The novel fungal-specific gene *FUG1* has a role in pathogenicity and fumonisin biosynthesis in *Fusarium verticillioides*

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SUMMARY

Fusarium verticillioides is a globally important pathogen of maize, capable of causing severe yield reductions and economic losses. In addition, *F. verticillioides* produces toxic secondary metabolites during kernel colonization that pose significant threats to human and animal health. *Fusarium verticillioides* and other plant-pathogenic fungi possess a large number of genes with no known or predicted function, some of which could encode novel virulence factors or antifungal targets. In this study, we identified and characterized the novel gene *FUG1* (*Fungal Unknown Gene 1*) in *F. verticillioides* through functional genetics. Deletion of *FUG1* impaired maize kernel colonization and fumonisin biosynthesis. In addition, deletion of *FUG1* increased sensitivity to the antimicrobial compound 2-benzoxazolinone and to hydrogen peroxide, which indicates that *FUG1* may play a role in mitigating stresses associated with host defence. Transcriptional profiling via RNA-sequencing (RNA-seq) identified numerous fungal genes that were differentially expressed in the kernel environment following the deletion of *FUG1*, including genes involved in secondary metabolism and mycelial development. Sequence analysis of the Fug1 protein provided evidence for nuclear localization, DNA binding and a domain of unknown function associated with previously characterized transcriptional regulators. This information, combined with the observed transcriptional reprogramming in the deletion mutant, suggests that *FUG1* represents a novel class of fungal transcription factors or genes otherwise involved in signal transduction.

Keywords: benzoxazinoids, DIMBOA, *Fusarium* ear rot, gene regulation, mycotoxin.

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (Nirenberg, 1976) is a versatile pathogen of maize (*Zea mays*), causing seedling disease, *Fusarium* stalk rot and *Fusarium* ear rot (Munkvold, 2003; White, 1999). Infected maize kernels are frequently contaminated with mycotoxins, such as fumonisins, fusaric acid and fusarins (Brown *et al.*, 2012; Desjardins *et al.*, 2002). Of these, fumonisins are

arguably the most important (Nelson *et al.*, 1993; Woloshuk and Shim, 2013). Fumonisins are polyketide-derived compounds linked to acute and chronic toxicoses in livestock, as well as neural tube birth defects and oesophageal cancer in humans (Proctor *et al.*, 2003; Rheeder *et al.*, 2002; Stockmann-Juvala and Savolainen, 2008). As a result of these health risks, the presence of fumonisins in grain and foodstuffs is regulated by governmental agencies worldwide (Woloshuk and Shim, 2013).

Despite the large number of filamentous fungi present in the environment, relatively few cause kernel-rotting diseases of maize (White, 1999). *Fusarium verticillioides* predominantly gains access to kernels via natural entry points (e.g. silk scars) or physical injury associated with growth cracks, mechanical damage or insect feeding (Duncan and Howard, 2010; Oren *et al.*, 2003; Sobek and Munkvold, 1999; Warfield and Davis, 1996). In addition, *F. verticillioides* is capable of systemically colonizing plant tissues and entering kernels through the rachis (Foley, 1962; Kedera *et al.*, 1994). Interestingly, *F. verticillioides* is frequently associated with maize as an asymptomatic endophyte (Bacon and Hinton, 1996; Bacon *et al.*, 2008); thus, disease is not necessarily the outcome of the interaction between the fungus and the plant. The biological mechanism underlying the transition from an endophytic to a pathogenic lifestyle in *F. verticillioides* is not known; however, the capacity of the fungus to be an endophyte or pathogen is probably predicated on extensive transcriptional reprogramming. Despite progress in the elucidation of the molecular basis of pathogenesis and fumonisin biosynthesis (Woloshuk and Shim, 2013), significant gaps persist in the current understanding of how *F. verticillioides* senses and responds to the maize kernel environment.

Although the number of sequenced fungal genomes has increased dramatically in recent years, functional characterization of novel fungal genes has not progressed at a similar rate. In the model fungus *Neurospora crassa*, approximately 40% of the predicted protein-encoding genes currently lack functional annotation (Ellison *et al.*, 2014). Similarly, only 55% of the predicted genes in *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch of wheat, are associated with a specific function (Goodwin *et al.*, 2011). A substantial number of functionally uncharacterized genes in plant-pathogenic fungi could represent novel virulence or pathogenicity factors. Thus, the characterization of genes of unknown function is critical to the elucidation of the genetic basis of plant pathogenesis and for the development of new strategies for disease control.

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The purpose of the current study was to identify and characterize novel genes in *F. verticillioides* involved in maize kernel pathogenesis. To this end, 12 uncharacterized genes in the *F. verticillioides* genome were selected based on a lack of sequence or structural homology with any functionally characterized gene or protein and evidence for expression [e.g. presence in microarray or RNA-sequencing (RNA-seq) datasets]. The 12 genes were functionally assessed via targeted gene deletion and molecular genetics. Preliminary analyses indicated that the gene FVEG_04008 was involved in maize kernel colonization. Subsequently, FVEG_04008 was designated as *Fungal Unknown Gene 1 (FUG1)* and its involvement in pathogenesis was characterized in detail.

RESULTS

***FUG1* of *F. verticillioides* encodes an uncharacterized, fungal-specific protein**

The *F. verticillioides* genome is predicted to contain 15 869 protein-encoding genes (Broad Institute), 50.3% of which have no known or predicted molecular function. In this study, 12 genes of unknown function were selected for analysis based on four criteria: (i) lack of overall sequence homology with any functionally characterized gene; (ii) lack of overall sequence or structural homology with any functionally characterized protein; (iii) absence of characterized functional domains, and (iv) evidence of expression based on the existence of expressed sequence tags (ESTs) or representation in RNA-seq datasets (Table S1, see Supporting Information). For functional analyses, each gene was targeted for deletion via homologous recombination. An initial assessment indicated that 11 of the 12 genes were not involved in pathogenesis on maize kernels (data not shown). However, deletion mutants of one gene, FVEG_04008, were substantially impaired in kernel colonization, and thus this gene was selected for further functional analysis.

Conceptual translation of FVEG_04008 revealed a protein of 561 amino acids with a molecular weight of 61.5 kDa and an isoelectric point of 7.2. Reciprocal protein–protein BLAST analyses (BLASTP; Altschul *et al.*, 1997) indicated that orthologues of the FVEG_04008 protein are well conserved among ascomycete fungi and are present predominantly as a single copy (data not shown). Phylogenetic analysis of putative FVEG_04008 orthologues produced a tree topography corresponding closely to the branching order expected for the Ascomycota (Schoch *et al.*, 2009) (Fig. 1). In addition, orthologue sequence identity clustered in a manner similar to the reconstructed phylogeny (Fig. 1). Notably, no putative orthologue of FVEG_04008 was identified in *Saccharomyces cerevisiae* or *Candida albicans* (Fig. 1). Thus, as a result of the lack of functional annotation or predicted orthology outside the

ascomycetes, FVEG_04008 was designated *Fungal Unknown Gene 1 (FUG1)*.

The Fug1 protein contains two domains of unknown function (DUFs)—DUF1752 and DUF3295 (Fig. S1A, see Supporting Information)—both of which are unique to fungal proteins. Homology searches revealed that DUF1752, in association with a GATA zinc finger domain, is present in several functionally characterized transcriptional regulators (e.g. Nit2 in *N. crassa*, AreA in *Aspergillus nidulans*, and Gat1, Reg1 and Mks1 in *S. cerevisiae*). However, the function of DUF1752 is not known. DUF3295 was not found in any functionally characterized protein. In *F. verticillioides*, DUF3295 is only present in the Fug1 protein. Similarly, in other ascomycetes (e.g. *Schizosaccharomyces pombe*, *N. crassa* and *F. graminearum*), DUF3295 is limited to putative orthologues of Fug1.

Cellular localization can provide important clues with regard to the molecular function of proteins. Sequence analysis of Fug1 did not identify an N-terminal signal peptide associated with secretion, and no evidence was found for membrane-spanning α -helices. However, the computational tools LocTree3, CELLO and WoLF PSORT predicted that Fug1 localizes to the nucleus (Goldberg *et al.*, 2014; Horton *et al.*, 2007; Yu *et al.*, 2006). In addition, Fug1 contains a putative bipartite nuclear localization signal near the C-terminus of the protein (amino acid residues 511–540) (Fig. S1A).

As a result of the presence of DUF1752 in transcriptional regulators known to bind DNA and evidence for nuclear localization, the Fug1 protein was assessed for DNA-binding potential. A majority consensus of 114 potential DNA-binding sites was identified with the programs BindN+, DBS-PRED and DP-Bind (Ahmad *et al.*, 2004; Hwang *et al.*, 2007; Wang *et al.*, 2010) (Fig. S1B). Notably, clusters of potential DNA-binding sites were identified in DUF1752, as well as in proximity to and overlapping the putative nuclear localization signal in Fug1 (Fig. S1B).

Deletion of *FUG1* in *F. verticillioides*

FUG1 was deleted via a split-marker approach, wherein the predicted open reading frame (ORF) was replaced with a hygromycin resistance cassette (Fig. S2A,B, see Supporting Information). Two independent deletion strains (FV020 and FV021; Table S2, see Supporting Information) were chosen for phenotypic analyses. Strain FV020 was genetically complemented with the wild-type *FUG1* locus to produce strain FV022 (Fig. S2B; Table S2). Deletion of *FUG1*, the absence of ectopic integration of the hygromycin resistance cassette and genetic complementation were confirmed by polymerase chain reaction (PCR) and Southern analyses (Fig. S2B). Deletion strains FV020 and FV021 were phenotypically indistinguishable on various growth media, and genetic complementation of the wild-type locus recapitulated the wild-type phenotypes (Fig. S2C,D). The phenotypic similarity between the independent *FUG1* deletion strains and restoration of the wild-type phenotype following complementation provided genetic

Fig. 1 Phylogenetic reconstruction reveals broad conservation of Fug1 within the Ascomycota. Branches having bootstrap values $\geq 70\%$ are presented in bold. Protein identity represents global amino acid sequence identity for each protein compared with Fug1.

evidence that deletion of *FUG1* was responsible for the phenotypes observed in this study.

***FUG1* is required for wild-type levels of maize kernel colonization**

In maize kernel pathogenicity assays, the wild type and genetic complement extensively colonized inoculated kernels,

as well as kernels in adjacent rows (Fig. 2A). In contrast, the *FUG1* deletion strain grew substantially less well in inoculated kernels, failed to colonize adjacent kernels and produced fewer aerial hyphae (Fig. 2A). In addition, the *FUG1* deletion strain was unable to colonize the germ tissues of the kernel (Fig. 2B). These results indicate that *FUG1* plays an important role in maize kernel colonization during pathogenesis.

Fig. 2 Deletion of *FUG1* (*Fungal Unknown Gene 1*) in *Fusarium verticillioides* impairs pathogenesis in maize kernels. Strains 7600, FV020 and FV022 were assessed for growth in maize kernels. (A) Visual assessment of external kernel colonization. (B) Visual assessment of internal kernel colonization. Arrows indicate location of germ tissue.

RNA-seq identifies numerous genes regulated by *FUG1* in the kernel environment

RNA-seq identified 1241 differentially expressed genes in the *FUG1* deletion background during maize kernel colonization [false discovery rate (FDR)-corrected $P \leq 0.01$ and fold change in expression ≥ 2] (Table 1). Of these, 493 genes were up-regulated and 748 were down-regulated (Table 1). Patterns of differential expression were similar among biological replicates of each genotype (Fig. 3A). The range of differential expression was broad, with approximately 52% of differentially expressed genes exhibiting fold changes of ≥ 4 (Table 1). Certain functional terms were significantly over-represented in the differentially expressed genes, including protein citrullination, carbohydrate metabolism and protein–arginine deiminase activity among the up-regulated genes, and electron carrier activity, haem binding and oxidoreductase activity among the down-regulated genes (Fig. 3B; Tables S5 and S6, see Supporting Information).

Table 1 Numbers of *Fusarium verticillioides* genes differentially expressed [false discovery rate (FDR)-corrected $P < 0.01$] in the *FUG1* (*Fungal Unknown Gene 1*) deletion strain during kernel colonization.

7600 (wild type) vs. FV020 (<i>FUG1</i> deletion)			
Fold change	Differentially expressed	Up-regulated	Down-regulated
≥ 2 to < 4	600	306	294
≥ 4 to < 6	258	102	156
≥ 6 to < 8	132	35	97
≥ 8	251	50	201
Total	1241	493	748

Deletion of *FUG1* alters the expression of genes involved in secondary metabolism

The RNA-seq data revealed the misregulation of key genes involved in secondary metabolism resulting from the deletion of *FUG1*. Three of 18 genes encoding polyketide synthase enzymes and three of 11 genes encoding non-ribosomal peptide synthetase-like enzymes were differentially expressed in the *FUG1* deletion strain (Table 2), including *FUM1* (FVEG_00316), the polyketide synthase responsible for fumonisin biosynthesis (Table 2). Furthermore, the *F. verticillioides* genome encodes seven non-ribosomal peptide synthetases, one terpene synthase and one dimethylallyl tryptophan synthase; however, the genes encoding these enzymes were not differentially expressed in the *FUG1* deletion strain (Table 2). With the exception of fumonisin, the secondary metabolites produced by the differentially expressed cluster genes have yet to be determined. These results indicate that the deletion of *FUG1* impacts the secondary metabolome of *F. verticillioides*.

FUG1 regulates the biosynthesis of fumonisin mycotoxins

RNA-seq indicated that *FUM* genes were among the most differentially expressed in the *FUG1* deletion strain during maize kernel pathogenesis (Fig. 3C,E), which was confirmed by subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses (Fig. 3D). To investigate the association between reduced *FUM* gene expression and fumonisin accumulation, fumonisins were quantified in colonized maize kernels. The biosynthesis of fumonisin B₁ (FB₁), FB₂ and FB₃ was substantially lower in the *FUG1* deletion strain than in the wild type (Fig. 4A). When normalized to fungal growth (as measured by ergosterol; Fig. 4B), levels of FB₁, FB₂ and FB₃ were approximately 35-fold lower, 15-fold lower and 22-fold lower, respectively, in the *FUG1* deletion strain (Fig. 4C). Interestingly, the percentage of FB₂ in relation to total fumonisins measured was greater in the *FUG1* deletion strain than in the wild type or the genetic complement (Fig. 4D). Taken together, these results indicate that *FUG1* is involved at some level in the regulation of fumonisin biosynthesis during the colonization of maize kernels.

Fig. 3 RNA-sequencing (RNA-seq) identifies numerous differentially regulated fungal genes in the kernel environment following *FUG1* (*Fungal Unknown Gene 1*) deletion. (A) Hierarchical clustering of the 1241 differentially expressed genes illustrates similar patterns in gene expression among biological replications. (B) Significantly over-represented functional terms in up- and down-regulated genes in the *FUG1* deletion strain compared with the wild type. Functional terms are represented as Gene Ontology (GO) terms. Significantly over-represented terms were reduced to most specific terms. Black bars represent GO terms associated with biological processes, and white bars represent GO terms associated with molecular functions. Vertical broken lines represent a statistical cut-off of $P < 0.05$ based on false discovery rate (FDR)-corrected *P* values. (C) Volcano plot comparing gene expression [$\log_2$ (fold change)] and significance ($-\log_{10}$ of FDR-corrected *P* values) in the *FUG1* deletion strain compared with the wild type. Significantly down-regulated genes are shown in green. Significantly up-regulated genes are shown in red. A statistical cut-off of $P < 0.01$ based on FDR-corrected *P* values is represented as a horizontal broken line. Fold changes of 2.0 and -2.0 are represented as vertical broken lines. Blue triangles represent fumonisin biosynthetic genes. The polyketide synthase (*FUM1*) and cluster-specific transcription factor (*FUM21*) are labelled. (D) Expression of *FUM1* and *FUM21* was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Bars indicate standard deviation. (E) Unclustered heatmap illustrating differential expression of genes in the fumonisin biosynthetic cluster following the deletion of *FUG1*. Expression values are presented as 'Row Z-score'.

Table 2 Effect of *FUG1* (*Fungal Unknown Gene 1*) deletion on the expression of key biosynthetic genes in secondary metabolite gene clusters.

Gene class		Backbone gene	Cluster product	log ₂ (fold change)	FDR-corrected <i>P</i> value
Non-reducing PKS		FVEG_03695.5	Perithecial pigment	nd	na
		FVEG_03379.5	Bikaverin	−0.19	0.83
Reducing PKS	Clade I	FVEG_10535.5*	Unknown	1.35	1.69E-04
		FVEG_05537.5*	Unknown	−5.41	7.15E-21
		FVEG_08425.5	Unknown	−0.12	na
		FVEG_14911.5 [†]	Unknown	0.40	na
		FVEG_00316.5*	Fumonisin	−4.28	5.31E-29
		FVEG_17587.5 [†]	Unknown	−0.53	na
	Clade II	FVEG_12610.5	Unknown	−0.76	na
		FVEG_11932.5	Unknown	−0.21	0.78
		FVEG_10497.5	Unknown	−0.12	na
		FVEG_11086.5	Fusarin	−0.14	0.85
	Clade III	FVEG_16698.5 [†]	Unknown	2.03	na
		FVEG_00079.5	Unknown	−0.36	0.71
		FVEG_12523.5	Fusaric acid	−0.24	0.73
		FVEG_01914.5	Unknown	−0.75	0.64
TS		FVEG_10718.5	Unknown	−1.79	0.13
NRPS		FVEG_06496.5	Unknown	−2.21	0.10
		FVEG_08697.5	Unknown	−0.22	0.74
		FVEG_09864.5	Unknown	1.31	na
		FVEG_16073.5 [†]	Unknown	nd	na
		FVEG_10547.5	Unknown	0.05	0.97
		FVEG_11762.5	Unknown	−0.74	0.17
		FVEG_12610.5	Unknown	−0.76	na
NRPS-like		FVEG_00238.5	Unknown	−1.44	1.04E-02
		FVEG_03243.5	Unknown	−0.84	0.30
		FVEG_03415.5	Unknown	nd	na
		FVEG_15444.5 [†]	Unknown	0.59	0.27
		FVEG_05643.5*	Unknown	1.76	7.89E-06
		FVEG_05723.5	Unknown	0.06	0.96
		FVEG_06502.5	Unknown	−1.31	na
		FVEG_08545.5	Unknown	−0.91	0.29
		FVEG_12503.5	Unknown	nd	na
		FVEG_14029.5*	Unknown	−3.71	5.59E-04
DMAT		FVEG_12218.5	Unknown	0.70	0.15

*Gene expression significantly different in the *FUG1* deletion strain compared with the wild-type strain based on a false discovery rate (FDR)-corrected $P < 0.05$ and an absolute log₂(fold change) ≥ 1.0 .

[†]Current gene annotation differs from previous version: FVEG_14911.5 = FVEG_01736.3; FVEG_17587.5 = FVEG_13715.3; FVEG_16073.5 = FVEG_09993.3; FVEG_15444.5 = FVEG_04296.3.

DMAT, dimethylallyl tryptophan synthase; na, not applicable because of variability among replications; nd, not detected; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; TS, terpene synthase; Unknown, cluster product has yet to be determined.

To investigate the potential mechanisms through which *FUG1* regulates *FUM* gene expression, the expression of genes previously shown to influence fumonisin biosynthesis was examined (Table S7, see Supporting Information; references listed therein). The majority of these genes (e.g. *FCC1*, *HXX1*, *SGE1*, *ZFR1*) were not differentially regulated (Table S7). Two genes (*AMY1* and *FLBA1*) were regulated in a manner that was not consistent with the expected effect on fumonisin biosynthesis. *AMY1* and *FLBA1* have been shown previously to exert a positive regulatory effect on fumonisin biosynthesis (Bluhm and Woloshuk, 2005; Mukherjee *et al.*, 2011). However, expression of both *AMY1* and *FLBA1* was up-regulated in the *FUG1* deletion strain compared with the wild type (Table S7). In contrast, the expression of *AREA*, a global regu-

lator of nitrogen metabolism that also positively regulates fumonisin biosynthesis (Kim and Woloshuk, 2008), was significantly down-regulated. Thus, it is possible that *FUG1* regulates fumonisin biosynthesis through the direct or indirect regulation of *AREA*.

Deletion of *FUG1* impacts the expression of genes involved in 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) biosynthesis in maize kernels and alters fungal tolerance to a DIMBOA derivative *in vitro*

Maize genes differentially expressed in response to the *FUG1* deletion strain versus the wild type were identified and analysed.

Fig. 4 Deletion of *FUG1* (Fungal Unknown Gene 1) in *Fusarium verticillioides* reduces fumonisin accumulation in maize kernels. Strains 7600, FV020 and FV022 were assessed for growth and fumonisin accumulation in maize kernels. (A) Quantification of fumonisin B₁ (FB₁; left), fumonisin B₂ (FB₂; centre) and fumonisin B₃ (FB₃; right) in infected maize kernels. (B) Quantification of growth in maize kernels as determined by ergosterol accumulation. (C) Determination of FB₁ (left), FB₂ (centre) and FB₃ (right) in infected maize kernels relative to fungal growth. All bars indicate standard deviation. (D) Determination of percentage fumonisin analogue relative to total fumonisin content in infected maize kernels. Black bars represent FB₁, grey bars represent FB₂ and white bars represent FB₃.

A total of 1871 maize genes were significantly differentially expressed in kernels infected with the *FUG1* deletion strain (FDR-corrected $P \leq 0.01$ and fold change ≥ 2) (Table 3). Of these, 142 genes were significantly up-regulated and 1729 were significantly down-regulated (Table 3).

Maize produces a suite of low-molecular-weight, cyclic hydroxamic acids [i.e. the benzoxazinoids DIMBOA and 2,4-dihydroxy-2H-1,4-benzoxazin-3-one (DIBOA)] that function as antimicrobials (Glenn *et al.*, 2001; Niemeyer, 1988). RNA-seq data revealed that genes involved in DIMBOA biosynthesis were highly differentially expressed in response to the *FUG1* deletion strain compared with the wild type (Table 4). Specifically, the majority of the DIMBOA biosynthetic genes were significantly down-regulated in maize infected with the *FUG1* deletion strain (Table 4).

Table 3 Numbers of maize genes differentially expressed [false discovery rate (FDR)-corrected $P < 0.01$] in kernel tissue infected with a *Fusarium verticillioides* *FUG1* (Fungal Unknown Gene 1) deletion strain.

Maize infected with 7600 (wild type) vs. maize infected with FV020 (*FUG1* deletion)

Fold change	Differentially expressed	Up-regulated	Down-regulated
≥ 2 to <4	1304	133	1171
≥ 4 to <6	369	8	361
≥ 6 to <8	110	1	109
≥ 8	88	0	88
Total	1871	142	1729

To explore the potential link between DIMBOA biosynthesis and *FUG1*, the deletion strain was assessed *in vitro* for sensitivity to 2-benzoxazolinone (BOA). DIMBOA and DIBOA are spontaneously converted to MBOA (6-methoxy-2-benzoxazolinone) and BOA (Niemeyer, 1988), respectively, both of which retain antimicrobial activity (Glenn *et al.*, 2001). The *FUG1* deletion strain was

Table 4 Effect of *FUG1* (Fungal Unknown Gene 1) deletion on the expression of genes required for the production of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA).

Gene	Gene accession	$\log_2$ (fold change)	FDR-corrected P value
<i>bx1</i> *	GRMZM2G085381	-6.52	3.54E-59
<i>bx2</i> *	GRMZM2G085661	-2.10	2.91E-03
<i>bx3</i> *	GRMZM2G167549	-2.38	2.99E-05
<i>bx4</i> *	GRMZM2G172491	-2.11	1.93E-04
<i>bx5</i> *	GRMZM2G063756	-2.73	8.90E-07
<i>bx6</i>	GRMZM6G617209	-2.14	na
<i>bx7</i> *†	GRMZM2G017557	-2.49	2.17E-04
<i>bx8</i> *	GRMZM2G085054	-1.37	1.03E-08
<i>bx9</i> *	GRMZM2G161335	-1.62	7.20E-05
<i>glu1</i> *	GRMZM2G016890	-3.46	1.78E-13
<i>glu2</i> *	GRMZM2G008247	-1.08	3.32E-02

*Gene expression significantly different in the *FUG1* deletion strain compared with the wild-type strain based on a false discovery rate (FDR)-corrected $P < 0.05$ and an absolute $\log_2$ (fold change) ≥ 1.0 .

†*bx7* is synonymous with *zrp4*.

na, not applicable because of variability among replications.

Fig. 5 Deletion of *FUG1* (*Fungal Unknown Gene 1*) alters tolerance to the antimicrobial 2-benzoxazinone (BOA) and hydrogen peroxide, but not cell wall stress. (A) Strains 7600, FV020 and FV022 were assessed for tolerance to the antimicrobial compound BOA. PDA, potato dextrose agar. (B) Strains 7600, FV020 and FV022 were assessed for tolerance to oxidative stress. Visual assessment of growth inhibition 24 h after exposure to H₂O₂. (C) Zone of inhibition, measured as the diameter of growth inhibition, in response to H₂O₂. Bars indicate standard error. (D) Strains 7600, FV020 and FV022 were assessed for tolerance to cell wall stress. CR, Congo Red; SDS, sodium dodecylsulfate; YMA, sucrose yeast extract malt extract agar.

significantly more sensitive than the wild type or the genetic complement to BOA (Fig. 5A).

Deletion of *FUG1* increases sensitivity to oxidative stress

A significant decrease in tolerance to oxidative stress, as determined by an increase in growth inhibition during exposure to H₂O₂, was observed in the *FUG1* deletion strain compared with the wild type or the genetic complement (Fig. 5B). When quantified, a 50% increase in growth inhibition in the presence of H₂O₂ was observed following *FUG1* deletion (Fig. 5C). Notably, no significant difference in growth was observed in the *FUG1* deletion strain under general cell wall stress (Fig. 5D). The reduction in tolerance to oxidative stress highlights another possible component of reduced kernel colonization observed following *FUG1* deletion.

Deletion of *FUG1* impairs conidiation

The *FUG1* deletion strain produced approximately 160-fold fewer microconidia than the wild type or the genetic complement (Fig. 6A). The reduction in microconidia resulted from the formation of fewer conidial chains and fewer conidia per chain (Fig. 6B). In addition, the *FUG1* deletion strain failed to produce macroconidia under conducive conditions, whereas the wild type and genetic complement produced macroconidia prolifically (data not shown).

To investigate the cause of impaired conidiation in the *FUG1* deletion strain, the expression of conidiation-related genes was examined in the RNA-seq data. Surprisingly, a number of genes

involved in conidiogenesis in *F. verticillioides* (e.g. *ZFR1*, *HTF1*, *STUA* and *GAP1*) were not differentially expressed in the *FUG1* deletion strain (Table S7; data not shown) (Bluhm *et al.*, 2008; Malapi-Wight *et al.*, 2014; Sagaram *et al.*, 2007; Zheng *et al.*, 2012). However, a notable exception was *ADA1*, which was significantly down-regulated in the *FUG1* deletion strain [$\log_2$ (fold change) = -1.11 and FDR-corrected $P = 5.86 \times 10^{-4}$]. *ADA1* encodes an orthologue of FlbC in *A. nidulans* that has been shown to play an important role in the regulation of asexual development in *F. verticillioides* (Malapi-Wight *et al.*, 2014). In addition, genes encoding putative orthologues of FluG and MedA, components of the *A. nidulans* conidiation pathway, were significantly up-regulated in the *FUG1* deletion strain [$\log_2$ (fold change) = 2.85 and FDR-corrected $P = 3.05 \times 10^{-4}$, and $\log_2$ (fold change) = 1.06 and FDR-corrected $P = 3.79 \times 10^{-3}$, respectively]. Lastly, a homologue of *con-10*, which encodes conidiation-specific protein 10 in *N. crassa*, was significantly down-regulated [$\log_2$ (fold change) = -3.22 and FDR-corrected $P = 1.91 \times 10^{-11}$]. Together, these results suggest that *FUG1* is important for asexual reproduction in *F. verticillioides* and identifies a potential link between *FUG1* and known regulators of conidiogenesis.

FUG1 is required for the maintenance of mycelial hydrophobicity

FUG1 deletion mutants formed fewer aerial hyphae and were less flocculent on defined media compared with the wild type or genetic complement (Figs S2C, 6C). Because conidiogenesis and

Fig. 6 Deletion of *FUG1* (*Fungal Unknown Gene 1*) in *Fusarium verticillioides* causes defects in asexual reproduction and mycelial hydrophobicity. (A) Strains 7600, FV020 and FV022 were assessed for differences in conidiation. Bars indicate standard deviation. (B) Strains 7600 and FV020 were assessed for the production of conidial chains. (C) Strains 7600, FV020 and FV022 were assessed for the development of aerial hyphae. (D) Strains 7600, FV020 and FV022 were assessed for the maintenance of mycelial hydrophobicity 10 min after the addition of water or detergent solution (DET). (E) Expression of hydrophobin-encoding genes (*HYD1–8*) was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Bars indicate standard deviation; nd, not detected (*Ct* value ≥ 37).

the formation of aerial hyphae are associated with the maintenance of cell surface hydrophobicity (Bayry *et al.*, 2012; Linder *et al.*, 2005; Whiteford and Spanu, 2002; Wosten, 2001), the *FUG1* deletion strain was examined for defects in hydrophobicity. Droplets of water or a detergent solution remained intact on the surface of colonies of the wild type and the genetic complement for more than 10 min (Fig. 6D). In contrast, both water and the detergent solution diffused into the mycelia of the *FUG1* deletion strain (Fig. 6D), which indicated that *FUG1* plays a role in the maintenance of mycelial hydrophobicity in *F. verticillioides*.

Eight hydrophobin-encoding genes (*HYD1–8*) have been identified in *F. verticillioides* (Fuchs *et al.*, 2004; Ridenour and Bluhm, 2014). RNA-seq revealed that the expression of *HYD1–7* was reduced by greater than two-fold and *HYD1–4* and *HYD7* were significantly down-regulated (FDR-corrected $P < 0.05$) in the *FUG1* deletion strain during kernel colonization (Table S8, see Supporting Information). Under the conditions examined, *HYD8* was not expressed at a quantifiable level in the wild-type strain or the *FUG1* deletion strain (Table S8). When the expression of hydrophobin-encoding genes was assessed *in vitro* by RT-qPCR, *HYD1–4*, *HYD6* and *HYD8* were down-regulated in the *FUG1* deletion strain compared with the wild type (Fig. 6E). In addition,

HYD5 was not expressed at a quantifiable level in the wild-type strain or the *FUG1* deletion strain, and *HYD7* was not expressed at a quantifiable level in the *FUG1* deletion strain (Fig. 6E).

DISCUSSION

FUG1 of *F. verticillioides* encodes a previously uncharacterized fungal-specific protein that is probably involved in transcriptional regulation. Multiple lines of evidence indicate that Fug1 represents a novel class of fungal transcription factor: (i) Fug1 shares an uncharacterized domain with known transcriptional regulators; (ii) Fug1 is predicted to localize to the nucleus; and (iii) regions of Fug1 are predicted to bind DNA. The Fug1 protein contains two putative functional domains, DUF1752 and DUF3295, both of which are unique to fungi. In *F. verticillioides* and other ascomycetes, DUF3295 only appears to be present in orthologues of Fug1 and is therefore absent from any characterized proteins. DUF1752, however, is present in two *F. verticillioides* proteins in addition to Fug1: AreA, a characterized GATA transcription factor that regulates the expression of genes involved in nitrogen catabolite repression (Kim and Woloshuk, 2008), and a putative orthologue of the *S. cerevisiae* Reg1, a regulatory subunit of a type-1 protein phosphatase that is involved in numerous processes,

including cell growth and carbon catabolite repression, in *S. cerevisiae* (Jiang *et al.*, 2000; Tu and Carlson, 1995). In *S. cerevisiae*, DUF1752 is also present in Mks1, a protein that is involved in transcriptional regulation via RAS-cyclic adenosine monophosphate (RAS-cAMP) signalling (Matsuura and Anraku, 1993). Notably, a clear orthologue of *MKS1* could not be identified in the *F. verticillioides* genome. Although the molecular function of the DUF1752 domain is unclear, an intriguing consideration is that DUF1752 appears to be found exclusively in regulatory proteins, which would suggest that Fug1 may be involved in transcriptional regulation via direct DNA binding, as is the case for AreA, or in fine-tuning mechanisms of signal transduction, as is the case for Reg1 or Mks1. The Fug1 protein exhibits additional hallmarks associated with transcriptional regulators, including nuclear localization and a propensity to bind DNA. Thus, multiple lines of evidence suggest that Fug1 represents a novel class of transcriptional regulator in fungi.

This study demonstrates that *FUG1* is a novel component of the genetic regulatory network underlying maize kernel pathogenesis. However, the exact role of *FUG1* in pathogenesis is not clear. One possible explanation is that *FUG1* plays a role in mitigating stresses associated with host defence responses. Maize utilizes a number of mechanisms to defend against kernel pathogens, including the production of hydrolases that degrade fungal cell walls, zeamatin, which increases the permeability of fungal cell membranes and inhibits α -amylase activity, and ribosome-inactivating proteins, which inactivate foreign ribosomes via a modification that blocks protein elongation (Guo *et al.*, 1997; Huynh *et al.*, 1992; Nielsen *et al.*, 2001; Roberts and Seli-trennikoff, 1990). In addition, benzoxazinoid metabolites of maize, such as DIMBOA and DIBOA, have antifungal activity (Glenn *et al.*, 2001). Long thought to be phytoanticipins that were absent in maize kernels, it has been shown recently that benzoxazinoids are present in kernels, and fungal pathogens and insect pests can induce the production of these compounds (Ahmad *et al.*, 2011; Basse, 2005; Cambier *et al.*, 2000). Interestingly, *F. verticillioides* is more tolerant to benzoxazinoids than other fungi associated with maize (Glenn *et al.*, 2001), which could potentially contribute to virulence during kernel colonization. Consistent with this idea, it has been demonstrated recently that benzoxazinoid detoxification in *F. pseudograminearum* contributes to virulence in wheat kernels (Kettle *et al.*, 2015). Thus far, *FUG1* is one of only a few genes known to impact benzoxazinoid tolerance in *F. verticillioides*. Glenn *et al.* (2002) identified two distinct genetic loci involved in benzoxazinoid metabolism, *FDB1* and *FDB2*, which are now known to represent xenobiotic resistance gene clusters (Glenn and Bacon, 2009; Glenn *et al.*, 2016). Recently, *MBL1*, a metallo- β -lactamase-encoding gene in the *FDB1* cluster, has been shown to be integral to the first step of benzoxazinoid detoxification (Glenn *et al.*, 2016). In the *FDB2* cluster, an arylamine

N-malonyltransferase encoded by *NAT1* (formerly *FDB2*) is required for benzoxazinoid detoxification (Glenn and Bacon, 2009; Glenn *et al.*, 2016). Lastly, *FDB3*, which is adjacent to *NAT1* in the *FDB2* cluster, encodes a Zn(II)₂Cys₆ transcription factor that has a minor contribution to benzoxazinoid tolerance (Glenn and Bacon, 2009). Although the involvement of *MBL1*, *NAT1* or *FDB3* in maize kernel pathogenesis has not been determined, increasing evidence suggests that benzoxazinoid detoxification may contribute to kernel pathogenesis in *F. verticillioides*.

The molecular regulation of fumonisin biosynthesis is becoming increasingly well understood, but significant gaps remain. *FUM* gene expression is coordinately regulated by the cluster-specific Zn(II)₂Cys₆ transcription factor *FUM21* that resides in the *FUM* cluster (Brown *et al.*, 2007), as well as a suite of trans-acting regulators (Woloshuk and Shim, 2013). Interestingly, the majority of genes known to influence fumonisin biosynthesis outside of the *FUM* cluster were not differentially expressed on deletion of *FUG1*. A notable exception was *AREA*, which was down-regulated in the *FUG1* deletion strain. *AREA* is a global regulator of nitrogen metabolite repression, a process through which easily assimilated nitrogen sources are preferentially utilized (Kim and Woloshuk, 2008). When favourable nitrogen sources are depleted or unavailable, *AREA* expression is induced, which triggers the induction of enzymes and transporters required to assimilate secondary nitrogen sources. Notably, the presence of favourable nitrogen sources is not conducive for fumonisin biosynthesis, and *FUM* gene expression is under the global regulatory control of *AREA* (Kim and Woloshuk, 2008). However, the results of this study provide little evidence to support the hypothesis that the misregulation of *AREA* and/or nitrogen metabolite repression is the underlying cause of fumonisin repression in the *FUG1* deletion strain. The *FUG1* deletion mutant displayed a degree of pleiotropy, with defects observed in growth and development, in addition to secondary metabolism; importantly, the phenotypic overlap with the *AREA* deletion strain is minimal. Thus, the mechanism through which *FUG1* regulates fumonisin biosynthesis is predicted to be indirect rather than direct, and requires further experimentation to be resolved.

Although reactive oxygen species (ROS) play a critical role in defending vegetative tissues of maize against pathogenic fungi (Lamb and Dixon, 1997), they have not been well studied in the context of maize kernel defence against fungal pathogens. Interestingly, recent evidence has indicated that ROS may contribute specifically to *F. verticillioides* resistance during kernel pathogenesis. Maize genes involved in ROS production and detoxification were induced in kernels more quickly and intensely in a resistant vs. susceptible line when inoculated with *F. verticillioides* (Lanubile *et al.*, 2014). Although additional work is required to elucidate the exact relationship between *FUG1*, ROS and kernel pathogenesis, an inability to efficiently detoxify ROS may contribute to the

reduction in invasive hyphal growth observed in the *FUG1* deletion strain. Regardless, if ROS detoxification is crucial for kernel pathogenesis, the *FUG1* mutant provides a novel tool to dissect the molecular mechanisms underlying how *F. verticillioides* is able to effectively overcome host defence in maize kernels.

Although a number of genes involved in sporulation have been identified in *F. verticillioides*, very few are known to positively regulate the production of both microconidia and macroconidia, as is the case for *FUG1*. *FAC1* (*F. verticillioides* adenylate cyclase 1), a key component of RAS-cAMP signalling, is important for the production of both spore types (Choi and Xu, 2010). Similar to the *FUG1* deletion strain, loss of *FAC1* resulted in reduced microconidiation and an absence of macroconidia (Choi and Xu, 2010). In addition to sporulation, Choi and Xu (2010) demonstrated that RAS-cAMP signalling regulates the expression of hydrophobin-encoding genes in *F. verticillioides*. Here, we found that *FUG1* is involved in the regulation of hydrophobin-encoding genes during growth in maize kernels and *in vitro*. Increasing evidence has shown the importance of hydrophobins in development (e.g. sporulation, formation of infection structures) and environmental adaptation (e.g. mycelial hydrophobicity, host evasion) in fungi (Bayry *et al.*, 2012; Linder *et al.*, 2005; Talbot *et al.*, 1996). We speculate that the regulation of hydrophobin-encoding genes by *FUG1* underpins the observed defects in sporulation and may also play a role in adaptation to the host environment. Although the exact mechanism by which *FUG1* is involved in the regulation of *HYD* genes remains unclear, one possibility is that *FUG1* regulates the expression of hydrophobin-encoding genes through the RAS-cAMP signalling pathway, as does *FAC1*. Interestingly, *MKS1*, which encodes a DUF1752-containing protein, plays a role in transcriptional regulation via the RAS-cAMP in *S. cerevisiae* (Matsuura and Anraku, 1993). Thus, *FUG1* may play a role in modulating the RAS-cAMP signalling cascade directly, similar to *MKS1* in *S. cerevisiae*, or in the direct regulation of downstream targets of the pathway.

Uncharacterized genes in pathogenic fungi represent potential virulence factors and/or antifungal targets. Therefore, the characterization of genes with no known function is significantly important to fully elucidate the mechanisms underpinning fungal pathogenesis. In the present study, we have identified and characterized a previously unknown gene, *FUG1*, in the maize pathogen *F. verticillioides*. We provide evidence that *FUG1* is a critical component of the genetic regulatory network underlying maize kernel pathogenesis. Moreover, we demonstrate that *FUG1* plays a role in mitigating stresses associated with the host environment and provide a compelling link between benzoxazinoid detoxification and maize kernel pathogenesis in *F. verticillioides*. *FUG1* is also required for wild-type levels of fumonisins and will thus serve as a novel molecular foothold in understanding how fumonisin biosynthesis is regulated. Lastly, we are investigating the role of *FUG1*

in other *Fusarium* spp., and it is expected that the characterization of *FUG1* orthologues will provide further insights into the genetic regulation of pathogenesis and secondary metabolism in fungi.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

The fungal strains used in this study are listed in Table S2. Strain 7600 (NRRL 20956) (Leslie, 1991; Ma *et al.*, 2010) served as the wild-type strain and the parental strain of *F. verticillioides* in this study. Strains were stored in 30% (v/v) glycerol at -80°C . Working cultures were maintained on potato dextrose agar (PDA), 0.2 $\times$ PDA or V8 agar medium (Leslie and Summerell, 2006).

Colony diameter was assessed on minimal and complete media (MM and CM, respectively) (Correll *et al.*, 1987), as described previously (Ridenour and Bluhm, 2014). Conidial production, mycelial hydrophobicity and aerial hyphae development were assessed as described by Ridenour and Bluhm (2014). Experiments were performed three times with similar results.

Nucleic acid manipulations

Plasmid DNA was isolated with a Zippy Plasmid Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA) or alkaline lysis midiprep (Sambrook and Russell, 2001). Genomic DNA was isolated from fungal cultures grown in yeast extract peptone dextrose (YEPD) medium (Kim *et al.*, 2011a) using a modified cetyltrimethylammonium bromide (CTAB) method (Leslie and Summerell, 2006). Southern blot analyses were performed as described by Sambrook and Russell (2001). For PCR-based screening, fungal DNA was prepared as described previously (Ridenour *et al.*, 2012). Primers were acquired from Integrated DNA Technologies (Coralville, IA, USA) (Table S3, see Supporting Information). DNA sequencing was performed at the University of Arkansas Core DNA Facility (Fayetteville, AR, USA).

Bioinformatics and phylogenetic reconstruction

Sequence data were obtained through the web portals of the Broad Institute, the Joint Genome Institute, Ensembl Plants and the National Center for Biotechnology Information (<http://www.broadinstitute.org>, <http://jgi.doe.gov>, <http://plants.ensembl.org> and <http://www.ncbi.nlm.nih.gov>, respectively). The current version of the *F. verticillioides* genome (FV3) was functionally annotated with BLAST2GO (Version 2.8) (Conesa *et al.*, 2005). Putative Fug1 orthologues were identified with reciprocal protein-protein BLAST analyses (BLASTP) (Altschul *et al.*, 1997). EMBOSS Needle was used to globally align protein sequences and to determine sequence identity (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). For phylogenetic reconstruction, sequences were initially aligned with Muscle (Version 3.8.31) (Edgar, 2004), and ambiguously aligned regions were removed with Gblocks (Version 0.91b) (Castresana, 2000; Talavera and Castresana, 2007). A neighbour-joining distance algorithm with 1000 bootstrap replicates was implemented in SeaView (Version 4.4.2) (Gouy *et al.*, 2010). Accession numbers for protein sequences used in tree reconstruction are listed in Table S4 (see Supporting Information). Predicted functional domains in protein sequences were identified with the Simple Modular

Architecture Research Tool (SMART) (Letunic *et al.*, 2012). The Fug1 protein sequence was analysed for secretory signals with TargetP and SignalP (Versions 1.1 and 4.1, respectively) (Emanuelsson *et al.*, 2000; Petersen *et al.*, 2011), for transmembrane α -helices with TMHMM (Version 2.0) (Krogh *et al.*, 2001), and for nuclear localization signals with cNLS Mapper (Kosugi *et al.*, 2009).

The R computing environment (Version 3.1.1) (<http://www.r-project.org>) was used. Bioconductor (Version 2.14) (Gentleman *et al.*, 2004) was used when applicable. Volcano plots and scatter plots were generated in the R package ggplot2 (Version 1.0.0). Heatmaps were generated in the R package gplots (Version 2.14.2) or Gtools (Version 2.2.1) (Perez-Llamas and Lopez-Bigas, 2011).

Targeted gene deletion and genetic complementation

A split-marker approach was used to replace *FUG1* with a hygromycin resistance cassette (Catlett *et al.*, 2003; Yu *et al.*, 2004). The deletion construct was generated as described previously (Ridenour *et al.*, 2014). Briefly, a region upstream of the *FUG1* ORF (*FUG1* 5' flank) and a region downstream of the *FUG1* ORF (*FUG1* 3' flank) were amplified from *F. verticillioides* genomic DNA (strain 7600) with primers FV04008_F1/FV04008_F2 and FV04008_F3/FV04008_F4, respectively. Overlapping marker fragments, HY and YG, were amplified from a hygromycin resistance cassette derived from pCB1003 (Carroll *et al.*, 1994) with primers M13F/HY and YG/M13R, respectively. The *FUG1* 5' flank and the HY marker fragment were joined and amplified by fusion PCR (Yu *et al.*, 2004) with nested primers FV04008_F1N/HYN. Similarly, the *FUG1* 3' flank and the YG marker fragment were joined and amplified with nested primers YGN/FV04008_F4N. Fungal transformation and screening were performed as described previously (Ridenour *et al.*, 2012).

For complementation, the *FUG1* ORF (plus 780 bp upstream and 1479 bp downstream of the ORF) was amplified with primers FV04008_C1/FV04008_C2 and cloned into pGEM-T Easy (Promega, Madison, WI, USA) to generate pBR0210. pBR0210 (25 μ g) and pKS-GEN, which contains a G-418 resistance cassette (5 μ g) (Bluhm *et al.*, 2008), were co-transformed into strain FV020. Transformants resistant to G-418 (150 μ g/mL) (Teknova, Hollister, CA, USA) were screened for integration of the *FUG1* complementation cassette with primers FV04008_PF/FV04008_PR.

Pathogenicity assays

A commercial maize hybrid was grown and self-pollinated in a glasshouse facility (University of Arkansas) at 19–25°C in a 14-h photoperiod. Maize ears were detached from the plant at the late dough stage of kernel development (growth stage R4, approximately 28 days after pollination) (Ritchie *et al.*, 1993). Infection and colonization of maize ears were evaluated as described by Kim and Woloshuk (2011). Inoculated ears were incubated at 25°C under humid conditions for 10 days. Four replicate ears were inoculated per strain; the experiment was repeated three times with similar results. For ergosterol and fumonisin analyses, infected kernels were removed from ears (five kernels per ear) and frozen in liquid nitrogen.

Analytical methods

Ergosterol extraction was performed as described previously (Bluhm and Woloshuk, 2005; Smith *et al.*, 2014). Ergosterol quantification was performed on a high-performance liquid chromatography (HPLC) system (Shimadzu, Columbia, MD, USA) with an ODS 5u column (250 mm $\times$ 4.6 mm) (Phenomenex, Torrance, CA, USA) and photodiode array detection (Shimadzu) at 282 nm. A mobile phase gradient comprising a linear increase from 100% solvent A (80% methanol) and 0% solvent B (100% dichloromethane) to 50% solvent A and 50% solvent B over 20 min, followed by a 15-min hold at 50% solvent A and 50% solvent B, was used to elute ergosterol (flow rate, 1.0 mL/min). Ergosterol concentrations were determined by comparing the sample peak areas with a standard curve generated by the analysis of HPLC-grade ergosterol (Sigma-Aldrich, St. Louis, MO, USA).

The accumulation of FB₁, FB₂ and FB₃ was measured by liquid chromatography-mass spectrometry (LC-MS). Briefly, ground kernels (400 mg) were extracted in 1 : 1 acetonitrile–water (2 mL) overnight at room temperature with shaking. The extract was filtered through a nylon syringe filter (0.2 μ m) (VWR International, Radnor, PA, USA) and chromatographic separation was performed with an HPLC system (LC-10AD vp, Shimadzu) equipped with a C18 5u column (150 mm $\times$ 4.6 mm) (Alltech, Deerfield, IL, USA). FB₁, FB₂ and FB₃ were detected with a ZMD quadrupole mass spectrometer (Micromass, Manchester, UK). The concentration of FB₁, FB₂ and FB₃ was determined by comparing the sample peak area with a standard curve generated by the analysis of HPLC-grade FB₁ (Sigma-Aldrich), FB₂ and FB₃ (Romer Labs, Union, MO, USA).

RNA isolation and RNA-seq analysis of gene expression

Total RNA was isolated from infected maize kernels as described previously (Bluhm *et al.*, 2008), 8 days after inoculation. RNA integrity was assessed on an Experion electrophoresis station (Bio-Rad, Hercules, CA, USA). Library preparation and RNA sequencing were performed through BGI (BGI Americas, Cambridge, MA, USA). Six libraries [three biological replicates of maize inoculated with the wild-type strain (7600) or the *FUG1* deletion strain (FV020)] were indexed and sequenced in one lane (single-end, 50-bp read length) on an Illumina HiSeq 2000 Genome Analyzer (Illumina Inc., San Diego, CA, USA).

RNA-seq reads were pre-processed with FASTQ Groomer, FASTQ Trimmer, Filter FASTQ and FASTQ Masker, essentially as described by Kellner *et al.* (2014). Pre-processing tools were accessed through the Galaxy web-server (<https://usegalaxy.org>) (Blankenberg *et al.*, 2010; Giardine *et al.*, 2005; Goecks *et al.*, 2010). Mapping of single-end RNA-seq reads to the *F. verticillioides* and *Zea mays* reference genomes was conducted with GSNAP (Version 2014-09-26) (Wu and Nacu, 2010; Wu and Watanabe, 2005). The asBAM function in the Rsamtools package (Version 1.18.3) was used to convert the output of GSNAP (SAM format) to BAM format. The summarizeOverlaps function (mode = Union) in the GenomicAlignments package (Version 1.2.2) (Lawrence *et al.*, 2013) was used to count the number of aligned reads. Notably, scatter plots comparing the variance between expression values indicated high correlation between biological replications (Fig. S3, see Supporting Information). The DESeq2

package (Version 1.6.1) was used to identify differentially expressed genes (Love *et al.*, 2014).

RT-qPCR analysis of gene expression

RT-qPCR was performed as described previously (Ridenour and Bluhm, 2014). Total RNA was isolated from infected maize kernels as described above. To assay gene expression *in vitro*, 50 mL of liquid CM was inoculated with conidia (50 µL of a 1×10^6 per millilitre conidial suspension) and incubated at 25°C in the dark with shaking at 150 rpm. Three days after inoculation, mycelia were collected by centrifugation and ground in liquid nitrogen. Total RNA was isolated with RiboZol reagent (Amresco, Solon, OH, USA) following the manufacturer's recommendations. RNA was treated with DNase I (Promega). Single-stranded cDNA was synthesized with M-MLV Reverse Transcriptase (Promega) following the manufacturer's recommendations. RT-qPCR was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Reaction and cycling conditions were as described by Bluhm *et al.* (2008). Three technical replicates were analysed per experiment; experiments were repeated twice with similar results.

Assessment of tolerance to BOA, H₂O₂ and cell wall stress

Tolerance to the antimicrobial compound BOA was assayed as described by Glenn *et al.* (2001) with minor modifications. Briefly, stock solutions of BOA (Sigma-Aldrich) were prepared in ethanol. Strains were grown on 0.2 × PDA amended with ethanol, 0.25 mg/mL BOA or 0.75 mg/mL BOA at room temperature in the dark and assessed after 4 days. Experiments were performed three times with similar results. Tolerance to hydrogen peroxide (H₂O₂) and cell wall stress [Congo Red (CR) or sodium dodecyl-sulfate (SDS)] was assayed as described by Ridenour and Bluhm (2014). The data presented represent the mean across four replicate plates per strain; experiments were performed three times with similar results.

Statistical analyses

Statistical differences were calculated in the JMP software package (Version Pro 11.0.0) (SAS Institute, Inc., Cary, NC, USA). Differences in ergosterol (Fig. 5B) and fumonisin accumulation (Fig. 5A,C) were determined according to Student's *t*-test. Statistical differences in Figs 6C and 7A were determined according to the Tukey–Kramer honestly significant difference (HSD) test. Letters indicate statistically significant differences ($P < 0.05$).

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AUTHOR CONTRIBUTIONS

Conception and design: J.B.R. and B.H.B.; analysis and interpretation of the data: J.B.R. and B.H.B.; drafting the article and/or critical revision: J.B.R. and B.H.B.; final approval of the article: J.B.R. and B.H.B.

REFERENCES

- Ahmad, S., Gromiha, M.M. and Sarai, A. (2004) Analysis and prediction of DNA-binding proteins and their binding residues based on composition, sequence and structural information. *Bioinformatics*, **20**, 477–486.
- Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., Glauser, G., Erb, M., Flors, V., Frey, M. and Ton, J. (2011) Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol.* **157**, 317–327.
- Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
- Bacon, C.W. and Hinton, D.M. (1996) Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Can. J. Bot.* **74**, 1195–1202.
- Bacon, C.W., Glenn, A.E. and Yates, I.E. (2008) *Fusarium verticillioides*: managing the endophytic association with maize for reduced fumonisins accumulation. *Toxin Rev.* **27**, 411–446.
- Basse, C.W. (2005) Dissecting defense-related and developmental transcriptional responses of maize during *Ustilago maydis* infection and subsequent tumor formation. *Plant Physiol.* **138**, 1774–1784.
- Bayry, J., Aimaniananda, V., Guijarro, J., Sunde, M. and Latge, J. (2012) Hydrophobins—unique fungal proteins. *PLoS Pathog.* **8**, e1002700.
- Blankenberg, D., Gordon, A., Von Kuster, G., Coraor, N., Taylor, J., Nekrutenko, A. and the Galaxy Team (2010) Manipulation of FASTQ data with Galaxy. *Bioinformatics*, **26**, 1783–1785.
- Bluhm, B.H. and Woloshuk, C.P. (2005) Amylopectin induces fumonisin B-1 production by *Fusarium verticillioides* during colonization of maize kernels. *Mol. Plant–Microbe Interact.* **18**, 1333–1339.
- Bluhm, B.H. and Woloshuk, C.P. (2006) Fck1, a C-type cyclin-dependent kinase, interacts with Fcc1 to regulate development and secondary metabolism in *Fusarium verticillioides*. *Fungal Genet. Biol.* **43**, 146–154.
- Bluhm, B.H., Kim, H., Butchko, R.A.E. and Woloshuk, C.P. (2008) Involvement of ZFR1 of *Fusarium verticillioides* in kernel colonization and the regulation of FST1, a putative sugar transporter gene required for fumonisin biosynthesis on maize kernels. *Mol. Plant Pathol.* **9**, 203–211.
- Brown, D.W., Butchko, R.A.E., Busman, M. and Proctor, R.H. (2007) The *Fusarium verticillioides* FUM gene cluster encodes a Zn(II)2Cys6 protein that affects FUM gene expression and fumonisin production. *Eukaryot. Cell*, **6**, 1210–1218.
- Brown, D.W., Butchko, R.A.E., Busman, M. and Proctor, R.H. (2012) Identification of gene clusters associated with fusaric acid, fusarin, and perithecial pigment production in *Fusarium verticillioides*. *Fungal Genet. Biol.* **49**, 521–532.
- Brown, D.W., Busman, M. and Proctor, R.H. (2014) *Fusarium verticillioides* SGE1 is required for full virulence and regulates expression of protein effector and secondary metabolite biosynthetic genes. *Mol. Plant–Microbe Interact.* **27**, 809–823.
- Carroll, A.M., Sweigard, J.A. and Valent, B. (1994) Improved vectors for selecting resistance to hygromycin. *Fungal Genet. Newsl.* **41**, 22.
- Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552.
- Catlett, N.L., Lee, B.-N., Yoder, O.C. and Turgeon, B.G. (2003) Split-marker recombination for efficient targeted deletion of fungal genes. *Fungal Genet. Newsl.* **50**, 9–11.
- Choi, Y.E. and Shim, W.B. (2008) Functional characterization of *Fusarium verticillioides* CPPI1, a gene encoding a putative protein phosphatase 2A catalytic subunit. *Microbiology*, **154**, 326–336.
- Choi, Y.E. and Xu, J.R. (2010) The cAMP signaling pathway in *Fusarium verticillioides* is important for conidiation, plant infection, and stress responses but not fumonisin production. *Mol. Plant–Microbe Interact.* **23**, 522–533.
- Conesa, A., Gotz, S., Garcia-Gomez, J., Terol, J., Talon, M. and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, **21**, 3674–3676.

- Correll, J.C., Klittich, C.J.R. and Leslie, J.F. (1987) Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology*, **77**, 1640–1646.
- Desjardins, A.E., Munkvold, G.P., Plattner, R.D. and Proctor, R.H. (2002) *FUM1*—a gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by *Gibberella moniliformis* in field tests. *Mol. Plant–Microbe Interact.* **15**, 1157–1164.
- Duncan, K.E. and Howard, R.J. (2010) Biology of maize kernel infection by *Fusarium verticillioides*. *Mol. Plant–Microbe Interact.* **23**, 6–16.
- Edgar, R. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797.
- Ellison, C.E., Kowbel, D., Glass, N.L., Taylor, J.W. and Brem, R.B. (2014) Discovering functions of unannotated genes from a transcriptome survey of wild fungal isolates. *mBio*, **5**, e01046–13.
- Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005–1016.
- Flaherty, J.E. and Woloshuk, C.P. (2004) Regulation of fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene, *ZFR1*. *Appl. Environ. Microbiol.* **70**, 2653–2659.
- Flaherty, J.E., Pirttilä, A.M., Bluhm, B.H. and Woloshuk, C.P. (2003) *PAC1*, a pH-regulatory gene from *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **69**, 5222–5227.
- Foley, D.C. (1962) Systemic infection of corn by *Fusarium moniliforme*. *Phytopathology*, **52**, 870–872.
- Fuchs, U., Czymmek, K.J. and Sweigard, J.A. (2004) Five hydrophobin genes in *Fusarium verticillioides* include two required for microconidial chain formation. *Fungal Genet. Biol.* **41**, 852–864.
- Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y. and Gentry, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.
- Giardine, B., Riemer, C., Hardison, R., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., Miller, W., Kent, W. and Nekrutenko, A. (2005) Galaxy: a platform for interactive large-scale genome analysis. *Genome Res.* **15**, 1451–1455.
- Glenn, A.E. and Bacon, C.W. (2009) *FDB2* encodes a member of the arylamine N-acetyltransferase family and is necessary for biotransformation of benzoxazolones by *Fusarium verticillioides*. *J. Appl. Microbiol.* **107**, 657–671.
- Glenn, A.E., Hinton, D.M., Yates, I.E. and Bacon, C.W. (2001) Detoxification of corn antimicrobial compounds as the basis for isolating *Fusarium verticillioides* and some other *Fusarium* species from corn. *Appl. Environ. Microbiol.* **67**, 2973–2981.
- Glenn, A.E., Gold, S.E. and Bacon, C.W. (2002) *Fdb1* and *Fdb2*, *Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from corn. *Mol. Plant–Microbe Interact.* **15**, 91–101.
- Goecks, J., Nekrutenko, A., Taylor, J. and the Galaxy Team (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* **11**, R86.
- Goldberg, T., Hecht, M., Hamp, T., Karl, T., Yachdav, G., Ahmed, N., Altmann, U., Angerer, P., Ansong, S., Balasz, K., Bernhofer, M., Betz, A., Cizmadija, L., Do, K.T., Gerke, J., Greil, R., Joerdens, V., Hastreiter, M., Hembach, K., Herzog, M., Kalemamov, M., Kluge, M., Meier, A., Nasir, H., Neumaier, U., Prade, V., Reeb, J., Sorokoumov, A., Troshani, I., Vorberg, S., Waldruff, S., Zierer, J., Nielsen, H. and Rost, B. (2014) LocTree3 prediction of localization. *Nucleic Acids Res.* **42**, W350–W355.
- Goodwin, S., Ben M'barek, S., Dhillon, B., Wittenberg, A., Crane, C., Hane, J., Foster, A., Van der Lee, T., Grimwood, J., Aerts, A., Antoniw, J., Bailey, A., Bluhm, B., Bowler, J., Bristow, J., van der Burg, A., Canto-Canche, B., Churchill, A., Conde-Ferraz, L., Cools, H., Coutinho, P., Csukai, M., Dehal, P., De Wit, P., Donzelli, B., van de Geest, H., Van Ham, R., Hammond-Kosack, K., Henrissat, B., Kilian, A., Kobayashi, A., Koopmann, E., Kourmpetis, Y., Kuzniar, A., Lindquist, E., Lombard, V., Maliepaard, C., Martins, N., Mehrabi, R., Nap, J., Ponomarenko, A., Rudd, J., Salamov, A., Schmutz, J., Schouten, H., Shapiro, H., Stergiopoulos, I., Torriani, S., Tu, H., de Vries, R., Waalwijk, C., Ware, S., Wiebenga, A., Zwieters, L., Oliver, R., Grigoriev, I. and Kema, G. (2011) Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genet.* **7**, e1002070.
- Gouy, M., Guindon, S. and Gascuel, O. (2010) SeaView Version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* **27**, 221–224.
- Guo, B.Z., Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S., Mehta, A.D., Selitrennikoff, C.P. and Widstrom, N.W. (1997) Germination induces accumulation of specific proteins and antifungal activities in corn kernels. *Phytopathology*, **87**, 1174–1178.
- Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**, W585–W587.
- Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R. and Shah, D.M. (1992) Antifungal proteins from plants – purification, molecular-cloning, and antifungal properties of chitinases from maize seed. *J. Biol. Chem.* **267**, 6635–6640.
- Hwang, S., Gou, Z. and Kuznetsov, I.B. (2007) DP-Bind: a web server for sequence-based prediction of DNA-binding residues in DNA-binding proteins. *Bioinformatics*, **23**, 634–636.
- Jiang, H., Tatchell, K., Liu, S. and Michels, C.A. (2000) Protein phosphatase type-1 regulatory subunits Reg1p and Reg2p act as signal transducers in the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **263**, 411–422.
- Kedera, C.J., Leslie, J.F. and Claflin, L.E. (1994) Genetic diversity of *Fusarium section Liseola* (*Gibberella fujikuroi*) in individual maize stalks. *Phytopathology*, **84**, 603–607.
- Kellner, R., Bhattacharyya, A., Poppe, S., Hsu, T., Brem, R. and Stukenbrock, E. (2014) Expression profiling of the wheat pathogen *Zymoseptoria tritici* reveals genomic patterns of transcription and host-specific regulatory programs. *Genome Biol. Evol.* **6**, 1353–1365.
- Kettle, A.J., Batley, J., Benfield, A.H., Manners, J.M., Kazan, K. and Gardiner, D.M. (2015) Degradation of the benzoxazolinone class of phytoalexins is important for virulence of *Fusarium pseudograminearum* towards wheat. *Mol. Plant Pathol.* **16**, 946–962.
- Kim, H. and Woloshuk, C.P. (2008) Role of *AREA*, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet. Biol.* **45**, 947–953.
- Kim, H. and Woloshuk, C.P. (2011) Functional characterization of *fst1* in *Fusarium verticillioides* during colonization of maize kernels. *Mol. Plant–Microbe Interact.* **24**, 18.
- Kim, H., Ridenour, J.B., Dunkle, L.D. and Bluhm, B.H. (2011a) Regulation of stomatal tropism and infection by light in *Cercospora zeae-maydis*: evidence for coordinated host/pathogen responses to photoperiod. *PLoS Pathog.* **7**, e1002113.
- Kim, H., Smith, J.E., Ridenour, J.B., Woloshuk, C.P. and Bluhm, B.H. (2011b) *HXX1* regulates carbon catabolism, sporulation, fumonisin B-1 production and pathogenesis in *Fusarium verticillioides*. *Microbiology*, **157**, 2658–2669.
- Kosugi, S., Hasebe, M., Tomita, M. and Yanagawa, H. (2009) Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. *Proc. Natl. Acad. Sci. USA*, **106**, 10 171–10 176.
- Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567–580.
- Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Biol.* **48**, 251–275.
- Lan, N., Zhang, H., Hu, C., Wang, W., Calvo, A., Harris, S., Chen, S. and Li, S. (2014) Coordinated and distinct functions of velvet proteins in *Fusarium verticillioides*. *Eukaryot. Cell*, **13**, 909–918.
- Lanubile, A., Ferrarini, A., Maschietto, V., Delledonne, M., Marocco, A. and Bellin, D. (2014) Functional genomic analysis of constitutive and inducible defense responses to *Fusarium verticillioides* infection in maize genotypes with contrasting ear rot resistance. *BMC Genomics*, **15**, 710.
- Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M. and Carey, V. (2013) Software for computing and annotating genomic ranges. *PLoS Comput. Biol.* **9**, e1003118.
- Leslie, J.F. (1991) Mating populations in *Gibberella fujikuroi* (*Fusarium section Liseola*). *Phytopathology*, **81**, 1058–1060.
- Leslie, J.F. and Summerell, B.A. (2006) *The Fusarium Laboratory Manual*. IA: Blackwell Publishing.
- Letunic, I., Doerks, T. and Bork, P. (2012) SMART 7: recent updates to the protein domain annotation resource. *Nucleic Acids Res.* **40**, D302–D305.
- Linder, M., Szilvay, G., Nakari-Setälä, T. and Penttilä, M. (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiol. Rev.* **29**, 877–896.
- Love, M.I., Huber, W. and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. *Genome Biol.* **15**, 550–571.
- Ma, L., van der Does, H., Borkovich, K., Coleman, J., Daboussi, M., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P.,

- Kang, S., Shim, W., Woloshuk, C., Xie, X., Xu, J., Antoniw, J., Baker, S., Bluhm, B., Breakspear, A., Brown, D., Butchko, R., Chapman, S., Coulson, R., Coutinho, P., Danchin, E., Diener, A., Gale, L., Gardiner, D., Goff, S., Hammond-Kosack, K., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C., Koehrsen, M., Kumar, L., Lee, Y., Li, L., Manners, J., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S., Proctor, R., Regev, A., Ruiz-Roldan, M., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D., Turgeon, B., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C., Kistler, H. and Rep, M. (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, **464**, 367–373.
- Malapi-Wight, M., Smith, J., Campbell, J., Bluhm, B. and Shim, W. (2013) Sda1, a Cys(2)-His(2) zinc finger transcription factor, is involved in polyol metabolism and fumonisin B-1 production in *Fusarium verticillioides*. *PLoS One*, **8**, e67656.
- Malapi-Wight, M., Kim, J. and Shim, W. (2014) The N-terminus region of the putative C2H2 transcription factor Ada1 harbors a species-specific activation motif that regulates asexual reproduction in *Fusarium verticillioides*. *Fungal Genet. Biol.* **62**, 25–33.
- Matsuura, A. and Anraku, Y. (1993) Characterization of the *MKS1* gene, a new negative regulator of the Ras-cyclic AMP pathway in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **238**, 6–16.
- Mukherjee, M., Kim, J.E., Park, Y.S., Kolomiets, M.V. and Shim, W.B. (2011) Regulators of G-protein signalling in *Fusarium verticillioides* mediate differential host–pathogen responses on nonviable versus viable maize kernels. *Mol. Plant Pathol.* **12**, 479–491.
- Munkvold, G.P. (2003) Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. *Eur. J. Plant Pathol.* **109**, 705–713.
- Myung, K., Li, S., Butchko, R.A.E., Busman, M., Proctor, R.H., Abbas, H.K. and Calvo, A.M. (2009) *FvVE1* regulates biosynthesis of the mycotoxins fumonisins and fusarins in *Fusarium verticillioides*. *J. Agric. Food Chem.* **57**, 5089–5094.
- Nelson, P.E., Desjardins, A.E. and Plattner, R.D. (1993) Fumonisin, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annu. Rev. Phytopathol.* **31**, 233–252.
- Nielsen, K., Payne, G.A. and Boston, R.S. (2001) Maize ribosome-inactivating protein inhibits normal development of *Aspergillus nidulans* and *Aspergillus flavus*. *Mol. Plant–Microbe Interact.* **14**, 164–172.
- Niemeyer, H.M. (1988) Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defense chemicals in the Gramineae. *Phytochemistry*, **27**, 3349–3358.
- Nirenberg, H.I. (1976) Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. *Mitt. Biol. Bundesanst. Land- u. Forstwirtschaft. (Berlin – Dahlem)*, **169**, 1–117.
- Oren, L., Ezrati, S., Cohen, D. and Sharon, A. (2003) Early events in the *Fusarium verticillioides*–maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Appl. Environ. Microbiol.* **69**, 1695–1701.
- Ortiz, C.S. and Shim, W.B. (2013) The role of MADS-box transcription factors in secondary metabolism and sexual development in the maize pathogen *Fusarium verticillioides*. *Microbiology*, **159**, 2259–2268.
- Perez-Llamas, C. and Lopez-Bigas, N. (2011) Gitoools: analysis and visualisation of genomic data using interactive heat-maps. *PLoS One*, **6**, e19541.
- Petersen, T.N., Brunak, S., von Heijne, G. and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods*, **8**, 785–786.
- Proctor, R.H., Brown, D.W., Plattner, R.D. and Desjardins, A.E. (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **38**, 237–249.
- Rheeder, J.P., Marasas, W.F.O. and Vismer, H.F. (2002) Production of fumonisin analogs by *Fusarium* species. *Appl. Environ. Microbiol.* **68**, 2101–2105.
- Ridenour, J.B. and Bluhm, B.H. (2014) The HAP complex in *Fusarium verticillioides* is a key regulator of growth, morphogenesis, secondary metabolism, and pathogenesis. *Fungal Genet. Biol.* **69**, 52–64.
- Ridenour, J.B., Hirsch, R.L. and Bluhm, B.H. (2012) Identifying genes in *Fusarium verticillioides* through forward and reverse genetics. In: *Plant Fungal Pathogens* (Bolton, M.D. and Thomma, B.P.H.J., eds), pp. 457–479. New York: Humana Press.
- Ridenour, J.B., Smith, J.E., Hirsch, R.L., Horevay, P., Kim, H., Sharma, S. and Bluhm, B.H. (2014) *UBL1* of *Fusarium verticillioides* links the N-end rule pathway to extracellular sensing and plant pathogenesis. *Environ. Microbiol.* **16**, 2004–2022.
- Ritchie, S.W., Hanway, J.J. and Benson, G.O. (1993) How a corn plant develops. *Special Report No. 48*. Ames, IA: Iowa State University of Science and Technology, Cooperative Extension Service.
- Roberts, W.K. and Selitrennikoff, C.P. (1990) Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. *J. Gen. Microbiol.* **136**, 1771–1778.
- Sagaram, U.S. and Shim, W.B. (2007) *Fusarium verticillioides* *GBB1*, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B1 biosynthesis and hyphal development but not with fungal virulence. *Mol. Plant Pathol.* **8**, 375–384.
- Sagaram, U.S., Butchko, R.A.E. and Shim, W.B. (2006) The putative monomeric G-protein *GBP1* is negatively associated with fumonisin B-1 production in *Fusarium verticillioides*. *Mol. Plant Pathol.* **7**, 381–389.
- Sagaram, U.S., Shaw, B.D. and Shim, W.B. (2007) *Fusarium verticillioides* *GAP1*, a gene encoding a putative glycolipid-anchored surface protein, participates in conidiation and cell wall structure but not virulence. *Microbiology*, **153**, 2850–2861.
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Schoch, C.L., Sung, G.H., López-Giráldez, F., Townsend, J.P., Miadlikowska, J., Hofstetter, V., Robertse, B., Matheny, P.B., Kauff, F., Wang, Z., Gueidan, C., Andrie, R.M., Trippe, K., Ciuffetti, L.M., Wynns, A., Fraker, B., Hodkinson, B.P., Bonito, G., Groenewald, J.Z., Arzanlou, M., de Hoog, G.S., Crous, P.W., Hewitt, D., Pfister, D.H., Peterson, K., Gryzenhout, M., Wingfield, M.J., Aptroot, A., Suh, S.O., Blackwell, M., Hillis, D.M., Griffith, G.W., Castlebury, L.A., Rossman, A.Y., Lumbsch, H.T., Lücking, R., Bidel, B., Rauhut, A., Diederich, P., Ertz, D., Geiser, D.M., Hosaka, K., Inderbitzin, P., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Mostert, L., O'donnell, K., Sipman, H., Rogers, J.D., Shoemaker, R.A., Sugiyama, J., Summerbell, R.C., Untereiner, W., Johnston, P.R., Stenroos, S., Zuccaro, A., Dyer, P.S., Crittenden, P.D., Cole, M.S., Hansen, K., Trappe, J.M., Yahr, R., Lutzoni, F. and Spatafora, J.W. (2009) The Ascomycota tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Syst. Biol.* **58**, 224–239.
- Shim, W.B. and Woloshuk, C.P. (2001) Regulation of fumonisin B1 biosynthesis and conidiation in *Fusarium verticillioides* by a cyclin-like (C-type) gene, *FCC1*. *Appl. Environ. Microbiol.* **67**, 1607–1612.
- Shin, J.H., Kim, J.E., Malapi-Wight, M., Choi, Y.E., Shaw, B.D. and Shim, W.B. (2013) Protein phosphatase 2A regulatory subunits perform distinct functional roles in the maize pathogen *Fusarium verticillioides*. *Mol. Plant Pathol.* **14**, 518–529.
- Smith, J., Mengesha, B., Tang, H., Mengiste, T. and Bluhm, B. (2014) Resistance to *Botrytis cinerea* in *Solanum lycopersicoides* involves widespread transcriptional reprogramming. *BMC Genomics*, **15**, 334.
- Sobek, E. and Munkvold, G. (1999) European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. *J. Econ. Entomol.* **92**, 503–509.
- Stockmann-Juvala, H. and Savolainen, K. (2008) A review of the toxic effects and mechanisms of action of fumonisin B1. *Hum. Exp. Toxicol.* **27**, 799–809.
- Talavera, G. and Castresana, J. (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* **56**, 564–577.
- Talbot, N.J., Kershaw, M.J., Wakley, G.E., De Vries, O.M., Wessels, J.G. and Hamer, J.E. (1996) *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell*, **8**, 985–999.
- Tu, J. and Carlson, M. (1995) REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 5939–5946.
- Wang, L., Huang, C., Yang, M.Q. and Yang, J.Y. (2010) BindN+ for accurate prediction of DNA and RNA-binding residues from protein sequence features. *BMC Syst. Biol.* **4**, S3.
- Warfield, C. and Davis, R. (1996) Importance of the husk covering on the susceptibility of corn hybrids to *Fusarium* ear rot. *Plant Dis.* **80**, 208–210.
- White, D.G. (1999) *Compendium of Corn Diseases*. MN: APS Press.
- Whiteford, J. and Spanu, P. (2002) Hydrophobins and the interactions between fungi and plants. *Mol. Plant Pathol.* **3**, 391–400.
- Woloshuk, C.P. and Shim, W.B. (2013) Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. *FEMS Microbiol. Rev.* **37**, 94–109.
- Wosten, H. (2001) Hydrophobins: multipurpose proteins. *Annu. Rev. Microbiol.* **55**, 625–646.
- Wu, T. and Nacu, S. (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics*, **26**, 873–881.
- Wu, T. and Watanabe, C. (2005) GMAP: a genomic mapping and alignment program for mRNA and EST sequences. *Bioinformatics*, **21**, 1859–1875.

- Yu, C.S., Chen, Y.C., Lu, C.H. and Hwang, J.K. (2006) Prediction of protein subcellular localization. *Proteins*, **64**, 643–651.
- Yu, J., Hamari, Z., Han, K., Seo, J., Reyes-Dominguez, Y. and Scazzocchio, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **41**, 973–981.
- Zhang, Y., Choi, Y.E., Zou, X. and Xu, J.R. (2011) The *FvMK1* mitogen-activated protein kinase gene regulates conidiation, pathogenesis, and fumonisin production in *Fusarium verticillioides*. *Fungal Genet. Biol.* **48**, 71–79.
- Zheng, W., Zhao, X., Xie, Q., Huang, Q., Zhang, C., Zhai, H., Xu, L., Lu, G., Shim, W.B. and Wang, Z. (2012) A conserved homeobox transcription factor Htf1 is required for phialide development and conidiogenesis in *Fusarium* species. *PLoS One*, **7**, e45432.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 *FUG1* (*Fungal Unknown Gene 1*) in *Fusarium verticillioides* encodes an uncharacterized, fungal-specific protein. (A) Translation of *FUG1* reveals a protein of 561 amino acids that is predicted to contain two domains of unknown function (DUFs; DUF1752 and DUF3295) and a bipartite nuclear localization signal (NLS). (B) The computational tools BindN+, DBS-PRED and DP-bind identified a majority consensus of 114 amino acid residues in the Fug1 protein sequence as potential DNA-binding sites. Clusters of DNA-binding sites were identified in DUF1752 and near the C-terminus of the protein. Light grey indicates the location of DUF1752; dark grey indicates the location of DUF3295. + indicates that a binding site was identified by the respective program, and B indicates a potential binding site based on majority consensus.

Fig. S2 Targeted deletion of *FUG1* (*Fungal Unknown Gene 1*) in *Fusarium verticillioides*. (A) Schematic representation of the *FUG1* locus in *F. verticillioides* and the split-marker strategy used for targeted deletion of *FUG1*. White blocks represent untranslated regions and dark grey blocks represent exons. Arrows indicate gene directionality. Black bars represent the locations of probes relative to genomic loci. (B) Strains 7600, FV020, FV021 and FV022 were validated by polymerase chain reaction (PCR) with the primer pairs: FV04008_PF/FV04008_PR (Probe 1), FV04008_A1/HSB (Probe 2) and HSC/FV04008_A2 (Probe 3). Genomic DNA was digested using the restriction enzyme *Xba*I

and probed with a hygromycin phosphotransferase-encoding gene (*hph*)-specific probe (Probe 4) by Southern blot. The presence of a single band indicates integration of a single copy of the deletion construct into the genome of strains FV020 and FV021. (C) Strains 7600, FV020, FV021 and FV022 were assessed for differences in growth. (D) Strains 7600, FV020, FV021 and FV022 grown on minimal medium (MM) (left panel) and complete medium (CM) (right panel) were assessed for differences in colony diameter. Data are presented as colony diameter in centimetres. All standard deviations for three replicates were less than 0.15 cm. Strain descriptions are presented in Table S2.

Fig. S3 High correlation between biological replicates was observed in expression data. (A) Scatter plots comparing the variance between expression values derived from biological replications of the wild-type strain. (B) Scatter plots comparing the variance between expression values derived from biological replications of the *FUG1* (*Fungal Unknown Gene 1*) deletion strain. Each dot represents a gene. Expression values are presented as the $\log_2$ of gene counts. Pearson's r is shown in the top left corner of each plot.

Table S1 Functionally unknown genes selected for analysis in this study.

Table S2 Fungal strains used in this study.

Table S3 Primers used in this study.

Table S4 Predicted Fug1 orthologues in select ascomycetes.

Table S5 Gene Ontology (GO) term enrichment in *Fusarium verticillioides* genes up-regulated in the *FUG1* (*Fungal Unknown Gene 1*) deletion strain during kernel colonization.

Table S6 Gene Ontology (GO) term enrichment in *Fusarium verticillioides* genes down-regulated in the *FUG1* (*Fungal Unknown Gene 1*) deletion strain during kernel colonization.

Table S7 Effect of *FUG1* (*Fungal Unknown Gene 1*) deletion on the expression of genes outside of the fumonisin biosynthetic cluster known to impact fumonisin production.

Table S8 Effect of *FUG1* (*Fungal Unknown Gene 1*) deletion on the expression of hydrophobin-encoding genes during kernel colonization.