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Localisation of the benzimidazole fungicide binding site of *Gibberella zeae* β_2-tubulin studied by site-directed mutagenesis

JianBo Qiu,[a†] JianQiang Xu,[a,b†] JunJie Yu,[a†] ChaoWei Bi,[a] ChangJun Chen[a] and MingGuo Zhou[a*]

Abstract

BACKGROUND: The efficacy of benzimidazole fungicides is often limited by resistance, and this is the case with the use of carbendazim for controlling *Fusarium* head blight caused by *Gibberella zeae* (Schwein.) Petch (anamorph *Fusarium graminearum*). Recent studies have shown that carbendazim resistance in field strains of *G. zeae* is associated with mutations in the β_2-tubulin gene. The aims of the present study were to validate this mechanism and research the binding sites of carbendazim on β_2-tubulin.

RESULTS: This work used site-directed mutagenesis followed by gene replacement to change the β_2-tubulin gene of a carbendazim-sensitive field strain of *G. zeae* at residues 50, 167, 198 or 200. The transformants were confirmed and tested for their sensitivity to carbendazim. All the mutants were resistant to carbendazim, but the level of resistance differed depending on the mutation. Biological characteristics did not differ between the field strain and the site-directed mutants. A three-dimensional model of β_2-tubulin was constructed, and the possible carbendazim binding site was analysed.

CONCLUSION: Mutations at codons 50, 167, 198 and 200 of *G. zeae* $\beta_2$tub could cause resistance to carbendazim, and these codons may form a binding pocket.

Keywords: *Gibberella zeae*; benzimidazole resistance; β_2-tubulin gene; site-directed mutagenesis

1 INTRODUCTION

Fusarium head blight (FHB), also called ear blight or scab, is a destructive disease affecting several hosts including wheat, maize and barley.[1,2] The disease occurs worldwide and is associated with several *Fusarium* spp., but *Fusarium graminearum* (the perfect state is *Gibberella zeae* Schwein.) is the prevalent species in many regions. The fungus reduces crop yield and produces mycotoxins that are harmful to humans and animals.[3] This reduction in quantity and quality of grain has adversely impacted upon the agricultural economy of small-grain-producing regions. For example, the total economic loss caused by FHB in the 1990s was estimated at $3 billion in North America.[4]

The economic impact of FHB highlights the necessity of an effective control strategy. Although various ways have been developed to control this disease, fungicides always play an important role in an integrated FHB control programme.[5] Carbendazim and other benzimidazole fungicides, which act by inhibiting mitosis, have been shown to be effective against a variety of plant pathogenic fungi, including most of the ascomycetes and some deuteromycetes, and have been used to control FHB over the past three decades.[6] The efficacy of these fungicides is often limited by resistance. Benzimidazole resistance often develops in many plant pathogenic fungi after these fungicides have been used for as few as 2–3 years.[7,8] Carbendazim had been widely used for decades in China and in many other parts of the world, however, before the first carbendazim-resistant strain of *G. zeae* was detected in the field in Zhejiang Province, China, in 1992.[9] Because of the problem, it is necessary to develop fungicides that can overcome resistance.

Benzimidazole resistance has been detected in many fungal species and usually results from certain point mutations in the β-tubulin gene that alter the protein's amino acid sequence; these amino acid substitutions have been documented at codons 6, 50, 165, 167, 198, 200, 241 and 257.[10] However, a few exceptions have also been observed; for instance, in *G. zeae* and *G. pulicaris* (Fr.) Sacc., the amino acid sequences of the β-tubulin gene from benzimidazole-resistant and benzimidazole-sensitive strains were identical.[11–13] In recent studies from the authors' laboratory,

* *Correspondence to: MingGuo Zhou, Jiangsu Key Laboratory of Pesticide Science, Nanjing Agricultural University, Nanjing 210095, People's Republic of China. E-mail: mgzhou@njau.edu.cn*

† *These authors contributed equally to this work.*

a *College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Nanjing, China*

b *College of Forestry, Henan University of Science and Technology, Luoyang, China*

point mutations in the β_2-tubulin gene at residues 167 (Phe to Tyr), 198 (Glu to Lys) and 200 (Phe to Tyr) were detected in carbendazim-resistant field strains of *G. zeae*, and these mutations were considered the likely molecular mechanism of carbendazim resistance in *G. zeae*.[14,15]

Because the carbendazim-resistant field strains of *G. zeae* have diverse genetic backgrounds, determining the relationship between carbendazim resistance and point mutations in the β_2-tubulin gene is difficult.[16] In other words, mechanisms other than mutations in the β_2-tubulin gene might be responsible for the resistance. Determining whether point mutations in the β_2-tubulin gene cause carbendazim resistance requires that other factors be excluded, i.e. that the strains compared differ only in the β_2-tubulin gene.

In the present study, site-directed mutagenesis was performed on a carbendazim-sensitive field strain of *G. zeae* to create mutations in the β_2-tubulin gene at residues 50 (TAC to TGC), 167 (TTT to TAT), 198 (GAG to CAG), 198 (GAG to AAG) or 200 (TTC to TAC). The sensitivities to benzimidazole fungicides of the resulting site-directed mutants were characterised to determine whether these mutations result in resistance. In addition, the three-dimensional structure of β_2-tubulin was modelled so as to identify the possible binding site for benzimidazoles on β_2-tubulin. Identification of this binding site is important because it will reveal the relative importance of these amino acid residues in binding with the fungicide molecule and will facilitate the rational design of new analogues that target tubulin.

2 MATERIALS AND METHODS

2.1 Strains and culture conditions

The *G. zeae* strains used are listed in Table 1. All *G. zeae* mutants used in this study were derived from the carbendazim-sensitive field strain 2021 and from strain DN83, in which the β_2-tubulin gene was replaced by two marker genes (Fig. 1C). The bacterial hygromycin B (hygB) phosphotransferase gene (*hph*) was used as a positive selectable marker, and the herpes simplex virus thymidine kinase gene (*hsv-tk*) was used as a conditional negative selection marker.[17] Four field-resistant strains of *G. zeae* were collected from winter wheat in China and stored in the laboratory (Table 1), and these strains were generated from a single conidium.

Mung bean broth (MBB) was prepared for conidia production.[18] Autoclaved potato sucrose agar (PSA) was used to culture the fungi and in routine assays for *in vitro* sensitivity to benzimidazoles. PSA amended with 100 µg mL^{-1} hygromycin B (hygB) was used to culture transformants with the hygromycin phosphotransferase (*hph*) gene, and was amended with 0.2 µmol mL^{-1} F_2dU to culture transformants that had lost the herpes simplex virus thymidine kinase (*hsv-tk*) gene.

2.2 DNA and RNA extraction

Genomic DNA was isolated from mycelia collected from three-day-old cultures grown in liquid potato sucrose (PS) medium using the CTAB procedure.[19] For RNA preparation from vegetative hyphae, each strain was grown in liquid PS for 36 h. Total RNA was extracted using TRIZOL Reagent (cat. no. 15 596-026, lot no. 1 401 902; Invitrogen, China) following the manufacturer's protocol and was quantified by spectrophotometry at 260/280 nm. RNA was treated with DNase I (Takara, Dalian, China), resuspended in 30 µL of DEPC-treated water and stored at −80 °C; the RNA was used as the template for reverse transcription-polymerase chain reaction (RT-PCR).

2.3 Site-directed mutagenesis of *Gibberella zeae* β_2-tubulin

Site-directed mutagenesis of the β_2-tubulin gene of *G. zeae* strain 2021 at codons 50, 167, 198 and 200 were performed by overlap extension PCR.[20,21] When used for the acquisition of mutagenic fragments, this method involves three separate PCRs by the primers (Table 2). In the first two rounds of PCR, upstream and downstream fragments are independently obtained. Four primers, including two flanking primers (P9 and P10) and two specially designed mutagenic primers (Pm F and Pm R, which were changeable at overlapping position), contained nucleotide mismatches at their centres. In a subsequent reaction driven by two flanking primers (P9 and P10), the two intermediate PCR products were fused, and the final product (Fig. 1D) containing the desired mutation was obtained.

2.4 Protoplast transformation

A double-replacement strategy[22] was used to introduce subtle changes into the $\beta_2$tub locus of the *G. zeae* wild-type strain (Fig. 1B). Replacement of the double markers was accomplished by transforming protoplasts of strain DN83 with the mutated fragments, as described above. Protoplasts were prepared and the transformation process was performed as described before,[23]

Table 1. *Gibberella zeae* strains used in this study

Strain	Parental strain	Genotype description	Source
2021		Wild type	Zhejiang Province, China
DN83	2021	$\triangle\beta_2$tub	Present study
Y50C	DN83	Mutation at codon 50 of $\beta_2$tub (Y → C)[a]	Present study
2052		Mutation at codon 167 of $\beta_2$tub (F → Y)	Zhejiang Province, China
F167Y	DN83	Mutation at codon 167 of $\beta_2$tub (F → Y)	Present study
NT-7		Mutation at codon 200 of $\beta_2$tub (F → Y)	Jiangsu Province, China
F200Y	DN83	Mutation at codon 200 of $\beta_2$tub (F → Y)	Present study
J2		Mutation at codon 198 of $\beta_2$tub (E → K)	Jiangsu Province, China
E198K	DN83	Mutation at codon 198 of $\beta_2$tub (E → K)	Present study
ZJ80		Mutation at codon 198 of $\beta_2$tub (E → Q)	Jiangsu Province, China
E198Q	DN83	Mutation at codon 198 of $\beta_2$tub (E → Q)	Present study

[a] Mutation was confirmed by sequence analysis.

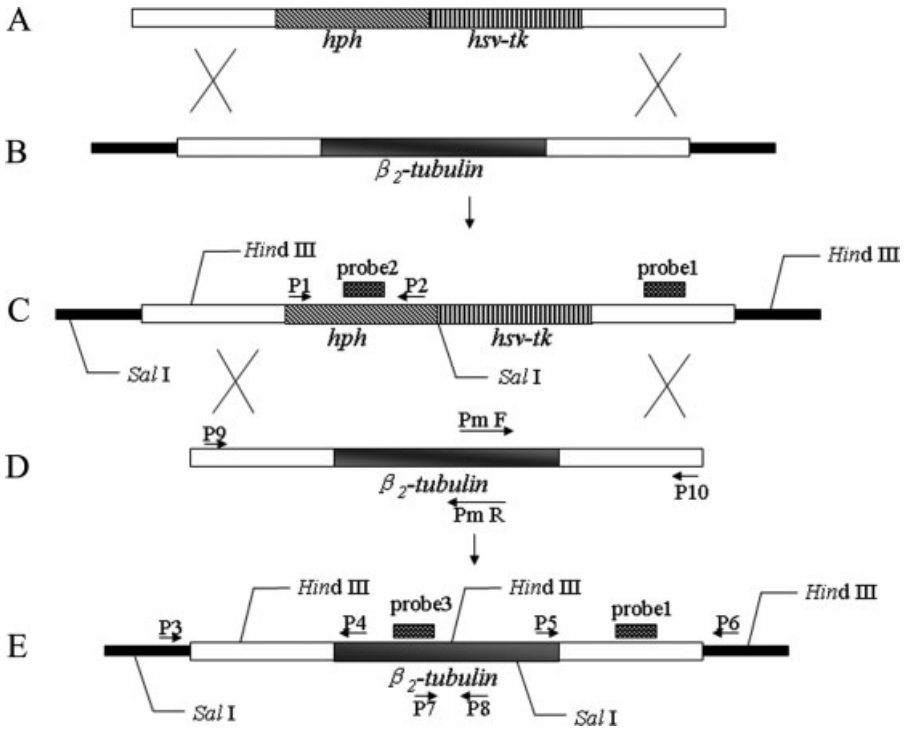

Figure 1. Gene replacement strategy for $\beta_2$tub of *Gibberella zeae*. Graphical representation of the structure of the $\beta_2$tub locus in the wild type (B), in $\beta_2$tub deletion mutant (C) and in site-directed mutant (E). The deletion cassette is composed of two selectable markers (A). The mutated fragment of $\beta_2$tub is shown in (D).

Table 2. PCR primers used in this study

Primer	Sequence (5′ to 3′)
P1	TGTCCTGCGGGTAAATAGC
P2	GTCCATCACAGTTTGCCAGT
P3	AGCCATTGCGGAGGAGGGAAAGA
P4	TTGGGCGAGGGCATAACGGAAAA
P5	AGCGAGGGTATGGACGAGATGGA
P6	TATCGTGAGTATTGCCCACCGCC
P7	CTTCATGGTCGGATTTGC
P8	TGTCGGCGTCTTGGTATT
P9	TTCCCATCGCCTGGATTA
PmR Y50C	CACCTCAGCAAAG[C][a]AGACGTTGAT
PmR F167Y	ACGGAA[T]AGGTGGCCATCAT
PmR E198K	TCGATACAGAAGGTCT[T]GTCAGAGT
PmR E198Q	TCGATACAGAAGGTCT[G]GTCAGAGT
PmR F200Y	CGATACAG[T]AGGTCTCGTCAGAGTTC
P10	GCAGACAGTGGTCCCAGAGATG
PmF Y50C	ATCAACGTCT[G]CTTTGCTGAGGTGA
PmF F167Y	GATCGCATGATGGCCACCT[A]TTCC
PmF E198K	TCGTCGAGAACTCTGAC[A]AGACCTT
PmF E198Q	TCGTCGAGAACTCTGAC[C]AGACCTT
PmF F200Y	GAACTCTGACGAGACCT[A]CTGTATCG
P11	CTCCTTCATTTTACTTTTAGGCACC
P12	TTTAGAAATCGTGCTTAAAAACACG

[a] Boxes indicate the subtle changes corresponding to codon 50, 167, 198 or 200 of β_2-tubulin in wild-type *G. zeae* strain 2021.

but some procedures were improved. To generate protoplasts, germlings were grown in liquid YEPD (10 mg mL^{-1} peptone, 3 mg mL^{-1} yeast extract, 20 mg mL^{-1} glucose), collected by filtration and centrifugation and mixed with protoplasting solution (5 mg mL^{-1} of lysing enzymes from *Tichoderma harzianum*, Sigma lot no. L1412; 25 mg mL^{-1} of driselase from *Basidiomycetes* sp., Sigma lot number D9515; 0.05 mg mL^{-1} of chitinase from *Streptomyces griseus*, Sigma C6137). Protoplasts were transformed using standard PEG-mediated procedures and were plated on regeneration medium (1 mg mL^{-1} yeast extract, 1 mg mL^{-1} casein hydrolysate, 1.0 M sucrose and 16 mg mL^{-1} granulated agar). After 12 h, regeneration plates were overlayed with 10 mL of water agar containing 0.2 µmol mL^{-1} F2dU. *hsv-tk* resistant colonies that contained mutated fragment (Fig. 1E) were collected 4–7 days later and cultured on PSA.

2.5 PCR amplification, RT-PCR, Southern blot analysis and sequencing of putative transformants

The putative transformants were transferred to PSA plates with/without 100 µg mL^{-1} hygB and 0.2 µmol mL^{-1} F2dU. The putative transformants that showed normal growth on PSA plates amended with/without 0.2 µmol mL^{-1} F2dU but no growth on PSA plates amended with 100 µg mL^{-1} hygB were tentatively considered to be the target transformants.

Various primers (Table 2) were used to verify the proper integration of the mutated fragment and the replacement of the double markers. Because the β_2-tubulin gene exhibited high sequence similarity (76% in putative amino acid sequence) to another member of the tubulin gene family from *G.* zeae, the β_1-tubulin gene, transformants were initially screened with primers P7/P8 specially designed to amplify $\beta_2$tub, which is predicted to be absent only in $\beta_2$tub deletion mutants. The primer pair P1/P2 was used to amplify the selectable marker hygromycin B phosphotransferase gene (*hph*), which was detected only in the strain DN83. The primer pairs P3/P4 and P5/P6 were used

for the amplification of the two homologous arms with a partial fragment of the connecting area in order to determine whether the β_2-tubulin gene was inserted into the correct site.

For RT-PCR, 0.5 μg of total RNA from each strain and mutant was reverse transcribed into first-strand cDNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Following separation by agarose gel electrophoresis, all RT-PCR products were analysed using Molecular Analyst software (Bio-Rad, Hercules, CA).

Subsequently, putative transformants and parent strains were selected for and analysed by Southern blot. Genomic DNA was first digested by *Hin*d III, and Probe1 was expected to hybridise to a single 3.4 kb band in the putative site-directed mutant transformants, instead of hybridising to a single 6.0 kb band in $\beta_2$tub deletion strains. Probe3 was generated from a 528 bp fragment amplified from the β_2-tubulin coding region and hybridised to *Sal* I-digested genomic DNA of mutants. The number of hybridised bands in the Southern blot indicated the copy number of the β_2-tubulin allele integrated into the host genome. Southern blotting was performed using the Dig Hight Primer DNA Labeling and Detection Starter Kit I (cat. no. 11 745 832 910; Roche, Germany) according to the instructions of the suppliers.

2.6 Biological characteristics

2.6.1 *Sensitivity to benzimidazole fungicides*

The sensitivities of the *G. zeae* parent strains and site-directed mutants were determined for the benzimidazole derivatives carbendazim, benomyl, thiabendazole and thiophanate-methyl. Carbendazim was dissolved in 0.1 M hydrochloric acid (HCl) at 10 mg mL^{-1} as a stock solution and was added to autoclaved media cooled to 45–50 °C. pH was adjusted with HCl to 6.8 in all media. Benomyl, thiabendazole and thiophanate-methyl were dissolved in acetone.

A mycelial plug (5 mm in diameter) taken from the edge of a four-day-old colony of strains was grown in the dark for 3 days at 25 °C in a 9 cm diameter petri plate containing PSA amended with different concentrations of fungicides. The radial growth from the edge of the plug to the edge of the colony of each strain was measured after 3 days at 25 °C. For each plate, the average colony radial growth, measured in two perpendicular directions, was used for calculation of the 50% effective concentration (EC_{50}), which is the fungicide concentration that results in 50% inhibition of mycelial growth. Three replicates were set, and the experiments were repeated twice. A linear equation, obtained by regressing the percentage of inhibition of mycelial growth on the $\log_{10}$ of fungicide concentration, was used to determine the EC_{50}.

Carbendazim-sensitive strains of *G. zeae* could not grow on PSA containing 1.4 μg mL^{-1} carbendazim, while carbendazim-resistant strains grew normally on this medium. Therefore, the concentration of 1.4 μg mL^{-1} has been used as the threshold for detecting resistant field strains *in vitro*. Based on differences in carbendazim sensitivity, tested strains were divided into four phenotypes according to the minimum inhibitory concentration (MIC) as follows: high carbendazim resistance, MIC > 100 μg mL^{-1}; moderate carbendazim resistance, 100 μg mL^{-1} > MIC > 25 μg mL^{-1}; low carbendazim resistance, 25 μg mL^{-1} > MIC > 1.4 μg mL^{-1}; carbendazim sensitive, MIC < 1.4 μg mL^{-1}.

2.6.2 *Mycelial linear growth assay*

Mycelial linear growth rate in the absence of fungicide was tested with the method described in Section 2.6.1. A mycelial plug was placed in a petri plate containing PSA without fungicide. Three replicates were used for each strain, and the experiment was repeated twice.

2.6.3 *Sporulation assay*

After the strains had been cultured on PSA at 25 °C for 2 days, ten mycelial plugs (5 mm in diameter) of each strain were cut and transferred to a conical flask containing 150 mL of 40 mL^{-1} MBB, and the flask was placed on a shaker for 7 days (150 rpm, 25 °C, 12 h photoperiod). The number of spores produced in the flask was determined with a hemacytometer. The numbers of spores produced by each strain and mutant were compared with Fisher's LSD test. Three replicates were used for each strain, and the experiment was repeated twice.

2.6.4 *Perithecium production*

Moist, autoclaved wheat seeds (cultivar Yangmai no. 56) in sterile 50 mL flasks (about 50 seeds per flask) were inoculated with mycelial plugs (5 mm in diameter, ten plugs per flask) of each mutant and strain and incubated at 25 °C. After 7–10 days, the seeds were placed in 9 cm diameter petri dishes, covered with sterile wet sand and incubated in a humid room (RH 80%) at 25 °C and 12 : 12 h light : dark photoperiod. After 15 days, 30 seeds per mutant or strain were scored as '+++', '++' or '+' when perithecia covered more than 2/3, between 1/3 and 2/3 or less than 1/3 of each seed respectively. Three replicates were used for each strain, and the experiment was repeated twice.

Wheat plants (cultivar Yangmai no. 49) were grown from seed in a greenhouse. Virulence of the mutants and isolates was measured as described before.[12] The two outer florets of the centre spikelet were injected with 10 μL of a macroconidia suspension (1×10^5 macroconidia mL^{-1} in 0.1 mg mL^{-1} Tween 20). Macroconidial suspensions were generated in liquid MBB as described in Section 2.6.3. Control heads were injected with distilled water. For each isolate or mutant, 20 replicate heads were injected. Disease severity was calculated as the percentage of blighted spikelets in each head 16 days after inoculation. Pathogenicity of isolate or mutant was compared using Fisher's LSD test.

2.7 Homology modelling of *Gibberella zeae* β_2-tubulin

Homology modelling was performed on the SWISS MODEL web server in the first approach mode (http://www.expasy.ch/swissmod/) by using the crystal structure of pig brain (*Sus scrofa*) β-tubulin (PDB ID: 1tubB); the sequence alignment between 1tubB and $\beta_2$tub shows an 83% identity; a three-dimensional model of the *G. zeae* $\beta_2$tub was built. The resulting structure was viewed with SwissPdb Viewer v.3.7 (http://www.expasy.org/spdbv/).

3 RESULTS

3.1 Confirmation of the site-directed mutants

To confirm each site-directed mutation, a split marker strategy was used to replace double markers with the mutated fragment. *Herpes simplex* virus thymidine kinase gene (*hsv-tk*), which acts as a conditional negative selection marker, is especially important. This split marker approach results in better homologous recombination and less ectopic integration in several species of filamentous fungi.

Replacement of the double selectable markers with the mutated fragment was confirmed by the primers specific to the $\beta_2$tub coding region and to regions beyond the expected homologous

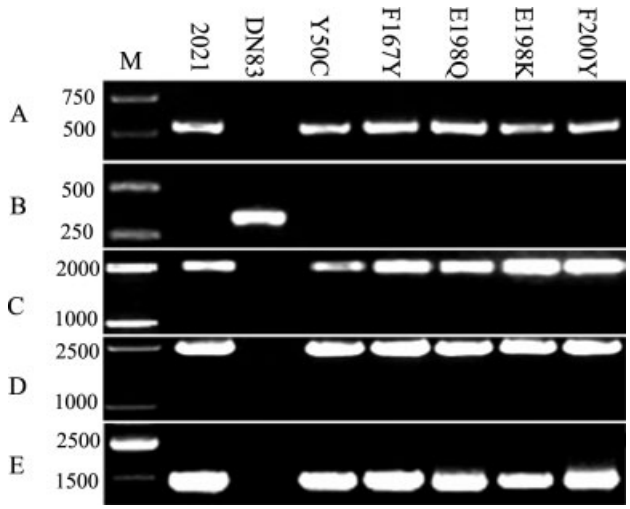

Figure 2. Identification of the mutants with primers shown in Table 2 and Fig. 1. (A) The primer pair P7/P8 was used specifically to amplify the partial $\beta_2$tub of *G. zeae* (550 bp). (B) The primer pair P1/P2 was used to validate the selectable marker *hph* (364 bp). (C), (D) The primer pairs P5/P6 and P3/P4 were used to amplify the two homologous arms with a partial fragment of connecting area (2.0kb in C and 2.5 kb in D). (E) $\beta_2$tub expression analysis in the field strain and mutants. Primers P11/P12 were used to amplify $\beta_2$tub of *G. zeae* transcript using RT-PCR. Numbers on the left refer to the migration of a standard DNA marker.

integration sites. The primer pair P7/P8, which amplifies a region in the $\beta_2$tub locus, generated a 550 bp fragment from genomic DNA of all transformants (Fig. 2A). The primer pair P1/P2 was used to amplify a 364 bp fragment of the selectable marker hygromycin B phosphotransferase gene (*hph*), which was detected only in the strain DN83 (Fig. 2B). The primer pair P3/P4, which amplifies the left homologous arm, generated a 2.0 kb fragment from genomic DNA of the transformants (Fig. 2C). Likewise, P5/P6 generated a 2.5 kb fragment, which was expected to be present in the right flanking region after homologous integration (Fig. 2D). This indicated that the mutated fragment had replaced the 3.5 kb double selectable markers in the $\beta_2$tub coding region after double-crossover homologous integration.

For further confirmation that the β_2-tubulin of *G. zeae* was transmitted to the mRNA of these transformants, the total RNA of these transformants was isolated and reversed to cDNA, which was used as the template for RT-PCR. The results showed that the mutated fragment was inserted into the genomes and was transmitted to transformants as expected (Fig. 2E).

Southern blot analysis of *Hin*d III-digested genomic DNA using Probe1 yielded a single 3.4 kb band in strain 2021 instead of a single 6.0 kb band in the β_2-tubulin deletion mutants (Fig. 3A). Southern blot analysis of *Sal* I-digested genomic DNA using Probe2 yielded a single 6.4 kb band in the β_2-tubulin deletion mutants (Fig. 3B). Southern blot analysis of *Hin*d III-digested genomic DNA using Probe1 yielded a single 6.0 kb band in the β_2-tubulin deletion mutants and a single 3.4 kb band in strain 2021 or site-directed mutants (Fig. 3C). Southern blot analysis of *Sal* I-digested genomic DNA using Probe3 yielded a single 6.1 kb band in strain 2021 or site-directed mutants (Fig. 3D).

Sequence analysis of transformants confirmed that codon 50 (TAC) for tyrosine was altered to a codon for cysteine (TGC) in Y50C, that codon 167 (TTT) for phenylalanine was altered to a codon for tyrosine (TAT) in F167Y and that codon 200 (TTC) for phenylalanine was altered to a codon for tyrosine (TAC) in F200Y.

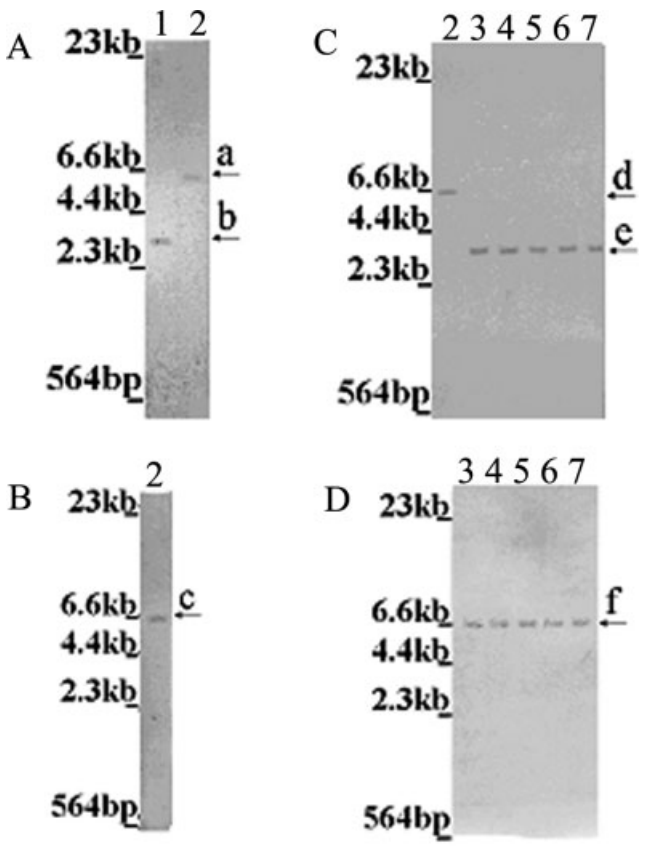

Figure 3. Southern blot analysis of site-directed mutants and parent strains of *Gibberella. zeae*. (A) Probe1 hybridised to *Hin*d III-digested genomic DNA of β_2-tubulin deletion mutants. (B) Probe2 hybridised to *Sal* I-digested genomic DNA of β_2-tubulin deletion mutants. (C) Probe1 hybridised to *Hin*d III-digested genomic DNA of site-directed mutants. (D) Probe3 hybridised to *Sal* I-digested genomic DNA of site-directed mutants. 1, 2021; 2, DN83; 3, Y50C; 4, F167Y; 5, E198Q; 6, E198K; 7, F200Y. a, 3.4 kb; b, 6.0 kb; c, 6.4 kb; d, 6.0 kb; e, 3.4 kb; f, 6.1 kb.

In addition, codon 198 (GAG) for glutamic acid was replaced by codons (CAG) and (AAG) for glutamine and lysine in E198Q and E198K respectively.

3.2 Biological characteristics of strains

3.2.1 Sensitivity to benzimidazole fungicides

Sensitivity to benzimidazoles (carbendazim, benomyl, thiabendazole and thiophanate-methyl) was tested for all mutants and parent strains. EC_{50} values were higher for mutants than for strains 2021 or DN83. The EC_{50} values of mutants F167Y and F200Y against carbendazim were $>$ 10 μg mL^{-1} and were identical to those of field strains 2052 and NT-7 respectively. Mutants F167Y and F200Y can grow on PSA amended with 25 μg mL^{-1} carbendazim and were inhibited completely at 100 μg mL^{-1}; they were classified as moderately resistant strains. The EC_{50} values of mutants Y50C and E198Q against carbendazim were about 3–4 μg mL^{-1}; these mutants, which can grow on PSA amended with 1.4 μg mL^{-1} carbendazim but cannot grow on PSA amended with 25 μg mL^{-1} carbendazim, were classified as low-resistance strains. It is interesting that, although the EC_{50} value of E198K (3.59 μg mL^{-1}) was not higher than that of Y50C and E198Q, E198K exhibited high resistance to carbendazim because it could grow on PSA amended with 100 μg mL^{-1} carbendazim (Table 3, Fig. 4). Similar results were obtained with the other benzimidazoles.

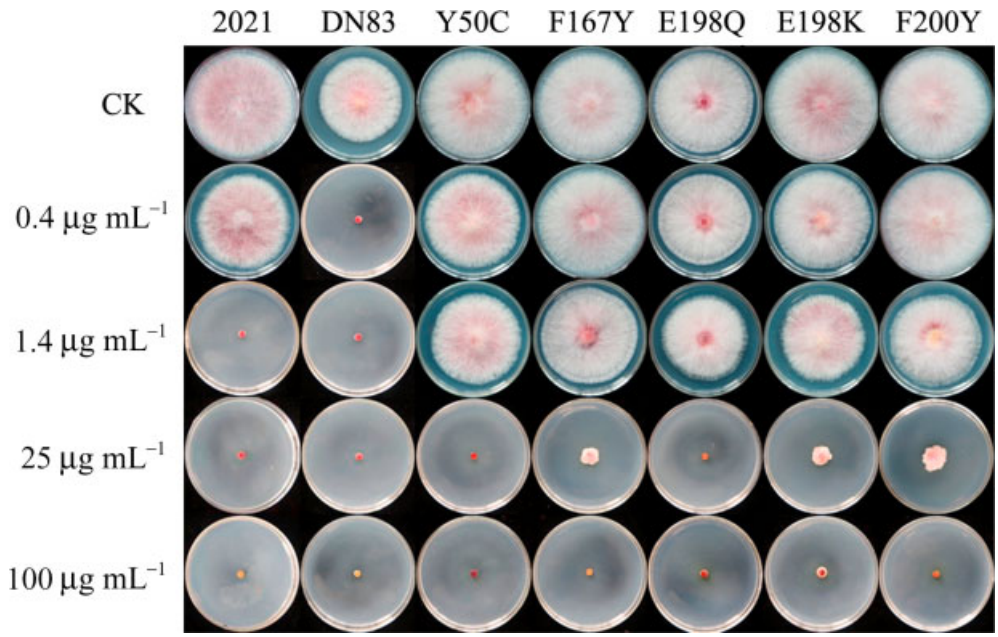

Figure 4. Effects of carbendazim on mycelial linear growth of site-directed mutants and parent strains of *Gibberella zeae*. All strains were grown for 3 days on PSA media amended with 0 (CK), 0.4, 1.4, 25 or 100 μg mL^{-1} carbendazim.

Table 3. Sensitivity of *Gibberella zeae* to four benzimidazoles

	EC_{50}[a] (μg mL^{-1})			
Strain	Carbendazim	Benomyl	Thiabendazole	Thiophanate-methyl
2021	0.47	0.61	1.13	11.02
DN83	0.10	ND[b]	ND	ND
Y50C	3.24	3.02	3.74	57.67
2052	12.31	17.68	10.75	113.94
F167Y	10.85	14.99	14.63	186.28
NT-7	10.92	8.83	6.76	181.42
F200Y	10.93	6.05	5.85	149.21
J2	3.59	5.81	3.86	38.17
E198K	3.52	4.46	3.56	33.47
ZJ80	3.52	3.19	4.52	42.92
E198Q	3.33	3.93	4.52	41.32

[a] EC_{50} indicates the fungicide concentration resulting in 50% inhibition of mycelial growth.
[b] ND: not determined.

3.2.2 Mycelial growth, pathogenicity, sporulation and production of perithecia

The wild-type strain 2021 and site-directed mutants did not differ in linear mycelial growth, pathogenicity, sporulation or production of perithecia. These biological properties did differ, however, between the $\beta_2$tub deletion mutant DN83 and complemented strains, which had the wild-type/mutant $\beta_2$tub allele (Table 4).

3.3 Homology modelling of *Gibberella zeae* β_2-tubulin

A three-dimensional model of the *G. zeae* $\beta_2$tub was constructed, and the ribbon diagram is shown in Fig. 5A. The modelled tertiary protein structure of the β_2-tubulin should provide a basis for understanding a significant body of research on tubulin's properties and interactions with antimicrotubule agents. The core of the structure contains two β-sheets of four and five strands, surrounded by 15 α-helices. In contrast, the three-dimensional model of *Trichoderma harzianum* Rifai β-tubulin contains ten β-strands and 14 α-helices.[24] Most β-tubulin mutations causing benzimidazole resistance in more than 16 fungi have been reported to cluster within a small volume of the molecular structure: residues 6, 50, 134, 165, 167, 198, 200 and 257 form a cluster within a compact region of the folded protein. The amino acids mutated in this study were marked in the β-tubulin model. It is interesting that mutations at amino acids 167, 198 and 200 are within the SDETFC motif of $\beta_2$tub in *G. zeae*, which is similar to the 'benzimidazole-resistant box' motif from other filamentous fungi.[25] Based on the distribution of these residues, it is clear that the fungicide-binding sites identified so far are located on the inside of the microtubule, although the sites of most reported interactions with other proteins occur on the outside. Although the position of amino acids that alter benzimidazole resistance varied among different fungi, and although the same residue with different substitutions may correlate with different resistance levels, the relative importance of the residues in these motifs in the *G. zeae* $\beta_2$tub was confirmed by site-directed mutagenesis here.

4 DISCUSSION

Classical genetics and nucleotide sequence analysis provided circumstantial evidence that mutations in key codons of the β_2-tubulin gene were responsible for resistance to carbendazim in *G. zeae*.[6,15] For codons 167, 198 and 200, site-directed mutagenesis has supported the hypothesis that these point mutations confer resistance to benzimidazoles in several plant pathogenic fungi other than *G. zeae*.[26–28] Here, based on site-directed mutagenesis and transformation experiments, direct evidence is provided that mutations at codons 50, 167, 198 and 200 of the β_2-tubulin gene are responsible for resistance to carbendazim in *G. zeae*.

The three-dimensional model of the β_2-tubulin demonstrates the possible binding site for carbendazim on the β_2-tubulin in

Table 4. Biological properties of the strains in this study

Strain	Mycelial growth[a] (mm)	Sporulation capacity[a] (10^6 CFU mL^{-1})	Perithecigerous capacity[b]	Pathogenicity[a]
2021	76.2 (±1.6) aA	3.37 (±0.31) aA	+++	66.5 (±10.14) aA
DN83	47.3 (±0.8) dC	0.82 (±0.18) dD	++	8.9 (±3.2) eD
Y50C	71.8 (±1.3) bcB	3.21 (±0.27) aAB	+++	63.2 (±11.22) bcBC
F167Y	70.7 (±0.6) cB	3.08 (±0.37) cC	+++	61.5 (±13.61) cdC
E198Q	71.0 (±0.9) bcB	3.32 (±0.13) aAB	+++	62.7 (±12.95) bcdBC
E198K	72.6 (±5.0) bB	3.14 (±0.33) bcBC	+++	61.1 (±15.18) dC
F200Y	70.5 (±1.7) cB	3.28 (±0.39) abABC	+++	64.2 (±11.42) bAB

[a] Values are the mean of three replicates (± standard error of the mean). Means followed by the same letter in each column are not significantly different between strains by the least significant difference (LSD) test at $P = 0.05$ and 0.01 respectively.
[b] +++ and ++ indicate that perithecia covered more than 2/3, between 1/3 and 2/3 and less than 1/3 of the seed surface respectively.

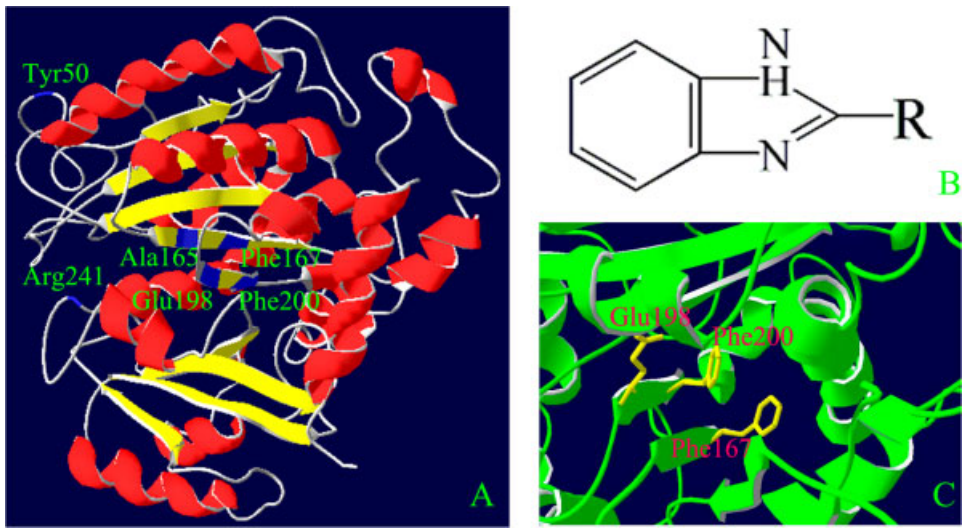

Figure 5. Ribbon models of the *Gibberella zeae* $\beta_2$tub. Model structure of β_2-tubulin with helices and strands labelled. (A) Helices are displayed in red and strands in yellow; amino acid Tyr50, Ala165, Phe167, Glu198, Phe200 and Arg241 of the β_2-tubulin are shown in blue. Amino acids at these sites have been identified as benzimidazole antimicrotubule agent binding targets in certain fungi. (B) The general chemical structure of benzimidazoles. (C) Amino acids 167, 198 and 200 are within a pocket domain that accommodates the fungicide of $\beta_2$tub; this pocket domain is similar to the 'benzimidazole-resistant box' motif from other filamentous fungi.

G. zeae. Phe167, Glu198 and Phe200 form a pocket domain that accommodates the fungicide and is a binding centre between carbendazim and β_2-tubulin. From these modelling results it is concluded that the amino acid side chains of mutation codons could play an important role in the interaction between benzimidazole and β-tubulin. The phenyl ring of Phe167 and Phe200 may interact with the phenyl ring of the benzimidazole,[29] and Glu198 might act as the anchor point when the fungicide enters the molecular target. Mutations in codons 167 and 200 involving change of amino acid from Phe to Tyr could change the binding site properties from hydrophobic to hydrophilic and decrease the binding of benzimidazole to the phenyl ring. When Glu198 was changed to Lys, Leu, Gln, or others, the anchor point would become so loose that it destabilised the binding site so that the benzimidazole molecule readily detached from the target. More will be known about the nature of the binding and the influence of codon mutation on the binding when the structure of the binding site is resolved.

In the present study, site-directed mutagenesis at codon 50 (Tyr to Cys) produced a low level of carbendazim resistance, while site-directed mutagenesis at codons 165 and 241 (from Ala to Val and from Arg to His respectively) did not produce carbendazim resistance (unpublished data). At first consideration, this finding seems inconsistent with the modelled structure shown in Fig. 5 because in that model Tyr50 is located further from the binding centre than Ala165 and Arg241. Why would a mutation at a site relatively distant from the binding centre cause resistance? This might be explained by the three-dimensional structure of β_2-tubulin and the R group on the benzimidazole fungicide. In the case of different fungi with resistance to the same benzimidazole, the mutated codons may have different effects on β-tubulin structure because the three-dimensional structure of β-tubulin differs among the fungi and affects how the fungicide binds to the β-tubulin. In the case of one species of fungus treated with different benzimidazoles, the codons for resistance to these fungicides would also be different because the benzimidazoles have different R groups that interact with different amino acid side chains on the β-tubulin. In the present study, codon 50 may interact with the R group of carbendazim and participate in the binding process while codons 165 and 241 do not. This would explain why Tyr50Cys can cause carbendazim resistance in *G. zeae* while Ala165Val and Arg241His do not alter carbendazim resistance.

Another possible reason is the reduction in binding force between fungicide molecule and target when one amino acid mutation at the binding site of the fungicide was mutated. The binding force includes covalent and non-covalent bonds, and the latter are more important. Non-covalent bonds mostly include charge interaction, hydrophobic accumulation and space location, and they play important roles in the interaction between fungicide molecule and target. Once one fungicide molecule enters the internal compartment of the target and interacts with it, the fungicide molecule brings its pharmacological effects into play. Mutations occurring at the binding site, such as Phe167Tyr, Phe200Tyr and Glu198Leu, lead to a reduction in the binding force as the binding environment changes from hydrophobicity to hydrophilicity, resulting in resistance. However, the cause of Glu198Lys mutation resistance may be the change in charge.

Although site-directed mutagenesis in the present study proved that the different codon mutations can cause carbendazim resistance in *G. zeae*, the affinity between the β_2-tubulin of different mutants to carbendazim is still not known. For *G. zeae*, the β_2-tubulin genes from the carbendazim-sensitive strain and the resistant strains Phe167Tyr and Glu198Lys have been cloned into the expression vector pET32a$^+$ and have been expressed in *E. coli* and purified.[30] Although the recombinant β_2-tubulin formed an inclusion body when expressed, the soluble protein dissolved in the buffer after dialysis and refolding. Characterisation of carbendazim binding with recombinant β_2-tubulin will be studied in future work.

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