The *snf1* Gene of *Ustilago maydis* Acts as a Dual Regulator of Cell Wall Degrading Enzymes

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ABSTRACT

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Many fungal plant pathogens are known to produce extracellular enzymes that degrade cell wall elements required for host penetration and infection. Due to gene redundancy, single gene deletions generally do not address the importance of these enzymes in pathogenicity. Cell wall degrading enzymes (CWDEs) in fungi are often subject to carbon catabolite repression at the transcriptional level such that, when glucose is available, CWDE-encoding genes, along with many other genes, are repressed. In *Saccharomyces cerevisiae*, one of the main players controlling this process is *SNF1*, which encodes a protein kinase. In this yeast, Snf1p is required to release glucose repression when this sugar is

depleted from the growth medium. We have employed a reverse genetic approach to explore the role of the *SNF1* ortholog as a potential regulator of CWDE gene expression in *Ustilago maydis*. We identified *U. maydis* *snf1* and deleted it from the fungal genome. Consistent with our hypothesis, the relative expression of an endoglucanase and a pectinase was higher in the wild type than in the $\Delta snf1$ mutant strain when glucose was depleted from the growth medium. However, when cells were grown in derepressive conditions, the relative expression of two xylanase genes was unexpectedly higher in the $\Delta snf1$ strain than in the wild type, indicating that, in this case, *snf1* negatively regulated the expression of these genes. Additionally, we found that, contrary to several other fungal species, *U. maydis* Snf1 was not required for utilization of alternative carbon sources. Also, unlike in ascomycete plant pathogens, deletion of *snf1* did not profoundly affect virulence in *U. maydis*.

Many plant pathogens actively force entrance into their host by employing mechanical or enzymatic methods or a combination of both. Many pathogenic fungi produce a specialized organ termed an appressorium able to generate enough turgor pressure to pierce the plant surface (9,15). The melanized appressorium of the rice blast fungus *Magnaporthe oryzae* can reach turgor pressures of 5.8 MPa (10). In addition to producing an appressorium, the corn leaf blight fungus *Cochliobolus carbonum* secretes a cocktail of cell wall degrading enzymes (CWDEs) that depolymerizes the different constituents of the plant cell wall, allowing the fungus to penetrate and spread within the host (45). The importance of CWDEs as virulence factors in fungi has only recently begun to be understood. Several CWDEs, including pectinases and endoxylanases, not only might aid pathogens during colonization of their hosts but the resulting enzymatic products may also function as elicitors to trigger plant defense mechanisms (3,25). Due to gene and enzymatic activity redundancy, reverse genetic approaches relying on single or even multiple gene deletions have not effectively addressed the importance of these enzymes in pathogenicity (17,35,46).

In the basidiomycete *Ustilago maydis*, causal agent of corn smut, when two compatible haploid sporidia mate on the plant surface, the resulting dikaryotic infective hypha produces a poorly differentiated appressorium at the site of entry (38). Within the host, the fungus spreads initially intracellularly by penetrating the host cell wall and invaginating the plasma membrane (39). The

occurrence of deformed rupture sites between plant cells suggests that some mechanical mechanism might be involved in the fungal ramification inside the plant. However, because there is no well-differentiated melanized appressorium, enzymatic digestion of plant cell wall components as a requisite for penetration is implied.

Using confocal microscopy, Doehlemann et al. (13) were able to demonstrate that, in *U. maydis* in later stages of gall formation, the fungal hyphae form aggregates that fill enlarged apoplastic cavities that result from degradation of the middle lamella between tumor cells. The authors also analyzed the pattern of expression of CWDE gene induction during pathogenic development and concluded that degradation of hemicellulose and cellulose may be critical for tissue colonization in all stages of disease development; however, pectin degradation might be required only for cavity development. Although this information points toward a pathogenic *U. maydis* employing an enzymatic approach, reverse genetics has not been successful in addressing the question, primarily due to gene redundancy but also because cell wall degradation might require coordinated activities of several CWDE classes rather than single enzyme types (13).

An interesting aspect of the function of CWDEs in fungi is that they are usually under transcriptional glucose repression (33). Glucose repression is a widespread mechanism exploited by organisms for which glucose is the preferred carbon source, such as that exhibited by baker's yeast, *Saccharomyces cerevisiae* (16). In *S. cerevisiae*, sucrose-nonfermenting (Snf1) serine-threonine protein kinase is required to release glucose transcriptional repression when this sugar is depleted (5,20). Snf1p forms a complex with the activating γ -subunit, Snf4p (7,34), and one of the three β -scaffolding subunits: Sip1p, Sip2p, or Gal83p (20,44,

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47). High levels of glucose inhibit the interaction between Snf1p and Snf4p (42). When glucose levels are low, Snf4p binds to the Snf1p regulatory domain, preventing the autoinhibition that this domain exerts on the Snf1p kinase domain. The β -scaffolding subunits interact with the Snf1p through a KIS domain and with Snf4p through its C-terminal ASC domain (24,48). Each β -scaffolding subunit is thought to confer specificity by mediating the binding of the Snf1 complex to a particular target or targets (44). One of the principal targets of the Snf1p catalytic α -subunit is the DNA-binding transcriptional repressor Mig1 (42). Phosphorylation of Mig1p by the Snf1 complex promotes its export to the cytoplasm, allowing transcription of the otherwise glucose-repressed genes (1,11,37).

In the human fungal pathogen *Cryptococcus neoformans*, the *SNF1* ortholog is required for alternative carbon source utilization, melanin production, and virulence; whereas, in the phytopathogenic ascomycete fungi, *Fusarium oxysporum* and *Cochliobolus carbonum*, disruption of the corresponding *SNF1* orthologs led to a reduction in the expression of several CWDE genes, accompanied by a major decrease in virulence (32,41). In this work, we explored the *SNF1* ortholog in the basidiomycete fungus *U. maydis* in connection to its role in CWDE expression and virulence. We present data indicating that, in *U. maydis*, Snf1 is not necessary for releasing glucose repression of some CWDE genes or to metabolize alternative carbon sources. Most importantly, deletion of *snf1*, unlike in plant-pathogenic species studied to date, did not profoundly affect virulence in *U. maydis*.

MATERIALS AND METHODS

Strains, media, and growth conditions. *U. maydis* strains used in this study are listed in Table 1. Fungal cultures were grown on potato dextrose agar (PDA) or in potato dextrose broth (PDB) (Difco Laboratories, Franklin Lakes, NJ). Nitrate minimal medium (MM) (23) amended with 1% (wt/vol) of either oat spelt xylan, citrus pectin, sucrose, glucose, or arabinose (Sigma-Aldrich, St. Louis) was employed for growth assays and gene expression experiments. *U. maydis* cultures for protoplast production were grown in YEPS (1% yeast extract, 2% bacto-peptone, and 2% sucrose). *U. maydis* transformants were selected on YEPS medium amended with 1 M sorbitol (YEPS-S) and carboxin at 3 μ g/ml (Gustafson, Inc., Plano, TX). Cultures were grown at 30°C and, for liquid cultures, agitation in a rotary shaker was kept at 250 rpm. *Escherichia coli* DH5 α cells were used for transformation during deletion construct assembly. Luria-Bertani (LB) medium containing ampicillin at 50 μ g/ml (Sigma-Aldrich) was employed for selecting *E. coli* transformants. Mating assays were performed on complete medium, consisting of 5 g of casamino acids, 1.5 g of ammonium nitrate, and 10 g of yeast extract per liter of H₂O; 62.5 ml of *U. maydis* salt solution (KH₂PO₄ at 16 g/liter, Na₂SO₄ at 4 g/liter, KCl at 8 g/liter, MgSO₄ · 7H₂O at 2 g/liter, and CaCl₂ · 2H₂O at 1 g/liter, pH 7); and 8 ml of trace elements (H₃BO₃ at 60 mg/liter, MnCl₂ · 4H₂O at 140 mg/liter, ZnCl₂ at 400 mg/liter, Na₂MoO₄ · 2H₂O at 40 mg/liter, FeCl₃ · 6H₂O at 100 mg/liter, and CuSO₄ · 5H₂O at 400 mg/liter) (23) containing 1% charcoal (Sigma-Aldrich) (22).

Nucleic acid procedures. The Δ *snf1* mutant strains were generated by completely replacing the *snf1* open reading frame (ORF) with a carboxin-resistant gene cassette (*cbx*). The 5' flank region of *snf1* (1 kb) was amplified from genomic DNA with primers MN1: CTCACAAGCAAAGCAGCGT and MN2: CAGCGAACGGCGTTCTCAATCCGTG, cloned into vector pCR 2.1-TOPO (Invitrogen, Carlsbad, CA), and then excised with *Eco*RI and introduced into the *Eco*RI site in pCBX5 (28) to generate pCBX55. The correct orientation of the 5' flank was confirmed by digesting the resulting plasmid with *Sac*I. The 3' flank (1 kb) was amplified with primers MN3: GGGCCC GAGCACCATTGAGCGTGAATGG and MN4: GGGCCCAT

GTTGGGCTGCTGCGAATGCG, TOPO-cloned, and then excised with *Apa*I. The resulting fragment was introduced into the *Apa*I site of pCBX55. Clones carrying the appropriately oriented 3' flank were identified by *Hind*III digestion. The resulting plasmid was named pCBX553. Correct assembly of the deletion construct was further confirmed by sequencing. A linear DNA fragment containing *cbx* flanked by 1 kb of the 5' and 3' *snf1* ORF flanks was obtained by digesting pCBX553 with *Sac*I and *Kpn*I. This fragment was gel purified and used to transform wild-type *U. maydis* strains 1/2 (mating type *a1b1*) and 2/9 (mating type *a2b2*) protoplasts as previously described (43). Transformants were initially tested by polymerase chain reaction (PCR) with primers SNF1Fw: CTCGAATTGGTTCGGTGAT and SNF1Rv: TACATCTTGACGACAGA for the absence of the *snf1* ORF, and with primers *cbx3*out: CATTCTCGTGATTG TCC and *snf13*out: TTGCCAAACGTAGCGTTG for homologous integration of the *cbx* resistance cassette within the *snf1* locus. Further confirmation was provided by Southern blot hybridization. Genomic DNA (10 μ g) from each strain was digested with *Bgl*III and resolved on a 0.7% agarose gel stained with ethidium bromide. DNA was transferred overnight by capillarity to Hybond XL (Amersham Pharmacia Biotech, Piscataway, NJ) nylon membrane in 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and crosslinked by UV light. For the probe, 1 kb of the *snf1* 3' flank was amplified and digoxigenin (DIG) labeled using Roche DIG-high Prime kit, and the Roche Wash and Block Buffer Set was used for hybridization steps (Roche, Indianapolis, IN). Probe labeling and hybridization procedures were performed according to the manufacturer's instructions. X-ray film (Kodak, Rochester, NY) was developed after 10 min of exposure.

Quantitative reverse-transcription PCR quantification of CWDE gene expression. Total RNA for quantitative reverse-transcription (qRT)-PCR quantification of relative transcript accumulation was extracted using a Spectrum Plant Total RNA kit (Sigma-Aldrich). cDNA was synthesized using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) using oligo-dT as primer and according to the manufacturer's recommendations. Transcript abundance was quantified by qRT-PCR using SYBR-GREEN methodology (Bio-Rad, Hercules, CA) with gene-specific primers designed through the Integrated DNA Technologies website (<http://www.idtdna.com/Home/Home.aspx>). Reactions were performed on a Cepheid SmartCycler I (Cepheid, Sunnyvale, CA). CWDE gene transcript relative expression levels were calculated according to Δ cycle threshold calculations ($2^{-\Delta\Delta CT}$ method) (31). Primer sequences are listed in Table 2. Relative gene expression was estimated by normalizing target transcript levels to the cyclophilin gene, *cpr1* (um03726) (12). Means of gene expression fold increase and their corresponding standard error were calculated based on three biological replicates.

Functional complementation of yeast Δ SNF1. Yeast reference strain W303 (40) (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15*) (wiki.yeastgenome.org/index.php/CommunityW303.html) and the derived Δ SNF1 MCY4908 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15*ΔSNF1) strain (21) were maintained in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% bacto-peptone, and 2% glucose).

TABLE 1. *Ustilago maydis* strains used in this study

Strain	Relevant genotype	Source
1/2	<i>a1b1</i> (also known as strain 521)	Gold et al. 1997
2/9	<i>a2b2</i> (near isogenic to 1/2)	Gold et al. 1997
18/9	<i>a1b1</i> Δ <i>snf1::cbx</i>	This study
18/10	<i>a1b1</i> Δ <i>snf1::cbx</i>	This study
18/11	<i>a2b2</i> Δ <i>snf1::cbx</i>	This study
18/12	<i>a2b2</i> Δ <i>snf1::cbx</i>	This study
ect7	<i>a1b1</i> ectopic <i>snf1::cbx</i>	This study

The *U. maydis* *snf1* and the *S. cerevisiae* *SNF1* ORFs were amplified from corresponding genomic DNA and directionally cloned into the *URA3* containing plasmid pWS28 (30) carrying the constitutively expressed *PGX* promoter at the *EcoRI* and *NotI* sites to generate plasmid pWS28*SNF1* and pWS28*Umsnf1*, respectively. Yeast strain MCY4908 was transformed with either empty vector pWS28, pWS28*SNF1* or pWS28*snf1* according to a standard yeast transformation protocol (14). Transformants were first selected on synthetic dextrose minus uracil (SD-URA) medium, and then tested on YEPS amended with antimycin at 1 µg/ml (Sigma-Aldrich) to block respiration.

***U. maydis* mating and pathogenicity analysis.** Mating plate assays were used to assess mutant mating abilities. Indicated strains were grown overnight in PDB and equal volumes of each of the mating strains were co-spotted on charcoal mating plates, sealed with parafilm, and incubated at room temperature in the dark for 24 h. White dikaryotic filaments indicated a successful mating reaction.

For pathogenicity tests, 7-day-old 'Golden Bantam' maize seedlings were co-inoculated with strain mixtures of 10⁶ cells/ml. Plants were kept in a growth chamber with cycles of 16 h of day at 28°C and 8 h of night at 20°C. Symptom development was scored 7, 10, and 14 days postinoculation (dpi) and each plant individually assigned a disease rating based on the following disease scale (18) 0 = no symptoms, 1 = anthocyanin production or chlorosis, 2 = small leaf galls, 3 = small stem galls, 4 = large stem galls, and 5 = plant death. Disease index was calculated as the average disease rating [(Σ disease rating for each plant)/total number of plants]. For each mutant strain considered, three independent biological replicates of pathogenicity tests were conducted. Severity rating of each pathogenicity test was analyzed by using a marginal effects nonparametric analysis based on ranks, as described in Shah and Madden (36).

RESULTS

Identification and deletion of the *U. maydis* *SNF1* ortholog.

In order to investigate the potential role of the *U. maydis* *SNF1* ortholog as a key regulator of glucose repression as well as CWDE expression in this fungus, we created *Δsnf1* mutant strains by completely removing the gene ORF. The *U. maydis* *SNF1* ortholog, *snf1* (um11293), was identified based on *S. cerevisiae* Snf1 protein sequence similarity by searching the genome (<http://mips.gsf.de/genre/proj/ustilago>) using the protein BLAST-homology search algorithm. The identified ortholog, *snf1* (e = 1.8e⁻¹⁴⁰) encodes an 841-amino-acid peptide with a predicted molecular mass of ≈92 kDa. National Center for Biotechnology Information alignment software indicates that the predicted protein encoded by um11293 shares 58 and 51% identity with Snf1p orthologs from *Cryptococcus neoformans* and *S. cerevisiae*, respectively. The serine/threonine (Ser/Thr) kinase catalytic domain extends from residue 52 to 303 and exhibits a high similarity to the Snf1 Ser/Thr catalytic domain in other fungi (Fig. 1). A well-conserved residue among Snf1p-related proteins is threonine 210 in the activation loop of the catalytic domain. Phosphorylation at this threonine by upstream kinases is indis-

pensable for activation of the catalytic kinase domain (29). In *U. maydis* Snf1, this threonine (Thr 207) and the sequence context in which it is located have been conserved. Gene replacement by homologous recombination at the *snf1* locus was initially tested in transformants by PCR and further confirmed by Southern blot (Fig. 2). No obvious morphological phenotype differences were observed under the light microscope between *Δsnf1* and wild-type strains grown in liquid PDB.

***U. maydis* *snf1* is sufficient to complement a *S. cerevisiae* *ΔSNF1* phenotype.** In *U. maydis*, the *snf1* gene and the protein it encodes have not previously been characterized. As a way to initially describe the *U. maydis* *snf1* gene function, we tested whether this gene is capable of rescuing the phenotype of a *S. cerevisiae* *ΔSNF1* mutant strain. In *S. cerevisiae*, Snf1 kinase is essential for releasing the transcriptional repression imposed on the *SUC2* gene by glucose when this sugar is depleted from the medium. *SUC2* encodes a secreted invertase responsible for hydrolyzing sucrose into glucose and fructose, allowing the cells to ferment these sugars (5). Consequently, deletion of *SNF1* abolishes the ability of the yeast cells to grow by fermentation on medium containing sucrose as the sole carbon source. Because the *U. maydis* *snf1* gene contains no introns, the complete ORF was cloned into yeast expression vector pWS28 to generate pWS28*snf1*. As a positive control, the *S. cerevisiae* *SNF1* ORF was cloned into pWS28 and the resulting plasmid named pWS28*SNF1*. Yeast transformants carrying these plasmids were initially selected on SD-URA medium for the presence of plasmid (see Materials and Methods) and then transferred to medium containing sucrose as the carbon source (yeast extract peptone sucrose [YPS] medium) to test their ability to grow on sucrose (Fig. 3). YPS plates were amended with antimycin A (1 µg/ml) to block respiration and any possible growth not due to fermentation. Only those strains carrying either pWS28*snf1* or pWS28*SNF1* grew on the YPS medium, showing that *U. maydis* *snf1* can functionally complement the *S. cerevisiae* *SNF1* mutation.

CWDE expression. The genome of *U. maydis* contains 33 potential hydrolytic enzymes able to work on living or dead plant tissues (26). Deletion of *snf1* orthologs in other plant-pathogenic fungi was shown to drastically reduce or completely abolish the expression of several CWDE genes. Based on the information concerning other plant-pathogenic fungi, we chose to test the expression patterns of several *U. maydis* CWDE orthologs for which the *snf1* serine threonine kinase is required to release the gene transcriptional repression imposed by glucose (32,41). A first attempt to score expression of several CWDE genes encoding xylanases (um06350 and um03411), glucanases (um04368, um00235), pectin methyltransferase (um03516), α-L-arabinofuranosidase (um04309), and polygalacturonase (um02510) in media containing either glucose or an alternative carbon source was carried out by Northern blots. The *Δsnf1* strain 18/9 and wild-type strain 1/2 were grown overnight in PDB, washed twice with sterile water, and then plated on MM amended with one of the following carbon sources: glucose, xylan, or pectin. After 2 days of incubation at 28°C, fungal cells were collected and total RNA extracted. Of the seven tested genes, we were only able to detect transcripts corresponding to the xylanase gene um03411. For this

TABLE 2. *Ustilago maydis* cell wall degrading enzyme genes tested by quantitative reverse-transcription polymerase chain reaction

Gene ^z	Enzyme	Forward primer	Reverse primer
um04422	Endo-β-1,4-xylanase	GACCATCACCGATTTCGTCACCA	AGTAGCCGTTCTTCTGAAGCCGT
um04897	Endo-β-1,4-xylanase	AGAAGAATGTGCGTCCAGAGGGTT	TGATGCATGTCATCCTCGTGCTCT
um06350	Endo-β-1,4-xylanase	AGTTTGCCACTGTCCTTGCTTTCG	TGGCAGCGTTACCGTTGTAGTTCT
um03411	Endo-β-1,4-xylanase	AAAGCTGATCCGAGGTCATACGCT	ACCTTGCCCTTGATATCTTCCACA
um02510	PGU1—endo-polygalacturonase	CCAACGTTTCGCAACCAAGATGACT	ACACTACCAATCGACAAGCCGTGA
um02523	Endoglucanase 1 precursor	GCCTGGTTGCAAGTGGAAGATGAA	TGACAACCAGTTCGGTCGATGTGA
um03726	Cyclophilin (reference)	ACGCCGATTCACCTTCGTC	AACGACGATCCCTCGTAACCGAA

^z *Ustilago maydis* gene annotation: <http://mips.helmholtz-muenchen.de/genre/proj/ustilago/>.

gene, no transcript accumulation was detected when wild-type or *Δsnf1* strains were grown on glucose. However, after cells were shifted to MM-xylan, transcript was detected at comparable levels in both the wild-type 1/2 and *Δsnf1* 18/9 strains. These results sug-

gested that the expression of um03411 encoding a potential xylanase is subject to glucose repression but, contrary to the initial hypothesis, *snf1* appears to be dispensable for releasing the transcriptional repression of this gene imposed by glucose (data not shown).

Fig. 1. Alignment showing homology among different Snf1 orthologs. Snf1 orthologs are shown in the following order from top to bottom: *Ustilago maydis*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Cochliobolus carbonum*, *Fusarium oxysporum*, and *Homo sapiens*. Black bars above the sequence indicate the serine/threonine kinase domain. The asterisk indicates the conserved threonine 210 of *S. cerevisiae* Snf1 critical for activation by upstream kinases. Conserved amino acids shown in black. Amino acid position within the protein is indicated to the right.

Because some of the CWDE genes might be expressed at levels difficult to detect by Northern blot, we sought to quantify the relative expression in wild-type and $\Delta snf1$ 18/9 strains using the more sensitive method of qRT-PCR. We initially included in our analysis all the genes tested by Northern blots as well as some additional genes encoding other potential CWDE enzymes. In agreement with our previous Northern results, for many of the tested CWDE genes, no transcripts were detected. Therefore, we decided to conduct our quantification analysis with those genes for which transcripts were detected (Table 2). For each strain, 200-ml PDB cultures were grown overnight to an optical density of ≈ 0.5 , washed, divided, and shifted to MM containing 1% of either glucose (MM-G), xylan (MM-X), pectin (MM-P), glucose plus xylan (MM-GX), or glucose plus pectin (MM-GP).

After 24 h, RNA was extracted from the cells and cDNA was synthesized. No changes in um03411 or um06350 relative tran-

script levels were observed when cells were shifted from MM-G to MM-GX. Similar results were obtained for genes um02510 and um02523 when cells were transferred from MM-G to MM-GP (data not shown). These results indicated that presence of the enzyme's principal substrate is not sufficient to induce the corresponding gene. We then compared the fold change in relative transcript levels of each gene when cells were shifted from MM-G to either MM-X or MM-P. The qRT-PCR results reinforce the hypothesis that expression of xylanases um03411 and um06350, pectinase um02510, and endoglucanase um02523 are strongly repressed by glucose, because their transcript accumulations were dramatically higher in the nonrepressive conditions in both strains than in glucose (Fig. 4). However, the relative expression of polygalacturonase gene um02510 and endoglucanase um02523 in MM-P was lower in the mutant 18/9 than in the wild-type strain 1/2, indicating that *snf1* is required for full

Fig. 2. Deletion of *snf1* in *Ustilago maydis*. **A**, Schematic representation of the wild type and the mutant allele generated by gene replacement. Horizontal arrows indicate the positions of polymerase chain reaction (PCR) primers used to screen transformants. Size of the expected PCR products is indicated below the primer sets. The Southern blot probe and position of the recognition sites of restriction enzymes used to digest fungal genomic DNA are indicated. Lengths of Southern blot hybridizing bands are indicated above the figure. **B**, Transformants of both mating types, *a1b1* and *a2b2*, were initially screened for *snf1* gene replacement by PCR. Primers: SNF10FW and SNF11RV were used to screen for the absence of the *snf1* open reading frame. Primers *cbx3out* and *3outSnf1* were used to screen for homologous integration of the carboxin (*cbx*) deletion construct. Length of PCR products is indicated to the right of images (arrows). **C**, Gene deletion was confirmed by Southern blot hybridization. Genomic DNA from wild-type and *snf1* transformants were digested with *Bgl*III. The size of hybridizing bands is indicated in kilobases to the left (arrows). Strains are indicated above; wt = wild type, ect = ectopic, 18/9 = *a1b1* $\Delta snf1::cbx$, 18/10 = *a1b1* $\Delta snf1::cbx$, 18/11 = *a2b2* $\Delta snf1::cbx$, 18/12 = *a2b2* $\Delta snf1::cbx$. Mating type of transformants, *a1b1* or *a2b2*, is indicated above (bars).

induction of these genes. On the other hand, in opposition to our prediction, the induction of xylanase genes um03411 and um06350 was 2.5 and 1.7 times higher, respectively, in the mutant 18/9 than in the wild-type 1/2 strain, indicating that the absence of *snf1* may marginally increase transcript accumulation of these two genes.

Fig. 3. Complementation of the Δ SNF1 *Saccharomyces cerevisiae* strain with the *Ustilago maydis* *snf1* gene. Yeast parental-ura (W303: *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15*) and derived Δ SNF1-ura (MCY4908: *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15ΔSNF1*) *S. cerevisiae* strains were transformed with either empty vector pWS28, pWS28*snf1* (pWS28 carrying *U. maydis* *snf1* open reading frame [ORF]), or pWS28SNF1 (pWS28 carrying *S. cerevisiae* SNF1 ORF). Transformants were scored for their ability to grow on medium containing sucrose as the sole carbon source. Strains were plated as indicated in the key; yeast extract peptone dextrose (YPD) medium, synthetic defined-ura (glucose medium lacking uracil), and yeast extract peptone sucrose (YPS) medium amended with antimycin (1 μ l/ml).

Carbon source utilization. In *S. cerevisiae*, *SNF1* is indispensable for growth on alternative carbon sources (other than glucose) and Δ SNF1 mutants are unable to utilize sucrose or other nonfermentable sugars. Similarly, the *SNF1* homolog of *Cochliobolus carbonum* and *F. oxysporum* are required for proper absorption or utilization of simple and complex carbon sources other than glucose (32,41). Based on this knowledge, we decided to test the growth efficiency of our Δ snf1 strains on different carbon sources to observe if the same held true for *U. maydis*. Two independent Δ snf1 mutants (18/9 and 18/10), the wild-type 1/2, and one ectopic integrant strain (ect7) were grown overnight in PDB and a serial dilution of each culture was spotted onto solid MM amended with either glucose, sucrose, galactose, xylan, or pectin or, alternatively, onto PDA. For each strain, the growth on a particular alternative carbon source was compared with the corresponding strain growth on MM containing solely glucose (Fig. 5). According to our results, the Δ snf1 mutation does not seem to impair *U. maydis*'s ability to grow on the alternative carbon sources tested. On any of the carbon sources, the Δ snf1 strains 18/9 and 18/10 can sustain growth similar to the wild-type or ectopic strains. Therefore, contrary to the case of *S. cerevisiae*, *F. oxysporum*, and *C. carbonum*, in the basidiomycete *U. maydis* the *snf1* gene appears dispensable for the utilization of alternative sugars.

Deletion of the *U. maydis* snf1 gene slightly reduces virulence. *U. maydis* is a plant-pathogenic fungus and much of its life cycle must occur within its maize host plant. Therefore, the ability of *U. maydis* to sustain a pathogenic life style is crucial for the survival of the species. In order to test the pathogenic potential of Δ snf1 strains, we co-inoculated 7-day-old maize seedlings with pairwise combinations of compatible wild-type or Δ snf1 strains and monitored disease progression based on severity of symptoms. The pathogenicity test corresponding to three independent biological replicates conducted with mutant strains 18/9 (a1b1) and 18/11 (a2b2) is summarized in Table 3. When plants were co-inoculated with compatible Δ snf1 strains (treatment 4), the average disease index at either 7, 10, or 14 dpi was lower than when they were infected with any other strain combinations. However, dikaryons formed by compatible Δ snf1 strains induced all the typical disease symptoms (chlorosis, anthocyanin production, and gall formation) observed in wild-type infections. Similar results were obtained when independent Δ snf1 strains 18/10 and 18/12 were used to inoculate plants. These results indicate that, in *U. maydis*, *snf1* is a relatively minor virulence determinant.

Fig. 4. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of relative expression of cell-wall-degrading enzyme genes in the *Ustilago maydis* wild-type and Δ snf1 mutant strains. For each gene, relative gene expression was estimated based on transcript level in the nonrepressive condition A, minimal medium (MM)-xylan or B, MM-pectin relative to the level in the repressive condition MM-glucose. Relative levels of A, xylanase (um04422, um04897, um06350, and um03411) and B, endo-polygalacturonase (um02510) and endoglucanase (um02523) were calculated by qRT-PCR methodology. Transcript levels were normalized to control gene *cpr1* (12). Indicated values correspond to means of three biological replicates; bars represent standard error of biological variation; wt = wild type and Δ snf1 = a1b1 Δ snf1::cbx indicated in dark and light gray, respectively. Fold increase values indicated in boxes below the corresponding gene. *U. maydis* gene (um gene number) is indicated.

DISCUSSION

The capacity of an organism to grow on a particular carbon source depends primarily upon the utilization of the appropriate enzyme set. Species that grow on a wide variety of carbon sources are able to do so because their genomes are equipped with the necessary genes encoding the enzymes that process each individual type of molecule. Nonetheless, it would be unnecessary and certainly wasteful to constitutively produce all those enzymes when the corresponding substrates are not available. Fortunately, cells have evolved strategies to avoid the energetically detrimental effect that such behavior would produce.

A common strategy among organisms is to adapt to their surroundings; that implies producing the required enzymes only when a particular carbon source becomes available. When multiple carbon sources are available, a particular carbon source is

usually preferred over others. This is exemplified in *S. cerevisiae*, where glucose is the preferred carbon source and the production of enzymes involved in the degradation of other carbon sources is repressed at the transcriptional level when this sugar is accessible. This phenomenon, known as “catabolite” or “glucose repression” has been extensively studied in this yeast where the *SNF1* gene, encoding a catalytic subunit of a protein-serine/threonine kinase (5,6), is a central regulator (4,16,20). The *S. cerevisiae SNF1* gene is required for the cell to utilize alternative carbon sources when glucose becomes limiting (5).

In many plant-pathogenic fungi, the enzymes involved in the depolymerization of the plant cell wall constituents (CWDE) are under glucose repression (2,8). Deletion of *SNF1* orthologs in *C. carbonum* and *F. oxysporum* impaired the ability of these fungi to release glucose repression of CWDE genes, affecting their virulence as well (32,41). In this work, we explored the potential

Fig. 5. Comparative growth of *Ustilago maydis* wild-type and $\Delta snf1$ mutant strains on various carbon sources. Serial 10-fold dilutions of indicated strain cultures were plated on potato dextrose agar and minimal medium containing indicated carbon sources and incubated at 28°C for 3 days. Dilution series indicated to the left. Strains are indicated above; wt = wild type, ect = ectopic, 18/9 = *alb1 Δsnf1::cbx*, and 18/10 = *alb1 Δsnf1::cbx*. The $\Delta snf1$ strains are indicated (bars).

TABLE 3. Effect of $\Delta snf1$ on *U. maydis* pathogenicity

Treatment ^y	Dikaryon	No. of plants	Disease index ^z		
			7 dpi	10 dpi	14 dpi
1	+/+	3 × 20	2.7 ± 0.04 (a)	2.9 ± 0.23 (a)	3.52 ± 0.4 (a)
2	+/-	3 × 20	1.9 ± 0.12 (b)	2.3 ± 0.08 (ab)	3.15 ± 0.1 (a)
3	-/+	3 × 20	2.2 ± 0.12 (a)	2.6 ± 0.22 (a)	3.68 ± .04 (a)
4	-/-	3 × 20	1.6 ± 0.05 (b)	1.9 ± 0.10 (b)	2.56 ± 0.1 (b)

^y Treatment: paired strains are as follows 1 = (1/2 × 2/9), 2 = (1/2 × 18/11), 3 = (18/9 × 2/9), and 4 = (18/9 × 18/11). Inoculation was of 10⁶ cells/ml for all strains.

^z Mean ± standard error were calculated for each treatment based on three independent biological replicates. Disease scale (18) 0 = no symptoms, 1 = anthocyanin production and/or chlorosis, 2 = small leaf galls, 3 = small stem galls, 4 = large stem galls, and 5 = plant death; dpi = days postinoculation. Disease index is calculated as the average disease rating [(Σ disease rating for each plant)/total number of plants]. Statistical analysis was performed using a nonparametric test of ordinal data in designed factorial experiments. Treatments with different letters at specific time points indicate a statistically significant difference at the 95% confidence interval.

functions of the *U. maydis* *SNF1* ortholog, *snf1*, as a determinant of CWDE expression and virulence in this fungus.

The *U. maydis* *snf1* gene um11293 was identified as the homolog of the *SNF1* α -catalytic subunit based on protein sequence similarity. Further examination of the *U. maydis* genome also indicated the presence of a homolog of the activating γ -subunit, Snf4p (um01350). Additionally, the *U. maydis* genome contains a single β -scaffolding subunit gene (um0668) instead of three paralogs as in *S. cerevisiae*. The fact that *U. maydis* *snf1* can functionally complement the phenotype of a yeast Δ *SNF1* mutant strain serves as strong evidence that this gene is, indeed, an *SNF1* ortholog. Therefore, *U. maydis* seems to conserve all the genetic elements that make up the *SNF1* complex involved in the regulation of glucose repression.

With the notion that the identified *snf1* gene can function as a *SNF1* ortholog, we then sought to investigate whether the gene was also involved in glucose repression of CWDE genes in *U. maydis*. We compared the expression patterns of several potential CWDE genes by Northern blot analysis but only detected transcripts corresponding to one of six CWDE genes tested. Because we failed to detect most of the CWDE transcripts by Northern blot, we used qRT-PCR, a more sensitive approach to evaluate gene expression. Our results revealed two different patterns depending on the nature of the CWDE tested. We first expanded our analysis of xylan-degrading enzyme genes by analyzing the expression of the four potential xylanase genes present in the *U. maydis* genome. Expression of xylanases um04422 and um04897 was independent of glucose availability, indicating that they are not under glucose repression. On the other hand, for xylanases um06350 and um03411, transcripts increased, on average, 85- and 16-fold, respectively, when wild-type cells were shifted to MM containing xylan as the sole carbon source (MM-X). The fact that no induction of these genes was observed when cells were moved from growth in repressive conditions (MM-G) to a growth medium in which both the repressor (glucose) and inducer (xylan) were simultaneously present (MM-GX) further reinforced the notion that the genes are, indeed, under glucose repression. Unexpectedly, and in accordance with the pattern revealed by the Northern results, the induction of um06350 and um03411 after the shift to MM-X was 2.5 and 1.8 times higher, respectively, in Δ *snf1* than in the wild-type cells. This result clearly argues against *snf1* acting as a "positive" regulator of um06350 and um03411 induction during growth in alternative carbon sources. Rather, it suggests that *snf1* somehow marginally negatively regulates these genes when glucose is depleted. This comes as a contradiction to the well-documented cases of *C. carbonum* and *F. oxysporum* where the corresponding *S. cerevisiae* *SNF1* orthologs are required for the expression of CWDE in the absence of glucose (32,41).

We also investigated the pattern of expression of two CWDEs involved in the degradation of other plant cell wall polymers. The fold increase of polygalacturonase um02510 and endoglucanase um02523 was 20 and 19, respectively, when wild-type cells were shifted to MM-P, pointing to transcriptional repression by glucose of these genes. As with the xylanase genes, the presence of the inducer is not sufficient to induce um02510 and um02523 because no change in relative transcript levels for these genes was observed when cells were shifted from MM-G to MM-GP. On the other hand, the relative transcript accumulation of these genes in the Δ *snf1* cells after the switch to MM-P was much lower than in the wild-type strain, indicating that, in *U. maydis*, *snf1* is required for full derepression of um02510 and um02523.

Therefore, in *U. maydis*, *snf1* does not appear to be a universal element controlling glucose repression, although the expression of many CWDE genes are repressed by this sugar. In the case of xylanase genes, *snf1* appears to negatively regulate glucose repression whereas, in the case of the other CWDEs analyzed (um02510 and um02523), it acts as a positive regulator, much in

the same way that has been described for *SNF1* orthologs in other systems. Regardless of its role in the regulation of CWDE gene expression, its deletion did not affect the capacity of the mutant strains to metabolize compounds other than glucose, indicating that *U. maydis* *snf1* is not critical for survival in the absence of glucose. This is in marked contrast to *S. cerevisiae*, *C. carbonum*, and *F. oxysporum*, where *snf1* deletion impairs the ability of these fungi to thrive on alternative carbon sources. However, our yeast complementation results clearly showed that *U. maydis* *snf1* was able to function as *S. cerevisiae* *SNF1*, at least in regard to carbon source utilization. *SNF1* has also been shown to be involved in the regulation of nitrogen metabolism, gluconeogenesis, and the glyoxylate and tricarboxylic acid cycles as well as respiration and β -oxidation (19,27). It is very possible that *U. maydis* *snf1* regulates these processes in *U. maydis*. However, preliminary microarray data (data not shown) indicated that, in *U. maydis*, *snf1* is not required for derepression of glucose-repressed genes. Moreover, many of the genes that were induced in the absence of glucose exhibited a higher degree of induction in the Δ *snf1* strain, reinforcing the notion that, in *U. maydis*, *snf1* may negatively regulate the expression of some genes. Further work is needed to accurately establish the full function of *snf1* in *U. maydis*.

U. maydis spends a great deal of its life living as a pathogen, prospering at the expense of its host, maize. It is only during this phase that sexual reproduction is possible in *U. maydis* and, therefore, pathogenic development is crucial for completing its life cycle. Because previous studies have demonstrated that *snf1* deletion affected virulence in other systems, we tested the capacity of Δ *snf1* strains to cause disease in maize seedlings. Our pathogenicity results showed that disease caused by Δ *snf1* strains was slightly less severe than that caused by the wild type, suggesting that *U. maydis* *snf1* is required for full virulence.

In this work, we generated data showing that *U. maydis* does not strictly follow the *S. cerevisiae*, *C. carbonum*, and *F. oxysporum* *snf1* paradigm. Rather, we showed that it may act as either a positive or negative regulator of CWDE expression in relation to glucose repression. We also demonstrated that *snf1* is dispensable for *U. maydis* haploid growth on alternative carbon sources.

However, the *U. maydis* Δ *snf1* strains did exhibit a minor reduction in virulence. This suggests that, during pathogenic development, *snf1* is required for some processes associated with the ability of the fungus to cause disease. Additionally, *snf1* could positively regulate CWDE genes not included in our analysis which, together with those mentioned above, are important for degradation of cell wall polymers during pathogenic growth. Microarray data showed that um02523 along with two other endoglucanase genes and endopolygalacturonase um02510 are highly induced during *U. maydis* infection (13). Our data indicates that induction of these genes is severely reduced in Δ *snf1* cells, which may be one of the causes of the observed reduction in virulence.

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