The HDF1 Histone Deacetylase Gene Is Important for Conidiation, Sexual Reproduction, and Pathogenesis in Fusarium graminearum

Yimin Li,1,2 Chenfang Wang,1 Wende Liu,2 Guanghui Wang,1 Zhensheng Kang,1 H. Corby Kistler,3 and Jin-Rong Xu1,2

1College of Plant Protection and Shaanxi Key Laboratory of Molecular Biology for Agriculture, Northwest A&F University, Yangling, Shanxi 712100, China; 2Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, U.S.A.; 3United States Department of Agriculture–Agricultural Research Service, Cereal Disease Laboratory, University of Minnesota, St. Paul, MN 55108, U.S.A.

Submitted 11 October 2010. Accepted 23 November 2010.

Head blight caused by Fusarium graminearum is an important disease of wheat and barley. Its genome contains chromosomal regions with higher genetic variation and enriched for genes expressed in planta, suggesting a role of chromatin modification in the regulation of infection-related genes. In a previous study, the FTL1 gene was characterized as a novel virulence factor in the head blight fungus. FTL1 is homologous to yeast SIF2, which is a component of the Set3 complex. Many members of the yeast Set3 complex, including Hos2 histone deacetylase (HDAC), are conserved in F. graminearum. In this study, we characterized the HDF1 gene that is orthologous to HOS2. HDF1 physically interacted with FTL1 in yeast two-hybrid assays. Deletion of HDF1 resulted in a significant reduction in virulence and deoxynivalenol (DON) production. The Δhdf1 mutant failed to spread from the inoculation site to other parts of wheat heads or corn stalks. It was defective in sexual reproduction and significantly reduced in conidiation. Expression of HDF1 was highest in conidia in comparison with wild type. Deletion of the other two class II HDAC genes had no obvious effect on vegetative growth and reproduction, respectively, over fivefold in the Δhdf1 mutant. Consistent with upregulation of putative catalase and peroxidase genes, the Δhdf1 mutant was more tolerant to H2O2 than the wild type. Deletion of the other two class II HDAC genes had no obvious effect on vegetative growth and resulted in only a minor reduction in conidiation and virulence in the Δhdf2 mutant. Overall, our results indicate that HDF1 is the major class II HDAC gene in F. graminearum. It may interact with FTL1 and function as a component in a well-conserved HDAC complex in the regulation of conidiation, DON production, and pathogenesis.

Fusarium head blight (FHB) or scab is one of the most important diseases of wheat and barley (Bai and Shaner 2004; Goswami and Kistler 2004). Fusarium graminearum (teleomorph Gibberella zeae) is the major causal agent of head blight in North America and other parts of the world. Plant infection is initiated when ascospores (the primary inoculum) ejected into the air by the pathogen (Mitter et al. 2006; Trail et al. 2005) are dispersed and deposited on flowering wheat or barley heads, which are susceptible to infection from the beginning of anthesis to the dough stage of kernel development. After initial colonization, the pathogen can spread from the infection site to other florets (Brown et al. 2010) and cause severe yield losses under favorable environmental conditions. In addition, F. graminearum produces harmful mycotoxins, such as deoxynivalenol (DON) and zearalenone. DON is also phytotoxic and an important virulence factor in the wheat scab fungus (Desjardins 2003; Proctor et al. 1995).

The F. graminearum genome has no active transposable elements and contains less than 0.5% repetitive sequences (Cuomo et al. 2007). Another unique feature of its genome is the presence of regions enriched for genes that are unique or highly expressed during plant infection (Cuomo et al. 2007). Some of these genes are known to be involved in fungus–plant interactions, suggesting that chromatin structure and modifications play a role in regulating infection-related genes. Reversible acetylation and deacetylation are common forms of histone modifications mediated by histone acetyltransferases (HAT) and histone deacetylases (HDAC). Classical HDAC include class I and class II HDAC that are sensitive to inhibition by trichostatin A and share similarity to yeast Rpd3 and Hda1, respectively (Yang and Grégoire 2005). In several fungi, histone modifications have been implicated in regulating genes important for pathogenicity, stress response, and secondary metabolism (Baidyaroy et al. 2001; Ding et al. 2009; Palmer et al. 2008; Tribus et al. 2010). In the corn pathogen Cochliobolus carbonum, the HDC1 HDAC gene is an important pathogenicity factor (Baidyaroy et al. 2001). In Aspergillus nidulans, the Rpd3-like HDAC is required for hyphal growth and conidiation (Tribus et al. 2010). HdaA is a class II HDAC important for the biosynthesis of sterigmatocystin and production of conidia in A. fumigatus (Lee et al. 2009). In the rice blast fungus Magnaporthe oryzae, the Tig1 HDAC complex is essential for invasive growth and blast lesion development (Ding et al. 2010).

In F. graminearum, the Δftl1 mutant was defective in colonizing wheat heads and causing typical head blight symptoms (Ding et al. 2009). FTL1 is homologous to yeast SIF2, which
is a component of the Set3 complex regulating genes important for late stages of sporulation (Cohen et al. 2008; Wang et al. 2002). Many members of the yeast Set3 complex, including the Hos2 HDAC and Snt1 protein, are conserved in _F. graminearum_ and may play a role in regulating plant infection and DON production. In this study, we identified and characterized the _F. graminearum_ ortholog of yeast HOS2 (named _HDF1_). Hdf1 physically interacted with Ftl1 in yeast two-hybrid assays. Mutants deleted of the _HDF1_ gene were significantly reduced in virulence and DON production. The _Δhdf1_ mutant was defective in sexual reproduction and significantly reduced in conidiation and HDAC activity. Deletion of the other two class II HDAC genes had no effect on vegetative growth. The _Δhdf3_ mutant was normal and the _Δhdf2_ mutant had only a minor reduction in conidiation and virulence. Overall, our results indicate that Hdf1 is the major class II HDAC gene in _F. graminearum_ and is involved in the regulation of conidiation, DON production, and plant infection.

**RESULTS**

**Identification of the HOS2 ortholog in the _F. graminearum_ genome.**

The genome of _F. graminearum_ contains three class II HDAC genes (FGSG_01353, FGSG_04324, and FGSG_05636), which are orthologous to yeast _HOS2, HDA1_, and _HOS3_, respectively. Phylogenetic analysis indicates that these three class II HDAC genes are well conserved in other filamentous fungi. The _HOS2_ ortholog was designated HDAC in _F. graminearum_ ( _HDF1_). It shares 78% identity at the amino acid level with _C. carbonum_ _HDC1_, which is the first HDAC gene known to be important for fungal pathogenicity (Baidyaroy et al. 2001). Hdf1 is also homologous to Hos2 in _M. oryzae_ (Ding et al. 2000) and HosA in _A. nidulans_ (Graessle et al. 2000).

Because Hos2 forms an HDAC complex with Sif2 in yeast, we constructed the _FTL1_ bait and _HDF1_ prey vectors. Growth on SD-His-Trp-Ura plates and LacZ activities were observed in transformants expressing both constructs (Fig. 1), indicating that Hdf1 interacts with Ftl1 in yeast two-hybrid assays.

**HDF1 is not essential for vegetative growth.**

To determine its function in _F. graminearum_, we generated the _Δhdf1_ mutant by the split-marker approach (Fig. 2A). Putative _HDF1_ deletion mutants were identified by polymerase chain reaction (PCR) and confirmed by Southern blot hybridization. When probed with the _HDF1_ fragment amplified with primers F5 and R6, the wild-type PH-1 had a 5.6-kb _HindIII_ band but transformants YM1 through YM6 lacked hybridization signals (Fig. 2B). When hybridized with a fragment of the hygromycin phosphotransferase (_hph_) gene, the wild type had no hybridization signals. The _Δhdf1_ mutants YM1, YM2, YM5, and YM6 (Table 1) had a 5.2-kb band resulting from the gene replacement event (Fig. 2B). All of the _Δhdf1_ mutants had the same phenotype, although only data for YM1 were described below. The _Δhdf1_ mutant was only slightly reduced in vegetative growth on potato dextrose agar (PDA) or complete medium (CM) plates but was reduced over 10-fold in conidiation (Table 2). In comparison with the wild type, colonies formed by the _Δhdf1_ mutant on PDA appeared to be less pigmented (Fig. 2C).

**The _Δhdf1_ mutant is significantly reduced in virulence.**

In wheat heads inoculated with the _Δhdf1_ mutant, only the inoculated spikelets developed typical disease symptoms (Fig. 3A) 14 days postinoculation (dpi). Under the same conditions, the wild-type strain was able to spread from the inoculation sites to nearby spikelets (Fig. 3A). The average disease index for YM1 and PH-1 was 1.1 and 11.6, respectively (Table 2), indicating that the _Δhdf1_ mutant was defective in disease spreading and colonization of wheat heads but not in the initial infection. Because _F. graminearum_ also is a pathogen on corn, we conducted infection assays with corn stalks. In stalk rot assays, the mutant caused only limited discoloration at the inoculation sites 14 dpi. Under the same conditions, extensive discoloration was observed in corn stalks inoculated with PH-1 (Fig. 3B), indicating the spreading and colonization in the pith by the wild type.

**Fig. 1.** Yeast two-hybrid assays for the interaction between _FTL1_ and _HDF1_. Transformants expressing the _HDF1_ bait and _FTL1_ prey constructs were assayed for growth on SD–Leu-Trp-His plates (SD-His) and β-galactosidase (LacZ) activities. Positive (+) and negative (−) controls were from the Stratagene HybridZap library construction kit.

**Fig. 2.** _HDF1_ gene and deletion mutants. **A,** _HDF1_ locus and gene replacement construct. The _HDF1_ and hygromycin phosphotransferase (_hph_) genes are marked with empty and black arrows, respectively. H, _HindIII_. **B,** Southern blots of _HindIII_-digested DNA hybridized with fragments of the _HDF1_ (probe 1, left) and _hph_ (probe 2, right) genes. Lanes 1 to 8: DNA samples from the wild type and transformants YM1–YM7. **C,** Colonies of the wild type (WT), _Δhdf1_ mutant (YM1), and an ectopic transformant (Ect) on potato dextrose agar plates.
To confirm that the defects of the Δhdf1 mutant in plant infection directly resulted from the gene deletion event, we cloned the wild-type HDF gene (containing 1.5 kb of promoter and 0.6-kb terminator regions) and transformed it into protoplasts of the Δhdf1 mutant YM1. Geneticin-resistant transformants, including YM11 (Table 1), were isolated and confirmed by PCR. Virulence of YM11 in infection assays with wheat heads (Table 2) and corn stalks (data not shown) were normal.

**The Δhdf1 mutant is defective in colonizing wheat rachis.**

To further determine the function of *HDF1* in plant infection, we examined the infection processes by scanning and transmission electron microscopy (SEM and TEM). At 24 h postinoculation (hpi), both wild-type and mutant strains directly penetrated the wheat epidermis (Fig. 4A). Fungal growth was observed in lemma tissue at 48 hpi (Fig. 4B), indicating that the Δhdf1 mutant was normal in the initial infection processes. However, *HDF1* appeared to be essential for spreading from the inoculated spikelet to the rachis and nearby spikelets. By 120 hpi, fungal growth was not observed in the rachis below or above the spikelets inoculated with the Δhdf1 mutant (Fig. 4C), whereas the wild type had colonized the vascular and other tissues of the rachis. Abundant intracellular hyphae were observed in rachis tissues (Fig. 4C). These observations were consistent with reduced virulence of the mutant (Table 2).

We then transformed the pRPE7-eGFP construct pRM7 (Mehrabi et al. 2008) into the wild-type strain PH-1 and Δhdf1 mutant YM1. The resulting transformants RM7PH1 and YM10 (Table 1) had strong green fluorescent protein (GFP) signals (data not shown) and were used to inoculate flowering wheat heads. At 5 dpi, fungal growth was examined with an epifluorescence microscope after removing the inoculated spikelets. Extensive GFP signals were observed in the rachis of wheat heads inoculated with RM7PH1 (Fig. 5). In contrast, YM10 failed to colonize the rachis tissues. GFP signals were restricted to the sites where the colonized spikelets were attached (Fig. 5), further indicating that the Δhdf1 mutant was defective in spreading from the inoculation site to the wheat rachis.

**Deletion of *HDF1* blocks sexual reproduction.**

*F. graminearum* is a homothallic fungus. On carrot agar plates, the wild type produced abundant perithecia 2 to 3 weeks after self fertilization. Many perithecia produced ascospore cirrhi that emerged through the ostiole (Fig. 6A). Under the same conditions, no perithecia were formed by the Δhdf1 mutant. Close examination revealed that the Δhdf1 mutant formed numerous sporodochia as tufts on carrot agar plates (Fig. 6). Abundant conidiophores and conidia were produced by the mutant in the sporodochia (Fig. 6B). These results indicate that *HDF1* is essential for sexual reproduction in self crosses of *F. graminearum*. Production of sporodochia instead of perithecia on mating plates suggests that the Δhdf1 mutant is defective in the proper regulation of sexual and asexual reproduction.

**HDAC activity is reduced in the Δhdf1 mutant.**

To confirm the biochemical function of the product of the *HDF1* gene, we isolated proteins from vegetative hyphae of the Δhdf1 mutant. HDAC activities were assayed with the colorimetric HDAC assay kit (as described below). HDAC activity in the Δhdf1 mutant was reduced approximately 60% compared with the wild type (Fig. 7), indicating that *HDF1* is the major type II HDAC gene in *F. graminearum*.

**Expression and localization of the *HDF1*-eGFP fusion construct.**

An *HDF1*-eGFP fusion construct was transformed into the Δhdf1 mutant YM1 (Table 1). The resulting transformant, YM9 (Table 1), had no or very weak GFP signals in the cytoplasm.

---

**Table 1.** Strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1</td>
<td>Wild type</td>
<td>Cuomo et al. 2007</td>
</tr>
<tr>
<td>11622</td>
<td>hdf1 mutant</td>
<td>Hou et al. 2002</td>
</tr>
<tr>
<td>YM1</td>
<td>Δhdf1 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM2</td>
<td>Δhdf1 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM3</td>
<td>Δhdf1 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM5</td>
<td>Δhdf1 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM6</td>
<td>Δhdf1 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM8</td>
<td>Ectopic transformant of the <em>HDF1</em> knockout construct</td>
<td>This study</td>
</tr>
<tr>
<td>YM9</td>
<td>Transformant of YM1 expressing the <em>HDF1</em>-eGFP fusion construct</td>
<td>This study</td>
</tr>
<tr>
<td>YM10</td>
<td>Transformant of YM1 expressing the RP27-eGFP construct</td>
<td>This study</td>
</tr>
<tr>
<td>YM11</td>
<td>Complemented transformant of YM1 (Δhdf1/HDF1)</td>
<td>This study</td>
</tr>
<tr>
<td>YM12</td>
<td>Δhdf3 deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM13</td>
<td>Δhdf2 deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM14</td>
<td>Ectopic transformant of the <em>HDF3</em> knockout construct</td>
<td>This study</td>
</tr>
<tr>
<td>YM15</td>
<td>Ectopic transformant of the <em>HDF2</em> knockout construct</td>
<td>This study</td>
</tr>
<tr>
<td>YM16</td>
<td>Δhdf1 Δhdf2 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YM17</td>
<td>Δhdf1 Δhdf3 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>RM7PH1</td>
<td>Transformant of YM1 expressing the RP27-eGFP construct</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2.** Vegetative growth, conidiation, and virulence of *Fusarium graminearum* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth rate (mm/day)</th>
<th>Conidiation (×10⁴/ml)</th>
<th>Disease index</th>
<th>DON (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1 (wild type)</td>
<td>11.1 ± 0.3</td>
<td>124.0 ± 6.7</td>
<td>11.6 ± 5.6</td>
<td>913.8 ± 151.2</td>
</tr>
<tr>
<td>YM1 (Δhdf1)</td>
<td>8.0 ± 0</td>
<td>9.8 ± 5.3</td>
<td>1.1 ± 4.8</td>
<td>617.5 ± 50.7</td>
</tr>
<tr>
<td>YM13 (Δhdf2)</td>
<td>10.7 ± 1.3</td>
<td>52.0 ± 4.5</td>
<td>7.5 ± 8.9</td>
<td>915.0 ± 258.5</td>
</tr>
<tr>
<td>YM12 (Δhdf3)</td>
<td>10.3 ± 1.1</td>
<td>104.0 ± 4.6</td>
<td>10.9 ± 7.9</td>
<td>358.3 ± 217.7</td>
</tr>
<tr>
<td>YM8 (ectopic)</td>
<td>10.3 ± 0</td>
<td>103.0 ± 8.6</td>
<td>9.7 ± 2.1</td>
<td>780.2 ± 146.3</td>
</tr>
<tr>
<td>YM11 (Δhdf1/HDF1)</td>
<td>11.0 ± 0.4</td>
<td>102.0 ± 7.9</td>
<td>11.2 ± 3.8</td>
<td>890.8 ± 201.5</td>
</tr>
</tbody>
</table>

* Mean and standard deviation were calculated from three independent replicates.

b Average daily extension in colony radius on potato dextrose agar plates.

© Conidiation in 5-day-old CMC cultures.

© Diseased spikelets per wheat head.

© Deoxynivalenol.
of vegetative hyphae or 18-h germlings (data not shown). Weak GFP signals were observed in the nucleus only in freshly harvested conidia (Fig. 8A).

The expression level of HDF1 in conidia, germlings (12 h), and vegetative hyphae of the wild-type strain was quantified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Transcripts of HDF1 were abundant in conidia, reduced in 12-h germlings, and lowest in vegetative hyphae, which were 2.4-fold lower than those of conidia (Fig. 8B). These results indicate that HDF1 had the highest expression level in conidia, which agreed with the observation that GFP signals were only observed in the nucleus of conidia in HDF1-eGFP transformant YM9.

**Microarray analysis with the Δhdf1 mutant.**

To identify genes regulated by HDF1, the whole-genome GeneChip of *F. graminearum* (Guldener et al. 2006) was used for microarray analysis with RNA samples isolated from 18-h germlings. In comparison with the wild type, 149 and 253 genes were down- and upregulated, respectively, over fivefold in the Δhdf1 mutant (Supplementary Tables 2 and 3). The largest MIPS (Munich Information Center for Protein Sequences) category (43%, n = 61) encodes proteins involved in metabolism (Supplementary Fig. 1). A number of genes involved in nitrogen metabolism, including the nitrate (FGSG_01947) and nitrite (FGSG_08402) reductase genes, were significantly reduced in the mutant. The second largest group of the downregulated genes (29%, n = 42) consists of hypothetical proteins with unknown biological functions.

The gene with the most significantly reduced expression level in the Δhdf1 mutant was FGSG_03168 (downregulated 124-fold), which is predicted to encode a putative fructose transporter. Interestingly, in total, 22 transport-related genes belonging to MIPS category 20 (Mewes et al. 2002), including eight additional sugar transporter and four putative amino acid permease or transporter genes, were reduced more than fivefold in their expression levels, which constituted the third largest group of genes downregulated in the Δhdf1 mutant. According to microarray data deposited in PlexDB, six of these transport-related genes—FGSG_00136 (9.1×), FGSG_04182 (3.4×), FGSG_04204 (2.0×), FGSG_05839 (6.1×), FGSG_07631 (3.4×), and FGSG_11480 (3.1×)—have increased expression levels in infected barley heads at 144 hpi. Another six had increased expression levels during colonization of wheat stems (Guenther et al. 2009). Some of these transport-related genes may play a role in plant infection.

To verify the microarray data, eight genes were selected for qRT-PCR analysis. All of them were confirmed to be significantly downregulated in the Δhdf1 mutant (Fig. 9A). FG03882 encodes a putative ABC transporter that is similar to ABC1, a
transporter gene known to be important for pathogenesis in M. oryzae (Urban et al. 1999). Although the exact molecular mechanism is not clear, ABC1 may play a critical role in the transportation or avoidance of plant defense compounds. FG03675 encodes the autophagy protein Atg22. In M. oryzae, autophagy plays an important role in appressorium penetration and plant infection (Kershaw and Talbot 2009). FG09921 encodes a fungal-specific transcription factor that is orthologous to a transcriptional regulator of acetate utilization in A. nidulans and Neurospora crassa (Bibbins, et al. 2002; Todd et al. 1997). The downregulation of some of these genes may contribute to reduced virulence of the Δhdf1 mutant.

For the genes that are upregulated in the Δhdf1 mutant, the top two categories were hypothetical proteins (73%) and proteins involved in metabolism (18%). Almost 75% of the genes upregulated in the Δhdf1 mutant encode proteins with unknown functions. Many of them are unique to filamentous fungi or Fusarium spp. Interestingly, six members of the auto-fusarin biosynthesis gene cluster (FGSG_2324 to FGSG_2329) were upregulated over 15-fold in the Δhdf1 mutant (Supplementary Table 4). Eight of the upregulated genes, including FGSG_06596 (putative catalase) and FGSG_08151 (putative peroxidase), were selected for qRT-PCR analysis (Fig. 9B). All but FGSG_07798 were confirmed to be significantly upregulated in the Δhdf1 mutant (Fig. 9B).

Because of the increased expression of FGSG_06596 and FGSG_08151 in the Δhdf1 mutant, we assayed hyphal growth in the presence of different concentrations of H₂O₂. On PDA...
with 0.05% H₂O₂, vegetative growth was inhibited in the wild type but the Δhdf1 mutant formed colonies with limited aerial hyphae (Fig. 9C). Under the same conditions, the complemented transformant, similar to the wild type, was completely inhibited in aerial hyphal growth (Fig. 9C). These data indicate that the Δhdf1 mutant was more tolerant to H₂O₂ than the wild type.

**Functional characterization of the HD1A and HOS3 orthologs in F. graminearum.**

To determine the functions of the other two class II HDAC genes that are homologous to yeast HD1A and HOS3 (designated HDF2 and HDF3 in this study), we used the split-marker approach to generate the Δhdf2 and Δhdf3 mutants. Unlike the HDF1 gene, HDF2 and HDF3 appeared to be dispensable for vegetative growth. The Δhdf2 and Δhdf3 mutants had a normal growth rate (Supplementary Fig. 2). The Δhdf2 mutant was slightly reduced in conidiation and virulence in infection assays with flowering wheat heads (Table 2; Fig. 10A). In contrast, the Δhdf3 mutant had no significant change in conidiation and virulence (Table 2; Fig. 10A).

To determine whether these two class II HDAC genes have overlapping functions with HDF1, we generated the Δhdf1 Δhdf2 and Δhdf1 Δhdf3 double mutants. Deletion of either HDF2 or HDF3 in the Δhdf1 mutant resulted in a more severe reduction in growth rate. On PDA plates, the growth rates of the Δhdf1 Δhdf2 and Δhdf1 Δhdf3 mutants were 6.9 ± 2.0 and 7.9 ± 1.0 mm/day, respectively, which were slower than that of the wild type and Δhdf1 mutant (Table 2). In wheat head infection assays, the Δhdf1 Δhdf2 and Δhdf1 Δhdf3 mutants were more significantly reduced in virulence (Fig. 10A). Typical head blight symptoms were observed in only approximately 50% of the inoculated spikelets. No spreading from the inoculated spikelets to other wheat kernels was observed in these two double mutants. Similar results were observed in infection assays with corn stalks (Fig. 10B). At 14 dpi, only limited discoloration was observed in the corn stalks at the inoculation sites inoculated with the Δhdf1 Δhdf2 and Δhdf1 Δhdf3 mutants. These results indicate that both HDF2 and HDF3 have a minor role in the regulation of vegetative growth and plant infection in F. graminearum.

**DISCUSSION**

The reversible acetylation and deacetylation of lysine residues in the tail of core histones are common forms of histone modifications mediated by HAT and HDAC, respectively. Whereas HAT act as transcriptional coactivators, HDAC function as transcriptional corepressors. HDAC genes are divided into three classes (Yang and Grégoire 2005). Class I and class II include the classical HDAC that are sensitive to inhibition with inhibitors such as TSA.
by trichostatin A and share similarity to yeast Rpd3 and Hda1, respectively. In addition to three class II HDAC genes characterized in this study, the F. graminearum genome has one predicted class I HDAC gene (FGSG_00870) that is highly homologous to yeast RPD3. Phylogenetic analysis indicates that these four class I and class II HDAC genes are well conserved in filamentous fungi and can be divided into four clades represented by yeast RPD3, HOS2, HDA1, and HOS3.

The Hdf1 HDAC is orthologous to yeast Hos2, which interacts with Sif2 as components of the Set3 complex (Cohen et al. 2008). In yeast two-hybrid assays, Hdf1 interacted with Ftl1, an ortholog of Sif2 in F. graminearum (Ding et al. 2009). Deletion of HDF1 significantly reduced the virulence in infection assays with flowering wheat heads and corn stalks. Similar to the Δsft1 mutant (Ding et al. 2009), the Δhdf1 mutant was defective in spreading from colonized spikelets into the rachis. In C. carbonum, the hdc1 mutant was significantly compromised in colonizing corn leaf tissues (Baidyaroy et al. 2001). In M. oryzae, the core components of the Tgf1 HDAC complex, including orthologs of yeast Hos2, Sif2, Snt1, and Set3, all are important for plant infection (Ding et al. 2010). Preliminary analysis with mutants deleted of the FgSNT1 gene indicated that the SNT1 ortholog also was important for pathogenesis in F. graminearum (S. Ding and J.-R. Xu, unpublished). These observations suggest that Hdf1 and Ftl1 are components of a well-conserved HDAC complex in the wheat scab fungus for regulating plant infection processes.

One common stress faced by hyphae of necrotrophic fungi in planta is reactive oxygen species (ROS) generated during oxidative burst. The Δsft1 mutant had increased sensitivity to H₂O₂, which may contribute to its defects in plant infection (Ding et al. 2009). To our surprise, the Δhdf1 mutant had increased tolerance to 0.05% H₂O₂ and upregulated expression of putative catalase and peroxidase genes, suggesting that FTL1 and HDF1 differ in subsets of genes that they regulate in F. graminearum. We also assayed responses of the Δhdf1 mutant to stresses. Although it had no obvious defects in growth or conidium germination in the presence of 0.7 M NaCl or 1 M sorbitol (data not shown), the mutant was more sensitive to 0.01% sodium dodecyl sulfate (SDS) than the wild-type strain (Supplementary Fig. 3A), suggesting a defect in the cytoplasm membrane. Five genes involved in ergosterol synthesis (FGSG_01836, FGSG_03686, FGSG_05740, FGSG_09031, and FGSG_11044) were upregulated in the Δhdf1 mutant, which is indicative of membrane stress. The Δhdf1 mutant also had increased sensitivity to cell wall stresses and cell-wall-degrading enzymes, suggesting that the Δhdf1 mutant was defective in cell wall integrity. In addition, the Δhdf1 mutant was reduced in the production of DON, which is one of the best-characterized virulence factors in F. graminearum (Maier et al. 2006; Proctor et al. 1997). It also had a defect in vegetative growth (reduced growth rate). All of these defects of the Δhdf1 mutant may contribute to its reduced virulence.

The Δhdf1 mutant was defective in sexual reproduction. On mating plates, instead of形成 perithecia or protoperithecia, the Δhdf1 mutant produced abundant sporodochia and macroconidia (Fig. 6). Although it was reduced in conidiation in liquid CMC cultures, asexual reproduction appeared to be stimulated in the Δhdf1 mutant on carrot agar plates under culture conditions conducive for sexual reproduction. It is possible that FgHOS2 plays a role in suppressing the production of sporodochia and conidia when sexual reproduction is activated on mating plates. In liquid cultures, F. graminearum does not produce sporodochia and conidia are formed on individual conidiophores. The difference between liquid CMC and mating cultures could be related to the regulation of sporodochium formation by FgHOS2. In M. oryzae, deletion of MoHOS2 also results in sexual reproduction defects (Ding et al. 2010) but conidiation was not stimulated in mating cultures of the Mohos2 mutant (data not shown). The difference between the Δhdf1 and Mohos2 mutants in conidiation on mating plates may be related to different mating systems in F. graminearum (homothallic) and M. oryzae (heterothallic).

Unlike the Mohos2 mutant of M. oryzae that formed abnormal conidia (Ding et al. 2010), the Δhdf1 mutant had no obvious defects in conidium morphology (Supplementary Fig. 4). The Δhdf2 and Δhdf3 mutants also produced normal conidia. However, majority of the conidia produced by the Δhdf1 Δhdf2 double mutant were shorter and wider than the wild-type conidia. In the Δhdf1 Δhdf3 mutant, most of the conidia had similar defects in morphology, although to a lesser extent. Therefore, HDF2 and HDF3 may have overlapping functions with HDF1 during conidiogenesis or conidium development.

Microarray analysis revealed that more genes were upregulated than downregulated (253 versus 149) in the Δhdf1 mutant. This is not surprising because HDAC usually function as transcriptional corepressors. However, it is unexpected that the expression of autofusarin biosynthesis genes was significantly increased because the Δhdf1 mutant formed whitish colonies on PDA plates (Fig. 2C). FGSG_03064 and FGSG_03065, orthologs of the AL-1 and AL-2 genes involved in carotenoid biosynthesis (Jin et al. 2010; Li and Schmidhauser 1995), also were upregulated over 15-fold in the mutant. The discrepancy between colony pigmentation and microarray data could be related to culture conditions. RNA samples for microarray experiments were isolated from 18-h germings grown in liquid CM. In fact, pigmentation in liquid cultures of the Δhdf1 mutant tended to be stronger than that of the wild type (Supplementary Fig. 5). In A. fumigatus, deletion of the HOS2 ortholog has not been reported but deletion of the hdaA HDAC gene results in the upregulation of secondary metabolism (Lee et al. 2009).

In comparison with HDF1, the other two class II HDAC genes appeared to be less important in F. graminearum. The Δhdf2 and Δhdf3 mutants were normal in growth rate. The Δhdf3 mutant was normal and the Δhdf2 mutant was only slightly reduced in conidiation and virulence. Significant reduction in conidiation and virulence was observed only in the Δhdf1 mutant, which had a 60% reduction in HDAC activity (Fig. 7). These results indicate that HDF1 is the major class II HDAC gene in F. graminearum and it plays a more important role than HDF2 and HDF3. In A. nidulans, the HdaA (ortholog of hdf2) was reported to be the major HDAC gene (Tribus et al. 2005), which is contradictory to the recent finding of RpdA being an essential HDAC gene (Tribus et al. 2010). Because the Δhdf1 Δhdf2 and Δhdf1 Δhdf3 double mutants displayed more severe defects in growth and plant infection than the Δhdf1 mutant, these three HDAC genes must have both overlapping and distinct functions. Therefore, it will be interesting to compare the expression profiles of these HDAC mutants. Comparative analysis will help to determine subsets of genes regulated by different class II HDAC genes and their interactions in regulating genes important for growth, conidiation, and pathogenesis.

### MATERIALS AND METHODS

#### Strains and culture conditions.

The wild-type strain and mutants of F. graminearum used in this study are listed in Table 1. Cultures were routinely grown on PDA plates at 25°C (Hou et al. 2002; Seong et al. 2006). Conidiation in 5-day-old CMC cultures and growth rate on PDA plates were measured as described (Ding et al. 2009; Zhou et al. 2010). For DNA, RNA, and protein extraction,
vegetative hyphae were harvested from liquid YEPD (1% yeast extract, 2% peptone, 2% glucose) cultures. proteoplast preparation and fungal transformation were performed as described (Hou et al. 2002; Proctor et al. 1995). For transformation, hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) and geneticin (Sigma-Aldrich, St. Louis) were added to the final concentration of 250 and 150 µg/ml, respectively. For testing sensitivities to various stresses, fungal growth was assayed after incubation at 25°C for 3 days on CM plates with 1 M sorbitol, 0.7% NaCl (wt/vol), 0.01% SDS (wt/vol), or different concentrations of H2O2 (Liu et al. 2011).

Plant infection assays.

For wheat head and corn stalk infection assays, freshly harvested conidia were resuspended to 10⁶ spores/ml in sterile water. Flowering wheat heads of cultivars Norm and Xiaoyan22 were inoculated with 10 µl of conidium suspensions at the fifth spikelet from the base of the spike (Gale et al. 2002; Kang and Buchenauer 1999). Spikelets with typical head blyight symptoms were counted 14 dpi. Diseased kernels were collected and assayed for DON and ergosterol production as described (Bluhm et al. 2007; Seong et al. 2006). Stalks of 8-week-old corn plants of cv. Pioneer 2375 were inoculated with toothpicks dipped in conidium suspensions as described (Choi and Xu 2010; Zhou et al. 2010). Stalk rot symptoms were examined after splitting the stalks longitudinally along the inoculation site 14 dpi.

Generation of the hdf1, hdf2, and hdf3 mutants.

The split-marker approach was used to generate the HDF1 gene replacement mutant. The 0.54-kb upstream and 0.55-kb downstream flanking sequences of the HDF1 gene were amplified with primer pairs Hdf1F1-Hdf1R2 and Hdf1F3-Hdf1R4 and fused with fragments of the hph gene amplified with primers HY/R, YG/F, HYGF, and HYGR by overlapping PCR as described (Catlett et al. 2003; Zhou et al. 2010). The resulting PCR products were transformed into protoplasts of the wild-type strain PH-1. Putative hdf1 mutants were identified by PCR with primer pairs Hdf1F5-df1R6, Hdf1F7-H555R, H856f-df1R8, and H852-H850 and confirmed by Southern blot hybridization. The same split-marker approach was used to generate the hdf2 and hdf3 mutants.?

Generation of the hdf1 hdf2 and hdf1 hdf2 double mutants.

To generate the double mutants, the geneticin-resistance (neoR) gene was used as the selectable marker to delete the HDF1 gene in the hdf2 or hdf3 mutant. Primer pairs GEN/F-GE/R and ENF-GEN/R were used to amplify the split-marker fragments of the neoR gene from plasmid pFL2, which was generated by replacing the hph gene on pDL2 with the geneticin-resistance marker (Bourett et al. 2002; Bruno et al. 2004). Primers pairs dm1F-dm2r and dm3F-dm4r were used to amplify the 0.83-kb upstream and 0.79-kb downstream flanking sequences of HDF1, respectively. The neoR fragment amplified with primers N850 and N852 was used as the probe for Southern blot analysis.

Complementation of the hdf1 mutant with HDF1 and HDF1-eGFP fusion.

For supplementation assays, a 3.4-kb fragment of the HDF1 gene (containing the 1.5-kb promoter region) was amplified with primers Gloc-h1F and Gloc-h1R and cloned into pFL2 by the gap repair method (Bourett et al. 2002; Bruno et al. 2004). The resulting complementation construct was transformed into the hdf1 mutant YM1. The HDF1-eGFP construct was generated by cloning the HDF1 fragment amplified with primers Gloc-h1F and Gloc-h1R into pKB04 (Bruno et al. 2004). After transforming into protoplasts of YM1, transformants expressing the HDF1 complementation and HDF1-eGFP constructs were analyzed by PCR. GFP signals were observed under an Olympus BX51 epifluorescence microscope.

qRT-PCR analysis.

RNA samples were isolated from conidia, germinals, and mycelia with the TRizol reagent (Invitrogen, Carlsbad, U.S.A.). First-strand cDNA was synthesized with the Fermentas 1st cDNA synthesis kit (Hanover, MD) following the instructions provided by the manufacturer. Primers used for qRT-PCR analysis are listed in Supplementary Table 1. The F. graminearum ß-tubulin (TUB2) gene was amplified with primers TubQF and TubQR (Bluhm et al. 2007). Relative changes in the expression level of each gene were calculated by the 2⁻ΔΔCt method (Livak and Schmittgen 2001) with TUB2 as the endogenous reference. For each gene, qRT-PCR data from three biological replicates were used to calculate the mean and standard deviation.

SEM and TEM observations.

Glumes and lemmas were collected from inoculated spikelets and fixed with 4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) overnight at 4°C and rinsed with the same buffer for 2 h. After dehydration in a graded series of acetone (30, 50, 70, 80, 90, and 100%; vol/vol), the samples were mounted on stubs, sputter coated with gold-palladium, and examined with a JEOL 6360 scanning microscope (Jeol Ltd., Tokyo). For TEM examination, slices of wheat glumes and rachis tissues were fixed, dehydrated, and embedded as described (Ding et al. 2009). Ultrathin sections were treated with uranyl acetate and lead citrate (Kang et al. 2008) and examined with a JEM-1230 electron microscope (Jeol Ltd.) at 80 KV. At least three independent biological replicates were examined for the wild-type and hdf1 mutant strains.

Microarray analysis.

Freshly harvested conidia of PH-1 and YM1 (Table 1) were resuspended to 10⁶ conidia/ml in 100 ml of CM. After incubation at 25°C for 18 h, germinated conidia were collected by filtration and used for RNA isolation with the TRizol reagent (Invitrogen). For each strain, RNA was isolated from three biological replicates. Probe labeling and hybridization of the Fusarium GeneChip microarrays (Guldener et al. 2006) were performed with standard Affymetrix procedures at the Purdue Core Genomics Facility. Hybridization signals were scanned with a GeneChip GCS 3000 scanner (Affymetrix, Santa Clara, CA, U.S.A.). The resulting CEL files were processed with the Affymetrix MAS5.0 system and analyzed with GeneSpring GX V7.2 (Agilent Technologies, Santa Clara, CA, U.S.A.).

HDAC activity assays.

Vegetative hyphae of the wild-type PH-1 and the hdf1 mutant were harvested from 100 ml of 3-day-old CM cultures and resuspended in 400 µl of lysis buffer (Ding et al. 2009) with 5 µl of protease inhibitor cocktail (Sigma-Aldrich). After homogenization with acid-washed glass beads in a Biospec mini-bead beater for three 40-s pulses with 1-min intervals on ice, the lysate was separated from the glass beads and centrifuged at 16,000 × g for 15 min at 4°C (Ding et al. 2009). The resulting supernatants were used to assay for HDAC activities with the colorimetric HDAC assay kit (Active Motif, Carlsbad, CA, U.S.A.) following the instructions provided by the manufacturer. Absorbance was detected with a plate reader (Synergy HT, Bio-TEK, Houston, TX, U.S.A.; 360-nm excitation and 465-nm emission). The concentration of deacetylated com-
pounds was calculated from the deacetylation standard curve and used to estimate the HDAC activity (pmoles per minute per milligram) with the formula provided with the Active Motif HDAC assay kit.

ACKNOWLEDGMENTS

We thank L. Dunkle and S. Goodwin at Purdue University for critical reading of this manuscript, Q. Hang and K. Zhang at Northwestern A&F University for technical assistance during this study, and Y. Dong for assistance with DON detection. This work was supported by the 111 Project from the Ministry of Education of China (B07049) and grants to J.-R. Xu from the National Research Initiative of the United States Department of Agriculture Cooperative State Research, Education and Extension Service (no. 2007-35319-102681) and the US WBSI.

LITERATURE CITED


AUTHOR-RECOMMENDED INTERNET RESOURCE

Plant Expression Database (PlexDB): www.plexdb.org