β1 tubulin rather than β2 tubulin is the preferred binding target for carbendazim in *Fusarium graminearum*

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Abstract

Tubulins are the proposed target of anticancer drugs, anthelminthics, 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 vs. 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*.

Introduction

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 anti-tumor drugs, herbicides, anthelminthics, 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 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 authors' 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; Chen *et al.*, 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, *ben*A and *tub*C, 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.

In the current study, we first developed dose-response curves to describe the carbendazim sensitivity (in terms of mycelial growth) of β1 and β2 tubulin deletion mutants of *F. graminearum.* To understand the functional significance of β1 and β2 tubulin isotypes in drug responses, we produced two antibodies that specifically

recognize these β tubulin isotypes and then characterized the protein accumulation level of β1 or β2 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 β1 and β2 tubulin and the hypothesis was supported by carbendazim sensitivity tests of a *F. graminearum* strain with the F240L mutation in β2 tubulin.

Materials and Methods

Strains, plasmids, primers, and culture conditions

The *F. graminearum* strains, plasmids, and primers used in this study are listed in 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 L 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 L 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 (w/v, 1% peptone, 0.3% yeast extract, and 2% glucose). *Escherichia coli* strain DH5α 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 L of distilled water) at 37 °C.

Production of recombinant tubulins in *E. coli* for antigens

Recombinant *F. graminearum* β1 and β2 tubulin were expressed and refolded as described previously (Zhang *et al.*, 2011). β1 and β2 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, USA) according to the manufacturer's instructions.

Preparation and purification of the polyclonal antibody specific to β1 tubulin

The polyclonal antibody specific to β1 tubulin was raised according to Bo et al. (2008). The recombinant β1 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, China). 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, USA), cross absorbed with a homemade NHS-β2 tubulin affinity column (NHS actived seahorse [GE, USA] coupled with recombinant β2 tubulin according to the manufacturer's instructions), and stored at -20℃. 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 β2 tubulin

A monoclonal antibody (McAb) specific to β2 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 β2 tubulin and Quick antibody adjuvant (Biodragon, Beijing, China) every 2 weeks. Each mouse received two intramuscular injections (100 μl per injection) containing 50 μg 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 micrititre plates (Nunc; 442404) (50 μl 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, USA). 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 2Δβ1 and 2Δβ2 (Table 1) were complemented with β1 and β2 tubulin full-length DNA (without the stop codon) of strain 2021 fused with the GFP gene, respectively (Fig. S2). Transformants were selected on a medium containing 0.2 μM floxuridine (Solarbio, Beijing, China). For analysis of the transformants by Southern blot, genomic DNA of 2β1-GFP (β1-GFP fusion) and 2β2-GFP (β2-GFP fusion) were digested by *Xho* Ⅰ and *Hind* Ⅲ (TaKaRa, Dalian, China), respectively, and hybridized with probe 1 and probe 2 (Figure S2), respectively.

MBC dose-response curves based on mycelial growth

The sensitivity of *F. graminearum* strains (2021, 2Δβ1, and 2Δβ2) 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: 0, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, 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 (EC_{50}) 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, USA) 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

199 excitation and emission wavelengths of 475 nm and 505 nm, respectively.

200 **Analysis of the β tubulin amino acid sequences from *F. graminearum*, *A. niduluns*,**

201 ***C. gloeosporioides*, and *T. viride***

202 Amino acid sequences for β tubulins from four fungal species were obtained from the

203 following references: *F. graminearum* (Qiu *et al*., 2012), *A. niduluns* (May *et al*.,

204 1987), *C. gloeosporioides* (Kongtragoul *et al*., 2011), and *T. viride* (Goldman *et al.*,

205 1993). The β tubulin amino acid sequences from the four fungi were aligned using

206 DNAMAN sequence analysis software (Lynnon Biosoft). The identities among the

207 sequences were calculated using DNAMAN, and a homology tree was constructed

208 using DNAMAN.

209 **Results**

210 **Antibodies specifically recognize the β-tubulin isotypes in *F. graminearum.***

211 β1 and β2 tubulin of *F. graminearum* were expressed in *E. coli* and purified as

212 antigens for antibody production. The purities of the recombinant proteins were > 95%

213 as determined by SDS-PAGE (Fig. S1). Rabbit polyclonal antiserum was generated

214 against purified recombinant *F. graminearum* β1 tubulin. The antiserum was applied

215 to a homemade β2 tubulin affinity column, and the flow-through fraction (which

216 specifically reacted with β1 tubulin without cross reacting with β2 tubulin) was

217 termed *β1-pAb*. The monoclonal antibody preparation generated two clones, *2F11* and

218 *4B5*, both of which appeared to be highly specific for β2 tubulin. *2F11* was selected

219 for further testing and was termed *β2-mAb*. The titers of *β1-pAb* and *β2-mAb* as

220 determined by indirect ELISA were 1.28×10^5 and 1×10^6, 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 EC_{50} 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 (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 and Fig. S3). The colony and hyphal morphologies of 2β1-GFP were similar to those of the parental strain 2021 (Fig. S3). 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 (Fig. S3). Furthermore, the EC_{50} 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 0.5 μg/mL carbendazim, 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 0.5 μg/mL carbendazim, the GFP-fused β2-tubulin formed normal-appearing microtubules in immature plastids (Fig. 5G). In the presence of 1.4 μg/mL carbendazim, 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. niduluns*,

287 ***C. gloeosporioides*, and *T. viride***

288 The identity between the amino acid sequence of *F. graminearum* β2 tubulin and the
289 sequences of *F. graminearum* β1 tubulin, *A. niduluns* benA, *C. gloeosporioides* tub2,
290 and *T. viride* tub2 were 76.4, 76.2, 76.0, and 75.5%, respectively (Fig. 6B). The
291 identity between the amino acid sequence of *F. graminearum* β1 tubulin vs *A.*
292 *niduluns* benA, *C. gloeosporioides* tub2, and *T. viride* tub2 were 94.4, 96.6, and
293 93.7%, respectively. A homology tree of the five β tubulin amino acid sequences was
294 generated using DNAMAN (Fig. 6B). Overall, the homologies for *A. niduluns* benA,
295 *C. gloeosporioides* tub2, and *T. viride* tub2 were higher for *F. graminearum* β1 tubulin
296 than for *F. graminearum* β2 tubulin.

297 **Discussion**

298 Tubulins are the primary target of a large and ever-growing number of small
299 molecules. β2 tubulin in *F. graminearum* has long been recognized as the
300 MBC-binding target. The effects of β1 tubulin on *F. graminearum* mycelial growth,
301 spore germination, and pathogenicity have been investigated by the gene knockout
302 method (Qiu *et al*., 2012). Additionally, the role of β1 tubulin in MBC resistance was
303 studied using comparisons of MBC EC_{50} and MIC values of β1 and β2 tubulin
304 deletion mutants. Furthermore, sequence alignment between *F. graminearum* β1 and
305 β2 tubulin and homology modeling of *F. graminearum* β1 tubulin were previously
306 performed, raising the hypotheses that β1 tubulin was the target of MBCs and the
307 difference of binding affinity of β1 and β2 tubulin to MBCs resulted from the 240
308 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 anti-tubulin drugs. For these reasons, the current study investigated the mechanism underlying the difference in the sensitivity of the two β tubulin subunits to the MBC fungicide carbendazim.

Although the EC_{50} 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 β1 and β2 tubulin deletion mutants to carbendazim. Most importantly, the deletion of β2 tubulin increased sensitivity to carbendazim, while the deletion of β1 tubulin greatly decreased sensitivity to low concentrations of carbendazim. These results are consistent with the inference that the carbendazim-binding affinity is greater for β1 than for β2 tubulin. Furthermore, we proposed the hypothesis that the MIC value of *F. graminearum* to carbendazim was determined by the low affinity target of carbendazim (β2 tubulin). The evidence supporting this hypothesis was that *F. graminearum* 2021 and 2Δβ1 strains have the same MIC value (Fig. 2). In addition, the *F. graminearum* strains with a β2 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 β2 tubulin, as indicated by mRNA level, increased MBC fungicide resistance in *F. graminearum* (Qiu *et al*., 2012). In the current study, to eliminate the possibility that the differences in carbendazim sensitivity among the three strains (2021, Δβ1 and Δβ2) 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 β1 tubulin did not differ between the β2 tubulin deletion mutant and the parental strain 2021, and the protein accumulation level of β2 tubulin did not significantly differ between the β1 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 β1 tubulin resulted in a two-fold increase in the mRNA expression level of β2 tubulin in *F. graminearum* (Qiu *et al*., 2012), a significant change in the protein expression of β2 tubulin was not detected in the 2Δβ1 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 β1 tubulin is more sensitive to carbendazim than β2 tubulin. Thus, when strain 2021 was

353 treated with a low concentration of carbendazim, the β1 tubulin level was reduced by
354 47%, in comparison to only 6% in the case of β2 tubulin; it may be due to the fact that
355 MBC fungicides preferentially bind to the β1 tubulin, which converts β1 tubulin to an
356 unstable conformation, and eventually leads to tubulin degradation (Garland, 1978);
357 on the other hand, the remaining carbendazim binding to β2 tubulin was relative very
358 low and caused a lower reduction of β2 tubulin level in contrast to β1 tubulin.
359 Similarly, a high concentration of carbendazim reduced the β1 tubulin level in strain
360 2021 by 87% but reduced the β2 tubulin level by only 24%; it is logical that the more
361 carbendazim binds to β1 and β2 tubulin, the higher the inhibition ratio it will be;
362 However, even treated with the MIC (minimum inhibitory concentration) dose of
363 carbendazim, there is still detectable level of β1 and β2 tubulin in the mycelia,
364 perhaps the degradation of β tubulin was a time consuming process. In conclusion,
365 this difference in sensitivity to MBC fungicides may result from the difference in
366 MBC binding affinity between β1 and β2 tubulin.

367 β2 tubulin in *F. graminearum* has long been recognized as the MBC-binding
368 target (Chen *et al.*, 2009; Qiu *et al.*, 2011). To observe the dynamic behavior of
369 microtubules and their associated proteins in living cells, researchers have fused the
370 genes encoding cytoskeleton proteins (such as tubulin) and green fluorescent protein
371 (GFP) in a variety of fungi including *A. nidulans* (Fernández-Ábalos *et al.*, 1998;
372 Horio and Oakley, 2005), *Magnaporthe grisea* (Czymmek *et al.*, 2005), and
373 *Neurospora crassa* (Freitag *et al.*, 2004). In this study, we generated one mutant of
374 2021 in which GFP was fused with β1 tubulin (2β1-GFP) and another in which GFP

was fused with β2 tubulin (2β2-GFP). We observed that both β1 and β2 tubulin assembled into cytoplasmic microtubules during spore germination, indicating that both β tubulin isotypes function well. When the strains were treated with 0.5 μg/ml carbendazim (the EC_{50} for 2021), 2β1-GFP exhibited scattered fluorescence and failed to form normal microtubules, but 2β2-GFP was not visibly affected. When the carbendazim concentration was increased to 1.4 μg/ml (the EC_{90} for 2021), normal microtubules were not evident in either 2β1-GFP or 2β2-GFP. These results indicate that carbendazim preferentially disrupts microtubules consisting of the β1 tubulin isotype rather than the β2 tubulin isotype, i.e., carbendazim binding affinity is greater for β1 tubulin than for β2 tubulin. These results were consistent with the effects of MBC on β tubulin levels that were discussed in the previous paragraph.

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, β1 tubulin but not β2 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* β1 tubulin clade consists of the canonical tubulin gene whereas β2 tubulin is the additional copy; one reasonable explanation is that the *Fusarium* β1 tubulin is under strong purifying selection but the β2 tubulin is under divergent selective pressure (Zhao *et al*., 2014). It is consistent with the observation that any polymorphism in the target gene β1 tubulin from field resistant strains of *F. graminearum* has not been detected yet; in contrast, all the field resistant strains of *F. graminearum* have β2 tubulin point mutations (Chen *et al.*, 2008). Furthermore, it is

speculated that β2 tubulin is more important than β1 tubulin in the life cycle of *F. graminearum* and point mutations protect the β2 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 β2 tubulin in *F. graminearum* results in the significantly slower mycelia growth and decrease in sporulation capacity; in contrast, deletion of β1 tubulin in *F. graminearum* results in the slightly slow growth and an increase in sporulation capacity, indicating the β2 tubulin is important for vegetative growth and asexual reproduction. Second, our group has demonstrated that the absolute protein content of β2 tubulin is about 11 fold to that of β1 tubulin in strain 2021 (data unpublished); the relatively abundant β2 tubulin in *F. graminearum* indicates that β2 tubulin has wider spatiotemporal distribution (indicated the more important functions). On the other hand, if the polymorphism occurs in β1 tubulin, MBCs preferentially bind to β2 tubulin, in consideration of the importance of β2 tubulin, these strains have lower survival rate under MBC pressure.

Sequence differences between the β1 and β2 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, Robinsona *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 β1 and β2 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 β2 tubulin but leucine in β1 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 β2 tubulin (strain Rβ2tub240Leu#3, preserved in our laboratory (Zhang *et al.*, 2009)) greatly increased the MBC sensitivity of *F. graminearum* strain Rβ2tub240Leu#3 (its EC_{50} was 0.05 μg/ml) relative to that of the parental strain ZF21 (its EC_{50} was 0.5 μg/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.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION LEGENDS

Table S1. Plasmids, vectors, and primers used in this study.

Figure S1. SDS-PAGE analysis of the recombinant tubulins expressed in *E. coli*. M, protein marker; β1, purified recombinant β1 tubulin expressed in the pET28a vector; β2, purified recombinant β2 tubulin expressed in the pET28a vector.

Figure S2. Gene complementation strategy for fusion of (A) *β1-GFP* and (B) *β2-GFP* into *β tubulin* deletion mutants of *F. graminearum* 2021. (1) Genotype of *β1* or *β2 tubulin* deletion mutants of *F. graminearum*. (2) Complementation construct containing upstream and downstream fragments of the *β1* or *β2 tubulin* flanking a *β tubulin-GFP* fusion cassette, and (3) Genotype of a *β1* or *β2 tubulin*-GFP fusion mutant in which the ORF is replaced by a *β tubulin-GFP* cassette. C and D: Southern blot analysis of the strain (2021) and *β1* and *β2 tubulin-GFP* fusion mutants hybridized with a β1 tub probe and a β2 tub probe, respectively.

Figure S3. Colony and hyphal morphology of *F. graminearum* GFP-β tubulin fusion mutants (2β1-GFP and 2β2-GFP) and their parental strain, 2021. (A) Colonies of the parental strain (2021) and the GFP-β tubulin fusion mutants (2β1-GFP and 2β2-GFP) were photographed after they had grown on solid media [potato sucrose agar (PSA)] for 3 days at 25 °C. (B) Hyphal morphology of 2021, 2β1-GFP, and 2β2-GFP after 36 h in YEPD medium. Bar, 40 μm.

559 **Table 1.** Some properties of the *Fusarium graminearum* strains in this study.

Strain	Genotype	[a]EC_{50} (μg/ml)	[b]MIC (μg/ml)	[c]Mycelial growth rate (mm/day)	Sporulation (10^6 CFU mL^{-1})	Reference
2021	Wild type	0.54	1.4	26.75±0.17	2.35±0.07	(Chen *et al*., 2008)
2Δβ1	β1 tub deletion mutant in 2021 genetic background	0.80	1.4	22.42±0.32	2.58±0.18	(Qiu *et al*., 2012)
2Δβ2	β2 tub deletion mutant in 2021 genetic background	0.10	0.5	13.67±0.54	0.20±0.05	(Qiu *et al*., 2012)
2β1-GFP	β1 tub-GFP fusion mutant in 2021 genetic background	0.51	1.4	26.24±0.21	2.52±0.11	This study
2β2-GFP	β2 tub-GFP fusion mutant in 2021 genetic background	0.57	1.4	25.14±0.19	2.23±0.09	This study
ZF21	Field type	0.50	1.4	26.45±0.18	2.40±0.08	(Zhang *et al*., 2009)
Rβ2tub240Leu#3	F240L mutation in β2-tubulin gene in ZF21 genetic background	0.05	-	25.30±0.16	2.26±0.11	(Zhang *et al*., 2009)

560 [a] Fungicide (MBC) concentration that resulted in 50% inhibition of mycelial growth.

561 [b] MIC: minimum inhibitory concentration.

562 [c] Measured after 3 days at 25°C.

563 - , undetermined.

564 Values are means of three experiments [differences among the experiments were not significant,

565 i.e., P >0.05, Fisher's least significant difference (LSD) test]. Standard errors are indicated for

566 growth rate and sporulation.

567

Figure legends

Figure 1. Specificity of the antibodies to the β tubulin isotypes in *F. 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, SDS-PAGE analysis of the total proteins of the three *F. graminearum* strains. WT: 2021. Actin expression was detected simultaneously as the loading control.

Figure 2. Dose-response curves of *F. graminearum* β1 and β2 tubulin deletion mutants (2Δβ1 and 2Δβ2) and their parental strain 2021 to MBC.

Figure 3. Protein accumulation levels of β tubulins in strains 2021, Δβ1, and Δβ2 of *F. 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).

Figure 4. Protein accumulation levels of β tubulins in *F. graminearum* strain 2021 in response to low and high concentrations of MBC. A, Protein accumulation level of β1 and β2 tubulins in 2021 in response to carbendazim at 0.0, 0.5, and 1.4 μg/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 non-treated culture (0 μg/ml MBC). B, Representative results of western blot analysis.

Figure 5. Characteristics of microtubules with expression of GFP-tagged β1-tubulin (2β1-GFP) or GFP-tagged β2-tubulin (2β2-GFP) in *F. graminearum*. The morphology of microtubules in conidia or germling untreated with MBC (A, B, E, and F), treated with 0.5 μg/ml MBC for 2 hours (C and G), or treated with 1.4 μg/ml MBC for 2 hours (D and H). Right panels with uppercase letters were photographed with fluorescence field (FF)

598 microscopy and corresponding left panels were photographed with bright field (BF)
599 microscopy. The representative microtubules in conidium (white arrow) and germling (red
600 arrow) were indicated. (scale bars = 10 μm).
601
602 **Figure 6.** Analysis of amino acid sequences of β tubulin from *F. graminearum* (*F. g.* β1 and
603 β2 tubulin), *A. niduluns* (*A. n* benA), *C. gloeosporioides* (*C. g* tub2), and *T. viride* (*T. v* tub2).
604 (A) Multiple sequence alignment of the five sequences using DNAMAN. Black dots indicate
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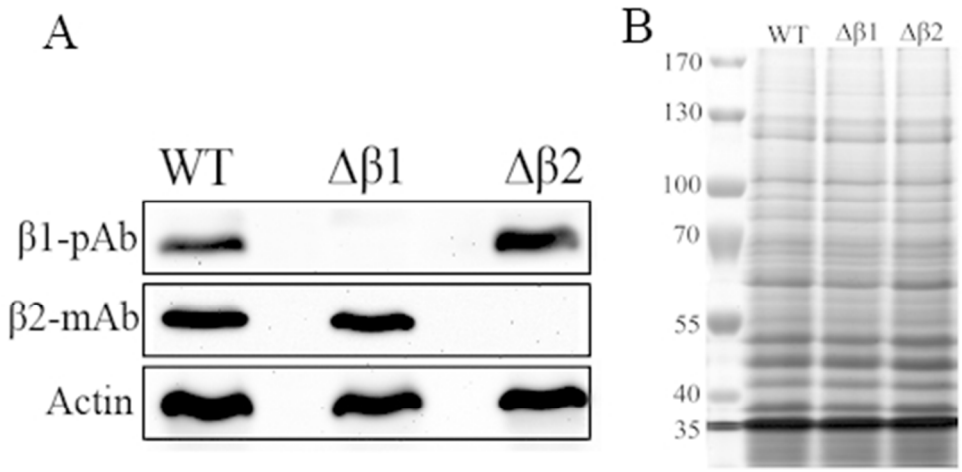
606 167, 198, 200, 240, 241, 250, and 257) that confer MBC resistance according to previous
607 reports. (B) Homology tree of β tubulin from selected fungal species. The percentage of
608 identity between the groups is indicated to the right of the forks. All sequences were from the
609 MBC-sensitive strain.
610



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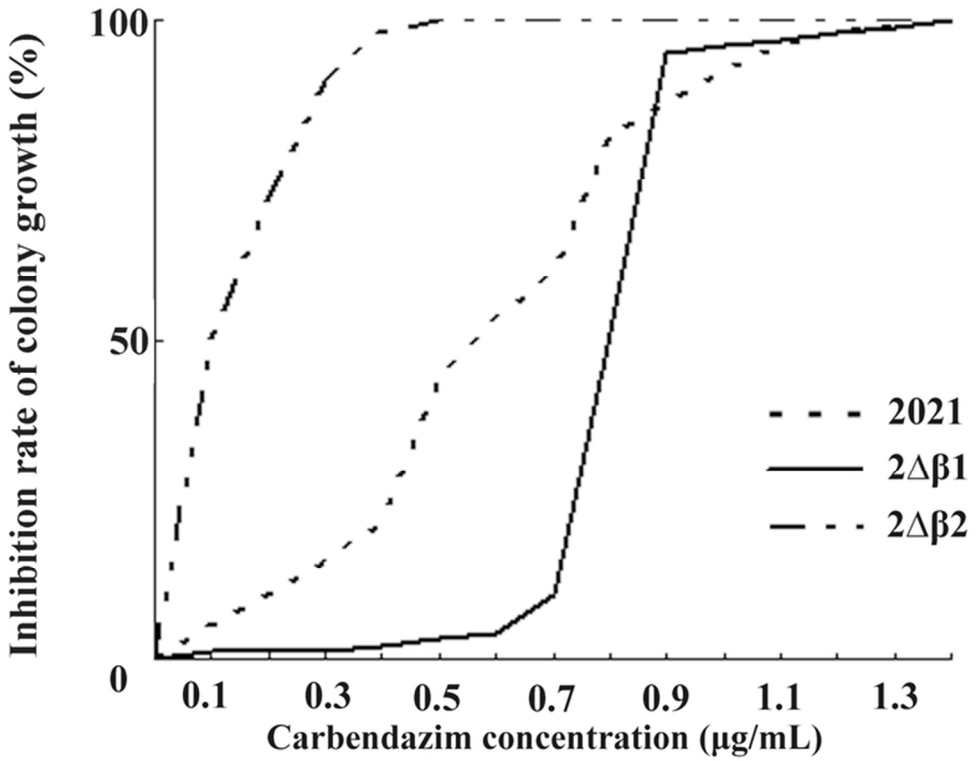

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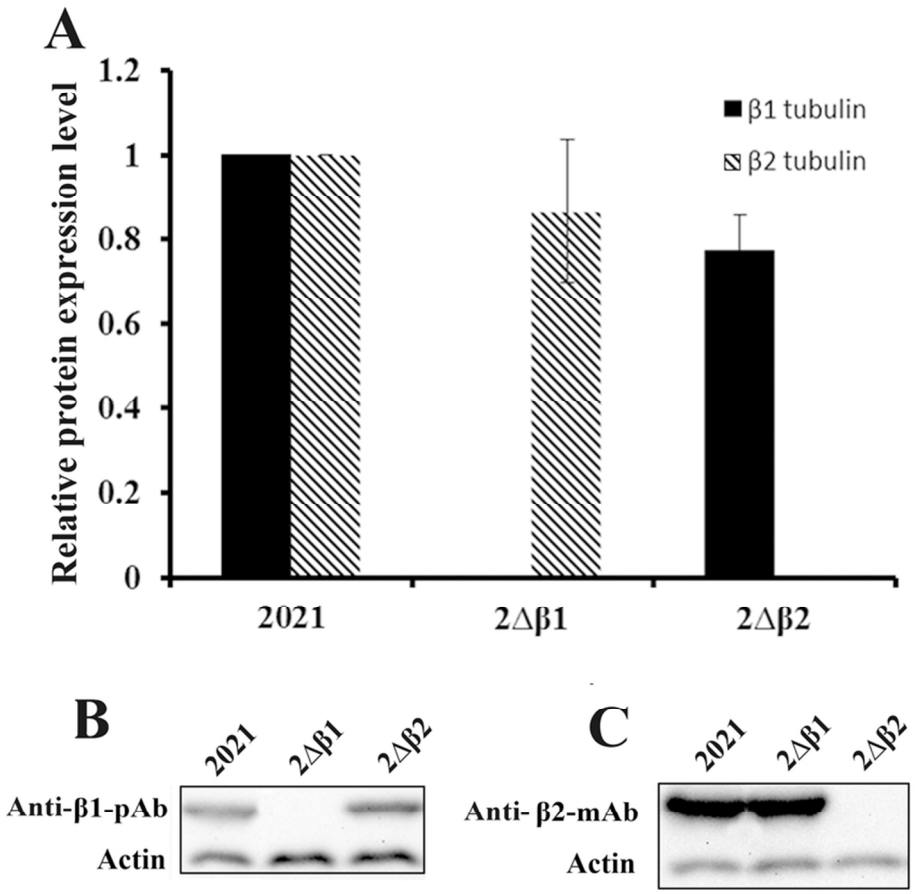
68x53mm (300 x 300 DPI)



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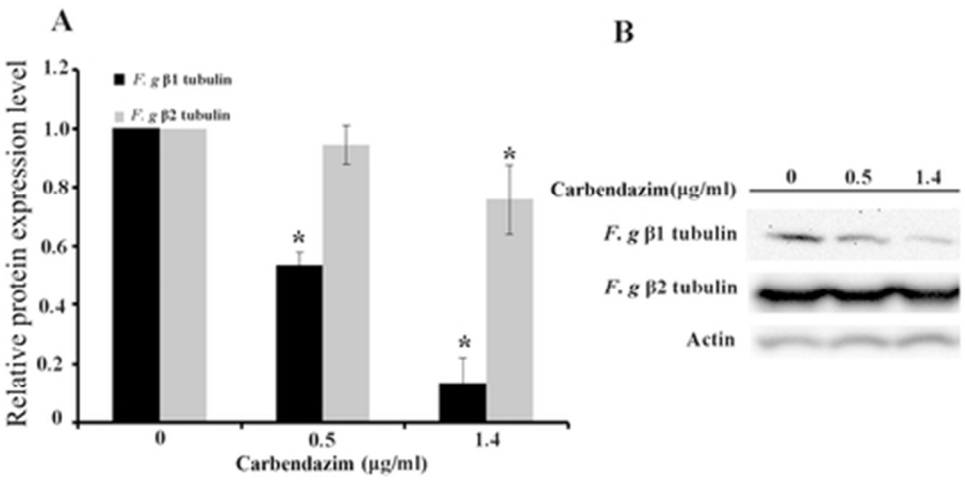
78x72mm (300 x 300 DPI)



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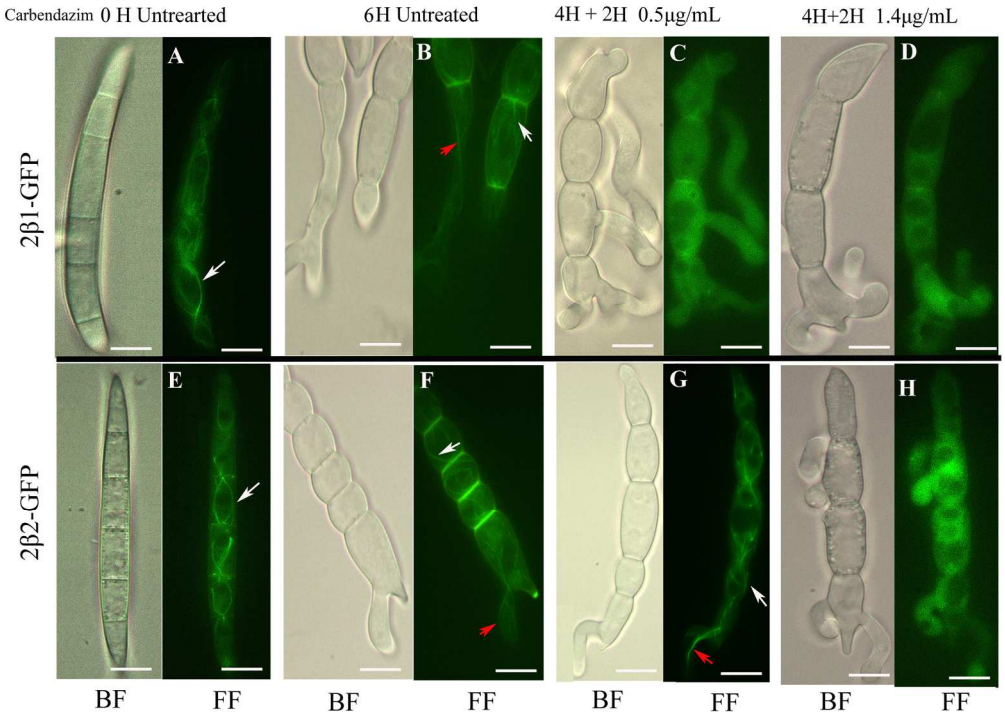
41x20mm (300 x 300 DPI)



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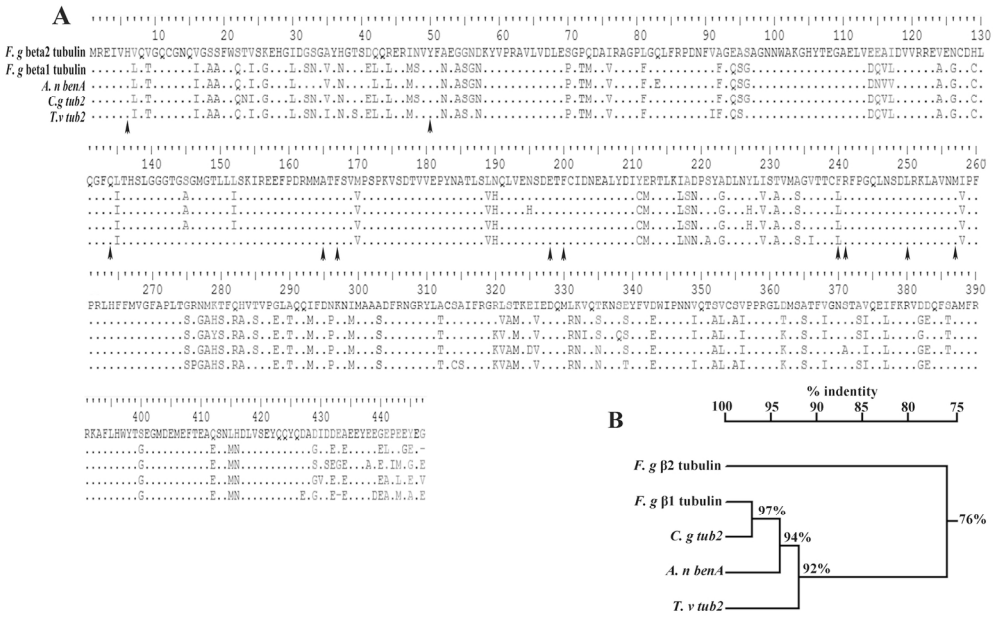
182x131mm (300 x 300 DPI)



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158x99mm (300 x 300 DPI)

β1 tubulin rather than β2 tubulin is the preferred binding target for carbendazim in *Fusarium graminearum*

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SUPPLEMENTARY INFORMATION includes
Supplementary Figures S1-S3 and Table S1

Figure S1. SDS-PAGE analysis of the recombinant tubulins expressed in *E. coli*. M, protein marker; β1, purified recombinant β1 tubulin expressed in the pET28a vector; β2, purified recombinant β2 tubulin expressed in the pET28a vector.

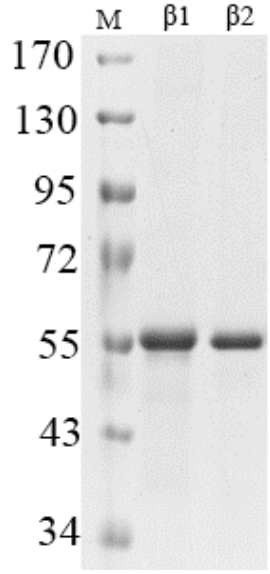

Figure S2. Gene complementation strategy for fusion of (A) *β1-GFP* and (B) *β2-GFP* into *β tubulin* deletion mutants of *F. graminearum* 2021. (1) Genotype of *β1* or *β2 tubulin* deletion mutants of *F. graminearum*. (2) Complementation construct containing upstream and downstream fragments of the *β1* or *β2 tubulin* flanking a *β tubulin-GFP* fusion cassette, and (3) Genotype of a *β1* or *β2 tubulin*-GFP fusion mutant in which the ORF is replaced by a *β tubulin-GFP* cassette. C and D: Southern blot analysis of the strain (2021) and *β1* and *β2 tubulin-GFP* fusion mutants hybridized with a β1 tub probe and a β2 tub probe, respectively.

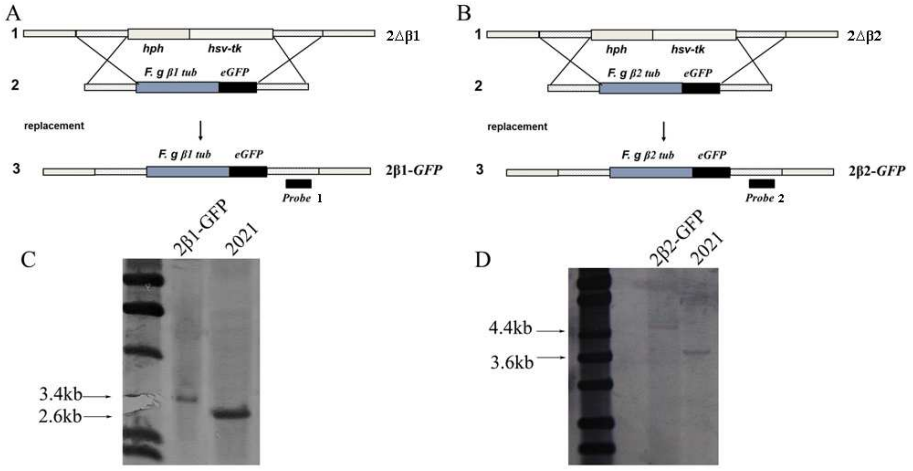

Figure S3. Colony and hyphal morphology of F. graminearum GFP-β tubulin fusion mutants (2β1-GFP and 2β2-GFP) and their parental strain, 2021. (A) Colonies of the parental strain (2021) and the GFP-β tubulin fusion mutants (2β1-GFP and 2β2-GFP) were photographed after they had grown on solid media [potato sucrose agar (PSA)] for 3 days at 25 °C. (B) Hyphal morphology of 2021, 2β1-GFP, and 2β2-GFP after 36 h in YEPD medium. Bar, 40 μm.

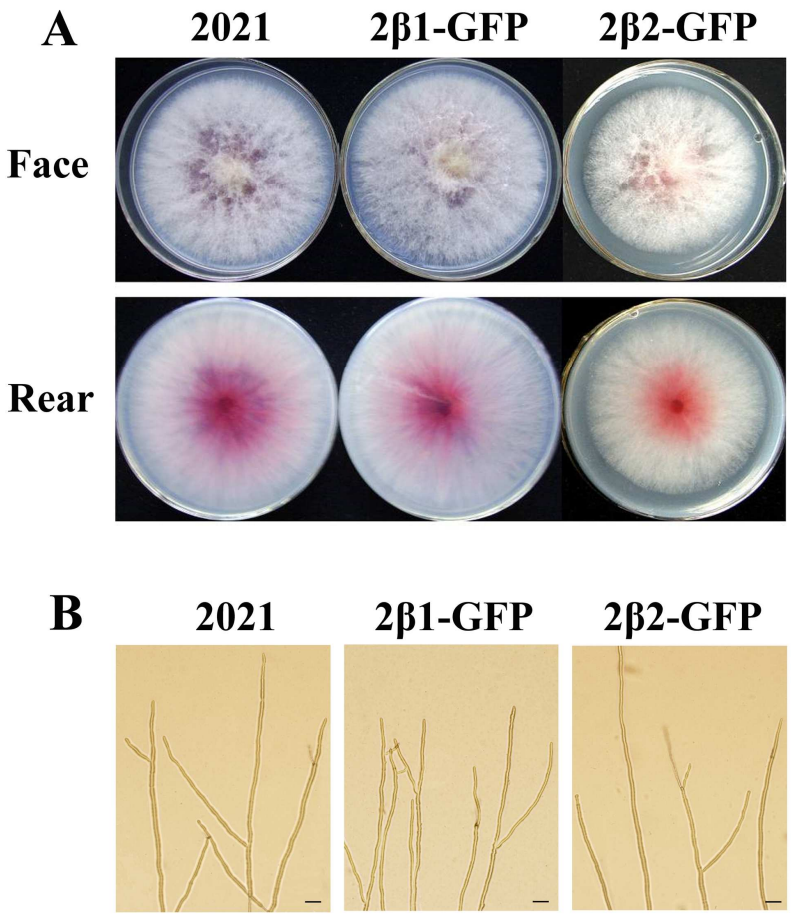

Table S1. Plasmids, vectors, and primers used in this study.

Plasmid	**Use**	
pET28a	Production of tubulin in *E. coli* for antigen	
pAcGFP1	Amplification of the *GFP* gene	
Primer	**Sequence (5'→ 3')**	**Use**
F1	ACGGGATCCATGCGCGAGATTGTTCACC	Clone *β1 tubulin* into pET28a
R1	AGCAAGCTTTTACTCCTCGCCCTCAGGCAG	
F2	GTCCCATGGCTATGCGTGAGATTGTCCACGTC	Clone *β2 tubulin* into pET28a
R2	GTCAAGCTTTCCACCCTCGTACTCCTCGGGCTC	
A1	TCCGTGTTACTTTGCTTTGC	Amplify upstream of *β1 tubulin*
A2	CCACCAGCCAGCCAACAGCTCCCCAGGGCGGTACTTTCTTGAC	
A3	CAATACGCAAACCGCCTCTCCCCGCCTGAGGGCGAGGAGTAAG	Amplify downstream of *β1 tubulin*
A4	CGAGACGGAGAATGGCTGTG	
A5	CGGTTTACTGTCTGGTTTCTGTTC	Amplify upstream of *β2 tubulin*
A6	GAAATACATACAATCTCACGCATCTTGACAGATTTAGTTGAT	
A7	CAATTGAGGGCGAGGAATAGATATTGGTTGGGAACGTTGG	Amplify downstream of *β2 tubulin*
A8	GGAGCGAGGTGCTACTTGGCGAAT	
A9	ATGGTGAGCAAGGGCGAGGAG	Amplify *GFP* from pAcGFP1 vector
A10	ATTACTTGTACAGCTCGTCCATGCCG	
P1F	GGGCGTAGCCGTTGTAAT	Probe 1 for Southern blotting
P1R	CTCGTCCTAATGCCTTCC	
P2F	AGCCGAGCACAAAGACAC	Probe 2 for Southern blotting
P2R	GAAAGAGCCAGCAATCCC	

The underscores indicate the sites for restriction digest.