β1 Tubulin Rather Than β2 Tubulin Is the Preferred Binding Target for Carbendazim in *Fusarium graminearum*

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ABSTRACT

Zhou, Y., Zhu, Y., Li, Y., Duan, Y., Zhang, R., and Zhou, M. 2016. β1 Tubulin rather than β2 tubulin is the preferred binding target for carbendazim in *Fusarium graminearum*. *Phytopathology* 106:978-985.

Tubulins are the proposed target of anticancer drugs, anthelmintics, and fungicides. In *Fusarium graminearum*, β2 tubulin has been reported to be the binding target of methyl benzimidazole carbamate (MBC) fungicides. However, the function of *F. graminearum* β1 tubulin, which shares 76% amino acid sequence identity with β2 tubulin, in MBC sensitivity has been unclear. In this study, MBC sensitivity relative to that of a parental strain (2021) was significantly reduced in a β1 tubulin deletion strain but increased in a β2 tubulin deletion strain, suggesting that β1 tubulin was involved in the MBC sensitivity of *F. graminearum*. When strain 2021 was grown in a medium with a low or high concentration of

the MBC fungicide carbendazim (0.5 or 1.4 μg/ml), the protein accumulation levels were reduced by 47 and 87%, respectively, for β1 tubulin but only by 6 and 24%, respectively, for β2 tubulin. This result was consistent with observations that MBC fungicides are more likely to disrupt β1 tubulin microtubules rather than β2 tubulin microtubules in GFP-β tubulin fusion mutants in vivo. Furthermore, sequence analysis indicated that a difference in tubulin amino acid 240 (240L in β1 versus 240F in β2) may explain the difference in MBC binding affinity; this result was consistent with the result that an F240L mutation in β2 tubulin greatly increased sensitivity to carbendazim in *F. graminearum*. We suggest that β1 tubulin rather than β2 tubulin is the preferred binding target for MBC fungicides in *F. graminearum*.

Additional keywords: benzimidazole resistance, drug-binding affinity.

Microtubules are found in all eukaryotes and are assembled by α and β tubulin heterodimers. The α tubulins and β tubulins are among the most highly conserved eukaryotic proteins and are involved in the maintenance of cell shape, mitosis, and a variety of other morphogenic events (Wilson and Jordan 1995). An integral part of microtubule function results from their constant assembly and disassembly, which involves tubulin filaments and various accessory proteins. Tubulin is also the target of many antitumor drugs, herbicides, anthelmintics, and fungicides (Jordan and Wilson 2004; Kiso et al. 2004).

Fusarium head blight (FHB), caused by the filamentous fungus *Fusarium graminearum*, can greatly reduce yields of wheat, maize, and barley worldwide (Goswami and Kistler 2004). To control FHB, farmers apply carbendazim (a methyl benzimidazole carbamate [MBC] fungicide) and other MBCs; these broad spectrum, systemic fungicides interact with β tubulin (Hollomon et al. 1998). Biochemical and genetic analyses have identified the β tubulin subunit as the primary MBC target. By isolating resistant mutants of susceptible fungi (e.g., *Aspergillus nidulans*) and sequencing their β tubulin genes, researchers have determined that MBC can interact with four regions of the tubulin molecule (Fujimura et al. 1992; Jung and Oakley 1990; Jung et al. 1992; Orbach et al. 1986; Thomas et al. 1985). These regions include amino acid residues 6, 165 to 167, 198 to 200, and 241. In recent studies in the our laboratory, point mutations in the β2 tubulin gene at codons 167 (Phe to Tyr), 198 (Glu to Lys), and 200 (Phe to Tyr) were detected in MBC-resistant field strains of *F. graminearum* (Chen et al. 2008,

2009). In addition, homology modeling indicated that these sites (amino acid 167, 198, and 200) in the β2 tubulin of *F. graminearum* constitute a hydrophobic pocket and may represent binding domains for MBC (Qiu et al. 2011).

Although most fungi contain only one β tubulin gene, *Aspergillus nidulans*, *Trichoderma* spp., and *Colletotrichum* spp. have two different β tubulin genes. *A. nidulans* produces two structural β tubulin genes, *benA* and *tubC*, and gene replacement has demonstrated that the highly different tubulins of *A. nidulans* are functionally interchangeable (May et al. 1987). The protein encoded by the *benA* gene, however, has been determined to be the MBC target (Jung et al. 1992). *Trichoderma* spp. have two β tubulin genes, *tub1* and *tub2*, and reverse genetics showed that a mutation in the *tub2* gene confers MBC resistance, indicating that the *tub2* is the MBC target (Goldman et al. 1993). Mutation at codon 198 of the *tub2* conferred MBC resistance in *Colletotrichum gloeosporioides* (Kongtragoul et al. 2011).

F. graminearum has two β tubulin isotypes, i.e., β1 tubulin (NCBI accession number XM_011329885.1) and β2 tubulin (NCBI accession number XM_011327927.1), and point mutations in amino acid residues 167, 198, or 200 of β2 tubulin conferred MBC resistance; no point mutations were identified in β1 tubulin of the MBC-resistant strains of *F. graminearum* examined (Chen et al. 2007). The two β tubulins in *F. graminearum* exhibit high sequence similarity (76%), especially at the MBC-binding sites (amino acid residues 167, 198, and 200). A recent knockout study of β1 tubulin isotypes reported that deletion of β1 tubulin reduced vegetative growth and pathogenicity but increased asexual reproduction in *F. graminearum* and that the β1 tubulin deletion mutant was more resistant to MBC than parent strains (Qiu et al. 2012). The possible MBC binding sites on β1 tubulin were analyzed by homology modeling, raising the hypothesis that β1 tubulin is a MBC target. However, there is little evidence, especially from in vivo experiments, testing this hypothesis. Another question of interest is whether there are differences of sensitivity between β1 tubulin and β2 tubulin in *F. graminearum* upon MBC binding.

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In the current study, we first developed dose-response curves to describe the carbendazim sensitivity (in terms of mycelial growth) of $\beta 1$ and $\beta 2$ tubulin deletion mutants of *F. graminearum*. To understand the functional significance of $\beta 1$ and $\beta 2$ tubulin isotypes in drug responses, we produced two antibodies that specifically recognize these β tubulin isotypes and then characterized the protein accumulation level of $\beta 1$ or $\beta 2$ tubulin in response to carbendazim treatment. We also determined the binding affinity of carbendazim for the two β tubulin isotypes by using GFP- β tubulin fusion mutants in vivo. Finally, sequence alignment was used to gain insight into why carbendazim binding affinity differed between $\beta 1$ and $\beta 2$ tubulin and the hypothesis was supported by carbendazim sensitivity tests of a *F. graminearum* strain with the F240L mutation in $\beta 2$ tubulin.

MATERIALS AND METHODS

Strains, plasmids, primers, and culture conditions. The *F. graminearum* strains, plasmids, and primers used in this study are listed in Supplementary Table S1. Mycelial growth, sporulation, and fungicide sensitivity were assessed as previously described (Zheng et al. 2014). For the mycelial growth assay, the strains were grown at 25°C on potato sucrose agar (PSA: 200 g of potato, 20 g of sucrose, 15 g of agar, and 1 liter of distilled water). For the sporulation assay, the strains were grown at 25°C in mung bean liquid medium (MBL: 30 g of mung beans was boiled in 1 liter of water for 20 min, and the mixture was filtered through cheesecloth). For total protein extraction from mycelia, the strains were grown at 25°C in liquid YEPD (wt/vol, 1% peptone, 0.3% yeast extract, and 2% glucose). *Escherichia coli* strain DH5 α was used for general cloning, and BL21 (DE3) was used for protein expression; these were cultured in Luria-Bertani broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract dissolved in 1 liter of distilled water) at 37°C.

Production of recombinant tubulins in *E. coli* for antigens. Recombinant *F. graminearum* $\beta 1$ and $\beta 2$ tubulin were expressed and refolded as described previously (Zhang et al. 2011). $\beta 1$ and $\beta 2$ tubulin were cloned into the pET28a vector using primers F1/R1 and F2/R2, respectively, and transformed into *E. coli* strain BL21 (DE3) for antigen production. Recombinant proteins were then isolated from inclusion bodies and refolded and purified in denatured form using Ni-NTA (GE) according to the manufacturer's instructions.

Preparation and purification of the polyclonal antibody specific to $\beta 1$ tubulin. The polyclonal antibody specific to $\beta 1$ tubulin was raised according to Bo et al. (2008). The recombinant $\beta 1$ tubulin was immunized on two male New Zealand white rabbits. The rabbits had free access to drinking water and a commercial,

standard laboratory diet (CZZ, Nanjing). They were housed according to the EEC 609/86 Directives regulating the welfare of experimental animals. Whole blood was obtained from the heart of each rabbit 7 days after the last immunization. The antiserum was purified with Protein G sepharose 4FF (GE), cross absorbed with a homemade NHS- $\beta 2$ tubulin affinity column (NHS activated seahorse [GE] coupled with recombinant $\beta 2$ tubulin according to the manufacturer's instructions), and stored at -20°C. The specificity and titer of the antibody were verified by an indirect enzyme-linked immunosorbent assay (indirect ELISA) and western blot analysis.

Preparation of a monoclonal antibody specific to $\beta 2$ tubulin. A monoclonal antibody (McAb) specific to $\beta 2$ tubulin was generated by typical cell fusion techniques (Kascsak et al. 1987). Five-week-old female BALB/c mice were immunized using the mixture of recombinant $\beta 2$ tubulin and Quick antibody adjuvant (Biodragon, Beijing, China) every 2 weeks. Each mouse received two intramuscular injections (100 μ l per injection) containing 50 μ g of protein and a single booster injection 4 days before fusion. Hybridoma cells were prepared as described elsewhere (Thornton 2001), and the supernatants were screened by ELISA against antigens immobilized to the wells of Maxisorp microtitre plates (Nunc; 442404) (50 μ l per well). McAb was prepared by injecting the fusion cells into syngeneic mice to induce antibody-containing ascites. The McAb was purified using Protein G sepharose 4FF (GE). The specificity and titer of the McAb were verified by indirect ELISA and western blot analysis.

Generation of GFP- β tubulin fusion mutants in *F. graminearum*. The GFP- β tubulin fusion mutants were generated as previously described with modification (Zhao et al. 2014). The GFP gene was amplified from vector pAcGFP1 using primers A9/A10. The mutants 2 $\Delta\beta 1$ and 2 $\Delta\beta 2$ (Table 1) were complemented with $\beta 1$ and $\beta 2$ tubulin full-length DNA (without the stop codon) of strain 2021 fused with the GFP gene, respectively. Transformants were selected on a medium containing 0.2 μ M floxuridine (Solarbio, Beijing). For analysis of the transformants by Southern blot, genomic DNA of 2 $\beta 1$ -GFP ($\beta 1$ -GFP fusion) and 2 $\beta 2$ -GFP ($\beta 2$ -GFP fusion) were digested by *Xho*I and *Hind*III (TaKaRa, Dalian, China), respectively, and hybridized with probe 1 and probe 2, respectively.

MBC dose-response curves based on mycelial growth. The sensitivity of *F. graminearum* strains (2021, 2 $\Delta\beta 1$, and 2 $\Delta\beta 2$) to carbendazim (methyl 1H-benzimidazol-2-ylcarbamate, was purchased from Sigma-Aldrich) was determined based on colony diameter as previously described (Chen et al. 2009). Each strain was cultured on PSA plates for 3 days at 25°C. A 5-mm-diameter mycelial plug taken from the margin of a 3-day-old PSA colony was placed on the center of a PSA plate amended with carbendazim at the following concentrations: 0, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95,

TABLE 1. Some properties of the *Fusarium graminearum* strains in this study^a

Strain	Genotype	EC ₅₀ (μ g/ml) ^b	MIC (μ g/ml) ^c	Mycelial growth rate (mm/day) ^d	Sporulation (10 ⁶ CFU ml ⁻¹)	Reference
2021	Wild type	0.54	1.4	26.75 $\pm$ 0.17	2.35 $\pm$ 0.07	(Chen et al. 2008)
2 $\Delta\beta 1$	$\beta 1$ tubulin deletion mutant in 2021 genetic background	0.80	1.4	22.42 $\pm$ 0.32	2.58 $\pm$ 0.18	(Qiu et al. 2012)
2 $\Delta\beta 2$	$\beta 2$ tubulin deletion mutant in 2021 genetic background	0.10	0.5	13.67 $\pm$ 0.54	0.20 $\pm$ 0.05	(Qiu et al. 2012)
2 $\beta 1$ -GFP	$\beta 1$ tubulin-GFP fusion mutant in 2021 genetic background	0.51	1.4	26.24 $\pm$ 0.21	2.52 $\pm$ 0.11	This study
2 $\beta 2$ -GFP	$\beta 2$ tubulin-GFP fusion mutant in 2021 genetic background	0.57	1.4	25.14 $\pm$ 0.19	2.23 $\pm$ 0.09	This study
ZF21	Field type	0.50	1.4	26.45 $\pm$ 0.18	2.40 $\pm$ 0.08	(Zhang et al. 2009)
R $\beta 2$ tub240Leu#3	F240L mutation in $\beta 2$ tubulin gene in ZF21 genetic background	0.05	—	25.30 $\pm$ 0.16	2.26 $\pm$ 0.11	(Zhang et al. 2009)

^a Values are means of three experiments (differences among the experiments were not significant, i.e., $P > 0.05$, Fisher's least significant difference test). Standard errors are indicated for growth rate and sporulation.

^b Fungicide (methyl benzimidazole carbamate [MBC]) concentration that resulted in 50% inhibition of mycelial growth.

^c MIC, minimum inhibitory concentration. —, undetermined.

^d Measured after 3 days at 25°C.

1.0, 1.2, or 1.4 µg/ml (strain 2Δβ1); 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.5, 0.8, 1.0, or 1.4 µg/ml (strain 2Δβ2); and 0, 0.33, 0.41, 0.52, 0.65, 0.80, 1.0, or 1.4 µg/ml (strain 2021). After incubation at 25°C for 3 days, the fungicide concentration that resulted in 50% mycelial growth inhibition (EC₅₀) and the minimum inhibitory concentration (MIC) were determined. The experiment was repeated at least twice and each experiment was done with triple replicate-plates.

Analysis of protein accumulation levels of β tubulins. Because MBCs disrupt the targeted β tubulin, relative changes in β tubulin protein accumulation levels can affect the degree of apparent sensitivity to MBC fungicides. For quantification of β tubulin isotype levels in the β tubulin deletion strains (2Δβ1 and 2Δβ2) and in the parent strain (2021) each strain were cultured in YEPD medium for 24 h at 25°C, and mycelia were harvested for western blot analysis.

To investigate the protein accumulation levels of the β tubulin isotype in response to a low and a high concentration of carbendazim (0.5 and 1.4 µg/ml) in strain 2021, the strain was cultured in YEPD medium in triplicate for 24 h at 25°C; the three cultures were treated with a carbendazim dose of 0.0, 0.5, or 1.4 µg/ml. After 6 h, the mycelia were harvested for western blot analysis. The β1 and β2 tubulin protein accumulation levels were determined by western blot using the anti-β1 tubulin antibody and anti-β2 tubulin antibody that were prepared as described earlier. The protein accumulation level of each tubulin isotype was calculated using Gel-Pro Analyzer 4.0 Image Analysis Software (Media Cybernetics, Inc., Bethesda, MD) and was normalized to the actin level. The protein accumulation level of actin was determined simultaneously as an internal control. All of the experiments were repeated at least twice.

Microscopic examination of mycelia and GFP-fusion β tubulins. For observation of mycelial morphology of the parental strains 2021 and of the β tubulin-GFP fusion mutants (2β1-GFP and 2β2-GFP), mycelial plugs were cultured in liquid YEPD medium for 36 h. Sections were prepared and were examined with an Olympus IX-71 microscope (Tokyo, Japan) as previously described (Zheng et al. 2014).

Conidial suspensions of β1 and β2 tubulin GFP-β tubulin fusion mutants (2β1-GFP and 2β2-GFP) were placed on a coverslip, treated with 0.01% polylysine, and air dried. When the preparations were dry, they were immediately placed (conidial-side down) on cellophane on the surfaces of PSA plates. After 4 h at 25°C, the coverslips were examined after they had been placed (conidia-side down) for 2 h on a new PSA plate containing carbendazim at 0, 0.5, or 1.4 µg/ml. The conidia and newly formed hyphae on the coverslips were examined for cytoplasmic microtubules using fluorescence microscopy (Leica Microsystems Wetzlar GmbH, Germany) with excitation and emission wavelengths of 475 and 505 nm, respectively.

Analysis of the β tubulin amino acid sequences from *F. graminearum*, *A. nidulans*, *C. gloeosporioides*, and *Trichoderma viride*. Amino acid sequences for β tubulins from four fungal species were obtained from the following references: *F. graminearum* (Qiu et al. 2012), *A. nidulans* (May et al. 1987), *C. gloeosporioides* (Kongtragoul et al. 2011), and *T. viride* (Goldman et al. 1993). The β tubulin amino acid sequences from the four fungi were aligned using DNAMAN sequence analysis software (Lynnon Biosoft). The identities among the sequences were calculated using DNAMAN, and a homology tree was constructed using DNAMAN.

RESULTS

Antibodies specifically recognize the β-tubulin isotypes in *F. graminearum*. β1 and β2 tubulin of *F. graminearum* were expressed in *E. coli* and purified as antigens for antibody production. The purities of the recombinant proteins were >95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE) (Supplementary Fig. S1). Rabbit polyclonal antiserum was generated against purified recombinant *F. graminearum* β1

tubulin. The antiserum was applied to a homemade β2 tubulin affinity column, and the flow-through fraction (which specifically reacted with β1 tubulin without cross reacting with β2 tubulin) was termed β1-pAb. The monoclonal antibody preparation generated two clones, 2F11 and 4B5, both of which appeared to be highly specific for β2 tubulin. 2F11 was selected for further testing and was termed β2-mAb. The titers of β1-pAb and β2-mAb as determined by indirect ELISA were 1.28 × 10⁵ and 1 × 10⁶, respectively.

The specificity of the polyclonal and monoclonal antibodies to the β tubulin isotypes was determined by reaction with three whole-cell lysates corresponding to three *F. graminearum* strains (2021, 2Δβ1, and 2Δβ2 in Table 1) in western blot analysis. The total protein of strains 2021 (WT) and 2Δβ2 reacted with β1-pAb, producing a single band at the ~50-kDa position, which matches the theoretical molecular weight of β1 tubulin; total protein of strain 2Δβ1, however, did not react with β1-pAb (Fig. 1A). In contrast, the total protein of strain 2021 and 2Δβ1 reacted with β2-mAb, producing a single band at the ~50-kDa position, which matches the theoretical molecular weight of β2 tubulin; total protein of strain 2Δβ2 did not react with β2-mAb (Fig. 1A). The three whole-cell lysates were analyzed by SDS-PAGE and with actin as the loading control (Fig. 1B).

β1 tubulin is involved in carbendazim sensitivity. β2 tubulin of *F. graminearum* has been reported to be the binding target of MBC fungicides. In this study, the involvement of β1 tubulin in the carbendazim sensitivity of *F. graminearum* was analyzed using β1 and β2 tubulin deletion mutants. The dose-response curves of β1 and β2 tubulin deletion mutants to carbendazim were determined based on colony diameter (Fig. 2). The sensitivity to carbendazim was much greater for the β2 tubulin deletion mutant 2Δβ2 than for the parental strain 2021. In contrast, the sensitivity to carbendazim was lower for the β1 tubulin deletion mutant 2Δβ1 than for the parental strain, especially when carbendazim concentrations were <0.7 µg/ml. Because EC₅₀ and MIC values (Table 1) are based on relatively high concentrations of carbendazim, they provided less information than the dose-response curves concerning the reduced sensitivity of 2Δβ1 to carbendazim. These results are consistent with a lower carbendazim binding affinity for β2 tubulin than for β1 tubulin. In addition, the protein accumulation level of β1 tubulin in the absence of carbendazim did not significantly differ between strains 2Δβ2 and 2021; the protein accumulation level of β2 tubulin did not significantly differ between strains 2Δβ1 and 2021 (Fig. 3). These results further indicate that the difference in carbendazim sensitivity between the β tubulin deletion mutants resulted from a difference in drug-binding affinity rather than to a difference in protein accumulation level.

Carbendazim reduced the β1 tubulin level more than the β2 tubulin level. To investigate the protein accumulation levels of the β tubulin isotypes in strain 2021 in response to a low and a high concentration of carbendazim (0.5 and 1.4 µg/ml), western blot analysis was performed. The low concentration reduced the level of β1 tubulin in strain 2021 by 47% but did not significantly affect the level of β2 tubulin in strain 2021 (Fig. 4). The high concentration of carbendazim reduced the level of β1 tubulin by 87% and that of β2 tubulin by 24%.

GFP-β tubulin fusions have fully functional β tubulin as indicated by mycelial growth, conidiation, and carbendazim sensitivity. We generated mutant strain 2β1-GFP by fusing GFP with β1 tubulin in *F. graminearum* 2021, and mutant strain 2β2-GFP by fusing GFP with β2 tubulin in *F. graminearum* 2021 (Supplementary Fig. S2). Sporulation of 2β1-GFP and 2β2-GFP were similar to that of the parental strain 2021 (Table 1). Mycelial growth rate of 2β1-GFP was similar to that of the parental strain 2021 but 2β2-GFP was slightly slower than that of the parental strain 2021 (Table 1). The colony and hyphal morphologies of 2β1-GFP were similar to those of the parental strain 2021. In addition, the hyphal morphology of 2β2-GFP was similar to that of the parental strain 2021 but the colony of 2β2-GFP was less pigmented

relative to that of the parental strain 2021 (Supplementary Fig. S3). Furthermore, the EC₅₀ and MIC values were the same for 2β1-GFP, 2β2-GFP, and 2021. These results indicated that the GFP labeling did not significantly change β tubulin functioning.

Carbendazim preferentially depolymerizes microtubules consisting of β1 tubulin in vivo. When the strains were cultured on PSA plates without carbendazim, the GFP-fusion β tubulins assembled into cytoplasmic microtubules in a net-like arrangement (Fig. 5A, B, E, and F). When strain 2β1-GFP and 2β2-GFP were cultured on PSA plates with carbendazim at 0.5 μg/ml, the GFP-fused β1-tubulin in strain 2β1-GFP appeared as scattered green fluorescence, and no normal microtubules (cytoplasmic microtubules) were visible (Fig. 5C). When strain 2β2-GFP was cultured with carbendazim at 0.5 μg/ml, the GFP-fused β2-tubulin formed normal-appearing microtubules in immature plastids (Fig. 5G). In the presence of carbendazim at 1.4 μg/ml, the β1 and β2 tubulin in the immature plastids seemed to be similar in that they only generated scattered green fluorescence, and no intact microtubules (cytoplasmic microtubules) were visible (Fig. 5D and H).

Analysis of the β tubulin amino acid sequences from *F. graminearum*, *A. nidulans*, *C. gloeosporioides*, and *T. viride*. The identity between the amino acid sequence of *F. graminearum* β2 tubulin and the sequences of *F. graminearum* β1 tubulin,

A. nidulans benA, *C. gloeosporioides* tub2, and *T. viride* tub2 were 76.4, 76.2, 76.0, and 75.5%, respectively (Fig. 6B). The identity between the amino acid sequence of *F. graminearum* β1 tubulin versus *A. nidulans* benA, *C. gloeosporioides* tub2, and *T. viride* tub2 were 94.4, 96.6, and 93.7%, respectively. A homology tree of the five β tubulin amino acid sequences was generated using DNAMAN (Fig. 6B). Overall, the homologies for *A. nidulans* benA, *C. gloeosporioides* tub2, and *T. viride* tub2 were higher for *F. graminearum* β1 tubulin than for *F. graminearum* β2 tubulin.

DISCUSSION

Tubulins are the primary target of a large and ever-growing number of small molecules. β2 tubulin in *F. graminearum* has long been recognized as the MBC-binding target. The effects of β1 tubulin on *F. graminearum* mycelial growth, spore germination, and pathogenicity have been investigated by the gene knockout method (Qiu et al. 2012). Additionally, the role of β1 tubulin in MBC resistance was studied using comparisons of MBC EC₅₀ and MIC values of β1 and β2 tubulin deletion mutants. Furthermore, sequence alignment between *F. graminearum* β1 and β2 tubulin and homology modeling of *F. graminearum* β1 tubulin were previously performed, raising the hypotheses that β1 tubulin was the target of MBCs and the difference of binding affinity of β1 and β2 tubulin to MBCs resulted from the 240 position amino acid diversity (Qiu et al. 2012). Except for the data described above, there are no reports further investigating the role of *F. graminearum* β1 tubulin relative to MBC fungicide resistance to test these hypotheses. Understanding the role of β1 tubulin in MBC resistance helps to reveal how the microtubule systems works (cooperation and competition between tubulin subunits) in the presence of antitubulin drugs. For these reasons, the current study investigated the mechanism underlying the difference in

Fig. 1. Specificity of the antibodies to the β tubulin isotypes in *Fusarium graminearum*. **A**, Western blot analysis of the β1 polyclonal antibody (β1-pAb) and β2 monoclonal antibody (β2-mAb) to total proteins of the three *F. graminearum* strains (WT, 2Δβ1, and 2Δβ2). **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the total proteins of the three *F. graminearum* strains. Wild-type (WT) 2021. Actin expression was detected simultaneously as the loading control.

Fig. 2. Dose-response curves of *Fusarium graminearum* β1 and β2 tubulin deletion mutants (2Δβ1 and 2Δβ2) and their parental strain 2021 to methyl benzimidazole carbamate.

Fig. 3. Protein accumulation levels of β tubulins in strains 2021, Δβ1, and Δβ2 of *Fusarium graminearum*. **A**, Protein accumulation level of β tubulins in 2021, Δβ1, and Δβ2 measured by western blot. Bars indicate standard errors from at least three independent experiments. **B** and **C**, Representative results of western blot analysis for accumulation levels of β tubulin in 2021, Δβ1, and Δβ2 as determined using β1-pAb antibody in **B** and β2-mAb antibody in **C**. The results indicate that β1 tubulin was not present in Δβ1 (but was present in the two other strains) and that β2 tubulin was not present in Δβ2 (but was present in the two other strains).

the sensitivity of the two β tubulin subunits to the MBC fungicide carbendazim.

Although the EC₅₀ and MIC values for carbendazim and *F. graminearum* β tubulin deletion mutants have been determined previously (Qiu et al. 2012), additional data concerning the responses of the mutants to carbendazim were provided in this study; namely the dose-response curves of $\beta 1$ and $\beta 2$ tubulin deletion mutants to carbendazim. Most importantly, the deletion of $\beta 2$ tubulin increased sensitivity to carbendazim, while the deletion of $\beta 1$ tubulin greatly decreased sensitivity to low concentrations of carbendazim. These results are consistent with the inference that the carbendazim-binding affinity is greater for $\beta 1$ than for $\beta 2$ tubulin. Furthermore, we proposed the hypothesis that the MIC value of

F. graminearum to carbendazim was determined by the low affinity target of carbendazim ($\beta 2$ tubulin). The evidence supporting this hypothesis was that *F. graminearum* 2021 and 2 $\Delta\beta 1$ strains have the same MIC value (Fig. 2). In addition, the *F. graminearum* strains with a $\beta 2$ tubulin 167, 198, or 200 position amino acid mutation (have lower binding affinity to MBCs, unpublished data in our lab) have higher MIC values in contrast to wild type 2021 (Qiu et al. 2011). Additionally, this conclusion was extensively supported by published data in other fungi that a 167, 198, or 200 position amino acid mutation in β tubulin could reduce MBC binding affinity (Hollomon et al. 1998). Recent studies demonstrated that the overexpression of $\beta 2$ tubulin, as indicated by mRNA level, increased MBC fungicide resistance in *F. graminearum* (Qiu et al.

Fig. 4. Protein accumulation levels of β tubulins in *Fusarium graminearum* strain 2021 in response to low and high concentrations of methyl benzimidazole carbamate (MBC). **A**, Protein accumulation level of $\beta 1$ and $\beta 2$ tubulins in 2021 in response to carbendazim at 0.0, 0.5, and 1.4 $\mu\text{g/ml}$ as measured by western blot. Bars indicate standard errors from at least three independent experiments. An asterisk indicates a significant difference relative to the nontreated culture (0 $\mu\text{g/ml}$ MBC). **B**, Representative results of western blot analysis.

Fig. 5. Characteristics of microtubules with expression of GFP-tagged $\beta 1$ -tubulin (2 $\beta 1$ -GFP) or GFP-tagged $\beta 2$ -tubulin (2 $\beta 2$ -GFP) in *Fusarium graminearum*. The morphology of microtubules in conidia or germling **A**, **B**, **E**, and **F**, untreated with methyl benzimidazole carbamate (MBC), **C** and **G**, treated with MBC at 0.5 $\mu\text{g/ml}$ for 2 h, or **D** and **H**, treated with MBC at 1.4 $\mu\text{g/ml}$ for 2 h. Right panels with uppercase letters were photographed with fluorescence field (FF) microscopy and corresponding left panels were photographed with bright field (BF) microscopy. The representative microtubules in conidium (white arrow) and germling (red arrow) are indicated (scale bars = 10 μm).

2012). In the current study, to eliminate the possibility that the differences in carbendazim sensitivity among the three strains (2021, $\Delta\beta 1$, and $\Delta\beta 2$) might be partly result from differences of the protein accumulation level of β tubulins, the protein accumulation level of β tubulins was examined; the results showed that the protein accumulation level of $\beta 1$ tubulin did not differ between the $\beta 2$ tubulin deletion mutant and the parental strain 2021, and the protein accumulation level of $\beta 2$ tubulin did not significantly differ between the $\beta 1$ deletion mutant and strain 2021 (Fig. 3); this result confirmed that drug-binding affinity rather than β tubulin accumulation level was responsible for the differences in MBC fungicide sensitivity among the three strains. Although the deletion of $\beta 1$ tubulin resulted in a twofold increase in the mRNA expression level of $\beta 2$ tubulin in *F. graminearum* (Qiu et al. 2012), a significant change in the protein expression of $\beta 2$ tubulin was not detected in the $2\Delta\beta 1$ strain relative to the parental strain (Fig. 3). mRNA levels and protein accumulation levels are not correlated to each other for many human and yeast proteins, especially for those that are regulated by posttranscriptional processes (Anderson and Seilhamer 1997; Gygi et al. 1999).

Protein accumulation levels determined by western blot also indicated that $\beta 1$ tubulin is more sensitive to carbendazim than $\beta 2$ tubulin. Thus, when strain 2021 was treated with a low concentration of carbendazim, the $\beta 1$ tubulin level was reduced by 47%, in comparison with only 6% in the case of $\beta 2$ tubulin; it may be due to the fact that MBC fungicides preferentially bind to the $\beta 1$ tubulin, which converts $\beta 1$ tubulin to an unstable conformation, and eventually leads to tubulin degradation (Garland 1978); on the other hand, the remaining carbendazim binding to $\beta 2$ tubulin was relative very low and caused a lower reduction of $\beta 2$ tubulin level in contrast to $\beta 1$ tubulin. Similarly, a high concentration of carbendazim reduced the $\beta 1$ tubulin level in strain 2021 by 87% but reduced the

$\beta 2$ tubulin level by only 24%; it is logical that the more carbendazim binds to $\beta 1$ and $\beta 2$ tubulin, the higher the inhibition ratio it will be; However, even treated with the MIC dose of carbendazim, there is still detectable level of $\beta 1$ and $\beta 2$ tubulin in the mycelia, perhaps the degradation of β tubulin was a time consuming process. In conclusion, this difference in sensitivity to MBC fungicides may result from the difference in MBC binding affinity between $\beta 1$ and $\beta 2$ tubulin.

$\beta 2$ tubulin in *F. graminearum* has long been recognized as the MBC-binding target (Chen et al. 2009; Qiu et al. 2011). To observe the dynamic behavior of microtubules and their associated proteins in living cells, researchers have fused the genes encoding cytoskeleton proteins (such as tubulin) and green fluorescent protein (GFP) in a variety of fungi including *A. nidulans* (Fernández-Abalos et al. 1998; Horio and Oakley 2005), *Magnaporthe grisea* (Czymmek et al. 2005), and *Neurospora crassa* (Freitag et al. 2004). In this study, we generated one mutant of 2021 in which GFP was fused with $\beta 1$ tubulin ($2\beta 1$ -GFP) and another in which GFP was fused with $\beta 2$ tubulin ($2\beta 2$ -GFP). We observed that both $\beta 1$ and $\beta 2$ tubulin assembled into cytoplasmic microtubules during spore germination, indicating that both β tubulin isotypes function well. When the strains were treated with carbendazim at 0.5 $\mu\text{g}/\text{ml}$ (the EC_{50} for 2021), $2\beta 1$ -GFP exhibited scattered fluorescence and failed to form normal microtubules, but $2\beta 2$ -GFP was not visibly affected. When the carbendazim concentration was increased to 1.4 $\mu\text{g}/\text{ml}$ (the EC_{90} for 2021), normal microtubules were not evident in either $2\beta 1$ -GFP or $2\beta 2$ -GFP. These results indicate that carbendazim preferentially disrupts microtubules consisting of the $\beta 1$ tubulin isotype rather than the $\beta 2$ tubulin isotype, i.e., carbendazim binding affinity is greater for $\beta 1$ tubulin than for $\beta 2$ tubulin. These results were consistent with the effects of MBC on β tubulin levels that were discussed in the previous paragraph.

Fig. 6. Analysis of amino acid sequences of β tubulin from *Fusarium graminearum* (*F.g* $\beta 1$ and $\beta 2$ tubulin), *Aspergillus nidulans* (*A.n benA*), *Colletotrichum gloeosporioides* (*C.g tub2*), and *Trichoderma viride* (*T.v tub2*). **A**, Multiple sequence alignment of the five sequences using DNAMAN. Black dots indicate conserved amino acids. Black arrows indicate mutation sites (amino acid residue 6, 50, 165, 167, 198, 200, 240, 241, 250, and 257) that confer methyl benzimidazole carbamate (MBC) resistance according to previous reports. **B**, Homology tree of β tubulin from selected fungal species. The percentage of identity between the groups is indicated to the right of the forks. All sequences were from the MBC-sensitive strain.

The following β tubulins have been identified as the MBC-binding target in these fungi: benA in *A. nidulans*, tub2 in *C. gloeosporioides*, and tub2 in *T. viride*. In this study, β 1 tubulin but not β 2 tubulin in *F. graminearum* has high identity with benA in *A. nidulans*, tub2 in *C. gloeosporioides*, and tub2 in *T. viride* (Fig. 6). Phylogenetic analysis shows that *F. graminearum* β 1 tubulin clade consists of the canonical tubulin gene whereas β 2 tubulin is the additional copy; one reasonable explanation is that the *Fusarium* β 1 tubulin is under strong purifying selection but the β 2 tubulin is under divergent selective pressure (Zhao et al. 2014). It is consistent with the observation that any polymorphism in the target gene β 1 tubulin from field resistant strains of *F. graminearum* has not been detected yet; in contrast, all the field resistant strains of *F. graminearum* have β 2 tubulin point mutations (Chen et al. 2008). Furthermore, it is speculated that β 2 tubulin is more important than β 1 tubulin in the life cycle of *F. graminearum* and point mutations protect the β 2 tubulin from MBC fungicides, in terms of survival rate. There is some evidence to support this hypothesis. First, Qiu et al. (Qiu et al. 2011 and Qiu et al. 2012) demonstrated that deletion of β 2 tubulin in *F. graminearum* results in the significantly slower mycelia growth and decrease in sporulation capacity; in contrast, deletion of β 1 tubulin in *F. graminearum* results in the slightly slow growth and an increase in sporulation capacity, indicating the β 2 tubulin is important for vegetative growth and asexual reproduction. Second, our group has demonstrated that the absolute protein content of β 2 tubulin is about 11-fold to that of β 1 tubulin in strain 2021 (unpublished data); the relatively abundant β 2 tubulin in *F. graminearum* indicates that β 2 tubulin has wider spatiotemporal distribution (indicated the more important functions). On the other hand, if the polymorphism occurs in β 1 tubulin, MBCs preferentially bind to β 2 tubulin, in consideration of the importance of β 2 tubulin, these strains have lower survival rate under MBC pressure.

Sequence differences between the β 1 and β 2 tubulin in *F. graminearum*, and especially differences in amino acid residues involved in the MBC-binding sites, may explain the differences in MBC sensitivity. Based on homology modeling of tubulin and a model of how MBCs dock with tubulin, Robinson et al. (2004) proposed that the MBC-binding domain consists of amino acid residues 6, 50, 134, 165, 167, 198, 200, and 257. In support of this, point mutations of β tubulin that conferred MBC resistance in field strains occurred in amino acids 6, 50, 134, 165, 167, 198, 200, 240, 241, 250, and 257 (Albertini et al. 1999; McKay et al. 1998; Orbach et al. 1986; Thomas et al. 1985). In this study, we compared the amino acid sequences of β 1 and β 2 tubulin in *F. graminearum*, benA in *A. nidulans*, tub2 in *C. gloeosporioides*, and tub2 in *T. viride* (Fig. 6). Except for amino acid 240, the amino acids listed earlier in this paragraph were consistent among the five β tubulins. In *F. graminearum*, codon 240 is phenylalanine in β 2 tubulin but leucine in β 1 tubulin. An L240F substitution has been reported to confer MBC resistance in fungi (Albertini et al. 1999). In addition, an L240I mutation in HM40 (class I) β tubulin has been identified in VCR-resistant cells (Kavallaris et al. 2001). An F240L substitution in β 2 tubulin (strain R β 2tub240Leu#3, preserved in our laboratory (Zhang et al. 2009)) greatly increased the MBC sensitivity of *F. graminearum* strain R β 2tub240Leu#3 (its EC₅₀ was 0.05 μ g/ml) relative to that of the parental strain ZF21 (its EC₅₀ was 0.5 μ g/ml). This result indicated that the difference in amino acid 240 in β tubulin determined the difference in the affinity to MBC fungicides. However, how the mutation in amino acid 240 in β tubulin alters MBC affinity is unclear and warrants additional research.

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