlier values were removed (*FUS1*:Wt-5 min C, Wt-360 min B, GMTcp-272-5 min A, GMTcp-272-30 min C; FVEG_00078-Wt-360 min B, Δ SGE-360 min B, and GMTcp-272-5 min A) so that log transformations could stabilize variances for all four genes before ANOVAs could be run.

Analysis of SM.

Fumonisin and fusarins were extracted from 2.5 g of cracked maize kernel cultures with 12 ml of acetonitrile/water (84:16, vol/vol). Cultures were allowed to steep for 2 h, with gentle shaking, and then extracts were filtered through a 0.45- μ m-pore-size Gelman filter. The culture extracts were then analyzed for fumonisin FB₁, FB₂, and FB₃ and fusarin C by LC-MS/MS, essentially as previously described (Brown et al. 2012; Plattner et al. 1996). In brief, 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 μ l of analyte were eluted from a Metachem (Torrance, CA, U.S.A.) Inertsil C18 3 by 150 mm column with a 300 μ l/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 to 10 min, 35% MeOH; 10 to 25 min, 35 to 95% MeOH; 25 to 26 min, 95% MeOH; 26 to 34 min, 95 to 35% MeOH; and 34 to 35 min, 35% MeOH. ESI-MS/MS detection of fusarin C was accomplished by monitoring three characteristic fragment ions (*m/z* 364, 382, and 396) of the *m/z* 432 [*M* + *H*]⁺ ion of fusarin C in multiple reaction monitoring mode. Quantization of fusarin C was done on the basis of the integrated intensity of the *m/z* 364 fragment compared with a calibration curve generated from fusarin C standard solutions. Signal for LC-MS/MS determination of fusarin C may contain multiple products from the decomposition of fusarin C during the LC-MS analysis. Fusaric acid was extracted from cracked maize cultures with 12 ml of acetonitrile/water (50:50, vol/vol) acidified to pH 2 with HCl and analyzed by LC-MS/MS. Extract analysis was accomplished essentially as described (Brown et al. 2012). Fusaric acid standard was obtained from Sigma-Aldrich.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Fusarium Comparative database:
www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html
- Dana-Farber Cancer Institute-Harvard School of Public Health
F. verticillioides Gene Index database:
compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=f_verticill