

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

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SUMMARY

Fumonisin comprises a class of carcinogenic mycotoxins produced by *Fusarium verticillioides* during colonization of maize kernels. In previous work, we identified *ZFR1*, which is predicted to encode a Zn(II)₂Cys₆ zinc finger transcription factor required for fumonisin B₁ (FB₁) production during growth on kernels. In this study, we characterized the role of *ZFR1* in colonizing maize kernels and inducing FB₁ biosynthesis. The *ZFR1* deletion strain ($\Delta zfr1$) grew approximately 2.5-fold less than the wild-type on endosperm tissue and a variety of other carbon sources, including glucose and amylopectin. However, the $\Delta zfr1$ strain displayed higher α -amylase activity and expression of genes involved in starch saccharification than the wild-type, thus indicating that the reduced growth of the $\Delta zfr1$ strain was not due to inhibition of amylolytic enzymes. In the wild-type strain, expression of six genes encoding putative sugar transporters was significantly greater on endosperm tissue than on germ tissue, and expression of at least three of the six genes was negatively affected by disruption of *ZFR1*. Intriguingly, disruption of *FST1* had no effect on growth, kernel colonization or kernel pH but decreased FB₁ production by approximately 82% on maize kernels. Based on these findings, we hypothesize that *ZFR1* controls FB₁ biosynthesis by regulating genes involved in the perception or uptake of carbohydrates.

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland, synonym *Gibberella fujikuroi* mating population A) is a ubiquitous fungal pathogen that causes ear, kernel, seedling and stem rots of maize (*Zea mays*). During

colonization of maize kernels, *F. verticillioides* produces a group of structurally related polyketide mycotoxins collectively referred to as fumonisins. FB₁, the predominant fumonisin produced by *F. verticillioides* (Vismer *et al.*, 2004), is a potent inhibitor of ceramide synthase (Desai *et al.*, 2002) and thus disrupts sphingolipid biosynthesis. Ingestion of FB₁ results in leukoencephalomalacia in horses and has been implicated as a cause of oesophageal cancer and birth defects in humans (Hendricks, 1999; Joffe, 1986). Because of health concerns related to consumption of FB₁, regulatory agencies throughout the world have established guidelines regarding acceptable levels of FB₁ in grains and food products.

FB₁ biosynthesis is influenced by components of the maize kernel (Shim *et al.*, 2003). One such component is starch, which changes dramatically in the kernel as it approaches maturity (Ingle *et al.*, 1965). Immature kernels lacking starch do not support FB₁ biosynthesis or expression of fumonisin biosynthetic (*FUM*) genes, but as kernels approach maturity and accumulate starch, they support high levels of FB₁ production (Bluhm and Woloshuk, 2005; Warfield and Gilchrist, 1999). Growth of *F. verticillioides* is comparable throughout each stage of kernel development (Bluhm and Woloshuk, 2005), indicating that immature kernels either repress FB₁ biosynthesis or lack the ability to induce *FUM* gene expression. In mature kernels, the starch-rich tissue of the endosperm supports 10- to 20-fold higher levels of FB₁ biosynthesis than germ tissue (Shim *et al.*, 2003), and disruption of the α -amylase gene of *F. verticillioides* results in low levels of FB₁ biosynthesis on endosperm fractions (Bluhm and Woloshuk, 2005). Analyses of FB₁ production in maize kernel mutants and in defined liquid media indicate that amylopectin, a component of starch, supports high levels of FB₁ biosynthesis (Bluhm and Woloshuk, 2005).

In a previous study, we identified *ZFR1*, a gene encoding a putative zinc-finger transcription factor required for wild-type levels of FB₁ production on maize kernels (Flaherty and Woloshuk, 2004). In the present study, we investigated the molecular mechanisms through which *ZFR1* regulates colonization of kernel tissues and *FUM* gene transcription. We evaluated the role of

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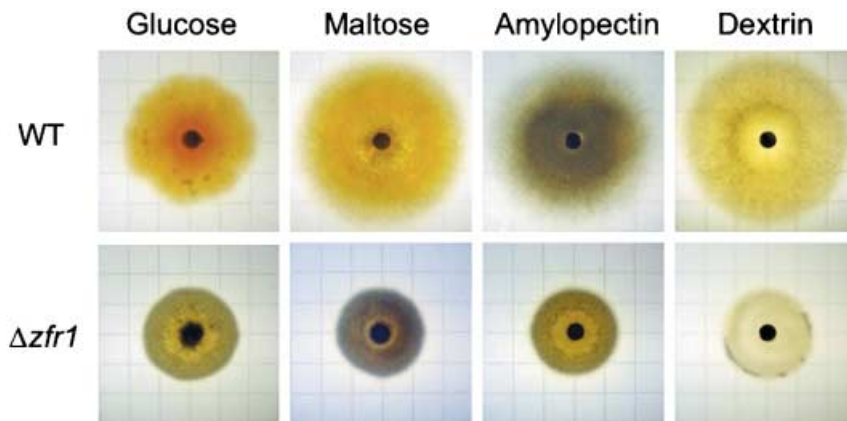


Fig. 1 Growth of the wild-type (WT) and $\Delta zfr1$ strains on minimal media containing either glucose, maltose, amylopectin or dextrin as the sole carbon source. Each square of the background grid represents 14×14 mm. Pictures were taken after 4 days of growth.

Table 1 Growth of the $\Delta zfr1$ strain on germ and endosperm fractions of maize kernels.

Fungal strain	Growth (ergosterol)*		
	Whole kernel	Germ	Endosperm
Wild type	110 ± 11	160 ± 17	100 ± 9
$\Delta zfr1$	90 ± 7	370 ± 33	40 ± 5

*Measurements were taken after 7 days of growth. Data were reported as micrograms of ergosterol per gram of kernel tissue \pm the standard error of the mean for three samples.

ZFR1 in regulating four distinct aspects of kernel colonization: growth in the presence of specific carbohydrates found in maize kernels, hydrolysis of starch, perception of glucose, and expression of putative mono- and oligo-saccharide transporters. From these experiments, we identified *FST1*, a putative sugar transporter regulated by *ZFR1* that is required for FB_1 biosynthesis but not growth during kernel colonization. The discovery that *ZFR1* regulates sugar transporter genes that in turn affect fumonisin biosynthesis during kernel colonization reveals a possible linkage between the perception of starch and the induction of fumonisin biosynthesis and provides new insight into the role of sugar transport proteins in plant pathogenesis.

RESULTS

Growth of the $\Delta zfr1$ mutant on carbohydrates found in maize kernels

Although visual assessments indicated that the $\Delta zfr1$ strain grew at approximately wild-type levels on whole kernels, ergosterol analysis indicated the mutant grew 20% less than the wild-type strain (Table 1). On endosperm fractions of kernels, growth of the $\Delta zfr1$ strain was approximately 60% less than wild-type (Table 1). In contrast, the $\Delta zfr1$ strain grew greater than twice as

much as wild-type in germ fractions of kernels (Table 1), indicating that the growth phenotype of the mutant is strongly influenced by kernel tissue. Growth of the $\Delta zfr1$ strain was substantially less than wild-type when provided with glucose, maltose, dextrin or amylopectin as the sole carbon source (Fig. 1). Together, these observations suggested that the $\Delta zfr1$ strain is impaired in the perception or utilization of carbohydrates.

Glucokinase activity in the $\Delta zfr1$ strain

In many organisms, hexokinases regulate carbohydrate metabolism by phosphorylating glucose and other hexose sugars and thus function as sugar sensors. To determine whether impairment of hexokinase activity accounted for the reduced growth of the $\Delta zfr1$ strain in the presence of carbohydrates, we measured the expression of putative hexokinase-encoding genes and glucokinase activity. Homology-based searches of the *F. verticillioides* genome revealed the presence of at least two genes encoding putative hexokinases (*HXK1* and *HXK2*). *HXK1* and *HXK2* were expressed at low levels in both the wild-type and $\Delta zfr1$ strains on germ tissue (Table 2). However, on the starch-rich tissue of the endosperm, expression of *HXK1* and *HXK2* increased by approximately 120- and 27-fold, respectively, in the wild-type, and by approximately 45- and 18-fold, respectively, in the $\Delta zfr1$ strain (Table 2). Consistent with these findings, glucokinase activity was substantially higher in both strains grown on endosperm tissue as compared with germ tissue with no significant differences observed between the two strains (Table 2). From these observations, we concluded that disruption of *ZFR1* has a negligible effect on carbohydrate perception by hexokinases.

Expression of genes encoding amylolytic enzymes in the $\Delta zfr1$ strain

To test the possibility that the $\Delta zfr1$ strain is impaired in its ability to hydrolyse starch, we measured the expression of six genes

Table 2 Glucokinase expression and activity in the wild-type and $\Delta zfr1$ strains grown on germ and endosperm fractions of maize kernels.

Fungal strain	<i>HXK1</i> expression*		<i>HXK2</i> expression		Glucokinase activity†	
	Germ	Endosperm	Germ	Endosperm	Germ	Endosperm
Wild-type	1.0 (0.7–1.3)	120 (100–150)	1.0 (0.7–1.3)	27.3 (27.0–27.7)	2.0 ± 0.2	131 ± 4.9
$\Delta zfr1$	1.5 (1.3–1.9)	45 (40–50)	-0.7 (-0.3 to -1.1)	18.2 (18.0–18.3)	1.7 ± 0.4	119 ± 3.7

*As analysed by qPCR. Expression of *HXK1* and *HXK2* was normalized to *TUB2* and was calculated relative to expression in wild-type growing on germ tissue. Data represent fold differences in expression. The relative expression of each gene was calculated as $2^{\Delta\Delta Ct}$. The range of expression, in parentheses, for each gene = $(2^{\Delta\Delta Ct-s} - 2^{\Delta\Delta Ct+s})$, where s = the standard deviation of the $\Delta\Delta Ct$ value.

†Data are reported as 1×10^{-4} units of glucokinase activity per gram total protein ± the standard error of the mean for three samples.

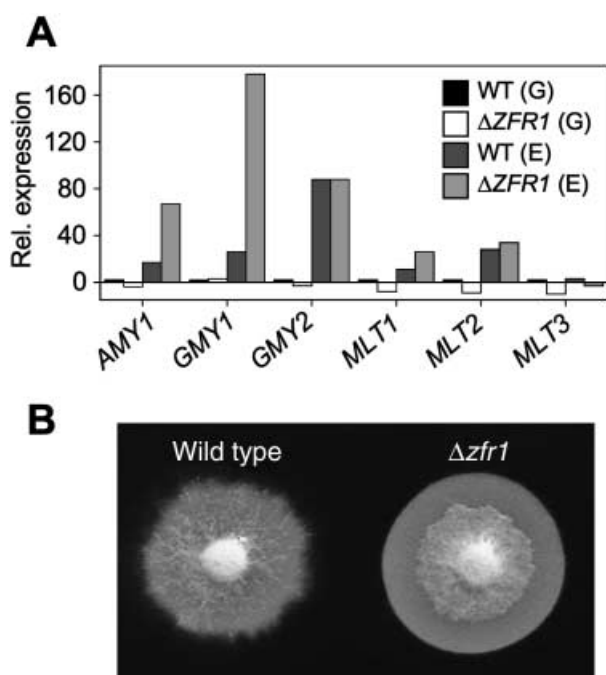


Fig. 2 Expression and activity of amyolytic genes in the wild-type and $\Delta zfr1$ strains. (A) Expression of an α -amylase (*AMY1*), two glucoamylases (*GMY1* and *GMY2*), and three maltases (*MLT1*, *MLT2* and *MLT3*) was compared between the strains after 6 days of growth on germ (G) and endosperm (E) tissues of mature maize kernels. For each gene, expression was normalized to *TUB2* expression and calculated as fold-changes in expression relative to expression in wild-type growing on germ tissue. (B) Amylase activity was measured by staining starch plates with iodine after 3 days of growth. Unstained areas are indicative of starch saccharification.

predicted to encode enzymes involved in starch saccharification. In both the wild-type and the $\Delta zfr1$ mutant, expression of all six genes was low when grown on germ tissue (Fig. 2A). Surprisingly, on endosperm tissue at least three amyolytic genes were upregulated in the $\Delta zfr1$ strain compared with the wild-type, including the α -amylase *AMY1* (three-fold higher), a putative glucoamylase designated *GMY1* (eight-fold higher) and a putative maltase designated *MLT1* (two-fold higher) (Fig. 2A).

To determine whether the increased levels of expression of amyolytic genes in the $\Delta zfr1$ strain corresponded to higher levels of starch hydrolysis, we grew the wild-type and $\Delta zfr1$ on plates containing amylose. By staining plates with an iodine solution, the amount of starch hydrolysis was determined qualitatively; unstained areas of the plate result from enzymatic degradation of amylose. Consistent with higher levels of amyolytic enzyme activity, the amount of clearing beneath mycelia of the $\Delta zfr1$ strain was much greater than beneath wild-type colonies (Fig. 2B). These results indicated that the $\Delta zfr1$ strain is able to perceive and hydrolyse starch at levels equal to or greater than the wild-type strain.

Regulation of putative sugar transporter genes by kernel tissue and *zfr1*

Observations that the $\Delta zfr1$ strain was impaired in growth on carbohydrates despite high levels of amyolytic and hexokinase activity suggested that *ZFR1* may regulate sugar uptake. In fungi, sugar uptake is facilitated in large part by membrane-bound transporters belonging to the major facilitator superfamily (MFS) (Pao *et al.*, 1998). The genome of *F. verticillioides* contains at least 381 genes predicted to encode MFS proteins, of which 147 share significant amino acid similarity with known sugar transporters. Using microarrays described previously (Sagaram *et al.*, 2006), we were able to measure the expression profiles of 50 of the 147 putative sugar transporter genes in the wild-type strain during growth on germ and endosperm tissues. Surprisingly, although endosperm tissues of kernels are rich in carbohydrates, only six putative sugar transporters were more highly expressed during growth on endosperm tissue (Fig. 3A). To verify the expression data obtained from microarrays, we arbitrarily selected three putative sugar transporter genes (*FST1*, *FST2* and *FST4*) for expression analysis by quantitative PCR (qPCR). Expression of *FST1*, *FST2* and *FST4* was two- to three-fold higher in the wild-type strain during growth on endosperm tissue compared with germ tissue (Fig. 3B). To determine if *ZFR1* regulates the expression of sugar transporters, we measured the expression of *FST1*, *FST2* and *FST4* on endosperm tissue. On germ tissue, transcription

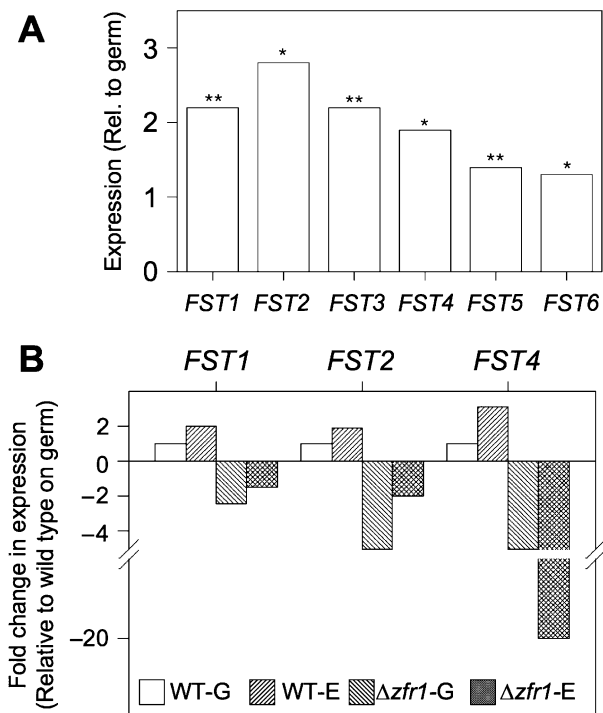


Fig. 3 Expression of putative sugar transporter genes in the wild-type strain during colonization of maize kernels. (A) Microarray analysis of six sugar transporter genes differentially expressed in germ and endosperm tissues. For each gene, expression was normalized to *TUB2* by dividing the spot intensity of the gene by the intensity recorded for *TUB2*. Measurements were taken after 4 days of growth on kernel tissues. Asterisks indicate a *P* value < 0.05 (*) or < 0.01 (**). (B) qPCR verification of microarray results and analysis of sugar transporter expression in the $\Delta zfr1$ strain. Expression of *FST1*, *FST2* and *FST4* was measured in wild-type growing on germ (WT-G) and endosperm (WT-E) and in the $\Delta zfr1$ strain growing on germ ($\Delta zfr1$ -G) and endosperm ($\Delta zfr1$ -E). For each gene, expression was normalized to *TUB2* expression and calculated as fold-changes in expression relative to expression in wild-type growing on germ tissue.

of *FST1*, *FST2* and *FST4* was reduced in the $\Delta zfr1$ strain by approximately two- to five-fold (Fig. 3B). On endosperm tissue, expression of *FST1* and *FST2* was reduced by approximately two-fold in the $\Delta zfr1$ strain, and expression of *FST4* was reduced by approximately 20-fold (Fig. 3B). These results showed that *ZFR1* regulated the expression of sugar transporters during kernel colonization.

Growth and FB₁ biosynthesis in a $\Delta fst1$ strain

To determine the function of *FST1* during kernel colonization, we disrupted the gene through double homologous recombination (Fig. 4). Growth of the $\Delta fst1$ strain was similar to the wild-type and complemented strains when provided glucose or maltose as the primary carbon source (Fig. 5), thus suggesting that the $\Delta fst1$

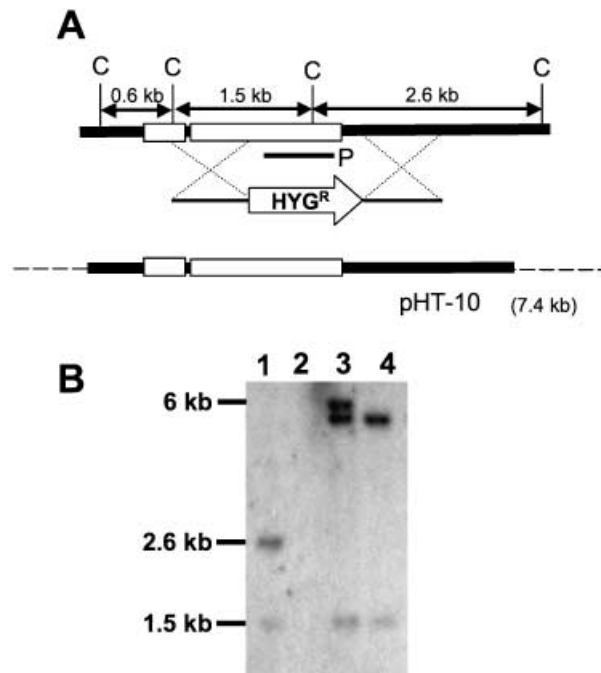


Fig. 4 Disruption and complementation of *FST1*. (A) A hygromycin-resistance cassette (HYG^R) was inserted into the *FST1* gene by homologous recombination to create a $\Delta fst1$ strain. For complementation, protoplasts of $\Delta fst1$ were co-transformed with pHT-10 (constructed by cloning the *FST1* locus into the pGEM-T-Easy vector; dashed lines represent vector sequence) and pKS-GEN, a vector harbouring a geneticin resistance cassette. For Southern analysis, genomic DNA was digested with *Cla*I (C) and probed with a 473-bp region of *FST1* (P). (B) The presence or absence of *FST1* was verified by Southern analysis in the wild-type (lane 1), $\Delta fst1$ (lane 2), FST-COMP1 (lane 3) and FST-COMP2 (lane 4) strains. Integration of pHT-10 was ectopic in the transformants, and the FST-COMP1 strain appeared to contain two copies of the complementation construct and thus was not used in subsequent experiments.

strain was not significantly impaired in the uptake or utilization of either carbohydrate. On whole kernels of maize, growth of the $\Delta fst1$ strain was slightly higher than either the wild-type or complemented strain (Table 3), which indicates that *FST1* is dispensable for kernel colonization. Additionally, disruption of *FST1* had no effect on kernel acidification after colonization (Table 3).

To determine if *FST1* affects fumonisin biosynthesis during kernel colonization, we measured production of FB₁ by the wild-type, $\Delta fst1$ and complemented strains after 10 days of growth on mature maize kernels. Despite robust growth on kernels, the $\Delta fst1$ strain produced approximately five-fold less FB₁ than the wild-type strain (Table 3). Production of FB₁ was restored in the complemented strain, thus confirming that the reduction in FB₁ biosynthesis is fully attributable to disruption of *FST1* (Table 3).

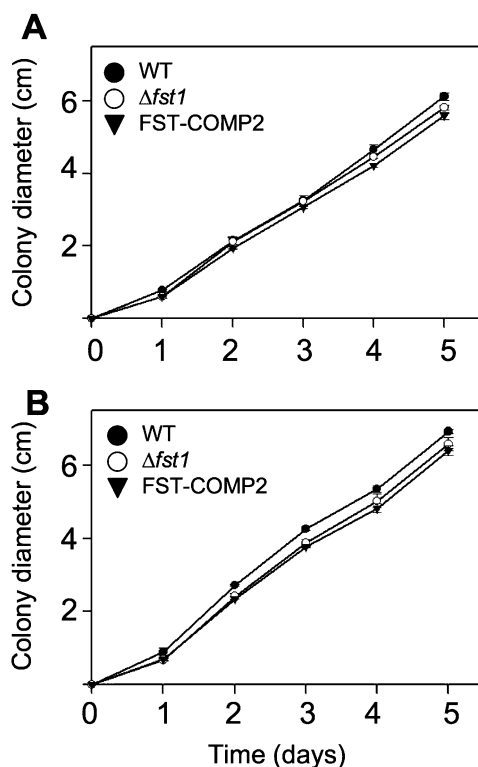


Fig. 5 Growth of the wild-type, $\Delta fst1$, and FST-COMP2 strains with either glucose (A) or maltose (B) as the sole carbon source. Radial growth was measured for three individual colonies of each strain. Error bars represent the standard error of the mean.

DISCUSSION

The discovery and characterization of *ZFR1* represented an important advance in understanding the genetic regulation of fumonisin biosynthesis during maize kernel colonization. However, the manner in which *ZFR1* regulates fumonisin biosynthesis is not fully understood. In many fungi, genes encoding enzymes that produce a specific class of secondary metabolites are physically grouped into clusters, and frequently these clusters contain a gene encoding a pathway-specific transcriptional regulator. Although *ZFR1* is predicted to encode a transcriptional regulator, it is not located within the *FUM* gene cluster, and recently a cryptic gene (*FUM21*) was discovered within the *FUM* cluster that functions as a pathway-specific regulator of *FUM* gene transcription (Brown *et al.*, 2007). Therefore, the regulation of fumonisin biosynthesis by *ZFR1* during kernel colonization is most probably an indirect consequence of other changes in the transcriptome of the fungus. Consistent with this hypothesis, the $\Delta zfr1$ strain grew less than the wild-type on endosperm fractions of mature kernels as well as various carbohydrates such as glucose and maltose. Initially, this finding suggested that the $\Delta zfr1$ strain was defective in the perception or utilization of starch, or possibly

Table 3 Growth, FB₁ production, and change in kernel pH of the $\Delta fst1$ strain.*

Strain	FB ₁ †	Ergosterol‡	pH (Δ pH)§
Wild type	111 ± 9	293 ± 15	4.8 ± 0.1 (−0.7)
$\Delta fst1$	20 ± 8	381 ± 88	5.0 ± 0.3 (−0.5)
FST-COMP2	100 ± 6	300 ± 58	5.0 ± 0.2 (−0.5)

*All measurements were taken from three experimental replicates after 10 days of growth on whole kernels of maize.

†As measured in micrograms of FB₁ per gram of maize, ± standard error.

‡As measured in micrograms of ergosterol per gram of maize, ± standard error.

§ Δ pH = [(the pH of the uninoculated material) − (the average pH after 10 days of growth)].

its amylolytic breakdown products such as maltose and glucose, in the endosperm tissue. However, our observations indicate that the $\Delta zfr1$ mutant perceives extracellular starch in the kernel environment. Expression of amylolytic genes in the $\Delta zfr1$ strain was substantially higher when grown on endosperm tissue than on germ tissue; unexpectedly, amylase activity was higher in the mutant than in the wild-type. The elevated levels of gene expression and amylolytic activity suggest that the $\Delta zfr1$ mutant is able to perceive and hydrolyse extracellular starch, but either cannot perceive or fully utilize the products of hydrolysis. Additionally, disruption of *ZFR1* had little effect on overall hexokinase activity or on the induction of *HXX1* and *HXX2* on endosperm tissue, thus leading us to conclude that the phenotypes of the $\Delta zfr1$ mutant are not caused by aberrant hexokinase expression or activity during kernel colonization. However, we cannot completely dismiss the possibility that *HXX1* and *HXX2* function as sugar sensors in *F. verticillioides* or that *ZFR1* is involved in regulating their expression. To clarify further the roles of *HXX1* and *HXX2* in fumonisin biosynthesis and kernel colonization, functional studies of these two genes are currently underway.

Although the molecular aspects of glucose sensing and uptake have been studied extensively in yeast, less is known about these pathways in filamentous fungi. Twenty sugar transporters have been characterized in *Saccharomyces cerevisiae* (Boles and Hollenberg, 1997), all of which belong to the sugar porter (SP) subfamily of the MFS. In yeast and filamentous fungi, glucose transport is facilitated by both constitutive low-affinity and glucose-repressible high-affinity systems that probably rely on high- and low-affinity glucose transporters such as HGT-1 (Xie *et al.*, 2004). In addition to transporting sugars across the plasma membrane, some members of the SP subfamily function as sugar sensors, including *SNF3* and *RGT2* in yeast, and *RCO-3* in *Neurospora crassa* (Madi *et al.*, 1994). A domain search of the genome of *F. verticillioides* reveals at least 147 putative genes with significant similarity to sugar transporters, none of which has been characterized as to function or substrate specificity. Of the 50 putative sugar transporter genes of *F. verticillioides* available for microarray analysis, six showed higher levels of

expression on endosperm tissue, including genes with high homology to hexose transporters, maltose transporters and other oligosaccharide transporters. Intriguingly, and at least three of these putative transporters induced on endosperm tissue were down-regulated in the $\Delta zfr1$ mutant. Based on these observations, we conclude that *ZFR1* is required for the expression of a crucial subset of the genes involved in perceiving or acquiring sugars. The exact regulatory mechanism through which *ZFR1* functions, however, is not clear. *ZFR1* may interact directly with the promoters of genes encoding sugar transport proteins, or conversely may play a more global role in regulating carbohydrate metabolism. It is also important to note that the microarray contained probes corresponding to only 34% of the putative sugar transporter genes in *F. verticillioides* and that 12% of the 50 genes that were represented had increased gene expression, thus making it likely that other sugar transporter genes also show increased expression on maize kernel endosperm. Future experiments will focus on identifying additional sugar transport genes involved in the regulation of secondary metabolism as well as determining the effect of *ZFR1* on the transcriptome of *F. verticillioides* during kernel colonization.

With respect to fumonisin biosynthesis, we hypothesize that one or more sugar transporter homologues of *F. verticillioides* sense specific molecules involved in pathway induction. Several lines of evidence suggest that *FST1* plays such a role during kernel colonization. Expression of *FST1* is induced by endosperm tissue, the fraction of maize kernels that supports the highest level of FB_1 biosynthesis. Disruption of *FST1* significantly reduced FB_1 biosynthesis on maize kernels, but had negligible effects on growth or kernel acidification during colonization. Also, the $\Delta fst1$ strain displayed no reduction in growth in defined media containing glucose or maltose as the sole carbon source, thus indicating that its role in uptake is negligible or compensated for by other transporters. Together, these observations suggest that disruption of *FST1* does not cause an overall reduction in sugar uptake, but rather a deficiency in specific aspects of sugar signalling. In earlier work, we found that amylopectin induces high levels of fumonisin biosynthesis during kernel colonization (Bluhm and Woloshuk, 2005). Our current effort is to test the hypothesis that *FST1* functions to sense specific breakdown products of starch during kernel colonization and activates as yet undescribed signal transduction pathways that induce fumonisin biosynthesis.

This study has shown that the uptake of sugars into the fungal cell is likely to be a central component of kernel colonization and the induction of fumonisin biosynthesis in *F. verticillioides*, and that *ZFR1* may affect fumonisin biosynthesis by regulating sugar uptake or perception. A thorough analysis of genes involved in sugar sensing and transport is anticipated to shed new light on pathogenesis and the accumulation of mycotoxins in maize kernels. For example, breeders of maize have documented a lack of correlation between disease severity of *F. verticillioides* and

fumonisin accumulation; often, asymptomatic kernels or ears contain relatively high levels of fumonisins. Further studies are needed to determine if the regulation of sugar transporters, possibly by *ZFR1*, plays a role in this phenomenon.

CONCLUSIONS

The regulation of fumonisin biosynthesis during maize kernel colonization is complex and poorly understood at the molecular level. The evidence presented in this study indicates that $\Delta zfr1$ strains display reduced growth on maize kernel endosperm tissue and a variety of carbohydrates including starch and breakdown products of starch.

Based on our findings, we hypothesize that *ZFR1* is required for the expression of *FST1* and other sugar transporters during kernel colonization. Our observation that *FST1* is required for fumonisin biosynthesis but not growth during kernel colonization suggests that *FST1* functions as a carbohydrate sensor and thus a key linkage between primary and secondary metabolism. This study provides new information on the function of sugar transporters in mycotoxigenesis and could provide novel approaches to control the accumulation of fumonisins in maize.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

F. verticillioides strain 7600 (Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City, KS) was used as wild-type in this study. The mutant strain $\Delta zfr1$ was described previously (Flaherty and Woloshuk, 2004). Mutant strain $\Delta fst1$ and complemented strains FST-COMP1 and FST-COMP2 were generated for this study as described below. Cultures were maintained on potato dextrose agar (PDA; B&D, Sparks, MD). Cracked maize kernels and kernel fractions were prepared as described by Shim *et al.* (2003). Radial growth was measured on BSAL-agar media [0.1 M KH_2PO_4 , pH 4.5; 0.01 M $MgSO_4$; 0.1 M NaCl; 0.1% bovine serum albumin; 2% (w/v) of a primary carbon source consisting of either glucose, maltose, amylopectin or dextrin].

Quantification of fungal growth

Radial growth measurements were performed as described by Bluhm and Woloshuk (2006). The ergosterol content of kernels or liquid cultures was analysed as a quantitative measure of fungal growth (Bluhm and Woloshuk, 2005; Seitz *et al.*, 1979). Ground kernels (100–400 mg) were extracted overnight at room temperature in 2 : 1 chloroform/methanol. Liquid cultures (10 mL) were pelleted by centrifugation, dried on sterile filter paper, ground in liquid nitrogen with a mortar and pestle, and extracted

in 1 mL of 2 : 1 chloroform/methanol. Ergosterol analysis was performed on an HPLC system with UV detection at 282 nm (Beckman Coulter, Fullerton, CA) and a 4.6 U ODS column (200 × 4.6 mm, Beckman Coulter). Compounds were eluted with 100% methanol at a flow rate of 1.0 mL/min. Quantities were determined by comparing peak areas of samples to a standard curve generated from HPLC-grade ergosterol (Sigma, St. Louis, MO).

Nucleic acid isolation and analysis

Bacterial plasmids were isolated with a Qiagen miniprep DNA purification system (Qiagen, Valencia, CA). Fungal genomic DNA was collected from cultures grown in YEPD media as described by Woloshuk *et al.* (1994). For Southern blot analysis, genomic DNA was digested with *Cla*I, fractionated by electrophoresis through 0.7% agarose gels and transferred to nylon membranes (Nytran; Schleicher and Schuell, Keene, NH). Probes were radiolabelled with the Rediprime II random prime labelling system (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and hybridizations were performed as described by Flaherty *et al.* (2003). For analyses of gene expression, total RNA was extracted from maize kernels as follows: ground colonized kernels (0.5–1 g) were suspended in 10 mL water-equilibrated phenol (pH 4.0) to which 10 mL Tris-HCl (pH 8.0) was added. Phases were separated by centrifugation (10 min at 12 000 *g*, 4 °C) and the aqueous phase was subsequently extracted with 10 mL phenol/chloroform (1 : 1) and 10 mL chloroform/isoamyl alcohol (24 : 1). RNA was precipitated overnight at –20 °C after the addition of ethanol to a final volume of 70%. Precipitated RNA was pelleted by centrifugation (30 min at 12 000 *g*, 4 °C), air-dried briefly and purified with the RNeasy RNA purification kit (Qiagen).

Identification of genes predicted to function in sugar sensing, amylolysis or sugar transport

Homology-based analyses of expressed sequence tags (ESTs) (<http://compbio.dfci.harvard.edu/tgi>) and genomic sequence of *F. verticillioides* (<http://www.broad.mit.edu>) were used to identify two genes predicted to function in sugar sensing (*HXX1*, GenBank accession no. EU247513; *HXX2*, GenBank accession no. EU247514) and six genes involved in starch hydrolysis (*AMY1*, GenBank accession no. DQ143884; *GMY1*, GenBank accession no. EU247508; *GMY2*, GenBank accession no. EU247509; *MLT1*, GenBank accession no. EU247510; *MLT2*, GenBank accession no. EU247511; and *MLT3*, GenBank accession no. EU247512). The genome of *F. verticillioides* is also predicted to contain 381 genes encoding MFS proteins, 147 of which are homologous to sugar transporters in yeast and other filamentous fungi. Of these 147 genes, 50 were represented on microarrays including *FST1* (GenBank accession no. EU152990), *FST2* (GenBank accession no. EU152991), *FST3* (GenBank accession no. EU152992), *FST4*

(GenBank accession no. EU152993), *FST5* (GenBank accession no. EU152994) and *FST6* (GenBank accession no. EU152995).

Determination of glucokinase activity

Germ and endosperm fractions of maize kernels (Beck's 5322) prepared as described above were inoculated with wild-type or the *Δzfr1* strain of *F. verticillioides*. After 6 days, kernels were harvested as described above. Glucokinase activity was determined essentially as described by Olsson *et al.* (2003). Briefly, ground colonized kernels were extracted for 10 min in buffer AT (50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100). Total protein concentration for each extract was determined with Bradford reagent (Sigma) and volumes were subsequently adjusted for each sample to achieve identical concentrations in assay buffer consisting of 100 mM HEPES-KOH, pH 7.8, 10 mM MgCl₂, 2 mM ATP, 0.8 mM NADP, 0.2 U glucose-6-phosphate dehydrogenase (Sigma) and 10 mM glucose (Sigma). After incubation at 30 °C for 20 min, the A₃₄₀ was measured for each sample. One unit of glucokinase activity was determined as the formation of 1 μmol NADPH/min. The concentration of NADPH was calculated based on an extinction coefficient of A = 6200/mol. To determine amylase activity, strains were grown on starch plates (0.3 M NaNO₃, 6 mM K₂HPO₄, 4 mM MgSO₄, 7 mM KCl, 66 μM FeSO₄ and 2% amylose) and stained with an iodine solution (0.5% iodine and 5% potassium iodine) to visualize α-amylase activity as described by Fakhoury and Woloshuk (1999).

Microarray analysis of gene expression

Germ and endosperm fractions of mature maize kernels (Beck's 5322) were separated as described by Shim *et al.* (2003). Five grams of each fraction were placed in 20 mL scintillation vials (RPI, Mt. Prospect, IL), autoclaved and inoculated with 1 × 10⁵ conidia of wild-type *F. verticillioides*. After 4 days of growth, vials were flash-frozen in liquid nitrogen and total RNA was extracted from samples. RNA samples were labelled and hybridized to microarrays by NimbleGen (Madison, WI). Oligonucleotide probes of 24 bp were derived from analysis of the entire suite of ESTs available from the *F. verticillioides* sequencing project (Brown, *et al.*, 2005). Each of the 11 126 unique ESTs is represented twice at randomly assigned positions on the microarray to provide technical replication. Data were normalized by robust multiarray average (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003) included in NimbleScan software. For each EST, expression was normalized to *TUB2* by dividing the spot intensity of each EST by the intensity recorded for *TUB2*. The experiment was conducted with two biological replicates. For each gene, the statistical significance of changes in gene expression was calculated with a *t*-test for equal and unequal variance using PROC TTEST from SAS. The array platform and experimental data were submitted to Gene Expression

Table 4 Primers used in this study.

Primer name*	Sequence (5'→3')
FST1-1	TCCTGCCTGATGATCTTGGTCTG
FST1-2	TTTGTGCTCACCGCTGGACTCGAACTGATAACTGACAC
FST1-3	GAATAGATGCCGACCGGAAACATCCTGGCTCTGAAT
FST1-4	ACCGTCACGTGCGCTACTTACTTC
FST1-5	CCGTCACGTGCGCTACTTACTCA
FST1-nF	AACTCTGGTATGCCGCTTGGTG
FST1-nR	TCTGTCATCTTCCCTGCTGGTAAA
FST1-ns5	TGCTCTTCTTCTCCGCTCTTT
FST1-ns3	AACACCAGCCCAATCTCCCTAAC
FST1-cF	CATGGGAATAGCAGTGCCTTTGAC
HYG5	GTCCAGGCGGTGAGCACAAA
HYG3	TTCCCGTCCGGCATCTATTC
HYG13	AATACGAGGTCCGCAACATCTTCT
FST1-pF	TCCTCGCTCTTTCATCGTCACTT
FST1-pR	TGCCAAACCGTCAAACATCTCCT
AMY1rtF	GGCATGGGATTTGATGCTATTTGG
AMY1rtR	GTCATGAGCAGATTTGACAAGACTC
GMV1rtF	CTGGTGTTATTCCTCCCTCTGGG
GMV1rtR	GAGAGCCGTCCTTCTTAACATTGATGTAC
GMV2rtF	ACAAGTCTCTCCAGCGCAAGATC
GMV2rtR	CAGAAGCTTTGCATGAAGCAGAGAAC
MLT1rtF	AGCCTCTTTGACGAGACTGAATCAG
MLT1rtR	TCGCAGACTTCTCTCTGACGAA
MLT2rtF	GACGCTCATTCCAAGGAAGTGC
MLT2rtR	TGACATCGTGAACCTGTTTTCTGACTGC
MLT3rtF	TCCAATTCGGTGGCGGTAGTG
MLT3rtR	TGAAGCATAAATGGCCCGACGC
HXK1rtF	GAGAACCAAGATATCTCTCTGCTACGC
HXK1rtR	TAAGGTTCTCCAGGGGTCTTC
HXK2rtF	ATCTCAACTCGAGCAGAACGACTG
HXK2rtR	GCCGCAACGGACATGATGGA
FST1rtF	CTTCTGATGCTCTTCTCTCCTCGC
FST1rtR	TCTGGTATATCTACCAATGAACGCGAT
FST2rtF	GGTATCTCTGAAAAGGAGGAGCCAG
FST2rtR	TGGCATCGCCTTTGAACTG
FST4rtF	TCATGAACTTCTTATCCAGGTTGCTC
FST4rtR	AAAGGCGAGCTCGACGGCAAT

*The rtF or rtR extension indicates the forward or reverse primer, respectively, for quantification of gene expression by real-time PCR.

Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession no. GSE8535.

Quantitative PCR (qPCR) analysis of gene expression

Primers used in this study are listed in Table 4; primers for *TUB2* were described previously by Bluhm and Woloshuk (2005). RNA was extracted from ground maize kernels and cDNA was generated as described by Flaherty *et al.* (2003). Reactions were performed in an ABI 7700 Sequence Detection System (Applied Biosystems) and data were collected with Sequence Detector Software version 1.7 (Applied Biosystems). Each reaction contained 10 µL of

QuantiTect SYBR[®]-green PCR Master mix (Qiagen, Valencia, CA), forward and reverse primers (500 nM of each), cDNA template and nuclease-free water to a final volume of 20 µL. PCR cycling conditions were 2 min at 50 °C (one cycle); 10 min at 95 °C (one cycle); 15 s at 95 °C followed by 1 min at 60 °C (40 cycles). Expression of genes was measured in triplicate. To verify that the efficiencies of the target and reference reactions were approximately equal, reactions were performed using the primers for each gene or *TUB2* with serial dilutions of cDNA as template. After verifying that the efficiencies of the primers were acceptable, expression levels were calculated by the comparative Ct method (Applied Biosystems) with *TUB2* as the endogenous reference for normalization.

Identification and disruption of *FST1*

A disruption vector for *FST1* was constructed by the double-joint PCR strategy (Yu *et al.*, 2004). DNA fragments corresponding to the 5' (1.0 kb) and 3' (1.6 kb) regions of *FST1* were amplified from genomic DNA of *F. verticillioides* with primer pairs FST1-1/FST1-2 and FST1-3/FST1-4, respectively, and a 1.4-kb hygromycin B phosphotransferase (HYG^R) gene cassette was amplified from pCB1003 (Carroll *et al.*, 1994) with primers HYG5/HYG3. A 3.6-kb fusion product was amplified from the three individual templates with nested primers FST1-nF/FST1-nR and was cloned into the pGEM-T-Easy vector (Promega) to create pFST1-KO. Protoplasts of *F. verticillioides* were transformed as described previously (Proctor *et al.*, 1999). Transformants were screened by PCR with primers FST1-ns5/FST1-ns3 and HYG13/FST1-5 to identify strains disrupted in *FST1*. One isolate that tested positive for disruption of *FST1* by PCR was confirmed by Southern analysis and designated $\Delta fst1$. For complementation of $\Delta fst1$, we amplified 4.4-kb DNA fragment, containing a 1.8-kb coding region of *FST1*, by primers FST1-cF/ FST1-4, and cloned the product into pGEM-T-Easy to generate pHT-10. Protoplasts of the $\Delta fst1$ strain were co-transformed with pHT-10 and pKS-GEN, a vector created by cloning the geneticin resistance cassette from pSM334 (Flaherty *et al.*, 2003) into the *Xba*I site of pBluescript-KS. Geneticin-resistant colonies were screened by PCR with primers FST1-ns5/FST1-ns3 and confirmed by Southern analysis for the presence of *FST1*.

FB, extraction and analysis

Maize kernels were ground and extracted as described by Bluhm and Woloshuk (2005), and 5 mL of the supernatant was passed over an equilibrated C18 column (Agilent Technologies, Palo Alto, CA) as described by Rice *et al.* (1995). Samples were derivatized with O-phthalaldehyde as described by Rice *et al.* (1995) and analysed with an HPLC system (Shimadzu, Kyoto, Japan) equipped with a C18 column (5 U, 150 × 4.6 mm; Alltech, Deerfield, IL) and a fluorescence detector (335 nm excitation, 440 nm emission).

For each sample, FB₁ was measured by comparing peak areas to a standard curve generated by analysis of HPLC-grade FB₁ (Sigma).

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