Sphingolipid C-9 Methyltransferases Are Important for Growth and Virulence but Not for Sensitivity to Antifungal Plant Defensins in *Fusarium graminearum*^

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The C-9-methylated glucosylceramides (GlcCers) are sphingolipids unique to fungi. They play important roles in fungal growth and pathogenesis, and they act as receptors for some antifungal plant defensins. We have identified two genes, *FgMT1* and *FgMT2*, that each encode a putative sphingolipid C-9 methyltransferase (C-9–MT) in the fungal pathogen *Fusarium graminearum* and complement a *Pichia pastoris* C-9–MT-null mutant. The *ΔFgmt1* mutant produced C-9-methylated GlcCer like the wild-type strain, PH-1, whereas the *ΔFgmt2* mutant produced 65 to 75% nonmethylated and 25 to 35% methylated GlcCer. No *ΔFgnt1ΔFgnt2* double-knockout mutant producing only nonmethylated GlcCer could be recovered, suggesting that perhaps C-9–MTs are essential in this pathogen. This is in contrast to the nonessential nature of this enzyme in the unicellular fungus *P. pastoris*. The *ΔFgnt2* mutant exhibited severe growth defects and produced abnormal conidia, while the *ΔFgnt1* mutant grew like the wild-type strain, PH-1, under the conditions tested. The *ΔFgnt2* mutant also exhibited drastically reduced disease symptoms in wheat and much-delayed disease symptoms in *Arabidopsis thaliana*. Surprisingly, the *ΔFgnt2* mutant was less virulent on different host plants tested than the previously characterized *ΔFgcs1* mutant, which lacks GlcCer synthase activity and produces no GlcCer at all. Moreover, the *ΔFgnt1* and *ΔFgnt2* mutants, as well as the *P. pastoris* strain in which the C-9–MT gene was deleted, retained sensitivity to the antifungal plant defensins MsDef1 and RsAFP2, indicating that the C-9 methyl group is not a critical structural feature of the GlcCer receptor required for the antifungal action of plant defensins.

Sphingolipids are ubiquitous membrane components of eukaryotes, including yeasts and filamentous fungi (21, 24, 34). Although classically viewed as structural components of membranes, sphingolipids and their metabolites are now recognized as important bioactive molecules (13, 14). *Saccharomyces cerevisiae*, because of its ease of genetic manipulation and isolation of mutants, has provided much of our current knowledge of sphingolipid biosynthesis, metabolism, and function (9, 11). Although little is known about sphingolipid functions in filamentous fungi, evidence is beginning to emerge that sphingolipids and their metabolites play important roles in signal transduction, cell-to-cell communication, and programmed cell death as well as pathogenesis (7, 15, 19, 20, 24). The in vivo functions of sphingolipids in the growth and differentiation of pathogenic filamentous fungi remain largely unexplored. In particular, the roles of different sphingolipids and their metabolites in the regulation of plant-fungus interactions have yet to be elucidated.

Sphingolipids are composed of a ceramide backbone that consists of a C18 long-chain base (LCB) bound to a fatty acid via an amide linkage. Complex sphingolipids are formed by the addition of various sugar residues or phosphate-containing headgroups to a ceramide backbone (9, 21). Some unicellular yeasts and filamentous fungi possess a few unique steps of sphingolipid biosynthesis and metabolism that are absent in *Saccharomyces cerevisiae*. Two classes of complex sphingolipids, phosphoinositolsphingolipids and glycosphingolipids, are present in fungi. The common occurrence of glucosylceramides (GlcCers), the simplest members of neutral glycosphingolipids, in the filamentous fungal and yeast pathogens and their absence in *S. cerevisiae* have recently sparked increased research interest in their biosynthesis and their functional roles in fungal pathogenesis. Previous study has shown that GlcCers induce fruiting-body formation in the fungus *Schizzyphillum commune* (17). More-recent studies have implicated these sphingolipids in plant-fungus interactions, where they enable plants to recognize fungal attack and initiate specific defense responses. For example, GlcCers isolated from the rice blast fungus, *Magnaporthe grisea*, elicit defense responses in rice cell suspension cultures (18). GlcCers sprayed on rice plants induce the synthesis of phytoalexins and expression of pathogenesis-related proteins, and the treated plants are protected from the rice blast disease under field conditions (18, 40, 41). Evidence is beginning to emerge that GlcCers play important roles in fungal pathogenicity. Thus, it has been shown recently that GlcCer is an essential regulator of pathogenicity in the human fungal pathogen *Cryptococcus neoformans*. The Δ*gcs1* strain, lacking the GlcCer synthase (GCS) function, becomes avirulent (28). In contrast, the effect of GCS1 mutation on the pathogenicity of *Fusarium graminearum* is host dependent. The virulence of the *F. graminearum Δgcs1* (*ΔFgcs1*) strain in wheat is highly compromised. However, in a model host plant, *Arabidopsis thali-
ana, the ΔFggs1 strain retains full virulence compared with the wild-type strain (25).

An unexpected discovery during the past few years has been the finding that some cysteine-rich cationic antifungal plant defensins interact directly with fungal GlcCers and cause fungal growth arrest in vitro. The plant defensin RsAFP2, from *Raphanus sativus*, interacts with GlcCers from *Pichia pastoris* and *Candida albicans* and requires the presence of this sphingolipid for its antifungal activity (37). We have recently found that the Δgcs1 strain of the fungal pathogen *F. graminearum*, which lacks GlcCer altogether, renders the fungus resistant to the plant defensin MsDef1, isolated from *Medicago sativa* (25). The mutant strain also exhibits a significant change in conidial morphology and a dramatic growth defect, and its mycelia become resistant to cell wall-degrading enzymes.

The structure of GlcCer in fungi is highly conserved. It is characterized by a ceramide moiety containing 9-methyl-4,8-sphingadienine linked to C16 or C18 α-hydroxy fatty acids. Importantly, fungal GlcCers have certain structural features that distinguish them from those found in mammals and plants (2, 37, 39, 43). These include the variable levels of unsaturation and the length of the fatty acid chain. However, the presence of C-9 methyl branching of the sphingoid LCB is a major distinguishing feature of fungal GlcCers. It is therefore likely that C-9 methylation of fungal GlcCers is functionally important for fungal growth and differentiation and for pathogenesis as well as for interaction with antifungal plant defensins. A gene encoding sphingolipid C-9-methytransferase (C-9-MT), which introduces a C-9 methyl branch into fungal GlcCer, has recently been identified in *P. pastoris*. A knockout strain of this fungus lacking this gene produced only nonmethylated GlcCer and was fully viable (35). This S-adenosylmethionine-dependent C-9–MT enzyme from *P. pastoris* uses C18 α-OH-Δ4,8-ceramide as a substrate. The C-9–MT enzymatic step follows the Δ8-desaturation of the LCB but precedes the Δ3-desaturation of the acyl moiety in the fungal GlcCer biosynthetic pathway (Fig. 1) (43). Homologs of genes encoding C-9–MT have been identified in several fungi by phylogenetic analysis, but their functional significance in the growth, differentiation, and pathogenicity of plant fungal pathogens remains to be determined (35).

Here we have characterized the biological functions of two genes, *FgMT1* and *FgMT2*, encoding putative C-9–MT in an economically important filamentous fungal pathogen, *F. graminearum*. We show that the *FgMT2* gene has a strong influence on the C-9-methylated GlcCer content of this fungus, whereas the *FgMT1* gene does not. Our results also point to the possibility that the presence of some C-9-methylated GlcCer in this fungus is essential for its survival. This is quite unexpected, since the null mutant of C-9–MT in the unicellular fungus *P. pastoris* is fully viable and has no apparent growth defect. We further show that the lack of C-9 methylation of the LCB of GlcCer has little influence on the antifungal activities of the plant defensins MsDef1 and RsAFP2. Our findings reveal for the first time the novel biological functions of sphingolipid C-9–MTs in the filamentous fungal pathogen *F. graminearum*.
resistant transformants by PCR with primers TM5 and TM6 and was confirmed by Southern blot analysis. Gene locus Fg05593.1, encoding C-9–MT2, was replaced with the hph gene in strain PH-1 (see Fig. S1B in the supplemental material). A 960-bp 5' flanking sequence of FgMT2 was amplified by PCR using primers H1 and H2 and was cloned into pBS-hph, containing the bacterial hph gene under the control of the Aspergillus nidulans trpC promoter, yielding pBS-hph-LF (26). A 1,201-bp 3' flanking sequence of FgMT2 was amplified by PCR using primers H3 and H4 and was cloned into pBS-hph-LF as pFgmt2, generating an FgMT2 gene replacement vector (see Table S1 in the supplemental material). pFgmt2 was linearized with Xhol and transformed into wild-type PH-1 protoplasts. A hygromycin-resistant transformant was isolated by single-colony purification and prescreened by PCR analysis of genomic DNA with primers T5 and T6 to determine the presence or absence of the FgMT2 fragment. The replacement of FgMT2 by the hph gene was verified by Southern blot analysis.

Complementation of the Δfmg2mt mutant. For complementation studies, the full-length FgMT2 gene, encompassing the protein coding sequence with 1.6-kb upstream and 500-bp downstream sequences, was amplified by PCR using primers Bcom1 and Bcom2 and was cloned into pHZ100 containing the neomycin resistance gene (26) to obtain pHZ100 containing the AMT1 and Bcom2 and was transformed into wild type (see Table S1 in the supplemental material). The gene sequences were verified by sequencing. The plasmid was linearized and transformed into the FgMT2 mutant, and transformed colonies were selected using neomycin resistance as a selectable marker and confirmed by PCR analysis using primers T5 and T6 and Southern blot analysis. P. pastoris C-9–MT gene replacement. The sphenolipid C-9–MT gene in P. pastoris was disrupted by a split-marker approach (see Fig. S2 in the supplemental material) (6). About 936-bp upstream and 376-bp downstream flanking sequences of PpMT1 (amplified with primers P1–P2 and P3–P4, respectively). The resulting upstream and downstream PCR products were fused with the HIS4 fragments H1 (amplified by HIS1–HIS2 primers) and IS (amplified by HIS3–HIS4 primers), respectively, and the resulting PCR products were transformed into P. pastoris GS115, which carries a HIS4 mutation. The Δpmpmt mutant was screened for His prototrophy. The absence of the C-9–MT gene was confirmed by PCR using primers P5 and P6. The deletion of the C-9–MT gene was further confirmed by analysis of GlcCer composition. Expression of FgMT1 and FgMT2 in P. pastoris lacking C-9–MT. The coding sequences of FgMT1 were amplified by PCR using primers MTACDSF and MTACDSR and were cloned into the pGAPZ-B vector (Invitrogen, CA) as pGAPZ-1. Similarly, the coding sequences of FgMT2 were amplified using primer pair MTBCDSF-MTBCDSR and were cloned into the pGAPZ-B vector as pGAPZ-2, pGAPZ-1, and pGAPZ-2 were linearized and transformed into the Δpmpmt mutant. Transformants were selected on YPD medium containing Zeocin, and the presence of the FgMT1 or FgMT2 gene was confirmed by PCR analysis for the presence of the corresponding coding sequence. Finally, the structural features of the GlcCers of the Δpmpmt mutants complemented with FgMT1 or FgMT2 were analyzed.

Fungal sphingolipid extraction and analysis. Fungal sphingolipids were extracted from the mycelia of fungal strains. GlcCer was purified, and thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and LC-MS analyses were performed, as described previously (25).

Plant infection and pathogenicity tests. The wheat head, corn silk, tomato fruit, and A. thaliana infection and disease evaluation experiments were carried out as described previously (25).

RESULTS

F. graminearum has two genes encoding putative sphingo- lipid C-9–MT. To determine the functions of C-9 methylation of the sphingolipid GlcCer, we first sought to identify genes encoding C-9–MT in F. graminearum. The likely presence of two genes encoding C-9–MT in the genome of this fungus has been reported previously (35). We performed a BLAST search of the F. graminearum genome database (http://www.broad. mit.edu/annotation/genome/fusarium_graminearum/MultiHome.html) using the P. pastoris C-9–MT gene sequence (GenBank accession number DQ070247) as a query. This search identified two genes, designated FgMT1 (Fg03951.1) and FgMT2 (Fg05593.1), encoding putative C-9–MT with E value of 0.0. The FgMT1 and FgMT2 proteins share 59% amino acid identity with each other and show 58% and 63% identities with a single C-9–MT of P. pastoris, respectively (Fig. 2). A single C-9–MT occurs in Neurospora crassa, but two are present in Aspergillus nidulans (35). The deduced amino acid sequences of these proteins share 58% to 68% amino acid identities with the amino acid sequences of FgMT1 and FgMT2. FgMT1 and FgMT2 complement the P. pastoris C-9–MT-null mutant. To determine if FgMT1 and FgMT2 encode functional C-9–MTs, we decided to test both genes for their abilities to complement the P. pastoris Δpmpmt mutant, which contains a disrupted C-9–MT gene. We first knocked out the C-9–MT gene in strain GS115 by using the HIS4 gene as a selectable marker and generated the Δpmpmt mutant (see Fig. S1A and B in the supplemental material) (35). HPLC analysis of the GlcCer-enriched fraction from strain GS115 showed that it contained both C-9-methylated GlcCers (9-methyl-sphinga-4,8-dienine and the psychosine form of 9-methyl-sphinga-4,8-dienine) and nonmethylated GlcCers (sphinga-4,8-dienine and the psychosine form of sphinga-4,8-dienine) (Fig. 3A). However, the Δpmpmt mutant contained only nonmethylated GlcCers (sphinga-4,8-dienine and the psychosine form of sphinga-4,8-dienine) (Fig. 3B). The FgMT1 and FgMT2 genes were then introduced individually into this mutant strain to determine if they would complement the mutant strain, which would indicate that they encode C-9–MT. The HPLC analysis of the GlcCer-enriched fractions of the mutant strains transformed with either the FgMT1 or the FgMT2 gene was similar to that of the wild-type strain, indicating that each gene encodes a bona fide sphingolipid C-9–MT capable of methylating the GlcCers produced in the Δpmpmt mutant (Fig. 3C and D). We conclude that FgMT1 and FgMT2 each encode a functional C-9–MT.

Sphingolipid C-9 methyltransferases are likely essential for growth and differentiation in F. graminearum. Next, we tested the biological functions of these genes individually by generating a knockout strain for each gene. The FgMT1 and FgMT2 gene replacement constructs were generated and transformed individually into strain PH-1 (see Fig. S2A and C in the supplemental material). The Δfmg1mt mutant was obtained at the first attempt. However, it took several attempts to obtain the Δfmg2mt mutant. Initially we tried a split-marker approach to knock out Fgmt2 but were unsuccessful. Therefore, we made a plasmid-based replacement construct as an alternative method. The Δfmg1mt and Δfmg2mt mutants were identified by PCR and confirmed by Southern blot analysis. Hybridization analysis of the Δfmg1mt transformant using an upstream flanking sequence probe (Probe upA) revealed a 1.8-kb SacI fragment in strain PH-1 but a 4.0-kb fragment in the Δfmg1mt strain. Hybridization with the gene-specific probe (probe A) revealed a 2.5-kb SacI fragment in strain PH-1 that was absent in the knockout strain, confirming replacement of the FgMT1 gene. The successful replacement of the FgMT1 gene was further confirmed using an hph gene-specific probe (probe Hph) (see Fig. S2B in the supplemental material). Similarly, hybridization analysis of the putative Δfmg2mt strain using an upstream flanking sequence probe (probe upB) revealed a 1.3-kb HindIII fragment in PH-1 but a 2.3-kb HindIII fragment in the Δfmg2mt strain. A gene-specific probe (probe B) hybridized to a 2.7-kb HindIII fragment in the wild-type strain that was absent in the mutant strain, confirming the replacement of the FgMT2 gene. The
successful replacement of the FgMT2 gene was further confirmed using an hph gene-specific probe (probe Hph) (see Fig. S2D in the supplemental material). A striking difference in the growth of the two knockout strains was observed on CM. The /H9004 Fg mt1 strain grew like strain PH-1, whereas the /H9004 Fg mt2 strain exhibited severe growth defects (see below).

To determine if FgMT1 and FgMT2 each methylate GlcCer in F. graminearum, analysis of GlcCer for the presence of a C-9 methyl moiety in LCB was carried out for the /H9004 Fg mt1 and /H9004 Fg mt2 strains, and the results were compared with those for strain PH-1. As reported previously (25), HPLC analysis of the derivatized and released LCBs following hydrolysis revealed the presence of two molecules in the wild-type strain: the psychosine form of C-9-methyl-d18:2 and 9-methyl-d18:2 (Fig. 4A). The psychosine form is the glucosylated LCB that arises from incomplete hydrolysis of GlcCers. Surprisingly, the HPLC profile for the /H9004 Fg mt1 mutant appeared similar to that for PH-1 (Fig. 4B), whereas for the /H9004 Fg mt2 mutant consisted of four molecules: the psychosine form of 9-methyl-d18:2, 9-methyl-d18:2, the psychosine form of d18:2, and d18:2 (Fig. 4C). Thus, the /H9004 Fg mt1 mutant produced GlcCer with a 9-methyl moiety, like PH-1, whereas the /H9004 Fg mt2 mutant produced 65 to 75% nonmethylated and 25 to 35% C-9-methylated GlcCer. LC-MS analysis of the molecules confirmed the identity of LCB with and without the 9-methyl moiety (Fig. 4E to H) and clearly indicated that the ΔFgmt1 mutant produces only C-9-methylated GlcCer but the ΔFgmt2 mutant produces both nonmethylated and C-9-methylated GlcCer. In order to confirm that the FgMT2 gene functions as C-9–MT, a wild-type copy of the FgMT2 gene was reintroduced into the /H9004 Fg mt2 mutant. The complemented strain produced only C-9-methylated GlcCers, like PH-1 (Fig. 4D). From these results, we conclude that FgMT2 encodes a C-9–MT that is capable of methylating GlcCers in the absence of the FgMT1-encoded enzyme and that FgMT2 is the predominant C-9–MT for methylating GlcCers. Our observation that FgMT1 is able to methylate a small fraction of GlcCer in the absence of FgMT2 indicates partial redundancy for sphingolipid C-9–MTs in F. graminearum.

We have reported previously that the ΔFgcs1 mutant of F. graminearum, which lacks GlcCers, is highly compromised in its growth and differentiation (25). In order to determine if the C-9 methylation of GlcCers is important for the normal growth and differentiation of F. graminearum, we analyzed the growth characteristics of the /H9004 Fg mt1 and /H9004 Fg mt2 knockouts relative to those of the wild-type. On solid CM, the /H9004 Fg mt1 knockout grew like the wild-type strain, PH-1. The ΔFgmt2 knockout, in contrast, exhibited severe growth defects on CM (Fig. 5A and Table 1). Surprisingly, the growth of this knockout was slower than that of the Fgcs1 knockout. The ΔFgmt2 knockout also grew poorly on different liquid media, such as CM, SFM (Fig. 6), and YPD (data not shown), and did not produce freely
made to generate a ΔFgmt1 ΔFgmt2 double-knockout mutant. Five transformation experiments for knocking out both genes were attempted, and a total of approximately 250 transformants were screened. The double-knockout mutant, however, could not be recovered, indicating perhaps its lack of viability. We therefore conclude that genes encoding C-9–MT are likely essential for the normal growth and differentiation of *F. graminearum*.

C-9 methylation of GlcCers is not essential for the sensitivity of *F. graminearum* to plant defensins MsDef1 and RsAFP2. We have previously reported that the absence of GlcCers in the ΔFggs1 mutant confers resistance to the antifungal plant defensins MsDef1 and RsAFP2 (25). In order to determine if the C-9 methyl group of GlcCers is required for the antifungal activities of MsDef1 and RsAFP2, we tested the ΔFgmt1 mutant, which produces 65 to 75% nonmethylated GlcCers, for its sensitivity to these defensins. The ΔFggs1 and PH-1 strains were used in these experiments as defensin-resistant and defensin-sensitive controls, respectively. When the ΔFgmt2 conidia were allowed to germinate in SFM without MsDef1 for 36 h, their phenotype resembled that of PH-1 treated with 1.5 μM MsDef1. In contrast, the ΔFggs1 mutant, which makes no GlcCers, grew like PH-1. MsDef1 inhibited the growth of PH-1 with a 50% inhibitory concentration of 1.5 to 3.0 μM, while inducing a strong hyperbranching effect (25). When exposed to these concentrations of MsDef1, the ΔFgmt1 mutant did not exhibit increased hyperbranching. Higher concentrations of this defensin caused some growth inhibition of this mutant, without a concomitant increase in hyperbranching typically observed with PH-1. The ΔFggs1 knockout, in contrast, showed strong resistance to MsDef1, as reported previously (Fig. 6) (25). As expected, the ΔFgmt1 mutant and the ΔFgmt2 mutant complemented with the FgMT2 gene were as sensitive to MsDef1 as the wild-type strain, PH-1. In addition, the ΔFgmt2 mutant did not show obvious resistance to RsAFP2, which has been previously shown to interact with GlcCers (Fig. 7).

Because of our inability to generate a ΔFgmt1 ΔFgmt2 mutant producing only nonmethylated GlcCers, it was important to test the antifungal activities of MsDef1 and RsAFP2 against the *P. pastoris* Ppmt1 knockout, which produces only nonmethylated GlcCers, as mentioned above (35). *P. pastoris* has previously been shown to be sensitive to RsAFP2, and the ΔPpmt1 mutant, which produces only nonmethylated GlcCers, has no apparent growth defects (Fig. 8A). Strain GS115 was used to generate the ΔPpmt1 mutant. For comparison, we also tested the ΔPpgcs1 mutant, which has previously been shown to be resistant to RsAFP2 (37). The antifungal activities of MsDef1 and RsAFP2 against the mutant strains and the isogenic strain GS115 were determined by measuring the growth of the strains in liquid cultures. As reported previously, RsAFP2 strongly inhibited the growth of strain GS115 in the nutrient-rich YPD medium. However, the ΔPpmt1 mutant showed no increase in the level of resistance to this defensin and was as sensitive to this defensin as strain GS115. As expected, the ΔPpgcs1 mutant showed strong resistance to this defensin at concentrations of 6, 9, and 12 μM (Fig. 8B). MsDef1 has weak antifungal activity against GS115 in YPD medium. We therefore decided to test its activities in SFM against the ΔPpmt1 and GS115 strains transformed with HIS4

![FIG. 3. The FgMT1 and FgMT2 genes complement the *P. pastoris* C-9–MT-null mutant (ΔPpmt mutant). C₁₈ reverse-phase HPLC analysis was performed on the o-phthaldialdehyde derivatives of sphingolipid C₉ CBs prepared from GlcCers isolated by TLC from cells of wild-type (Wt) *P. pastoris* GS115 (A), the ΔPpmt mutant, lacking sphingolipid C-9-ΔMT (B), the ΔPpmt mutant complemented with FgMT1 (C), and the ΔPpmt mutant complemented with FgMT2 (D).](image-url)
strain GS115 carries a mutation in HIS4 and therefore does not grow in SFM. MsDef1 inhibited the growth of the HIS4/H11001 wild-type strain. The HIS4/Ppmt1 mutant, again, failed to exhibit increased resistance to MsDef1 in SFM (data not shown). We therefore conclude that C-9 methylation of GlcCers is not required for the antifungal action of the plant defensins MsDef1 and RsAFP2.

C-9 methylation of GlcCer is important for fungal virulence. We have reported previously that the role of GlcCers in the virulence of F. graminearum is host dependent (25). Deletion of the FgGCS1 gene adversely affected the growth and development of this pathogen in vitro and markedly reduced its virulence in wheat. However, in the model host A. thaliana, the ability of this pathogen to cause disease was FgGcs1 independent, and the ΔFggs1 mutant retained full virulence, like PH-1 (25). We therefore sought to determine if the C-9 methylation of GlcCers was an important regulator of the virulence of F. graminearum in different host plants and if its impact on the virulence of this fungus was also host dependent.

F. graminearum causes infection of flowering wheat heads, leading to bleaching of the infected spikelets (12). ΔFgmt1 and ΔFgmt2 strains were used to point inoculate the ears of the susceptible wheat cultivar Norm as described previously (25). The ΔFgmt1 strain infected the inoculated spikelet and spread to the adjacent five to six spikelets. The disease index of 29% for this mutant was similar to that for PH-1. In contrast, the disease index for the ΔFgmt2 strain was 6% (Table 2). This mutant infected only the inoculated spikelet and rarely spread to the adjacent spikelet. Complementation of the ΔFgmt2 mutant with the wild-type FgMT2 gene restored the wild-type levels of virulence and aggressiveness in the complemented strain (data not shown). For comparison, we also tested the ΔFgcs1 mutant, which had previously been shown to be less pathogenic than PH-1 on wheat heads (25). As expected, the disease index for the ΔFgcs1 mutant in wheat was 13% (Fig. 9). The ΔFgmt1 and ΔFgmt2 mutants were also tested for their abilities to infect and colonize corn silk and tomato fruit. The
ΔFgmt2 mutant exhibited much reduced virulence in these host tissues, whereas the ΔFgmt1 mutant was similar to PH-1 (see Fig. S3A and B in the supplemental material).

Since the disease-causing ability of the ΔFgcs1 mutant lacking GlcCers in A. thaliana was earlier reported to be markedly different from that in wheat (25), we tested the virulence of the ΔFgmt1 and ΔFgmt2 mutants in A. thaliana. The virulence of mutant strains in susceptible A. thaliana (Col-O) was determined using a leaf infiltration assay as described previously (22). In plants infected with either a mutant or PH-1, yellowing and water-soaked lesions around the site of infection were observed at 3 days postinoculation. Five days postinoculation, as symptoms advanced, the spreading of the mycelia was more prominent on the leaves of plants infected with the ΔFgmt1 mutant or PH-1 (Fig. 10A). However, in ΔFgmt2-infected plants, fungal hyphae spread much less and significantly more slowly than in PH-1-infected plants. At 7 days postinoculation, ΔFgmt2 mutant-infected plants showed only chlorosis of the leaves; no prominent mycelia or detachment of the leaves was observed. In contrast, the progression of disease in ΔFgmt1 mutant-infected plants was similar to that in PH-1-infected plants (Fig. 10A). As the disease progressed through the ninth day after inoculation, the disease symptoms of the ΔFgmt2 mutant-infected plants were almost similar to those of the ΔFgmt1 mutant- and PH-1-infected plants (Table 3). The infiltrated leaves became dry and covered with mycelia, leading to the detachment of some of the leaves (Fig. 10A). Thus, the development of disease symptoms is significantly delayed in ΔFgmt2 mutant-infected plants.

The fungal biomass from infiltrated leaves was quantified as previously described at 2, 5, and 7 days postinoculation (25). When ΔFgmt1 conidia were examined microscopically, at 2 days postinoculation, germination was similar to that of PH-1, with no significant branching of the germ tubes observed. Interestingly, in leaves inoculated with the ΔFgmt2 mutant, germination of the conidia was much slower, and hyphae were very short. At 5 days postinoculation, several clusters of secondary conidia and patches of cell death were found in the inoculated leaves of ΔFgmt1 mutant- and PH-1-infected plants, leading to complete collapse of the entire leaf by 10 days postinoculation. In leaves inoculated with the ΔFgmt2 mutant, the hyphae were just branching and started to spread inter- and intracellularly, with the aerial hyphae invading the leaves predominantly through the stomatal pores, forming a dark stain around the stomata (Fig. 10B). At 7 days postinoculation, the hyphae spread rapidly throughout the host tissue, and severe cell death was observed in the inoculated leaves of ΔFgmt1 mutant- and PH-1-infected plants, forming extensive patches of dark staining in the intercellular space. However, in plants inoculated with the ΔFgmt2 mutant, no rapid spread of the mycelia was observed. In addition, very few clusters of secondary conidia and patches of cell death were found in the inoculated leaves (Fig. 10B). In contrast, the ΔFgcs1 mutant, lacking GlcCer altogether, exhibited full virulence on foliar tissues, as reported previously (25). In conclusion, disease severity caused by the ΔFgmt1 mutant was similar to that caused by PH-1 both visually and microscopically. However, the virulence of the ΔFgmt2 mutant was severely impaired, and it was

![Image](https://example.com/image.png)

**TABLE 1.** Comparison of growth patterns of the wild-type strain, PH-1, and mutants

| Strain          | Mycelial growth* (±SD) | No. of cells per conidium* | Conidial length (µm)* |
|-----------------|------------------------|---------------------------|-----------------------|
|                 | 3rd day                | 5th day                   |                       |
| PH-1            | 40.8 (±1.5)            | 88.3 (±1.3)               | 4.8                   |
| ΔFgmt1          | 42.8 (±2.2)            | 89.0 (±1.2)               | 4.7                   |
| ΔFgmt2          | 16.3 (±1.7)            | 23.0 (±1.2)               | 2.7                   |
| ΔFgmt2 + FgMT2  | 41.0 (±2.5)            | 88.3 (±1.0)               | 4.7                   |
| ΔFgcs1          | 21.0 (±0.8)            | 32.8 (±1.3)               | 3.4                   |

*Expressed as the mean colony diameter (in centimeters) for four replicates of each strain.

*Means for 50 conidia of each strain.
very slow in colonizing the infected leaves. We conclude that the ΔFgmt2 mutant, which produces largely nonmethylated GlcCer, is much less virulent than the ΔFgcs1 mutant, which results in complete loss of GlcCer.

**DISCUSSION**

Because of their unique structural characteristics, fungal GlcCers have sparked much interest in understanding their biological functions in fungal growth, differentiation, and pathogenesis by using forward- and reverse-genetics approaches (27, 43). At present, the roles of GlcCers in plant-pathogenic fungi remain relatively unknown. We recently reported that the growth of the ΔFgcs1 mutant of *F. graminearum*, devoid of GlcCers, was retarded but that its ability to cause disease was strongly influenced by the host plant. This null mutant also exhibited a high level of resistance to the plant defensin MsDef1 (25). A C-9 methyl substituent of the LCB is a unique structural feature distinguishing fungal GlcCers from their plant and mammalian counterparts (2, 43). In this report, we have examined critical functions of the C-9 methylation of this sphingolipid in *F. graminearum*. We have identified two genes, *FgMT1* and *FgMT2*, encoding a C-9–MT enzyme in this fungus. Whereas the unicellular fungi *P. pastoris* and *C. neoformans* apparently have a single gene for this enzyme, some fungi, including *Aspergillus nidulans*, have two (35). The *FgMT1* and *FgMT2* genes encode C-9–MTs that share substantial sequence identity with their homologs from other fungi. Both genes complement the *P. pastoris* mutant *Ppmt1*, lacking C-9–MT activity, indicating that they encode functional enzymes. *FgMT1* and *FgMT2* are likely to be *S*-adenosyl-L-methionine (SAM)-dependent membrane-bound enzymes, since their deduced amino acid sequences contain the two predicted N-terminal transmembrane domains as well as the SAM-binding motifs present in the C-9–MT enzyme of *P. pastoris* (35).

The characterization of the *Fgmt1* and *Fgmt2* knockout
strains reported here has provided significant insight into the functional roles of these enzymes in vivo. Our results indicate partial redundancy of the C-9–MT gene function in *F. graminearum*. Surprisingly, deletion of the FgMT1 gene alone produced no detectable changes in the levels of C-9-methylated GlcCer from those in the wild-type, PH-1. Moreover, no obvious deleterious effects on the growth and differentiation of *F. graminearum* could be detected. In contrast, deletion of the FgMT2 gene had a dramatic impact on the GlcCer composition of the fungus, resulting in a 65 to 75% reduction in the level of C-9-methylated GlcCer. This alteration of GlcCer composition was accompanied by severe growth defects in the fungus, as indicated by extremely retarded growth in both solid and liquid media, lack of aerial hyphae, and the production of extremely small 2- to 3-celled conidia as opposed to the 5- to 6-celled conidia produced by PH-1. It is particularly intriguing that the ΔFgges1 mutant, which produces no GlcCer at all (25), exhibits less severe growth defects than the ΔFgmt2 mutant, which produces 75 to 80% nonmethylated and 20 to 25% methylated GlcCer. This observation is significant because it indicates that the growth and differentiation of a filamentous fungus are greatly influenced by the degree of C-9 methylation of GlcCer. Further support for the essential nature of C-9-methylated GlcCer comes from the fact that a ΔFgmt1 ΔFgmt2 double-knockout strain could not be generated, indicating perhaps its lack of viability. The C-9-methylated GlcCer may also be essential for the model ascomycetous fungus *N. crassa*, since a knockout mutant of its C-9–MT gene (Broad Institute Neurospora crassa database accession number NCU07859.3) is available only as a heterokaryon (http://www.dartmouth.edu/~neurosporagenome/obtaining_knockouts.html). The purity of the heterokaryon, however, needs to be confirmed before a definitive statement can be made regarding the essential nature of C-9–MT in *N. crassa*. C-9–MT is not essential in *P. pastoris*, since the mutant strain with its gene deleted is fully viable and exhibits no apparent growth defects (35). Filamen-

FIG. 7. Sensitivities of the ΔFgmt1 and ΔFgmt2 mutants to the plant defensin RsAFP2. Conidia of the wild-type strain (PH-1), the ΔFgmt1 and ΔFgmt2 mutants, the ΔFgmt2 mutant complemented with FgMT2, and the ΔFgges1 mutant, suspended in SFM, were incubated with twofold serially diluted RsAFP2 in the dark. Images were taken after 36 h of incubation. Bar, 50 μm.

FIG. 8. Sensitivities of the *P. pastoris* ΔPpges1 and ΔPpmnt mutants to the plant defensin RsAFP2. (A) The *P. pastoris* strains were grown on solid YPD medium. The initial population loads spotted onto the medium are given on the right. (B) Cells were grown in the liquid medium in the presence of the indicated concentrations of RsAFP2 for 36 h. Growth was monitored by measuring absorbance at 595 nm. Growth inhibition was measured quantitatively. The values are means of three replications. Error bars indicate standard deviations.
tous fungi exhibit greater morphological complexity during their life cycle and produce differentiated cells such as conidia, the fruiting body, and infection structures. One can speculate that perhaps FgMT2 is involved in morphological changes associated with the more complex filamentous life cycle of F. graminearum and FgMT1 plays a role similar to that of PpMT1 in P. pastoris. The functional roles of C-9–MT need to be examined in more fungi in order to determine if they perform critical functions that are unique to multicellular filamentous fungi.

In the proposed overall biosynthetic pathway of fungal GlcCer, the C-9–MT enzymatic step follows the Δ3-desaturase enzymatic step but precedes the Δ3-desaturase step and the last GCS enzymatic step (27, 43). The structure of C-9-methylated GlcCer of F. graminearum is identical to that of P. pastoris, and the enzymatic steps leading to the synthesis of GlcCer seem to be conserved between the two fungi. The P. pastoris SAM-dependent C-9–MT specifically uses C18 α-OH-Δ4,8-ceramide as a substrate (35). Since both FgMT1 and FgMT2 complement the C-9–MT-deficient mutant of this yeast, we believe that C18 α-OH-Δ4,8-ceramide is the endogenous substrate for these enzymes in F. graminearum. In this study, we have not identified the endogenous substrates of the FgMT1 and FgMT2 enzymes. However, based on our characterization of the ΔFgmt1 and ΔFgmt2 mutants, the possibility that the FgMT1 and FgMT2 enzymes use alternate substrates besides C18 α-OH-Δ4,8-ceramide cannot be ruled out. Fungal C-9–MTs belong to a superfamily of SAM-dependent MTs that are capable of methylating a large variety of substrates and show striking similarity to cyclopropane lipid synthases from plants and bacteria (35). A SAM-dependent O-MT (OMT2) enzyme from the hop (Humulus lupulus) was recently shown to accept a broad range of substrates (23). Even though C-9–MT activity and the ability to synthesize GlcCer have been lost in certain yeasts and, furthermore, the P. pastoris strain lacking this enzyme is fully viable and does not show impaired growth, it is likely that deletion of both FgMT1 and FgMT2 is lethal to F. graminearum. Our data indicate that FgMT2 is more important than FgMT1. This may be due to differences in the expression patterns of FgMT1 and FgMT2. It is likely that FgMT2 is expressed constitutively at a higher level than FgMT1. Alternatively, FgMT1 may be expressed only during certain stages of the fungal life cycle, or perhaps in response to specific environmental cues. In the F. graminearum expressed sequence tag (EST) database (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html), two ESTs for FgMT2 exist, but none for FgMT1. An in-depth analysis of the expression of these two genes in F. graminearum is necessary to further address this issue.

One of the unexpected findings during the past decade has been that certain plant defensins, as part of their antifungal action, bind to fungal sphingolipids (36, 38). RsAFP2 interacts specifically with GlcCer in P. pastoris and C. albicans (37). We have recently shown that a null mutation of the FgGCS1 gene results in a mutant strain of F. graminearum that makes no GlcCer and exhibits strong resistance to MsDef1 (25). Since the presence of a C-9 methyl group is a unique feature of fungal GlcCer, we investigated whether C-9 methylation of GlcCer is required for the antifungal activities of MsDef1 and RsAFP2 against F. graminearum in vitro. Although the ΔFgmt2 strain at least partially mimics the phenotype of MsDef1-treated PH-1 (Fig. 6), the data presented here have revealed that the presence of a C-9 methyl group in GlcCer is not required for the antifungal activities of these defensins against F. graminearum as well as P. pastoris. Thus, C-9 methylation of GlcCer does not account for the lack of defensin toxicity against plant and mammalian cells. It has been reported that some fungi, especially euascomycetes, contain GlcCers with a Δ3-desaturated fatty acid (43). GlcCers of F. graminearum also contain a Δ3-desaturated fatty acid. Thus, it will be of interest to determine the importance of this double bond in the antifungal activities of MsDef1 and RsAFP2. To our knowledge, no fungal Δ3-desaturase gene has been cloned yet. The presence or absence of this double bond in the fatty acid of fungal GlcCers may also explain why these defensins show potent antifungal activity against some but not all fungi. It has also been reported recently that plant and insect defensins with similar 3-dimensional structures also bind to different structural motifs of fungal GlcCer. Thus, the interaction of GlcCer with RsAFP2 could not be competed for by the insect defensin heliomycin, and vice versa (34). In addition, heliomycin binds

\[ \text{PH1} \quad \Delta \text{Fgmt1} \quad \Delta \text{Fgmt2} \quad \Delta \text{gcs1} \]

FIG. 9. Pathogenicities of the ΔFgmt1 and ΔFgmt2 mutants and PH-1 in wheat. Wheat heads were infected by injecting conidial suspensions of the indicated strains into the fifth spikelet from the base of the wheat head. The plants were photographed at 14 days postinoculation.

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**TABLE 2. Disease severity in wheat inoculated with wild-type PH-1 or mutant strains**

| Strain | Disease index (±SE) | t test significance |
|--------|---------------------|-------------------|
| PH-1   | 28.71 (±1.05)       |                   |
| ΔFgmt1 | 29.35 (±1.32)       | 0.83              |
| ΔFgmt2 | 6.34 (±0.43)        | <0.0001           |
| ΔFgcs1 | 13.22 (±0.91)       | <0.0001           |

* Determined by the mean number of symptomatic spikelets at 21 days after inoculation.

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to both plant and fungal GlcCers, whereas RsAFP2 binds only to fungal GlcCer. Apparently, some defensins have evolved to bind to different sphingolipids, whereas other defensins interact with different structural motifs of a specific sphingolipid. The modes of action of many other defensins do not involve binding to any sphingolipids (1). More studies are needed to fully understand the specificity of the antifungal action of plant defensins.

Filamentous fungi use a wide variety of lipid molecules for signaling involved in physiological or developmental processes (32). It is therefore not surprising that GlcCers are important regulators of pathogenicity in human fungal pathogens (27). However, their roles in plant-fungus interactions are poorly understood. We recently reported that GlcCers are not required for the pathogenicity of *F. graminearum* in plants but that their roles in fungal virulence were greatly influenced by the host plant used (25). The \( \Delta gcs1 \) mutant of *F. graminearum* exhibits greatly reduced virulence in wheat but retains full virulence on the foliar and floral tissues of *A. thaliana*. Although Fg*GCS1* affects the growth and development of *F. graminearum* in vitro, this study revealed that full disease formation in *A. thaliana* tissues occurs even in the absence of GlcCer synthesis. A comparison of the virulence of the \( \Delta fgmt1 \) and \( \Delta fgmt2 \) mutants, producing different levels of C-9-methylated GlcCer on these host plants, is therefore of particular importance. Because the \( \Delta fgmt1 \) mutant exhibits PH-1-like characteristics, it is not surprising that its virulence was similar to that of the wild-type strain and the \( \Delta fgmt2 \) mutant.

| Strain          | Disease severity* (±SD) on the following day after inoculation: |
|-----------------|---------------------------------------------------------------|
|                 | 2                | 5                | 7                | 9                |
| PH-1            | 0.42 (±0.04)     | 0.88 (±0.08)     | 0.94 (±0.06)     | 0.95 (±0.05)     |
| \( \Delta gcs1 \) | 0.45 (±0.04)     | 0.78 (±0.09)     | 0.82 (±0.1)      | 0.90 (±0.06)     |
| \( \Delta fgmt1 \) | 0.52 (±0.08)     | 0.86 (±0.08)     | 0.87 (±0.09)     | 0.87 (±0.08)     |
| \( \Delta fgmt2 \) | 0.45 (±0.06)     | 0.65 (±0.1)      | 0.68 (±0.09)     | 0.80 (±0.07)     |

* Mean disease severity values for 80 infiltrated leaves.
on both wheat and *A. thaliana*. The ∆fgmt2 mutant, however, exhibits greatly diminished virulence on wheat and much-delayed symptom development in *A. thaliana*. In plant-pathogenic fungi, a majority of genes important for pathogenicity have been found to be involved in signaling cascades and metabolic pathways. It is likely that C-9-methylated GlcCer plays an important role in signaling mechanisms governing the successful interaction of *F. graminearum* with its host. In *C. neoformans*, GlcCer is a component of the extracellular vesicles used to transport capsular polysaccharide and proteins synthesized inside the cell (29, 30). It is known that *F. graminearum* secretes various extracellular enzymes that are hypothesized to be important for host infection. Thus, a secreted triacylglycerol lipase is an important virulence factor of this fungus (42). It will be of interest to determine if there is a connection between C-9-methylated GlcCer and the secretion of this lipase and other enzymes in *F. graminearum*. Surprisingly, the ability of this knockout mutant to infect and colonize both host plants is significantly less than that of the ∆fgmcs1 mutant, which makes no GlcCer. The inability of the ∆fgmcs1 mutant to cause disease symptoms during the early stages of interaction with *A. thaliana* may not be the consequence of its poor ability to grow on the solid or liquid media in vitro, because the ∆fgmcs1 mutant, which also grows poorly on solid medium, grows vigorously in *A. thaliana* and causes disease symptoms like those caused by PH-1. It is clear that FgMT2 is required for full expression of disease in both wheat and *A. thaliana*. In contrast, FgGCS1 is required for full expression of disease in wheat but not in *A. thaliana* (25). The contribution of C-9 methylation of GlcCers to fungal virulence thus appears not to be influenced by the host. How *A. thaliana* is able to compensate for the complete loss of the C-9-methylated GlcCers in this pathogen, allowing it to be fully virulent, but is not able to compensate for the loss of the C-9 methylation of GlcCers remains unclear. Two possibilities are supported by our data: (i) the knockout mutant unable to synthesize GlcCer is resistant to endogenous defensins expressed in the foliar tissue of *A. thaliana*, whereas the mutant with FgMT2 deleted is sensitive to them, or (ii) the ∆fgmcs1 mutant has more pronounced growth defects than the ∆fgmcs1 mutant in planta (Fig. 6). The *F. graminearum- A. thaliana* model pathosystem is well suited for further addressing this issue, even though functional roles of some fungal genes in this pathosystem may turn out to be different from those in the wheat-*F. graminearum* pathosystem (10). Collectively, our study documents, for the first time, to our knowledge, critical biological roles of the sphingolipid C-9–MTs in an economically important filamentous fungal pathogen of plants.

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