Transducin Beta-Like Gene FTL1 Is Essential for Pathogenesis in Fusarium graminearum

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Fusarium head blight caused by Fusarium graminearum is an important disease of wheat and barley. In a previous study, we identified several mutants with reduced virulence by insertional mutagenesis. A transducin beta-like gene named FTL1 was disrupted in one of these nonpathogenic mutants. FTL1 is homologous to Saccharomyces cerevisiae SIF2, which is a component of the Set3 complex involved in late stages of ascospore formation. The Δftl1 mutant was significantly reduced in conidiation and failed to cause typical disease symptoms. It failed to colonize the vascular tissues of rachis or cause necrosis on the rachis of inoculated wheat heads. The Δftl1 mutant also was defective in spreading from infected anthers to ovaries and more sensitive than the wild type to plant defensins MsDef1 and osmotin. However, the activation of two mitogen-activated protein kinases, Mgv1 and Gpmk1, production of deoxynivalenol, and expression of genes known to be important for plant infection in F. graminearum were not affected, indicating that the defect of the Δftl1 mutant in plant infection is unrelated to known virulence factors in this pathogen and may involve novel mechanisms. The Δftl1 deletion mutant was significantly reduced in histone deacetylation, and many members of the yeast Set3 complex are conserved in F. graminearum. FTL1 appears to be a component of this well-conserved protein complex that plays a critical role in the penetration and colonization of wheat tissues.

The filamentous ascomycete Fusarium graminearum (teleomorph Gibberella zeae) is the main causal agent of Fusarium head blight (FHB), or scab, which is an important disease on wheat and barley throughout the world (18). It also causes stalk and ear rots of maize and infects other small grains. In addition to causing yield losses, this pathogen often contaminates infested grains with trichothecene and estrogenic mycotoxins, such as deoxynivalenol (DON) and zearalenone. Unfortunately, complete resistance to F. graminearum is lacking in wheat, and fungicide application is not cost-effective for FHB control in wheat and barley. F. graminearum overwinters in infected plant debris and produces ascospores in the spring. Ascospores are forcibly discharged from mature perithecia (52) and function as the primary inoculum for FHB. The multicellular conidia or macrogametes are important for spreading the disease in the field and colonizing plant vegetative tissues. Wheat spikes are most susceptible to FHB at anthesis (34a). Although F. graminearum can colonize glumes, anthers are the main site of primary infection on flowering wheat heads (3, 38). Earlier studies indicated that wheat anther extracts stimulate F. graminearum virulence on wheat. Choline and glycine betaine were identified as two major components in anthers that stimulate fungal growth and predispose wheat to F. graminearum infection (50, 51). Under conducive conditions, the fungus can spread from the infected floret along the rachis and cause severe damage. The production of DON, the first virulence factor identified in F. graminearum (11, 42), is not necessary for the initial infection but is important for the spread of FHB on infected wheat heads (2).

In the past few years, genetic and genomic studies of F. graminearum have advanced significantly. The genome of F. graminearum has been sequenced (10) and a whole-genome microarray of this haploid homothallic fungus is commercially available (21). A number of pathogenicity or virulence factors have been identified by insertional mutagenesis or targeted gene deletion approaches. Two mitogen-activated protein (MAP) kinase genes, MGV1 and GPMK1, are essential for pathogenicity in F. graminearum (23, 24). Genes that are important for full virulence in F. graminearum on wheat include FGL1 (54), GzCPS1 (31), FBP1 (22), FSR1 (48), SID1 (19), NPS6 (37), RAS2 (5), GzGPA2 and GzGPB1 (56), and HMR1 (47). These virulence-associated genes encode proteins with various biochemical activities, such as lipase, nonribosomal peptide synthase, Ras protein, and 3-hydroxy-3-methylglutaryl coenzyme A reductase. Several genes involved in the primary metabolism, such as the CBL1, RSY1, GzHIS7, ADE5, and ARG2 genes (29, 44, 46) that are required for methionine, histidine, and arginine syntheses, also have been implicated in plant infection in F. graminearum. Overall, molecular mechanisms underlying F. graminearum pathogenesis appear to be complex and remain to be fully understood.

In a previous study, we identified 11 restriction enzyme-mediated integration (REMI) mutants that are defective in plant infection (46). In one of these mutants, the transforming

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vector was inserted in a predicted gene named FTL1 (for *Fusarium graminearum* beta-like gene 1). FTL1 is homologous to the mammalian TBL1 or TBLR1 genes (40, 55) and the *Sacccharomyces cerevisiae* SIF2 gene (8). The products of these genes are components of protein complexes involving histone deacetylases (HDACs). In mammalian cells, TBL1 and TBLR1 are parts of the N-CoR/SMRT/HDAC complexes (40). In yeast, SIF2 is a part of the Set3 complex regulating ascosporogenesis. In *F. graminearum*, the Δftl1 gene replacement mutant was significantly reduced in conidiation and failed to cause typical head blight symptoms on flowering wheat heads. It failed to colonize vascular tissues or cause necrosis on the rachis of inoculated wheat heads. The Δftl1 mutant also was defective in spreading from infected anthers to ovaries and was more sensitive than the wild type to plant defensins MsDef1 and osmotin. Although it was normal in the production of deoxynivalenol and the expression of known virulence factors, the Δftl1 mutant was significantly reduced in HDAC activities. FTL1 appears to be a component of this well-conserved HDAC complex that plays a critical role in the penetration and colonization of wheat tissues.

**Materials and Methods**

**Culture conditions and fungal transformation.** The *F. graminearum* wild-type strain PH-1 (NRRL 31084) and transformants generated in this study (see Table 3, below) were cultured at 25°C on V8 juice agar or minimal medium (MM) plates (23, 53). Transformant GZT501 expressing the green fluorescent protein (GFP) construct pTEFEGFP (49) was kindly provided by Robert Proctor at USDA Agricultural Research Service. Protofibril preparation and fungal transformation were performed as described previously (42). Complete medium (CM) supplemented with 250 μg/ml hygromycin B (Calbiochem, La Jolla, CA) or 150 μg/ml Genticin (Sigma, St. Louis, MO) was used for selection of transformants (23). DON and ergosterol production were measured with rice grain cultures as described previously (43). Sensitivities to MsDef1 (43) and osmotin (9) were measured by assaying for each test and all tests were repeated four times.

**Generation of the Δftl1 mutant.** The *FTL1* gene replacement vector pKY56 was constructed by the ligation-PCR approach (58). A 0.96-kb upstream fragment and a 0.92-kb downstream fragment were amplified with primers DL1F/ H9004 and DL2R/ H9004 and cloned into pGEM-T Easy as pYK56, which was used to transform PH-1 protoplasts. Hygromycin-resistant transformants were screened by PCR with primers TN1F and TN2R (see Fig. 2A, below) and further characterized by Southern blot analysis. For complementation assays, a 4.5-kb fragment containing the *FTL1* gene was amplified with primers TC3F and TC4R (Table 1) and cloned into pGEM-T Easy as pZMH12 (see Fig. 2A), which was cotransformed with pSM289 (23) into the Δftl1 mutant T1. To construct the GFP reporter strain, the enhanced GFP (EGFP) cassette under the control of the *Magnaporthe grisea* RP27 promoter was amplified from pKB04 (7) with primers GFP-R and GFP-F (Table 2). The Geneticin resistance gene was amplified from pSM289 with primers NeoF and NeoR. About 10 μl of each of the resulting PCR products was cotransformed into protoplasts of the Δftl1 mutant T1. Geneticin-resistant transformants expressing the EGFP cassette were examined for GFP signals and analyzed by Southern blot analysis.

**FTL1-GFP fusion and FTL1Δftl1 constructs.** The FTL1-GFP and FTL1Δftl1 constructs were generated by the yeast gap-repair method (7). A 4.5-kb fragment of the *FTL1* gene was amplified with primers TDDYC3F and TDD1R (Table 1) and cotransformed with *XhoI*-digested pKB04 into *S. cerevisiae* X1K1-25 (7). Plasmid pZH12 was rescued from the Trp* yeast transformant. The FTL1-GFP fusion construct was confirmed by sequence analysis and transformed into PH-1. For constructing the FTL1Δftl1 allele, primers TDDYC3F and TDD1R were used to amplify the first seven codons and 1.6-kb upstream promoter region of FTL1. The rest of *FTL1* was amplified with primers TDD2F and TDDYC4R. The resulting PCR products were cotransformed into X1K1-25 with *XhoI*-digested pKB04. Plasmid pZMH12 was rescued from the resulting Trp* yeast transformant and confirmed by sequence analysis to contain the expected deletion of amino acid residues 8 to 40.

**Wheat infection assays.** Conidia were collected from 5-day-old CMC (1.5% carboxymethylcellulose, 0.1% NH4NO3, 0.1% KH2PO4, 0.05% MgSO4, 7H2O, 0.1% yeast extract) cultures and resuspended in 0.01% (vol/vol) Tween 20 to a concentration of 106 conidia/ml. Six-week-old plants of wheat cultivar Norm were used for inoculation at flowering as described previously (15, 26). The fifth, full-sized spikelet from the base of the inlorescence was injected with 10 μl of the spore suspension. Inoculated plants were placed in a humidity chamber for 3 days and then transferred to ambient growth conditions in a greenhouse. Discoloration in the rachis was examined by removing wheat florets. For assaying disease indices, symptomatic spikelets in each head were counted 14 days postinoculation (dpi). At least nine inoculated wheat heads per treatment were used for each test and all tests were repeated four times.

For inoculation assays with detached flowering wheat heads, conidia were

### Table 1. Wild-type and mutant strains of *Fusarium graminearum* used in this study

| Strain    | Genotype description             | Reference |
|-----------|----------------------------------|-----------|
| PH-1      | Wild type                        | Traill et al. (53) |
| 11622     | nit1 mutant of the wild-type strain G3639 | Hou et al. (23) |
| GZT501    | Transformant of G3639            | Skadsen and Hohn (49) |
| M75       | RE1 mutant of PH-1               | Seong et al. (46) |
| T1        | Δftl1 mutant                     | This study |
| T2        | Δftl1 mutant                     | This study |
| T3        | Δftl1 mutant                     | This study |
| TC1       | Complementation strain of Δftl1 mutant T1 | This study |
| TC3       | Complementation strain of Δftl1 mutant T1 | This study |
| TDD1      | *FTL1Δftl1-GFP* (pZMH13) in Δftl1 mutant T1 | This study |
| TDD2      | *FTL1Δftl1-GFP* (pZMH13) in Δftl1 mutant T1 | This study |
| TDD3      | *FTL1Δftl1-GFP* (pZMH13) in Δftl1 mutant T1 | This study |
| RM1       | Transformant of Δftl1 mutant expressing EGFP | This study |
| TGN1      | *FTL1-GFP* (pZMH12) in PH-1      | This study |
resuspended to 10^5 conidia/ml in 0.25% gelatin. A drop of 1 to 2 μl of the conidium suspension was inoculated onto each exposed anther on detached wheat heads. After incubation at 25°C for 4 to 5 days in a moist chamber, fungal growth and infection of wheat florets were examined under a dissecting microscope. For inoculation assays with unexposed anthers, wheat ovaries with intact paleae were punched with a dissecting needle and inoculated with 1 to 2 μl of conidium suspension on the anther and incubated at 25°C. For infection through wounding, wheat ovaries and paleae were punched with a rotating puncher (50 mm HEPS, pH 7.5, 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 10% glycerol, 200 μM phenylmethylsulfonyl fluoride). After sonication for 20 s and incubation on ice for 30 min, the nuclear suspension was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant containing crude nuclear extracts was used for assaying HDAC activities with the HDAC activity assay kit (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer's instructions. The deacetylation reaction was carried out at 37°C for 30 min and stopped by addition of 40 μl of the HDAC developer. Fluorescent signals were detected with a plate reader (360 nm excitation and 465 nm emission; Synergy HT; Bio-Tek). For assaying HDAC activities with the HDAC activity assay kit (Cayman Chemical Company), the disease index is reported as the mean (± standard deviation) number of symptomatic spikelets at 14 dpi in four independent infection assays.

RESULTS

A transducin beta-like gene FTL1 was disrupted in REMI mutant M75. The REMI mutant M75 was generated by transforming EcoRI-digested pKY37 vector DNA into the F. graminearum strain PH-1 (46). It was defective in plant infection and had a slightly reduced growth rate (Table 3). Plasmids pKY46 and pKY47 (Fig. 1A) were recovered by self-ligation of KpnI- and PstI-digested genomic DNA of M75, respectively. Sequence analysis of these two plasmids revealed that pKY37 was inserted at an EcoRI site (Fig. 1A) of a predicted gene, FGSG_00332.3, which was named FTL1 (for F. graminearum TBLRI-like gene 1). We sequenced the reverse transcription-first-strand cDNA of M75, and had a slightly reduced growth rate (Table 3). Phosphat pKY46 and pKY47 (Fig. 1A) were recovered by self-ligation of KpnI- and PstI-digested genomic DNA of M75, respectively. Sequence analysis of these two plasmids revealed that pKY37 was inserted at an EcoRI site (Fig. 1A) of a predicted gene, FGSG_00332.3, which was named FTL1 (for F. graminearum TBLRI-like gene 1). We sequenced the reverse transcription-first-strand cDNA of M75, and had a slightly reduced growth rate (Table 3).

FIG. 1. Mutant M75 was disrupted in the FTL1 gene. (A) The transforming vector pKY37 was inserted into the EcoRI site (marked by an asterisk) of the FTL1 gene (empty arrow). Plasmids pKY12 and pKY25 were recovered from M75 by self-ligation with PstI- and KpnI-digested genomic DNA, respectively. B, BamH I; E, EcoRI; K, KpnI; P, PstI. (B) The FTL1 gene contains one N-terminal LisH domain (empty box) and WD40 repeats (solid box) at the C-terminal half of the protein.

TABLE 2. PCR primers used in this study

| Primer | Sequence (5′→3′) |
|--------|-----------------|
| DL1F   | CCGAATTCAAAAAACACCAGCATCGG |
| DL2R   | CAGTTTGGCCCGCCACAGATCTAAAGGCTTCG |
| DL3F   | AAGGTTGGCCCGCCGGAAGATACGCTAGGATCTG |
| DL4R   | TTAAATCTGCAACGAGGATC |
| TN1F   | GTAGGATGTTTCGCCTCACG |
| TN2R   | AGGATAGAGGCGTTCTGG |
| TC3F   | AGGAGGATATTCTCTCTGTCATC |
| TC4R   | CAAGAAAATCCTTCAACCACAA |
| TDDYC3F | GGCGAATTGGTCATCTAATTTGGAAGAGCTTCATT |
| TDD1R  | AAAAGCGAACTCTTCAATGAGAAAAAGTTCATT |
| TDD2F  | ATGTTGCATGAAAGATTCTCCATATAGAGATT |
| TDDYC2R | ATTCACCGACGGATTGATTCCTCAAGAAGTCATT |
| NeoF   | ATACCGGGATTAAAGCTTACAATT |
| NeoR   | ATAACCGGGATATAAGGCTTT |
| GFF-P  | GTGACAGGGGCGGAGGCGT |
| GFF-R  | CTAAAGACAGATTATCATATCATATAGC |
| M27F-GFP | GTGCACGAGTTTGTATAGTTTCG |
| M27R-GFP | AAACGACCTGCCGTCCTCATTTATAGGATGTT |
| TY1F   | GGTCGAGAGGTGTCGTTAAGAAGCTCTCGAGG |
| TY2R   | GGCTGCTAGCTGGTCTGTTCCCTTCAT |
| TDDYC1F | CTATATGGGCACTTAGCTCAATAGGTAAGAAG |
| AGCATTCCTT |
| TGD2R  | GAAGCTCCTGCCGGTCTACATGTTGGT |
| TRITF  | GACCAAGGAGGAGGATCTG |
| TB-RM-R2 | TTAATGTTCTGGTCTGCTT CAT |
| FSN1F  | CTACAGACCGAGCAGACAGCCTGCGAAGAC |
| FSN2R  | TTAGCCCTACACTGCTAGCAGAGCAGCCTGAAAT |
| FSN3F  | GTAAGAGGACATGACATAGCGCACGCGGTTTTTGATG |
| FSN4R  | ACCACCTCTCCTCATACAGCATCTCAGTC |

TABLE 3. Defects of the Δftl1 mutants in growth, conidiation, and plant infection

| Strain          | Growth rate (cm/day) | Conidiation (10^6 conidia/ml) | Disease index* |
|-----------------|----------------------|-------------------------------|----------------|
| PH-1 (WT)       | 1.33 ± 0.04          | 293 ± 21.7                   | 6.8 ± 1.2      |
| M75 (REMI mutant) | 0.80 ± 0.03          | 0.7 ± 0.2                    | 0.0 ± 0.0      |
| T1 (Δftl1)      | 0.83 ± 0.04          | 1.3 ± 0.3                    | 0.2 ± 0.1      |
| TDD2 (Δftl1/FYL1Δsat*) | 0.80 ± 0.04   | 0.5 ± 0.1                    | 0.0 ± 0.0      |
| T1C (Δftl1/FYL1Δsat*) | 1.32 ± 0.03   | 290.7 ± 12.7                 | 7.1 ± 0.8      |

* The disease index is reported as the mean (± standard deviation) number of symptomatic spikelets at 14 dpi in four independent infection assays.

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Disease index a
The \\( \Delta ftl1 \) mutants were caused by deletion of the \( FTL1 \) gene, we reintroduced the wild-type allele amplified with primers TC3F and TC4R (Fig. 2A) into mutant T1. The resulting Geneticin-resistant transformant TC1 had a normal vegetative growth rate, conidiation, and virulence on flowering wheat heads (Table 3). \( F. graminearum \) is a homothallic fungus, but it can be forced to outcross. On selfing plates, no protoperithecia or perithecia were observed. When crossing with a \( nit1 \) mutant, \( \Delta ftl1 \) nit1 recombinant progeny were isolated, indicating that the \( \Delta ftl1 \) mutant was female sterile but fertile when mated as the male. From outcross perithecia between mutant T1 and a \( nit1 \) mutant 11622 (23), we isolated 31 hygromycin-resistant ascospore progeny. All of these progeny displayed characteristic phenotypes of the \( \Delta ftl1 \) mutant (data not shown). When scored for growth on CM with 1% potassium chlorate, 14 of them were \( NIT1^+ \) and 19 were \( NIT1^- \), indicating that the observed defect of the \( \Delta ftl1 \) mutant, but not the \( nit1 \) mutation, cosegregated with the \( hph \) marker.

The \( \Delta ftl1 \) mutant was defective in colonizing the rachis. To further characterize the defect of the \( \Delta ftl1 \) mutant in plant infection, we removed the spikelets on the same side of drop-inoculated florets 3 or 6 dpi and examined for necrosis in the rachis (Fig. 3A). In wheat heads inoculated with PH-1, discoloration was observed in the rachis both above and below the inoculation site, indicating that the fungus had spread from the inoculated floret through the rachis to other spikelets. In contrast, the \( \Delta ftl1 \) mutant caused only limited necrosis at the bases of the inoculated floret. Discoloration was not observed in the rachis (Fig. 3A). These observations indicate that the \( \Delta ftl1 \) mutant was defective in spreading from infected florets to the rachis and neighboring florets on wheat heads.

When examined at 6 dpi by TEM, intercellular and intracellular hyphae were readily observed in the cortical and vascular tissues of the wheat rachis next to the kernels inoculated with the wild type (Fig. 3B). In wheat heads inoculated with the \( \Delta ftl1 \) mutant, intercellular, but not intracellular, hyphae were occasionally observed in the cortical tissues (Fig. 3B). However, no hyphal growth was observed in the vascular tissues of the rachis (Fig. 3B). The \( \Delta ftl1 \) mutant appeared to be blocked in intracellular growth and colonization of vascular tissues in the rachis of inoculated wheat heads. This defect may be directly related to the failure of the \( \Delta ftl1 \) mutant in causing typical FHB symptoms and spreading from spikelet to spikelet.

To further confirm this observation, we transformed an RP27-EGFP construct into the \( \Delta ftl1 \) mutant T1. EGFP was constitutively expressed in the conidia and hyphae of the resulting transformant, RM1 (data not shown). In wheat heads inoculated with RM1, hyphal growth was not or only rarely observed in the rachis by confocal microscopy (Fig. 3C). In the wheat heads inoculated with a GFP-tagged wild-type strain, hyphal growth was readily observed in cortical and vascular tissues (Fig. 3C). These results further confirmed that the \( \Delta ftl1 \) gene replacement construct pKY56 (Fig. 2A) was generated by replacing the entire \( FTL1 \) ORF with the hygromycin phosphotransferase \( (hph) \) gene and transformed into the wild-type strain PH-1. Three \( \Delta ftl1 \) mutants, T1, T2, and T3 (Table 2), were identified by PCR with primers TN1F and TN2R and confirmed by Southern blot analysis (data not shown). Similar to REMI mutant M75, the \( \Delta ftl1 \) mutants were significantly reduced in condiation and defective in plant infection (Table 3) but formed conidia of normal morphology. They were normal in DON production in cracked corn cultures and accumulated a reddish pigment in liquid CMC culture (data not shown). Typical WHB symptoms were not observed on spikelets inoculated with the \( \Delta ftl1 \) mutants (Fig. 2B). These three \( \Delta ftl1 \) mutants had the same phenotype, and only one of them, T1, was selected for further characterization.

To determine whether the observed phenotypes of \( \Delta ftl1 \) mutants were caused by deletion of the \( FTL1 \) gene, we re-introduced the wild-type allele amplified with primers TC3F and TC4R (Fig. 2A) into mutant T1. The resulting Geneticin-resistant transformant TC1 had a normal vegetative growth rate, conidiation, and virulence on flowering wheat heads (Table 3). \( F. graminearum \) is a homothallic fungus, but it can be forced to outcross. On selfing plates, no protoperithecia or perithecia were observed. When crossing with a \( nit1 \) mutant, \( \Delta ftl1 \) nit1 recombinant progeny were isolated, indicating that the \( \Delta ftl1 \) mutant was female sterile but fertile when mated as the male. From outcross perithecia between mutant T1 and a \( nit1 \) mutant 11622 (23), we isolated 31 hygromycin-resistant ascospore progeny. All of these progeny displayed characteristic phenotypes of the \( \Delta ftl1 \) mutant (data not shown). When scored for growth on CM with 1% potassium chlorate, 14 of them were \( NIT1^+ \) and 19 were \( NIT1^- \), indicating that the observed defect of the \( \Delta ftl1 \) mutant, but not the \( nit1 \) mutation, cosegregated with the \( hph \) marker.

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FIG. 3. The Δfll mutant was blocked in colonizing the wheat rachis. (A) Rachises of wheat heads inoculated with the wild type (PH-1) and the Δfll mutant strain were examined at 3 (left) or 6 (right) dpi. Wheat kernels on the inoculation side were removed to expose the rachis. The inoculation sites are marked with arrows. (B) TEM images of the cortical (top row) and vascular (bottom row) tissues of the wheat rachis next to the florets inoculated with the wild type (PH-1) or the Δfll mutant (Δfll) at 6 dpi. H, inter- and intracellular hyphae; CW, plant cell wall. Bar, 2 μm. Hyphal growth was only observed in the xylem vessels in wheat heads inoculated with the wild type. (C) Microscopic images of longitudinal sections of rachises and adjacent nodes from wheat heads infected with transformants of the wild type (GZTS01) and Δfll mutant (RM1) that expressed GFP constitutively. At 6 dpi, intracellular hyphae of the wild type colonized the parenchyma tissue in the rachis. Hyphal growth of the Δfll mutant was rare or not observed in the rachis. Left and right panels depict the same areas observed under confocal and differential interference contrast microscopy. A basal level of autofluorescence was visible in the plant cell wall.
mutant is defective in the penetration and colonization of vascular tissues in the rachis.

The Δftl1 mutant failed to infect wheat florets through anthers. Because anthers are the main site of primary infection, we also inoculated detached wheat heads by placing drops of conidium suspension from PH-1 or the Δftl1 mutant on exposed anthers. After incubating at 25°C for 4 to 5 days, extensive fungal growth was visible on florets inoculated with PH-1 (Fig. 4A). When the glumes were removed, discolored ovaries and fungal hyphae were observed inside wheat florets inoculated with PH-1 (Fig. 4A). Under the same conditions, hyphal growth was limited to the anthers inoculated with the Δftl1 mutant and the ovaries remained healthy (Fig. 4A).

We also isolated ovaries with unexposed anthers with drops of conidium suspensions of the wild-type and Δftl1 mutant strains that constitutively expressed GFP. After incubation at 25°C for 3 days, the wild-type colonized the anthers, filaments, and ovaries. Discoloration of the ovaries and abundant hyphal growth (evidenced by strong GFP signals) were observed (Fig. 4B). In contrast, the Δftl1 mutant had limited growth on the inoculated anthers (Fig. 4B). However, the ovaries remained healthy and GFP signals were relatively weak. These observations indicate that the Δftl1 mutant was defective in colonizing wheat ovaries through the anthers and filaments. To further confirm this observation, we examined fungal growth in the filaments connecting inoculated anthers to the ovaries (Fig. 4C). While the wild-type colonized the entire length of the filaments at 2 dpi, the Δftl1 mutant only had limited hyphal growth at the ends next to the anthers (inoculation sites). In most of the samples examined, it failed to spread more than 1/10 of the filaments at 2 dpi.

The Δftl1 mutant is defective in infectious growth in wheat tissues. To further examine defects of the Δftl1 mutant in plant colonization, we wounded developing seeds with a needle and inoculated with F. graminearum conidia. After incubating for 2 days, hyphae growing on the plant surface were gently removed. When examined by epifluorescence microscopy, the wild type had extensive intracellular and extracellular hyphal growth in seed coat tissues (data not shown). Under the same conditions, conidia of the Δftl1 mutant germinated and grew on plant surfaces. However, intracellular growth of infectious hyphae was rarely observed. While the wild type was able to penetrate and colonize neighboring plant cells, hyphal growth of the Δftl1 mutant was limited. In similar infection assays with wounded wheat paleae, extensive growth of infectious hyphae also was only observed in tissues colonized by the wild type at 2 dpi (data not shown). These observations indicate that the Δftl1 mutant was defective in the penetration and invasive growth in seed coat and palea cells.

Increased sensitivity of the Δftl1 mutant to plant defensin MsDef1. Because the Δftl1 mutant was defective in infectious growth, we tested its sensitivity to plant defensin MsDef1 (43). In the presence of 0.5 μM MsDef1, conidium germination and germ tube growth were not affected in either the wild-type or Δftl1 mutant strains. When the concentration of MsDef1 was increased to 2 μM, conidial germination and germ tube growth were normal in the wild-type strain. Under the same conditions, the Δftl1 mutant produced short germ tubes and displayed a hyperbranching phenotype (Fig. 5A). At 10 μM, MsDef1 completely blocked conidium germination in the Δftl1 mutant. Conidial germination and germ tube growth were not affected in the Δftl1 mutant at 10 μM MsDef1, indicating that the Δftl1 mutant was defective in infectious growth in wheat tissues. To confirm this observation, we examined fungal growth in the filaments connecting inoculated anthers to the ovaries (Fig. 4C). While the wild-type colonized the entire length of the filaments at 2 dpi, the Δftl1 mutant only had limited hyphal growth at the ends next to the anthers (inoculation sites). In most of the samples examined, it failed to spread more than 1/10 of the filaments at 2 dpi.
mutant but only stunted germ tube growth in PH-1 (data not shown). These results indicated that the \textit{ftl1} mutant was more sensitive to MsDef1 than the wild type in conidium germination and germ tube growth. MsDef1 may interfere with calcium signaling and polarized tip growth in \textit{F. graminearum}. The \textit{ftl1} mutant also was more sensitive to osmotin, although \textit{F. graminearum} appeared to be more tolerant to this antifungal protein. In the presence of 32\,M osmotin, conidium germination was blocked in the \textit{ftl1} mutant. Under the same conditions, germination was normal for the vast majority of conidia, but germ tube growth was slightly reduced in the wild-type strain PH-1 (data not shown).

In \textit{F. graminearum}, Mgv1 and Gpmk1 are two MAP kinases known to be essential for pathogenesis. The defects of the \textit{ftl1} mutant in wheat infection and enhanced sensitivity to MsDef1 were similar to those of the \textit{gpmk1} and \textit{mgv1} mutants (43). To determine whether Ftl1 is involved in the activation of Mgv1 or Gpmk1, we assayed the activation of these two MAP kinases. In both the \textit{ftl1} mutant and transformant TDD2, phosphorylation of Gpmk1 and Mgv1 was detectable in total proteins isolated from vegetative hyphae (Fig. 5B). The expression and phosphorylation levels of Mgv1 were lower than those of Gpmk1. However, a similar pattern was observed in PH-1 and the \textit{ftl1} mutant. Therefore, deletion of \textit{FTL1} had no obvious effect on the expression or activation of Gpmk1 and Mgv1. Increased sensitivities of the \textit{ftl1} mutant to MsDef1 were unlikely to be related to Gpmk1 or Mgv1 activities.

The LisH domain is essential for \textit{FTL1} function. To determine the role of the LisH domain in Ftl1, we generated a \textit{FTL1}^{Lish} allele and transformed it into the \textit{ftl1} mutant T1. Three resulting transformants expressing the \textit{FTL1}^{Lish} allele, including TDD2 (Table 1), were isolated and confirmed by Southern blot analysis to contain a single copy of the transforming vector (data not shown). Similar to the \textit{ftl1} mutant, TDD2 was defective in growth, conidiation, and plant infection (Table 3; Fig. 6A). It also accumulated a reddish pigment in liquid CMC cultures (data not shown). A 0.55-kb cDNA band was amplified by RT-PCR in both PH-1 and transformant TDD2 (Fig. 7C) with primers TRT5F and TB-RM-R2 (Table 2). The same primer pairs amplified a 0.7-kb band from genomic DNA. These results indicated that the \textit{FTL1}^{Lish} allele was expressed in TDD2 but it failed to complement the defects of the \textit{ftl1} mutant. Therefore, the LisH domain is essential for the function of the \textit{FTL1} gene in \textit{F. graminearum}.

The \textit{ftl1} mutant has reduced HDAC activities. Because the \textit{FTL1} homologs in yeast and mammalian cells are components of a conserved HDAC complex (41, 55), we assayed the HDAC activities in the \textit{ftl1} mutant with an HDAC activity assay kit (Cayman Chemical Company). The \textit{ftl1} mutant was significantly reduced in HDAC activities.
Microarray analysis with the Δftl1 mutant. To determine what genes are regulated by FTL1, we used the F. graminearum GeneChip (21) to conduct microarray analysis of the Δftl1 mutant. In comparison with the wild type, 448 and 168 genes were down- and upregulated, respectively, over fivefold in the Δftl1 mutants in three independent biological replicates. The majority (over 70%) of those genes with significantly altered expression patterns in the Δftl1 mutant encoded hypothetical proteins with unknown biological functions. Thirty-seven of them were unique to F. graminearum. Fourteen putative hydrolase genes were among the downregulated genes, but none of them was homologous to known fungal virulence factors. The polyketide synthase gene PKS12 (FGSG_02324.3), which is responsible for aurofusarin synthesis, was increased 18-fold in the Δftl1 mutant. Interestingly, several other members of the aurofusarin synthesis gene cluster (28, 33), including FGSG_02321.3 (5.1×), FGSG_02325.3 (8.7×), FGSG_02326.3 (9.6×), FGSG_02327.3 (7.8×), FGSG_02328.3 (11.5×), and FGSG_02329.3 (11.2×), also were expressed at higher levels in the Δftl1 mutant, suggesting that FTL1 negatively regulates aurofusarin biosynthesis.

We also checked the expression levels of known virulence factors, including GPMK1, MGV1, FBPI, S1D1, ADE5, ARG2, NPS6, RSY1, CBL1, FSR1, RAS2, ZIFI, TR15, FGL1, GzHSI7, GzCPS1, GzGPA2, GzGPB1, and HMRI. None of these genes had more than twofold changes in their expression levels in the Δftl1 mutant. Therefore, the defect of the Δftl1 mutant in plant infection may involve novel mechanisms. However, with the number of genes affected in the Δftl1 mutant, it was impossible to determine which genes are directly involved in plant infection. To verify the microarray data, we selected eight genes for quantitative RT-PCR (qRT-PCR) analysis. Six of them, FGSG_08347.3, FGSG_01767.3, FGSG_06223.2, FGSG_05973, FGSG_07582.3, and FGSG_11080.3, encoding a putative choline permease, cytochrome P450, trehalase, hoxo transporter, triacylglycerol lipase, and membrane receptor, respectively, were confirmed by qRT-PCR to be significantly downregulated in the Δftl1 mutant (data not shown). Some of these genes may be involved in plant infection processes.

DISCUSSION

In the REMI mutant M75, the transforming vector was inserted at the N terminus of the FTL1 gene. In this study, we generated the Δftl1 gene replacement mutant. Phenotypically, M75 and the Δftl1 mutant were identical, indicating that the original insertion event completely eliminated the function of the FTL1 gene. FTL1 homologs are well conserved in filamentous ascomycetes. The Fusarium oxysporum (FO00731) and Fusarium verticillioides (FO00784) homologs share 79% and 78% identity with FTL1, respectively. However, the functions of this well-conserved gene in hyphal growth and plant infection are not clear, because none of the FTL1 homologs has been functionally characterized in filamentous fungi.

FTL1 homologs also are conserved in yeast and mammals. FTL1 is homologous to the mammalian TBL1 (transducin beta-like) and TBL1R (TBL1-related) genes. TBL1 and TBL1R are two paralogous genes with overlapping functions, and they both interact with N-CoR and SMRT (55, 57). N-CoR and SMRT were initially identified as corepressors for the retinoic acid receptor and thyroid hormone receptor, but they have also been implicated, in association with HDACs, in repression of other transcription factors (17, 20, 30). In S. cerevisiae, the Set3 complex contains Sif2, which is a homolog of TBL1 (8). Interestingly, FTL1 shares higher homology with mouse TBL1R than with yeast SIF2. The Set3 complex is the yeast analog of the mammalian HDAC/SMRT complex (55). It represses early and middle sporulation genes, including key meiotic regulators such as the IDE2 protein kinase and the NDT80 transcription factor (41). In F. graminearum, the Δftl1 mutant was female sterile but was able to mate as a male and produced ascospore progeny. We were able to isolate normal Δftl1 ascospore progeny from a Δftl1 NIT1 × FTL1 nit1 cross. Yeast has no distinction in male or female fertility. Defects in ascospore formation could be observed in the sif2 × sif2 cross. Because of the loss of female fertility, we could not test the fertility of the Δftl1 × Δftl1 cross in this study. Therefore, it remains possible that FTL1 is important for sexual reproduction in F. graminearum. Conidiation is an asexual reproduction process that is absent in yeast. In F. graminearum, conidiation was significantly reduced in the Δftl1 mutant. There is a distinct homolog of Set3 in F. graminearum, M. grisea, and Neurospora crassa. Other members of the yeast Set3 complex include an NAD-dependent HDAC, Hst1, and an NAD-independent HDAC, Hos2. In mammalian cells, the HDAC gene HDAC3 is a member of the N-CoR complex (20). Our data indicated that the Δftl1 mutant was significantly reduced in HDAC activities. The F. graminearum genome has one putative Hst1 homolog and two putative Hos2 homologs. These genes also are well conserved in M. grisea and other filamentous ascomycetes. To date, there are only limited studies on HDACs in phytopathogenic fungi. In Cochliobolus carbonum, the yeast HOS2 homolog HDC1 is required for full virulence (44). Another member of the Set3 complex is the SNT1 gene, which encodes a putative DNA-binding protein and directly interacts with SIF1 (8). The equivalent of Snt1 in the mammalian N-CoR/SMRT/HDAC3 complex is N-CoR or SMRT (57). All Sif2 homologs, including FgSnt1 in F. graminearum and a predicted M. grisea protein (MG09174), have a SANT DNA-binding domain (SM00717) that functions as a unique histone tail-binding module coupling histone binding to enzyme catalysis (6). In F. graminearum, our preliminary data have indicated that deletion of FgSNT1 resulted in similar defects as the Δftl1 mutant (S. Ding and J.-R. Xu, unpublished data). Because these genes are conserved from yeast to human, it is likely that FTL1 and FgSNT1 are components of this well-conserved complex, which may have been adapted to regulate plant infection processes, such as infectious or in planta growth in F. graminearum and other plant pathogenic fungi.

The Δftl1 mutant was normal in DON production, which is the first virulence factor that has been characterized in F. graminearum. The activation of Gpmk1 and Mgv1 was not affected in the Δftl1 mutant either. In the microarray analysis, the expression levels of all the F. graminearum genes that are known to be required for pathogenicity or full virulence were normal in the Δftl1 mutant. Therefore, FTL1 is likely involved in novel mechanisms regulating plant infection processes.
However, the \( \Delta f t l 1 \) mutant had pleiotropic defects. It is difficult to determine which genes are responsible for observed defects of the \( \Delta f t l 1 \) mutant in plant infection based on transcript profiling. In addition, microarray analysis was conducted with RNA isolated from vegetative hyphae, which may not reflect in planta growth conditions.

We noticed that 14 hydrolytic enzyme genes had reduced expression levels in the \( \Delta f t l 1 \) mutant, including three putative triacylglycerol lipase genes that are unrelated to \( F G L 1 \) (54). No putative hydrolase genes were upregulated in the \( \Delta f t l 1 \) mutant. Among eight genes selected for verification by qRT-PCR, \( F G S G _ { 0 8 3 4 7 . 3 } \) encodes a choline permease that may be involved in the uptake of choline, a compound abundant in wheat anthers (51). \( F G S G _ { 0 1 7 6 7 . 3 } \) encodes a \( P 4 5 0 \) monoxygenase that is highly homologous to pisatin demethylase (32). \( F G S G _ { 0 5 6 2 2 . 3 } \) shares strong similarity to a trehalase precursor. In \( M . g r i s e a \) and \( C . a l b i c a n s \), trehalase plays an important role in pathogenesis (14, 39). \( F G S G _ { 0 7 5 8 2 . 3 } \) encodes a putative low-affinity hexose transporter. In \( S c l e r o t i n i a s c l e r o t i o n a t u m \), two hexose transporter genes are upregulated during plant infection (25). \( F G S G _ { 0 5 5 9 7 . 3 } \) is a putative triacylglycerol lipase gene. \( F G L 1 \) and other lipase genes are important for full virulence in \( F . g r a m i n e a r u m \) (35, 54). \( F G S G _ { 1 1 0 8 0 . 3 } \) is homologous to \( P T H 1 1 \), encoding a putative membrane receptor that is important for plant infection in \( M . g r i s e a \) (12). Changes in their expression levels in the \( \Delta f t l 1 \) mutant were confirmed by qRT-PCR for all six of these candidate virulence factors. However, none of them has been functionally characterized, and their functions in the virulence of \( F . g r a m i n e a r u m \) remain to be analyzed.

Interestingly, \( P K S 1 2 \) and six other members of the aurofusarin synthesis gene cluster (28, 33) were upregulated in the \( \Delta f t l 1 \) mutant. The production of aurofusarin is likely to be elevated in the \( \Delta f t l 1 \) mutant. We noticed that deletion of the entire \( F T L 1 \) or the LisH domain resulted in the accumulation of a reddish pigment in the CMC cultures. However, not all genes in the aurofusarin synthesis cluster were expressed at higher levels in the \( \Delta f t l 1 \) mutant, and the chemical identity of this reddish pigment was not clear. The LisH domain is known to be involved in protein-protein interactions and the regulation of microtubule dynamics and nuclear migration (13, 16, 34). In \( F . g r a m i n e a r u m \), the LisH domain is essential for the function of \( F T L 1 \) (Fig. 6). In the original REMI mutant M75, the transforming vector was inserted in the LisH domain (Fig. 1). The \( F . g r a m i n e a r u m \) genome appears to have six additional genes containing the putative LisH domain (IPR006594). To date, the \( A s p e r i l l u s n i d u l a n s n u d F \) gene, which is known to be involved in nuclear migration (1), is the only LisH domain-containing gene that has been functionally characterized in filamentous fungi.

It is possible that a reduction in the growth rate may contribute to the defects of the \( \Delta f t l 1 \) mutant in plant infection. However, while the growth rate was only reduced about 40% in the \( \Delta f t l 1 \) mutant (Table 3), its disease index was near zero. Deletion of \( F T L 1 \) must have adversely affected other pathogenicity factors in \( F . g r a m i n e a r u m \). Based on microscopic examination, loss of full virulence in the \( \Delta f t l 1 \) mutant was related to its defects in penetration and infectious growth in wheat tissues. In nature, anthers are the main site of primary infection on flowering wheat heads (3, 38). The \( \Delta f t l 1 \) mutant was defective in the colonization of filaments and spreading from infected anthers to ovaries. In \( C l a v i c e p s p u r p e a r e a \), deletion of two endopolygalacturonase genes (\( c p p g 1 \) and \( c p p g 2 \)) also resulted in a near loss of pathogenicity and the mutant was blocked for the colonization of oat ovaries (36). However, the \( c p p g 1 \) \( c p p g 2 \) mutant was normal in growth and conidiation, and \( C . p u r p e a r e a \) is a biotrophic pathogen. Our TEM examination indicated that rare intercellular hyphae developed for the \( \Delta f t l 1 \) mutant in the cortical layer of wheat rachises. However, the \( \Delta f t l 1 \) mutant failed to penetrate and grow intracellularly in the cortical layer and was defective in the colonization of palea or seed coat cells. Because colonization of vascular tissues of the rachis may be a critical factor in the development and spread of wheat growth and colonization of vascular tissues.

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