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Deletion and Complementation of the Mating Type (*MAT*) Locus of the Wheat Head Blight Pathogen *Gibberella zeae*

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***Gibberella zeae*, a self-fertile, haploid filamentous ascomycete, causes serious epidemics of wheat (*Triticum aestivum*) head blight worldwide and contaminates grain with trichothecene mycotoxins. Anecdotal evidence dating back to the late 19th century indicates that *G. zeae* ascospores (sexual spores) are a more important inoculum source than are macroconidia (asexual spores), although the fungus can produce both during wheat head blight epidemics. To develop fungal strains to test this hypothesis, the entire mating type (*MAT1*) locus was deleted from a self-fertile (*MAT1-1*/*MAT1-2*), virulent, trichothecene-producing wild-type strain of *G. zeae*. The resulting *MAT* deletion (*mat1-1*/*mat1-2*) strains were unable to produce perithecia or ascospores and appeared to be unable to mate with the fertile strain from which they were derived. Complementation of a *MAT* deletion strain by transformation with a copy of the entire *MAT* locus resulted in recovery of production of perithecia and ascospores. *MAT* deletion strains and *MAT*-complemented strains retained the ability to produce macroconidia that could cause head blight, as assessed by direct injection into wheat heads in greenhouse tests. Availability of *MAT*-null and *MAT*-complemented strains provides a means to determine the importance of ascospores in the biology of *G. zeae* and perhaps to identify novel approaches to control wheat head blight.**

All *Gibberella* species are sexual states of *Fusarium* species. Many *Gibberella* species are destructive plant pathogens, although most of them are more familiar to plant pathologists under the *Fusarium* names of their asexual states (10). *Gibberella zeae*, asexual state *Fusarium graminearum*, causes head blight of wheat (*Triticum aestivum* L.), barley, and other small grains and also infects maize ears and stalks and a diversity of other plants worldwide. *G. zeae* reduces yields and also contaminates grain with deoxynivalenol and other trichothecene toxins that can be harmful to humans and animals that consume infected grain. Since 1990, epidemics of *G. zeae* head blight on wheat and barley have increased in frequency and severity in the United States, Canada, and elsewhere (20). Reemergence of this plant disease has been associated with the use of conservation tillage practices that leave large amounts of maize stalk pieces and other crop residues on the soil surface (30).

G. zeae was first isolated by Schweinitz in 1822 from perithecia and ascospores (sexual spores) on maize and was described as *Sphaeria zeae*, which was amended to *Gibberella zeae* in 1936 (24). By the late 19th century, the importance of *G. zeae* spores in wheat head blight had been recognized in Europe and in the United States (1). In Wisconsin in 1920, Atanasoff (2) observed that both ascospores and macroconidia (asexual spores) of *G. zeae* could infect wheat heads and that both types of spores were abundant during wheat head blight epidemics. During the past 80 years, both of Atanosoff's observations have been confirmed in numerous studies in the laboratory and in the field, but the relative contribution of *G. zeae* ascospores and macroconidia to wheat head blight epidemics is still debated (30). On the one hand, differentiated hyphae and perithecia formed in crop residues during autumn can, over winter, mature rapidly as temperatures warm and produce ascospores for early infections in the spring. Furthermore, ascospores often are recovered at higher counts than macroconidia from traps that sample airborne spores in the field (23), perhaps because ascospores are forcibly discharged, whereas macroconidia are not (32). On the other hand, some *Fusarium* species such as *Fusarium avenaceum* and *Fusarium culmorum*, which rarely or never produce ascospores, can produce severe wheat head blight epidemics in Europe and Canada, indicating that macroconidia are sufficient under some conditions (30).

Mating type (*MAT*) loci have been shown to control sexual development and ascospore production in a number of ascomycete fungi (34). Production of non-ascospore-producing strains of *G. zeae* became feasible after isolation and sequencing of the entire *G. zeae MAT* locus (36). In this homothallic fungus, the *MAT* locus comprises four genes: the *MAT1-1* idiomorph with three genes (*MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*) and the *MAT1-2* idiomorph with one gene (*MAT1-2-1*). In a previous study, differential deletion of each *MAT1* idiomorph resulted in strains that were self-sterile yet able to cross with each other (18). The objectives of this study were to

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delete and complement the entire *MAT* locus to confirm its function in *G. zeae* and to develop *MAT*-null non-ascospore-producing strains that could be used to assess the importance of ascospores in wheat head blight epidemics.

MATERIALS AND METHODS

Fungal strains and inoculum preparation. The *G. zeae* strains used in this study were maintained and cultured as previously described (28). A virulent strain isolated from scabby wheat in Kansas (6) and designated GZ3639 (*MAT1-1*/*MAT1-2*) was the ascospore-producing, deoxynivalenol-producing progenitor strain of all the transformants in this study and served as the wild-type strain for all virulence tests. This strain is available as FGSC 8630 from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City). Non-trichothecene-producing strain GZT40 (*MAT1-1*/*MAT1-2 hygB*^{R} *tri5*), previously derived from GZ3639 (27), served as a low-virulence control. Strains were routinely cultured on V8 juice agar medium (V8 juice, 200 ml; $CaCl_2$, 3 g; agar, 20 g/liter). For head injection tests in the greenhouse, a macroconidium inoculum was harvested from strains grown in mung bean liquid medium for 4 days at 28°C (3). If necessary, mung bean cultures were stored at 4°C for up to 10 days prior to use.

Self-fertility and mating assays. For clarity, as there is no convention for *MAT* nomenclature in homothallic ascomycetes (35), we used *MAT1-1*/*MAT1-2* as the wild-type designation, to indicate that both types of *MAT* sequence are present, and we used *mat1-1*/*mat1-2* to indicate that both have been deleted. Self-fertility was tested by culturing strains on 2 to 5 replicate 60-mm-diameter petri plates containing fresh carrot agar medium (17) in an incubator at 20 ± 1°C under continuous illumination provided by an equal mixture of fluorescent white and black (40 W; General Electric BLB) light bulbs. After 1 week, 2 ml of sterile water containing 2.5% (vol/vol) Tween 20 or Tween 60 (Sigma Chemical Co., St. Louis, Mo.) was added to the culture and spread with a plastic loop or rubber policeman to flatten the mycelium. Cultures were incubated for an additional 8 weeks, during which time individual cirri were picked and perithecia were squash mounted on microscope slides and checked by microscope for the presence of immature or mature asci and ascospores. The self-fertility of 15 selected strains (including the wild-type *MAT1-1*/*MAT1-2* strain, *MAT* deletion *mat1-1*/*mat1-2* strains, nondeletion transformants, and mock transformants) was tested further on 60-mm-diameter petri plates of water agar with pieces of sterile carnation leaf (22) or on slants of water agar with autoclaved pieces of maize husk, rice straw, or mulberry twigs. For photography, perithecia were observed with an M3C dissecting microscope (WILD, Heerbrugg, Switzerland) and asci were observed with a BH-2 compound microscope (Olympus America, Melville, N.Y.).

Mating tests were conducted as previously described (7). Spontaneous non-nitrate-utilizing (*nit*) mutants were generated on minimal agar medium supplemented with 1.5% (wt/vol) chlorate, and their phenotypes were scored on basal agar medium amended with different nitrogen sources (8). Two *nit* mutants were placed on opposite sides of plates of carrot agar, spread with Tween solution as described above, and incubated for up to 6 weeks until cirri were observed oozing from perithecia. To obtain single ascospore strains, individual cirri were picked from perithecia at the interface of the two parental colonies and the ascospores were suspended by vortexing each cirrus in 2 ml of sterile water. The resulting suspension was poured over a plate of 5% (wt/vol) water agar and incubated for 2 to 4 h. Ascospores were picked from these plates before they germinated, and ascospore germination was determined. With the aid of a stereomicroscope, small agar blocks were cut around individual ascospores and 24 to 30 ascospores from each perithecium were transferred to 150-mm-diameter plates of minimal agar medium. After 1 week, plates were scored for the presence of prototrophic colonies and selected progeny were analyzed for resistance to hygromycin. Prototrophic colonies result only from perithecia that were not formed homothallically.

Nucleic acid manipulations. Standard methods were used for PCR analyses, restriction enzyme digests, and Southern analyses (29). Hybridization probes used in Southern analyses were labeled with [^{32}P]CTP via the Prime-A-Gene kit (Promega, Madison, Wis.). Plasmid DNA was purified from *Escherichia coli* with the UltraClean mini plasmid prep kit (MoBio Laboratories, Solana Beach, Calif.). *G. zeae* genomic DNA for PCR and Southern analyses was isolated from lyophilized mycelia with the UltraClean kit (MoBio) as previously described (26).

Construction of *MAT* locus deletion vector pGzMAT-H. To simultaneously delete the four genes within the *G. zeae MAT* locus, we constructed deletion vector pGzMAT-H, which consisted of a 2.8-kb fragment (designated 2.8-up) of DNA flanking one side of the *MAT* locus, a 1.2-kb fragment (designated 1.2-down) flanking the other side of the locus, the hygromycin B resistance gene *hygB*, and the PCR cloning vector pCR XL TOPO (Fig. 1). pGzMAT-H was constructed by first amplifying fragments 2.8-up and 1.2-down via an inverse PCR strategy that employed two SalI restriction sites located 13.7 kb apart on either side of the *MAT* locus. To prepare the inverse PCR template, *G. zeae* strain GZ3639 genomic DNA was digested with SalI, purified by phenol extraction and ethanol precipitation, and then self-ligated. The resulting self-ligated DNA was purified by phenol extraction and ethanol precipitation and then used as a PCR template with *ExTaq* DNA polymerase (TaKaRa, Madison, Wis.) and oligonucleotide primers 01 and 02 (Table 1 and Fig. 1). The resulting 4-kb inverse PCR product consisted of the 2.8-up and 1.2-down fragments linked at a SalI site and in the opposite orientation relative to their original orientation in the *G. zeae* genome.

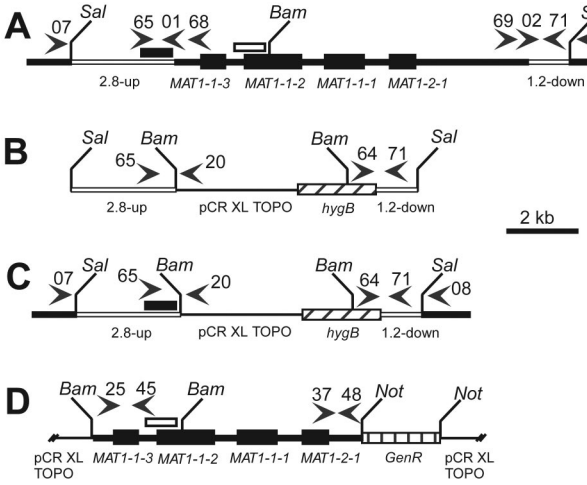

FIG. 1. (A) Wild-type *G. zeae MAT* locus; (B) *MAT* locus deletion vector, pGzMAT-H, shown in the linear form that would result from digestion with SalI; (C) *MAT* locus in which the 9.6-kb region with the four *MAT* genes has been deleted and replaced by a 5.1-kb fragment carrying the *hygB* and pCR XL TOPO portions of pGzMAT-H; (D) *MAT* locus complementation vector pGzMAT-Gen. Numbered arrows indicate relative positions and orientations of PCR primers used in this study. The black bars in panels A and C and the white bars in panels A and D indicate the regions of DNA used to prepare the hybridization probe for Southern analysis of transformants carrying pGzMAT-Gen.

The PCR product was cloned into cloning vector pCR XL TOPO (Invitrogen Life Technologies, Carlsbad, Calif.) to generate vector pGzMAT. The *hygB* gene was cloned into this vector via a unique XbaI restriction site within the pCR XL TOPO-derived sequence to generate the deletion vector pGzMAT-H (Fig. 1B). The *hygB* gene, which consisted of the *E. coli* hygromycin phosphotransferase gene coding region flanked by the *Aspergillus nidulans trpC* gene promoter and terminator sequences (9), was located on a 2.1-kb XbaI fragment excised from plasmid pCRATPH. In pCRATPH, *hygB* was modified by removing a unique SalI site in the *trpC* promoter sequence by digesting pCRATPH with SalI, filling in the protruding 5′ ends by incubation with Klenow DNA polymerase, and then religating. The deletion vector, pGzMAT-H, was linearized by digestion with SalI prior to its use in transformation experiments. In the linearized vector, the orientation of the 2.8-up and 1.2-down fragments to one another was the same as it is in the *G. zeae* genome. This orientation facilitated *MAT* locus deletion via homologous recombination between 2.8-up and 1.2-down in the deletion vector with the corresponding DNA flanking the *MAT* locus in *G. zeae*.

Transformation of wild-type strain. During the course of the study, four batches of protoplasts were prepared from germinating macroconidia of GZ3639 as previously described (28). Aliquots of protoplast batches 2, 3, and 4 were frozen at −80°C. Transformation was performed by placing a 100-μl aliquot of freshly prepared or frozen protoplasts on ice and diluting them with 100 μl of STC (0.8 M sorbitol, 50 mM Tris [pH 8.0], 50 mM $CaCl_2$) containing 1 to 10 μg of vector pGzMAT-H DNA and 50 μl of 30% polyethylene glycol 6000 (Sigma Chemical Co.). Following a 20-min incubation at room temperature, an additional 2 ml of 30% polyethylene glycol was added and incubation was continued at room temperature for 5 min. The transformation mixture was diluted further with 4 ml of STC, and then 600-μl aliquots were mixed with 4.5 ml of regener-

TABLE 1. Oligonucleotide primers used for PCR analysis of transformants and for preparation of probes for Southern hybridization

Primer no.	Sequence (5′ to 3′)
01	CCAGTGGACGCGGGCAGATGTAAAAG
02	TACACCCCTTGATCCCATTACTTAACATACAC
07	GCCTCCAATTGACCCAATTCGATG
08	GGCTTGTCTGCACAGATCAGGAAC
20	GGACAGGAAACAGCTATGACC
25	ACAGGATGTGGACCTAATGTG
27	GCTCAGTAGCGTGACGAAGAAG
37	CCAGAAGAATGGCACATGGTAC
41	GGATCCTGCAAGCATGCTCAGA
45	AGAGCCTACAAGGAGGTCTGCT
48	CACCTGCCAACTTCATCTGAAG
64	CCAGAATGCACAGGTACACTTG
65	CAAGTCGGCCACTCAAGAAGAC
67	CCACCTTAGCATGTCCTATTGC
68	TGGATTCGACACCAGCACACTG
69	GGGAGCCTATTGAATGACTCTG
71	ATTGGCACTGGCGGTTGAGTAC
89	CAAGAGCTGGTTCAACTTCTCGA
90	GTTCGTCGTGCTCGCATCTTGGT
92	CTAAGTACCAAATGATTACTGC
93	GGTAACCACCATGGATACCTCC
106	CCAGGCTTTGTGCTCCTTGGG
107	GACGTACCACAATTGACTCCC
611	CTGGTGTCGAATCCAGAGCAAGCAT
614	CCAGCTGATGTTCAGCTTCCTGAG
619	CATGCGGCCGCATGCCAGTTGTTCCCAGTGATCT
620	CATGCGGCCGCAGAGTAAAGAAGAGGAGCATGTC

ation medium and spread on agar plates of regeneration medium (15). After 24 h of incubation at room temperature, plates were overlaid with hygromycin B at 150 μg/ml in 1% water agar to yield a final concentration of 50 μg/ml in the agar plate. Putative transformants were transferred to V8 medium agar slants containing hygromycin B at 300 μg/ml, reisolated from single macroconidia, and maintained on V8 medium agar slants with hygromycin B. For production of mock transformants, the same protocol was followed, except that no DNA was added to the transformation mixture and strains were generated and maintained in the absence of hygromycin B.

Protoplast batch 1 yielded one transformant, a *MAT* deletion strain that contained two or more copies of the vector. Protoplast batch 2 yielded three transformants that contained a wild-type *MAT* locus and one or more ectopic copies of the vector; these were designated nondeletion transformants. Protoplast batch 3 yielded mock transformants and 72 transformants, including *MAT* deletion strains and nondeletion transformants. Protoplast batch 4 yielded 101 transformants, including *MAT* deletion strains and nondeletion transformants. In the text, strain numbers are preceded by ΔMAT for *MAT* deletion strains.

PCR amplification and Southern blot analysis of putative *MAT* deletion transformants. The first 24 putative *MAT* deletion transformants were analyzed by Southern blotting. Genomic DNA of each strain was digested with EcoRI, electrophoresed, blotted, and hybridized to three ^{32}P-labeled probes. Probe 1 was prepared from a 1.5-kb EcoRI fragment from pGZMAT-H and corresponded to the 1.2-down *MAT* locus flank. A 2.3-kb band was observed in strain GZ3639 and in transformants in which the vector integrated ectopically. A 0.24-kb band was observed in seven transformants that lacked the 2.3-kb band. Probe 2 was prepared from a 1.3-kb fragment generated by using PCR primers 106 (GzExnF) and 107 (GzExnR) (Table 1) and corresponding to the 2.8-up *MAT* locus flank.

A 3.3-kb band was observed in strain GZ3639 and in transformants in which the vector integrated ectopically. A 1.5-kb band was observed in the seven putative *MAT* deletion transformants (identified above) that lacked the 3.3-kb band. One of the seven transformants was not considered further because the intensity of its 1.5-kb band suggested that more than one copy of the vector was present. Probe 3 corresponds to *MAT1-1-2* and was prepared from a 1.6-kb fragment generated with primers 92 (FgMAT2R) and 93 (FgMAT2F) and genomic DNA of strain GZ3639 as a template. None of the seven putative *MAT* deletion transformants contained a hybridizing band corresponding to *MAT1-1-2*. This preliminary analysis identified six candidate *mat1-1*/*mat1-2* strains that were subjected to further analysis by PCR and Southern blotting.

These six candidate *MAT* deletion strains plus 145 additional transformants were then analyzed by an initial PCR protocol that employed two three-primer combinations to test for the presence of the wild-type *MAT* locus and pGzMAT-H-specific sequence junctions (Fig. 1; Tables 1 and 2). The first three-primer combination consisted of oligonucleotides 65, 68, and 20 and was used to detect sequence junctions in the region upstream of the *MAT* locus. For the purposes of this study, DNA sequences to the left of the four *MAT* genes in Fig. 1 are considered upstream and sequences to the right of the *MAT* genes are considered downstream. The second three-primer combination consisted of PCR primers 64, 69, and 71 and was used to detect sequence junctions in the region downstream of the *MAT* locus. A second PCR screen of transformants employed four two-primer combinations and was used to confirm the integration of pGzMAT-H via homologous recombination at the *MAT* locus. The first two sets of two-primer combinations consisted of oligonucleotide 07 paired with oligonucleotide 68 and oligonucleotide 07 paired with oligonucleotide 20, and they tested for integration of pGzMAT-H via recombination with DNA upstream of the *MAT* locus. The second two sets of two-primer combinations consisted of oligonucleotide 08 paired with oligonucleotide 69 and oligonucleotide 08 paired with oligonucleotide 64, and they tested for integration of the vector via recombination with DNA downstream of the *MAT* locus. Homologous integration of

TABLE 2. Predicted PCR amplicon sizes for *MAT* deletion strains of *G. zeae*

Primer combination	Amplicon size (bp) for[a]:	
	MAT1-1/*MAT1-2*	*mat1-1*/*mat1-2*
65-68	875	None
65-20	None	700
71-69	950	None
71-64	None	530
07-68	3,155	None
07-20	None	2,990
08-69	1,834	None
08-64	None	1,320

[a] Expected and observed sizes of PCR products amplified with the primer combinations indicated and DNA templates consisting of genomic DNA from the wild-type *MAT1-1*/*MAT1-2* strain GZ3639 and *MAT* deletion *mat1-1*/*mat1-2* strains.

vector DNA in all *mat1-1*/*mat1-2* strains was confirmed by additional Southern blot analysis. BamHI/SalI-restricted genomic DNA of each strain was electrophoresed, blotted, and hybridized to ^{32}P-labeled probes prepared from fragments 2.8-up and 1.2-down.

Construction of MAT locus complementation vector pGzMAT-Gen. To simultaneously complement the four genes within the *G. zeae MAT* locus, we constructed complementation vector pGzMAT-Gen. We amplified a 7.5-kb fragment carrying *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2* with *EXL* DNA polymerase (Stratagene, La Jolla, Calif.) from genomic DNA of strain GZ3639. Primers for this PCR amplification were 611 and 614 (Table 1). The resulting PCR product contained 12 differences in its nucleotide sequence compared with the GZ3639 sequence deposited in GenBank (accession no. AF318084). However, only one of these differences was located within a *MAT* gene coding region, and this difference did not affect the amino acid specificity of the codon in which it occurred.

The amplification product was cloned into the PCR cloning vector pCR-XL-TOPO (Invitrogen Life Technologies, Carlsbad, Calif.) to yield pGzMAT-7 (Fig. 1D). A 2.5-kb fragment carrying the Geneticin resistance gene (*GenR*) was then ligated into pGzMAT-7 via the NotI site derived from the pCR-XL-TOPO multiple cloning region. The resulting plasmid was designated pGzMAT-Gen. The *GenR* fragment was obtained by NotI digestion of pGEN-Not, a plasmid constructed by PCR amplification of *GenR* from pII99 (31) with primers 619 and 620 (Table 1) and subsequent cloning of the PCR product into pT7Blue (Novagen, Madison, Wis.). Primers 619 and 620 included NotI recognition sequences (GCGGCCGC) so that, in pGEN-Not, *GenR* was flanked by NotI sites.

Transformation of a *MAT* deletion strain. One batch of protoplasts was prepared from germinating macroconidia of *MAT* deletion strain ΔMAT#78 and used for transformation as described above for the wild-type strain, with the following modifications. Protoplasts were incubated with vector pGzMAT-GEN DNA, regeneration plates were overlaid with Geneticin at 300 μg/ml in 1% water agar, and putative transformants were transferred to V8 juice medium agar slants containing Geneticin at 300 μg/ml.

PCR amplification and Southern blot analysis of putative *MAT*-complemented transformants. PCR analysis of putative pGzMAT-Gen transformants employed two two-primer combinations to detect regions near both ends of the 7.5-kb locus fragment in pGzMAT-Gen. The first primer combination, primers 25 and 45, amplified a 0.8-kb fragment that spanned the entire *MAT1-1-3* coding region (Fig. 1). The second primer combination, primers 37 and 48, amplified a 0.6-kb fragment that spanned the 5′ half of the *MAT1-2* coding region (Fig. 1). Southern analysis of putative pGzMAT-Gen transformants employed a SalI/BamHI digestion and a hybridization probe prepared from equal amounts of two DNA templates. The first template corresponded to nucleotides 1758 to 2620 of the *G. zeae MAT* locus in GenBank accession no. AF318048 and was amplified from wild-type genomic DNA with primers 89 and 90 (Table 1). This region of DNA was not present in pGzMAT-Gen but was present in wild-type and *MAT* deletion genomic DNA (Fig. 1). The second template corresponded to nucleotides 4450 to 5301 of accession no. AF318048 and was amplified from wild-type DNA with primers 27 and 41 (Table 1). This region was present in pGzMAT-Gen and wild-type genomic DNA but not in *MAT* deletion genomic DNA (Fig. 1). A probe prepared from the two templates was expected to hybridize to a single 5.3-kb fragment of DNA from wild-type strains, a single 2.8-kb fragment of DNA from *MAT* deletion strains, and 2.2- and 2.8-kb fragments of DNA from a *MAT* deletion strain transformed with pGzMAT-Gen.

Wheat head blight assays and deoxynivalenol analysis. Five greenhouse virulence tests were conducted: test 1 in 2000, test 2 in 2001, tests 3 and 4 in 2002, and test 5 in 2003. Virulence tests were conducted on cultivar Wheaton as previously described (11). Heads were inoculated by injecting a drop of macroconidium suspension containing approximately 10^3 macroconidia into one floret of a central spikelet of each head. Control heads were injected with mung bean medium. For each treatment, 20 replicate heads were injected in tests 1, 2, and 3, and 10 replicate heads were injected in tests 4 and 5. Disease severity was calculated as the percentage of blighted spikelets in each head 17 to 21 days after inoculation. Mature heads were threshed individually to ensure that all scabby seeds were collected. Seed samples were ground and extracted with 4 ml of extraction solvent (acetonitrile-water, 86:14 vol/vol) per g in Erlenmeyer flasks with shaking for 2 h. The solvent extract was filtered through Whatman 2-V filter paper. Extracts were stored in capped vials at 4°C until analyzed. The concentration of deoxynivalenol in the extracts was determined by liquid chromatography-mass spectrometry as previously described (25).

Statistical analyses. A randomized complete block design with 10 or 20 replications was utilized in five greenhouse tests comparing percentages of head blight for various treatments. Levene's homogeneity of variance tests (21) at the 5% alpha level were performed to determine whether any datum transformations of the dependent variables were necessary. One-way analysis of variance tests were performed for each greenhouse test to detect percent head blight differences among treatments. F-test statistical results were considered significant at $P \leq 0.05$. Duncan's multiple range tests were used as the multiple comparison procedure for finding mean differences in dependent variables if a significant F-test statistic was obtained from a one-way analysis of variance. All Duncan analyses were performed at the 5% alpha level of significance.

RESULTS

Deletion of the *MAT* locus. To delete the G. *zeae MAT* locus, the linearized deletion vector pGzMAT-H was transformed into wild-type GZ3639. Deletion of the locus was expected to occur following a homologous recombination event between both fragments of *G. zeae*-derived DNA in the vector and the corresponding regions of DNA flanking the *MAT* locus in the genome. Such recombination events result in the replacement of a 9.6-kb region of DNA carrying the four *MAT* genes with a 5.6-kb fragment carrying the *hygB* and pCR XL TOPO sequences from the deletion vector (Fig. 1A, B, and C).

Hygromycin-resistant transformants were screened via PCR amplification for the presence of pGzMAT-H and wild-type sequences with two three-primer combinations (Fig. 1A, B, and C, and Tables 1 and 2). Ninety-four transformants yielded pGzMAT-H-specific amplicons but no wild type-specific amplicons. Most of the remaining transformants yielded both vector and wild type-specific amplicons. Rare transformants had more complex patterns, but DNA of these transformants was not characterized further. The 94 transformants that yielded only pGzMAT-H-specific amplicons were subjected to a second PCR screen with four two-primer combinations to determine whether pGzMAT-H had integrated via homologous recombination at the *MAT* locus or via nonhomologous recombination elsewhere in the genome. All 94 transformants examined with the two-primer combinations yielded amplicons consistent with integration of pGzMAT-H at the *MAT* locus.

Twenty-seven of the 94 putative *MAT* deletion strains were examined by Southern blot analysis to confirm homologous integration of the vector. The Southern analysis was predicted to yield hybridization patterns consisting of 5.3- and 8.4-kb bands with genomic DNA from the wild-type *MAT1-1*/*MAT1-2* strain and 2.0- and 2.8-kb bands with DNA from *mat1-1*/*mat1-2* strains. Of the 27 candidate *mat1-1*/*mat1-2* strains examined, 23 had the *MAT* deletion banding pattern with only one copy of vector DNA (Fig. 2A). Four candidate *mat1-1*/*mat1-2* strains gave more complex hybridization patterns, but DNA of these strains was not characterized further.

Self-fertility and mating ability of *mat1-1*/*mat1-2* strains. Forty-one candidate *mat1-1*/*mat1-2* strains, generated from protoplast batches 3 and 4, were tested for self-fertility on carrot agar medium (Fig. 3). On this medium, nearly all candidate *MAT* deletion strains produced the characteristic red pigmentation. Fifty-one percent of the strains produced perithecium-like structures that were superficially similar to perithecia, but in most cases, they were smaller in size and more variable in shape than perithecia produced by wild-type GZ3639. Usually, these perithecium-like structures clumped together (Fig. 3B). Examination of hundreds of squash mounts of the perithecium-like structures produced by *mat1-1*/*mat1-2* strains, however, indicated that these structures contained neither asci nor ascospores (Fig. 3E). Six *mat1-1*/*mat1-2* strains

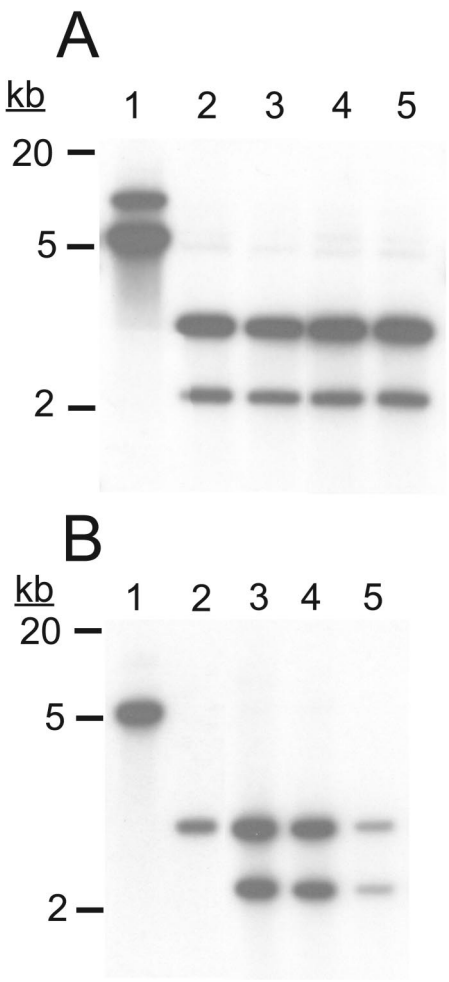

FIG. 2. (A) Southern blot analysis of *G. zeae mat1-1*/*mat1-2* strains conducted by using BamHI/SalI-digested DNA and a mixture of probes prepared from fragments 2.8-up and 1.2-down as described in Materials and Methods. Lane 1, wild-type strain GZ3639; lanes 2 to 5, *mat1-1*/*mat1-2* strains ΔMAT#9, ΔMAT#14, ΔMAT#40, and ΔMAT#43, respectively. The gel image has been modified from the original blot by deleting one lane between lanes 3 and 4. (B) Southern blot analysis of *G. zeae mat1-1*/*mat1-2* strains transformed with the complementation vector pGzMAT-Gen, conducted by using BamHI/SalI-digested DNA and a mixture of probes described in Materials and Methods and illustrated in Fig. 1. Lane 1, wild-type strain GZ3639; lane 2, *mat1-1*/*mat1-2* strain ΔMAT#78; lanes 3 to 5, *MAT*-complemented transformants 1, 2, and 9, respectively.

(ΔMAT#9, ΔMAT#14, ΔMAT#17, ΔMAT#20, ΔMAT#21, and ΔMAT#24) were self-sterile in two additional independent tests on carrot agar and in tests on media containing other natural substrates, carnation leaves, maize husks, rice straw, and mulberry twigs, that have been reported to stimulate sexual development in *Gibberella* (4, 12, 14). Wild-type GZ3639 and five nondeletion transformants produced perithecia with ascospores on carrot agar and carnation leaf agar but rarely produced them on the other natural substrates.

Five *mat1-1*/*mat1-2* strains that were generated from protoplast batch 3 were tested for their ability to mate with wild-type GZ3639. Because GZ3639 is self-fertile, non-nitrate-utilizing (*nit*) mutants of this wild-type strain and of the five *mat1-1*/*mat1-2* strains were used to facilitate identification of heterozygous perithecia. Perithecia were picked from matings of GZ3639 with strains ΔMAT#9 (24 perithecia), ΔMAT#14 (40 perithecia), ΔMAT#17 (12 perithecia), ΔMAT#20 (12 perithecia), and ΔMAT#24 (42 perithecia). Of the 3,741 random ascospore progeny isolated, 2,364 germinated but none could utilize nitrate. When tested for resistance to hygromycin B, 494 progeny representing the five crosses were sensitive to the antibiotic.

Forty nondeletion transformants that were generated from protoplast batch 3 were tested at least once for self-fertility on carrot agar medium, but only 15% of these transformants produced perithecia and ascospores. Eight mock transformants prepared from the same batch of protoplasts were tested for self-fertility on carrot agar; 50% were self-fertile and produced perithecia and ascospores. These data suggest that mock transformants are more fertile than nondeletion transformants.

Wheat head blight after injecting macroconidia from *mat1-1*/*mat1-2* strains. Although *mat1-1*/*mat1-2* strains produced no ascospores, they produced wild-type numbers of macroconidia after 4 days culture in mung bean liquid medium. Twenty-seven candidate *mat1-1*/*mat1-2* strains produced suspensions with 1.0×10^5 to 10.5×10^5 macroconidia per ml and a mean ± standard deviation (SD) of $3.3 \times 10^5 \pm 2.3 \times 10^5$ macroconidia per ml. Ten strains with a wild-type *MAT* locus, including GZ3639, nondeletion transformants, and mock transformants, produced suspensions with 2.6×10^5 to 13.3×10^5 macroconidia per ml and a mean ± SD of $5.4 \times 10^5 \pm 3.4 \times 10^5$ macroconidia per ml.

Macroconidia from GZ3639 and from 27 *mat1-1*/*mat1-2* strains that were generated from protoplast batch 3 were tested for the ability to cause wheat head blight in the first three greenhouse tests. Wheat heads were scored for the average percentage of blighted spikelets per head at 17 to 21 days after fungal inoculation. Strain GZ3639 produced 91, 97, and 85% head blight in tests 1, 2, and 3, respectively. Control wheat heads inoculated only with mung bean medium showed head blight percentages of 23, 5, and 19% in tests 1, 2, and 3, respectively. All 27 *mat1-1*/*mat1-2* strains that were compared in greenhouse tests 1, 2, and 3 were virulent, but most strains produced less head blight than strain GZ3639 ($P < 0.05$). For six *MAT* deletion strains that were tested twice, the percentage of head blight was 44% ± 9% (mean ± SD) in test 1 and 36% ± 9% (mean ± SD) in test 2. For the 21 remaining *MAT* deletion strains that were tested only once, the percentage of head blight was 40% ± 13% (mean ± SD) in test 3. Six mock transformants and two nondeletion transformants were tested for the ability to cause wheat head blight in the second and third greenhouse tests. The percentage of head blight was 55% ± 10% (mean ± SD) for four of these strains in test 1 and 33% ± 11% for eight strains in test 2.

MAT deletion strains and nondeletion transformants that were generated from protoplast batch 4 were tested for virulence in greenhouse test 4. Macroconidia from GZ3639, 38 *MAT* deletion strains, six nondeletion transformants, and the non-trichothecene-producing, low-virulence strain GZT40 were tested for the ability to cause wheat head blight. In this test, GZ3639 produced 97% head blight when scored 17 to 18 days after inoculation. For the 38 *MAT* deletion strains, the percentage of head blight was 92% ± 7% (mean ± SD), which

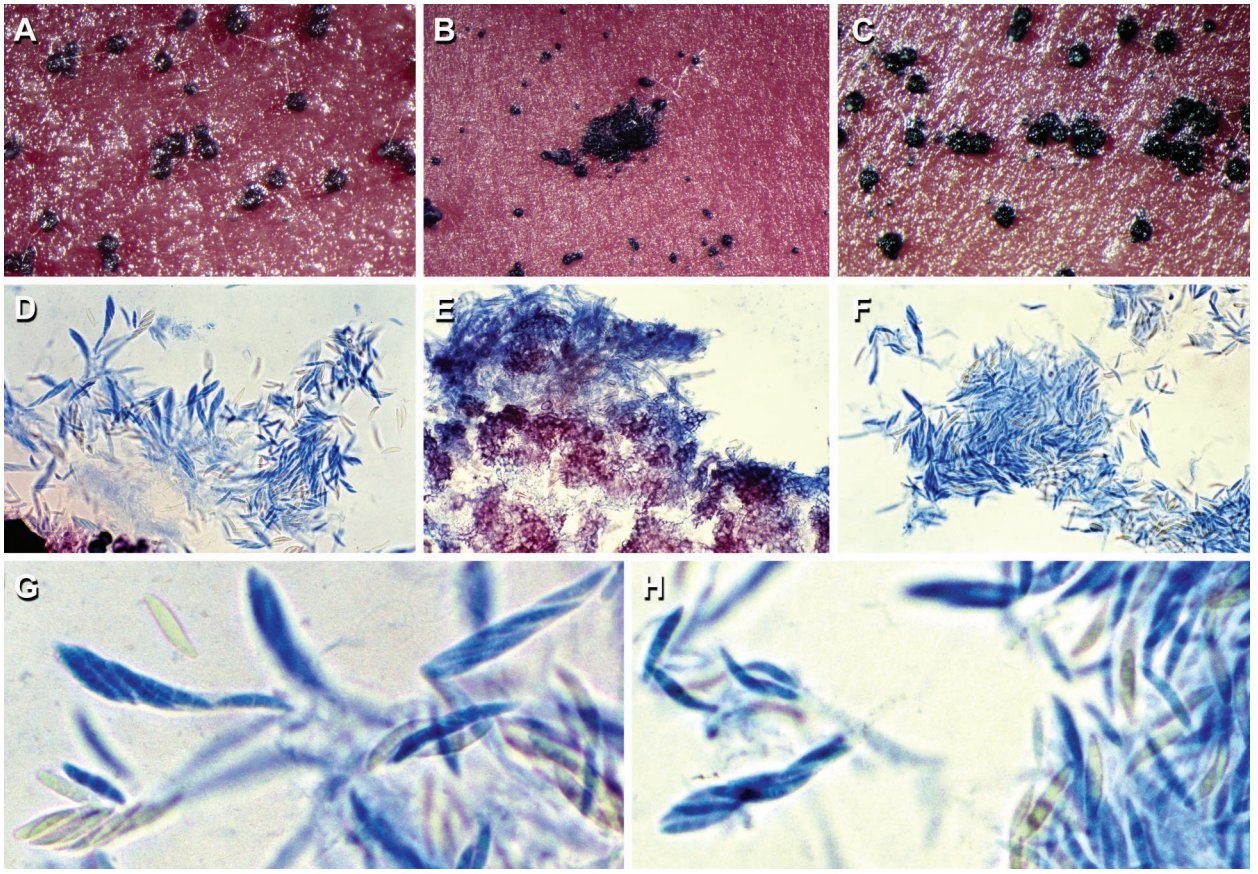

FIG. 3. Self-fertility of *G. zeae* wild-type GZ3639 (A, D, and G), *MAT* deletion strain ΔMAT#78 (B and E), and a representative *MAT* add-back strain (C, F, and H). The top row shows the morphology and distribution patterns of perithecia on carrot agar plates. The middle row shows the contents of squashed perithecia and perithecium-like structures, stained with cotton blue. The bottom row shows a higher magnification view of asci and individual ascospores produced by wild-type and add-back strains.

was not significantly different from that for the wild-type strain ($P < 0.05$). For the six nondeletion transformants, the percentage of head blight was 94% ± 6% (mean ± SD), which was not significantly different from that for the wild-type strain. Control wheat heads inoculated only with mung bean medium or with the low-virulence strain GZT40 showed only 6 to 7% head blight in this test. The wild-type virulence of the new *MAT* deletion strains and nondeletion transformants indicated that the reduced virulence of some of the previous *MAT* deletion strains was not due to specific deletion of the *MAT* locus.

Complementation of the *MAT* locus. pGzMAT-Gen was transformed into *MAT* deletion strain ΔMAT#78, which was generated from protoplast batch 4. This strain was selected as the recipient because of its wild-type virulence on wheat in greenhouse test 4. Thirty-nine Geneticin-resistant strains recovered following transformation were analyzed by PCR with primer pairs 25-45 and 37-48 (Fig. 1D). All 39 transformants yielded the expected 0.8- and 0.6-kb amplification products, a result consistent with the presence of both ends of the 7.5-kb *MAT* fragment in pGzMAT-Gen. A subset of 10 candidate *MAT*-complemented transformants was analyzed by Southern blotting to confirm the presence of the *MAT* locus. All 10 transformants had a hybridization pattern consisting of the expected 2.2- and 2.8-kb bands in a manner consistent with the presence of a single copy of pGzMAT-Gen (Fig. 2B).

Self-fertility of *MAT1-1*/*MAT1-2* complemented strains. Thirty candidate *MAT1-1*/*MAT1-2* strains were tested for self-fertility on carrot agar medium. All of the strains produced enlarged perithecia on this medium, and 97% of the strains produced asci and ascospores (Fig. 3C, F, and H). In general, *MAT1-1*/*MAT1-2* complemented strains were able to produce fertile perithecia in abundance, similar to wild-type GZ3639.

Wheat head blight after injecting macroconidia of *MAT1-1*/*MAT1-2* complemented strains. Macroconidia from GZ3639 and from 39 *MAT1-1*/*MAT1-2* strains were tested for the ability to cause wheat head blight in greenhouse test 5. In this test, GZ3639 produced 81% head blight when scored 17 to 18 days after inoculation. All of the *MAT*-complemented strains tested were highly virulent; the percentage of head blight for the 39 strains was 92% ± 12% (mean ± SD), which was not significantly different from that for the wild-type strain ($P < 0.05$).

The wild-type GZ3639 and all classes of strains generated during this study, including mock transformants, nondeletion transformants, *MAT* deletion strains, and *MAT*-complemented strains, could produce deoxynivalenol in wheat heads in greenhouse virulence tests (data not shown). However, deoxynivale-

nol was not used as a disease parameter in greenhouse tests because the amount of seed available from individual heads was usually too low for accurate measurement of deoxynivalenol.

DISCUSSION

More than a century of careful field observations have identified the complex nature of the interactions among the three elements of the disease triangle (host, pathogen, and environment) in the development of a wheat head blight epidemic (30). To date, efforts to control wheat head blight with resistant cultivars, fungicides, biological control, and other practices have had limited success. Identification of traits that contribute to the pathogenicity of *G. zeae* under field conditions could identify potential novel targets for disease control, but the identification of such traits has been hindered by the polygenic nature of wheat resistance to head blight and the high impact of environmental variability on expression of disease.

Targeted gene deletion provides a useful experimental tool for testing the importance of a particular trait in a complex agroecosystem. For example, deletion of *TRI5*, a gene required for trichothecene biosynthesis in *G. zeae*, had a large effect on the development of wheat head blight both in the greenhouse and in the field (13, 27). Identification and deletion of *G. zeae* homologs of virulence factors known from other fungi has demonstrated the importance of the product of the peptide synthetase-like *CPS1* gene and the protein kinase MGV1 in wheat head blight under controlled conditions in the greenhouse (16, 19). The availability of databases of expressed sequence tags (33) and the recent public release of the complete genome sequence of *G. zeae* (www.genome.wi.mit.edu/annotation/fungi/fusarium/index.html) will provide countless new targets for gene deletion strategies.

The construction of *MAT*-null, non-ascospore-producing strains of *G. zeae* initiates examination of a trait that may be important in pathogen fitness in the wheat head blight agroecosystem. In this study, we analyzed the pathogenicity of *MAT* deletion *G. zeae* and showed that macroconidia of such strains can cause blight following direct injection into wheat heads in the greenhouse. Although some *MAT* deletion strains and other transformants were less virulent than the wild-type strain from which they were derived, the reduced virulence of these transformants was linked to the source of protoplasts and not to the transforming DNA. Thus, it is likely that procedures used for protoplast production and transformation reduced virulence by causing alterations at loci other than the targeted gene, a phenomenon previously observed in transformation-mediated gene disruption in *G. zeae* (11).

G. zeae mat1-1/*mat1-2* strains produced neither true perithecia nor ascospores, which is consistent with the previous observation that *mat1-1*/*MAT1-2* strains and *MAT1-1*/*mat1-2* strains are self-sterile (18). In the present study, however, some *mat1-1*/*mat1-2* deletion transformants, including the strain we used for add-back experiments, produced perithecium-like structures, in contrast to transformants carrying a deletion of one or the other *MAT* idiomorph (18). We do not have an explanation for this apparent phenotypic difference. When *G. zeae MAT* deletion strains were mated with GZ3639, none of the perithecia were heterozygous and all of the progeny were derived from GZ3639. Definitive proof that *MAT* deletion strains cannot mate requires analysis of more ascospore progeny than were tested in this study. Even among highly self-fertile strains of *G. zeae*, the frequency of heterozygous perithecia can be highly variable, ranging from 0 to 60% in previous studies that included GZ3639; thus, the absence of mating in vitro does not necessarily prove that the strains are unable to mate (7, 11; A. E. Desjardins, unpublished data).

In the *MAT* deletion experiment in the present study, some transformants with an apparently wild-type *MAT* locus were self-sterile. We cannot explain these results. One possibility is that vector DNA inserted at a site close to the wild-type *MAT* locus and affected its function in nondeletion transformants. The vector insertion sites in these self-sterile nondeletion transformants remain to be identified.

In 1981, the *Fusarium* taxonomist Colin Booth (5) concluded, "The presence of the perfect or perithecial state (teleomorph) in *Fusarium*, appears, superficially at least, to be of more value to the taxonomist than the fungus." Because *G. zeae* is homothallic and can reproduce both asexually and sexually in nature, this species provides an excellent system for comprehensive analysis of the value of the *Gibberella* sexual state to a *Fusarium* species. The availability of a series of virulent, isogenic *G. zeae* strains with wild-type, deleted, and complemented *MAT* loci should allow rigorous tests of the importance of ascospores in the wheat head blight agroecosystem and in other aspects of the biology of this important plant pathogen.

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