A Chitin Synthase with a Myosin-Like Motor Domain Is Essential for Hyphal Growth, Appressorium Differentiation, and Pathogenicity of the Maize Anthracnose Fungus Colletotrichum graminicola

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Chitin synthesis contributes to cell wall biogenesis and is essential for invasion of solid substrata and pathogenicity of filamentous fungi. In contrast to yeasts, filamentous fungi contain up to 10 chitin synthases (CHS), which might reflect overlapping functions and indicate their complex lifestyle. Previous studies have shown that a class VI CHS of the maize anthracnose fungus Colletotrichum graminicola is essential for cell wall synthesis of conidia and vegetative hyphae. Here, we report on cloning and characterization of three additional CHS genes, CgChsI, CgChsIII, and CgChsV, encoding class I, III, and V CHS, respectively. All CHS genes are expressed during vegetative and pathogenic development. ΔCgChsI and ΔCgChsIII mutants did not differ significantly from the wild-type isolate with respect to hyphal growth and pathogenicity. In contrast, null mutants in the CgChsV gene, which encodes a CHS with an N-terminal myosin-like motor domain, are strongly impaired in vegetative growth and pathogenicity. Even in osmotically stabilized media, vegetative hyphae of ΔCgChsV mutants exhibited large balloon-like swellings, appressorial walls appeared to disintegrate during maturation, and infection cells were nonfunctional. Surprisingly, ΔCgChsV mutants were able to form dome-shaped hyphopodia that exerted force and showed host cell wall penetration rates comparable with the wild type. However, infection hyphae that formed within the plant cells exhibited severe swellings and were not able to proceed with plant colonization efficiently. Consequently, ΔCgChsV mutants did not develop macroscopically visible anthracnose disease symptoms and, thus, were nonpathogenic.

Additional keywords: force measurement, fungal cell wall.


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Chitin, the β-1,4-linked polymer of N-acetylglucosamine, represents a major component of the cell walls of higher fungi and is essential for hyphal growth and rigidity (Bartnicki-Garcia 1968; Cabib et al. 1996). After trans-membrane polymerization of chitin by membrane-localized glycosyl transferases called chitin synthases (CHS), chitin strands are covalently linked with β-glucan to contribute to a network of four structural cell wall components (i.e., chitin, β-1,3- and β-1,6-glucan, and mannoprotein) (Kollar et al. 1997; Sietsma and Wessels 1994; Wessels 1993).

The yeast Saccharomyces cerevisiae contains three CHS with distinct cell cycle functions in cell wall expansion, septum formation, and budding (Cabib et al. 1996, 2001). In comparison, the genome of filamentous fungi may contain as many as 10 CHS genes (Miyazaki and Ootaki 1997), grouped in six classes, with CHS of classes III, V, and VI being typical for filamentous fungi (Munro and Gow 2001). Because certain CHS are active during defined developmental stages of Aspergillus nidulans (Lee et al. 2004), one may argue that individual enzymes are required during different stages of complex life cycles and to adapt to different ecological niches (Ruiz-Herrera et al. 2002). Alternatively, different CHS may concertedly contribute to certain processes in vegetative or pathogenic development, with overlapping roles (Roncero 2002). This view is supported by the fact that mutations in single CHS genes often do not cause obvious phenotypes (Motoyama et al. 1994, 1997; Specht et al. 1996), but double and triple mutations result in drastic cell wall defects (Fujisawa et al. 2000; Ichinomiya et al. 2002; Motoyama et al. 1997; Shaw et al. 1991; Wang et al. 2001). With the exceptions of the maize smut fungus Ustilago maydis, the vascular wilt fungus Fusarium oxysporum, and the gray mold fungus Botrytis cinerea, the role of individual CHS in the infection process of plant-pathogenic fungi has not been studied (Madrid et al. 2003; Soulis et al. 2006; Weber et al. 2006). These detailed investigations revealed essential roles of class V CHS (Madrid et al. 2003; Weber et al. 2006), suggesting that these myosin-like CHS are of particular importance for virulence of fungal pathogens.

Colletotrichum graminicola, the causal agent of maize leaf anthracnose and stalk rot, is a severe pathogen that caused considerable yield losses throughout the north-central and eastern United States (Bergstrom and Nicholson 1999; Warren et al. 1973). Differentiation of an appressorium is essential for invasion and penetration of the host, followed by formation of a melanized conidium. After germination, the hyphal tip forms an appressorium and exhibits a persistent appressorial wall with contractile activity that is crucial for infection (Sugui et al. 2004). During differentiation, hyphal tip appressoria and infections cells exhibit strong appressorial wall disorganization and disintegration as evidenced by electron microscopy (Sugui et al. 2004). Previous studies have shown that a class VI CHS of the maize anthracnose fungus Colletotrichum graminicola is essential for cell wall synthesis of conidia and vegetative hyphae. Here, we report on cloning and characterization of three additional CHS genes, CgChsI, CgChsIII, and CgChsV, encoding class I, III, and V CHS, respectively. All CHS genes are expressed during vegetative and pathogenic development. ΔCgChsI and ΔCgChsIII mutants did not differ significantly from the wild-type isolate with respect to hyphal growth and pathogenicity. In contrast, null mutants in the CgChsV gene, which encodes a CHS with an N-terminal myosin-like motor domain, are strongly impaired in vegetative growth and pathogenicity. Even in osmotically stabilized media, vegetative hyphae of ΔCgChsV mutants exhibited large balloon-like swellings, appressorial walls appeared to disintegrate during maturation, and infection cells were nonfunctional. Surprisingly, ΔCgChsV mutants were able to form dome-shaped hyphopodia that exerted force and showed host cell wall penetration rates comparable with the wild type. However, infection hyphae that formed within the plant cells exhibited severe swellings and were not able to proceed with plant colonization efficiently. Consequently, ΔCgChsV mutants did not develop macroscopically visible anthracnose disease symptoms and, thus, were nonpathogenic.

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fection of intact host tissue (Deising et al. 2000; Mendgen and Deising 1993; Mendgen et al. 1996). During maturation of appressoria of Colletotrichum spp., melanin is incorporated into the cell wall and osmotically active compounds are synthesized to high concentrations. Measurements with optical waveguides indicated that appressoria of C. graminicola generate considerable turgor pressure, which is translated into force that is exerted onto an epidermal cell in order to penetrate the host (Bastmeyer et al. 2002; Bechinger et al. 1999). Conceivably, rigid fungal cell walls are thought to be essential to support the high osmotic pressure and direct force during the infection process. Moreover, invasive growth of fungal hyphae in planta requires cell wall synthesis, which further suggests that wall-synthesizing enzymes are of high importance for fungal virulence. Random mutagenesis experiments allowed identifying a mutant of C. graminicola with conidia that burst and hyphal apices that swell in media with low osmotic potential (Epstein et al. 1998). According to recent classification (Munro and Gow 2001), the tagged gene encodes a class VI CHS, which is responsible for synthesis of approximately 30% of the chitin in conidial cell walls (Amnuaykanjanasin and Epstein 2003).

In this article, we report on genetic dissection of chitin synthesis in C. graminicola and show that CgChsV, a chitin synthase with a myosin-like motor domain, is essential for hyphal growth, infection structure differentiation, and pathogenicity.

RESULTS

Chitin synthesis is required for hyphal morphology, appressorium formation, and pathogenicity of C. graminicola.

Chitin represents a major cell wall polymer in filamentous fungi and is synthesized at the apex of a growing hypha. To localize chitin, vegetative hyphae of C. graminicola were stained with the tetramethylrhodamine isothiocyanate (TRITC)-conjugated chitin-specific lectin wheat germ agglutinin (WGA) (Fig. 1). Hyphae growing with few branches (Fig. 1A) as well as highly branched hyphae (Fig. 1B) showed strongly fluorescing apices (arrows), indicating chitin biosynthesis and deposition of newly formed chitin at hyphal tips. However, some apices were not decorated by WGA (compare hyphae marked by arrowheads in fluorescence images of Figure 1A and B), suggesting either that these hyphae were not actively growing or that the chitin was not accessible to the agglutinin, due to modification or masking of the polymer.

To investigate the importance of chitin for rigidity and functionality of cell walls of the maize pathogen C. graminicola, saprophytic and pathogenic development of the fungus was assessed in the presence of the competitive CHS inhibitor nikkomycin Z (Fig. 2). In liquid media, branched vegetative hyphae developed (Fig. 2A, left image). When grown in medium containing 50 μM nikkomycin Z, large hyphal swellings filled with vacuoles occurred along hyphae at irregular intervals (Fig. 2A, middle image), suggesting that the inhibition of CHS resulted in cell wall defects that were unevenly distributed along the hypha. Occasionally, swellings were closely associated and formed chains of protrusions (Fig. 2A, right image). These observations clearly indicate that chitin represents an essential structural polymer of vegetative hyphae. Next, we assessed the importance of chitin synthesis in infection structures differentiated in vitro and for infection competence on maize leaves (Fig. 2B through D). On hydrophobic hard surfaces such as polyester, conidia germinate, form a short germ tube, and differentiate in vitro and for infection competence on maize leaves (Fig. 2B through D). On hydrophobic hard surfaces such as polyester, conidia germinate, form a short germ tube, and differentiate in vitro and for infection competence on maize leaves (Fig. 2B through D).

Because differentiation of functional appressoria is essential for invasion of host leaves, we anticipated that inhibition of chitin synthesis would affect not only infection-related morphogenesis but also infection rates and disease symptom de-

Fig. 1. Localization of chitin by labeling with wheat germ agglutinin (WGA). A, Newly synthesized chitin localized at the apices of rapidly growing hyphae was stained with tetramethylrhodamine isothiocyanate-labeled WGA (right image, arrow). Some hyphae did not show apical fluorescence (compare arrowheads in left differential interference contrast (DIC) and right fluorescence images). B, Localization of chitin in older, heavily branched parts of the mycelium. Arrows indicate WGA-labeled hyphal tips; arrowheads in upper DIC and lower fluorescence images indicate nonfluorescing apices. Bars are 10 μm.
velopment. Indeed, increasing inhibitor concentrations resulted in reduction of anthracnose symptom severity, and no symptoms were visible when the infection droplets contained 100 μM nikkomycin Z (Fig. 2D). As expected, clear anthracnose symptoms occurred in the absence of the chitin synthase inhibitor, and control leaves mock inoculated with distilled water did not develop disease symptoms (Fig. 2D). Taken together, these data suggest that chitin synthesis is indispensable for vegetative growth and pathogenicity of *C. graminicola*.

**Identification of CHS from *C. graminicola***

To isolate CHS genes of *C. graminicola*, we performed polymerase chain reaction (PCR) studies with degenerate primers designed to amplify class I to III and class IV to VI genes. Four PCR fragments of 619, 638, 660, and 710 bp were obtained. Sequence analyses of the 710-bp PCR fragment amplified with primers CHS3 and CHS4 showed that it corresponds to a part of the class VI *CgChsVI* gene originally designated *chsA* (Amnuaykanjanasin and Epstein 2003). This gene has been studied in detail previously; therefore, it was not further analyzed here. The 619- and 638-bp PCR fragments amplified with primers CHS1 and CHS2 were used to screen a cosmid library. Purified cosmid clones contained the complete coding regions of a class I (*CgChsI*) and a class III (*CgChsIII*) CHS gene (classification was done according to Munro and Gow [2001]; see also Mellado and associates [2003] and Takeshita and associates [2006]). The complete sequences of the CHS gene of *C. graminicola* studied here are available under GenBank accession numbers AY052545 (*CgChsI*), AY052546 (*CgChsIII*), and AY052547 (*CgChsV*). Phylogenetic analyses showed that the class I and class III CHS are most closely related with the corresponding enzymes NcChs3 and NcChs1 of *Neurospora crassa*, with 74 and 71% amino acid identity, respectively (Fig. 3A). The proteins consist of 899 and 912 amino acids, respectively, and show a typical CHS core region (Nagahashi et al. 1995). Hydropathy plots suggest that both enzymes have eight transmembrane domains (Fig. 3B). In addition to the class I and III CHS genes, we identified a class V CHS gene of *C. graminicola*, designated *CgChsV*. *CgChsV* has a 5,709-bp open reading frame, which is interrupted by two introns (nucleotides 311 to

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**Fig. 2.** Effect of the chitin synthase inhibitor nikkomycin Z on vegetative development and pathogenesis of the maize pathogen *Colletotrichum graminicola*.  
A. Effect of nikkomycin Z on vegetative hyphae. Hyphae of the wild-type strain CgM2 were grown in liquid complete medium in the absence (control) or in liquid complete medium supplemented with 50 μM inhibitor (nikkomycin Z). Individual hyphal swellings (middle) or chains of bulbs (right) can be observed in the presence of the inhibitor. Bar represents 20 μm.  
B and C. Effect of nikkomycin Z on infection structure differentiation. Wild-type conidia were allowed to germinate and differentiate appressoria in the presence of different nikkomycin Z concentrations for 24 h, and rates of distorted structures were scored. Melanized appressoria formed at the tip of short germ tubes of untreated controls (control). In the presence of the inhibitor at 10 μM or higher concentrations, germ tubes (nikkomycin Z, black arrowhead) or appressoria (nikkomycin Z, white arrowhead) swell and appressoria sometimes fail to melanize and collapse. Bar represents 10 μm.  
D. Inhibition of disease symptom development by nikkomycin Z. Leaves were inoculated with conidial suspensions containing increasing concentrations of the chitin synthase inhibitor nikkomycin Z. In the presence of 100 μM nikkomycin, anthracnose disease symptoms did not occur. Likewise, leaves mock inoculated with water did not show disease symptoms.
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367 and 5,624 to 5,677). This derived protein, like CgChsVI, has an N-terminal myosin-like motor domain and a CHS core region at the C-terminus (Fig. 3B). The myosin motor domain of CgChsV shows strong similarity (2.2 e-115) with the myosin motor consensus sequence and has a P-loop ATP binding site (GESGSG) as well as the regulatory IQ motif. In comparison, the myosin-like domain of CgChsVI is much shorter and shows only limited similarity with the myosin motor consensus sequence (2.9 e-7). For both CHS of C. graminicola with myosin-like motor domains, six transmembrane domains have been predicted (Fig. 3B). In total, the proteins contain 1,866 (CgChsV) and 1,783 (CgChsVI) amino acids. The expression profile of the four CHS genes identified in C. graminicola was studied by reverse-transcription (RT)-PCRs with RNA isolated from vegetative hyphae and pre- and post-penetration stages of pathogenic development (i.e., from in vitro infection structures that had formed appressoria and from infected leaves). All genes were expressed at all stages of fungal development investigated (Fig. 3C).

**Generation of ΔCgChsI, ΔCgChsIII, and ΔCgChsV mutants.**

To functionally characterize CgChsI, CgChsIII, and CgChsV, mutants were generated in which a part of the CHS coding region was replaced by the hygromycin phosphotransferase (hph) gene of Escherichia coli. As an example, targeted inactivation of CgChsV is shown (Fig. 4). The knock-out (KO) vector consisted of 974 bp of the CgChsV gene 5′ and 1,621 bp 3′ of the hph gene (Fig. 4A) and was transformed into the C. graminicola wild-type strain CgM2. In order to discriminate between transformants with homologous and ectopic integration of the KO vector, Southern blot experiments were performed with HindIII-digested DNA from the wild-type CgM2 and different independent transformants. While the wild-type strain showed a single 5.6-kb band hybridizing with the CgChsV probe, KO mutants exhibited a 7.7-kb band. In addition to the 5.6-kb CgChsV wild-type band, a high molecular weight band was detected in transformants with ectopically integrated KO vector (Fig. 4B). To demonstrate that partial replacement of the CgChsV gene resulted in failure to synthesize the CgChsV transcript, RT-PCR experiments were performed with total RNA from vegetative hyphae of the wild-type strain CgM2 and the transformants used in Southern blot experiments. As expected, CgChsV transcripts were present in the wild-type strain and transformants with ectopically integrated KO vector, but absent from four transformants with homologous integration of the KO vector (Fig. 4B). In corresponding gene-deletion experi-

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**Fig. 3.** Sequences and expression pattern of the chitin synthases (CHS) of Colletotrichum graminicola. A, Phylogenetic analysis of fungal CHS. The amino acid sequences of the four CHS of C. graminicola (the class VI CgChsVI has been described previously as chsA) (Amnuaykanjanasin and Epstein 2003) were compared with typical enzymes of all known CHS classes. Af, Aspergillus fumigatus; An, A. nidulans; Ao, A. oryzae; Bg, Blumeria graminis; Ca, Candida albicans; Cg, Colletotrichum graminicola; Mg, Magnaporthe grisea; Nc, Neurospora crassa; Pc, Phanerochaete cincta; Sc, Saccharomyces cerevisiae; Um, Ustilago maydis; Sp, Schizosaccharomyces pombe. B, Domain organization of the CHS of C. graminicola. Class I and III CHS show a typical CHS core region containing parts that most likely provide the enzymatic activity, and eight transmembrane domains. Class V and class VI CHS also have a typical CHS core region and six transmembrane domains. Both enzymes exhibit a myosin-like motor domain; whereas this domain shows high similarity with the myosin motor consensus sequence in CgChsV, it is truncated in CgChsVI. C, Expression pattern of CHS genes of C. graminicola. Reverse-transcription polymerase chain reaction assays were performed with total RNA isolated from vegetative hyphae (veg. Hyphae), conidia that had formed appressoria (appressoria), infected leaves 3 days postinfection (inf. leaves (3 dpi.)), and noninfected leaves (healthy leaves) to visualize transcripts of individual CHS genes. Control PCR reactions were performed with genomic DNA as template (genomic DNA).
ments, transformants carrying homologous and ectopic integrations of the KO vector for CgChsI and CgChsIII also were obtained (data not shown). However, C. graminicola mutants defective in CgChsI and CgChsIII did not display any obvious phenotype (growth rate, hyphal morphology, and conidiation rates) differing from the wild type and their virulence on maize leaves was not altered (data not shown).

CgChsV confers rigidity to vegetative hyphae of C. graminicola.

Inactivation of CgChsV drastically affected vegetative growth of C. graminicola (Fig. 5). Although the wild-type strain CgM2 and transformants with ectopically integrated KO vector grew comparably well on oatmeal agar (OMA), the ΔCgChsV mutant failed to grow on this substrate. However, on OMA osmotically stabilized with 150 mM KCl, growth of the ΔCgChsV mutant was partially rescued (Fig. 5A). Under these conditions, wild-type hyphae grew straight with some branching (Fig. 5B). Even in the presence of high osmolyte concentrations, significant cell wall defects were obvious in the ΔCgChsV mutant. In some parts of the mycelium, hyphae grew with swellings occurring occasionally (Fig. 5C) whereas, in others, chains of protrusions almost completely lacking filamentous stretches were visible (Fig. 5D). Cell wall defects were reminiscent of those caused by the chitin synthase inhibitor nikkomycin Z, although the latter primarily occurred at the hyphal tips (compare Figs. 2A and 5B through D). Determination of the N-acetyl glucosamine contents suggested that chitin content is reduced by approximately 30% in ΔCgChsV compared with wild-type hyphae (S. Werner, H. B. Deising, and B. Morschbacher, data not shown). Complementation of a ΔCgChsV strain with the entire CgCHSV gene driven by the glyceraldehyde-3-phosphate promoter of A. nidulans complemented growth and hyphal morphology defects of ΔCgChsV, but did not fully restore its ability to produce conidia (data not shown). These data demonstrate that CgChsV has essential roles in wall formation in C. graminicola.

CgChsV is essential for appressorium formation but dispensable for hyphopodium development and function.

Because appressoria need very rigid cell walls to control the high osmotic pressure, it was of interest to see whether or not the ΔCgChsV mutant would be able to differentiate morphologically and functionally normal infection cells (Fig. 6). ΔCgChsV mutants failed to form acervuli with falcate conidia in regular complete medium (CM); however, in liquid CM supplemented with 0.5 M sucrose, both the wild type and the ΔCgChsV mutant produced conidia. Although wild-type conidia were falcate (Fig. 6A), the ΔCgChsV mutant formed both falcate and shorter cylindrical conidia (Fig. 6B and C). Even in the presence of 0.5 M sucrose, the wild-type conidia formed a short germ tube (Fig. 6A, arrowheads) and differentiated a melanized appressorium on the surface of polyester sheets (Fig. 6A, arrows). Conidia of the ΔCgChsV mutant also germinated in CM medium with 0.5 M sucrose and formed appressoria. However, ΔCgChsV appressoria were severely distorted and the appressorial cell wall appeared to be disintegrated (Fig. 6B, arrow). The fact that germ tubes formed (Fig. 6B, arrowhead) suggests that ΔCgChsV conidia do not rupture and release cytoplasm upon germination. Moreover, the size of the distorted cells is comparable with that of wild-type appressoria (compare cells in Figure 6A to C, arrows). The distorted appressoria showed some melanization.
ΔCgChsV is essential for hyphal growth in planta.

ΔCgChsV null mutants were able to differentiate hyphopodia, suggesting that they are able to infect plants. To investigate the role of CgChsV in pathogenic development, infection assays were performed with the ΔCgChsV mutant and wild-type strains (Fig. 7). Microscopical analyses of infection by wild-type cells showed that hyphopodia (Fig. 7A, asterisk) invaded the host leaf to form thick, primary hyphae (Fig. 7A, large arrowhead) from which thin, destructive secondary hyphae (Fig. 7A, small arrowhead) grew out to colonize the host tissue. Thus, infection hyphae initiating from hyphopodia closely resemble those developing from appressoria. Consistent with the in vitro studies described above, both wild-type and ΔCgChsV mutants formed hyphopodia on the plant surface when osmotically stabilized (Fig. 7A; black bars), and post-penetration infection structures formed at comparable rates (Fig. 7B; gray bars, infection vesicles; hatched bars, primary hyphae; white bars, secondary hyphae). However, the infection hyphae formed by ΔCgChsV mutants within the first host cell showed large swellings (Fig. 7A, arrows). These hyphae were able to penetrate host cell walls to grow into neighboring cells, but the ability to colonize the host tissue was severely reduced.

Consequently, when the inoculum was applied to host leaves in the presence of osmolytes, the ΔCgChsV mutant did not cause any disease symptoms, whereas wild-type and transformants with ectopically integrated KO vector were able to cause anthracnose lesions on wounded and unwounded leaves (Fig. 7C).

These results show that CgChsV is required for post-penetration pathogenic development of C. graminicola. In the absence of osmotic stabilization, however, the ΔCgChsV mutant was unable to differentiate hyphopodia on the maize leaf surface (Fig. 7B) and, therefore, infections did not occur under these conditions.

In order to test whether the pathogenicity defect of ΔCgChsV mutants also was due to increased sensitivity to plant defense
compounds such as \( \text{H}_2\text{O}_2 \), growth assays were performed on CM amended with 0.5 M sucrose and approximately 0.01\% (vol/vol) \( \text{H}_2\text{O}_2 \). At 4 days postinoculation, growth of the wild-type strain was inhibited by 88.5 ± 2.4\%. For comparison, growth inhibition measured for \( \Delta \text{CgChsV} \) and for ectopic transformants were 83.6 ± 15.0\% and 87.3 ± 2.3\%, respectively (data not shown). Differences in inhibition were not statistically different (\( p = 0.05 \)). Although these data indicate that pathogenicity defects are primarily due to cell wall defects rather than increased sensitivity to \( \text{H}_2\text{O}_2 \) (Madrid et al. 2003), it should be noted that vegetative hyphae analyzed in growth assays cannot directly be compared with infection hyphae.

**DISCUSSION**

Chemically, the fungal cell wall consists of 80 to 90\% polysaccharides (Bartnicki-Garcia 1968), and the gross monomeric composition of an average ascomycete cell wall shows a predominance of glucose and amino sugars, predominantly N-acetylglucosamine (Sietsma and Wessels 1994). Chitin may contribute more than 50\% of the dry weight of the cell wall (Aronson and Machlis 1959) and is thought to be essential for fungal cell wall rigidity. In several filamentous fungi, newly synthesized chitin localizes to the hyphal tip (Weber et al. 2006; Wessels 1993) and, by WGA labeling, we demonstrated that this also holds true for *C. graminicola* (Fig. 1). Strongly stained hyphal tips suggest that chitin is a prominent polymer in this fungus. The fact that only hyphal tips but not older, subapical regions expose chitin at the surface may be due to enzymatic modification of chitin that could be mediated by secreted chitin deacetylases (El Gueddari et al. 2002). Alternatively, lack of hyphal decoration by WGA could be due to apposition of other cell wall layers such as hydrophobin coatings (Wösten et al. 1994). Some hyphal tips of *C. graminicola* were not labeled by WGA, suggesting either that these hyphae were not actively growing or that the entire hyphal surface may have been modified (Sietsma and Wessels 1994), rendering them inaccessible for the lectin.

Studies involving CHS inhibitors, namely nikkomycins and polyoxins, demonstrated the importance of these enzymes and the polymers they synthesize for cell wall rigidity and form and function of fungal cell walls (Cabib et al. 1996). In contrast to yeasts, large numbers of CHS genes have been found in the genomes of filamentous fungi (Munro and Gow 2001) and, although inhibitor studies allow the determination of the importance of chitin synthesis (Zhang et al. 2000), they do not lead to an understanding of the role of individual CHS genes or enzymes. Targeted gene inactivation experiments made it possible to genetically dissect chitin synthesis and to investigate the one or more roles of individual genes in plant (Madrid et al. 2003; Weber et al. 2006) and human-pathogenic fungi (Munro and Gow 2001).

We have cloned and functionally characterized a class I, a class III, and a class V CHS gene of the filamentous ascomycete and maize pathogen *C. graminicola*. This is the first report that a class V CHS with a myosin-like motor domain is indispensable for vegetative growth, differentiation of infection structures, and pathogenic development of this plant-pathogenic fungus.

**Structure of the *C. graminicola* CHS.**

Due to their high degree of conservation, class I to III CHS genes have been isolated with degenerate PCR primers (Bowen et al. 2002). The *C. graminicola* CHS is a class V enzyme with a myosin-like C-terminal domain.

Fig. 6. Infection structure differentiation and hyphopodial force exertion by *Colletotrichum graminicola* wild type and the \( \Delta \text{CgChsV} \) mutant strain. Appressorium differentiation by A, the wild-type strain CgM2 and B through D, the \( \Delta \text{CgChsV} \) mutant strain. On a hard, hydrophobic surface, wild-type conidia germinate, form a short germ tube (A, arrowheads), and differentiate a melanized appressorium (A, arrows). Likewise, conidia of the \( \Delta \text{CgChsV} \) mutant form a germ tube (B, arrowhead) and an appressorium (B, arrow), but the infection cell is drastically distorted. All fully enlarged appressoria differentiated by \( \Delta \text{CgChsV} \) conidia show lyzed cell walls (C, arrows). Appressorial initials (D, arrow) develop from germ tubes (D, white arrowhead) but exhibit cell wall defects after maturation, as indicated by a second appressorium (D, black arrowhead) formed by the same conidium. Differentiation of hyphopodia by E, the wild-type strain CgM2 and F and G, the \( \Delta \text{CgChsV} \) mutant strain. Although the wild-type strain forms lobed, melanized hyphopodia (E, arrow), hyphopodia of the mutant also are melanized, but dome-shaped (F and G, arrows). Occasionally, larger, melanized swollen hyphopodia can be observed (G, arrow). Hyphopodia (G, arrow) do not exhibit cell wall defects comparable with those found in appressoria (G, arrowhead) developing in close vicinity. Infection cells were observed 48 h after inoculation of polyester sheets. Bars indicate 10 \( \mu \text{m} \). H, Force measurement using optical waveguides. Force exerted by hyphopodia of the wild-type (WT) and \( \Delta \text{CgChsV} \) mutant strain (KO) were measured by optical waveguides.
et al. 1992). The homologous region is restricted to approximately two-thirds of the carboxyl terminus and, in most cases, hydrophobic membrane-spanning domains are found near this end (Cabib et al. 1996). Although these features have been established with the yeast *S. cerevisiae*, they also hold true for other fungi (Weber et al. 2006). In *C. graminicola*, class I and III CHS exhibit eight hydrophobic membrane-spanning domains at their carboxyl ends and, thus, are typical examples of these enzyme classes. Whether or not they are zymogenic like other class I to III CHS (Mellado et al. 2003) has not been determined.

Class IV to VI CHS genes have been isolated either using PCR primers based on the *ScCHS3* sequence, with primers specifically designed to amplify fragments of CHS with a myosin-like motor domain, or by random insertional mutagenesis (Annuaykanjanasin and Epstein 2003; Madrid et al. 2003; Munro and Gow 2001). In contrast to class IV CHS, enzymes classified as class V CHS have a myosin-like motor domain with significant similarity to the consensus sequence of single-headed myosins, including a classical ATP-binding motifs (P-loop and switch I and II motifs) and the IQ motif. The myosin motor of some CHS (e.g., Chs4 of *Paracoccidioides brasiliensis*, AoChsZ of *A. oryzae*, and UmChs6 of *U. maydis*) are somewhat degraded and, thus, distinct from class V CHS. Nino-Vega and associates (2004) proposed that these CHS should be classified as a new class of enzymes, and enzymes with a shorter myosin-like binding domain lacking classical ATP-binding motifs or the IQ motif have now been designated as class VI CHS (Mellado et al. 2003; Munro and Gow 2001; Takeshita et al. 2006). In *C. graminicola*, typical class V (this study) and VI (Annuaykanjanasin and Epstein 2003) CHS exist, each consisting of approximately 1,800 amino acids and exhibiting six transmembrane domains and either a complete or a shorter myosin-like motor domain (Fig. 3).

**Filamentous growth requires a class V CHS with a myosin-like motor domain.**

Class III, V, and VI CHS have been identified only in filamentous and some dimorphic fungi that are able to differentiate true hyphae. Individual replacement mutagenesis of the class I and III genes *CgChsI* and *CgChsIII* of *C. graminicola* did not cause alterations in hyphal development or conidiation. This may not necessarily be due to lack of function in filamentous growth but, rather, could be due to overlaps in gene and enzyme function. Also, in *A. fumigatus*, deletion of the class I, II, and IV CHS genes *AfchsA*, *AfchsB*, and *AfchsF* had little effect on morphology and growth (Munro and Gow 2001). A class III CHS was not important in the dimorphic basidiomy-

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**Fig. 7.** Penetration competence and pathogenicity of *Colletotrichum graminicola* wild-type strain, ΔCgChsV mutant strain, and a strain with ectopic integration of the knock-out (KO) vector. A, Microscopical analysis of the penetration process. Wild-type and ΔCgChsV hyphopodia (asterisks) formed in the presence of 0.5 M sucrose and invaded the host epidermal cell. The wild type formed thick primary hyphae (large arrowhead) and thin secondary hyphae (small arrowhead). Intracellular hyphae of the ΔCgChsV mutant showed balloon-like swellings (arrows) and grew more slowly than corresponding wild-type hyphae. B, Osmolyte effect on infection structure differentiation by wild-type and ΔCgChsV mutant on maize leaves. In the absence of the osmolyte, the ΔCgChsV mutant is unable either to grow and form hyphopodia on the leaf surface or to differentiate infection structures in planta. In the presence of 150 mM KCl, rates of infection structure differentiation are comparable. Black bars, hyphopodia; gray bars, infection vesicles; hatched bars, primary hyphae; white bars, secondary hyphae. Error bars indicate standard deviation. Infection structure formation was scored 48 h postinoculation. C, Leaf infection assays. Mycelia of the wild type (WT), a ΔCgChsV mutant strain, and a strain with ectopic integration of the KO vector were placed onto the surface of intact or wounded maize leaves. Non-inoculated control leaves (C) also are shown. Photographs were taken 6 days postinoculation.
cete *U. maydis* (Gold and Kronstad 1994; Weber et al. 2006) and class III CHS do not even exist in *Phycomyces blakesleeanus* (Miyazaki and Ootaki 1997). In contrast, mutants of the gray mold fungus *B. cinerea* deficient in the class III CHS BcChs3a were severely impaired in growth on solid substratum (Soulé et al. 2006). In *U. maydis*, two class IV CHS, Chs5 and Chs7, have been shown to be essential for fungal morphogenesis and filamentous growth (Weber et al. 2006), and class IV CHS also have important roles in hyphal growth of the dimorphic fungus *C. albicans* (Mio et al. 1996) and in the yeast *S. cerevisiae* (Shaw et al. 1991). However, in the filamentous fungus *N. crassa*, deletion of class IV CHS was without significant effect (Beth Din et al. 1996). These findings raised doubts about specific cellular functions of individual classes of CHS, and the situation is even more complicated by the fact that CHS of different classes cooperate to mediate hyphal growth and conidiation (Fujiwara et al. 2000; Motoyama et al. 1997).

A growing body of literature shows that mutations in individual class V and VI genes drastically affect filamentous growth. *C. graminicola ΔCgChsV* replacement mutants showed swollen hyphal tips and balloon-like structures along the hyphae, similar to those seen in *A. nidulans* mutants deficient in the class V CHS gene *csmA* (Horiuchi et al. 1999). In *F. oxysporum* class V CHS mutants, occurrence of balloon-like structures was suppressed in solid synthetic media complemented with osmotic stabilizers, indicating that swellings result from cell wall weakening and, in turn, that class V CHS play a major role in maintenance of hyphal wall integrity and polarized cell wall synthesis (Madrid et al. 2003). As indicated by the inability of *C. graminicola ΔCgChsV* mutants to form conidia on the surface of solid substrata, the class V CHS also plays a role in asexual sporulation. This also has been observed in Δ*csmA* mutants of *A. nidulans*. In addition to cell wall weakening, inactivation of the *csmA* gene led to formation of hyphae within previously formed older hyphae (intrahyphal growth) (Horiuchi et al. 1999).

Mutating the *A. nidulans* class VI CHS gene *csmB* caused formation of balloons, intrahyphal hyphae, lysis in subapical hyphal regions, and drastically reduced conidiation; thus, *csmA* and *csmB* mutants show a similar phenotype. Staining experiments indicated that CsmA and CsmB co-localized with actin, but immuno-precipitation experiments did not suggest a physical interaction between these enzymes (Takeshita et al. 2006). In *C. graminicola*, inactivation of the class VI CHS gene caused conidial budding and swelling of hyphal tips in media with low osmotic pressure, indicating the role of this enzyme in cell wall strength of conidial and vegetative hyphae (Amnuayakanjanasin and Epstein 2003). These data suggest that filamentous growth in several fungi, including *C. graminicola*, requires class V or class VI CHS with a myosin-like motor domain.

**Role of chitin synthases in pathogenesis.**

Apical growth is a hallmark of filamentous fungi, allowing invasion and colonization of solid substrata, including living tissues of either plant or animal origin (Wessels 1993). Fungal pathogenesis requires invasive growth (Deising et al. 2000; Stoldt et al. 1997); therefore, one may expect that CHS exclusively found in filamentous fungi are required for pathogenicity or virulence. Functional characterization of enzymes such as chitin and β-1,3-glucan synthases is essential not only to understand fungal pathogenicity but also to identify novel fungicide targets to be used in agriculture and medicine (Debono and Gordee 1994; Georgopapadakou 2001).

In comparison with information on the role of chitin synthases in vegetative development, little is known about the role of chitin synthesis and CHS during pathogenic growth. CHS with myosin motor domains have been found in filamentous fungi, including plant pathogens such as *F. oxysporum* (Madrid et al. 2003), *B. cinerea* (Choquer et al. 2004), *Magnaporthe grisea* (Vidal-Cros and Boccarda 1998), and *U. maydis* (Weber et al. 2006), and also in human pathogens such as *A. fumigatus* (Auffacure-Brown et al. 1997), *Paracoccidioides brasiliensis* (Nino-Vega et al. 2004), and *Wangiella dermatitides* (Liu et al. 2004). The *mcs1* gene of *U. maydis* is specifically required for invasive growth in planta. However, the class VI CHS of the corn smut fungus *U. maydis*, Chs6, also contributes to fungal pathogenicity (Garcera-Teruel et al. 2004; Weber et al. 2006). Likewise, targeted chsV replacement mutants of *F. oxysporum* failed to colonize the vascular system of tomato plants and to invade wounded tomato fruit (Madrid et al. 2003).

In contrast to *U. maydis* and *F. oxysporum*, the corn antracnose fungus *C. graminicola* differentiates distinct appressoria. These infection cells are melanized and previous studies have shown that they exert significant force to invade an epidermal host cell (Bechinger et al. 1999). Because nonmelanized mutants are unable to infect corn (Rasmussen and Hanau 1989), generation of turgor and its translation into force are thought to be essential for pathogenesis. On polyester sheets, Δ*CgChsV* mutants formed germ tubes and appressorial initials, but failed to differentiate functional (i.e., plant cell wall-penetrating) appressoria (Fig. 6). These data clearly indicate that CgChsV is indispensable for synthesis of rigid appressorial cell walls and for appressorium-mediated plant infection. Surprisingly, Δ*CgChsV* mutants were able to synthesize hyphopodia, which exhibited a distinct morphology, but exerted force and penetrated the host epidermis at rates comparable with wild-type hyphopodia (Figs. 6 and 7), allowing the study of infection structure formation in planta. After entering the epidermal host cell, *C. graminicola ΔCgChsV* primary hyphae swell markedly, reminiscent of the phenotype of the infection hyphae of *U. maydis Δmcs1* mutants (Weber et al. 2006), and antracnose disease symptoms did not develop. Thus, the class V chitin synthase CgChsV of *C. graminicola* is essential for both pre- and post-invasive stages of plant infection. Class V mutants of neither *C. graminicola* nor *U. maydis* exhibit increased sensitivity toward H2O2, suggesting that reduced virulence is due to cell wall defects per se, rather than to increased sensitivity to plant defense compounds (Weber et al. 2006; this study). Thus, increased sensitivity to reactive oxygen species may be specific for the class V chitin synthase mutant of *F. oxysporum* (Madrid et al. 2003).

Class V CHS of the plant pathogens *C. graminicola*, *U. maydis*, and *F. oxysporum*, as well as of the black fungal pathogen of humans, *W. dermatitidis* contribute to virulence (Liu et al. 2004; Madrid et al. 2003; Weber et al. 2006; this study); therefore, it would be tempting to investigate the role of these enzymes in a broader spectrum of pathogenic fungi. It is important to note, however, that pathogenicity assays in plants and animal models are difficult to compare, as indicated by studies with the tomato pathogen *F. oxysporum*. chsV mutants of this fungus are nonpathogenic on plants but show increased virulence in immunodepressed mice (Ortoneda et al. 2004).

Our study has shown that a class V CHS is a viability and pathogenicity factor in the maize antracnose fungus *C. graminicola* and suggests that this class of fungal CHS may be important for infection of plants. Although general conclusions cannot be drawn so far, increasing evidence suggests that class V CHS have important roles in fungal pathogenicity (Madrid et al. 2003; Weber et al. 2006), and one may speculate that these enzymes could represent excellent novel fungicide targets. Entire fungicide classes have been lost due to development of fungicide resistance in fungal pathogen populations; therefore, appropriate fungicide mixtures may not durably be available in...
agriculture, and the search for new fungicides will become increasingly important (Deising et al. 2002).

**MATERIALS AND METHODS**

**Fungal cultures and in vitro differentiation of infection structures.**

*C. graminicola* (Ces.) G. W. Wilson (teleomorph *Glomerella graminicola* D. J. Politis) strain M2 was kindly provided by R. L. Nicholson, Purdue University, IN, and used as wild-type strain in this study. The wild-type strain and Δ*CgChsI* and Δ*CgChsIII* mutants were grown on OMA (50 g of chopped oatmeal, 200 g of blended malt, 1 g of agar-agar [Difco Laboratories, Augsburg, Germany] per liter) at 23°C under continuous fluorescent light (Climas Control CIR, UniEquip, Weiterstadt, Germany). Alternatively, strains were grown in CM (Leach et al. 1982) in an incubation shaker (Unitron, Infors AG, Bottmingen, Switzerland) at 110 rpm and 23°C. To solidify CM, 1.5% (vol/vol) agar-agar (Difco Laboratories, Augsburg, Germany) was added. Δ*CgchsV* mutants were kept on oatmeal or CM agar plates or in CM liquid medium supplemented with 0.15 M KCl, 1 M sorbitol, or 0.5 M sucrose.

To determine H₂O₂ sensitivity, strains were grown on solidified CM containing 0.5 M sucrose and approximately 0.01% (vol/vol) H₂O₂. Plates without H₂O₂ served as controls.

To induce differentiation of infection structures in vitro, aqueous spore suspensions were adjusted to a concentration of 10⁵ conidia/ml, and 10- to 20-μl droplets were inoculated onto polyester transparency sheets (no. 3558; Avery Dennison, Madison, WI, U.S.A.). BLAST search was carried out on the National Center for Biotechnology Information server. Sequence analyses were performed using the software available by ABI and the DNAStar programs (DNASTAR Inc., Madison, WI, U.S.A.). BLAST search was carried out on the National Center for Biotechnology Information server.

**Targeted gene disruption.**

To generate the *CgChsI* KO vector, the *CgChsI* gene was digested with *Cfr*I and *Bam*HI, removing a 1,037-bp fragment of the coding region. After treatment with the Klenow fragment, the 2,395-bp *Sal*I fragment of pUCATPH (Lu et al. 1994) carrying the hygromycin resistance cassette was inserted into *CgChsI* to replace the *Cfr*I–*Bam*HI fragment. A DNA fragment consisting of the hygromycin resistance cassette flanked by 1,000 bp of *CgChsI* at the 5′ end and 971 bp at the 3′ end was ampliﬁed using the Expand Long-Template PCR system (Roche Diagnostics) and the primers CHSI1 (5′-GTC ACA AAC ATC AGA AAC CAG CCY TTT T-3′) and CHSI4 (5′-CAC CAC GGT AGT AGT GAG TTA TCA CAA T-3′). The amplified fragment was transformed into CgM2 to generate Δ*CgchsI* mutants.

The 2,691-bp *Ace651-NdeI* fragment of pUCATPH carrying the hygromycin resistance cassette was ligated into the Δ*CgChsIII* gene digested with the same enzymes, so that 1,093 bp of the coding region of this CHS gene was replaced. A 4,91-kbp fragment of this construct was ampliﬁed using the Expand Long-Template PCR system (Roche Diagnostics). To generate Δ*CgchsIII* mutants, the amplified fragment was transformed into CgM2.
Transformation of *C. graminicola* basically followed the protocol described by Epstein and associates (1998), with some modifications. SYE medium (0.5 M sucrose and 0.1% [wt/vol] yeast extract) was inoculated to contain 10^6 falcate conidia/ml and incubated in an incubation shaker (Unitron, Infors AG, Bottmingen, Switzerland) at 23°C and 110 rpm for 3 days. Cultures were filtered through two layers of cheesecloth and the filtrate containing oval conidia was centrifuged at 4,260 × g at 4°C for 10 min. The conidia were resuspended in 10 ml of a protoplasting solution containing lysing enzyme from *Trichoderma harzianum* at 20 mg/ml (Sigma, Deisenhofen, Germany) and 0.1% (vol/vol) β-mercaptoethanol in 0.7 M NaCl, and incubated at 30°C for 3 h with slight agitation. Formation of protoplasts was controlled microscopically. The protoplasts were sedimented by centrifugation (800 × g at 4°C for 10 min), washed in 10 ml of SCT (1 M sorbitol; 50 mM CaCl₂; and 50 mM Tris-HCl, pH 8.0), centrifuged again, and resuspended in 1 ml of SCT. The KO vector (0.5 to 10 μg) was added to 100 μl of the protoplast suspension and incubated on ice for 30 min. After adding 1 ml of PEG solution (40% polyethylene glycol 4000; 0.6 M KC1; 50 mM CaCl₂; and 50 mM Tris-HCl, pH 8.0) and incubation at room temperature for 20 min, 3 to 4 ml of liquid regeneration medium (1 M sucrose, 0.1% [wt/vol] yeast extract, 0.1% [wt/vol] casein, and 0.6% [wt/vol] agar; 45°C) was added and poured onto selection plates containing hygromycin B at 400 μg/ml and 1.5% (wt/vol) agar. After 7 to 10 days, growing colonies were transferred to new selection plates containing hygromycin B at 100 μg/ml. To obtain homokaryotic transformants, colonies that had developed on selection plates were allowed to conidiate on OMA. Conidia again were plated out on selection plates containing hygromycin B at 100 μg/ml.

To identify transformants with homologous and ectopic integration of the KO vector, DNA was extracted and digested with *BamH I* (CgChsI), Acc65I plus XhoI (CgChsIII), or HindIII (CgChsV). After electrophoresis, DNA was blotted onto positively charged nylon membranes (Roche Diagnostics) by an alkaline downward procedure (Brown 1999), and hybridized with a DIG-labeled probe, as suggested by the manufacturer (DIG user's guide; Roche Diagnostics). Probes of 696 bp with a DIG-labeled probe, as suggested by the manufacturer (DIG user's guide; Roche Diagnostics), were am-plified with primer combinations CHSIIr1 and CHSIII7; and CHSVI2.1 and CHSVIr1 (5′-GCC GTC GTG CCG CCA TCA GGT AGT-3′) were used to identify the KO vector and the wild-type control. The KO vector contained a fragment of the CgChsIII gene, and the wild-type control contained a fragment of the CgChsII gene.

**RNA isolation and gene expression analyses.** The KO vector, DNA was extracted and digested with *BamH I* (CgChsI), Acc65I plus XhoI (CgChsIII), or HindIII (CgChsV). After electrophoresis, DNA was blotted onto positively charged nylon membranes (Roche Diagnostics) by an alkaline downward procedure (Brown 1999), and hybridized with a DIG-labeled probe, as suggested by the manufacturer (DIG user's guide; Roche Diagnostics). Probes of 696 bp with a DIG-labeled probe, as suggested by the manufacturer (DIG user's guide; Roche Diagnostics), were am-plified with primer combinations CHSIIr1 and CHSIII7; and CHSVI2.1 and CHSVIr1 (5′-GCC GTC GTG CCG CCA TCA GGT AGT-3′) were used to identify the KO vector and the wild-type control. The KO vector contained a fragment of the CgChsIII gene, and the wild-type control contained a fragment of the CgChsII gene.

**Microscopy and chitin staining.** Microscopy was done with either a Nikon Eclipse E600 microscope (Nikon, Düsseldorf, Germany) equipped with bright-field and differential interference contrast optics or a Zeiss Axioplan Imaging II microscope (Oberkochen, Germany) and standard rhodamine or FITC filters. Digital images were taken with a DS-5M (Nikon) or a CoolSNAP-HQ CCD camera (Photometrics, Tucson, AZ, U.S.A.) controlled by the imaging software MetaMorph (Universal Imaging, Downingtown, PA, U.S.A.). Image processing was done with the software package Lucia 4.61 (Nikon) and the imaging software MetaMorph. For staining of chitin, cells were grown overnight in CM liquid medium supplemented with 0.5 M sucrose. Under these conditions, both wild-type and ΔCgChsV mutant differentiated hyphopodia, and force was measured 24 h after inoculation of the waveguides.

**Measurement of hyphopodal force exertion by optical waveguides.** Force measurement was performed with optical waveguides as described previously (Bechinger et al. 1999). Because ΔCgChsV mutants produced neither falcate conidia nor intact hyphae in the absence of osmolytes, pieces of mycelia (approximately 1 mm²) were placed onto optical waveguides in 0.5 M sucrose. Under these conditions, both wild-type and ΔCgChsV mutant differentiated hyphopodia, and force was measured 24 h after inoculation of the waveguides.

**Inhibitor studies with nikkomycin Z.** Nikkomycin Z (Sigma) was added to conidial suspensions at different concentrations (0 to 500 μM) prior to inoculation of polyester transparency sheets or maize leaf segments as described above. In vitro differentiated infection structures were evaluated 24 h after inoculation of transparency sheets; symptoms developed on leaf segments were photographed 3 to 4 days postinoculation.

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