Mutations in a β -Tubulin Confer Resistance of *Gibberella zeae* to Benzimidazole Fungicides

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ABSTRACT

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Wheat head blight caused by *Gibberella zeae* (anamorph: *Fusarium graminearum*) is a threat to food safety in China because of mycotoxin contamination of the harvested grain, the frequent occurrence of the disease, and the failure of chemical control in some areas due to benzimidazole resistance in the pathogen population. The molecular resistance mechanism, however, of *G. zeae* to benzimidazole fungicides (especially carbendazim; active ingredient: methyl benzimidazol-2-yl carbamate [MBC]) is poorly understood. DNA sequences of a β -tubulin gene (β_2tub) (GenBank access number FG06611.1) in *G. zeae* were analyzed. Mutations in β_2tub in moderately resistant strains (MBC^{MR}) included TTT (Phe)→TAT (Tyr) at codon 167 or TTC (Phe)→TAC (Tyr)

at codon 200. A highly resistant strain (MBC^{HR}) had two point mutations, one at codon 73, CAG (Gln)→CGG (Arg), and the other at codon 198, GAG (Glu)→CTG (Leu). To confirm that mutations in the β_2tub confer resistance to benzimidazole fungicides, the entire β_2Tub locus was deleted from MBC^{MR} and MBC^{HR} strains of *G. zeae*. The resulting $\Delta\beta_2tub$ mutants from both MBC^{MR} and MBC^{HR} strains grew normally on MBC-free potato dextrose agar medium and were supersensitive to MBC. Complementation of the $\Delta\beta_2tub$ mutants by transformation with a copy of the intact β_2Tub locus from their parent strains exhibited less resistance than the original strains, and complementation of the $\Delta\beta_2tub$ mutants by transformation with a copy of the intact β_2Tub locus from sensitive strains restored MBC sensitivity. The results indicated that the mutations in the β_2tub gene conferred resistance of *G. zeae* to benzimidazole fungicides and this gene can be used as a genetic marker in *G. zeae*.

Additional keywords: gene-deletion and -complementation mutants.

Benzimidazoles and thiophanate are a group of broad-spectrum systemic fungicides that interact with tubulin (11), especially β -tubulin. The repeated use of these fungicides, whose active ingredient or metabolite is generally methyl benzimidazol-2-yl carbamate (MBC or carbendazim), often selects for resistance, which is a major practical problem in the use of these fungicides. Benzimidazole resistance has developed in many plant-pathogenic fungi after the fungicides have been used for 2 or 3 years (5,32). Mutations at Glu198Ala, Val, Gly, Phe200Tyr, and other codons in β -tubulin have been associated with resistance (1). Mutations in codons 198 and 200 are also encountered in veterinary medicine, where parasitic nematodes have become resistant to treatment with benzimidazole nematicides (19).

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum*) is a filamentous ascomycete that causes economically important diseases on a variety of cereal crops (23,28). On wheat, *G. zeae* can cause seedling blight, root rot, and head blight (or scab) resulting in yield loss, as well as contamination of grain with trichothecene and estrogenic mycotoxins (3,7).

In China, the first case of resistance to MBC in *G. zeae* was detected in 1992 in Zhejiang Province after these fungicides had been used widely for 20 years (35). Previous studies showed that a β -tubulin gene (FG09530.1) (β_1tub) in *G. zeae* had high amino acid identity with that of the MBC-resistant gene from other filamentous fungi but none of mutations at this locus were found relating to resistance to benzimidazole fungicides (7). A similar

lack of mutations in the β_1 -tubulin associated with benzimidazole resistance has been reported in *G. pulicaris* (15). Yuan et al. reported that a major gene in *G. zeae* confers benzimidazole resistance based on the results of crossing analysis (35). The tubulin gene family in *F. graminearum* has five members, including α_2 -tubulin (FG00639.1), α -tubulin (FG00397.1), β_1 -tubulin (FG09530.1), β_2 -tubulin (FG06611.1) and the γ -tubulin gene (FG09993.1). The genes coding α_2 -tubulin (FG00639.1) (9), α -tubulin (FG00397.1) (6), the γ -tubulin gene (8), and β_1 -tubulin (FG09530.1) (7), however, do not confer benzimidazole resistance in *G. zeae*. Thus, the current research is aimed at examining whether the mutations in β_2tub , the other known gene in the family, can confer resistance to benzimidazoles.

The objectives of this article were to (i) sequence the β_2tub from strains differing in MBC sensitivity to determine if mutations are present that may be correlated with differences in phenotype and (ii) confirm the identity of the resistant phenotypes by construction of gene-deletion and -complementation mutants.

MATERIALS AND METHODS

Fungicide, media, and pathogens. Potato dextrose agar (PDA) (20 g of dextrose, 15 g of agar, and infusion of 200 g of potato in 1 liter of distilled water) was used in routine in vitro assays for MBC sensitivity, and technical grade MBC (MBC, 93% a.i.) was kindly provided by Dr. Si N-G (Shenyang, China). MBC was dissolved in 0.1 M HCl at 10 mg/ml as a stock solution and was added to autoclaved media after cooling to 45 to 50°C. The pH was adjusted to 6.8 in all media using HCl.

Fifteen MBC-sensitive (MBC^S) strains of *G. zeae* previously characterized at the discriminating concentration of MBC at 1.4 μ g/ml (35), 16 strains moderately resistant to MBC (MBC^{MR}), and 1 strain highly resistant to MBC (MBC^{HR}) were collected from

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winter wheat in Zhejiang and Jiangsu Provinces. All strains tested were generated from single conidia dissected from contaminated heads (Table 1). To test the sensitivity of *G. zeae* to MBC, PDA was amended with MBC at 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 µg a.i./ml for sensitive strains or isolates or at 0, 3.125, 6.25, 12.5, 25, 50, and 100 µg a.i./ml for resistant strains or isolates. A mycelial plug (5 mm in diameter) from the growing edge of a 5-day-old colony was transferred onto each plate amended with MBC as listed above. After incubation at 25°C for 5 days, the radial growth (colony diameter) was measured. A linear regression of the percent inhibition (relative to the 0 µg a.i. control) versus the log₁₀ transformation of each MBC concentration was obtained, and the effective concentration giving 50% inhibition (EC₅₀) was calculated. The minimum inhibitory concentration (MIC) was defined as that concentration resulting in 100% mycelial growth inhibition. Each treatment had four replicates and assays were performed twice. The significance of the two assays was determined by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) using Statview (SAS Institute, Cary, NC). Based on differences in MBC sensitivity, tested strains were divided into three phenotypes as follows: high MBC resistance, MIC > 100 µg/ml; intermediate MBC resistance, 100 µg/ml > MIC > 50 µg/ml; MBC sensitive, MIC < 1.4 µg/ml (35).

Cloning, sequencing, and analyses. For cloning the whole β_2 Tub locus, eight primer pairs (30-31, 32-33, 34-35, 36-37, 38-39, 40-41, 42-43, and 44-45) (Table 2) were used to amplify products from genomic DNA of *G. zeae*. *LA Taq* DNA polymerase (TaKaRa, Dalian, China), a high-fidelity DNA polymerase, was used in all polymerase chain reaction (PCR) reactions except for reverse-transcriptase (RT)-PCR and the procedure of constructing the β_2 tub deletion vector pMD-B2T. Genomic DNA was extracted from $\approx$ 1 g of freeze-dried mycelia of *G. zeae* strains which were grown in a potato dextrose broth (PDB) (20 g of dextrose and infusion of 200 g of potato in 1 liter of distilled water) at 25°C for 4 days. The DNA extraction method was adapted by a modified cetyltrimethylammonium bromide method (27). The genomic DNA was precipitated with $\approx$ 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate, washed twice with 70% ethanol, and dissolved in 50 to 100 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The PCR products were cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI). *Escherichia coli* strain DH5 α was used as the bacterial host for the propagation of constructs. Plasmid DNA was extracted with the Axyprep Plasmid Miniprep Kit (Axygen, China) following the manufacturer's instructions. DNA was sequenced

by dideoxy chain-termination method (29) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA) with the ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Three clones were sequenced for each isolate and both DNA strands were sequenced for each clone. Sequence analysis and multiple alignments were carried out with the Bioedit Sequence Alignment Editor software (14). Sequence homology of tubulin genes from *G. zeae* with other fungi was analyzed using the BLAST algorithm (2), and sequences from strains of *G. zeae* differing in MBC sensitivity were compared to determine mutations in the nucleotides associated with MBC resistance. The sequences were submitted to GenBank.

Nucleic acid manipulations. Total RNA was isolated from fresh mycelia ($\approx$ 100 mg) using the TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was precipitated with 2-propanol, washed with 70% ethanol, dried, resuspended in 20 µl of RNase-free d₂H₂O, and finally treated with RNase-free DNaseI (TaKaRa). All nucleic acids were analyzed by electrophoresis on 0.7% agarose gels and spectrophotometrically (DU640: Beckman Coulter, Fullerton, CA).

Southern blotting was performed following the instructions of the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Germany). Briefly, the hybridization probes used in Southern analyses were labeled with digoxigenin (DIG). The *EcoRV*-digested genomic DNA of *G. zeae* was electrophoresed on 1% agarose gels and capillary blotted onto positively charged nylon membranes (Roche Applied Science, Germany). After being hybridized at 48°C for 6 h, the membranes were washed two times in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 20°C for 15 min and then two times in 0.5 $\times$ SSC–0.1% SDS at 65°C for 15 min.

Phylogenetic analysis. Amino acid sequences of β -tubulins from *G. zeae* and other plant pathogens were obtained from the public database GenBank. For phylogenetic analysis, the protein sequences were aligned with Clustal W (31) using the Blosom matrix and standard default parameters. The phylogenetic tree was constructed using MEGA software (18) with the neighbor-joining method, excluding positions with gaps. The confidence of the branches was determined by bootstrap analysis in 1,000 samplings.

Construction of β_2 tub deletion vector pMD-B2TK. This deletion vector was constructed in two steps. Vector pMD-B2T (Fig. 1A) was first constructed using a double-jointed PCR strategy (34) to amplify flanking sequences from the β_2 tub gene.

TABLE 1. Origin of strains or mutants and sensitivity of the strains to benzimidazole used in this study

Resistant phenotype of strains ^a	No.	Codes of strains	Origin
MBC ^S	15	ZF-43, ZF-32, ZF-22, 2021,T17 and S1~S10	ZF-43, ZF-32, ZF-22, and 2021 were separated from Zhejiang province of China in 2000; S1~S10 from Zhejiang in 2003; and T7 from Jiangsu province of China in 2001
MBC ^{MR}	16	ZF-52, ZF-54, SP-2, R1~R10, r2, NT-7 and t1	ZF-52, ZF-54, and SP-2 were separated from Zhejiang in 2000; R1~R10 also from Zhejiang but in 2003; and r2, NT-7, and t1 from Jiangsu in 2006
MBC ^{HR}	1	JT04	From Jiangsu in 2000
β_2 Tub-deletion mutants	9	$\Delta\beta_2$ tub-2021D51 $\Delta\beta_2$ tub-2021D81 $\Delta\beta_2$ tub-2021D96, $\Delta\beta_2$ tub-R9D4 $\Delta\beta_2$ tub-R9D32 $\Delta\beta_2$ tub-R9D49, $\Delta\beta_2$ tub-JT04D47 $\Delta\beta_2$ tub-JT04D144 $\Delta\beta_2$ tub-JT04D151	...
β_2 Tub-complementation mutants	18	β_2 tub-2021D96-JT04C2, β_2 tub-R9D49-R9C1, β_2 tub-JT04D144JT04C4, β_2 tub-2021D96-JT04C3, β_2 tub-R9D49-R9C4, β_2 tub-JT04D144JT04C6, β_2 tub-2021D96-JT04C5, β_2 tub-R9D49-R9C6, β_2 tub-JT04D144JT04C7, β_2 tub-2021D96-R9C1, β_2 tub-R9D49-2021C1, β_2 tub-JT04D144-2021C2, β_2 tub-2021D96-R9C3, β_2 tub-R9D49-2021C3, β_2 tub-JT04D144-2021C5, β_2 tub-2021D96-R9C8, β_2 tub-R9D49-2021C5, β_2 tub-JT04D144-2021C7	...

^a MBC^S, MBC^{MR}, and MBC^{HR} indicate that the strain is sensitive, moderately resistant, or highly resistant, respectively, to carbendazim (methyl benzimidazol-2-yl carbamate [MBC]).

Genomic DNA of *G. zeae* strain JT04 was used as a PCR template and sequences consisted of a 1-kb upstream fragment (designated 1-up) and a 1.2-kb downstream fragment (designated 1.2-down). Amplification was done using the primer pairs 7-8 and 9-10, respectively. The two fragments with *Sma*I restriction sites were joined by double-jointed PCR using primers 1-2 and cloned into cloning vector pMD19T-Simple (TaKaRa). Second, a 1.4-kb *PtpC-hpt* cassette containing the hygromycin phosphotransferase gene (*hpt*) under the control of a *Aspergillus nidulans trpC* gene promoter (*PtpC*) was amplified from plasmid pKht (26) with a polymerase that yields blunt-ended PCR products (Prime STAR HS DNA polymerase) (TaKaRa) and primers 11-12. The PCR product was cloned into the *Sma*I site of pMD-B2T to generate the deletion vector pMD-B2TK (Fig. 1B). The orientation of the inserted *PtpC-hpt* cassette was confirmed by a three-primer combination, 1-2-6. The 3.6-kb 1-up:*PtpC-hpt*:1.2-down fragment used to transform was amplified from pMD-B2TK using primers 1-2.

Transformation of the wild-type strain. Protoplasts were prepared by a modified method based on Maier et al. (23). Modifications were as follows: conidia of *G. zeae* strains harvested from 7-day-old mung bean broth cultures (3% mung bean extract) were used to inoculate 100 ml of yeast extract peptone dextrose (YEPD) (wt/vol: 1% peptone, 0.3% yeast extract, 2% glucose) at 1 to 5 × 10⁶ conidia/ml. The inoculum was incubated at 28°C for 8 to 9 h at 200 rpm. Germlings were harvested by filtration on a 37-μm nitrate filter, washed with 50 ml of 0.7 M NaCl, and suspended in 20 ml of 0.7 M NaCl with a protoplasting enzyme mix (100 mg of lysing enzymes, 500 mg of driselase, and 1 mg of chitinase). The mixture was incubated at 28°C for 100 to 120 min at 80 rpm. Protoplasts were collected by centrifugation at 1,300 × g for 5 min, and then washed once in 10 ml of ice-cold 0.7 M NaCl and twice in STC (0.8 M sorbitol; 50 mM Tris-HCl, pH 8.0; and 50 mM CaCl₂) buffer. The washed protoplasts were suspended in STC and stored on ice until transformation.

MBC^S strain 2021, MBC^{MR} strain R9, and MBC^{HR} strain JT04 were transformed with the 3.6-kb 1-up:*PtpC-hpt*:1.2-down fragment amplified from pNT-B2TK as described by Maier et al. (23). Putative transformants were transferred to PDA containing hygromycin B at 150 μg/ml, reisolated as single macroconidia, and maintained on PDA slants with hygromycin B.

Putative transformants (*n* = 192 for MBC^S strain 2021, 181 for MBC^{MR} strain R9, and 209 for MBC^{HR} strain JT04) were obtained for two transformation experiments, including β_2 tub deletion mutants and ectopic transformants.

PCR amplification and southern blot analysis of putative β_2 tub deletion transformants. In all, 582 transformants of 2021, JT04, and R9 were analyzed to test for the presence of the wild-type β_2 tub coding region and pMD-B2TK-specific sequence junctions. Primers 3-4 were used to detect the sequence of the β_2 tub gene coding region (Fig. 1A to D). Two three-primer combinations, 16-26-27 and 6-28-29, were used to detect the two sequence junctions upstream and downstream of the β_2 tub coding region, respectively. The second PCR screen used another two three-primer combinations to confirm the integration of pMD-B2TK via homologous recombination at the β_2 Tub locus. The first set of three-primer combination consisted of oligonucleotides 15-16-27, which tested for integration of pMD-B2TK via recombination with DNA upstream of the β_2 Tub locus. The second set of three-primer combination consisted of oligonucleotides 5-6-28, which tested for integration of the vector via recombination with DNA downstream of the β_2 tub coding region. Forty-two putative $\Delta\beta_2$ tub mutants were identified for 2021, 32 for JT04, and 27 for R9.

Putative β_2 tub deletion mutants (three derived from 2021, three from JT04, and three from R9) were selected for further analysis by Southern blot to confirm the homologous integration of the 1-up:*PtpC-hpt*:1.2-down fragment. Probe 1 was prepared from a

0.3-kb fragment generated by using PCR primers 17-18 (Table 2) and corresponded to 1.2-down. Genomic DNA of each mutant was digested with *Eco*RV, separated by electrophoresis, blotted, and hybridized to probe 1. The probe was expected to hybridize to a single 3.4-kb band of DNA from the $\Delta\beta_2$ tub mutant, a single 1.4-kb fragment from starting strains, and both fragments from transformants with an ectopic integration.

Construction of β_2 tub complementation vector pAMDS- β_2 Tub. To simultaneously complement $\Delta\beta_2$ tub within the *G. zeae*

TABLE 2. Oligonucleotide primers used for plasmid construction, polymerase chain reaction analysis of transformants, and Southern hybridization blot analysis

Primer no.	Sequence (5'–3')
1 ^a	TTTGGGGTTTGCTTTTGGGGA
2	GGAGCGAGGTGCTACTTGGCG
3 ^b	CCACCGTCTCCAAGGAGCAC
4	CGCAGATCCGAGTTGAGCTG
5	GCAAGCTCCTCGACCTCATC
6	TGCGACGCAATCGTCCGATC
7	CTGATAAAGCGGTGAGATGAC
8	CAATCTCACGCATCTTGACAG
9	CTGTCAAGATGCGTGAGATTGCCCGGGATACCAAGA CGCCGACATCG
10	TGACGATCCAGCCGAACAC
11	ACAGAAGATGATATTGAAGGAGC
12	TACTCTATTCCTTTGCCCTCG
13	ATATCTAGACTGCCAACAGCCACTATGTC
14	ATACTCGAGGTGCTACCCGTAACCTCGT
15	TTCGGGTGGTTGGGATCAAG
16	ATCAGGTGCGAGACGCTGTC
17 ^c	AGTTACGGGTAGCGACAGAG
18	TAGGTCTCGGACCTCGTCTG
19 ^d	CTTACTGCCTCCACCAACTG
20	TGACGTTGGAAGGAGCGAAG
21	CTCTGACTGGTCGCAACATG
22	AGTGGCGGACATGTCGAGAC
23 ^e	CCTCGGGATCTTGGACAG
24	GGCAGACCCTCTCTGGCGAG
25	CGGACGACATCGAGAAGCTTG
26	GACACCTAATAAGTGACCTC
27	CAGAAGCTGGAACCGACTTG
28	ACGACCAAGTCTCAGCCATG
29	TCGTGCAGAGTCTGGTGCTC
30 ^f	CTGCCAACAGCCACTATGT
31	GTCAGCAAGCCACCTAT
32	ATTGGTGTAGTTGTTTCGTG
33	ATCGCTGTATGTTCTACTGATT
34	GATAAAGCGGTGAGATGA
35	AACGGAAGGAAAGAAGAG
36	AGCCTGAACCTACAACAA
37	CATCAATACCGTGCTCCT
38	GACCAGACCCAAAGACAC
39	GAAGAAGCGTTCCTATAC
40	CTGTTCTGGTTCGATCTTG
41	GGCTGGGAATTTTCATTAG
42	GAACCTTACAACGCCACTT
43	TCCATCTCGTCCATACCC
44	TCCGTCTGTTCCGTGCTT
45	CTACCCGTAACCTCGTGGG

^a Primer pairs 1-2, 7-8, 9-10, and 11-12 were used in construction of pMD-B2TK; Primer combination 1-2-6 was used to detect orientation of *PtpC-hpt* cassette inserted into pMD-B2T.

^b Primer pair 3-4 and combinations 16-26-27, 6-28-29, 15-16-27, and 5-6-28 were used to analysis of β_2 -tubulin deletion mutants. Primer pair 3-4 was used to analysis of β_2 -tubulin complementation mutants.

^c Primer pair 17-18 was used to amplify the fragment to make probe 1.

^d Primer pairs 19-20, 21-22, and 24-25 were used to amplify the mitochondrial RNA transcript of elongation factor 1- α - and the β_2 - and β_1 -tubulin gene, respectively.

^e Primer pair 3-23 was used to amplify the fragment to make probe 2.

^f Primer pairs 30-31, 32-33, 34-35, 36-37, 38-39, 40-41, 42-43, and 44-45 were used to amplify products (range from 2.2 kb upstream to 444 bp downstream of β_2 -tubulin coding region) from genomic DNA of *Gibberella zeae* strains.

β_2Tub locus, pAMDS- β_2Tub -2021, pAMDS- β_2Tub -R9, and pAMDS- β_2Tub -JT04 were constructed. Fragments *Xba*I-*Xho*I (4.3 kb), containing the β_2Tub locus digested from strains 2021, R9, and JT04, were amplified by using primers 13-14 and ligated to the *Spe*I-*Sal*I pAMDS4-1 (28) large fragment, respectively. pAMDS4-1 (provided by R. H. Proctor, Bioactive Agents Unit, National Center for Agricultural Utilization Research, United States Department of Agriculture-Agricultural Research Service, Peoria, IL), containing an *Aspergillus nidulans* acetamidase gene (*amdS*) under the control of the *Cochliobolus heterostrophus* Promoter I, facilitated the selection of transformants by their ability to grow on media in which acetamide is the sole source of nitrogen.

Transformation of the β_2tub deletion mutants. Protoplasts were prepared as described above. The transformation process (23,28) was modified as follows: protoplasts were suspended at a concentration of 1 to 2×10^8 ml⁻¹ in four parts STC and one part

SPTC (e.g., STC with 40% polyethylene glycol 6000). Then, 5 to 20 μ g of pAMDS- β_2Tub and 5 μ l of heparin (5 mg/ml in STC) were added into 100 μ l of the protoplast suspension. After samples were mixed and incubated on ice for 30 min, 1 ml of SPTC was mixed with the suspension and incubated at room temperature for 20 min. The samples were diluted further with 4 ml of STC; then, 600- μ l aliquots were mixed with 6.5 ml of molten SL medium (28) containing 15 mM CsCl and 1% agarose and spread on SL medium. SL medium consisted of a previously described basal medium (10) plus 10 mM acetamide (Sigma). After incubation at 28°C for 2 days, putative transformants were transferred to SL agarose slants and single-spore isolates were picked using inoculation needles. The pAMDS- β_2Tub -2021 was transformed into $\Delta\beta_2tub$ -R9D49 and $\Delta\beta_2tub$ -JT04D144; the pAMDS- β_2Tub -R9 was transformed into $\Delta\beta_2tub$ -R9D49 and $\Delta\beta_2tub$ -2021D96; and the pAMDS- β_2Tub -JT04 was transformed into $\Delta\beta_2tub$ -JT04D144 and $\Delta\beta_2tub$ -2021D96.

Fig. 1. Vectors constructed for this study. **A**, pMD-B2T. This fragment contained 1 kb upstream- (1-up) and 1.2 kb downstream (1.2-down) of β_2 -tubulin gene (β_2tub) coding region, a *Sma*I restriction site between 1-up and 1.2-down, and pMD19T-simple vector backbone (TaKaRa, Dalian, China). **B**, β_2 -Tubulin deletion vector pMD-B2TK. A blunt-ended polymerase chain reaction (PCR) fragment containing the hygromycin phosphotransferase gene (*hpt*) under the control of *Aspergillus nidulans* *trpC* gene promoter (*PtrpC*) was inserted into the *Sma*I site of pMD-B2T to generate pMD-B2TK. A three-primer (1-2-6) combination PCR was used to determine the orientation of 1-up:*PtrpC-hpt*:1.2-down. **C**, β_2 -Tubulin locus (β_2Tub) of wild-type *Gibberella zeae* strain. Primer pairs 3-4, 26-27, and 28-29 were used to detect the presence of β_2Tub . Probe1 (P1) and Probe2 (P2) labeled by digoxigenin (DIG) were used to hybridize to the 1.2-down and β_2tub coding region, respectively. *EcoRV* was used to digest genomic DNA of the strains or mutants in Southern blot. **D**, β_2tub Coding region of wild-type *G. zeae* strain replaced by *PtrpC-hpt* cassette. Primer pairs 16-26 and 6-29 were used to detect the presence of the 1-up:*PtrpC-hpt*:1.2-down cassette in the genomic DNA of transformants. Two primer pairs (15-16 and 5-6) were used to detect homologous recombination of 1-up:*PtrpC-hpt*:1.2-down cassette with β_2Tub in *G. zeae*. *EcoRV* was used to digest genomic DNA of the strains or mutants in the Southern blot. **E**, β_2Tub -complementation vector pAMDS- β_2Tub . A 4.3-kb fragment of *G. zeae* β_2Tub was inserted into the pAMDS4-1 to generate pAMDS- β_2Tub . pAMDS4-1 consisted of a Promoter I-*amdS* cassette (*Aspergillus nidulans* acetamidase gene under the control of *Cochliobolus heterostrophus* promoter I) and the pBluescript II KS (-) (Stratagene, La Jolla, CA) backbone.

PCR amplification and Southern blot analysis of putative β_2 tub-complementation transformants. This was done as above, except that the 0.8-kb fragment which carries half of the β_2 tub coding region (Fig. 1C) was amplified by primer 3-4, and the 528-bp probe 2, which was amplified from pAMDS- β_2 Tub-R9 with primers 3-23, was expected to hybridize to a single 2.1-kb DNA fragment from wild-type strains and β_2 tub-complementation mutants.

RNA extraction and RT-PCR assay. Three strains differing in sensitivity to MBC (MBC^S strain 2021, MBC^{MR} strain R9, and MBC^{HR} strain JT04), three $\Delta\beta_2$ tub mutants ($\Delta\beta_2$ tub-2021D96, $\Delta\beta_2$ tub-R9D49, and $\Delta\beta_2$ tub-JT04D144), and six β_2 tub-complementation mutants (β_2 tub-R9D49-2021C3, β_2 tub-JT04D144-2021C5, β_2 tub-2021D96-R9C1, β_2 tub-2021D96-JT04C3, β_2 tub-R9D49-R9C1, and β_2 tub-JT04D144-JT04C4) were used to evaluate gene expression differences in β_1 tub and β_2 tub. cDNA was produced from 0.8 μ g of total RNA from each strain using M-MLV RT (Invitrogen) following the manufacturer's instructions and stored at -20°C . PCR was performed in a 25- μ l reaction mixture containing 2 μ l of RT reaction product, 1.25 units of *Taq* DNA polymerase (TaKaRa), 0.2 mM dNTP, and 0.4 mM each of forward and reverse primer in 1 $\times$ PCR reaction buffer provided by the *Taq* enzyme supplier. A 253-bp mitochondrial (m)RNA transcript of the elongation factor 1- α gene (EF-1- α , a house-keeping gene) was amplified with primers 19-20 and used as a control. The 301-bp β_2 tub- and 283-bp β_1 tub-mRNA transcripts were amplified with primer pairs 21-22 and 24-25, respectively. The reaction mixture was denatured at 94°C for 5 min; amplified for 35 cycles of 30 s at 94°C for denaturation, 30 s at 60°C for annealing, and 30 s at 72°C for extension; followed by a single 10-min extension at 72°C .

Sensitivity of β_2 tub-deletion and -complementation mutants to MBC. The following strains and mutants were analyzed: 3 strains differing in MBC sensitivity (MBC^S 2021, MBC^{MR} R9, and MBC^{HR} JT04), 9 β_2 tub-deletion mutants, and 18 β_2 tub-complementation mutants (Table 1). MBC was added to autoclaved PDA to produce a series of concentrations of 0, 0.4, 1.4, 2.5, and 100 μ g a.i./ml for mycelial growth assays and 0, 1.25, 2.5, 5, 10, 20, and 40 μ g a.i./ml for sensitivity assays with highly resistant strains; 0, 0.018, 0.036, 0.072, 0.14, and 0.28 μ g a.i./ml for sensitivity assays with deletion mutants; and 0, 0.625, 1.25, 2.5, 5, 10, and 20 μ g a.i./ml for sensitivity assays with complementation mutants. Each treatment had four replicates. The other experimental procedures were the same as those described above for the sensitivity assay.

RESULTS

Isolation and sequence analysis. The complete DNA sequences of a β_2 Tub locus (ranging from 2.2 kb upstream to 444 bp downstream of the β_2 tub coding region) from 32 strains of *G. zeae* were obtained following PCR amplification using four primer pairs (Table 2). There was an open reading frame (ORF) of 1,713 bp interrupted by six introns. The positions of the introns were confirmed by comparison with the gene sequence from the PH-1 genomic database (<http://www.broad.mit.edu/cgi-bin/annotation/fusarium/findfeatures>). The ORF encodes a putative protein

of 447 amino acids, with a calculated molecular weight of 50 kDa, a G+C content of 53%, and an estimated isoelectric point of 4.5. There were 12 nucleotide differences, in total, between the β_2 tub from all of the Chinese MBC^S strains and the sequenced strain PH-1.

Correlation between MBC-sensitive phenotypes and mutations in β_2 tub. Nucleotide changes occurred at positions 73 (CAG), 167, 198, and 200 in the predicted deduced amino acid sequence of the β_2 tub gene from the Chinese strains of differing sensitivity to MBC (Table 3). MBC^S strains had a glutamine at position 73, a phenylalanine at position 167 (TTT), a glutamic acid at position 198 (GAG), and a phenylalanine at position 200 (TTC). Of the 16 MBC^{MR} strains, 13 had a tyrosine at position 167 (TAT) and 3 had a tyrosine at position 200 (TAC). MBC^{HR} strain JT04 had an arginine at codon 73 (CGG) and a leucine at codon 198 (CTG). The GenBank accession numbers of β_2 tub from different MBC-sensitivity strains are FJ214662.1 to FJ214665.1.

Phylogenetic analysis. The analysis for β -tubulins from *G. zeae* and other selected plant pathogens was performed on the amino acid sequence. The alignment and cluster analysis were used to generate the phylogenetic tree. This first analysis grouped β_1 tub of *G. zeae* in a branch together with MBC-resistant β -tubulins from some other plant pathogens, while β_2 tub of *G. zeae* belonged to another branch with only one member.

Validation of the mutations correlated with resistance.

Deletion of the β_2 tub. Hygromycin-resistant transformants were screened via PCR amplification for the presence of 1-up:*PtpC-hpt*:1.2-down sequences and the β_2 Tub locus sequences of the three strains (MBC^S 2021, MBC^{MR} R9, and MBC^{HR} JT04) with two three-primer combinations (Fig. 1B to D; Tables 2 and 4). In all, 42 transformants of MBC^S 2021, 27 transformants of MBC^{MR} R9, and 32 transformants of MBC^{HR} JT04 yielded 1-up:*PtpC-hpt*:1.2-down-homologous recombination-specific amplicons (with primer pairs 16-26, 6-29, 15-16, and 5-6) but no wild-type-specific amplicons (with primer pairs 3-4, 26-27, 28-29, 15-27, and 5-28). Some transformants had more complex banding patterns but these transformants were not characterized further. Nine of the transformants which were derived from 2021, R9, and JT04 were

TABLE 4. Predicted polymerase chain reaction (PCR) amplicon sizes for β_2 -tubulin (β_2 tub) deletion mutants of *Gibberella zeae*

Primers	Amplicon size (bp) for β_2 tub ^a	
	Wild-type strains	Deletion mutants
3-4	804	None
15-16	None	1,874
5-6	None	1,440
16-26	None	758
6-29	None	586
26-27	491	None
28-29	559	None
15-27	1,607	None
5-28	1,413	None

^a Expected and observed sizes of PCR products amplified with the primer combinations indicated and DNA templates consisting of genomic DNA from carbendazim (methyl benzimidazol-2-yl carbamate [MBC])-sensitive strain 2021, moderately MBC-resistant strain R9, highly MBC-resistant strain JT04, and β_2 tub-deletion mutants.

TABLE 3. Correlation between phenotype of carbendazim (methyl benzimidazol-2-yl carbamate [MBC]) resistance and mutations within the β_2 tub gene (at codons 73, 167, 198, and 200) in *Gibberella zeae*^a

Phenotype ^b	No.	Names of strains	Codon73	Codon 167	Codon 198	Codon 200
MBC ^S	15	ZF-43, ZF-32, 2021, ZF-22, T17, S1-S10	CAG(Gln)	TTT(Phe)	GAG(Glu)	TTC(Phe)
MBC ^{MR}	13	ZF-52, ZF-54, SP-2, R1-R10	CAG(Gln)	TAT(Tyr)	GAG(Glu)	TTC(Phe)
MBC ^{MR}	3	NT-7, r2, t1	CAG(Gln)	TTT(Phe)	GAG(Glu)	TAT(Tyr)
MBC ^{HR}	1	JT04	CGG(Arg)	TTT(Phe)	CTG(Leu)	TTC(Phe)

^a Amino acid corresponding to each codon is presented in parentheses and nucleotides in boxes indicate the particular point mutation in the given codon.

^b MBC^S, MBC^{MR}, and MBC^{HR} indicate that the strain is sensitive, moderately resistant, or highly resistant, respectively, to carbendazim.

randomly chosen and analyzed. Southern blots showed the predicted β_2tub -deletion banding pattern (Fig. 2A).

Complementation of the β_2Tub from MBC^R strains into β_2tub deletion mutants of MBC^R strains. pAMDS- β_2Tub -R9 and pAMDS- β_2Tub -JT04 were transformed into β_2tub -deletion mutants $\Delta\beta_2tub$ -R9D49 and $\Delta\beta_2tub$ -JT04D144. These two β_2tub -deletion mutants were selected because they produced large numbers of conidia. In total, 14 acetamide-utilizing transformants were recovered from $\Delta\beta_2tub$ -R9D49 and 11 from $\Delta\beta_2tub$ -JT04D144. All of the transformants from $\Delta\beta_2tub$ -R9D49 and 8 of 11 transformants from $\Delta\beta_2tub$ -JT04D144 had the expected 0.8-kb amplification product. Six of these restored β_2tub transformants analyzed by Southern blotting carried the β_2Tub locus (Fig. 2B). Two bands were detected in the Southern blot analysis, of which the 2.1-kb band was determined to be from the β_2Tub locus (Fig. 2B to D).

Complementation of the β_2Tub of MBC^R strains into β_2tub deletion mutants of MBC^S strain. pAMDS- β_2Tub -R9 was transformed into the β_2tub -deletion mutant $\Delta\beta_2tub$ -2021D96. The same procedure was done for pAMDS- β_2Tub -JT04 for transformation into $\Delta\beta_2tub$ -2021D96. In all, 17 acetamide-utilizing transformants which transformed with pAMDS- β_2Tub -R9, and 14 acetamide-utilizing transformants with pAMDS- β_2Tub -JT04 were analyzed by PCR with the primers 3-4 (Fig. 1). In total, 13 of 17

transformants using pAMDS- β_2Tub -R9 and 13 of 14 transformants using pAMDS- β_2Tub -JT04 contained the expected 0.8-kb amplification product. Six transformants analyzed by Southern blotting contained the β_2Tub locus (Fig. 2C).

Complementation of the β_2Tub from MBC^S strains into β_2tub deletion mutants of MBC^R strain. pAMDS- β_2Tub -2021 was transformed into $\Delta\beta_2tub$ -R9D49 and $\Delta\beta_2tub$ -JT04D144, respectively. In all, 15 and 9 acetamide-utilizing transformants were recovered from $\Delta\beta_2tub$ -R9D49 and $\Delta\beta_2tub$ -JT04D144, respectively. In total, 13 of the 15 transformants using $\Delta\beta_2tub$ -R9D49 and 8 of the 9 transformants using $\Delta\beta_2tub$ -JT04D144 had the expected 0.8-kb amplification product. All six of the β_2tub -complementation mutants analyzed by Southern blotting contained the β_2Tub locus (Fig. 2D).

Sensitivity of deletion- and complementation-mutants to MBC. Sensitivity to MBC (EC_{50}) differed significantly among β_2tub -deletion and -complementation mutants. The 9 $\Delta\beta_2tub$ mutants and the 18 β_2tub -complementation mutants derived from MBC^S strain 2021, MBC^{MR} strain R9, and MBC^{HR} strain JT04 were considered as four groups. There were significant differences in EC_{50} between these groups (Fig. 3; Table 5). EC_{50} values of the $\Delta\beta_2tub$ mutants (which were 0.08 to 0.1 μ g/ml) were much lower than that of MBC^S strain 2021 (0.6 μ g/ml). These $\Delta\beta_2tub$ mutants were considered to be supersensitive to MBC. Comple-

Fig. 2. Southern blot analysis of *Gibberella zeae* β_2tub deletion ($\Delta\beta_2tub$) mutants and β_2tub -tubulin (β_2tub) complementation mutants. Genomic DNA of all the strains were digested by *EcoRV*. The size markers in the figures were based on λ -Hind III digest (TaKaRa, Dalian, China). Shown are **a**, 1.4-kb bands; **b**, 3.4-kb bands; **c**, 2.1-kb bands; and **d**, 4.8-kb bands. **A**, Detected using probe 1, which was prepared from the 1.2-downstream fragment of the β_2tub coding region. **B–D**, Detected using probe 2, which was prepared from the β_2tub coding region. **A**, Analysis of β_2tub -deletion mutants. Probe 1 hybridized to a 1.4-kb band of the original *G. zeae* strains and to a 3.4-kb band of *G. zeae* β_2tub -deletion mutants. Lane 1: Methyl benzimidazol-2-yl carbamate-sensitive (MBC^S) strain 2021, lane 2: moderately resistant (MBC^{MR}) strain R9, lane 3: highly resistant (MBC^{HR}) strain JT04, lane 4: $\Delta\beta_2tub$ -2021D51, lane 5: $\Delta\beta_2tub$ -2021D81, lane 6: $\Delta\beta_2tub$ -2021D96, lane 7: $\Delta\beta_2tub$ -R9D4, lane 8: $\Delta\beta_2tub$ -R9D32, lane 9: $\Delta\beta_2tub$ -R9D49, lane 10: $\Delta\beta_2tub$ -JT04D47, lane 11: $\Delta\beta_2tub$ -JT04D144, lane 12: $\Delta\beta_2tub$ -JT04D151. **B–D**, Southern blot analysis of *G. zeae* β_2tub -complementation mutants. Probe 2 hybridized to a 2.1-kb band of β_2tub -tubulin locus (β_2Tub) and a weak 4.8-kb band of β_1 -tubulin locus (β_1Tub). **B**, Lane 1: Polymerase chain reaction (PCR) product of β_1Tub digested by *EcoRV*, lane 2: MBC^{MR} strain R9, lane 3: MBC^{HR} strain JT04, lane 4: $\Delta\beta_2tub$ -R9D49, lane 5: $\Delta\beta_2tub$ -JT04D144, lanes 6–11: β_2tub -complementation mutants β_2tub -R9D49-R9C1, β_2tub -R9D49-R9C4, β_2tub -R9D49-R9C6, β_2tub -JT04D144-JT04C4, β_2tub -JT04D144-JT04C6, and β_2tub -JT04D144-JT04C7, respectively. **C**, Lane 1: PCR product of the β_1Tub digested by *EcoRV*, lane 2: MBC^S strain 2021, lane 3: MBC^{MR} strain R9, lane 4: MBC^{HR} strain JT04, lane 5: $\Delta\beta_2tub$ -2021D96, lanes 6–11: β_2tub -complementation mutants β_2tub -2021D96-R9C1, β_2tub -2021D96-R9C3, β_2tub -2021D96-R9C8, β_2tub -2021D96-JT04C2, β_2tub -2021D96-JT04C3, and β_2tub -2021D96-JT04C5, respectively. **D**, Lane 1: PCR product of the β_1Tub digested by *EcoRV*, lane 2: MBC^S strain 2021, lane 3: MBC^{MR} strain R9, lane 4: MBC^{HR} strain JT04, lane 5: $\Delta\beta_2tub$ -R9D49, lane 6: $\Delta\beta_2tub$ -JT04D144, lanes 7–12: β_2Tub -complementation mutants β_2tub -R9D49-2021C1, β_2tub -R9D49-2021C3, β_2tub -R9D49-2021C5, β_2tub -JT04D144-2021C2, β_2tub -JT04D144-2021C5, and β_2tub -JT04D144-2021C7, respectively.

mentation of the $\Delta\beta_2tub$ mutants derived from MBC^S strain 2021 with the β_2Tub from MBC^{MR} R9 or MBC^{HR} strain JT04 conferred the phenotype of moderate or high resistance, respectively, to MBC. Complementation of $\Delta\beta_2tub$ mutants derived from MBC^{HR} strain JT04 and MBC^{MR} strain R9 with the β_2Tub locus from MBC^S 2021 restored the phenotype of sensitivity to MBC. Decreased sensitivity was observed in all complementation mutants, no matter what resistant strain was initially used to complement the β_2Tub locus in the deletion mutants.

RT-PCR analysis of the expression of β_1tub and β_2tub genes in MBC^S, MBC^{MR}, and MBC^{HR} strains of *G. zeae* and their β_2tub -deletion and -complementation mutants. The RT-PCR fragments of β_1tub and β_2tub genes were detected in MBC^S strain 2021, MBC^{MR} strain R9, MBC^{HR} strain JT04, and the six β_2tub -complementation strains, whereas the RT-PCR fragments of β_2tub were not detected in the three β_2tub -deletion mutants (Fig. 4).

DISCUSSION

In this study, the molecular basis for resistance to MBC in *G. zeae* is described. Based on transformation experiments with *G. zeae*, we provide direct evidence that mutations at the β_2tub gene are responsible for resistance to benzimidazoles. In addition, this gene may be used as a genetic marker in *G. zeae* for biological research, similar to the genetic marker *hpt* in *G. zeae*.

The deduced amino acid sequences of the β_2tub and the β_1tub (another member of the tubulin gene family) in *G. zeae* were 76% identical (Fig. 5). β_1tub has higher similarity than β_2tub to the β -tubulin genes that have been shown to confer benzimidazole resistance in other plant pathogens (7,15–17,30).

In the Southern blot analysis of wild-type strains and β_2tub -complementation mutants (Fig. 2B to D), two bands were detected by probe 2. The 2.1-kb band is for β_2tub and another faint band of $\approx$ 4.8 kb was identified as a fragment of β_1tub , which was 58% identical to probe 2 and spanned from 3,038 bp upstream to 104 bp downstream of the β_1tub -coding region (data not shown).

Mutations at different codons in the β_2tub resulted in altered amino acid sequences in the protein and different resistance to MBC. A single-point mutation (Phe167Tyr or Phe200Tyr) yielded moderate resistance to MBC, and the double-point mutations (Gln73Arg and Glu198Leu) were detected in the MBC^{HR}

strain JT04. This MBC^{HR} phenotype, however, was due to the mutation Glu198Leu rather than the mutation Gln73Arg and was confirmed by separately constructing the mutants with Gln73Arg and with Glu198Leu (data not shown). Of the three carbendazim-

TABLE 5. Carbendazim (methyl benzimidazol-2-yl carbamate [MBC]) sensitivity of β_2 -tubulin (β_2tub)-deletion and -complementation mutants of *Gibberella zeae*

Strains or mutants ^a	EC ₅₀ /MIC (μg/ml) ^b
2021 (S)	0.6/1.4
$\Delta\beta_2tub$ -2021D51	0.1/0.5
$\Delta\beta_2tub$ -2021D81	0.09/0.5
$\Delta\beta_2tub$ -2021D96	0.1/0.5
β_2tub -2021D96-R9C1	6.5/60
β_2tub -2021D96-R9C3	6.8/60
β_2tub -2021D96-R9C8	7.0/60
β_2tub -2021D96-JT04C2	4.8/300
β_2tub -2021D96-JT04C3	4.6/300
β_2tub -2021D96-JT04C5	4.7/300
R9 (MR)	7.4/70
$\Delta\beta_2tub$ -R9D4	0.09/0.5
$\Delta\beta_2tub$ -R9D32	0.1/0.5
$\Delta\beta_2tub$ -R9D49	0.1/0.5
β_2tub -R9D49-R9C1	6.9/60
β_2tub -R9D49-R9C4	7.1/60
β_2tub -R9D49-R9C6	6.9/60
β_2tub -R9D49-2021C1	0.5/1.4
β_2tub -R9D49-2021C3	0.5/1.4
β_2tub -R9D49-2021C5	0.6/1.4
JT04 (HR)	6.9/400
$\Delta\beta_2tub$ -JT04D47	0.08/0.5
$\Delta\beta_2tub$ -JT04D144	0.08/0.5
$\Delta\beta_2tub$ -JT04D151	0.1/0.5
β_2tub -JT04D144JT04C4	4.4/300
β_2tub -JT04D144JT04C6	4.3/300
β_2tub -JT04D144JT04C7	4.7/300
β_2tub -JT04D144-2021C2	0.5/1.4
β_2tub -JT04D144-2021C5	0.5/1.4
β_2tub -JT04D144-2021C7	0.5/1.4

^a $\Delta\beta_2tub$ -D* and β_2tub -D*C* represent β_2tub -deletion and -complementation mutants, respectively. S, MR, and HR indicate sensitive, moderately resistant, and highly resistant to MBC, respectively.

^b EC₅₀ indicates 50% effective concentration, which is the fungicide concentration resulting in 50% mycelial growth inhibition. The value of each EC₅₀ is the mean of four replicates. Minimum inhibitory concentration (MIC) results in 100% mycelial growth inhibition.

Fig. 3. Effects of carbendazim (methyl benzimidazol-2-yl carbamate [MBC]) on mycelial linear growth of *Gibberella zeae* wild-type strains (2021, R9, and JT04) and β_2 -tubulin (β_2tub)-deletion and -complementation mutants on potato dextrose agar (PDA). The mycelial plugs (5 mm in diameter) taken from the edge of a 3-day-old colony of strains or mutants were grown at 25°C for 3 days on the PDA plates, which were amended with MBC at 0, 1.4, 50, or 100 μg a.i./ml. From left to right, lane 1: MBC-sensitive strain 2021; lane 2: MBC moderately resistant strain R9; lane 3: MBC highly resistant strain JT04; lanes 4–6: β_2tub -deletion mutants $\Delta\beta_2tub$ -2021D96, $\Delta\beta_2tub$ -R9D49, and $\Delta\beta_2tub$ -JT04D144, respectively; lanes 7–12: β_2tub -complementation mutants β_2tub -R9D49-2021C3, β_2tub -JT04D144-2021C5, β_2tub -2021D96-R9C1, β_2tub -2021D96-JT04C3, β_2tub -R9D49-R9C1, and β_2tub -JT04D144-JT04C4, respectively.

sensitive phenotypes found (MBC^S, MBC^{MR}, and MBC^{HR}), the MBC^{HR} phenotype took up <1% of the >10,000 carbendazim-resistant and -sensitive isolates tested in the laboratory, while the MBC^{MR} phenotype accounted for >99% of the selected resistant population. Of >100 MBC^{MR} isolates sequenced (only some of isolates and strains are shown in Table 3), those with the mutation Phe167Tyr accounted for >90% and isolates with the mutation Phe200Tyr accounted for <10%. Overall, <10 MBC^{HR} isolates were detected from the nearly 2,000 MBC-resistant isolates collected (data not shown).

The mechanism of resistance to benzimidazole fungicides in *G. zeae* is different from that in other plant-pathogenic fungi that have been characterized. There are two main differences between them, as follows. First, in *G. zeae*, the substitution of Glu198Leu at β_2tub was unique, conferring a high resistance to MBC, ditho-

fencarb (DFC), and methyl N-(3,5-dichlorophenyl) carbamate (MDPC). This substitution is distinctly different from the Glu198Gln in the same gene, conferring resistance of the *F. graminearum* complex to benzimidazole (33). In other plant pathogens, however, five different substitutions at the same codon are found as follows. (i) The substitution of Glu198Ala in *Botrytis cinerea* (20), *Helminthosporium solani* (25), *Monilinia fructicola* (22), *Penicillium expansum* (17), *Tepesia acuformis* (1), *T. yal-hundae* (1), and *Venturia inaequalis* (17) confers high resistance to carbendazim as well as insusceptibility to MDPC and DFC. (ii) The substitution of Glu198Gln in *H. solani* (25), *T. acuformis* (1), and *T. yal-hundae* (1) confers low resistance to carbendazim and insusceptibility to MDPC as well as susceptibility to DFC. (iii) The substitution of Glu198Gly in *V. inaequalis* (17), *T. acuformis* (1), and *T. yal-hundae* (1) confers moderate resistance to car-

Fig. 4. Reverse transcriptase polymerase chain reaction (RT-PCR) fragments of β_1 -tubulin (β_1tub) and β_2 -tubulin (β_2tub) genes in methyl benzimidazol-2-yl carbamate-sensitive (MBC^S) strain 2021, moderately resistant (MBC^{MR}) strain R9, highly resistant (MBC^{HR}) strain JT04, and β_2tub -deletion and -complementary mutants derived from R9 and JT04 by RT-PCR. Elongation factor 1- α gene (*EF-1- α*), a housekeeping gene, was employed as a control. RT-PCR was performed with total RNA extracted from the strains or mutants. From left to right, lane 1: MBC^S strain 2021; lane 2: MBC^{MR} strain R9; lane 3: MBC^{HR} strain JT04; lanes 4–6: β_2tub -deletion mutants $\Delta\beta_2tub$ -2021D96, $\Delta\beta_2tub$ -R9D49, and $\Delta\beta_2tub$ -JT04D144, respectively; lanes 7–12: β_2tub -complementation mutants β_2tub -R9D49-2021C3, β_2tub -JT04D144-2021C5, β_2tub -2021D96-R9C1, β_2tub -2021D96-JT04C3, β_2tub -R9D49-R9C1, and β_2tub -JT04D144-JT04C4, respectively.

Fig. 5. Phylogenetic relationship of β -tubulin in *Gibberella zeae* and other plant-pathogenic fungi based on deduced amino acid sequences. Bootstrap values ($n = 1,000$) are shown at the branch points.

bendazim susceptibility to MDPC and DFC. (iv) The substitution of Glu198Lys in *M. fructicola* (17), *P. expansum* (17), *T. aciformis* (1), and *T. yallundae* (1) confers moderate resistance to carbendazim susceptibility to MDPC as well as insusceptibility to DFC. (v) The substitution of Glu198Val in *P. expansum* (17) confers high resistance to carbendazim susceptibility to MDPC as well as insusceptibility to DFC. Second, the benzimidazole resistance in the other pathogens was conferred by mutations in a β -tubulin gene that has high identity to the β_1 tub gene of *G. zeae*, whereas the MBC resistance in *G. zeae* reported in the current article was conferred by mutation in the β_2 tub. This may result in not only the much lower resistance factor (mean EC₅₀ of resistant population/mean EC₅₀ of sensitive population) of *G. zeae* compared with *B. cinerea* (13), *Sclerotinia homoeocarpa* (12), *Venturia inaequalis* (17), and others (21) but also the similar EC₅₀ values and the different MIC values of MBC^{MR} and MBC^{HR} strains of *G. zeae*.

Both β_1 tub and β_2 tub of *G. zeae* are expressed during mycelial growth, which differs from the pattern observed for *benA* and *tubC* of *A. nidulans* (24).

β_1 tub-deletion mutants of *G. zeae* showed supersensitivity to MBC but also reduced growth on MBC-free PDA. This result suggests that β_1 Tub can provide what is necessary for growth without β_2 Tub. The β_1 tub-deletion mutants of *G. zeae*, however, grew normally on MBC-free PDA (data not shown). Thus, this difference of β_2 tub- and β_1 tub-deletion mutants implies that the functions of β_1 Tub and β_2 Tub of *G. zeae* differ in mycelial growth.

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LITERATURE CITED

- Albertini, C., Gredt, M., and Leroux, P. 1999. Mutations of the b-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eye spot fungi *Tapesia yallundae* and *Tapesia acutormis*. *Pestic. Biochem. Physiol.* 64:17-23.
- Altschul, S. F., Madden, T. L., Shaffer, A. A., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Atanassov, Z., Nakamura, C., Mori, N., Kaneda, C., Kato, H., Jin, Y. Z., Yoshizawa, T., and Murai, K. 1994. Mycotoxin production and pathogenicity of *Fusarium* species and wheat resistance to *Fusarium* head blight. *Can. J. Bot.* 72:161-167.
- Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., and Pratella, G. C. 2003. Studies on thiabendazole resistance of *Penicillium expansum* of pears, pathogenic fitness and genetic characterization. *Plant Pathol.* 52:362-370.
- Bollen, G. J., and Scholten, G. 1971. Acquired resistance to benomyl and some other systemic fungicides in a strain of *Botrytis cinerea* in cyclamen. *Neth. J. Plant Pathol.* 77:83-90.
- Chen, C. J., Li, J., Qi, Z. Q., Wang, J. X. and Zhou, M. G. 2005. Cloning of α -tubulin gene from *Fusarium graminearum* and analyzing its relationship with MBC resistance. *Acta Microbiol. Sin.* 45:288-291.
- Chen, C. J., Wang, J. X., Luo, Q. Q., Yuan, S. K., and Zhou, M. G. 2007. Characterization and fitness of carbendazim-resistant strains of *Fusarium graminearum* (wheat scab). *Pest Manage. Sci.* 63:1201-1207.
- Chen, C. J., Wang, J. X., and Zhou, M. G. 2005. Cloning of γ -tubulin gene from *Fusarium graminearum* and analyzing its relationship with carbendazim resistance. *Acta Phytopathol. Sin.* 35:161-167.
- Chen, C. J., Zhou, L. B., Bi, Z. W., Luo, Q. Q., Wang, J. X., Li H. X., and Zhou, M. G. 2008. Resistant mechanism of *Fusarium graminearum* against carbendazim, un-relative to alpha2-tubulin gene. *Acta Microbiol. Sin.* 48:1356-1361.
- Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640-1646.
- Davidse, L. C. 1986. Benzimidazole fungicides: Mechanism of action and biological impact. *Annu. Rev. Phytopathol.* 24:43-65.
- Detweiler, A. R., Vargas, J. M., and Danneberger, T. K. 1983. Resistance of *Sclerotinia homoeocarpa* to iprodione and benomyl. *Plant Dis.* 67:627-630.
- Faretra, F., and Pollastro, S. 1991. Genetic basis of resistance to benzimidazole and dicarboximide fungicides in *Botryotinia fuckeliana* and *Botrytis cinerea*. *Mycol. Res.* 95:943-951.
- Hell, T. A. 1999. Bioedit A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95-98.
- Kawchuk, L. M., Hutchison, L. J., Verhaeghe, C. A., Lynch, D. R., Bains, P. S., and Holley, J. D. 2002. Isolation of the b-tubulin gene and characterization of thiabendazole resistance in *Gibberella pulicaris*. *Can. J. Plant Pathol.* 24:233-238.
- Koenraadt, H., and Jones, A. L. 1993. Resistance to benomyl conferred by mutations in codon 198 or 200 of the beta-tubulin gene of *Neurospora crassa* and sensitivity to diethofencarb by codon 198. *Phytopathology* 83:850-854.
- Koenraadt, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology* 82:1348-1354.
- Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinf.* 5:150-163.
- Kwa, M. S. G., Veenstra, J. G., vanDijk, M., and Roos, M. H. 1995. β -Tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *J. Mol. Biol.* 246:500-510.
- Luck, J. E., and Gillings, M. R. 1995. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. *Mycol. Res.* 99:1483-1488.
- Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Prot.* 24:853-863.
- Ma, Z., Yoshimura, M., and Michailides, T. J. 2003. Identification and characterization of benzimidazole resistance in *Monilinia fructicola* from stone fruit orchards in California. *Appl. Environ. Microbiol.* 69:7145-7152.
- Maier, F. J., Malz, S., Losch, A. P., Lacour, T., and Schafer, W. 2005. Development of a highly efficient gene targeting system for *Fusarium graminearum* using the disruption of a polyketide synthase gene as a visible marker. *FEMS Yeast Res.* 5:653-662.
- May, G. S. 1989. The highly divergent β -tubulins of *Aspergillus nidulans* are functionally interchangeable. *J. Cell Biol.* 109:2267-2274.
- McKay, G. J., and Cooke, L. R. 1997. A PCR-based method to characterize and identify benzimidazole resistance in *Helminthosporium solani*. *FEMS Microbiol. Lett.* 152:371-378.
- Mullins, E. D., Chen, X., Romaine, P., Raina, R., Geiser, D. M., and Kang, S. 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* 91:173-180.
- Park, Y. S., Choi, I. D., Kang, C. M., Ham, M. S., Kim, J. H., Kim, T. H., Yun, S. H., Lee, Y. W., Chang, H. I., Sung, H. C., and Yun, C. W. 2006. Functional identification of high-affinity iron permeases from *Fusarium graminearum*. *Fungal Genet. Biol.* 43:273-282.
- Proctor, R. H., Hohn, T. M., and McCormick, S. P. 1997. Restoration of wild-type virulence to Tri5 disruption mutants of *Gibberella zeae* via gene reversion and mutant complementation. *Microbiology* 143:2583-2591.
- Sanger, F., Nicklens, S., and Coulson, A. R. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Seip, E. R., Woloshuk, C. P., Payne, G. A., and Curtis, S. E. 1990. Isolation and sequence analysis of a β -tubulin gene from *Aspergillus flavus* and its use as a selectable marker. *Appl. Environ. Microbiol.* 56:3686-3692.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gaps penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
- Wicks, T. 1974. Tolerance of the apple scab fungus to benzimidazole fungicides. *Plant Dis. Rep.* 58:886-889.
- Yin, Y., Liu, X., Li, B., and Ma, Z. 2009. Characterization of Sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology* 99:487-497.
- Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Yazmid, R. D., and Scaccocchio, C. 2004. Double-joint PCR: A PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* 41:973-981.
- Yuan, S. K., and Zhou, M. G. 2005. A major gene for resistance to carbendazim, in field isolates of *Gibberella zeae*. *Can. J. Plant Pathol.* 27:58-63.