Gene Encoding a c-Type Cyclin in *Mycosphaerella graminicola* Is Involved in Aerial Mycelium Formation, Filamentous Growth, Hyphal Swelling, Melanin Biosynthesis, Stress Response, and Pathogenicity

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Mycosphaerella graminicola is an important wheat pathogen causing Septoria tritici blotch. To date, an efficient strategy to control *M. graminicola* has not been developed. More significantly, we have a limited understanding of the molecular mechanisms of *M. graminicola* pathogenicity. In this study, we attempted to characterize an *MCC1*-encoding c-type cyclin, a gene homologous to *FCC1* in *Fusarium verticillioides*. Four independent *MCC1* knock-out mutants were generated via *Agrobacterium tumefaciens*-mediated transformation. All of the *MCC1* mutants showed consistent multiple phenotypes. Significant reductions in radial growth on potato dextrose agar (PDA) were observed in all of the *MCC1* mutants. In addition, *MCC1* gene-deletion mutants produced less aerial mycelium on PDA, showed delayed filamentous growth, had unusual hyphal swellings, produced more melanin, showed an increase in their stress tolerance response, and were reduced significantly in pathogenicity. These results indicate that the *MCC1* gene is involved in multiple signaling pathways, including those involved in pathogenicity in *M. graminicola*.

The ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is one of the most important pathogens on wheat, causing a serious foliar disease known as Septoria tritici blotch (STB). This disease has been reported in most wheat-growing regions around the world, including the United States (Polley and Thomas 1991). When environmental conditions are favorable for the growth of *M. graminicola*, STB can cause significant yield loss of wheat. In spite of the importance

of STB, strategies for efficient management of *M. graminicola* have not been developed. Currently, breeding for resistant cultivars and treatments with fungicides are used to manage STB. However, neither of these methods provides complete control of STB due to rapid evolution of *M. graminicola* populations and frequent sexual recombination. New haplotypes with resistance to fungicides and that can overcome resistant cultivars arise rapidly. Furthermore, recent concerns over environmental issues can restrict fungicide applications and limit the number of compounds available. Therefore, it is necessary to better understand *M. graminicola* biology which, in turn, will help to establish an efficient strategy to manage STB.

Cyclins are a highly conserved family of proteins which are involved in cell cycle progressions via the activation of cyclin-dependent protein kinases (CDK). There are several types of cyclins which control different parts of cell cycles through activation of appropriate CDK partners. Among these cyclins, c-type cyclin is distantly related to the others and is unique in that it is involved in transcriptional regulation (Kuchin et al. 1995; Liao et al. 1995). In *Saccharomyces cerevisiae*, the c-type cyclin protein Ume3p acts as a repressor of gene transcription. In response to appropriate environmental cues such as stress conditions, Ume3p is destroyed at the protein level via ubiquitin-mediated proteolysis. After Ume3p is degraded, the expression of many downstream genes is initiated at the transcriptional level (Cohen et al. 2003; Cooper et al. 1997). Recently, an emerging body of information indicates that c-type cyclin in filamentous fungi is implicated in many different signaling pathways, including fungal development, toxin metabolism, and pathogenicity. In *Fusarium verticillioides*, the *FCC1* gene encoding a c-type cyclin was identified as an important regulator in the biosynthesis of the mycotoxin fumonisin as well as the asexual development of microconidia (Shim and Woloshuk 2001). In *F. graminearum*, the *FCC1*-homologous gene *CID1* is involved in conidiation, vegetative growth, pigmentation, and pathogenicity (Zhou et al. 2010). Therefore, it is obvious that c-type cyclins in *F. verticillioides* and *F. graminearum* are involved in multiple important signaling pathways. However, to our knowledge, except for *FCC1* and *CID1*, no other c-type cyclin gene has been reported in filamentous fungi.

To determine the roles of c-type cyclin in *M. graminicola*, we identified and characterized the *MCC1* gene that is homologous to *F. verticillioides* *FCC1* and *F. graminearum* *CID1*. As with *FCC1* and *CID1*, *MCC1* gene-deletion mutants

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showed pleiotropic effects on growth, filamentous growth rate, hyphal swelling, melanin biosynthesis, stress tolerance, and pathogenicity. Our results indicate that the *MCC1* gene encoding a c-type cyclin in *M. graminicola* impacts multiple signaling pathways, including those for growth, differentiation, secondary metabolism, and pathogenesis.

RESULTS

The *MCC1* gene encodes a c-type cyclin.

We searched for c-type cyclin genes in *M. graminicola* using the sequence of the *F. verticillioides* c-type cyclin, *FCC1* (GenBank accession number AF294431), as query. A putative homolog of *FCC1* was identified in the *M. graminicola* genomic database. This gene was located on *M. graminicola* chromosome 9, specifically at the sequence from bases 1,594,202 to 1,595,191. Analysis of the identified sequence revealed that it had significant similarity to various c-type cyclin genes across different fungal species. For example, the predicted gene shares high similarity with *FIC1* (XP_755914.1) in *Aspergillus fumigatus* (E value, 2e-86), *CID1* (XP_384531.1) in *F. graminearum* (E value, 1e-76), and *FCC1* (AAK30047.1) in *F. verticillioides* (E value, 5e-76). Based on this similarity, we designated the identified c-type cyclin gene in *M. graminicola* as *MCC1* (probable *M. graminicola* c-type cyclin 1). Amino acid alignments of *MCC1* with c-type cyclin

proteins in *F. verticillioides* (*FCC1*) and *F. graminearum* (*CID1*) indicated high similarity in the sequences (Supplementary Fig. 1). Further in silico analysis showed that the *MCC1* gene encodes 327 amino acids with cyclin domains from amino acids 53 to 142 and 155 to 236. An RXXL domain previously shown to be required for self destruction also was found from amino acids 25 to 28.

MCC1 gene-deletion mutants were generated via *Agrobacterium tumefaciens*-mediated transformation.

To test the functional roles of *MCC1* in *M. graminicola*, we generated *MCC1* gene-deletion mutants by *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Mullins et al. 2001). Among 144 hygromycin-resistant transformants, four independent *MCC1* gene-deletion mutants were identified by polymerase chain reaction (PCR) screens with primers *MCC1*-che-F1 and R1. The *MCC1*-che-F1 and R1 primers are located on the upstream and downstream flanking regions of the *MCC1* open reading frame (ORF) and the resultant *MCC1* gene-deletion mutants should have a gene-replacement event substituting the entire *MCC1* ORF with a hygromycin phosphotransferase (*HPH*) gene (Supplementary Fig. 2a). Based on that, we identified gene-deletion mutants of *MCC1* by the size of PCR reaction amplicons. The wild-type and ectopic control strains had a 1.3-kb band originating from the *MCC1* ORF, whereas *MCC1* gene-deletion mutants had instead a 1.6-kb band from the *HPH* gene. Transformation was confirmed with another set of primers, *MCC1*-neg-F1 and R1, located within the *MCC1* ORF. The wild-type and ectopic control strains generated a 0.7-kb band from the *MCC1* ORF, whereas *MCC1* gene-disruption strains did not produce detectable PCR amplicons, indicating that the gene was absent in the *MCC1* gene-deletion mutants. We designated the four independent mutants as $\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4. We also included an isolate containing the DMC1 construct integrated elsewhere in the genome as an ectopic control and designated it "Ect".

The *MCC1* gene-deletion mutants are defective in growth on agar plates.

We first tested whether *MCC1* gene deletion can cause morphological differences on yeast-sucrose agar (YSA) or potato dextrose agar (PDA) plates. Four independent *MCC1* gene-disruption strains showed consistent differences in growth and produced darker pigmentation compared with the wild-type progenitor (Fig. 1A). Regardless of the incubation temperature, whether 18°C or room temperature, we observed the same phenotypic differences between the wild type and $\Delta mcc1$ mutants (data not shown). We observed consistent growth reduction in the four independent *MCC1* gene-deletion mutants compared with the wild-type and ectopic control strains. Measurement of the radial growth of *M. graminicola* strains on PDA plates revealed that $\Delta mcc1$ mutants grew at approximately 50% the rate of the wild-type progenitor and the ectopic control strain (Fig. 1B). From these data, we concluded that *MCC1* in *M. graminicola* affects radial growth on agar plates in a positive manner.

The *MCC1* gene-deletion mutants are defective in formation of aerial mycelium on agar plates.

When we observed the growth patterns on PDA under a dissecting microscope, we noticed remarkable differences between the wild-type or ectopic control strains and $\Delta mcc1$ mutants. The wild-type and ectopic control strains developed long aerial mycelia, whereas $\Delta mcc1$ mutants produced limited aerial mycelia (Fig. 2). These phenotypic differences appeared consistently across four independent *MCC1* gene-disruption mutants ($\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4). The

Fig. 1. Morphology and radial growth on agar plates analysis. **A**, Growth morphology on agar plates was tested with the wild type (WT), ectopic transformant (Ect), and four independent *MCC1* gene-deletion mutants. All strains were grown on potato dextrose agar (PDA) or yeast-sucrose agar (YSA) for 20 days. Growth was reduced and dark pigmentation was significantly increased in four *MCC1* mutant isolates ($\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4). **B**, Radial growth on PDA was measured as the diameter of colonies and presented as a bar graph. Three biological replications were performed. Thin lines above the bars indicate standard errors.

wild-type and ectopic control strains developed thick, white mycelia. In contrast, aerial mycelium was almost nondetectable in $\Delta mcc1$ mutants (Fig. 2). We extracted aerial mycelium with 0.15% Tween 20 and quantified its amount with a hemacytometer. The $\Delta mcc1$ mutants produced approximately less than 10% of the aerial mycelium of the wild-type or ectopic control strains (data not shown). Our data clearly indicated that *MCC1* influences the formation of aerial mycelium in *M. graminicola* in a positive manner.

***MCC1* gene deletion resulted in a significant increase in melanin biosynthesis.**

Deletion of the *MCC1* gene resulted in increased dark pigmentation compared with the wild-type progenitor, which

showed a lack of dark pigment (Figs. 1A and 3A). Melanin is commonly responsible for the dark pigmentation in filamentous fungi. We followed an extraction and measurement protocol specifically designed for melanin (Babitskaya et al. 2000) to estimate the amounts of melanin produced by each strain with three biological replications on agar plates (YSA), as well as in liquid medium (yeast-sucrose broth [YSB]). We observed more than a 15-fold increase in melanin production in $\Delta mcc1$ mutants ($\Delta mcc1-1$, $\Delta mcc1-2$, $\Delta mcc1-3$, and $\Delta mcc1-4$) on YSA culture when compared with the wild-type progenitor or ectopic control (Fig. 3B). In addition, we also observed an approximately sevenfold increase in production of melanin in four independent *MCC1* disruption mutants in YSB (Fig. 3B). Increased melanin production in $\Delta mcc1$ mutants in YSB was

Fig. 2. Aerial mycelia in the *MCC1* mutants. **A**, Aerial mycelium was photographed with a dissecting microscope. All strains were grown on potato dextrose agar after 20 days at room temperature or at 18°C (indicated on the left). In the wild type (WT) and ectopic transformant (Ect), abundant aerial mycelia were present. However, aerial mycelium was rarely observed in four independent *MCC1* mutants.

Fig. 3. Assays for melanin production. **A**, Yeast-sucrose broth (YSB) cultures of the wild type (WT), an ectopic transformant (Ect), and four independent *MCC1* mutants ($\Delta mcc1-1$, $\Delta mcc1-2$, $\Delta mcc1-3$, and $\Delta mcc1-4$) were photographed after incubation for 30 days. Black pigmentation was observed in *MCC1* gene-deletion mutants. **B**, Measurements of melanin production in the WT, Ect, and four *MCC1* gene-deletion mutants grown on yeast-sucrose agar for 30 days. Data from three independent replicates were analyzed to calculate means and standard errors, which are indicated by thin lines above the bars. **C**, Melanin production measurement in the WT, Ect, and four *MCC1* gene-deletion mutants grown on YSB for 30 days. Data from three independent replicates were analyzed to calculate means and standard errors, indicated by thin lines above the bars.

verified in microscopic images (Fig. 3A). Our results clearly indicate that the *MCC1* gene in *M. graminicola* is associated negatively with melanin biosynthesis.

***MCC1* gene deletion resulted in defects in filamentous growth.**

Interestingly, we noticed significant defects of filamentous growth in the four independent $\Delta mcc1$ mutants. Observation under the dissecting microscope clearly revealed that the full development of filamentous hyphae in $\Delta mcc1$ mutants was lacking compared with the wild-type or ectopic controls. The wild-type and ectopic controls had long, well-developed hyphae extending to the margin of the colonies, whereas four $\Delta mcc1$ mutants produced short, aggregated hyphae and very restricted vegetative growth (Fig. 4A). These phenotypic characteristics were independent of growth temperature (Fig. 4A).

To confirm our observation, we tested germination morphology from single, isolated spores of the wild type, ectopic control, and four independent $\Delta mcc1$ mutants. After the same time

period under identical environmental conditions, we observed consistent vegetative germination defects in all four *MCC1* gene-deletion mutants compared with the wild type or ectopic controls (Fig. 4B). As before, the wild type and ectopic controls produced elongated filaments. In contrast, all four *MCC1* gene-deletion mutants produced compact, small filaments with limited development (Fig. 4B). These vegetative germination defects in $\Delta mcc1$ mutants appeared consistently in every single cell (more than 200) examined on water agar (WA) (data not shown). Measurement of vegetative growth also demonstrated that growth of $\Delta mcc1$ mutants was reduced 60% on WA compared with that of the wild type (Fig. 4C). Our results indicate that *MCC1* plays a critical role in vegetative filamentous growth of *M. graminicola*.

***MCC1* gene deletion resulted in hyphal swellings.**

In contrast to the wild type and ectopic controls, the *MCC1* gene-deletion mutants ($\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4) developed distinguishable hyphal swelling pheno-

Fig. 4. Filamentous growth assays. **A**, Filamentous growth was photographed with a dissecting microscope. All strains were grown on potato dextrose agar (PDA) for 20 days at room temperature or at 18°C (indicated on the left). In the wild type (WT) and ectopic transformant (Ect) controls, invasive filamentous growth was present. However, *MCC1* mutants ($\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4) were defective in filamentous growth, regardless of temperature. **B**, Defects in germination from a single cell in *MCC1* mutants. Single cells of all strains were isolated and spread on water agar. After 20 days of incubation at room temperature, germination patterns were photographed with a dissecting microscope. **C**, Germination growth in B was measured and presented as a bar graph. Results are means of three biological replications. Standard errors are indicated by thin lines above the bars; those for mutants $\Delta mcc1$ -1, $\Delta mcc1$ -2 and $\Delta mcc1$ -3 are too small to be visible at this scale.

types when grown in liquid media (Fig. 5). These phenotypes in $\Delta mcc1$ mutants became apparent in the late growth stages (data not shown). The wild-type and ectopic control strains were capable of maintaining a filamentous growth pattern and similar size of cells even during the late stages, whereas $\Delta mcc1$ mutants could not. Compared with the wild type and ectopic controls, all of the $MCC1$ gene-deletion mutants had consistent, abnormal balloon-shaped hyphae, which did not sustain a filamentous growth pattern after a certain time point (Fig. 5). Our results provide evidence that $MCC1$ in *M. graminicola* is involved in suppressing hyphal swelling or maintaining hyphal growth in late-stage cells.

Disruption of $MCC1$ increases stress tolerance to reactive oxygen species.

Because the gene encoding c-type cyclin in *F. graminearum* is known to be involved in stress responses (Zhou et al. 2010), we assayed the effect of reactive oxygen species (ROS) on $\Delta mcc1$ mutants as described previously (Choi and Xu 2010). When grown on WA plates supplemented with 400 mM concentrations of H_2O_2 , growth of the wild-type and ectopic control strains was negligible and they did not survive (Fig. 6). However, all $MCC1$ gene-deletion mutants survived on WA containing 400 mM H_2O_2 , indicating that they had an increased tolerance to ROS. We also measured and quantified the relative growth of each strain by dividing its growth on media containing H_2O_2 by that on control media. The $\Delta mcc1$ mutants had approximately 5- to 10- fold increases in growth on media containing H_2O_2 compared with the wild-type or ectopic control strains. These results support the hypothesis that $MCC1$ in *M. graminicola* is associated with ROS stress-response signaling in a negative manner.

$MCC1$ disruption significantly reduces pathogenicity.

To determine the effect of $MCC1$ deletion on virulence of *M. graminicola*, two infection assays were performed on the highly susceptible wheat 'Taichung 29'. Wheat seedlings at the three- to five-leaf stage were inoculated separately with 10^7 spores of each strain. Both stem and spray inoculations were performed and plants were incubated until the appearance of STB disease symptoms, such as pycnidia indicated by black spots within tan lesions. We obtained consistent results from all four independent $MCC1$ mutants; STB caused by $MCC1$

mutants was significantly limited, compared with that caused by the wild-type and ectopic control strains (Fig. 7A and B). Wild-type and ectopic control strains were capable of successfully colonizing the leaves of Taichung 29 wheat, showing the typical black spots of pycnidia, and these symptoms were spread across the wheat leaves. However, we observed significant reductions in the progression of STB in four $\Delta mcc1$ strains (Fig. 7A and B). STB symptoms from $MCC1$ gene-deletion strains were barely detectable or even nondetectable, and these defects in pathogenicity were consistent, regardless of inoculation method (Fig. 7A and B). Taken together, we concluded that the $MCC1$ gene in *M. graminicola* is associated with pathogenicity and virulence on wheat.

DISCUSSION

Cyclins are a group of well-conserved proteins which are crucial for the progressions of cell cycles and are important determinants of cell fates (Fung and Poon 2005). Cyclins act by forming complexes with CDK, and these cyclin-CDK complexes direct the activation of cell cycle progressions. Several different types of cyclins and matching CDK are known, tightly regulating different phases of cell cycle transitions. To do so, the concentrations of cyclins oscillate at the protein level throughout the cell cycle which, in turn, leads to cell cycle progressions (Ekholm and Reed 2000; Morgan 1995; Sherr 1993).

Of the different cyclins, c-type cyclin has distinct features because it is a component of RNA polymerase II involved in transcriptional regulation (Liao et al. 1995). The carboxy-terminal domain (CTD) of RNA polymerase II has been implicated in transcriptional regulation (Dahmus 1996). In *S. cerevisiae*, c-type cyclin Srb11p (Ume3p) and a CDK, Srb10p complex, is physically associated with the CTD of RNA polymerase II. The Srb11p-Srb10p pair phosphorylates CTD accordingly, allowing transcription of many downstream genes to be activated or repressed (Liao et al. 1995). In addition, unlike the other cyclins, protein concentration of c-type cyclin does not fluctuate throughout the cell cycle (Cooper et al. 1997; Molz and Beach 1993).

Thus far, most of the information obtained on c-type cyclin originated from yeast, *Drosophila* spp., and human oncogenes. However, recent research has begun to reveal interesting functional roles of c-type cyclin in filamentous fungi. Emerging evidence from *Fusarium* spp. suggested that c-type cyclins

Fig. 5. Hyphal swelling phenotype associated with $MCC1$ mutation in *Mycosphaerella graminicola*. Each strain was inoculated in defined liquid medium with the same amount of conidia (1×10^5 fungal cells ml^{-1}). After 30 days of incubation at $18^\circ C$, photos were taken under the microscope. WT: wild type; Ect: ectopic transformant control; $\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4: four independent $MCC1$ mutants.

Fig. 6. Increased H_2O_2 tolerance in $\Delta mcc1$ mutants. Relative growth of each strain was calculated by comparing its radial growth on potato dextrose agar plates with 400-mM concentrations of H_2O_2 to those on plates with no H_2O_2 . Wild type (WT) and ectopic transformant (Ect) control strains could not survive on 400 mM H_2O_2 , whereas all of the $\Delta mcc1$ mutants ($\Delta mcc1$ -1, $\Delta mcc1$ -2, $\Delta mcc1$ -3, and $\Delta mcc1$ -4) were able to survive and grow.

have fundamental roles in fungal development, production of secondary metabolites, and pathogenicity (Shim and Woloshuk 2001; Zhou et al. 2010). Nevertheless, we have limited knowledge about c-type cyclin, because only two c-type cyclin genes, *FCC1* in *F. verticillioides* and *CID1* in *F. graminearum*, have been characterized in filamentous fungi.

In this study, we attempted to characterize the c-type cyclin gene, *MCC1*, in *M. graminicola*. As with other c-type cyclin genes in different species, *MCC1* gene-deletion mutants were not lethal. Moreover, we identified multiple phenotypes in *MCC1* mutants, indicating that *MCC1* is involved in multiple signaling in *M. graminicola*. Four independent $\Delta mcc1$ mutants were generated and exhibited consistent phenotypes, suggesting that *MCC1* is truly associated with all of the phenotypes observed in this study.

Deletion of the *MCC1* gene led to phenotypes that were very similar to those of the *FCC1* and *CID1* gene deletions (Shim and Woloshuk 2001; Zhou et al. 2010). Phylogenetically, *M. graminicola* is placed in the order Capnodiales (Schoch et al. 2006; Torriani et al. 2008), distantly related to the order Hypocreales, in which *Fusarium* spp. are placed (Goodwin 2004; Schoch et al. 2006). In spite of the evolutionary divergence between the Hypocreales and Capnodiales, we observed very similar consequences of c-type cyclin gene deletions. Moreover,

amino acid sequence analysis of *MCC1*, *FCC1*, and *CID1* demonstrated that 78% of Mcc1p amino acids were either identical or similar to those of Fcc1p and Cid1p. Therefore, we propose that functional roles of c-type cyclin genes in filamentous fungi are well conserved across many divergent fungal species.

Deletion of the *MCC1* gene resulted in significant reduction in growth, which also was reported in *Fusarium* spp. In *Fusarium* spp., deletion of the c-type cyclin gene led to an approximately 25% reduction in growth (Shim and Woloshuk 2001; Zhou et al. 2010), whereas approximately 50% reduction occurred in *M. graminicola* (Fig. 1B). When we searched for putative homologs of c-type cyclin genes, we found two and three copies in the genomes of *F. verticillioides* and *F. graminearum*, respectively. However, only one putative homolog of a c-type cyclin gene was identified in the genome of *M. graminicola* (data not shown). Therefore, the absence of additional redundant c-type cyclin genes in the genome of *M. graminicola* may explain the more severe growth defects in *MCC1* deletion mutants compared with those in knock-out strains of homologous genes of *Fusarium* spp.

We also observed defects in filamentous growth in $\Delta mcc1$ mutants, with a phenotype of short and condensed germination tubes (Fig. 4). The *CID1* mutant in *F. graminearum* also

Fig. 7. Effect of *MCC1* gene deletion on pathogenicity. **A**, Stem infection assays. Sheaths of the susceptible wheat ‘Taichung 29’ were punctured with a sterile needle and infected with 3×10^7 spores of the wild type (WT), an ectopic transformant (Ect) control strain, and four independent *MCC1* gene-deletion mutants ($\Delta mcc1-1$, $\Delta mcc1-2$, $\Delta mcc1-3$, and $\Delta mcc1-4$). Infected wheat plants were incubated in a Purdue University greenhouse facility for 21 days. Stem infection assays were performed with at least three biological replications for each fungal isolate. Symptoms are visible only near the sites of inoculation. **B**, Spray infection assays. In all, 10^8 spores of WT, Ect, and four independent *MCC1* gene-deletion mutants ($\Delta mcc1-1$, $\Delta mcc1-2$, $\Delta mcc1-3$, and $\Delta mcc1-4$) were spray inoculated onto seedling leaves of highly susceptible Taichung 29 wheat. After inoculation, plants were placed in the dark for 48 h under high humidity. Subsequently, the infected wheat plants were incubated in a Purdue University greenhouse facility with cycles of 12 h of light and 12 h of darkness for 21 days. Spray infection assays were performed with at least three biological replications for each fungal isolate.

showed a delay in germ tube elongation (Zhou et al. 2010). Therefore, c-type cyclin in fungi is likely responsible for filamentous growth or hyphal elongation, which are likely to involve similar processes. However, because c-type cyclin null mutants are still able to grow filamentously, even though impaired, there should be one or more additional and indispensable signals for filamentous growth. Presumably, c-type cyclin may be involved in full development of filamentous growth in the late phases of elongating germ tubes. Further study will be necessary to understand the functional role or roles of c-type cyclin in filamentous growth.

Interestingly, we observed hyphal swelling phenotypes in *Δmcc1* mutants (Fig. 5). Previously, Choi and Shim (2008) showed that knock-out mutation of the protein phosphatase gene *CPPI* resulted in hyphal swelling similar to that in *Δmcc1* mutants, and *CPPI* was identified initially as a downstream gene of c-type cyclin *FCCI* (Pirttilä et al. 2004; Shim and Woloshuk 2001). Thus, it is likely that *MCC1* is associated with hyphal swelling via a Cpp1p-homologous protein in *M. graminicola*. Although hyphal swelling was not reported in the other studies on the c-type cyclin gene, we believe that hyphal swelling phenotypes probably occur in the other cyclin gene mutants, considering the high conservation of functional roles of c-type cyclins. Additionally, a previous report on deletion mutants of *MgSlr2*, encoding a mitogen-activated protein (MAP) kinase in *M. graminicola*, also showed large swollen cells (Mehrabi et al. 2006a). Further study is required to reveal the molecular relationships between *MCC1* and *MgSlr2*.

We also found that *MCC1* deletion led to significant decreases in development of aerial mycelium (Fig. 2). Previous reports have documented that fungal c-type cyclin influences conidiation positively (Shim and Woloshuk 2001; Zhou et al. 2010). However, in this study, *MCC1* was instead positively associated with development of aerial mycelium. Because fungal conidia develop on the tips of aerial hyphae, it is likely that the conidial defects observed in the *FCCI* and *CID1* gene-deletion mutants also may have originated from lack of aerial mycelium rather than a defect in conidia formation itself. In *A. nidulans*, three genes (*brlA*, *abaA*, and *wetA*) are involved in key regulatory pathways for conidiation, controlling conidia-specific gene expression or activation in a specific sequence (Adams et al. 1998). Of these, *brlA* is responsible for the early development of conidiophore vesicles and regulating transcript accumulation of *abaA* and *wetA* (Boylan et al. 1987; Clutterbuck 1969). Because *MCC1* deletions affect formation of aerial mycelium, which should take place before the development of conidiophore vesicles, we propose that c-type cyclin signaling in filamentous fungi might be upstream of *brlA*, influencing all of the regulatory genes controlling conidia development.

Another possibility might be found in the relationship between c-type cyclin and hydrophobins. For many fungal species, secreted proteins known as hydrophobins enable aerial hyphae to develop by decreasing the water surface tension (Wösten and Willey 2000). Thus, the relationship between the hydrophobins and c-type cyclin also should be determined in the future.

We noticed that *Δmcc1* mutants had increased melanin production (Figs. 1A and 3). Previously, c-type cyclin genes in *Fusarium* spp. were associated with mycotoxins or secondary metabolites (Shim and Woloshuk 2001; Zhou et al. 2010). In *M. graminicola*, *MCC1* directs production of another secondary metabolite, melanin, indicating functional conservation of c-type cyclins in the secondary metabolism of fungi. Furthermore, we could not eliminate the possibility that *MCC1* also might be implicated in production of mycotoxins or secondary metabolites as yet unidentified in *M. graminicola*. Evidence from *Fusarium* spp. tells us that the same c-type cyclin gene is implicated in production of several secondary metabolites,

including mycotoxins as well as pigments (Zhou et al. 2010). Based on that, we suspected that it also might be true in *M. graminicola*.

Another phenotype of *Δmcc1* mutants was their increased stress tolerance, indicating that *MCC1* might function as a repressor of stress responses (Fig. 6). In *S. cerevisiae*, the c-type cyclin protein Ume3p functions as a repressor of stress-response gene transcription. To escape from this repression, Ume3p must be destroyed via ubiquitin-mediated proteolysis in response to environmental stresses (Cohen et al. 2003; Cooper et al. 1997). In this regard, it is conceivable that *MCC1* gene deletion activates many downstream stress-response genes which, in turn, lead to more stress tolerance. Interestingly, the *CID1* mutant in *F. graminearum* was defective in stress responses (Zhou et al. 2010). Therefore, c-type cyclin genes in *F. graminearum* and *M. graminicola* seem to have opposite roles in stress responses, although most of the phenotypes observed indicate well-conserved functional roles. In addition, several reports demonstrated that fungal melanin has antioxidant properties (Jacobson and Tinnell 1993; Shcherba et al. 2000). It remains to be determined whether increased melanin biosynthesis in *Δmcc1* mutants is directly related to ROS tolerance.

We also observed significant reductions in pathogenicity in *MCC1* gene-deletion mutants, regardless of inoculation method (Fig. 7A and B). This is consistent with the previous results with the *CID1* mutant, suggesting c-type cyclin involvement in pathogenicity in filamentous fungi (Zhou et al. 2010). Therefore, it will be interesting to elucidate signal-transduction convergence or the relationship of fungal c-type cyclin genes with well-known signaling pathways for pathogenicity such as the MAP kinase or cAMP signaling pathways. It also is noteworthy that there may be a connection between defects in filamentous growth in *MCC1* deletion mutants and pathogenicity.

As with *ume3* in *S. cerevisiae*, c-type cyclin in filamentous fungi supposedly has regulatory roles in gene transcription, including multiple signaling genes to generate the variety of phenotypes observed in this study. However, we still have limited knowledge of genes that act downstream of c-type cyclin. So far, only genes downstream of *FCCI* have been studied extensively via microarray or cDNA-subtraction-library techniques (Pirttilä et al. 2004; Shim and Woloshuk 2001). To better understand *MCC1* regulation on gene transcription, it will be important to investigate genome-wide mRNA transcription with microarrays or other approaches. Furthermore, *MCC1* undoubtedly regulates the phenotypes observed in this study via CDK, similar to the CDK protein Fck1 in *F. verticillioides*, which has a pleiotropic effect on several morphological processes through its interactions with Fck1 (Bluhm and Woloshuk 2006). Further study with *M. graminicola* CDK will be necessary to unravel *MCC1*-mediated signaling pathways.

In summary, we characterized *MCC1*, a c-type cyclin gene in *M. graminicola*, and demonstrated that it plays important roles in multiple phenotypes: development of aerial mycelium, filamentous growth, hyphal swellings, melanin biosynthesis, stress tolerance response, and pathogenicity. Further research will be necessary to elucidate the exact mechanism of each type of c-type cyclin-mediated signaling pathway. Because many of the phenotypes described in this study are consistent with those in previous reports on c-type cyclin mutants in *Fusarium* spp., we concluded that the functional roles of *MCC1*-homologous genes are well conserved across species in the fungal kingdom.

MATERIALS AND METHODS

Fungal strains and culture media.

Highly virulent *M. graminicola* strain IPO323 was used as the wild type throughout this study and was the subject of gene

disruptions. All of the strains used or generated in this study were stored at -80°C after desiccation on strips of Whatman filter paper (Whatman Inc., Piscataway, NJ, U.S.A.) overnight in a lyophilizer. Cultures grown in YSB (10 g each of sucrose and yeast extract [Difco Laboratories, Detroit] per liter of distilled water) were used for genomic DNA extraction or ATMT. For phenotyping, we used various agar media: YSA (YSB with 15 g of agar per liter); PDA (4%; Difco Laboratories); and WA (15 g of agar per liter). For hyphal swelling studies, defined liquid medium consisted of 22 mM KH_2PO_4 , 2.5 mM MgSO_4 , 85 mM NaCl , 10 mM NH_4NO_3 , and 117 mM sucrose.

Nucleic acid isolation and manipulation.

All plasmid DNAs used in this study were prepared with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, U.S.A.). The *MCC1* gene knock-out construct, DMC1, was made via double-joint PCR as described previously (Yu et al. 2004). All primers used for PCR are listed in Supplementary Table 1. Primer pairs MCC1-dis-A and MCC1-dis-B and MCC1-dis-C and MCC1-dis-D were used to amplify flanking regions of approximately 1 kb around the *MCC1* gene from *M. graminicola* genomic DNA. Simultaneously, an *HPH* gene was amplified from plasmid vector pBHT2 (Mullins et al. 2001) with HPH-F and HPH-R primers using AccuSure DNA Polymerase, which has proof-reading activity (Bioline, Taunton, MA, U.S.A.). Subsequently, the three amplicons were mixed in a single tube in a 1:3:1 (5' fragment: marker: 3' fragment) molar ratio and joined by PCR without using primers as described previously (Yu et al. 2004). Finally, nested primers MCC1-nest-F and MCC1-nest-R were used to amplify the 3.5-kb DMC1 construct. Primers MCC1-nest-F and MCC1-nest-R contain recognition sites for the restriction enzymes *EcoRI* and *KpnI*, respectively. With these enzyme sites, the DMC1 construct was cloned into pBHT2 vector (Mullins et al. 2001) for ATMT. The created vector, designated pMC1, was transformed into competent cells of *Agrobacterium* strain LBA1100 by electroporation following the standard protocols for further use of ATMT.

ATMT.

An *Agrobacterium* strain containing the *MCC1* knock-out ATMT construct, pMC1, was used for transformation into *M. graminicola*. Cells for gene disruption were grown in YSB at 18°C for 2 weeks in an orbital incubated shaker. ATMT was carried out using fungal spores adjusted to 1×10^7 spores/ml by essentially following the protocol described previously (Zwiers and de Waard 2001). PDA containing 100 μM hygromycin and 200 μM cefotaxime was used for the selective medium. After 2 to 3 weeks, hygromycin-resistant colonies appeared on selective medium and every isolated colony was transferred for subsequent subculture on selective medium for further verification of DMC1 incorporation into the genome.

Single-spore isolations and germination study.

We isolated single spores of *M. graminicola* by spreading extremely low concentrations of fungal spores (fewer than 50 spores/plate) on WA. Hemacytometer counts were used for adjustments of fungal spore concentration and serial dilutions were performed to obtain the expected concentration of fungal spores of each strain. After incubating at room temperature for 20 days, shapes of the germination were investigated. Three or more biological replications were performed.

Microscopy.

Microscopic observations were made with either a dissecting or compound microscope (Olympus America Inc., Melville, NY, U.S.A.). For images of aerial mycelia and filamentous

growth, we used dissecting microscope model SZX12 and Qcapture software. Analysis of hyphal growth phenotypes was performed using DP controller software and an Olympus BX41 microscope equipped with a DP70 camera.

Melanin assays.

Melanin was extracted from total mycelium grown on YSA and YSB at room temperature for 30 days. Fungal strains grown in 5 ml of YSB in 14-ml polypropylene tubes (BD Biosciences, San Jose, CA, U.S.A.) were used for melanin analysis. Melanin was extracted following the protocol described previously (Babitskaya et al. 2000). Briefly, melanin was extracted with 2% NaOH at 100°C for 2 h in a water bath. The cooled extracts were acidified to pH 2.0 with HCl, precipitating the melanin pigments. Subsequently, extracts were centrifuged to separate melanin from extract solution and the resulting pellets were dissolved in 2% NaOH. The absorbance of the extract was measured spectrophotometrically at a wave length of 459 nm and the amount of melanin was determined by a calibration curve plotted from the results of a correlation between melanin dry weight and absorbance. All assays were replicated at least three times.

Stress tolerance assays.

To assay the responses to oxidative stress, identical 0.2-cm-diameter agar blocks excised from actively growing cultures were inoculated onto PDA (4%; Difco Laboratories) plates containing 100, 200, or 400 mM H_2O_2 . Radial growth was analyzed after incubation at room temperature for 21 days. All assays were repeated at least three times.

Virulence assays.

The susceptible wheat line Taichung 29 was inoculated by either stem or spray inoculations. Briefly, we grew Taichung 29 in a Purdue University greenhouse facility until the wheat seedlings reached the three- to five-leaf stage. Fungal inoculum was prepared from 2-week-old cultures of *M. graminicola* grown in YSB at room temperature on an orbital shaker adjusted to 120 rpm. For the stem-infection assay, wheat stems were punctured with a sterile needle and 3×10^7 spores of each strain were inoculated into the wound. For spray inoculations, we followed the protocol described previously (Mehrabi et al. 2006a and b). Wheat at the above-mentioned stage was inoculated by spraying 10^7 spores suspended in water per pot. After inoculation, wheat pots were placed in the dark for 48 h while tightly maintaining full humidity. Subsequently, infected wheat plants were incubated in cycles of 12 h of light and 12 h of darkness. After 21 days, when unmistakable STB disease symptoms of clear black pycnidia spots appeared, we analyzed the extent of the infections. All assays were repeated at least three times.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Joint Genome Institute *M. graminicola* genomic database:
genome.jgi-psf.org/Mycgr3/Mycgr3.home.html
 SMART (Simple Modular Architecture Research Tool) database:
smart.embl-heidelberg.de