Random Insertional Mutagenesis Identifies Genes Associated with Virulence in the Wheat Scab Fungus *Fusarium graminearum*

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ABSTRACT

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Fusarium graminearum is an important pathogen of small grains and maize in many areas of the world. To better understand the molecular mechanisms of *F. graminearum* pathogenesis, we used the restriction enzyme-mediated integration (REMI) approach to generate random insertional mutants. Eleven pathogenicity mutants were identified by screening 6,500 hygromycin-resistant transformants. Genetic analyses indicated that the defects in plant infection were tagged by the transforming vector in six of these mutants. In mutant M8, the transforming plasmid was integrated 110-bp upstream from the start codon of the cystathionine beta-lyase gene (*CBL1*). Gene replacement mutants deleted for *CBL1* and the methionine synthase gene *MSY1* were also obtained. Both the *cbl1* and *msy1* deletion mutants were methionine auxotrophic and significantly reduced in virulence on corn silks and wheat heads. We also identified genes disrupted by the transforming DNA in three other REMI mutants exhibiting reduced virulence. In mutants M68, the transforming vectors were inserted in the NADH: ubiquinone oxidoreductase. The putative b-ZIP transcription factor gene and the transducin beta-subunit-like gene disrupted in mutants M7 and M75, respectively, had no known homologs in filamentous fungi and were likely to be novel fungal virulence factors.

Additional keywords: auxotrophic mutant, cystathionine, *Gibberella zeae*, methionine.

Fusarium head blight (FHB) or scab is a disease of wheat and barley that can reduce crop yield and grain quality (28). In North America, the primary pathogen causing this disease is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*), lineage 7 (31,32). *F. graminearum* also causes stalk and ear rots of maize and infects other small grains (28,34,44). Infested cereals may be contaminated with trichothecenes and estrogenic mycotoxins that are harmful to humans and animals and make the grain unsuitable for food or feed (9,28,33). Trichothecene toxins, such as deoxynivalenol (DON), are potent inhibitors of eukaryotic protein biosynthesis.

F. graminearum overwinters in infected plant debris as saprophytic mycelia and produces spores in the spring when weather conditions favor fungal development. Ascospores are believed to be the primary inoculum for the disease and are forcibly discharged from mature perithecia and dispersed by wind (12,41). Although it is naturally a haploid homothallic ascomycete, heterothallic *F. graminearum* strains have been generated by deleting the *MAT1-1* or *MAT1-2* mating-type locus (22). *F. graminearum* is amenable to classical and molecular genetic manipulations (4,36). Thousands of expressed sequence tags (ESTs) (42) and the genome sequence of *F. graminearum* are now available (provided online by the Broad Institute, Massachusetts Institute of Technology [MIT]). However, most of the molecular studies in this fungus have focused on trichothecene biosynthesis and population structure (5,7,27,32,45,48). There are only limited studies on identification and characterization of fungal virulence factors in *F. graminearum* (7).

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The first virulence factor characterized in *F. graminearum* was the *TRI5* trichodiene synthase gene that catalyzes the first committed step in the trichothecene biosynthetic pathway (36). The *tri5* deletion mutants are normal in growth and development but reduced in virulence on seedlings of Wheaten wheat, common winter rye, and maize (8,9,16). Further studies with *tri5* mutants indicate that DON production is not necessary for initial infection but is important for allowing the fungus to spread within colonized spikes (1). In addition to *TRI5*, two mitogen-activated protein kinase genes, *MGV1* and *GPMK1*, have been shown to be essential for pathogenicity of *F. graminearum* (18,19,43). The *mgv1* deletion mutants are substantially reduced in virulence and DON production, and rarely spread beyond the inoculated floret (18). The *mgv1* mutant is female sterile and self-incompatible in heterokaryon formation. The *gpmk1* mutants have no defect in vegetative growth and conidiation, but they are defective in colonizing flowering wheat heads and in spreading from inoculated florets to neighboring spikelets (19,43). Disruption of the homolog of *Cochliobolus heterostrophus CPS1*, which represents a novel virulence factor in fungal pathogens, also causes reduced virulence in *F. graminearum* (23).

Restriction enzyme-mediated integration (REMI) mutagenesis has been used in several plant-pathogenic fungi, including *Ustilago maydis*, *C. heterostrophus*, and *Magnaporthe grisea* (2,3, 24,40). REMI remains one of the most efficient approaches for identifying pathogenicity genes in several fungi (15,21). In this study, we generated and screened 6,500 REMI transformants of *F. graminearum*. Eleven pathogenicity mutants defective in colonizing corn silks and flowering wheat heads were isolated. Genetic analyses indicated that the defects in plant infection were tagged by the transforming vector in six of these mutants. In mutant M8, the transforming vector was inserted in the cystathionine beta-lyase gene (*CBL1*). We further generated mutants

deleted for *CBL1* and the methionine synthase gene *MSY1*, and demonstrated that methionine synthesis is an important virulence factor in *F. graminearum*. In addition, we have identified genes disrupted by the transforming DNA in three other REMI mutants. Two of them appeared to be novel fungal virulence factors in *F. graminearum*.

MATERIALS AND METHODS

Strains and culture condition. *F. graminearum* wild-type strain PH-1 (NRRL 31084) and all the mutants (Table 1) generated in this study were cultured at 25°C on V8 agar or liquid carboxymethylcellulose (CMC) medium for conidiation (18). A *nit1* mutant of PH-1 (11622) was obtained from R. Bowden at Kansas State University. For genomic DNA and RNA extraction, mycelia were cultivated in complete medium (CM) (17) for 3 days at 25°C. Standard molecular biology procedures were followed for Southern blot analyses (38). CM supplemented with hygromycin B (Calbiochem, La Jolla, CA) at 150 μg/ml or neomycin (Amresco, Solon, OH) at 200 μg/ml was used for selecting transformants. All the REMI mutants were preserved by resuspending conidia and mycelia mixtures scraped off V8 agar culture in 15% glycerol and stored at –80°C. For assaying auxotrophic phenotypes, minimal medium (MM) (17) was supplemented with methionine at 0.5 mg/ml or 0.5 homocysteine at mg/ml. Genetic crosses and random ascospore progeny isolation were performed as described previously (18).

Fungal transformation and REMI mutants. Protoplast preparation and fungal transformation were performed as described previously (18). For REMI mutagenesis, plasmids pCB1003 (6) and pKY37 were digested with *Bam*HI and *Eco*RI, respectively, and directly used for transforming PH-1 protoplasts. To generate pKY37, the hygromycin phosphotransferase (*hph*) gene was amplified from pCB1003 with primers 5′-AAGAATTCGGCGCGCCGTCGACGCTGGAGCTAGTGGAGGTCA-3′ and 5′-TTGGATCCGGCCGGCCGTCGACCGGTCGGCATCTACTCTATTS-3′. The β-lactamase gene (*amp*) and replication origin (*ori*) were amplified from pUC19 with primers 5′-AGAATTCGGCGCGCCATCGATGCCTCGTGATACGCCTATTT-3′ and 5′-CCGGAATTCATCGATACGAAAACTCACGTTAAGGG-3′, and 5′-CCGGAATTCGGATCCGATATCCAGACCCCGTAGAAAAGATC-3′ and 5′-TTGGATCCGGCCGGCCGATATCGGGGATAACGCAGGAAAGAA-3′, respectively. The amplified *hph*, *amp*, and *ori* fragments were digested with *Asc*I-*Fse*I, *Asc*I-*Eco*RI, and *Eco*RI-*Fse*I, respectively, and then ligated to construct plasmid pKY36. A polylinker generated by annealing oligoes 5′-AATTCTGCAGAGCTCGAGGTACCTCTAGAAGCTTGTCGACG-3′ and 5′-GATCCGTCGACAAGCTTCTAGAGGTACCTCGAGCTCTGCAG-3′ was inserted between the *Eco*RI and *Bam*HI sites of pYK36. In the resulting plasmid pKY37, *Pst*I, *Sac*I, *Xho*I, *Kpn*I, *Xba*I, *Hin*dIII, *Eco*RI, and *Bam*HI had single digestion sites and were suitable for plasmid rescue. For complementation assays, the wild-type *CBL1* and *MSY1* genes were reintroduced into the corresponding mutants by cotransformation with pSM334 (provided by S. Kang at Pennsylvania State University), a vector containing the neomycin phosphotransferase as described previously (18). Neomycin-resistant transformants were screened by polymerase chain reaction (PCR) with primers K1R and 13F (18).

Corn silk infection assay. Fresh corn silks were collected from young corn ears of cv. LH 132 and sliced into 5-cm fragments. Exposed portions of corn silks were discarded and only the portions originating from inside husks were used for infection assays. Three to four pieces of corn silk fragments were aligned with each other evenly as a bundle over a Whatman No. 1 filter paper soaked with sterile distilled water. The lower ends of corn silks were covered with a 4-mm^2 block of *F. graminearum* V8 agar culture. Infection of corn silks was scored by the extent of discoloration after incubation at 25°C in a moisture chamber for 4 to 5 days.

Infection assays on flowering wheat inflorescence. Conidia were collected from 7- to 10-day-old cultures on mung bean agar and resuspended in sterile distilled water to a concentration of 10^6 conidia per ml (13). Approximately 6-week-old plants of wheat cv. Norm were chosen for inoculation at flowering. The fifth full-sized spikelet from the base of the inflorescence was injected with 10 μl of the spore suspension containing 0.01% (vol/vol) Tween 20; control plants were inoculated with 10 μl of 0.01% Tween alone. Inoculated plants were placed in a humidity chamber for 3 days and then transferred to ambient growth conditions in a greenhouse. Symptomatic spikelets in each head were counted 14 days after inoculation. Student's *t* tests (two-tailed, two sample unequal variance) were used to compare the mean number of symptomatic spikelets for plants inoculated with PH-1 or mutant strains. The number of inoculated wheat heads per treatment was 9 or 10 for each test, and all tests were repeated at least four times.

Generating the *cbl1* deletion mutant. To construct the *CBL1* gene replacement vector, a 0.6-kb upstream flanking sequence of *CBL1* was amplified with primers cb1 (5′-CGCGGATCCTCGATGGGCCATGATAGTGT-3′) and cb2 (5′-AAGGTTGGCGCGCCTGGATGCAGGATGTAGTAG-3′) and digested with *Asc*I. A 0.7-kb downstream fragment was amplified with primers cb3 (5′-AAGGTTGGCCGGCCTTCAAGCTGGTGCTGTCACT-3′) and cb4 (5′-CGCTCTAGAGAGTTTGTGTGGAAGCGTTG-3′) and digested with *Fse*I. These flanking sequences of *CBL1* were then ligated with a 1.4-kb *Asc*I-*Fse*I *hph* released from pCX62 (49). Using the ligation mixture as the template, a 2.7-kb fragment containing the gene replacement construct was amplified with primers cb1 and cb4 and cloned between *Bam*HI-*Xba*I sites on pBCSK as pKY41. pKY41 was digested with *Bam*HI and transformed into PH-1 protoplasts. The resulting transformants were screened by PCR with primers cb5 (5′-ATTCAACCCTCTGGATGTGG-3′) and cb6 (5′-TCTGACAAGGCATGCTGATC-3′). All putative *cbl1* gene replacement mutants were further confirmed by Southern blot analyses. For complementation of the *cbl1* mutant, a 4.7-kb *Xho*I fragment containing the full-length *CBL1* gene was amplified with primers cb7 (5′-CTCGAGCGAATGAATCGGAAC-3′) and cb8 (5′-CTCGAGCTGCCACCCAGAGTC-3′) and cloned into the pGEM-T easy vector (Promega, Madison, WI) as pKY49. Primers cb9 (5′-CGTTCCAAAGCTACTC-3′) and cb10 (5′-CGCACTAGGGTGTACA-3′) are 408- and 118-bp upstream from the *CBL1* open reading frame (ORF), respectively.

Generating the *msy1* deletion mutant. To generate the *MSY1* gene replacement construct, a 0.9-kb upstream fragment was amplified with primers m1 (5′-ATCGGATCCTTTTCTGATGCCCTGAGAGC-3′) and m2 (5′-AAGGTTGGCGCGCCCAAG-

TABLE 1. Pathogenicity phenotypes of restriction enzyme-mediated integration mutants

Strain	Disease index (mean ± SD)[a]	*t* test significance
PH-1	8.4 ± 1.4	Wild-type control
CX8	0	<0.001
M28	0	<0.001
M30	0	<0.001
M75	0	<0.001
25C3	0.5 ± 0.5	<0.001
CX3	0.1 ± 0.1	<0.001
222	0.8 ± 0.6	<0.001
2G8	0.2 ± 0.4	<0.001
M68	1.0 ± 0.3	0.01
M7	4.7 ± 0.7	0.006
M8	3.7 ± 1.0	0.002

[a] Disease index is represented by the mean and standard deviation of numbers of symptomatic spikelets at 14 days after inoculation in four independent infection assays.

GTTGGTTAGTCGTTG-3′) and digested with *Asc*I. A 0.7-kb downstream fragment amplified with primers m3 (5′-AAGGTTGGCCGGCCTGTGTGTAGAGGTTGATGGG-3′) and m4 (5′-ATCAAGCTTTTGTACCTTCGGCTTCAGCT-3′) was digested with *Fse*I. After ligation of these PCR products with the *Asc*I-*Fse*I *hph* fragment, a 3.0-kb *MSY1* gene replacement construct was amplified with primers m1 and m4 and cloned into pGEM-T easy as pKY43. After being linearized with *Eco*RI, pKY43 was transformed into protoplasts of PH-1. Hygromycin-resistant transformants were isolated and examined for their growth on MM with or without methionine. Putative methionine auxotrophic *msy1* mutants were further confirmed by Southern hybridizations with probe 1 amplified with primers m1/m2 and probe 2 amplified with primers m5/m6. For complementation of the *msy1* mutant, a 4.3-kb fragment containing the *MSY1* gene was amplified with primers m7 (5′-AGGCATTGCAATGCCACAAC-3′) and m8 (5′-TTCAGACAGTGACGCATCAC-3′) and cloned into pGEM-T easy as pKY52.

GenBank accession numbers are as follows: EAA68828 for *CBL1* (FG01932.1), EAA75179 for *MSY1* (FG10825.1), EAA68510 for *ZIF1* (FG01555.1), EAA69636 for *NOS1* (FG00376.1), and EAA67363 for *TBL1* (FG00332.1).

RESULTS

Generating REMI transformants. We isolated 6,500 hygromycin-resistant transformants by transforming PH-1 with either *Bam*HI-digested pCB1003 or *Eco*RI-digested pKY37. To determine the integration events in these transformants, 22 randomly selected pKY37 transformants were analyzed by Southern blot hybridization. When blots of DNA samples digested with *Hin*dIII were probed with the *hph* fragment, 13 transformants showed a single band larger than 3.3 kb (Fig. 1) and likely contained one copy of pKY37 integrated at a single site. Seven of these transformants had a 3.3-kb band and one additional band (Fig. 1), indicating that more than one copy of pKY37 was tandem integrated at a single site. The single 3.3-kb band observed in transformant 7 (Fig. 1) was likely a doublet band. Two (<10%) of them, transformants 13 and 19, had two or more bands in addition to the 3.3-kb band (Fig. 1). When blots of *Stu*I-digested DNAs were probed with the *hph* gene, transformants 13 and 19 had more than one band (data not shown), suggesting that pKY37 was inserted at two different loci in these two transformants. Similar Southern blot analyses with 20 randomly selected pCB1003 transformants indicated that 18 of them (90%) had the transforming vector integrated at a single genomic site (data not shown).

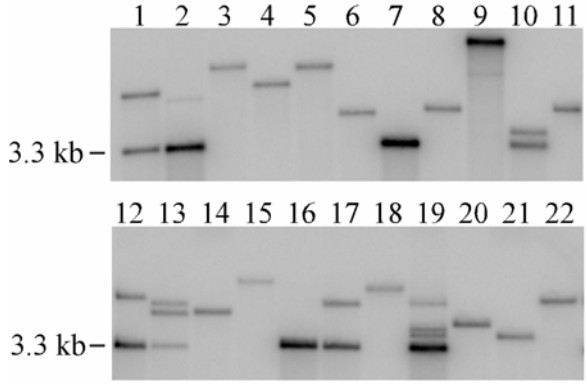

Fig. 1. Southern blot analyses of restriction enzyme-mediated integration (REMI) transformants. Blots of *Hin*dIII-digested DNAs from 22 randomly selected REMI transformants (1 to 22) were hybridized with the *hph* gene. Nine transformants had the 3.3-kb band resulting from the tandem integration of more than one copy of the transforming vector pKY37, which had a single *Hin*dIII site.

Screening for mutants defective in plant infection. To screen for mutants defective in plant infection, we devised a corn silk infection assay as described previously. *F. graminearum* is a causal agent of the corn ear rot disease and can infect corn kernels through silks (16,37). The wild-type strain PH-1 colonized fresh corn silks and caused discoloration (Fig. 2A). The corn silk infection was used as the primary screen to assay the virulence of all 6,500 REMI transformants. REMI mutants identified in the primary screen were tested in three additional corn silk infection assays. Only 20 transformants with reduced virulence in repeated corn silk infection assays were used to inoculate flowering wheat heads. Table 1 lists 11 REMI mutants that were significantly reduced in virulence on both corn silks and flowering wheat heads (Table 1). REMI mutants CX8 (Fig. 2B), M28, M30, and M75 usually did not cause any symptoms in inoculated florets (Table 1). Mutants 25C3 (Fig. 2B), CX3, 222, 2G8, and M68 colonized the inoculated florets but usually failed to spread to other florets on the same spikelets (Table 1). In contrast, mutants M7 (Fig. 2B) and M8 were still pathogenic but the number of infected kernels was significantly reduced in inoculated wheat heads, indicating that these REMI mutants were less virulent and might be defective in spreading through infected wheat heads (Table 1).

These REMI mutants were crossed with the *nit1* mutant strain 11622. Outcross perithecia were identified by the ability of ascospore progeny to grow on MM with hygromycin and 1% potassium chlorate. Random mono-ascospore cultures were isolated from outcross perithecia and assayed for their resistance to hygro-

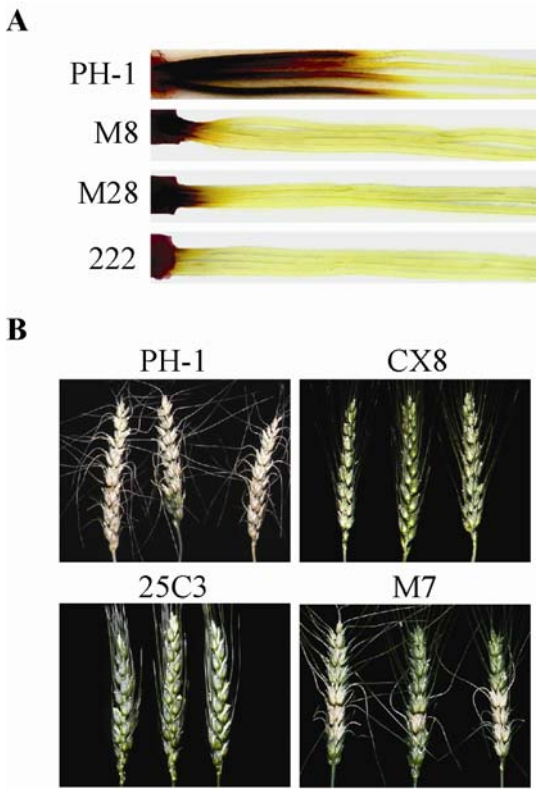

Fig. 2. Restriction enzyme-mediated integration (REMI) mutants with reduced virulence. **A,** Corn silk infection assay. A small block of V8 agar cultures of wild-type PH-1 and REMI mutants M8, M28, and 222 were placed on freshly cut ends of corn silks. PH-1 colonized and caused discoloration in infected corn silks. Discoloration was significantly reduced in mutants M8, M28, and 222. Photos were taken 4 days after inoculation. **B,** Infection assay on wheat heads. Scab disease development on wheat heads inoculated in the fifth spikelet from the bottom with conidia from the wild-type strain (PH-1) and REMI mutants (25C3, CX8, and M7). Photos were taken 2 weeks postinoculation. Mutants CX8 and 25C3 were reduced in their ability to cause necrosis on wheat kernels beyond the point of inoculation.

mycin and virulence on wheat plants and corn silks (data not shown). Cosegregation analysis indicated that the phenotypes of REMI mutants CX3, 222, M7, M8, M68, and M75 were tagged by the transforming hygromycin-resistant vector. In crosses between 11622 and CX8, 2G8, M28 or M30, we had isolated progeny that were sensitive to hygromycin but displayed mutant phenotypes, indicating that defects observed in these mutants were not caused by transforming DNA. Cosegregation analysis was not performed with mutant 25C3 because it contained multiple integrations of the transforming vector (data not shown).

Mutant M8 was disrupted in the cystathionine β-lyase gene. Mutant M8 grew at a normal rate on V8 agar plates but was significantly reduced in virulence (Table 1). To identify the gene disrupted in M8, genomic DNA was digested with *Xho*I and transformed into *Escherichia coli* after self-ligation. Five identical clones containing a 2.2-kb *Eco*RI-*Xho*I fragment of *F. graminearum* genomic DNA were recovered, and a representative was designated pKY40 (Fig. 3A). Sequence analysis of the fragment recovered in pKY40 indicated that the transforming vector pKY37 was inserted at the *Eco*RI site located 110-bp upstream from the start codon of the *CBL1* (for cystathionine beta-lyase 1) gene (Fig. 3A). *CBL1* is highly homologous to the *Neurospora crassa MET-2* and *Aspergillus nidulans metG* genes. We searched the available *F. graminearum* EST sequences but could not identify the *CBL1* cDNA sequence. However, using first-strand cDNA synthesized from RNA of PH-1 mycelia grown on CM, a 290-bp fragment upstream from the *CBL1* ORF (–408 to –118) was amplified with primers cb9 and cb10 (data not shown). Therefore, the transcription of *CBL1* must be initiated upstream from the integration site of pKY37 in mutant M8. The insertion of pKY37 was in the 5′-untranslated region and likely abolished the expression of the *CBL1* gene.

To confirm that the phenotype of M8 was caused by the disruption of *CBL1* expression, we generated a *CBL1* gene replacement construct pKY41 (Fig. 3A) and transformed it into PH-1. Two *cbl1* gene replacement mutants, KC1 and KC2, were identified and confirmed by Southern blot analyses (Fig. 3B). The wild-type strain PH-1 had a 1.6-kb *Eco*RI and a 1.8-kb *Sal*I band when hybridized with probe 1 and probe 2, respectively. The *cbl1* mutants KC1 and KC2 had no signal when hybridized with probe 1 (Fig. 3B) and contained a 5.1-kb *Sal*I band when hybridized with probe 2. Similar to REMI mutant M8, the *cbl1* deletion mutant was significantly reduced in plant infection (Fig. 4A). The colony formed by the *cbl1* mutant was morphologically identical to that of M8 and had much less aerial hyphae than PH-1 (Fig. 4B). Similar to the *A. nidulans* mutants that are blocked in conversion of cystathionine to homocysteine (39), mutant M8 and *cbl1* mutants were methionine auxotrophic (Fig. 4C). Supplementation of homocysteine or methionine rescued the growth defect of M8 and the *cbl1* deletion mutants on MM. When a wild-type *CBL1* gene was cotransformed into the *cbl1* mutant KC1, the resulting transformants KCC1 and KCC2 harboring pKY49 were prototrophic and normal in virulence on corn silks and flowering wheat heads (data not shown).

Deletion of the methionine synthase gene also reduced virulence in *F. graminearum*. Since the cystathionine β-lyase catalyzes the conversion of cystathionine to homocysteine, which is a precursor for methionine synthesis, we also generated a gene replacement construct pKY43 (Fig. 5A) for deleting the methionine synthase gene *MSY1* identified in the *F. graminearum* genome sequence. After transformation of PH-1 with pKY43, 25 hygromycin-resistant transformants were isolated and screened for their inability to grow on MM without methionine. Two methionine auxotrophic transformants, KM1 and KM2, were identified and confirmed by Southern blot analyses to be deleted of the *MSY1* gene (Fig. 5B). Similar to the *cbl1* mutant, the *msy1* mutants were defective in wheat head infection (Fig. 4A) and methionine auxotrophic (Fig. 4C), indicating that defects in methionine synthesis significantly reduced the virulence in *F. graminearum*. The *cbl1* and *msy1* mutants had no defects in conidiation in the liquid CMC medium (data not shown).

Although both *msy1* and *cbl1* mutants displayed methionine auxotrophic phenotypes, in contrast to the *cbl1* mutant, the *msy1* deletion mutant failed to grow on MM supplemented with homocysteine (Fig. 4C). The colony morphology of the *msy1* mutant was also distinct from that of the *cbl1* mutant. On V8 agar or MM plus methionine plates, the *msy1* mutants produced much less aerial hyphae than the *cbl1* mutants (Fig. 4B and C). When the wild-type *MSY1* gene was amplified and re-introduced into the *msy1* mutant KM2 by cotransformation, the resulting transformant KMC1 harboring pKY52 was prototrophic and normal in plant infections. Transformant KMC1 was also normal in colony morphology and aerial hyphae growth (data not shown). The difference between the *msy1* and *cbl1* mutants in aerial hypha production, even in the presence of methionine, indicated that robust aerial hyphal growth requires the methionine synthase gene.

Genes disrupted in other REMI mutants. To identify genes disrupted by the transforming vector, genomic DNAs of M7, M68, and M75 were digested by *Pst*I, *Hin*dIII, and *Kpn*I, respectively, and transformed into *E. coli* after self-ligation. Sequence analyses of the genomic fragments recovered by plasmid rescue indicated that the transforming vector pKY37 was integrated in mutants M7, M68, and M75 at the recognition site of *Eco*RI, which was the restriction enzyme used in transformation to generate these mutants.

The insertion site of pKY37 in mutant M7 was located at the 3′ end (amino acid residue 487) of a putative b-ZIP transcription factor gene FG01555.1 (named *ZIF1* for bZIP transcription factor 1).

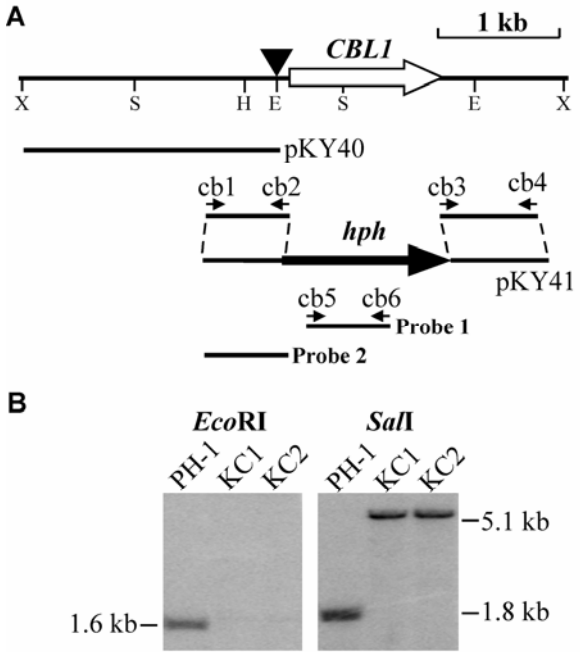

Fig. 3. The *CBL1* locus of mutant M8 and gene replacement of *CBL1*. **A,** The filled triangle indicated the *Eco*RI site where pKY37 was inserted upstream of *CBL1* in M8. The *Xba*I-*Eco*RI fragment was recovered from M8 in the plasmid rescue clone pKY40. The *CBL1* gene replacement vector pKY41 was generated by ligating a 0.6-kb upstream and a 0.7-kb downstream flanking sequence of *CBL1* with the *hph* gene. The empty and filled arrows indicate the direction of the *CBL1* and *hph* genes, respectively. The positions and directions of primers cb1, cb2, cb3, cb4, cb5, and cb6 are indicated with small arrows. Probe 1 and probe 2 indicate polymerase chain reaction products amplified with primers cb1/cb2 and cb5/cb6, respectively. Restriction enzymes *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sac*I (S), *Xho*I (X), and *Xba*I (Xb). **B,** Southern blots of the wild-type strain PH-1 and the *cbl1* deletion mutants KC1 and KC2. DNA samples were digested with *Eco*RI and hybridized with probe 1 (left) or digested with *Sal*I and hybridized with probe 2 (right).

The protein encoded by *ZIF1* contained a basic region leucine zipper domain. In mutant M68, the insertion site was 103-bp upstream from the ORF of the predicted gene FG00376.1 (named *NOS1*) that is homologous to the 49-kDa subunit of NADH: ubiquinone oxidoreductase (complex I) from *Neurospora crassa* (30). In mutant M75, pKY37 was integrated at amino acid residue 6 of the predicted ORF FG00332.1 (named *TBL1* for transducin beta-subunit-like gene 1), which encodes a 662-amino acid protein homologous to the beta-subunits of transducins (11,25). The *TBL1* gene product has seven WD repeats (14) at the C terminus and an N-terminal LisH domain that is known to be involved in protein–protein interactions (10).

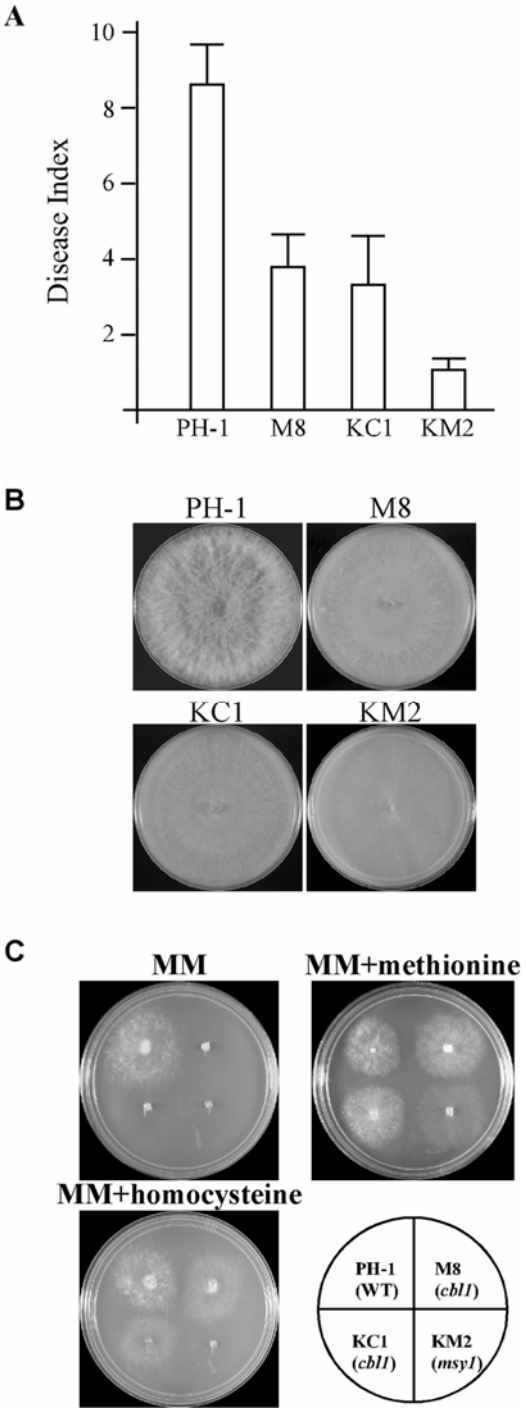

Fig. 4. The *cbl1* and *msy1* mutants were defective in plant infection and methionine auxotrophic. **A,** Flowering wheat heads were inoculated with conidia from PH-1, restriction enzyme-mediated integration (REMI) mutant M8, and the *cbl1* (KC1) or *msy1* (KM2) deletion mutants. Disease index is represented by the mean and standard deviation of numbers of symptomatic spikelets in four independent infection assays. Virulence of the *cbl1* and *msy1* mutants, similar to that of mutant M8, was significantly reduced. **B,** V8 agar cultures of the wild-type strain PH-1, REMI mutant M8, and the *cbl1* and *msy1* deletion mutants KC1 and KM2, respectively. **C,** The same set of strains grown on minimal medium (MM) and MM supplemented with methionine (MM+M) or homocysteine (MM+H). Photos were taken after 5 days of incubation at 25°C.

DISCUSSION

REMI has been used in several filamentous fungi for insertional mutagenesis (15,21). Although the random nature of plasmid integration is a matter of debate, REMI has been used effectively in recovering novel virulence factors in many plant-pathogenic fungi (3,24,40). In this study, we isolated 11 pathogenicity mutants after screening 6,500 *F. graminearum* REMI transformants. Approximately 90% of the REMI transformants were estimated to have the transforming vector integrated at a single locus. In *M. grisea*, it has been shown that removal of polyethylene glycol (PEG) during regeneration favors single-plasmid insertion events at endonuclease-cleaved sites (2). In our protocol, PEG added to the transformation mixtures was removed by centrifugation before the protoplasts were plated out in regeneration media for selecting hygromycin-resistant transformants.

Since wheat head infection assays are time-consuming and labor-intensive, we devised a corn silk infection assay. Our data suggested that the corn silk infection assay is useful as the primary screen to rapidly identify mutants that are defective in wheat

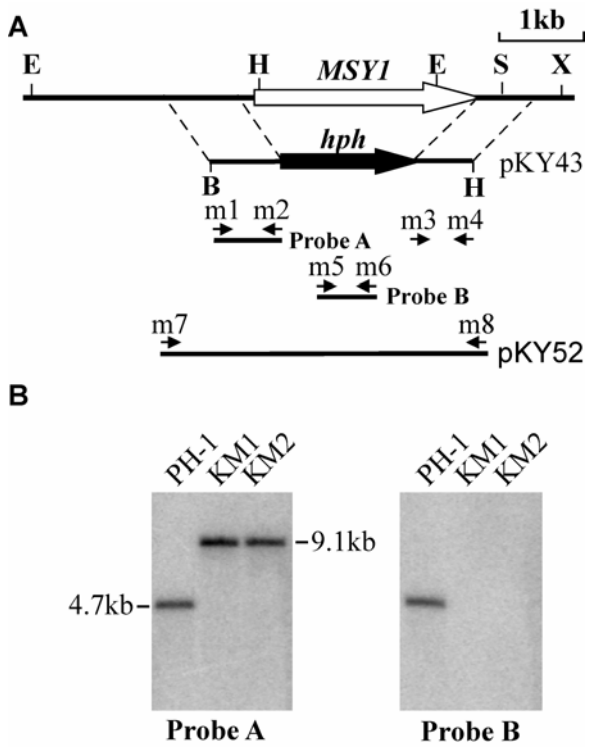

Fig. 5. Gene replacement of the methionine synthase *MSY1*. **A,** The gene replacement construct pKY43 was generated by ligating a 0.9-kb upstream and a 0.7-kb downstream flanking sequence of *MSY1* with the *hph* gene. The empty and filled arrows indicate the direction of the *MSY1* and *hph* genes, respectively. The positions and directions of primers m1, m2, m3, m4, m5, m6, m7, and m8 are indicated with small arrows. Probe A and probe B are polymerase chain reaction products amplified with primers ms1/ms2 and ms5/ms6, respectively. *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S), *Xho*I (X). **B,** Southern blots of *Eco*RI-digested genomic DNAs of PH-1, the *msy1* deletion mutants KM1 and KM2 were hybridized with probe A (left) and probe B (right). The *msy1* mutants KM1 and KM2 had a 9.1-kb *Eco*RI band that hybridized with probe A but no signal when hybridized with probe B. The wild-type strain PH-1 had a 3.7-kb *Eco*RI band when hybridized with both probes.

head infection. The *mgv1* and *gpmk1* mutants (18,19) that are known to be defective in wheat infection were nonpathogenic on corn silks (data not shown). However, not all the REMI transformants that were defective in colonizing corn silks were significantly reduced in infecting wheat heads. In fact, 9 out of 20 REMI mutants with reduced virulence on corn silks were as virulent as the wild-type strain when assayed on flowering wheat heads (data not shown). It is possible that different virulence factors in *F. graminearum* are involved in infecting corn silks versus wheat heads. However, we attempted to include all putative virulence mutants identified in the primary screens for wheat head infection assays. Some of them were only slightly reduced in virulence on corn silks and possibly not true mutants defective in plant infection. On the other hand, it is also possible that some *F. graminearum* genes are specific for wheat head infection. Mutants disrupted in this type of gene are likely to be normal in colonizing corn silks and unidentified in the primary screen with corn silk infection assays.

In mutant M8, the transforming vector was inserted 110-bp upstream from the start codon of the *CBL1* gene. The transforming vector might have disrupted the transcription of the *CBL1* gene resulting in the methionine auxotrophic phenotype of mutant M8. Mutants deleted of the *CBL1* or the methionine synthase gene *MSY1* were, similar to M8, significantly reduced in virulence on corn silks and flowering wheat heads, indicating that methionine synthesis is critical for plant infection in *F. graminearum*. In *M. grisea*, the methionine auxotrophic mutant 130 (*met1*) was also reduced in virulence on rice seedlings (2). However, it is not clear which gene was disrupted in this mutant. In *Cryptococcus neoformans*, mutants deleted of the methionine synthase gene *MET6* are nonpathogenic (35). Auxotrophic mutants of other fungal pathogens defective in other primary metabolism, such as histidine synthesis in *M. grisea* (40) and arginine synthesis in *F. oxysporum* (29), have also been reported to be reduced in virulence.

Although both the *cbl1* and the *msy1* mutants were methionine auxotrophic, we noticed that the *msy1* mutant was more severely reduced than the *cbl1* mutant in aerial hyphal growth and virulence (Fig. 4). In *N. crassa* and *A. nidulans*, homocysteine can be synthesized from homoserine by homoserine acetyltransferase and homocysteine synthase (20). Homologs of the homoserine acetyltransferase and homocysteine synthase genes found in the *F. graminearum* genome (FG05658.1 and FG01417.1) may be functional and responsible for homocysteine synthesis in the *cbl1* deletion mutant and REMI mutant M8. The *msy1* mutant displayed a growth defect on MM supplemented with methionine (Fig. 4C). A similar phenomenon has been reported in *C. neoformans* (35,47). The *met3* mutants of *C. neoformans* blocked in the sulfate-assimilation arm of the methionine biosynthetic pathway are reduced in virulence and have a growth defect even in the presence of abundant exogenous methionine (47). The *met6* mutant also grew poorly in media supplemented with methionine (35). It is possible that deletion of the methionine synthase gene leads to the accumulation of homocysteine, a toxic intermediate that interferes with ergosterol biosynthesis in *F. graminearum* (26,35).

In addition to M8, we have identified genes disrupted in mutants M7, M68, and M75. The growth rates of these REMI mutants were similar to that of the wild-type strain (data not shown). The putative b-ZIP transcription factor gene *ZIF1* disrupted in mutant M7 had no significant homology in yeasts and appeared to be unique to filamentous fungi. The *TBL1* gene disrupted in mutant M75 is highly conserved among filamentous fungi, including *N. crassa*, *A. nidulans*, and *M. grisea*. However, all of its homologs are predicted genes that have not been functionally characterized. Different from the G-beta subunits characterized in various fungi, the transducin beta-subunit-like protein has the LisH domain. In *A. nidulans*, NudF has the LisH domain and is required for nuclear movement (46). However, the *F. graminearum* genome has distinct homologs of the G-beta subunit and *NudF* genes that are not related with *TBL1*. The wild-type *ZIF1* and *TBL1* genes were transformed into mutants M7 and M75, respectively. Preliminary analyses indicated that re-introduction of the wild-type alleles of *ZIF1* and *TBL1* complemented the phenotypes of mutants M7 and M75, respectively (K. Seong and J.-R. Xu, *unpublished data*). Since there are no homologs of *ZIF1* and *TBL1* that have been characterized in filamentous fungi, the putative b-ZIP transcription factor and transducin beta-subunit-like protein genes identified in this study are likely to be novel fungal virulence factors. It will be important to further characterize the *ZIF1* and *TBL1* genes and to determine their roles in plant infection processes.

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