Effect of Carbendazim Resistance on Trichothecene Production and Aggressiveness of *Fusarium graminearum*

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Fusarium graminearum (teleomorph, *Gibberella zeae*) causes head blight of cereals and contaminates grains with trichothecene mycotoxins that are harmful to humans and domesticated animals. Control of *Fusarium* head blight relies on carbendazim (MBC) in China, but resistance to MBC in *F. graminearum* is now widespread. Sixty-seven strains were evaluated for trichothecene production in shake culture or in the field. The strains included 60 wild-type strains (30 MBC-resistant and 30 MBC-sensitive), three MBC-resistant site-directed mutants at codon 167 in β_2 -tubulin, three MBC-sensitive site-directed mutants at codon 240 in β_2 -tubulin, and their MBC-sensitive wild-type progenitor strain ZF21. The incidence of infected spikelets and the amount of *F. graminearum* DNA in field grain (AFgDNA) also were evaluated for all strains. MBC resistance increased trichothecene production in shake culture or in the field. Although MBC resistance did not change the incidence of infected spikelets, it did increase AFgDNA. *Tri5* gene expression increased in MBC-resistant strains grown in shake culture. We found a significant exponential relationship between trichothecene production and *Tri5* gene expression in shake culture and a linear relationship between the incidence of infected spikelets or AFgDNA and trichothecene production in field grain.

Fusarium head blight (FHB), also known as scab, is a destructive fungal disease of wheat and other small grain cereals worldwide, and is particularly favored by high humidity and warm temperatures during flowering (McMullen et al. 1997; Parry et al. 1995). Recent outbreaks in Asia, Canada, Europe, and South America suggest that FHB is a growing threat to the world's grain supply (Goswami and Kistler 2004). Although FHB can also be caused by other *Fusarium* spp., *Fusarium graminearum* (teleomorph: *Gibberella zeae* (Schwein.) Petch) is by far the most important causal organism in China. In an extensive survey of 21 Chinese provinces, 95% of *Fusarium* cultures isolated from 2,450 diseased wheat heads were determined to be *F. graminearum* (Gale et al. 2002). In China, FHB was initially reported in 1936 and, since then, FHB epi-

demics have become more severe and frequent in the middle and lower regions of the Yangtze River and in Heilongjiang Province in northeastern China (Chen et al. 2000). More recently, FHB has gradually spread toward the Yellow River and the Huai River in northern China (Qu et al. 2008). This pathogen poses a double threat to cereals. First, seed quality and yield are significantly reduced (diseased plants produced discolored, shriveled kernels). Second, the scabby grain is often contaminated with trichothecene mycotoxins, making it unsuitable for human food or animal feed. The trichothecene toxins produced by this fungus pose a serious hazard to animal and plant health in that these sesquiterpenoids are potent inhibitors of eukaryotic protein biosynthesis (Desjardins 2006).

The principal trichothecene mycotoxins associated with FHB are deoxynivalenol (DON) and nivalenol (NIV). Three strain-specific profiles of trichothecene metabolites (chemotypes) have been identified in *F. graminearum*: i) NIV and acetylated derivatives (NIV chemotype), ii) DON and 3-acetyldeoxynivalenol (3ADON chemotype), and iii) DON and 15-acetyldeoxynivalenol (15ADON chemotype) (Miller et al. 1991). In China, more than 90% of FHB isolates have the 3ADON chemotype (Qu et al. 2008). Trichothecene production contributes to the virulence of *F. graminearum* toward wheat (Proctor et al. 1995). DON biosynthesis requires approximately 15 biochemical steps (Kimura et al. 2007). The initial unique step in the trichothecene biosynthetic pathway is catalyzed by the enzyme trichodiene synthase to produce the intermediate trichodiene (Proctor et al. 1995). Trichodiene synthase is encoded by the *Tri5* gene. In three *Fusarium* spp., at least 10 trichothecene biosynthesis genes, including *Tri5*, are located near one another in the trichothecene gene cluster (Kimura et al. 2007).

Various strategies have been developed to control FHB and to reduce mycotoxin contamination of cereals, with chemical control having an important role in an integrated FHB control program (Müllenborn et al. 2008). Benzimidazole fungicides, particularly carbendazim (MBC), have been used to control FHB in China for over 30 years (Yuan and Zhou 2005). MBC resistance develops in many fungi after the fungicide has been used for only 2 to 3 years (Bollen and Scholten 1971; Wicks 1974). However, MBC resistance in *F. graminearum* was neither detected nor reported worldwide until 1992, when the first MBC-resistant (MBC^R) field isolate of *F. graminearum* was recorded in the Zhejiang Province of China (Zhou et al. 1994). MBC-sensitive (MBC^S) isolates of *F. graminearum* could not grow on potato sucrose agar (PSA) containing MBC at 1.4 $\mu\text{g ml}^{-1}$ whereas MBC^R isolates grew normally on this

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medium (Zhou and Wang 2001). Therefore, the concentration of 1.4 $\mu\text{g ml}^{-1}$ has been used as the cut-off for detecting resistant field isolates in vitro. The frequency of resistant isolates in some regions has been increasing gradually, and the efficacy of MBC against *F. graminearum* decreased dramatically after 1998 (Wang et al. 2002).

The most studied and best understood resistance mechanism to benzimidazole fungicides is a replacement of glutamic acid with either alanine or lysine at codon 198 or 200 of the β_1 -tubulin gene (Catherine et al. 1999; Faretra and Pollastro 1991). In some fungi, this mutation reduces fitness under field conditions (Jung and Oakley 1990). Previous studies did not find any mutations in the β_1 -tubulin gene of resistant isolates of *F. graminearum* (Li et al. 2003). More recently, the resistance of *F. graminearum* to MBC has been attributed to mutations at codon 167, 198, or 200 in the β_2 -tubulin gene, but mutation at codon 167 was the main mutation type found in the field (Chen et al. 2008). Both MBC^R and MBC^S strains had similar fitness based on mycelial linear growth in vitro, sporulation capacity in vitro, FHB severity, and capacity to produce perithecia in vitro (Chen et al. 2007). Moreover, trichothecenes (including T-2 toxin, 4,15-diacetoxyscirpenol, and neosolaniol) produced by *F. sporotrichioides* were affected by the MBC resistance (D'Mello et al. 2000). However, there are no data on the effect of MBC resistance on trichothecene production by *F. graminearum* strains. These data are needed to assess the risk of resistance of *F. graminearum* to MBC.

In this article, we measured trichothecene production and *Tri5* gene expression by MBC^S and MBC^R *F. graminearum* wild-type strains in shake culture or in the field. Although our goal was to measure the effect of MBC resistance on trichothecene production of *F. graminearum* strains, wild-type strains of this fungus have diverse genetic backgrounds that make it difficult to compare the effect of MBC resistance on trichothecene production. Thus, we also evaluated trichothecene production and *Tri5* gene expression by several site-directed mutants (derived from a single wild-type MBC^S strain). Because the aggressiveness of the *F. graminearum* strain toward wheat is closely correlated with trichothecene production by each strain (Goswami and Kistler 2005), we also compared the effect of MBC resistance on the aggressiveness of *F. graminearum* strains.

RESULTS

Confirmation and MBC sensitivity test of site-directed mutants.

Twenty-three putative β_2 -tubulin deletion mutants for ZF21 had homologous recombination-specific amplicons but no wild-type-specific amplicons (data not shown). Southern blot analysis

of *Hind*III-digested genomic DNA using probe 1 yielded a single 3.4-kb band in the wild-type progenitor ZF21, instead of a single 6.0-kb band in the β_2 -tubulin deletion mutants (Fig. 1A). Southern blot analysis of *Sal*I-digested genomic DNA using probe 2 yielded a single 6.4-kb band in the β_2 -tubulin deletion mutants (Fig. 1B). Twenty-six putative β_2 -tubulin site-directed mutants at codon 167 and 21 putative β_2 -tubulin site-directed mutants at codon 240 were confirmed by polymerase chain reaction (PCR). Southern blot analysis of *Hind*III-digested genomic DNA using probe 1 yielded a single 6.0-kb band in the β_2 -tubulin deletion mutants and a single 3.4-kb band in the wild-type progenitor ZF21 or β_2 -tubulin site-directed mutants (Fig. 1C). Southern blot analysis of *Sal*I-digested genomic DNA using probe 3 yielded a single 6.1-kb band in the wild-type progenitor ZF21 or β_2 -tubulin site-directed mutants (Fig.

Fig. 1. Southern blot analysis of *Fusarium graminearum* wild-type progenitor ZF21, β_2 -tubulin deletion mutants, and β_2 -tubulin site-directed mutants. **A**, Probe 1 hybridized to *Hind*III-digested genomic DNA of β_2 -tubulin deletion mutants; **B**, Probe 2 hybridized to *Sal*I-digested genomic DNA of β_2 -tubulin deletion mutants; **C**, Probe 1 hybridized to *Hind*III-digested genomic DNA of β_2 -tubulin site-directed mutants; **D**, Probe 3 hybridized to *Sal*I-digested genomic DNA of β_2 -tubulin site-directed mutants. 1, ZF21; 2, $\Delta\beta_2$ tub#6; 3, $\Delta\beta_2$ tub#9; 4, $\Delta\beta_2$ tub#11; 5, R β_2 tub167Tyr#25; 6, R β_2 tub167Tyr# 42; 7, R β_2 tub167Tyr#45; 8, R β_2 tub240Leu#3; 9, R β_2 tub240Leu#10; 10, R β_2 tub240Leu#23. a = 3.4 kb, b = 6.0 kb, c = 6.4 kb, d = 6.0 kb, e = 3.4 kb, and f = 6.1 kb.

Table 1. *Fusarium graminearum* strains used in this study^z

Strain	Mutation type	Sensitivity to MBC	Number	Origin
ZF21,ZF22,ZF43,S3-S8,U1,JF0656-JF0658,JF0662,O3,O4,AT81,JX03,JX102,FG10, FG11, 2HR,4HR,Q6,Q8-Q10,B2, P1,P2	Wild type	MBC ^S	30	Wheat field
ZF52,R3,R5,S1,S2,JT04,JJ05-1,JJ05-2,JF062,JF063,RD05-1, NT7,L1,L2,U2,U3,X3, X4, EII2, GII2, Y3,C,D,HII,I,T1, AII2,J3,E1, III3	Wild type	MBC ^R	30	Wheat field
R β_2 tub240Leu#3,R β_2 tub240Leu#10,R β_2 tub240Leu#23	Codon 240 in the β_2 -tubulin gene	MBC ^S	3	Derived from ZF21
R β_2 tub167Tyr#25,R β_2 tub167Tyr#42,R β_2 tub167Tyr#45	Codon 167 in the β_2 -tubulin gene	MBC ^R	3	Derived from ZF21

^z Carbendazim (MBC)-sensitive (MBC^S) *F. graminearum* strains cannot grow on potato sucrose agar (PSA) containing MBC at 1.4 $\mu\text{g ml}^{-1}$, whereas MBC-resistant (MBC^R) strains can grow in this medium (Wang and Zhou 2002). Mutation at codon 167 in the β_2 -tubulin gene, conferring the resistance of strains to MBC, was the main mutation type found in the field. Mutation at codon 240 was used as a negative control. All strains were used to test trichothecene production and aggressiveness in 2007 and 2008. The following strains were arbitrarily selected and used to test *Tri5* gene expression: MBC^S wild-type strains (ZF43, AT81, JF0662, Q6, and JX03), MBC^R wild-type strains (R5, JT04, U3, J3, and E1), and wild-type progenitor ZF21 and its site-directed mutants (R β_2 tub240Leu#3, R β_2 tub240Leu#10, R β_2 tub240Leu#23, R β_2 tub167Tyr#25, R β_2 tub167Tyr# 42, and R β_2 tub167Tyr#45).

1D). Site-directed mutants at codon 167 or 240 of β_2 -tubulin were MBC^R or MBC^S, respectively (Table 1).

Effect of MBC resistance on trichothecene production and aggressiveness of *F. graminearum* wild-type strains in shake culture or in the field.

More trichothecenes ($P < 0.05$) were produced by MBC^R wild-type strains than MBC^S wild-type strains both in shake culture and in the field (Table 2). Although the incidence of infected spikelets did not differ between MBC^R and MBC^S wild-type strains, the amount of *F. graminearum* DNA in field grain was significantly higher with MBC^R wild-type strains than with MBC^S wild-type strains in both 2007 and 2008 (Table 2).

Effect of MBC resistance on trichothecene production and aggressiveness of *F. graminearum* site-directed mutants in shake culture or in the field.

We also measured trichothecene production by *F. graminearum* site-directed mutants and their wild-type progenitor ZF21 (Table 3). The site-directed mutants at codon 167 in the β_2 -tubulin gene produced significantly ($P < 0.05$) more trichothecenes than did the site-directed mutants at codon 240 or the

wild-type progenitor ZF21 in shake culture and in the field. There was no significant difference in the amount of trichothecenes produced by the site-directed mutants at codon 240 and the wild-type progenitor ZF21. The aggressiveness trait, the incidence of infected spikelets, did not significantly differ among the site-directed mutants and the wild-type progenitor ZF21. The quantity of *F. graminearum* DNA in field grain was greater with the site-directed mutants at codon 167 than with the site-directed mutants at codon 240 or with the wild-type progenitor ZF21 in both 2007 and 2008; however, there was no significant difference between the site-directed mutants at codon 240 and the wild-type progenitor ZF21.

Effect of MBC resistance on the *Tri5* gene expression of *F. graminearum* wild-type strains or site-directed mutants in shake culture.

The expression of the *Tri5* gene was evaluated relative to that of the elongation factor 1- α (*EF1- α*) gene. *Tri5* gene expression was significantly higher ($P < 0.05$) in the five MBC^R wild-type strains than in the five MBC^S wild-type strains 72, 96, 120, and 144 h after flasks were inoculated (Fig. 2A). Results were similar with the site-directed mutants (i.e., *Tri5* gene expression was greater in the MBC^R site-directed mutants at codon 167 than in the MBC^S site-directed mutants at codon 240 or the MBC^S wild-type progenitor ZF21 72, 96, 120, and 144 h after inoculation) (Fig. 2B). *Tri5* gene expression, however, did not significantly differ between the MBC^S site-directed mutants at codon 240 and the wild-type progenitor ZF21.

Relationship between trichothecene production and *Tri5* gene expression or aggressiveness of *F. graminearum*.

Based on regression analysis, there was a significant ($P < 0.05$) relationship between trichothecene production and *Tri5* gene expression in shake culture (Fig. 3) but the relationship was exponential rather than linear. For both MBC^S and MBC^R strains, the incidence of infected spikelets and trichothecene production in field grain both were linearly related (Fig. 4A). For both MBC^S and MBC^R strains, the amount of *F. graminearum* DNA and trichothecene production in field grain were linearly related (Fig. 4B). However, the correlation coeffi-

Fig. 2. Time course of the *Tri5* gene expression by *Fusarium graminearum* in shake cultures containing glucose–yeast extract–peptone medium. **A**, Wild-type strains. **B**, Site-directed mutants (mutants at codon 167 are carbendazim resistant and mutants at codon 240 are carbendazim sensitive) and its wild-type progenitor ZF21. Values are the means $\pm$ standard error of three replicates.

Table 2. Trichothecenes production, the incidence of infected spikelets, and the amount of *Fusarium graminearum* DNA in field grain by MBC^R and MBC^S *F. graminearum* wild-type strains in shake culture or under field conditions^z

Strain type	TPSC	Field experiment 1 (2007)			Field experiment 2 (2008)		
		TPFG	IIS	AFgDNA	TPFG	IIS	AFgDNA
MBC ^R wild type	88 $\pm$ 12 a	17 $\pm$ 1.8 a	27 $\pm$ 2.6 a	223 $\pm$ 22 a	21 $\pm$ 2.3 a	31 $\pm$ 3.3 a	300 $\pm$ 24 a
MBC ^S wild type	23 $\pm$ 2.6 b	9.1 $\pm$ 1.3 b	26 $\pm$ 2.2 a	170 $\pm$ 13 b	12 $\pm$ 1.6 b	29 $\pm$ 2.6 a	218 $\pm$ 19 b

^z Carbendazim (MBC)-resistant and -sensitive (MBC^R and MBC^S, respectively). Trichothecene production was measured in shake culture (TPSC, $\mu\text{g g}^{-1}$ of dry mycelia) in the laboratory and in field grain (TPFG, mg kg^{-1} of grain) in two field experiments. The incidence (%) of infected spikelets (IIS) and the amount of *F. graminearum* DNA in field grain (AFgDNA, $\mu\text{g kg}^{-1}$ of grain) were measured in the field experiments. For statistical analysis, IIS data were arcsine transformed and the values presented are back-transformed means. Data for TPSC, TPFG, and AFgDNA did not require transformation. Values are the means $\pm$ standard error of 30 strains. Means followed by the same letter in a column are not significantly different by the least significant difference (df = 29; $P > 0.05$).

Table 3. Trichothecenes production, the incidence of infected spikelets, and the amount of *Fusarium graminearum* DNA in field grain by ZF21 and its site-directed mutants in shake culture or under field conditions^z

Strain type	TPSC	Field experiment 1 (2007)			Field experiment 2 (2008)		
		TPFG	IIS	AFgDNA	TPFG	IIS	AFgDNA
Wild-type progenitor ZF21	28 $\pm$ 1.8 b	7.5 $\pm$ 0.38 b	24 $\pm$ 1.4 a	149 $\pm$ 16 b	9.8 $\pm$ 1.4 b	31 $\pm$ 1.9 a	194 $\pm$ 20 b
Mutants at codon 240	26 $\pm$ 1.6 b	7.3 $\pm$ 0.36 b	25 $\pm$ 1.5 a	141 $\pm$ 15 b	8.6 $\pm$ 1.7 b	34 $\pm$ 2.1 a	191 $\pm$ 21 b
Mutants at codon 167	54 $\pm$ 7.5 a	16 $\pm$ 3.5 a	22 $\pm$ 1.7 a	218 $\pm$ 17 a	22 $\pm$ 4.9 a	32 $\pm$ 2.4 a	306 $\pm$ 24 a

^z Trichothecene production was measured in shake culture (TPSC, $\mu\text{g g}^{-1}$ of dry mycelia) in the laboratory and in field grain (TPFG, mg kg^{-1} of grain) in two field experiments. The incidence (%) of infected spikelets (IIS) and the amount of *F. graminearum* DNA in field grain (AFgDNA, $\mu\text{g kg}^{-1}$ of grain) were measured in the field experiments. For statistical analysis, IIS data were arcsine transformed, and the values presented are back-transformed means. Data for TPSC, TPFG, and AFgDNA did not require transformation. Values are the means $\pm$ standard error of three replicates. Means followed by the same letter in a column are not significantly different by the least significant difference (df = 4; $P > 0.05$).

cients (R) were higher for the relationship between the amount of *F. graminearum* DNA and trichothecene production in field grain than for the relationship between the incidence of infected spikelets and trichothecene production in field grain. Moreover, in the linear equations ($y = ax + b$) between the incidence of infected spikelets or the amount of *F. graminearum* DNA and trichothecene production in field grain, the slopes (a) were higher for MBC^R strains than for MBC^S strains.

DISCUSSION

The present study is the first report of the effect of MBC resistance on trichothecene production by *F. graminearum* strains. Trichothecene production by *F. graminearum* was generally higher in the MBC^R wild-type strains than in the MBC^S wild-type strains. The *Tri5* gene also was expressed at higher levels in the MBC^R wild-type strains than in the MBC^S wild-type strains. However, trichothecene production by *F. graminearum* wild-type strains varied widely regardless of MBC^R or MBC^S status. These results are consistent with previous results (Mirocha et al. 1989; Walker et al. 2001). Therefore, the effect of MBC resistance on trichothecene production was apparent only when large numbers of MBC^R and MBC^S strains were compared.

To accurately determine the effect of MBC resistance on trichothecene production, we compared trichothecene production and *Tri5* gene expression in MBC^R β_2 -tubulin site-directed mutants at codon 167, MBC^S β_2 -tubulin site-directed mutants at codon 240, and their wild-type progenitor strain ZF21. Mutants at codon 167 produced significantly more trichothecenes and had higher *Tri5* gene expression than did their wild-type progenitor ZF21. However, mutants at codon 240, which were sensitive to MBC, produced amounts of trichothecenes and had levels of *Tri5* gene expression similar to those of their wild-type progenitor ZF21. This indicated that increased trichothecene production was due to MBC resistance rather than to the process of site-directed mutagenesis. There also was a significant exponential relationship between trichothecene production and *Tri5* gene expression in shake culture. Consequently, we hypothesize that MBC resistance results in increased trichothecene production by *F. graminearum* strains through increased *Tri5* gene expression.

Previous work has shown that FHB severity on wheat was identical when caused by MBC^R or MBC^S strains of *F. graminearum* (Chen et al. 2007). We also found that the aggressiveness trait, the incidence of infected spikelets, did not differ between MBC^R and MBC^S strains. This result supports the hy-

pothesis that MBC resistance does not significantly reduce the fitness of *F. graminearum* strains. Previous reports indicated a positive correlation between the trichothecene production in grain and head blight rating (Cumagun et al. 2004; Goswami and Kistler 2005). The data in our trial are consistent with a relationship between the incidence of infected spikelets and trichothecene production in field grain. However, the linear equations between the incidence of infected spikelets and trichothecene production in field grain differed for MBC^R and MBC^S strains. The slopes of this relationship were higher for MBC^R than for MBC^S strains. It seems that MBC resistance can increase the efficiency of trichothecene production per unit of pathogen.

Because samples of grain with higher fungal DNA content usually had higher levels of trichothecenes, the differences in trichothecene production might reflect only the different amounts of fungal biomass in field grain. Thus, we also quantified the mycelium in grain, as indicated by the amount of *F. graminearum* DNA. There was a significant relationship between the amount of *F. graminearum* DNA and trichothecene production in field grain. Moreover, we also found that the correlation coefficients were significantly higher for the relationship between the amount of *F. graminearum* DNA and trichothecene production in field grain than for the relationship between the incidence of infected spikelets and trichothecene production in field grain. This may be due to the fact that the quantification was more accurate for *F. graminearum* DNA in harvested grain than for the incidence of infected spikelets. In addition, the slopes of the linear equation were higher for MBC^R strains than for MBC^S strains. However, the difference between the slopes for MBC^R and MBC^S strains was less when the first variable was quantity of *F. graminearum* DNA rather than the incidence of infected spikelets. This indicated that MBC resistance might be associated with increased spread of

Fig. 3. Relationship between relative *Tri5* gene expression and trichothecene production by *Fusarium graminearum* strains 144 h after inoculation of shake cultures containing glucose–yeast extract–peptone medium.

Fig. 4. Relationship between **A**, the incidence of infected spikelets and **B**, the amount of *Fusarium graminearum* DNA and trichothecene production in field grain of wheat heads inoculated with MBC-sensitive (MBC^S) or MBC-resistant (MBC^R) strains.

the fungus within the spike but not with increased incidence of infected spikelets.

Although our results do not provide deep insight into the mechanism by which MBC resistance increases trichothecene production, all of the data from shake culture and from the field indicated that MBC resistance increases trichothecene production by *F. graminearum* strains. This finding increases our understanding of the harmfulness of MBC resistance and should make pest managers think carefully about the strategy of chemical control for FHB disease.

MATERIALS AND METHODS

Fungal material.

F. graminearum wild-type strains were collected from wheat spikes showing FHB symptoms during 2004 to 2006 in regions with a known history of FHB epidemics in China. Strains were obtained by single-spore isolation, morphologically identified (identifications were confirmed by sequence characterized amplified region analysis), and assessed for MBC sensitivity as previously described (Nelson et al. 1983; Nicholson et al. 1998; Wang and Zhou 2002). In all, 30 MBC^S and 30 MBC^R wild-type strains were arbitrarily selected and used in this study (Table 1). Also used were six site-directed mutants derived from a single MBC^S wild-type progenitor, ZF21 (Table 1). These site-directed mutants, which had the same genetic background as their wild-type progenitor strain ZF21 except for the site-directed mutation, were obtained through the following steps and confirmed to be resistant or sensitive to MBC as described for the wild-type strains.

Production and confirmation of site-directed mutants.

The knockout construct pB2U-hpt-tk-D was generated by a modified double-joint PCR (Yu et al. 2004). In the first round

of PCR reaction, 1.1 kb upstream (1.1-up) and 1.2 kb downstream (1.2-down) of β_2 -tubulin (FGSG06611) coding region was amplified from genomic DNA of wild-type progenitor ZF21 with primer pairs 1-2 and 5-6 (Table 4), respectively. Also, a 3.5-kb *hpt-tk* cassette, containing *Aspergillus nidulans* *trpC* promoter (P_{trpC}), hygromycin phosphotransferase cassette (*hpt*), polyadenylation signal from *Cauliflower mosaic virus* (CaMV PolyA), promoter I from *Cochliobolus heterostrophus*, and *Herpes simplex virus thymidine kinase* cassette (*tk*) in a tail-head orientation, was amplified from pTrpChptA-P_{Itk} (unpublished) with primer pairs 3-4 as well. In the second round of PCR reaction, the three fragments were subsequently joined in a tail-head orientation (1.1-up:*hpt-tk*:1.2-down). In the third round of PCR reaction, a 5.7-kb fragment (*Bam*HI:1.1-up:*hpt-tk*:1.1-down:*Bam*HI) containing a *Bam*HI recognition site, 1.1-up, 3.5-kb *hpt-tk* cassette, 1.1-down, and a *Bam*HI recognition site in head-tail orientation was amplified from the second-round PCR products with a pair of nest primers 7 and 8. Then, the 5.7-kb fragment was digested with *Bam*HI and inserted into the *Bam*HI site of pUC19 plasmid to form pB2U-hpt-tk-D.

The DNA fragment 1.1-up:*hpt-tk*:1.1-down was cut from pB2U-hpt-tk-D with *Bam*HI and purified before the transformation (Fig. 5A). Protoplasts of wild-type progenitor ZF21 were prepared using Lysing Enzymes/Driselase/Chitinase mix (Sigma-Aldrich, Shanghai, China) and PEG-mediated transformation was performed as in the method described previously (Maier et al. 2005). Genomic DNA of 182 β_2 -tubulin deletion transformants was extracted and analyzed by PCR to screen β_2 -tubulin deletion mutants, in which the β_2 -tubulin coding region was replaced by positive and negative selectable markers (*hpt-tk*). Three-primer combinations 13-15-17 and 14-16-18 were used to test for the presence of the β_2 -tubulin coding region and the 1.1-up:*hpt-tk*:1.1-down fragment, while other three-primer combinations 15-17-19 and 16-18-20 were used to confirm the integration of the 1.1-up:*hpt-tk*:1.1-down fragment via homologous recombination (Fig. 5B and C). Finally, 23 putative β_2 -tubulin deletion mutants for ZF21 were preliminarily screened out. The single-spore isolates of the mutants were maintained on potato dextrose agar containing hygromy-

Table 4. Oligonucleotide primers used for production and confirmation of site-directed mutants

No.	Sequence of primers (5' to 3')
1	AAGACATTCTGATAAAGCGGTGAGATGAC
2	GCTCCTTCAATATCATCTTCTGTCAATCTACGCATCTTGACAG
3	ACAGAAGATGATATTGAAGGAGC
4	TGTTCTTCCGGTATTGTCTC
5	GAGACAATACCGGAAGGAACATACCAAGACGCCGACATCG
6	GTCATCTCACCGCTTTATCAGAAATGTCTT
7	AAGGATCC TTTGGGGTTTGCTTTTGGGGA (<i>Bam</i> HI) ^a
8	AAGGATCCGGAGCGAGGTGCTACTTGGCG (<i>Bam</i> HI)
9	CATAACGGAAAGGTGGCCATCATGCGATC
10	CATGATGGCCACCTATCCGTTATGCCCTC
11	GGGAATCGAAACATGTCTGCACACCGGCC
12	ACGACATGTTTACGATTCCCCGGACAGCTCA
13	GACACCTAATAAGTGACCTC
14	TCGTGCAGAGTCTGGTGCTC
15	CAGAAGCTGGAACCGACTTG
16	ACGACAGTTTCTCAGCCATG
17	TATCGTGACCAAGCAGCAG
18	ATGATCCAGACCCACGTCAC
19	TTCGGGTGGTTGGGATCAAG
20	GCAAGCTCTCGACCTCATC
21	AGTTACGGGTAGCGACAGAG
22	TAGGTCTCGGACCTCGTCTG
23	GCGAAGAATCTCGTGCTTTC
24	GATGTGGCGACCTCGTATT
25	CCACCGTCTCCAAGGAGCAC
26	TCCTCGCGGATCTTGGACAG

^aSequence of the restriction sites designed in primers is underlined and the site mutations at codon 167 or 240 of β_2 -tubulin of *F. graminearum* are boxed.

^bRestriction enzymes corresponding to the restriction sites in primers.

Fig. 5. Site-directed mutagenesis of β_2 -tubulin in *Fusarium graminearum* by double replacement strategy. Primers, restriction sites of *Hind*III and *Sal*I, probe 1 (P1), probe 2 (P2), and probe 3 (P3) used in the polymerase chain reaction or Southern blot were also marked. **A**, The “1.1-up:*hpt-tk*:1.1-down” fragment used in the first replacement; **B**, wild-type β_2 -tubulin locus in *F. graminearum*; **C**, wild-type β_2 -tubulin coding region in *F. graminearum* replaced by *hpt-tk* cassette; **D**, fragment of site-mutated β_2 -tubulin allele used in the second replacement; **E**, site-mutated β_2 -tubulin locus in *F. graminearum* mutants.

cin B at 150 mg/liter at 4°C. Three putative β_2 -tubulin deletion mutants were randomly selected and further analyzed by two rounds of Southern blot analysis. In the first round, a 324-bp fragment amplified from the 1.1-down with primers 21 and 22 was labeled with digoxigenin to generate probe 1 (Fig. 5C). *Hind*III-digested genomic DNA of mutants was electrophoresed, blotted, and hybridized to probe 1. A single 6.0-kb band was expected to be observed in the β_2 -tubulin deletion mutants instead of a single 3.4-kb band in the wild-type progenitor ZF21. In the second round, probe 2 was made from a 605-bp fragment amplified from the *hpt* cassette with primers 23 and 34, and then used to hybridize to *Sal*I-digested genomic DNA of mutants (Fig. 5C). The number of the hybridized bands in the Southern blot indicated the copy number of the 1.1-up:*hpt*-*tk*:1.1-down fragment in the host genome. In the text, these β_2 -tubulin deletion mutants were named as $\Delta\beta_2\text{tub}\#$.

The complementary constructs were generated by an improved procedure of gene splicing by overlap extension (Sambrook and Russell 2001). The substitutions for site-directed mutagenesis were introduced into the internal primers used in the first round PCR. Primer pairs 1-9 and 6-10 were used for site-directed mutation at codon 167 (Phe to Tyr) and primer pairs 1 and 11 and 6 and 12 were used for site-directed mutation at codon 240 (Phe to Leu) of the β_2 -tubulin gene, respectively. The strands of overlapping fragments hybridized at their 3' ends and extended to generate a full-length product in the second-round PCR. Finally, the 3.8-kb full-length fragments were amplified from the product of the second-round PCR with a pair of nest primers 7 and 8, then inserted into the *Bam*HI site of pUC19 plasmid to form pB2SR-Phe167Tyr and pB2SR-Phe240Leu.

The fragments containing site-directed mutant β_2 -tubulin alleles were cut from the pB2SR-Phe167Tyr and pB2SR-Phe240Leu with *Bam*HI and purified before they were transferred into the β_2 -tubulin deletion mutant $\Delta\beta_2\text{tub}\#6$ (Fig. 5D). The 1% water agar amended with 5-fluoro-2'-deoxyuridine at 50 mg liter⁻¹ was used in regeneration of transformants. Genomic DNA of 87 β_2 -tubulin site-directed mutant transformants (47 for the mutation at codon 167 and 40 for the mutation at codon 240) were extracted and analyzed by PCR to screen β_2 -tubulin site-directed mutants as described in screening β_2 -tubulin deletion mutants. Finally, 26 putative β_2 -tubulin site-directed mutants at codon 167 and 21 putative β_2 -tubulin site-directed mutants at codon 240 were preliminarily screened out. Subsequently, three putative β_2 -tubulin site-directed mutants in each type were analyzed by the Southern blot. Probe 1 was hybridized to *Hind*III-digested genomic DNA of mutants. A single 3.4-kb band was expected in the putative β_2 -tubulin site-directed mutants instead of a single 6.0-kb band in the β_2 -tubulin deletion mutants. Probe 3 was generated from a 528-bp fragment amplified from a β_2 -tubulin coding region with primers 25 and 26 and hybridized to *Sal*I-digested genomic DNA of mutants (Fig. 5E). The number of the hybridized bands in the Southern blot indicated the copy number of the β_2 -tubulin allele integrated in the host genome. These β_2 -tubulin site-directed mutants were named R $\beta_2\text{tub}167\text{Tyr}\#$ for the mutants at codon 167 and R $\beta_2\text{tub}240\text{Leu}\#$ for the mutants at codon 240. Finally, all β_2 -tubulin site-directed mutants were sequenced to confirm the mutagenesis.

Trichothecene production in shake culture.

Experiments were performed in glucose-yeast extract-peptone (GYEP) medium (Miller et al. 1983). Erlenmeyer flasks (250 ml, containing 100 ml of GYEP) were inoculated with 5×10^4 spores and incubated in darkness at 28°C and at 200 rpm on a rotary shaker. After 7 days, the GYEP cultures (three flasks per strain) were filtered (Whatman filter paper no. 4) under

vacuum. A 30-ml sample of each filtrate was extracted twice with an equal volume of ethyl acetate. The organic phases were combined, evaporated to dryness, dissolved in 1 ml of acetonitrile, transferred to a new vial, evaporated to dryness, and derivatized with 100 μ l of trimethylsilylimidazole-trimethylchlorosilane (TMSI-TMSC) mixture (100:1). The trimethylsilyl (TMS) derivations of trichothecenes were identified and quantified by gas chromatography electron capture detector as previously described (Zhang et al. 2009). Results were quantified by comparison with standard solutions of DON, 3ADON, and 15ADON (Sigma-Aldrich). The amount of trichothecenes produced by each strain in liquid GYEP culture was the sum of DON and 3ADON or DON and 15ADON. Fungal biomass production in liquid GYEP culture was measured by weighing mycelia after freeze drying at -40°C for 48 h (Miller et al. 1983). Trichothecene production in shake culture was expressed as the amount of trichothecenes produced/dry weight of mycelia ($\mu\text{g g}^{-1}$ of dry mycelia).

Aggressiveness and trichothecene production in the field.

A moderately susceptible winter wheat cultivar, Nannong No. 9918, was planted according to normal agronomic practices at the Jiangpu Farm (Experiment Farm of Nanjing Agricultural University) in 2007. When more than half of the wheat spikes reached anthesis at Zadoks growth stage (ZGS) 65 (Zadoks et al. 1974), 200 flowering heads in each of 201 plots (67 strains $\times$ three replicates; each plot was 1.0 by 1.0 m) were selected and tagged with colored tape right below the heads. On the same day, the tagged heads were inoculated by injecting 10^3 conidia into a central floret of a spike (injection inoculation). After inoculation, the plots were misted twice daily if it did not rain during the day (Hilton et al. 1999). The experiment was repeated in the same field in the growing season of 2008. A randomized complete block design was used. All plots were arranged in a chess-cross design: each plot inoculated with a strain was surrounded by four border plots of similar size and planted with triticale to reduce plot-by-plot interference.

Aggressiveness was assessed as the incidence of infected spikelets or the amount of *F. graminearum* DNA in field grain (AFgDNA). The incidence of infected spikelets was determined visually by counting the number of infected spikelets per head 21 days after inoculation (Zhang et al. 2009) and was expressed as a percentage. *F. graminearum* DNA in field grain was quantified by real-time quantitative PCR as described in the next section. For trichothecene production analysis, each tagged head was harvested and threshed individually at maturity at ZGS 92. The kernels from each plot were bulked to form a 200-g homogeneous sample. Subsamples (100 g each) of bulked kernels were ground for 5 min in an all-purpose grinder (Tianjin Traditional Chinese Medicine Machinery Factory, Tianjin, China). Three finely ground subsamples (20 g per subsample) for each plot were extracted for 1 h with 80 ml of acetonitrile/water (84:16, vol/vol) on a reciprocating shaker. A 4-ml extract was eluted through a 1-g cleanup column containing C-18/aluminum oxide (3:1, wt/wt). Elution (1 ml) was evaporated to dryness and derivatized with the TMSI-TMSC (100:1) mixture. The TMS derivatives of trichothecene were identified and quantified as previously described (Zhang et al. 2009). The amount of trichothecenes produced by each strain in harvested grain was the sum of DON, 3ADON, and 15ADON.

DNA and RNA extraction.

DNA was extracted from pure fungal cultures and field grain as previously described (Doohan et al. 1998). DNA from grain was diluted 500-fold in Tris-EDTA for use in a PCR amplification reaction. The DNA content in 1 μ l of DNA so-

lution was equal to the DNA content in 400 µg of grain. For *Tri5* gene expression analysis, 17 strains were used: the wild-type progenitor ZF21, its six site-directed mutants, five MBC^S wild-type strains, and five MBC^R wild-type strains (the latter 10 strains were arbitrarily selected from the 30 wild-type resistant strains and the 30 wild-type susceptible strains) (Table 1). Erlenmeyer flasks (250 ml) containing 100 ml of GYEP were inoculated with a conidial suspension to give 5×10^4 conidia/ml. Flasks were incubated at 28°C and 200 rpm and were harvested after 72, 96, 120, and 144 h post inoculation (three flasks per strain per time point). Total RNA of pure fungal cultures was extracted using TRIZOL Reagent (Sigma-Aldrich) and quantified by spectrophotometry at 260 or 280 nm. RNA was treated with DNase I (Takara, Dalian, China) (Covarelli et al. 2004), resuspended in 30 ml of diethylpyrocarbonate-treated water, and stored at -70°C.

Primer design.

The *F. graminearum* species-specific primers (Fg16F [5'-CTCCGGATATGTTGCGTCAA-3'] and Fg16R [5'-GGTAGGTATCCGACATGGCAA-3']) amplify a 410-bp DNA fragment (Nicholson et al. 1998). Primers for the *Tri5* gene (Tr5F [5'-AGCGACT ACAGGCTTCCCTC-3'] and Tr5R [5'-AAACCATCCAGTTCTC CATCTG-3']) amplify a 485-bp cDNA fragment from conserved regions of the *Tri5* gene in *F. graminearum* (Doohan et al. 1999). *EF1-α* was used as an internal control (Dean et al. 2002). Primers for the *EF1-α* gene (EF1F [5'-CTTACTGCCTCCACCAACTG-3'] and EF1R [5'-TGACGTTGGAAGGAGCG AAG-3']) amplify a 253-bp cDNA fragment from a region conserved in the *F. graminearum EF1-α* gene (Transcript: FGST-09307). Primers were synthesized by Sangon (Shanghai, China) and purified by high-performance liquid chromatography. Lyophilized primers were dissolved in water to a concentration of 10 µM and stored at -20°C.

Real-time quantitative PCR.

PCR reactions were set up in 0.2-ml white, opaque, low-profile polypropylene tubes covered with ultra clear caps (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Each tube contained 12 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, U.S.A.), 1 µl of internal passive reference dye (ROX), 1 µl of primers Fg16F and -R mix (containing the forward and reverse primers, each at a concentration of 10 µM), and 1 µl of either water (negative control) or sample DNA. Distilled water was added to give a total volume of 25 µl. Samples were run in an ABI PRISM 7000 PCR (Applied Biosystems) with the following cycling protocol. Samples were preheated at 50°C for 2 min followed by 2 min at 95°C. Subsequently, 35 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C were run. Fluorescent products were measured after the last step of each cycle. Following the final amplification cycle, a melting curve was determined by one cycle of heating at 95°C, cooling to 60°C, and then slowly heating to 95°C with continuous measurement of optical density at 520 nm (OD₅₂₀). The amount of *F. graminearum* DNA (AFgDNA) present in a sample was calculated from a standard curve using the ABI PRISM 7000 software package (Applied Biosystems). The quantity of the AFgDNA in a grain sample was expressed as micrograms of AFgDNA per kilogram of grain (µg kg⁻¹ of grain).

The standard curve was produced by the following method. First, the amount of purified *F. graminearum* DNA solution was measured at OD₂₆₀ and the concentration of the purified DNA solution was determined from a calibration curve (Dyer et al. 2007). Second, a 10-fold dilution series of purified fungal DNA (100 to 0.001 ng µl⁻¹) was run on real-time PCR, and

threshold cycle (CT) values at which the amplification curves of each solution crossed the crossing line were recorded. The crossing line was set manually to 0.26 and gave the standard curve with the lowest standard deviation. Finally, the standard curve was generated by plotting the CT values as a function of the log₁₀ of the amount of fungal DNA. The standard curve gave a linear relationship ($y = -3.5x + 21$, $R = 0.98$) over the range of DNA concentrations examined. The detection limit of the assay was determined to be 1 pg of genomic DNA per reaction. No signal was measured in the uncontaminated wheat sample used as the negative control.

Quantitative *Tri5-EF1-α*-specific reverse-transcription PCR.

Reverse transcription (RT) was performed as previously described (Doohan et al. 1999), except that the reaction mixtures contained 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 µg of oligo (dT₁₂₋₁₈) (Invitrogen), and the mixtures were incubated at 37°C for 50 min. Reactions were terminated by incubating at 70°C for 15 min. PCR reactions were conducted in a volume of 25 µl containing 1-µl aliquots of RT products as template, 2.5 µl of 10x buffer, 1.5 µl of 25 mM MgCl₂, 2 µl of 1.25 mM dNTPs, 1 µl of primers Tr5F/R mixture, and 1 U of *Taq* polymerase (Takara, Dalian, China). For each reaction, *Tri5* cDNA and *EF1-α* cDNA were coamplified. PCR was performed in a Bio-Rad PTC200 thermocycler under the following conditions: 95°C for 5 min; followed by 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 8 min. Following separation by agarose gel electrophoresis (1.5% [wt/vol] agarose), all quantitative RT-PCR products were analyzed by using Molecular Analyst Software (Bio-Rad Laboratories, Inc.). The ratio of *Tri5* to *EF1-α* PCR product was used as an index of the relative *Tri5* gene expression in samples. All RT-PCR amplifications were performed at least twice.

Statistical analysis.

Data from the 60 wild-type strains and from the site-directed mutants were analyzed separately. All data were processed as an analysis of variance; when effects were significant, a least significant difference test ($P = 0.05$) was used to determine whether MBC resistance had a significant effect on trichothecene production, *Tri5* gene expression, or aggressiveness. Simple regression analysis was also performed to determine whether significant relationships existed between the incidence of infected spikelets, the amount of *F. graminearum* DNA, and trichothecene production in field grain or levels of *Tri5* gene expression and trichothecene production in shake culture and in the field. All data analysis was carried out with the statistical software SAS GLM (SAS Institute Inc., Cary, NC, U.S.A.).

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