**HXXK1** regulates carbon catabolism, sporulation, fumonisin B$_1$ production and pathogenesis in *Fusarium verticillioides*

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In *Fusarium verticillioides*, a ubiquitous pathogen of maize, virulence and mycotoxigenesis are regulated in response to the types and amounts of carbohydrates present in maize kernels. In this study, we investigated the role of a putative hexokinase-encoding gene (HXXK1) in growth, development and pathogenesis. A deletion mutant (Δhxk1) of HXXK1 was not able to grow when supplied with fructose as the sole carbon source, and growth was impaired when glucose, sucrose or maltotriose was provided. Additionally, the Δhxk1 mutant produced unusual swollen hyphae when provided with fructose, but not glucose, as the sole carbon source. Moreover, the Δhxk1 mutant was impaired in fructose uptake, although glucose uptake was unaffected. On maize kernels, the Δhxk1 mutant was substantially less virulent than the wild-type, but virulence on maize stalks was not impaired, possibly indicating a metabolic response to tissue-specific differences in plant carbohydrate content. Finally, disruption of HXXK1 had a pronounced effect on fungal metabolites produced during colonization of maize kernels; the Δhxk1 mutant produced approximately 50% less trehalose and 80% less fumonisin B$_1$ (FB$_1$) than the wild-type. The reduction in trehalose biosynthesis likely explains observations of increased sensitivity to osmotic stress in the Δhxk1 mutant. In summary, this study links early events in carbohydrate sensing and glycolysis to virulence and secondary metabolism in *F. verticillioides*, and thus provides a new foothold from which the genetic regulatory networks that underlie pathogenesis and mycotoxigenesis can be unravelled and defined.

**INTRODUCTION**

*Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a ubiquitous pathogen of maize and is endemic virtually everywhere maize is grown in the world (Nelson et al., 1993). *F. verticillioides* is remarkably versatile, capable of infecting seedlings, roots, stalks and ears of maize plants. Although yield losses can be considerable due to poor stand establishment (seedling infections) and lodging before harvest (stalk infections), kernel infections present a unique set of problems for agricultural production due to the accumulation of fumonisin mycotoxins in colonized kernels. At least 28 fumonisin analogues have been identified, the B series of which is most commonly associated with *F. verticillioides* (Rheeder et al., 2002); of these, fumonisin B$_1$ (FB$_1$) is predominant during kernel infections and is also the most toxic to humans and livestock (Desai et al., 2002).

Ingestion of FB$_1$ leads to the obstruction of sphingolipid biosynthesis by inhibiting ceramide synthase (sphinganine N-acyltransferase) (Desai et al., 2002), and results in leukoencephalomalacia in horses and oesophageal cancer and birth defects in humans (Hendricks et al., 1999). The biochemical and genetic basis of fumonisin biosynthesis is increasingly well understood. Fumonisins are derived from polyketides, the backbone chains of which are elongated through incorporation of acetyl-CoA or malonyl-CoA via Claisen condensations, followed by a series of condensations, reductions and esterifications that result in the formation of structural analogues within a series (Gerber et al., 2009). In *F. verticillioides*, fumonisin biosynthetic (*FUM*) genes are physically clustered within the genome and have been studied extensively. Currently, 17 *FUM* genes are known to constitute the fumonisin biosynthetic gene cluster (Brown et al., 2007; Proctor et al., 2003); genes encoding proteins similar to a polyketide synthase (*FUM1*), cytochrome P450 monoxygenases (*FUM6, FUM12, FUM15*), dehydrogenases (*FUM7, FUM13*), an aminotransferase (*FUM8*), a dioxygenase (*FUM9*), fatty acyl-coenzyme A synthetases...
(FUM10, FUM16), a tricarboxylate transporter (FUM11), a peptide synthetase (FUM14), longevity assurance factors (FUM17, FUM18), an ABC transporter (FUM21), a putative pathway-specific transcription factor (FUM24), and a gene of unknown function (FUM20). In addition, a small number of regulatory genes have been identified in F. verticillioides that affect FB1 biosynthesis, including FCC1 (a C-type cyclin), PAC1 (a pH-responsive transcription factor gene), ZFR1 (a zinc finger transcription factor gene) and AREA (a nitrogen metabolism regulatory gene) (Flaherty et al., 2003; Flaherty & Woloshuk, 2004; Kim & Woloshuk, 2008; Shim & Woloshuk, 2001). However, genes that specifically regulate the induction of FB1 biosynthesis in response to the plant environment have not been described.

Important environmental cues have been identified that modulate the genetic regulatory network underlying fumonisin biosynthesis. For example, the sources of carbon available to F. verticillioides play a critical role in the induction of fumonisin biosynthesis. The availability of amylopectin, the major constituent of starch in maize kernels, correlates strongly with high levels of FB1 biosynthesis during kernel colonization. FB1 biosynthesis increases dramatically with kernel maturity, peaking when starch accumulates in endosperm tissues during kernel development (Bluhm & Woloshuk, 2005; Warfield & Gilchrist, 1999), and is substantially higher in the starch-rich endosperm of mature kernels than the lipid- and protein-rich germ tissues (Shim et al., 2003). Additionally, FB1 biosynthesis is repressed by nitrogen sources such as ammonium and glutamine (Kim & Woloshuk, 2008; Shim & Woloshuk, 1999), and high pH (Flaherty et al., 2003). The ability of diverse environmental cues to influence fumonisin biosynthesis hints at the existence of a complex genetic regulatory network in F. verticillioides.

In F. verticillioides, the signal transduction networks that regulate FB1 biosynthesis in response to carbohydrate availability have not been elucidated at the molecular level. Of critical importance is to understand how F. verticillioides perceives and utilizes starch and other carbohydrates during kernel colonization. Comparative analyses of gene expression in F. verticillioides during growth on germ versus endosperm tissues have led to the identification of FST1, which is predicted to encode a member of the major facilitator superfamily (MFS), homologous to sugar transporters in other filamentous fungi (Bluhm et al., 2008). Expression of FST1 is induced on endosperm tissue and regulated by ZFR1, a gene encoding a zinc finger transcription factor that is required for FB1 biosynthesis (Bluhm et al., 2008). Disruption of FST1 causes a severe reduction in FB1 biosynthesis and virulence. Intriguingly, the protein encoded by FST1 may not function as a transporter, but rather as a carbohydrate sensor, analogous to RCO3 in Neurospora crassa (Kim & Woloshuk, 2011). However, additional mechanisms of carbohydrate detection are likely to exist in filamentous fungi. In higher eukaryotes, hexokinases are multifunctional enzymes, with roles in catabolism (e.g. required for the first step of glycolysis) and signalling (e.g. glucose sensors) (Harrington & Bush, 2003; Santangelo, 2006). Although hexokinases have been implicated in sugar sensing/signalling in plants and animals (Clayssen & Rivoal, 2007; Wilson, 2003; Xiao et al., 2000), the extent to which hexokinases function as sugar sensors is unknown among filamentous fungi, and has not been investigated in F. verticillioides.

The purpose of this study was to investigate the potential function of hexokinase genes in F. verticillioides during pathogenesis. We focused on HXK1, a gene predicted to encode a putative hexokinase, whose expression was reduced significantly in a Δzfr1 strain (Bluhm et al., 2008). Here, a HXK1 deletion mutant (Δhxk1) was characterized, and defects were observed in growth, development, pathogenesis and secondary metabolism, including FB1 biosynthesis. Several phenotypes were most evident when fructose was supplied as the sole carbon source, possibly indicating that HXK1 encodes a fructokinase. Taken together, the results support the hypothesis that hexokinases such as the HXK1 product serve important roles in the transduction of external environmental cues that regulate pathogenesis and mycotoxigenesis in F. verticillioides.

**METHODS**

**Fungal strains and media.** F. verticillioides strain 7600 (Fungal Genetics Stock Center) was the wild-type in this study, and Δhxk1 (HXK1 deletion strain) and Δhxk1-comp (strain Δhxk1 complemented with the wild-type copy of HXK1) were created as described below. All strains were stored at −80 °C in 25% (v/v) glycerol. Conidia were obtained from cultures grown on potato dextrose agar medium (PDA; BD) by rinsing plates with sterile water, and conidia were quantified with a haemocytometer prior to inoculation. To extract fungal genomic DNA, strains were grown in yeast extract peptone (YPE) medium (5 g yeast extract, 10 g peptone) containing 20 g glucose l⁻¹. To quantify sugar uptake, cultures were resuspended into defined liquid (DL) medium (per litre: 3 g KH₂PO₄, 0.3 g MgSO₄, 1 g BSA, 5 g NaCl, 20 g of either glucose or fructose) from YEP medium.

**Nucleic acid isolation and analysis.** Bacterial plasmids were isolated with a Qiagen Miniprep DNA purification system. PCR primers were obtained from Integrated DNA Technologies. Isolation of protoplasts from F. verticillioides and transformation were performed as described previously (Shim & Woloshuk, 2001). Standard procedures were followed for isolation of fungal genomic DNA and Southern blotting (Kim & Woloshuk, 2008).

**Phylogenetic analysis.** A dataset of amino acid sequences encoding putative proteins was assembled from the genome of F. verticillioides (Broad Institute) by BLASTP (Altschul et al., 1990). Sequences were initially aligned withCLUSTAL_X (Larkin et al., 2007). Subsequently, ambiguously aligned regions were removed manually. The amino acid dataset was analysed by distance and neighbour-joining methods with a Dayhoff PAM matrix in PHYLP v3.68 (http://evolution.genetics.washington.edu/phylib.html). Default settings were used and internal branch support was evaluated based on 100 bootstrap replicates.

**Deletion and complementation of HXK1.** To delete HXK1, a DNA fragment consisting of a selective resistance gene cassette (HPH, hygromycin B phosphotransferase) fused between the 5’ and 3’
flanking sequences of HXK1 was generated by double-joint PCR (Yu et al., 2004). To this end, DNA fragments corresponding to 5′ (1.4 kb) and 3′ (1.4 kb) regions flanking the ORF of HXK1 were amplified from genomic DNA with the primer pairs HXK1-1 (5′-CCATTGGCCGTCAGAGA-3′)/HXK1-2 (5′-TTGTTGCTCCACCGTTGAGCAGTGT-3′) and HXK1-3 (5′-GATGATGCGCCGAGCTGCTCCTCACCAGCCTTT-3′) /HXK1-4 (5′-TCATCATCGAACCCAGACTGTTGA-3′), respectively. The HPH cassette was amplified from pCB1003 (Fungal Genetics Stock Center) with primers HYG5 (5′-GTCAGCGGTGAGACAAAGAGAGAAGGGAATCTCCCTCACCAGCCTTT-3′) and HYG3 (5′-TCCCGTGCACGACCTCA-3′). Sequences complementary to primers HYG5 and HYG3 were incorporated into the 5′ ends of primers HXK1-2 and HXK1-3 (underlined above) to facilitate fusion of the three PCR products. For double-joint PCR, the 5′ flanking region of HXK1, the HPH cassette and the 3′ flanking region of HXK1 were fused as described by Yu et al. (2004). Finally, the fusion product was amplified with nested primers HXK1-NF (5′-ACCTACAGCAGAAGGCACCCACATGATC-3′) and HXK1-NR (5′-TCCCGGACCCAGACAGCAGCAACAGACAACAAGAGAAGGGAATCTCCCTCACCAGCCTTT-3′) to create a 3.6 kb product containing the HPH cassette fused to the HXK1 flanking regions. The resulting PCR product was concentrated and transformed into the wild-type strain of F. verticillioides as described by Kim & Woloshuk (2008). The deletion strains were confirmed by Southern analysis by probing for either the absence or the presence of the HXK1 ORF. The probe used for Southern analysis was amplified with the primer pair HXK1-NSF (5′-TCCCGGACCCAGACAGCAGCAACAGACAACAAGAGAAGGGAATCTCCCTCACCAGCCTTT-3′) and HXK1-NSR (5′-GTCAGCGGTGAGACAAAGAGGGAATCTCCCTCACCAGCCTTT-3′). To complement the Δhxk1 mutant, a PCR product containing the wild-type copy of HXK1 was amplified with primers HXK1-NF and HXK1-NR from strain 7600, and co-transformed into the Δhxk1 mutant with plasmid pXS-GEN, which confers resistance to geneticin (Flaherty et al., 2003). The resulting transformants were screened by PCR with primers HXK1-NSF and HXK1-NSR for the deletion region, and the growth of complemented strains was confirmed on a defined medium containing fructose as the sole carbon source.

Quantification of FB$_3$, and ergosterol during kernel colonization. Maize kernels (inbred B73) were inoculated as described by Kim & Woloshuk (2008), and FB$_3$, and ergosterol were quantified after 7 days growth, as described by Bluhm & Woloshuk (2005). Briefly, FB$_3$ was extracted from ground maize kernels (500 mg) with 2 ml acetone/ water (1:1, v/v). The extracts were passed through equilibrated C-18 solid phase extraction columns (Agilent Technologies), and FB$_3$ was eluted with 2 ml acetone/water (7:3, v/v). Analysis was performed on an HPLC (Shimadzu Scientific Instruments) equipped with an analytical C-18 column (150 x 4.6 mm, Alltech) and a fluorescence detector (excitation 335 nm/emission 440 nm). FB$_3$ was quantified by comparison of peak areas with those of an FB$_3$ standard (Sigma-Aldrich). To quantify ergosterol, ground kernels (~500 mg) were extracted in 2 ml chloroform/methanol (2 : 1, v/v). Ergosterol was measured with a GC-2010 gas chromatograph (Shimadzu) equipped with a ZB-5 column (30 m x 0.25 mm, Phenomenex) and a flame-ionization detector (FID) set at 340 °C. To separate metabolites, the oven temperature was held constant at 120 °C for 5 min, increased to 300 °C by 4 °C per 1 min, and then held constant at 300 °C for 15 min. GC-FID data were normalized to the internal standard by multiplying each metabolite peak area by a normalization factor. To generate the normalization factor, the largest internal standard peak area was divided by the internal standard peak area for each fingerprint. To minimize differences due to slight variation in starting material, peak areas from each fingerprint were divided by the tissue weight used for extraction. Finally, to normalize each metabolite to fungal growth, peak areas from each fingerprint were divided by ergosterol peak area of the sample. Arabitol, mannnitol and trehalose were quantified based on comparison with standard curves derived from analytical standards (respectively: Alfa Aesar, Fisher Scientific and TCI America).

RESULTS

Identification and disruption of HXK1

The genome of F. verticillioides is predicted to contain at least six genes encoding putative hexokinases: CVEG_00957, CVEG_0632, CVEG_0642, CVEG_01889 and CVEG_05775 (Broad Institute; http://www.broadinstitute.org). Conceptual translation of these genes indicated a similar domain architecture, consisting of two characteristic hexokinase domains: hexokinase 1 (Pfam accession no. (10$^3$ ml$^{-1}$) were injected into internodes of maize stalks with a syringe attached to a needle. Ear and stalk rot assays were repeated at least twice, with at least three replications per strain in each independent experiment.

Quantification of sugar uptake. Total sugar amounts were measured with a modified anthrone method (Laurentin & Edwards, 2003). Briefly, fungal strains were grown in YEP medium for 3 days, and mycelia were harvested by centrifugation. Mycelia (0.22 g) from each strain were then transferred to D-glucose or D-fructose medium, with at least three replications per strain. From each culture, 50 μl medium was collected at 0, 24, 48, 72 and 96 h, and diluted 200-fold with water for quantification of sugar. Samples (300 μl) were mixed with 600 μl anthrone reagent [2 μg anthrone (ml sulfuric acid)$^{-1}$] in test tubes. After boiling for 5 min, A$_{520}$ was measured with a spectrophotometer (Beckman Instruments). A standard curve was prepared with either glucose or fructose (Sigma-Aldrich).

Metabolic fingerprinting of colonized kernels. Kernels of inbred B73 were selected arbitrarily for metabolic fingerprinting at 7 days after inoculation with the wild-type, Δhxk1 and Δhxk1-comp strains. Metabolic fingerprinting was performed essentially as described by Smith & Bluhm (2011), with minor modifications. Briefly, kernels were ground under liquid nitrogen in a pre-chilled mortar, and metabolites were extracted with methanol (500 mg kernel tissue per 2 ml methanol) containing phenyl-β-D-glucopyranoside (130 μg ml$^{-1}$) as an internal standard for normalization. After 16 h incubation, samples were centrifuged, and part of the supernatant (150 μl) was transferred to a 2 ml autosampler vial and dried by nitrogen at ambient temperature. Dried extracts were resuspended in 100 μl trimethylsilylimidazole/trimethylchlorosilane (TMS; 100 : 1, v/v), and incubated at 37 °C for 1 h. The TMS products were partitioned into the organic phase after the addition of 100 μl isooctane and 200 μl water. Of the organic phase, 1 μl was analysed with a GC-2010 gas chromatograph (Shimadzu) equipped with a ZB-5 column (30 m x 0.25 mm, Phenomenex) and a flame-ionization detector (FID) set at 340 °C. To separate metabolites, the oven temperature was held constant at 120 °C for 5 min, increased to 300 °C by 4 °C per 1 min, and then held constant at 300 °C for 15 min. GC-FID data were normalized to the internal standard by multiplying each metabolite peak area by a normalization factor. To generate the normalization factor, the largest internal standard peak area was divided by the internal standard peak area for each fingerprint. To minimize differences due to slight variation in starting material, peak areas from each fingerprint were divided by the tissue weight used for extraction. Finally, to normalize each metabolite to fungal growth, peak areas from each fingerprint were divided by ergosterol peak area of the sample. Arabitol, mannnitol and trehalose were quantified based on comparison with standard curves derived from analytical standards (respectively: Alfa Aesar, Fisher Scientific and TCI America).
PF0349) and hexokinase 2 (Pfam accession no. PF03727; Fig. 1a). A phylogenetic analysis of the putative hexokinase proteins by neighbour-joining methods revealed three distinct groups, each containing two proteins that shared substantial similarity (Fig. 1a and Supplementary Fig. S1). An additional phylogenetic analysis of hexokinases, non-catalytic hexokinases and glucokinases from *F. verticillioides* and selected other fungi indicated that FVEG_00957 and FVEG_10632 group with hexokinases, FVEG_01889 and FVEG_05775 are most similar to other fungal glucokinases, and FVEG_02067 and FVEG_06423 group with non-catalytic or regulatory hexokinase-like proteins (Supplementary Fig. S1). Gene sequence FVEG_00957 (GenBank accession no. EU247513), which was designated previously as *HXK1* (Bluhm et al., 2008), was selected for characterization based on its expression profile during kernel colonization (Bluhm et al., 2008) and the similarity of the predicted Hxk1 protein to a functionally characterized hexokinase from *Kluyveromyces lactis* (KHxk1, GenBank accession no. CAA08922.1; Kuettner et al., 2010). *HXK1* has an ORF of 2329 bp with five introns, and is predicted to encode a protein of 493 aa containing two structurally similar domains. Ribbon diagrams depicting the tertiary structure of KHxk1 and the predicted tertiary structure of Hxk1 indicated a high degree of structural similarity (Fig. 1b). In addition, the amino acid sequence of Hxk1 shares 71.8% identity with the characterized hexokinase HxkA of *Aspergillus nidulans* (GenBank accession no. XP_680728.1) and 80.1% identity with a hexokinase in *Botrytis cinerea* (GenBank accession no. ABM30191.1). The other *F. verticillioides* gene (FVEG_10632) that grouped with Hxk1 in the phylogenetic analyses (Fig. 1a and Supplementary Fig. S1) shared substantially less identity with KHxk1 and HxkA than Hxk1 (46 and 52.4%, respectively), which suggests that the protein has a substantially different tertiary structure or phosphorylates other substrates.

To functionally characterize *HXK1*, the gene was deleted in the wild-type strain via homologous recombination (Fig. 2a). We obtained 32 transformants that were resistant to hygromycin, and these transformants were screened by PCR with primers HXK1-1 and HYG3 (data not shown) to identify putative deletion mutants. Two individual *HXK1* deletion mutants were obtained and confirmed by Southern analysis (Fig. 2b), and one of these mutants was chosen for further study. To complement the mutant, the *HXK1* gene was amplified via PCR from the wild-type
strain and transformed directly into the Δhxk1 mutant. A complemented strain (Δhxk1-comp) containing the wild-type copy of HXK1 was confirmed by PCR with primers specific to HXK1 (Fig. 2b).

**HXK1 is required for utilization of fructose**

The specific substrate(s) of a hexokinase cannot be determined conclusively from conceptual translation and homology-based analyses of the putative protein sequence. To evaluate substrate specificity for Hxk1, we compared growth and sugar uptake of the Δhxk1 mutant with those of the wild-type and Δhxk1-comp. On a medium containing fructose as the sole carbon source, the Δhxk1 mutant was unable to grow (Fig. 2c). Complementation of the Δhxk1 mutant restored growth on fructose, albeit not completely compared with the wild-type strain (Fig. 2c). However, the Δhxk1 mutant was able to grow on a medium containing glucose as the sole carbon source (Fig. 2c). These results indicated that HXK1 is required specifically for utilization of fructose, and suggest that other putative hexokinases are unable to compensate for Hxk1 function in the presence of fructose, despite moderate to substantial sequence similarity (Fig. 1a).

Phosphorylation of a hexose by a hexokinase prevents diffusion out of the cell and commits the hexose molecule to a specific cellular process, i.e. glycolysis (Romano, 1982). To determine whether HXK1 is required for fructose uptake and/or retention, the amount of free sugar was quantified in resuspension cultures of the wild-type, mutant and complemented strain. The Δhxk1 mutant was unable to take up and/or retain fructose, as evidenced by the relatively constant amount of soluble fructose present in the resuspension medium; in comparison, a 50% depletion of fructose was observed in resuspension cultures of the wild-type or complemented strain (Fig. 3). Furthermore, dry weight measurements indicated that Δhxk1 did not grow in DL medium containing fructose, whereas both the wild-type and Δhxk1-comp grew considerably (Table 1). In contrast, the three strains had similar growth in DL medium containing glucose as the sole carbon source (Table 1). Together, these results

**Fig. 2.** Disruption of HXK1 in *F. verticillioides.*

(a) To disrupt HXK1, a hygromycin-resistance cassette (HYG<sup>R</sup>) was inserted into the HXK1 locus by homologous recombination. (b) For Southern analysis (left panel), genomic DNA was digested with XhoI (X) and probed with a 600 bp fragment (P) shown in (a). For the complementation strain, PCR (right panel) was performed with primers specific to the deleted region of the HXK1 locus, and the resulting band indicates the presence of the internal fragment (623 bp) of HXK1. M, 1 kb size marker. (c) Wild-type, Δhxk1 and Δhxk1-comp were grown on various media: YEP, and DL containing either 2% glucose or 2% fructose as the sole carbon source.

**Fig. 3.** Sugar analysis. All strains were grown on YEP medium for 3 days. Mycelia were transferred to defined medium containing either 2% fructose or 2% glucose as the sole carbon source. At each time point, sugars that remained in the medium were measured in three biological replicates. The experiment was performed twice with similar results (data from a representative experiment are shown).
Table 1. Growth of the wild-type, Δhxk1 mutant and complemented strain on maize kernels and liquid media

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maize kernel medium*</th>
<th>DL medium†</th>
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<tbody>
<tr>
<td></td>
<td>Whole kernel</td>
<td>Endosperm tissue</td>
</tr>
<tr>
<td>Wild-type</td>
<td>744 ± 53</td>
<td>566 ± 6</td>
</tr>
<tr>
<td>Δhxk1</td>
<td>482 ± 59</td>
<td>207 ± 2</td>
</tr>
<tr>
<td>Δhxk1-comp</td>
<td>742 ± 42</td>
<td>550 ± 15</td>
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</tbody>
</table>

*Growth on maize kernel medium is represented by ergosterol concentration (values show mean ± SD of three replicates). Ergosterol values are µg ergosterol (g maize kernels)-1.
†After transferring mycelia grown on YEP medium, growth as dry mycelial weight (g) was measured at 65 h (DL-glucose) or 96 h (DL-fructose). Values in parentheses indicate the increase in weight compared with the initial inoculum. Similar results were obtained in two independent experiments.

indicated that HXK1 is involved primarily in the uptake and/or utilization of fructose.

**Deletion of HXK1 impairs conidiation and alters hyphal morphology**

To identify roles for HXK1 in processes other than primary metabolism, the impact of HXK1 disruption on fungal development was examined. The Δhxk1 mutant produced fewer conidia than the wild-type or the Δhxk1-comp strain when provided with glucose, sucrose or maltotriose as the sole carbon source (Fig. 4a). Also, the mutant grew more slowly than the wild-type or complemented strain when provided with the aforementioned carbon sources (Fig. 4a). Furthermore, when grown on YEP + fructose medium, the mutant produced swollen hyphae and conidia, although the wild-type grew normally (Fig. 4b). Interestingly, when grown on YEP + glucose medium, the mutant was morphologically indistinguishable from the wild-type (Fig. 4b), which suggested a specific response to fructose.

**HXK1 is required for infection and colonization of maize kernels, but not stalks**

Observations that the Δhxk1 mutant was impaired in growth when provided with specific sugars or carbohydrates (Fig. 4) gave rise to the question of whether the mutant would be reduced in virulence during infection of...
distinct maize tissues, e.g., kernels versus stalks. To evaluate virulence on maize kernels, ears of inbred B73 were inoculated with the wild-type, Δhxk1 mutant or Δhxk1-comp strain in greenhouse assays. At 7 days after inoculation, the wild-type and the complemented strain aggressively colonized maize kernels, whereas growth of the Δhxk1 mutant was restricted to wound sites (Fig. 5a). Dissection of colonized kernels revealed that the endosperm of kernels inoculated with Δhxk1 remained yellow and firm, but the endosperm tissues inoculated with the wild-type and complemented strain were completely colonized (data not shown). Additionally, on autoclaved cracked whole-kernel and endosperm tissue media, Δhxk1 produced 64 and 36%, respectively, of the ergosterol produced by the wild-type (Table 1); ergosterol is commonly used as a quantitative measure of fungal growth. However, when inoculated on germs excised from mature kernels, growth of the mutant was similar to that of the wild-type and Δhxk1-comp strain (Table 1). These results indicated that Δhxk1 was impaired in its ability to utilize carbohydrates found specifically in maize endosperm tissues.

To evaluate virulence on maize stalks, a commercial hybrid was inoculated with the wild-type, Δhxk1, and Δhxk1-comp in greenhouse assays. Somewhat surprisingly, the wild-type, Δhxk1, and Δhxk1-comp were consistently indistinguishable (Fig. 5b), which indicates that HXK1 does not play an important role during colonization of stalk tissue.

**HXK1 is required for wild-type levels of FB₁ biosynthesis during kernel colonization**

To determine whether HXK1 affects fumonisin biosynthesis, we measured FB₁ production by the wild-type, the Δhxk1 strain and the Δhxk1-comp strain during growth on cracked maize kernel medium. FB₁ levels were normalized to fungal growth [ergosterol content (g maize)⁻¹]. The mutant produced fivefold less FB₁ than the wild-type, and production of FB₁ was restored in the complemented strain (Fig. 6, Supplementary Table S1). These results showed that the reduction in FB₁ biosynthesis was fully attributable to disruption of HXK1.

**Metabolic profiling reveals that deletion of HXK1 impairs trehalose biosynthesis and utilization of hexose sugars during colonization of maize kernels**

Because of the growth impairment of Δhxk1 on maize kernels, metabolic profiling was performed to determine whether disruption of HXK1 incurred global changes throughout the metabolome during kernel colonization. To this end, maize kernels were inoculated with the wild-type, the Δhxk1 strain and the Δhxk1-comp strain, and metabolic profiles of polar metabolites such as carbohydrates were obtained. A total of 22 metabolites were detected in the colonized maize kernels (Supplementary Table S2). Comparisons of relative, normalized concentrations of each metabolite indicated that disruption of HXK1 did not cause widespread changes in the metabolome (Supplementary Fig. S2). However, consistent with a role for HXK1 in sugar uptake and/or retention, disruption of HXK1 significantly increased the amount of sucrose, fructose and glucose present in colonized kernels compared with the wild-type or Δhxk1-comp strain (Supplementary Table S2). Presumably, this difference resulted from the rapid catabolism of the sugars during growth and colonization of the wild-type and the complemented strain. Additionally, disruption of HXK1 consistently reduced the concentration of a metabolite eluting at 36.448 min (Fig. 7), which has previously been identified by MS as trehalose (Smith et al., 2011). This identification was confirmed by spiking experiments and

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**Fig. 5.** Effect of HXK1 on pathogenesis. (a) Ear rot assay. Conidia (10⁶ ml⁻¹) of the wild-type, Δhxk1 or Δhxk1-comp were inoculated into maize kernels with a needle. Pictures were taken 7 days after inoculation. (b) Stalk rot assay. Maize stalks (internodal regions) were inoculated with 10⁵ spores of fungal strains and incubated in a greenhouse. After 5 days, the maize stalks were split longitudinally to assess stalk rot severity. A maize stalk inoculated with sterile water is shown as the negative control.
Among filamentous fungi, hexokinases have been postulated to play important roles in the biosynthetic pathways of trehalose and sugar alcohols, both of which are important in mitigating environmental stresses such as heat and oxidative shock (Fillinger et al., 2001). Our analysis indicated that arabitol and mannitol levels were similar in the wild-type and the Δhxk1 strain (Fig. 7, Supplementary Table S2). However, accumulation of trehalose in kernels colonized by Δhxk1 was twofold less than that of kernels colonized by either the wild-type or the complemented strain (Fig. 7), whereas in uninoculated kernels, trehalose was not detected (Supplementary Fig. S2). Additionally, the Δhxk1 strain produced 30% less trehalose than the wild-type during colonization of autoclaved maize kernels (data not shown), and 35% less trehalose than the wild-type when grown on YEP liquid medium containing 10% glucose (Supplementary Fig. S3). Intriguingly, levels of glucose 6-phosphate were comparable between the mutant and the wild-type (Supplementary Fig. S3) during growth on YEP liquid medium containing 10% glucose, which suggests that the reduction in trehalose biosynthesis observed in the Δhxk1 strain may be related to carbohydrate sensing rather than the ability to synthesize glucose 6-phosphate, a precursor of trehalose biosynthesis. Taken together, these results showed that trehalose biosynthesis is consistently reduced by disruption of HXK1 across a range of environmental conditions.

Deletion of HXK1 reduces osmotic stress tolerance

The observation that trehalose biosynthesis was reduced in the Δhxk1 mutant gave rise to the question of whether or not the mutant displayed increased sensitivity to environmental stresses. In many fungi, trehalose is essential for virulence and tolerance of environmental stresses, including heat, cold, starvation, desiccation, and osmotic and oxidative damage (Gadd et al., 1987; Lewis et al., 1995; Ngamskulrungroj et al., 2009; Thevelein, 1984). To investigate osmotic stress tolerance in the Δhxk1 mutant, we compared growth (colony diameter) of the wild-type and Δhxk1 mutant on YEP media containing glucose, fructose or glycerol in the presence or absence of 1 M NaCl (Fig. 8). In the presence of 1 M NaCl, growth of the mutant was approximately 65% less than that of the wild-type when fructose was the sole carbon source (Fig. 8a, b), but when glucose or glycerol was supplied to the YEP medium, the mutant exhibited approximately 10% reduced growth compared with the wild-type (Fig. 8b). In the absence of NaCl, the reduced growth rate of the mutant was similar on all YEP media, regardless of carbon source (Fig. 8). We confirmed that the complemented strain restored the growth defect on YEP + fructose medium in the presence of 1 M NaCl (data not shown). Additionally, tolerance of oxidative stress was evaluated by measuring growth on YEP medium (containing glucose or fructose as the sole carbon source) supplemented with H2O2 (10 mM) and Congo red (50 μg ml−1). Under these conditions, growth rates of the mutant and wild-type were similar, regardless of carbon source (data not shown).
DISCUSSION

The molecular events underlying fungal responses to sugar, particularly glucose, are understood best in the yeast Saccharomyces cerevisiae (Santangelo, 2006). Three components are known to be involved in the sensing of glucose and the initiation of a molecular response in yeast: glucose sensors (Snf3p and Rgt2p), hexokinase (Hxk2p) and the G-protein-coupled receptor (Gpr1p) (Hohmann et al., 1999; Kraakman et al., 1999; Ozcan et al., 1998). Filamentous fungi also have mechanisms to perceive and respond to sugar, some of which are unique to higher fungi, and some of which are similar to those found in yeast. Evidence from A. nidulans indicates that a system involving heterotrimeric G-proteins coupled with adenylate cyclase is the major mechanism for glucose sensing and the subsequent changes in the transcriptome (Lafon et al., 2005). Also, two kinases that phosphorylate hexoses have been identified in A. nidulans (GlkA and HxkA) and B. cinerea (Glk1 and Hxk1). However, these enzymes are not involved in carbon catabolite repression, which has a different regulatory mechanism from that of yeast (Filiphi et al., 2003; Rui & Hahn, 2007). Hexokinases in fungi and plants are known to be involved in signalling networks for controlling growth and development in response to the changing environment (Fleck & Brock, 2010; Moore et al., 2003).

Beyond the crucial role that hexokinase plays in glycolysis, the regulatory functions of hexokinases, including responding to and signal transduction by sugars, are poorly understood in filamentous fungi. Rui & Hahn (2007) have shown that a B. cinerea mutant disrupted in HXK1 fails to grow on fructose, and grows poorly on media containing a variety of other carbon sources. In addition, the mutant is virulent on tomato leaves, but produces small lesions and reduced conidiation on apple fruit, which suggests that the regulation of sugar catabolism plays a major role in pathogenicity and disease development. Furthermore, Fleck & Brock (2010) have shown that during infection of mouse lung, GLKA and HXKA of Aspergillus fumigatus are highly expressed, suggesting the involvement of the genes in virulence. These results are consistent with our observation that strain Δhxk1 is substantially impaired in pathogenicity during infection of maize. Moreover, our results indicating that HXK1 is involved in ear rot but not stalk rot suggest that F. verticillioides possesses distinct, carbohydrate-dependent mechanisms for pathogenicity that depend heavily on the types and amount of carbon sources present in a particular plant tissue.

Our observations that HXK1 is required for FB1 biosynthesis establish an important new genetic link between primary and secondary metabolism in F. verticillioides. Keller & Sullivan (1996) reported that glucose did not repress FB1 production in Fusarium proliferatum, a close relative of F. verticillioides, when glucose concentrations were maintained between 30 and 40 g l−1 at pH 3.5 in a bioreactor. These results suggest that genes involved in carbon catabolite repression do not regulate fumonisin biosynthesis, which is consistent with the lack of consensus binding sites for CreA in the promoters of FUM1 and FUM21. However, in F. verticillioides, CreA may indirectly regulate fumonisin biosynthesis through the regulation of sugar transporters and sugar kinases (Bhatnagar et al., 2003). This hypothesis is supported by our observation that HXK1 has four putative CreA binding sites (−356, −245, −42 and −24 bp) within 500 bp upstream of the start codon. However, the exact mechanism through which HXK1 affects FB1 production remains to be determined and will require further genetic dissection.

In fungi, trehalose and polyols such as mannitol serve as protectants against environmental stress. For example, conidia produced by an A. nidulans mutant disrupted in TP5, which encodes a trehalose-6-phosphate synthase catalysing the first step in trehalose biosynthesis, show a rapid loss of viability when subjected to heat and oxidative stress, whereas conidia of the wild-type retain viability
under the same treatments (Fillinger et al., 2001). Houansa et al. (1998) showed that in S. cerevisiae, mutant strains (Δtps1Δtps2 and Δtps1Δhxk2) are unable to produce trehalose and are also more sensitive to osmotic stress. Additionally, disruption mutants in TPS1 of A. nidulans and B. cinerea are more sensitive to osmotic stress and heat stress, respectively, than the wild-type strains (Doehlemann et al., 2006; Fillinger et al., 2001). Trehalose biosynthesis is catalysed by the action of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase from glucose 6-phosphate. However, our observation that Δhxk1 was not able to take up fructose or grow on fructose as a sole carbon source gave rise to a question: why does Hxk1 have an effect on trehalose biosynthesis, but not on mannitol biosynthesis, considering that mannitol is produced by the action of mannitol-1-phosphate dehydrogenase and mannitol-1-phosphate phosphatase from fructose 6-phosphate? We cannot exclude the possibility that Hxk1 of F. verticillioides is directly or indirectly involved in the phosphorylation of glucose; this possibility is supported by our observations of reduced growth of the mutant on DL medium containing glucose. Furthermore, we observed that mannitol accumulation was increased up to threefold in the Δhxk1 strain compared with the wild-type when grown on autoclaved maize kernels (data not shown). These results suggest that disruption of HXK1 in F. verticillioides has a conditional effect on mannitol biosynthesis, although the underlying mechanism is not clear at this time.

In some fungi, trehalose has undetermined functions in pathogenesis. For example, trehalose metabolism regulates development, in yeast, trehalose 6-phosphate has been made by those authors with relevance to our findings in secondary metabolism in F. verticillioides. Characterization of HXK1 creates new insights into how F. verticillioides senses distinct tissues of maize during pathogenesis, and also provides tools for further molecular dissection of the complex signalling pathways governing pathogenesis and mycotoxigenesis in F. verticillioides.

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