The CID1 cyclin C-like gene is important for plant infection in *Fusarium graminearum*

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**Abstract**

Head blight or scab caused by *Fusarium graminearum* is a destructive disease of wheat and barley. The pathogen can cause severe yield losses and contaminates infested kernels with harmful mycotoxins. In this study, we characterized the CID1 gene in *F. graminearum* that is an ortholog of the *Fusarium verticilloides* FCC1 and yeast UME3 genes. The protein encoded by CID1 has typical structural features of C-type cyclins. Deletion of CID1 resulted in a reduction in conidiation and vegetative growth but an increase in pigmentation. The Δcid1 mutant was female sterile but could outcross as a male. It was significantly reduced in DON production and virulence on wheat heads and corn stalks. Only about 50% of inoculated spikelets developed scab symptoms and scab disease rarely extended to nearby florets, suggesting that the Δcid1 mutant was defective in colonizing and spreading in wheat heads. Deletion of CID1 resulted in reduced expression levels of TRI5 and TRI101 but increased PKS12 expression. When expressed in *F. verticilloides*, the CID1 gene complemented the defects of the Δfcc1 mutant in conidiation, hyphal growth, and fumonisin production. Our data indicate that the CID1 C-type cyclin gene plays multiple roles in the regulation of vegetative growth, sexual development, conidiation, DON production, and pathogenicity.

1. Introduction

*Fusarium graminearum* (Schwabe) infects a broad range of crop plants including wheat, barley, and maize (Bai and Shaner, 2004; Goswami and Kistler, 2004). In addition to yield losses, infested cereals are often contaminated with mycotoxins, such as deoxynivalenol (DON), that are harmful to humans and animals (Desjardins et al., 1996; McMullen et al., 1997). DON is a potent inhibitor of protein synthesis in eukaryotic organisms and causes a variety of acute and chronic toxicoses. Ascospores that are forcibly discharged from mature perithecia are the primary inoculum for the infection of flowering wheat heads in the spring (McMullen et al., 1997; Trail et al., 2005). Infection of wheat can occur from the beginning of anthesis until the dough stage of kernel development, and is initiated when ascospores or conidia are deposited on or inside flowering spikelets. The fungus penetrates through stomata or floral bracts (Pritsch et al., 2000). From the infected floret, the fungus can spread up and down the spike and cause severe damage.

Molecular mechanisms underlying pathogenesis in *F. graminearum* are complex and not fully understood. In addition to genes related to well conserved signal transduction pathways (Hou et al., 2002; Jenczmionka et al., 2003; Jenczmionka and Schafer, 2005; Urban et al., 2003; Yu et al., 2008), a number of pathogenicity genes with diverse biological or biochemical functions have been characterized (Han et al., 2007; Lee et al., 2005; Lu et al., 2003; Seong et al., 2005, 2006; Shim et al., 2006). The first pathogenicity factor most thoroughly characterized by molecular studies in *F. graminearum* is the trichodiene synthase gene TRI5 (Desjardins et al., 2000; Proctor et al., 1995). Although trichothecene mycotoxins are not necessary for the initial infection, they are important for the spread of the fungus within colonized spikes (Bai et al., 2002; Desjardins et al., 2000; Harris et al., 1999; Proctor et al., 1995). In the past decade, the trichothecene biosynthetic gene cluster and biosynthesis pathway have been extensively studied. Most genes encoding enzymes for metabolic steps in DON biosynthesis have been characterized, including TRI3, TRI5, TRI6, TRI7, TRI10, and TRI110 (Kimura et al., 2003, 2007; Lee et al., 2002). TRI6 and TRI10 are pathway-specific transcription factors for the TRI genes (Hohn et al., 1999; Seong et al., 2009; Tag et al., 2001). However, global regulatory systems that control the expression of TRI genes and DON synthesis in *F. graminearum* are not well characterized.

In *F. verticilloides*, a close relative of *F. graminearum*, the FCC1 C-type cyclin-like gene was identified by random insertional mutagenesis to be important for fumonisin biosynthesis (Shim and Woloshuk, 2001). On corn kernels and defined minimal medium at pH 6, the fcc1 deletion mutant was reduced in conidiation and blocked in fumonisin production. Further studies indicated that the Fck1 cyclin-dependent kinase directly interacts with Fcc1. Deletion of FCK1 also lead to a significant reduction in conidiation and fumonisin production on corn kernels (Bluhm and Woloshuk,
2. Materials and methods

2.1. Fungal strains and growth conditions

The Fusarium graminearum wide type strain PH-1 and transformants generated in this study were routinely maintained at 25°C on V8 juice agar and incubated in liquid CMC medium for conidium production as described (Hou et al., 2002; Tail et al., 2003). To assay the effects of pH on conidiation, the CMC medium was adjusted to pH 3.0, 5.0, 7.0, and 9.0 with phosphoric acid as described (Shim and Woloshuk, 2001). Conidia were collected by filtration through two layers of Miracloth (Calbiochem, La Jolla, CA) and counted with a hemacytometer (Hou et al., 2002). Protoplast preparation and fungal transformation were performed as described (Proctor et al., 1995). TB3 medium (0.3% yeast extract, 0.3% casamino acids, and 20% sucrose) supplemented with 200 μg/ml G418 (Sigma, St. Louis, MO) was used for selection of transformants (Seong et al., 2006). Growth rate on V8 juice agar was assayed with race tube cultures incubated at 25°C for 16 days. For assaying defects of the Δcid1 mutant in stress responses, cultures grown on YEPD medium (0.3% yeast extract, 1% peptone, 2% glucose, 1.8% agar) with different concentrations of NiCl₂, CdCl₂, and H₂O₂ was measured for colony radius after incubation for 7 days. Selfing and outcrossing with the nit1 mutant 11622 were performed on carrot agar plates (Hou et al., 2002). DON and ergosterol production in infected wheat kernels were measured as previously described (Bluhm and Woloshuk, 2005; Bluhm and Woloshuk, 2006; Goswami and Kistler, 2005). To assay the effect of acidic pH on DON production, the defined medium containing 0.2% agmatine (Gardiner et al., 2009a) was buffered with phosphoric acid to different pH values as described (Shim and Woloshuk, 2001). Culture conditions for DON production followed what were described by Gardiner and colleagues (2009a,b).

For F. verticillioides, the wild-type strain FT536, the Δfuc1 mutant (Shim and Woloshuk, 2001), and the Δfuc1/C176 complemented strain were cultured on V8 juice agar for assaying vegetative growth. For conidium production, F. verticillioides strains were grown in the BSM6.0 medium (Shim et al., 2006) at 25°C.

2.2. The CID1 gene replacement vector and mutant

A 0.7-kb upstream sequence and a 0.8-kb downstream flanking sequence of the CID1 gene were amplified with primer pairs CID1-F1/CID1-R2 and CID1-F3/CID1-R4 (Table 4), respectively. Primers HYGF, HYR, YGF, and HYGR (Table 4) were used to amplify PCR fragments for generating the Δcid1 deletion construct by the split-marker approach (Catlett et al., 2003). After fungal transformation, hygromycin-resistant transformants were screened by PCR with primers CID1-F5 and CID1-R6 (Table 4). The putative Δcid1 mutant was further confirmed by Southern blot analysis following standard molecular protocols (Sambrook and Russell, 2001). Genomic DNA was digested with Xhol and separated on 0.8% agarose gel before being transferred to a nylon membrane.

For complementation assays, a 3.4-kb fragment containing the entire CID1 gene, including the open reading frame, 1.7-kb promoter region, and 0.6-kb terminator region, was amplified with primers CID1-FC and CID1-CR (Table 4) and cloned between the BamHI and NotI sites of pHZ100 (Bluhm et al., 2007) to generate the complementation construct pCID1. In pHZ100, the neomycin-resistant cassette derived from pSM334 was inserted into the vector backbone. Plasmid pCID1 was transformed into the Δcid1 mutant CH1 as described (Proctor et al., 1995).

2.3. Corn stalk and wheat infection assays

Conidia were collected from 5-day-old CMC cultures by filtration through two layers of Miracloth (Calbiochem) and resuspended to 5 × 10⁸ conidia/ml in sterile distilled water. For infection assays with corn plants of cultivar Pioneer 2375, sterile wooden toothpicks were briefly dipped in the spore suspension and then slowly droved into the stalk between the tassel and node immediately below. Stalk rot symptoms were examined 14 days post-inoculation (dpi) by removing the toothpick and slicing corn stalks into halves along the inoculation sites. Six-week-old plants of wheat cultivar Norm were used for inoculation as described (Gale et al., 2002; Kang and Buchenauer, 1999). The second, full-sized spikelet from the base of the inflorescence was injected with 10 μl of the spore suspension. Inoculated wheat heads were covered with a small plastic bag for 2 days. Infected plants were then cultured at ambient greenhouse conditions. Symptomatic spikelets in each head were counted 14 dpi to calculate the disease index as described (Gale et al., 2002; Seong et al., 2006). At least nine inoculated wheat heads per treatment were used for each test and all tests were repeated four times. Diseased kernels were harvested from inoculated spikelets and assayed for DON and ergosterol as described (Bluhm et al., 2007).

2.4. Quantitative RT-PCR (qRT-PCR)

A total of 10⁶ conidia of each strain was inoculated into 100 ml of YEPD medium and incubated at 25°C for 48 h with shaking at 150 rpm. Mycelia were harvested by filtration through two layers of Miracloth. RNA was isolated from mycelia with the Trizol reagent (Invitrogen, Carlsbad, CA). The Stratascript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA) was used for cDNA synthesis following the instruction provided by the manufacturer. The primer pairs used for qRT-PCR were Tri5QF/Tri5QR for Tri5, PKS12QF/PKS12QR for PKS12 (Table 4), TubQF/TubQR (Bluhm et al., 2007) for the beta-tubulin gene TUB2. PCR was performed with a MX3000 (Stratagene) System with the program of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C followed by 1 min at 60°C. Data were collected with the sequence detector software MXPro. All expression data were normalized to TUB2 expression and relative changes in gene expression levels were calculated by the comparative Ct method (Applied Biosystems, Foster City, CA). For each gene assayed by qRT-PCR, data from three biological replicates were used to calculate the mean and standard deviation.
2.5. Complementation of the F. verticilloides Afcc1 mutant with the CID1 gene

Plasmid pCID1 containing the full length CID1 gene was transformed into the F. verticilloides Afcc1 mutant FTS36 as described (Shim and Woloshuk, 2001). Neomycin-resistant transformants were isolated and verified by PCR with primers CID-5F and CID-6R to contain the CID1 gene integrated in the F. verticilloides genome. Cracked corn cultures incubated at 25°C for 14 days were used for assaying fumonisin B1 (FB1) production as described (Shim and Woloshuk, 2001).

3. Results

3.1. The CID1 gene is well conserved in filamentous ascomycetes

BLASTP search revealed that the homolog of the F. verticilloides FCC1 gene in F. graminearum is FGSG_04355.3, which was named the CID1 gene (for Cyclin C-like gene required for infection and DON production) in this study. CID1 also is homologous to UME3 of Saccharomyces cerevisiae that encodes a C-type cyclin or cyclin C-like protein. Ume3p is one component of the RNA polymerase II holoenzyme, and it is required for the repression of genes involved in meiosis and stress response (Kuchin et al., 1995).

The CID1 gene is predicted to encode a 321 amino acid peptide. Like Ume3 and Fcc1, the Cid1 protein had the cyclin-box interactions and the holoenzyme associating domain (Fig. 1A). It also contains the PEST-rich region and RQLK motif that are known to be responsible for Ume3 degradation in S. cerevisiae (Cooper et al., 1997). Cid1 protein is 85% identical to Fcc1 and its ortholog from F. oxysporum (FOXG_13879). All these Fusarium cyclin-C-like proteins are highly conserved in the cyclin box and the cyclin destruction domains.

3.2. The Acid1 mutant is defective in hyphal growth and conidiation

To determine the function of CID1 in F. graminearum, we generated mutants in which the CID1 gene was deleted with the split-marker approach (Catlett et al., 2003) (Fig. 1B). After transformation of wild-type PH-1 protoplasts, hygromycin-resistant transformants were isolated and screened by PCR with primers CID-F5 and CID-R6 (Fig. 1B). One Acid1 mutant named CH1 was identified and further confirmed by Southern blot analysis (Fig. 1C). When hybridized with the downstream flanking fragment amplified with primers F3 and F4 (probe 1), a 1.5 kb band was detected in PH-1. The Acid1 mutant had a 7.0 kb band that was diagnostic for the gene replacement event. When hybridized with the CID1 ORF region amplified with primers F5 and R6 (probe 2), the 1.5 kb band detected in PH-1 was absent in the Acid1 mutant (Fig. 1C).

The growth rate of the Acid1 mutant was measured with race tube cultures grown at 25°C for 16 days. On average, it grew about 30% slower than the wild-type strain (Table 1). The mutant also was reduced about 50% in conidiation (Table 1). Conidia produced by the Acid1 mutant had only four cells (three septa) instead of 5–6 celled wild-type conidia (Fig. 2A). Some of them also lacked the typical morphology of Fusarium conidia. In addition, conidium germination was slightly delayed in the mutant but germ growth and hyphal branching appeared to be normal (Fig. 2A). The Acid1 mutant had increased pigmentation in aerial hyphae of aging cultures. Five-day-old V8 agar cultures of the mutant appeared to be more reddish than those of the wild-type strain (Fig. 2B).

To confirm that deletion of CID1 was responsible for observed defects in vegetative growth and conidiation, we transformed the full-length wild-type CID1 allele into the Acid1 mutant CH1. The resulting transformant CH1comp (Acid1/CID1) had the wild-type growth rate and was normal in conidiation (Table 1), indicating that the defects of the mutant were complemented by the ectopic copy of the CID1 gene.

**Table 1**

Vegetative growth, conidiation, and DON production in Fusarium graminearum strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Growth rate (mm/day)</th>
<th>Conidiation (5 × 10^6/ml)</th>
<th>DON (ppm)</th>
<th>Erg (ppm)</th>
<th>DON/Erg ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1</td>
<td>12.5 ± 0.3</td>
<td>133.0 ± 0.6</td>
<td>318.4 ± 1.2</td>
<td>103.4 ± 2.1</td>
<td>3.01 ± 0.1</td>
</tr>
<tr>
<td>CH1 (Acid1)</td>
<td>9.2 ± 0.2</td>
<td>67.0 ± 0.6</td>
<td>20.5 ± 0.6</td>
<td>91.5 ± 0.2</td>
<td>0.25 ± 0.0</td>
</tr>
<tr>
<td>CH1comp (Acid1/CID1)</td>
<td>12.5 ± 0.5</td>
<td>132.0 ± 0.6</td>
<td>290.5 ± 2.3</td>
<td>97.9 ± 2.1</td>
<td>2.99 ± 0.1</td>
</tr>
</tbody>
</table>

* Growth rate was measured with race tube cultures (V8 juice agar) after incubating at 25°C for 16 days. Means and standard errors were calculated from six replicates for each strain.

* Conidiation was assayed with 5-day-old CMC cultures as described (Hou et al., 2002).

* DON and ergosterol (Erg) was assayed with the wheat kernels that were harvested from the inoculated florets 14 dpi.

* The same letter indicated that there was no significant difference. Different letters were used to mark statistically significant difference (P = 0.05).
3.3. CID1 is essential for female fertility

F. graminearum is a homothallic fungus but it can be forced to outcross. On selfing carrot agar plates, the Δcid1 mutant failed to form perithecia (Fig. 2B). Close examination indicated that the mutant was blocked in the differentiation of protoperithecia. Although it was female sterile, the Δcid1 mutant was able to mate as the male. When mating with a nit1 mutant, Δcid1 nit1 recombinant progeny were isolated from outcross perithecia. The complemented transformant CH1comp (Δcid1/CID1) produced fertile perithecia on selfing plates (Fig. 2C), further indicating that CID1 is essential for female fertility in F. graminearum.

**Fig. 2.** Defects of the Δcid1 mutant in conidium morphology, colony pigmentation, and mating. (A) Conidia of the wild-type PH-1, Δcid1 mutant, and Δcid1/CID1 complemented (comp) strains were examined after incubation in liquid CM for indicated times. Bar = 10 μm. (B) Colonies of PH-1, Δcid1 mutant, and comp strains grown on V8 agar for 5 days. (C) Selfing plates of PH-1, Δcid1 mutant, and Δcid1/CID1 complemented strain. Black dots on the surface were perithecia.
3.4. *The Acid1 mutant is defective in colonizing flowering wheat heads*

To determine the function of *CID1* in plant infection, flowering wheat heads were point-inoculated with conidium suspensions. On wheat heads inoculated with the wild-type strain, typical scab symptoms were observed in the inoculated and nearby spikelets 14 days post-inoculation (dpi). Under the same conditions, only about 50% of the spikelets inoculated with Δ*cid1* conidia developed scab symptoms. On heads with disease symptoms caused by the Δ*cid1* mutant, wheat scab was limited to the inoculated spikelets (Fig. 3A). Spikelets nearby the inoculation site remained healthy 14 dpi. On average, the Δ*cid1* mutant had a disease index score of 0.5 (Fig. 3B). In comparison with the disease index of the wild-type strain, the mutant had a 16-fold reduction in virulence. Re-introduction the wild-type *CID1* gene complemented the defects of the Δ*cid1* mutant. In wheat head infection assays, the Δ*cid1*/*CID1* complemented strain was as virulent as PH-1 (Fig. 3). These data indicated that *CID1* is important for full virulence on wheat.

3.5. *The CID1 gene also is important for full virulence on maize*

*F. graminearum* is also a corn stalk rot pathogen. In corn plants inoculated with the Δ*cid1* mutant, stalk rot symptoms were observed at the inoculation site (Fig. 4). When measured at 14 dpi, the average length of stalk rot lesions caused by the Δ*cid1* mutant was 1.6 ± 0.2 cm. Under the same conditions, the length of stalk rot lesions were 6.3 ± 0.5 cm and 6.3 ± 0.7 cm (Table 1), respectively, for the wild-type and complemented strains. Therefore, the Δ*cid1* mutant had a 75% reduction in virulence on corn stalks. These results suggest that the *CID1* gene might be important for *F. graminearum* to colonize and spread in the corn pith.

3.6. *The Acid1 mutant is defective in stress responses*

In yeast, the *ume3* mutant has increased sensitivities to various stresses (Cohen et al., 2003; Cooper et al., 1997). To test whether the Δ*cid1* mutant had similar defects, Ni²⁺, Cd²⁺, and H₂O₂ were added to YEPD medium (Table 2). In the presence of 500 μM Ni²⁺, the growth rate of the mutant was about 30% of that of the wild-type strain. Colony growth was completely inhibited by 1 mM Ni²⁺ in the Δ*cid1* mutant, but the wild-type strain (PH-1) still grew under the same conditions (Table 2). The Δ*cid1* mutant was also more sensitive to the CdCl₂ and H₂O₂ treatments than the wild-type strain. In the presence of 100 μM Cd²⁺ or 0.04% H₂O₂, it grew about 50% slower than the wild-type strain (Table 2). The Δ*cid1* mutant, but not the wild-type strain, failed to grow on medium with 500 μM Ni²⁺ or 1% H₂O₂. These results suggest that the *CID1* gene may be important for responses to oxidative and heavy metal stresses.

3.7. DON production and TRI5 expression are reduced in the Δ*cid1* mutant

To determine the role of *CID1* in DON production, we isolated the diseased wheat kernels from the inoculated spikelets and measured DON content. On average, only about 20 ppm DON (Table 1) was detected in wheat kernels infected by the Δ*cid1* mutant. Kernels inoculated with the wild-type and complemented strains contained approximately 300 ppm DON. Because the Δ*cid1* mutant was reduced in growth rate, the same samples also were assayed...
for ergosterol for a measure of fungal biomass. After normalization with ergosterol, it was clear that DON production was significantly reduced by deletion of the \( CID1 \) gene (Fig. 5A). The DON/ergosterol ratio in wheat kernels colonized by the \( \Delta cid1 \) mutant was about nine times less than that of the wild-type and complemented transformant (Fig. 5A), indicating that \( CID1 \) plays an important role in regulating DON synthesis in wheat kernels.

To further support the conclusion that the \( CID1 \) gene affects DON synthesis, we assayed the expression of the trichodiene synthase gene \( TRI5 \) by quantitative RT-PCR (qRT-PCR). RNA samples were isolated from hyphae grown in YEPD medium for 2–3 days. In the \( \Delta cid1 \) mutant, the expression level of \( TRI5 \) was reduced about four times in comparison with that of the wild-type or complemented strain (Fig 5B). We also assayed the expression of the \( TRI101 \) gene that encodes a trichothecene acetyltransferase. The expression level of \( TRI101 \) was reduced in the \( \Delta cid1 \) mutant but to a lesser extent than that of \( TRI5 \) (Fig. 5B).

Because of increased pigmentation in the mutant deleted for \( CID1 \), we also assayed the expression levels of two polyketide synthase genes, \( PKS12 \) and \( PKS10 \). In comparison with the wild type, the expression level of \( PKS12 \), which is responsible for aurofusarin synthesis in \( F. graminearum \) was increased about three times in the \( \Delta cid1 \) mutant (Kim et al., 2005; Malz et al., 2005). Expression of \( PKS12 \) in the complemented transformant was comparable to the wild-type level (Fig. 6B). In contrast, \( PKS10 \) was expressed at similar levels in all these three strains assayed (Fig. 6B). These results indicated that deletion of \( CID1 \) had different effects on the expression of different PKS genes. Increased expression of \( PKS12 \) might result in increased production of aurofusarin and contribute to enhanced pigmentation observed in the \( \Delta cid1 \) mutant.

3.8. Acidic pH failed to suppress the defects of the \( \Delta cid1 \) mutant in conidiation and toxin production

Because acidic pH has a significant impact on conidiation and fumonisin synthesis in \( F. verticillioides \) (Shim and Woloshuk, 2006), we examined the ability of acidic pH to suppress the conidiation defect of the \( \Delta cid1 \) mutant. The \( \Delta cid1 \) mutant failed to form conidia at acidic pH, whereas the wild type was able to form conidia even at pH 4.5 (Fig. 5B).

### Table 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration</th>
<th>Reduction in the growth rate (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni** (µM)</td>
<td>100</td>
<td>15.6 ± 3.1, 17.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>19.7 ± 2.9, 20.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>33.2 ± 2.1, 66.5 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>79.4 ± 1.0, 100</td>
</tr>
<tr>
<td>Cd** (µM)</td>
<td>10</td>
<td>3 ± 2.9, 23.0 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>31.5 ± 0.9, 60.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>68.3 ± 2.2, 82.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>84.4 ± 0.8, 100</td>
</tr>
<tr>
<td>H2O2 (%)</td>
<td>0.01</td>
<td>12.6 ± 2.3, 27.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>39.1 ± 3.1, 62.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>50.9 ± 1.9, 97.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>61.2 ± 0.9, 100</td>
</tr>
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</table>

*The radius of colonies was measured after incubating at 25°C for 7 days. Reduction in the growth rate was estimated by comparing colonial growth on medium with each treatment with that of PH-1 and the \( \Delta cid1 \) mutant on regular YPEG medium. Means and standard deviation were calculated from three replicates.
2001), we also assayed conidiation and DON production in Δcid1 cultures buffered at different pH. In *F. graminearum*, acidic pH appeared to have an inhibitory effect on conidiation (Table 3). Both the wild-type and Δcid1 mutant strains produced fewer conidia at pH 5 than at pH 7. At pH 3, conidiation was completely blocked (Table 3). Similar to what was reported recently (Gardiner et al., 2009b), we noticed that acidic pH had a stimulatory effect on DON production in *F. graminearum*. In both the wild-type and Δcid1 mutant strains, DON production was increased about two folds in cultures grown at pH 3 in comparison with those grown at pH 7 (Table 3). These results indicate that acidic pH failed to rescue the defects caused by deletion of the CID1 gene in *F. graminearum*.

3.9. CID1 functionally complements the *F. verticillioides* Δfcc1 mutant

To determine its function in *F. verticillioides*, we transformed the CID1 gene (under the control of its native promoter) into the Δfcc1 mutant FT536 (Shim and Woloshuk, 2001). Geneticin-resistant transformants were isolated and confirmed by PCR for the transforming CID1 gene. Unlike the original Δfcc1 mutant, the Δfcc1/CID1 transformant grew as fast as the wild-type strain on V8 agar plates (Fig. 7A). It also produced similar amount of conidia as the wild-type strain (Fig. 7B). While the Δfcc1 mutant produced 80% less FB1 fumonisin than the wild-type strain, the Δfcc1/CID1 transformant was normal in FB1 production (Fig. 7C), indicating that when expressed in *F. verticillioides*, the CID1 gene fully complemented the Δfcc1 mutant. It is likely that these two genes are functionally conserved between *F. graminearum* and *F. verticillioides*, two closely related species that produce different mycotoxins and cause different diseases.

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH</th>
<th>Conidiation (&lt;i&gt;5 × 10⁴&lt;/i&gt; conidia/ml)</th>
<th>DON (ppm)</th>
</tr>
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<tbody>
<tr>
<td>PH-1</td>
<td>3</td>
<td>0</td>
<td>2950 ± 58</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>112.0 ± 0.9</td>
<td>1276 ± 34</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>138.0 ± 0.7</td>
<td>1585 ± 67</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>100.0 ± 0.3</td>
<td>1124 ± 23</td>
</tr>
<tr>
<td>Δcid1</td>
<td>3</td>
<td>0</td>
<td>856 ± 24</td>
</tr>
<tr>
<td>Mutant</td>
<td>5</td>
<td>28.0 ± 1.5</td>
<td>356 ± 31</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>56.0 ± 1.2</td>
<td>418 ± 16</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>49.0 ± 0.6</td>
<td>165 ± 13</td>
</tr>
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</table>

Means and standard errors were calculated from three independent replicates.

### Table 4

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′ – 3′)</th>
</tr>
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<tbody>
<tr>
<td>CIDI-F1</td>
<td>GCTTTAACTGCGCGGAGGACC</td>
</tr>
<tr>
<td>CIDI-R2</td>
<td>GACCTCCTAAGCTTCCAGCCAAGCCGCTACTGTCGACAGACATCGTGCTG</td>
</tr>
<tr>
<td>CIDI-F3</td>
<td>ATAGAGTAGATGCCGACCG</td>
</tr>
<tr>
<td>CIDI-R4</td>
<td>GCCGCAAGGATGACTGTCG</td>
</tr>
<tr>
<td>CIDI-R5</td>
<td>CACCCATGCCTGCCGCCC</td>
</tr>
<tr>
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<tr>
<td>HYR</td>
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<td>YGF</td>
<td>GATTTAGGAGGCCCAGTATGTCCCT</td>
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<tr>
<td>TRISOF</td>
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</tr>
<tr>
<td>TRISOQ</td>
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<tr>
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<td>TACGCGCAAGCCGAGGAA</td>
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<tr>
<td>PKS12QR</td>
<td>CTACGGCGCATCAGCTGTTT</td>
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<tr>
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<tr>
<td>TubQR</td>
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<tr>
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</tr>
<tr>
<td>TR101QR</td>
<td>TGACTCCTGTCGAGATGAGCT</td>
</tr>
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</table>

Nucleotide sequences from the hygromycin resistance gene were underlined.

4. Discussion

*FCC1* and *CID1* are homologous to the *S. cerevisiae* UME3 gene, which also is known as SSN8 or SRB11. Ume3 and its cyclin-dependent kinase (Cdk) Ume5 are components of the RNA polymerase II holoenzyme and are required for the repression of genes...
involved in stress responses, meiosis, and other metabolic or developmental processes (Ansari et al., 2005; Cohen et al., 2003; Cooper and Strich, 2002). This cyclin-Cdk kinase can modify the general transcriptional machinery by phosphorylation of the carboxy-terminal domain of RNA polymerase II. Deletion of UME3 is not lethal but results in reduced glycogen accumulation and increased sensitivities to various stresses, such as alkaline pH and heavy metals (Cohen et al., 2003; Cooper et al., 1997). The use of UME3 is reduced in diploid filamentous growth, blocked in haplo- loid invasive growth, and has increased telomere length and reduced sporulation (Cooper and Strich, 2002). In addition, it produces small buds with nuclei (budding growth defects) on sporulation medium.

Like UME3 in yeast, CID1 and FCC1 are not essential genes. However, the growth rate of the Δfcc1 and Δcid1 mutants was reduced although no obvious changes in conidium germination and hyphaal branching were observed in the Δcid1 mutant. Because Cid1p is likely a component of the RNA polymerase II holoenzyme, deletion of CID1 may result in a general reduction in transcription. However, the expression of PKS12, TRIS, and PKS1 in the Δcid1 mutant was increased, decreased, and unaltered, respectively. Therefore, the effect of Δcid1 mutation on transcription must be specific for different genes. Deletion of CID1 may result in the derepression of genes that are normally repressed. Abnormal or unbalanced transcription of different genes may disturb normal physiology and reduce fungal growth.

In yeast, the Ume3-Ume5 kinase system is required for entry into and execution of meiosis (Cooper and Strich, 2002). Ume3 is required for the normal exit from the mitotic cell cycle prior to meiotic induction and is degraded before meiosis I. The cid1 mutant was female sterile but it could function as the male. We were able to isolate normal Δcid1 ascospore progeny from a Δcid1 × CID1 cross. Because the Δcid1 mutant is female sterile, it is impossible to test the fertility of the Δcid1 × Δcid1 cross. Therefore, it remains possible that CID1 is important for meiosis in F. graminearum. Loss of female fertility in the Δcid1 mutant may be related to a possible role of CID1 in switching from asexual to sexual reproduction. However, many mutants deleted for genes of unrelated functions are known to be female sterile, such as the mgv1, gpmk1, and ftl1 mutants in F. graminearum (Jenczmonka and Schafer, 2005; Urban et al., 2003; Hou et al., 2002).

Our data indicate that the Δcid1 mutant produced a very low amount of DON on wheat kernels and it was reduced significantly in TRIS expression. In F. verticilloides, the Δfcc1 mutant also was reduced in FB1 fumonisin production when cultured on cracked corn kernels. However, unlike the Δcid1 mutant with detectable TRIS expression, the expression of FUMS was undetectable in the fcc1 mutant (Shim and Woloshuk, 2001). In the Δcid1 mutant, the TRIS101 gene also had a reduced expression level. TRIS101 is not located in the same TRI cluster with TRIS and its expression is not regulated by the pathway-specific transcription factor TRIS6. Therefore, the effect of CID1 deletion on trichothecene synthesis must be at a global or semi-global level. Nevertheless, it is unlikely that deletion of CID1 has a universal effect on secondary metabolism because the Δcid1 mutant had increased expression of PKS12 and pigmentation. Various secondary metabolites may have different biological functions in F. graminearum and are regulated by different mechanisms.

Like the Δfcc1 mutant, the Δcid1 mutant was reduced in conidiation. The Fcc1 orthologs may have a conserved role in filamentous fungi to positively regulate asexual reproduction. Interestingly, conidia formed by the Δcid1 mutant had fewer septa and cell numbers. Conidium germination also was delayed in the Δcid1 mutant. These defects were not described for the Δfcc1 mutant. However, it is worth noting here that unlike most Fusarium species, F. graminearum does not form microconidia. Conidiation was assayed with macroconidia in F. graminearum. For F. verticilloides, microconidia are formed more abundantly than macroconidia. It was microconidia that were examined in this study and the original characterization of the Δfcc1 mutant (Shim and Woloshuk, 2001).

In F. verticilloides, acidic pH could suppress the defects of the Δfcc1 mutant in fumonisin production and conidiation (Shim and Woloshuk, 2001). In contrast, acidic pH had a negative impact on the production of macroconidia in both the wild-type and Δcid1 mutant strains in F. graminearum. No conidia were produced when cultured at pH3 (Table 3). Therefore, the effect of acidic pH on conidiation must be different between these two Fusarium species. In F. graminearum, low pH increases the expression of TRIS and DON production in axenic cultures (Gardiner et al., 2009b). The Δcid1 mutant produced more DON at pH3 than at pH5 or pH7 (Table 3), indicating that it still responded to acidic pH. However, in comparison with the wild-type strain, DON production at lower pH was still significantly reduced in the Δcid1 mutant. Therefore, although acidic pH had a stimulatory effect on DON production, it could not suppress the defect caused by the deletion of CID1. The relationship between CID1 and pH regulation on DON synthesis in F. graminearum must be different from that of FCC1 and acidic pH on fumonisin synthesis in F. verticilloides.

Although its homologs are well conserved in filamentous asco- mycetes, the CID1 gene is the first one to be shown to be important for plant infection. The Δcid1 mutant was significantly reduced in virulence on flowering wheat heads (Fig 3A). While the 30% reduction in growth rate may contribute to the defects in plant infection, other important pathogenicity factors are probably affected in the mutant because its disease index was reduced more than 16-fold. The Δcid1 mutant had a disease index score of 0.5 (near non-pathogenic). In stalk rot assays, it had a 75% reduction in virulence (Fig. 4), which also was much greater than its reduction in growth rate. However, the Δcid1 mutant appeared to be more virulent on corn stalks than on wheat heads. One possible reason is that corn pith mainly consists of dead plant tissues. The mutant may face more effective defense responses on flowering wheat heads than inside corn stalks. In addition, DON is an important virulence factor in F. graminearum and DON production was significantly reduced in the Δcid1 mutant. In F. verticilloides, the Δfcc1 mutant was not examined for its defects in plant infection. While it is possible that the Δfcc1 mutant also is reduced in virulence, fumonisin is not an important virulence factor in F. verticilloides.

In yeast, Ume5 is the Cdk associated with Ume3. The Ume3-Ume5 kinase can bind to and phosphorylate various transcriptional regulators, including Gal4, Gcn4, Ste12, Msn2, Sip4, and Med2, that are involved in different metabolic and developmental processes (Ansari et al., 2005; Hallberg et al., 2004; Larschan and Winston, 2005). In F. verticilloides, Fck1 is the Cdk that is homologous to Ume5 and directly interacts with Fcc1 (Bluhm and Woloshuk, 2006). The F. graminearum genome has the orthologous gene (FGSG_04484) of UME5 and FCK1. It is tempting to speculate that the normal function of Cid1 and the corresponding Cdk is to repress the expression of stage-specific regulatory genes that negatively control sexual reproduction, conidiation, plant infection, and mycotoxin production. Deletion of CID1 may result in the improper expression of these negative regulators and the phenotypes observed in the Δcid1 mutant. Although conidiation and mycotoxin production may be co-regulated, it is unlikely that loss of female fertility and reduced virulence are controlled by the same regulatory mechanism. Therefore, multiple negative regulators may be regulated by CID1 in F. graminearum. The F. graminearum genome contains homologs of many yeast transcription factors regulated by Ume3-Ume5, including Gal4, Gcn4, Ste12, and Msn4. It will be important to characterize their functions and regulation by CID1 in F. graminearum.
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References