F240 of β_2 -Tubulin Explains why *Fusarium graminearum* is Less Sensitive to Carbendazim than *Botrytis cinerea*

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ABSTRACT

β -Tubulin is the target of benzimidazole fungicides, the most widely used of which is carbendazim (methyl benzimidazol-2-ylcarbamate [MBC]). MBC sensitivity is determined by the differential affinity of MBC for β -tubulins. However, the mechanism of less sensitivity of *Fusarium graminearum* to MBC compared with other fungi, including *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Sclerotinia sclerotiorum*, remains exclusive. Alignment of β -tubulin amino acid sequences showed that position 240 of β -tubulins is leucine (L) in most pathogenic fungi but is phenylalanine (F) in the $Fg\beta_2$ -tubulin of the *F. graminearum* wild type. The effective concentration resulting in 50% inhibition (EC_{50}) value of MBC against the $Fg\beta_2$ F240L mutant of *F. graminearum* is 0.047 μ g/ml, which was 10-fold lower than that of wild-

type strain 2021. Moreover, The EC_{50} value of MBC against the $Bc\beta L$ “240”F (actually position 232) mutant of *Botrytis cinerea* was 0.44 μ g/ml, which was ninefold higher than that of *B. cinerea* wild-type strain Bt4-1. In response to MBC treatment (0.15 μ g/ml), microtubules were clearly visible in $Fg\beta_2$ -enhanced green fluorescent protein (EGFP) but not in $Fg\beta_2$ F240L-EGFP. Moreover, a molecular docking assay indicated that F240L mutation created a pi-pi interaction between $Fg\beta_2$ -tubulin and MBC and increased the binding affinity of $Fg\beta_2$ -tubulin to MBC. Our results suggest that F240 is responsible for the naturally less MBC sensitivity in *F. graminearum* compared with *B. cinerea*, *C. gloeosporioides*, and *S. sclerotiorum* by decreasing the binding affinity between $Fg\beta_2$ -tubulin and MBC.

The fungus *Fusarium graminearum* (sexual state *Gibberella zeae*; phylum Ascomycota) causes wheat Fusarium head blight (FHB), resulting in yield loss and contamination of wheat products with the mycotoxin deoxynivalenol (DON) (Atanassov et al. 1994; Bai and Shaner 2004). Over the last 40 years in China, control of FHB has mainly depended on the benzimidazole fungicide carbendazim (methyl benzimidazol-2-ylcarbamate [MBC]). The molecular target of MBC has been determined as β -tubulin (Davidse 1986; Davidse and Flach 1977).

Tubulins include α -, β -, γ -, δ -, ϵ -, and η -tubulin families (Ludueña and Banerjee 2008; McKean et al. 2001). The α - and β -tubulins are major components of microtubules, which are important for many intracellular activities, including cell division, material transport, and cell morphology (Huffaker et al. 1988; Niwa et al. 2013). Microtubules are assembled in a head-to-tail manner by α/β -heterodimers (Oakley 2000). In addition, microtubules can rapidly switch between growing and shrinking states, a behavior which is termed dynamic instability (Desai and Mitchison 1997). Microtubule dynamics and structural stability play a critical role in many cellular events, especially in cell division, which involves changes in microtubule architecture. Consequently, tubulins and microtubules have become important targets of anticancer drugs and pesticides (Davidse 1986; Giannakakou et al. 1999; Jordan and Wilson 1998). Some of these compounds, such as paclitaxel, stabilize the microtubule (Hari et al. 2006), whereas others, such as vinblastine and benzimidazole fungicides destabilize the microtubule (Davidse 1986; Hari et al. 2003). Mutations found in some kinds of β -tubulin isotypes from cancer cell lines and plant-pathogenic fungi affect the

sensitivity to these chemicals (Chen et al. 2007; Kanakkanthara et al. 2011, 2015).

In many plant-pathogenic fungi, benzimidazole fungicides inhibit microtubule assembly by binding to β -tubulin of the α/β -heterodimer (Davidse 1986). Mutations in the β -tubulin confer resistance to benzimidazole fungicides in various pathogenic fungi (Cai et al. 2015; Davidse 1986). Some of the pathogenic fungi, however, contain two divergent β -tubulins such as *benA* and *tubC* of *Aspergillus nidulans*, *Tub1* and *Tub2* of *Colletotrichum gloeosporioides*, and $Fg\beta_1$ and $Fg\beta_2$ of *F. graminearum* (Zhao et al. 2014). Mutations leading to benzimidazole fungicide resistance were found only in *benA*, *Tub2*, and $Fg\beta_2$ which indicated that these β -tubulin isotypes regulated the sensitivity to benzimidazole fungicides (Buhr and Dickman 1994; Chen et al. 2009; Jung and Oakley 1990; Peres et al. 2004).

In the β -tubulin isotypes involved in benzimidazole resistance, the most frequent mutations were at position 6, 50, 134, 165, 167, 198, 200, 240, 241, or 257 (Albertini et al. 1999; Buhr and Dickman 1994; Chen et al. 2009; Jung and Oakley 1990; Jung et al. 1992; Ma et al. 2005; Thomas et al. 1985; Yan and Dickman 1996; Yarden and Katan 1993), which indicated that mutations at these sites could affect the interaction between β -tubulin and benzimidazole fungicides.

According to previous studies, the L240F mutation in β -tubulin confers resistance to benzimidazole fungicides in field strains of *Monilinia laxa* and *Tapesia yallundae* (Albertini et al. 1999; Ma et al. 2005). According to another study, the sensitivity to MBC was much lower in wild-type strain 2021 of *F. graminearum* than wild types of *B. cinerea*, *C. gloeosporioides*, and *Sclerotinia sclerotiorum* (Li et al. 2002) (Supplementary Table S1). Why *F. graminearum* is naturally less sensitive to MBC than the other fungi, however, has not been determined. In this study, we identified approximately 100 amino acids in $Fg\beta_2$ -tubulin of *F. graminearum* that are different from the benzimidazole-relevant β -tubulin isotypes of other pathogenic fungi, including *A. nidulans*, *Botrytis cinerea*, *C. gloeosporioides*, *F. moliniforme*, *G. pulicaris*, *Neurospora crassa*, *Rhynchosporium secalis*, *S. sclerotiorum*, and *Venturia inaequalis*. Consistent with the earlier reports for *M. laxa* and *T. yallundae*, we then determined that F240 is responsible for the naturally less MBC sensitivity in *F. graminearum* compared with *B. cinerea*, *C. gloeosporioides*, and

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S. sclerotiorum. Position 240 in *Fg* β ₂-tubulin is phenylalanine (F) but is leucine (L) in the β -tubulin isotypes of other pathogenic fungi. We then used site-directed mutagenesis, enhanced green fluorescent protein (EGFP) tagging, and molecular docking studies to determine the mechanism of this sensitivity of *F. graminearum* to MBC.

MATERIALS AND METHODS

Strains, culture conditions, and fungicides. The *F. graminearum* and *B. cinerea* strains and other pathogenic fungi used in this study are listed in Table 1. The wild-type strains 2021 and Bt4-1 were used as reference strains. For transformation of *F. graminearum*, we used the *Fg* β ₁- and *Fg* β ₂-tubulin deletion mutant strains 2 $\Delta\beta$ ₁ and 2 $\Delta\beta$ ₂, containing the hygromycin resistance gene (*hph*) connected with herpes simplex virus thymidine kinase gene (*hph*+*hsv-tk*). *Fg* β ₁L240F-1 and -2 were generated from strain 2 $\Delta\beta$ ₁, *Fg* β ₂F240L-7 and -8 were generated from strain 2 $\Delta\beta$ ₂, and *Bc* β -*hph* and *Bc* β L232F mutants were generated from Bt4-1. All of the strains and mutants were stored at 4°C in test tubes containing potato dextrose agar (PDA) (200 g of potato, 20 g of glucose, 15 g of agar, and distilled water to 1 liter) for short-term preservation. For long-term preservation, the strains and mutants were stored at -70°C in 2-ml centrifuge tubes containing PDA. For mycelial growth, all strains were grown at 25°C on liquid yeast extract-peptone-dextrose (YEPD) (0.3% yeast extract, 1% peptone, and 2% dextrose [wt/vol]) medium or PDA. To induce production of conidia, all *F. graminearum* strains were grown on PDA plates for 3 days and then transferred into mung bean liquid (MBL) medium (30 g of mung bean boiled in 1 liter of water) for 2 to 5 days. Benomyl, MBC, and thiabendazole were dissolved in 0.1 M HCl at 10 mg/ml as stock solutions (Chen et al. 2009).

Multiple alignment of β -tubulins. Alignment of amino acid sequences of β -tubulin of *F. graminearum* (β ₁, GenBank ID: 23556480; β ₂, GenBank ID: 23553735), *A. nidulans* (benA, GenBank ID: AN6838.2), *B. cinerea* (tubA, GenBank ID: 5441652; in tubA of Bt4-1, position 190 is glutamic acid), *C. gloeosporioides* (tub2, GenBank ID: AAA62875), *F. moliniforme* (tub2, GenBank ID: AAB18275), *G. pulicaris* (tub2, GenBank ID: AAN03787), *N. crassa* (tub2, GenBank ID: AAA33617), *R. secalis* (β -tubulin, GenBank ID: CAA56936), *S. sclerotiorum* (β -tubulin, GenBank ID: 5490658), and *V. inaequalis* (β -tubulin, GenBank ID: AAA34230) was performed using ClustalW multiple alignment function from BioEdit Sequence Alignment Editor software (version 7.0.5).

Vectors construction, protoplast preparation, and transformation. To investigate the effect of mutations at position 240 of *Fg* β ₁- and *Fg* β ₂-tubulin of *F. graminearum*, we constructed the gene replacement cassettes with point mutations (Li et al. 1996). Schematic representations of construction vectors are described in Supplementary Figures S1 and S2). For construction of *Fg* β ₁L240F vector, a 2.6-kb left fragment (left side of position 240 of *Fg* β ₁-tubulin) and a 1.6-kb right fragment (right side of position 240 of *Fg* β ₁-tubulin) were amplified from strain 2021 by primers A1/A2 and A3/A4 (A2/A3 containing the L240F mutation), respectively. For construction of *Fg* β ₂F240L vector, a 2.1-kb left fragment (left side of position 240 of *Fg* β ₂-tubulin) and a 1.8-kb right fragment (right side of position 240 of *Fg* β ₂-tubulin) were amplified from strain 2021 by primers B1/B2 and B3/B4 (B2/B3 containing F240L mutation), respectively. The left and right fragments were mixed at a 1:1 molar ratio and used as templates for double-joint polymerase chain reaction (PCR) to generate a 4.2-kb fragment (*Fg* β ₁L240F vector) and a 3.9-kb fragment (*Fg* β ₂F240L vector) (Yu et al. 2004), which were used to transform protoplasts of strains 2 $\Delta\beta$ ₁ and 2 $\Delta\beta$ ₂, respectively.

To determine whether phenylalanine in position “240” (actually position 232) of *Bc* β -tubulin could decrease the sensitivity of *B. cinerea* to MBC, we used site-directed mutagenesis to construct a *Bc* β L232F mutant from *B. cinerea* wild-type strain Bt4-1 (Supplementary Fig. S3). In brief, the wild-type and L232F mutant *Bc* β -tubulin sequences connected to the *hph* were introduced individually into wild-type

sensitive strain Bt4-1 to generate *Bc* β -*hph* (wild-type strain Bt4-1 with *hph* inserted at the 3' end of the *Bc* β -tubulin locus) and *Bc* β L232F mutants. First, a 1.1-kb left fragment (left side of position 232 of *Bc* β -tubulin) and a 0.7-kb right fragment (right side of position 232 of *Bc* β -tubulin) were amplified from strain Bt4-1 by primers C1/C2 and C3/C4 (C2/C3 containing L232F mutation), respectively. The left and right fragments were mixed at a 1:1 molar ratio and used as templates for double-joint PCR to generate a 1.7-kb L232F mutant *Bc* β -tubulin. Second, a 1.7-kb fragment of *hph* with *A. nidulans* *trpC* promoter (*trpC*-*hph*) was amplified from plasmid pKHt (Mullins et al. 2001) by primers D1/D2. Third, a 1.5-kb *Bc* β -tubulin downstream fragment was amplified from Bt4-1 by primers C5/C6. The three fragments (L232F mutant *Bc* β -tubulin sequence, *trpC*-*hph*, and downstream of *Bc* β -tubulin) were mixed in a molar ratio of 1:3:1 and used as a template for fusion reaction. Finally, the 5.0-kb L232F vector was amplified from fusion product by primers C1/C6. PCR were performed in PCR Thermal Cycler (TaKaRa) with the following parameters: 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 55 to 58°C for 30 s, and 72°C for 1 to 5 min (according to the length of the fragment); and a final extension at 72°C for 10 min. All PCR products were purified by a gel extraction kit (Omega Bio-Tek). *F. graminearum* and *B. cinerea* protoplasts were prepared and transformed as previously described (Gronover et al. 2001; Zheng et al. 2014).

Benzimidazole sensitivity assays. Radial growth was used to determine the sensitivity of strains in Table 1 to benzimidazole, as previously described (Zheng et al. 2014). In brief, a 5-mm mycelial plug was transferred from the margin of a 3-day-old colony to the center of a PDA plate amended with benomyl, MBC, or thiabendazole (rates are listed in the next paragraph) and kept at 25°C. Then, colony diameters were measured after 2 days for the *S. sclerotiorum* strain, 3 days for the *F. graminearum* strains, 3.5 days for the *B. cinerea* strains, and 5 days for the *C. gloeosporioides* strain. The dose response curve for each strain was generated by regressing the probability of the

TABLE 1. Strains and mutants of *Fusarium graminearum*, *Botrytis cinerea*, and other pathogenic fungi used in this study

Strains and mutants	Genotype ^a	Reference
2021	<i>F. graminearum</i> wild-type sensitive to MBC	Chen et al. 2009
2 $\Delta\beta$ ₁	<i>Fg</i> β ₁ <i>tub</i> deletion mutant in 2021 genetic background	Qiu et al. 2012
2 $\Delta\beta$ ₂	<i>Fg</i> β ₂ <i>tub</i> deletion mutant in 2021 genetic background	Qiu et al. 2012
2 $\Delta\beta$ ₁ -C	<i>Fg</i> β ₁ <i>tub</i> complementary mutant	This study
2 $\Delta\beta$ ₂ -C	<i>Fg</i> β ₂ <i>tub</i> complementary mutant	This study
<i>Fg</i> β ₁ L240F-1	<i>Fg</i> β ₁ <i>tub</i> L240F mutant generated from 2 $\Delta\beta$ ₁	This study
<i>Fg</i> β ₁ L240F-2	<i>Fg</i> β ₁ <i>tub</i> L240F mutant generated from 2 $\Delta\beta$ ₁	This study
<i>Fg</i> β ₂ F240L-7	<i>Fg</i> β ₂ <i>tub</i> L240F mutant generated from 2 $\Delta\beta$ ₂	This study
<i>Fg</i> β ₂ F240L-8	<i>Fg</i> β ₂ <i>tub</i> L240F mutant generated from 2 $\Delta\beta$ ₂	This study
<i>Fg</i> β ₂ -EGFP	<i>Fg</i> β ₂ <i>tub</i> -EGFP mutant generated from 2 $\Delta\beta$ ₂	This study
<i>Fg</i> β ₂ F240L-EGFP	<i>Fg</i> β ₂ <i>tub</i> F240L-EGFP mutant generated from 2 $\Delta\beta$ ₂	This study
Bt4-1	<i>B. cinerea</i> wild-type sensitive to MBC	This study
<i>Bc</i> β - <i>hph</i>	<i>B. cinerea</i> wild-type with hygromycin resistance gene at 3' end of β -tubulin gene	This study
<i>Bc</i> β L232F-1	<i>Bc</i> β <i>tub</i> L232F mutant generated from Bt4-1	This study
<i>Bc</i> β L232F-2	<i>Bc</i> β <i>tub</i> L232F mutant generated from Bt4-1	This study
CG-21	<i>Colletotrichum gloeosporioides</i> wild-type sensitive to MBC	This study
HA61S	<i>Sclerotinia sclerotiorum</i> wild-type sensitive to MBC	This study

^a MBC = methyl benzimidazol-2-ylcarbamate.

percentage of inhibition of average radial growth (colony diameters – 5 mm) on the log₁₀ of benzimidazole concentration, as previously described (Zheng et al. 2014). The effective concentration resulting in 50% inhibition (EC₅₀) was obtained from the dose response curve. This experiment was repeated three times.

Benomyl was added to PDA plates at 0, 0.04, 0.06, 0.08, 0.10, and 0.12 µg/ml or at 0, 0.4, 0.6, 0.8, 1.0, and 1.2 µg/ml. MBC was added to PDA plates at 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, and 0.14 µg/ml; at 0, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.48 µg/ml; at 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 µg/ml; or at 0, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 µg/ml. Thiabendazole was added to PDA plates at 0, 0.08, 0.16, 0.24, 0.32, 0.4, and 0.48 µg/ml or at 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 µg/ml.

Quantitative real-time PCR. According to studies in cancer cells, altered expression levels of β-tubulin can confer resistance to microtubule binding agents (Kanakkanthara et al. 2011; Kavallaris et al. 1997; Ranganathan et al. 1998). Therefore, to exclude the possibility that variation of sensitivity of *Fgβ2F240L* and *BcβL232F* mutants to MBC is caused by an altered expression level of β-tubulin, we measured the relative expression levels of *Fgβ2*-tubulin and *Bcβ*-tubulin. Five 5-mm mycelial plugs were transferred from the margin of a 3-day-old colony of *F. graminearum* or *B. cinerea* strains on a PDA plate to 100 ml of YEPD liquid medium. The cultures were shaken at 175 rpm at 25°C for 36 h for

F. graminearum and 48 h for *B. cinerea* (Shao et al. 2016; Zheng et al. 2014). The mycelia were then collected for total RNA extraction by RNA sample kits (Tiangen). First-stand cDNA was synthesized with the Prime Script RT reagent kit (TaKaRa). Quantitative real-time (qRT)-PCR were performed with a CFX Connect Real-Time System (Bio-Rad) with the following parameters: 95°C for 30 s, 40 cycles of 95°C for 10 s and 60°C for 30 s, a melt curve step of 95°C for 15 s and 60°C for 60 s, followed by 71 cycles of gradual increase in temperature from 60 to 95°C for 13

TABLE 2. Hyphal growth rate and sensitivity of *Fusarium graminearum* strains and mutants to methyl benzimidazol-2-ylcarbamate (MBC)^x

Strain	EC ₅₀ (µg/ml) ^y	Growth rate (cm/day) ^z
2021	0.53 ± 0.05 a	2.45 ± 0.07 a
2Δβ ₁ -C	0.49 ± 0.03 a	2.16 ± 0.13 b
2Δβ ₂ -C	0.48 ± 0.08 a	2.21 ± 0.07 b
<i>Fgβ₁L240F-1</i>	0.50 ± 0.02 a	2.22 ± 0.11 b
<i>Fgβ₂F240L-8</i>	0.047 ± 0.006 b	2.22 ± 0.15 b

^x Values are means and standard deviations. Means in a column followed by the same letter are not significantly different (*P* > 0.05) according to Fisher's least significant difference test.

^y MBC concentration that resulted in 50% mycelial growth inhibition.

^z Growth rate was measured after incubation of three replicates for 3 days.

Fig. 1. Alignment of amino acid sequences of β-tubulin. Rectangles indicate positions related to benzimidazole resistance.

s/cycle. The primers used for qRT-PCR analysis are listed in Supplementary Table S2. The expression of *Fgβ₂-tubulin* and *Bcβ₂-tubulin* in each sample was normalized to expression of *Fgβ₂-actin* and *Bcβ₂-actin* genes.

Fig. 2. Effects of methyl benzimidazol-2-ylcarbamate (MBC) on mycelial linear growth on potato dextrose agar (PDA) of *Fusarium graminearum* strain 2021 and $\Delta\beta_1$ -C, $\Delta\beta_2$ -C, *Fgβ₁L240F-1*, *Fgβ₁L240F-2*, *Fgβ₂F240L-7*, and *Fgβ₂F240L-8* mutants. Mycelial plugs were transferred from the margins of 3-day-old PDA colonies to fresh PDA plants containing MBC at 0 (CK) to 1.4 $\mu\text{g/ml}$. Plates were photographed after 3 days at 25°C.

Test for conidial, hyphal morphology, and sporulation. To compare the hyphal morphology of the *Fgβ₂F240L* mutant with that of the wild-type strain 2021, a 5-mm mycelial plug was transferred from the margin of a 3-day-old colony on a PDA plate onto a piece of glassine on a fresh PDA plate. After 24 h at 25°C, the glassine margin was cut, and hyphae on the glassine were photographed. Conidia that were freshly produced in MBL were also photographed. Both hyphae and conidia were examined using an Olympus IX-71 inverted microscope. Conidial production in MBL was assessed as previously described using a hemacytometer and microscope (Zheng et al. 2014). Each assay was repeated three times.

Pathogenicity assays. To assess the virulence of the *Fgβ₂F240L* mutant, we carried out infection assays using coleoptiles, as previously described (Zheng et al. 2014). Briefly, Yunong 035 wheat seed were planted in 24-well plates, one seed per well. The 24-well plates were placed in a plastic case containing filter paper to retain moisture. At 3 days after sowing, the top 2 to 3 mm of the coleoptiles were cut out, and 2 μl ($1 \times 10^6/\text{ml}$) of fresh spore suspension was pipetted onto the wound seedlings. Sterilized water was used as controls. After inoculation at 25°C in a growth chamber (12 h/day and 12 h/night) for 7 days, the lengths of disease lesions were measured. At least 20 wheat seedlings for each strain were measured. This experiment was repeated three times.

To evaluate the virulence of *B. cinerea* mutants, we carried out pathogenicity assays on apple and tomato, as previously described (Ren et al. 2017). Briefly, mycelial plugs taken from the periphery

Fig. 3. **A**, Expression levels of *Fgβ₂-tubulin* in 2 $\Delta\beta_2$ -C, *Fgβ₂F240L-8*, *Fgβ₂-enhanced green fluorescent protein (EGFP)*, and *Fgβ₂F240L-EGFP* relative to that of 2021. **B**, Expression levels of *Bcβ₂-tubulin* in *Bcβ-hph*, *BcβL232F-1* and *BcβL232F-2* relative to that of Bt4-1. Values are the means and standard deviation of three repeated experiments. Means of the column with the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

of a 3-day-old colony were used to inoculate the fruit, and agar plugs without fungal mycelia were used as negative controls. Fruit were wounded with a sterilized needle to facilitate the penetration of the fungus into fruit tissue before inoculation. Fruit were incubated in high relative humidity (approximately 95%) at 25°C with 16 h of daylight. The diameters of disease lesions were measured after 3 to 5 days. The experiment was repeated three times.

Statistical analysis. Data were analyzed with the Data Processing System computer program (Hangzhou Reifeng Information Technology Ltd.). The gene expression, conidiation, hyphal growth, sensitivity, and pathogenicity of wild-type strains and mutants were compared using Fisher's least significant difference test ($P > 0.05$).

Construction of the *Fgβ₂-tubulin-EGFP* fusion mutant. To increase our understanding of why the F240L mutation in *Fgβ₂-tubulin* increases the sensitivity of *F. graminearum* to MBC, we have generated *Fgβ₂-tubulin-EGFP* fusion mutants (*Fgβ₂-EGFP* and *Fgβ₂F240L-EGFP*) to visualize cellular microtubules (Jaglin et al. 2009; Ti et al. 2016; Tischfield et al. 2010). The *Fgβ₂-tubulin* genes amplified by primers E1/E2 from the *Fgβ₂F240L* mutant and 2021 were cloned into pYF11 plasmid by the yeast gap repair approach (Bruno et al. 2004). The recombinant plasmid was verified by sequencing and then transformed into protoplasts of

Fgβ₂-tubulin deletion mutant 2Δβ₂. Transformants were first selected by PDA plates containing G418 (geneticin) at 100 μg/ml and were then verified by fluorescence under a long-pass EGFP filter set (488 nm excitation and 492 to 550 nm emission) and PCR (primers E3/E4 were used for identification of *Fgβ₂-tubulin* in *Fgβ₂-tubulin-EGFP* mutants) with the following parameters: 95°C for 5 min; follow by 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Confocal microscopy of microtubules. To visualize tubulin-EGFP in living hyphae, strains containing EGFP were placed in MBL medium to induce sporulation. Fresh spores (200 μl, adjusted to 5 × 10⁵ conidia/ml) were germinated into 20 ml of YEPD medium to germinate for 10 h and then incubated for a further 5 min after first adding MBC (final concentration 0.15 or 0.3 μg/ml). Images of living hyphae were obtained at room temperature by a LEICA TCS SP8 confocal laser-scanning microscope (LEICA). Culture (10 μl at 5 × 10³ conidia/ml) was spotted directly onto slides and using a ×100 oil objective to visualize the microtubules in the hyphae of germinated spores. This experiment was repeated three times.

Molecular docking assay. We used a bioinformatics analysis to investigate the molecular docking of MBC with *Fgβ₂-tubulin* with or without the F240L mutation. A homology model of *Fgβ₂-tubulin* with or without F240L mutation was constructed using the online server I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Roy et al. 2010; Zhang 2008). Molecular docking of MBC with *Fgβ₂-tubulin* was performed using the DS-CDocker function from Discovery Studio software (version 3.0) (Du et al. 2013; Ye et al. 2014). The three-dimensional structure of MBC was constructed by Chem. 3D ultra 12.0 software (Chemical Structure Drawing Standard; Cambridge Soft Corporation). Then, the *Fgβ₂-tubulin* model and molecule of MBC were energetically minimized by using MMFF94 with 5,000 iterations and minimum RMS gradient of 0.10. All bound ligands and waters were excluded from the model of *Fgβ₂-tubulin*, and the Kollman-united charges and the polar hydrogens were added to the proteins.

Fig. 4. Hyphal and conidial morphology and sporulation of *Fusarium graminearum* strain 2021 and 2Δβ₂, 2Δβ₂-C, and *Fgβ₂F240L-8* mutants. **A**, Hyphal morphology was photographed after strains were incubated on potato dextrose agar for 24 h; conidia were photographed immediately after they were harvested from 3-day-old mung bean liquid (MBL) medium. Bar = 20 μm. **B**, Number of conidia produced per MBL culture after 5 days at 25°C. Values are means ± standard deviation of three repeated experiments. Means of the column with the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

TABLE 3. Hyphal growth rates and sensitivities of *Botrytis cinerea* strains and mutants to methyl benzimidazol-2-ylcarbamate (MBC)^x

Strain	EC ₅₀ (μg/ml) ^y	Growth rate (cm/day) ^z
Bt4-1	0.051 ± 0.007 b	1.86 ± 0.08 a
<i>Bcβ-hph</i>	0.052 ± 0.003 b	1.53 ± 0.15 b
<i>BcβL232F-2</i>	0.44 ± 0.05 a	1.66 ± 0.07 ab

^x Values are means and standard deviations. Means in a column followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

^y MBC concentration that resulted in 50% mycelial growth inhibition.

^z Growth rate was measured after incubation of three replicates for 4 days.

Fig. 5. Effects of methyl benzimidazol-2-ylcarbamate (MBC) on mycelial linear growth on potato dextrose agar (PDA) of *Botrytis cinerea* strain Bt4-1 and *Bcβ-hph*, *BcβL232F-1*, *BcβL232F-2* mutants. Mycelial plugs were transferred from the margins of 3-day-old PDA colonies to fresh PDA plants containing MBC at 0 (CK) to 1.4 μg/ml. Plates were photographed after 4 days at 25°C.

RESULTS

Alignment of amino acid sequences of β -tubulin isoforms.

Comparison of amino acid sequence β -tubulin isoforms related to benzimidazole resistance indicated that approximately 100 amino acids of $Fg\beta_2$ -tubulin are different from those of *F. graminearum* $Fg\beta_1$ -tubulin and from those of the β -tubulins of other pathogenic fungi (Fig. 1). Moreover, the following positions related to benzimidazole resistance of $Fg\beta_2$ -tubulin were identical with $Fg\beta_1$ -tubulin and β -tubulins of other pathogenic fungi: 6, 50, 134, 167, 198, 200, 241, and 257 (Fig. 1, rectangles). In addition, among the 100 positions that differed between $Fg\beta_2$ -tubulin and the β -tubulin of other isoforms, only the position 240 is relevant to benzimidazole resistance (Fig. 1, third row). Position 240 in $Fg\beta_2$ -tubulin is phenylalanine but is leucine in other β -tubulin isoforms. This suggests but does not prove that the phenylalanine at position 240 of $Fg\beta_2$ -tubulin makes *F. graminearum* naturally less sensitive to benzimidazole fungicides.

Construction of $Fg\beta_1$ L240F and $Fg\beta_2$ F240L mutants of *F. graminearum*. To verify that phenylalanine in position 240 of $Fg\beta_1$ - and $Fg\beta_2$ -tubulin is more beneficial than leucine against MBC for *F. graminearum*, we constructed the $Fg\beta_1$ L240F and $Fg\beta_2$ F240L mutants by site-directed mutagenesis. In brief, the $Fg\beta_1$ and $Fg\beta_2$ deletion mutants were transformed with the $Fg\beta_1$ L240F and $Fg\beta_2$ F240L cassettes. The gene replacement events were verified in the selected transformants by PCR and sequencing. Single-copy insertions at the loci of $Fg\beta_1$ -tubulin and $Fg\beta_2$ -tubulin were verified by Southern blotting. Finally, the results indicated that the mutations had occurred as designed.

Sensitivities of $Fg\beta_1$ L240F and $Fg\beta_2$ F240L mutants to MBC. The EC_{50} value of MBC against $Fg\beta_1$ L240F was equivalent to that of 2021 but was greatly reduced for $Fg\beta_2$ F240L (Table 2). The sensitivities of the $Fg\beta_1$ L240F and $Fg\beta_2$ F240L mutants to MBC were also indicated by colony morphology (Fig. 2). These results confirmed that phenylalanine at position 240 of $Fg\beta_2$ -tubulin conferred *F. graminearum* resistance to MBC.

Expression level of the $Fg\beta_2$ -tubulin gene in the $Fg\beta_2$ F240L mutant. We measured the expression level of the $Fg\beta_2$ -tubulin gene in 2021 and the $Fg\beta_2$ F240L mutant by qRT-PCR. The results showed that the F240L mutation in β_2 -tubulin did not affect the expression level of $Fg\beta_2$ -tubulin (Fig. 3A).

Morphology (for hyphae and conidia) and spore production of $Fg\beta_2$ F240L. According to previous studies, disruption of $Fg\beta_2$ -tubulin severely decreased mycelium growth rate and sporulation, resulting in twisted hyphae (Chen et al. 2009; Zhao et al. 2014). To determine whether the F240L mutation disrupts the function of

$Fg\beta_2$ -tubulin, we assessed the morphology and sporulation of the $Fg\beta_2$ F240L mutant. The morphology of hyphae and conidia was similar to that of 2021 (Fig. 4A). Spore production was also normal for $Fg\beta_2$ F240L (Fig. 4B). These results indicated that the F240L mutation does not affect the function of $Fg\beta_2$ -tubulin.

Construction and MBC sensitivity of the $Bc\beta$ L232F mutant of *B. cinerea*. We constructed the $Bc\beta$ L232F mutant by gene replacement strategy. The transformants were verified by PCR, Southern blot, and sequencing. The results indicated that the selected transformants of the $Bc\beta$ L232F mutant had the designed mutations. In addition, the qRT-PCR results showed that L232F mutation in β -tubulin does not affect the expression level of $Bc\beta$ -tubulin (Fig. 3B).

Based on inhibition of mycelial growth, the EC_{50} value of MBC against $Bc\beta$ L232F was ninefold higher than that of wild-type sensitive strain Bt4-1 and the $Bc\beta$ -hph mutant (Table 3; Fig. 5). Moreover, the patterns in sensitivities of $Fg\beta_2$ F240L and $Bc\beta$ L232F mutants to benomyl and thiabendazole were consistent with those for MBC (Supplementary Table S3).

Pathogenicity of $Fg\beta_2$ F240L and $Bc\beta$ L232F mutants. We tested the pathogenicity of $Fg\beta_2$ F240L by a coleoptile infection assay (Fig. 6A). Ten days after inoculation, the lesions caused by $Fg\beta_2$ F240L and $2\Delta\beta_2$ -C mutants were identical with those of 2021 (Fig. 7A).

The pathogenicity of $Bc\beta$ L232F mutants was tested by fruit infection (Fig. 6B). Four days after inoculation, the lesions caused by $Bc\beta$ L232F and $Bc\beta$ -hph mutants were smaller than those of Bt4-1. However, the pathogenicity of $Bc\beta$ L232F mutants was identical with $Bc\beta$ -hph (Fig. 7B).

Microscopic observation of microtubules in the $Fg\beta_2$ F240L mutant. To visualize cellular microtubules, we generated $Fg\beta_2$ -tubulin-EGFP fusion mutants ($Fg\beta_2$ -EGFP and $Fg\beta_2$ F240L-EGFP). The qRT-PCR results indicated that the expression level of the

Fig. 6. Virulence of $Fg\beta_2$ F240L and $Bc\beta$ L232F mutants. **A**, Infected wheat coleoptiles were photographed at 10 days after inoculation. **B**, Disease symptoms on wounded apple and tomato fruit were photographed at 4 days after inoculation.

Fig. 7. **A**, Lengths of brown lesions of wheat coleoptiles caused by 2021, $2\Delta\beta_2$, $2\Delta\beta_2$ -C and $Fg\beta_2$ F240L-8 were measured at 10 days after inoculation. **B**, Diameter of disease lesions on apple and tomato fruit caused by Bt4-1 and $Bc\beta$ -hph and $Bc\beta$ L232F mutants at 4 days after inoculation. Values are means $\pm$ standard deviation of three repeated experiments. Means of the column with the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

Fgβ₂-tubulin gene was not affected by the EGFP tag (Fig. 3A). Moreover, in the absence of MBC treatment, microtubules were evident in the germlings of both *Fgβ₂*-EGFP and *Fgβ₂*F240L-EGFP (Fig. 8A). With a treatment of MBC at 0.15 μg/ml for 5 min, microtubules were easy to observe in *Fgβ₂*-EGFP but difficult to observe in *Fgβ₂*F240L-EGFP (Fig. 8B). When the concentration of MBC was increased to 0.3 μg/ml, microtubules were still evident in *Fgβ₂*-EGFP but not in *Fgβ₂*F240L-EGFP (Fig. 8C). These results

indicated that MBC inhibited microtubule assembly to a greater degree in the *Fgβ₂*F240L mutant than in the wild-type strain 2021.

Molecular docking of MBC with *Fgβ₂*-tubulin. Our microscopic observation of microtubules indicated that the F240L mutation changed the interaction between *Fgβ₂*-tubulin and MBC. To determine whether the F240L mutation affects the binding affinity of MBC for *Fgβ₂*-tubulin, we carried on molecular docking of MBC with *Fgβ₂*-tubulin by Discovery Studio 3.0 software (Ye et al.

Fig. 8. Effect of methyl benzimidazol-2-ylcarbamate (MBC) on microtubules of *Fgβ₂*-enhanced green fluorescent protein (EGFP) and *Fgβ₂*F240L-EGFP mutants. Fresh spores (10^5) of *Fgβ₂*-EGFP and *Fgβ₂*F240L-EGFP mutants were first incubated into 20 ml of yeast extract-peptone-dextrose medium on an orbital shaker (175 rpm) at 25°C to germinate for 10 h and MBC was added at **A**, 0 μg/ml (CK); **B**, 0.15 μg/ml; and **C**, 0.3 μg/ml. After 5 min, 5 μl of culture was spotted directly onto slides and using a ×100 oil objective to visualize the germ tubes. Bar = 50 μm.

2014). In the wild-type *Fg* β_2 -tubulin, the MBC bound to the *Fg* β_2 -tubulin via three H-bonds with Ser138 and Asp177 (Fig. 9, top). The F240L mutation, however, altered the configuration of *Fg* β_2 -tubulin and changed the number and locations of the H-bonds (Fig. 9, bottom). Moreover, F240L mutation created a pi-pi interaction between two benzene rings of Tyr222 and MBC and increased the -cdock_interaction_energy score from 25.537 to 28.764 (Δ Gb [kcal/mol]).

DISCUSSION

In many plant-pathogenic fungi, MBC inhibits microtubule assembly by binding to the β -tubulin subunit of the α/β -heterodimer (Davidse 1986). Even though β -tubulin is highly conserved among fungi, MBC sensitivity differs among plant-pathogenic fungi, perhaps because of differences in β -tubulin sequence (Davidse 1986; Li et al. 2002). In this study, we found that difference in position 240 of β -tubulins affected the sensitivity of *F. graminearum* and other plant-pathogenic fungi. According to previous studies, *F. graminearum* was naturally less sensitive than other plant-pathogenic fungi, including *B. cinerea*, *C. gloeosporioides*, and *S. sclerotiorum* (Li et al. 2002). Through alignment of amino acid sequences of β -tubulins, we identified 100 amino acids that differ between the β_2 -tubulin of *F. graminearum* and β -tubulin isotypes of other plant-pathogenic fungi. Among the 100 sites, only position 240 had been previously reported to be related to benzimidazole resistance (Albertini et al. 1999; Ma et al. 2005). In the current study, we found that position 240 is leucine in the *Fg* β_1 -tubulin but is phenylalanine in the *Fg* β_2 -tubulin of the *F. graminearum* wild-type strain. The β -tubulins of the benzimidazole-resistant field isolates of *M. laxa* and *T. yallundae* also had a phenylalanine instead of a leucine at position 240 (Albertini et al. 1999; Ma et al. 2005). Moreover, position 240 in β -tubulin of *B. cinerea*, *C. gloeosporioides*, and *S. sclerotiorum* wild-type sensitive strains is a leucine. Together,

these findings indicated that the β -tubulin of the wild-type strains of many pathogenic fungi contains phenylalanine or leucine in position 240. The phenylalanine in position 240 confers resistance to benzimidazoles in at least some pathogenic fungi.

To test the hypothesis that phenylalanine in position 240 of *Fg* β_2 -tubulin gives rise to the naturally less MBC sensitivity in *F. graminearum*, we constructed the *Fg* β_2 F240L mutant from *F. graminearum* strain $\Delta\beta_2$. We found that the *Fg* β_2 F240L mutant was hypersensitive to MBC. Moreover, F240L mutation also increased the sensitivity to benomyl and thiabendazole. This result, identical with the research in *M. laxa* and *T. yallundae* (Albertini et al. 1999; Ma et al. 2005), was consistent with our hypothesis.

F. graminearum contains two β -tubulin isotypes: *Fg* β_1 - and *Fg* β_2 -tubulin. Position 240 is leucine in *Fg* β_1 -tubulin. The sensitivity of the *Fg* β_1 L240F mutant, however, is identical with that of 2021, suggesting that phenylalanine in position 240 of *Fg* β_1 -tubulin does not decrease sensitivity to MBC. Interestingly, the identity between *Fg* β_1 -tubulin and β -tubulin of other plant pathogens fungi is 93 to 99%. For *Fg* β_2 -tubulin, in contrast, the identity is only approximately 70 to 77% (Chen et al. 2009). Moreover, the preferred binding target for MBC in *F. graminearum* is *Fg* β_1 -tubulin rather than *Fg* β_2 -tubulin (Zhou et al. 2016b). This explains why mutations had been found only in *Fg* β_2 -tubulin rather than in *Fg* β_1 -tubulin in MBC-resistant field strains of *F. graminearum* (Chen et al. 2007, 2009). Perhaps *Fg* β_2 -tubulin has experienced divergent and positive selective pressure and *Fg* β_2 -tubulin rather than *Fg* β_1 -tubulin is sufficient for viability even though *Fg* β_1 -tubulin is the preferred binding target for MBC (Qiu et al. 2012; Zhao et al. 2014). *F. graminearum* is not unique in containing two β -tubulin isotypes. For example, *A. nidulans* also contains two β -tubulin isotypes, *benA* and *tubC* (May et al. 1987), and mutations that conferred resistance to benzimidazole fungicides were only found in *benA* (Jung et al. 1992), which resembles the situation with *F. graminearum*.

Fig. 9. Molecular docking of methyl benzimidazol-2-ylcarbamate (MBC) to *Fg* β_2 -tubulin. Homology models of wild-type and F240L mutant *Fg* β_2 -tubulins were constructed using the online server at I-TASSER. Molecular docking of MBC with β_2 -tubulin was carried out using Discovery Studio 3.0 software and the results were shown in two-dimensional (2D) and three-dimensional (3D) forms. The pi-pi interactions are shown as lines and hydrogen bond interactions are represented by dashed arrows directed toward the electron donor.

To further demonstrate the effect of phenylalanine in position 240 of β -tubulin, we constructed the *Bc* β L232F mutant from *B. cinerea* strain Bt4-1 (position 232 in β -tubulin of strain Bt4-1 is equivalent to position 240 in β -tubulin of *F. graminearum*). Strangely, both the *Bc* β L232F and *Bc* β -*hph* mutants showed decreased growth rate and virulence. It might be due to the negative effect of protoplast transformation. However, the virulence of the *Bc* β L232F mutant was identical with *Bc* β -*hph*, which meant that L232F mutation in *Bc* β -tubulin did not affect the virulence. More importantly, the EC₅₀ value of the *Bc* β L232F mutant was ninefold higher than that of Bt4-1 and the *Bc* β -*hph* mutant, which indicates that the L232F mutation in the β -tubulin of strain Bt4-1 confers resistance to MBC. Moreover, the *Bc* β L232F mutant also showed increased resistance to benomyl and thiabendazole. These results are once again consistent with those obtained with *M. laxa*, *T. yallundae*, and the *Fg* β ₂F240L mutant and also confirmed that phenylalanine at position 240 of *Fg* β ₂-tubulin makes *F. graminearum* naturally less sensitive to MBC.

After confirming that the F240L mutation in *Fg* β ₂-tubulin increases the sensitivity of *F. graminearum* to MBC, we wanted to figure out whether the F240L mutation could affect the function of *Fg* β ₂-tubulin. Neither the F240L nor L232F mutations affected the expression level of *Fg* β ₂-tubulin and *Bc* β -tubulin, which means that differences in MBC sensitivity cannot be explained by expression level. Moreover, disruption of *Fg* β ₂-tubulin in *F. graminearum* led to a severely decreased growth rate, twisted hyphae, decreased sporulation, and decreased virulence (Chen et al. 2009; Zhao et al. 2014). However, growth rate, mycelium morphology, sporulation, and virulence of the *Fg* β ₂F240L mutant were identical with those of 2021, indicating that the F240L mutation does not affect the viability of *F. graminearum*. Taken together, these studies suggested that leucine in position 240 does not affect the function of *Fg* β ₂-tubulin, which is consistent with the natural occurrence of leucine in position 240 of other β -tubulin isotypes. Interestingly, in human leukemia cells resistant to vincristine (a drug that can destabilize microtubules), position 240 is leucine located at H7 and H8 helices and near the intradimer surface of the α/β -heterodimer; the L240I mutation affected the longitudinal interaction between α - and β -monomers and the conformation of the H7 and H8 helices and, therefore, decreased the stability of microtubules (Kavallaris et al. 2001). In yeast, systematic mutation of β -tubulin in yeast at position 249 to 252 generated a recessive-lethal phenotype (Reijo et al. 1994). Human nerve cells expressing the R262C mutant *TUB3B* (neuron-specific β -tubulin isotype III) had reduced microtubule incorporation (Tischfield et al. 2010). These results indicate that mutation near the region of position 240 may decrease the stability of microtubules. In our study, however, *Fg* β ₂-tubulin with the F240L mutation could polymerize into microtubules. Therefore, we suspect that, although tubulin is a highly conserved protein, structural differences among species may account for the different effects of mutations in position 240 of β -tubulin.

To determine the mechanism of increased sensitivity of the *Fg* β ₂F240L mutant to MBC, we studied the microtubule dynamics of *Fg* β ₂-EGFP and *Fg* β ₂F240L-EGFP mutants treated with MBC. We found that MBC greatly inhibited microtubule formation and stability in *Fg* β ₂F240L-EGFP but not in *Fg* β ₂-EGFP. According to a previous study, MBC can prevent free *Fg* β ₂-tubulin from polymerizing into microtubules but it cannot disassemble polymerized microtubules (Zhou et al. 2016a). In addition, microtubules are highly dynamic structures that can grow or contract at both the plus and minus ends (Mitchison and Kirschner 1984). Once growth state is inhibited by physical and chemical factors, microtubules become less stable and their functions will be disrupted. Therefore, we infer that free *Fg* β ₂-tubulin polymerization must be inhibited more easily by MBC in the *Fg* β ₂F240L mutant than in the wild-type strain 2021. In addition, according to our molecular docking assay, the binding affinity between MBC and *Fg* β ₂-tubulin was significantly greater with leucine than with phenylalanine at position 240, which is consistent with a previous in vitro finding that an E198G

mutation increased the binding affinity between β -tubulin and carbendazim (Hollomon et al. 1998). Taken together, these results indicate that polymerization of *Fg* β ₂-tubulin is more sensitive to MBC in *Fg* β ₂F240L than in 2021 because the binding affinity between MBC and *Fg* β ₂-tubulin is greater in *Fg* β ₂F240L.

In summary, our study revealed that a difference in position 240 of β -tubulin gives rise to different sensitivity of multiple fungi to benzimidazole fungicides. This information could increase our understanding about selectivity of benzimidazole fungicides among multiple plant-pathogenic fungi and could be useful for optimizing the molecular structure of fungicides. However, the molecular mechanisms by which point mutations affect the interaction between benzimidazole fungicides and tubulin remain elusive. Future research should analyze the crystal structure of tubulin complexed with benzimidazole fungicides, which would be helpful for overcoming fungicide resistance.

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