Identification and Characterization of Carbendazim-Resistant Isolates of *Gibberella zeae*

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ABSTRACT

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Sensitivity of *Gibberella zeae* to carbendazim was determined by measuring mycelial growth in fungicide-amended media. Among 1,529 isolates tested, 31 isolates showed a high level of resistance (HR) to carbendazim (fungicide concentration that results in 50% inhibition of mycelial growth [EC₅₀] of 10.35 to 30.26 mg a.i. liter⁻¹) and 10 isolates were moderately resistant (MR) (EC₅₀ of 4.50 to 7.28 mg a.i. liter⁻¹). The remaining 1,488 isolates were sensitive to carbendazim and were unable to grow on potato dextrose agar amended with carbendazim at 2 mg a.i. liter⁻¹. Analysis of DNA sequences of the β 2-tubulin (*Tub2*) gene showed that all 10 MR isolates had a point mutation at codon 198 causing a replacement of glutamic acid by glutamine. At the codon position 167, the amino acid phenylalanine was replaced by tyrosine in 28 of 31 HR isolates. The remaining three HR isolates had a point mutation at codon 200 which converted phenylalanine to tyrosine. Based on these point mutations in the *Tub2* gene, allele-specific polymerase chain reaction primers were developed for rapid detection of the point mutations. The rapid molecular method will be a valuable tool for the monitoring of carbendazim resistance in *G. zeae*. Additionally, deletion of the β 1-tubulin gene (*Tub1*) in the HR isolate GJ33 resulted in increased resistance to carbendazim. These results indicate that *Tub1* plays a role in the sensitivity of *G. zeae* to carbendazim.

Fusarium head blight (FHB), caused by *Gibberella zeae*, is a devastating disease of wheat in China. Disease epidemics can lead to significant yield loss in the more than 5 million hectares of wheat grown each year. From 1991 to 2007, there were two severe and seven moderate epidemics of FHB in the middle to lower reaches of the Yangtze River. In 2003, the proportion of scabbed spikes was 60 to 80% in this region, which resulted in greater than 20% yield losses (18). Because most wheat cultivars planted in China are susceptible to *G. zeae*, methyl benzimidazole carbamate (MBC) fungicides, particularly carbendazim and thiophanate-methyl, have been used extensively to control FHB during the past three decades. Because MBC-resistant isolates of *G. zeae* were first detected in 1992 in China, a decline in the control of FHB by MBC has been observed frequently in the Zhejiang and Jiangsu Provinces (3). Therefore, in order to control FHB effectively, it is necessary to monitor the development of MBC-resistant *G. zeae* populations in China.

Resistance to MBC fungicides has been reported for many phytopathogenic fungi, including *Botrytis cinerea*, *Cladobotryum dendroides*, *Helminthosporium solani*, *Monilinia fructicola*, *M. laxa*, *Tapesia yallundae*, and *Venturia inaequalis* (1,7,8,12). In most cases, the resistance is caused by point mutations in the β -tubulin gene which result in altered amino acid sequences at the MBC-binding site (12). In *G. zeae*, two homologous β -tubulin genes (*Tub1* and *Tub2*) exist in the fungal genome. A previous study showed that resistant and sensitive isolates of *G. zeae* had identical sequences of *Tub1* (3). The resistance of *G. zeae* to MBC is primarily due to a single point mutation at codons 167 or 200 in *Tub2* (FGSG 06611) (4,11). Double mutations at codons 73 and 198 in *Tub2* were also detected from a highly resistant isolate but the frequency of this genotype was very low in field populations (4). Many previous studies have shown that changes at codons 6, 50, 167, 198, 200, and 240 in the β -tubulin gene could cause MBC resistance in several pathogenic fungi (12). Thus, it is necessary to determine whether such mutations also occur in *G. zeae*.

The objectives of this study were to (i) screen carbendazim-resistant isolates from a collection of 1,529 isolates of *G. zeae*, (ii) investigate molecular mechanisms of carbendazim resistance in field isolates of *G. zeae*, (iii) develop a molecular technique for the rapid detection of alleles mediating MBC resistance in *G. zeae*, and

(iv) investigate the contribution of *Tub1* to the sensitivity of *G. zeae* to carbendazim.

MATERIALS AND METHODS

Survey of sensitivity of *G. zeae* to carbendazim. In total, 1,529 single-spore isolates of *G. zeae* used in this study were collected from six provinces of China, including Anhui, Jiangsu, Hebei, Henan, Shandong, and Zhejiang, in 2008 and 2009 (Table 1). Each isolate was recovered from a single infected wheat head and no more than 10 isolates were collected from a single field.

Carbendazim (50% a.i.; Sicuan Guo-Guang Agricultural Chemical Company Ltd., China) was suspended in sterilized water and adjusted to 100 mg a.i. liter⁻¹. The fungicide was added to potato dextrose agar (PDA) after autoclaving at the following concentrations: 0, 2, 10, 50, 100, or 500 mg a.i. liter⁻¹. Single 5-mm mycelial plugs were taken from the edge of a 2-day-old colony of each isolate and placed on each concentration of fungicide-amended PDA. Four replicated plates were used for each fungicide concentration. Petri plates were incubated at 25°C for 3 days in the dark and then radial growth (in millimeters) of each isolate was measured, with the original mycelial plug diameter (5 mm) subtracted from this measurement. The experiment was repeated.

The fungicide concentration that results in 50% inhibition of mycelial growth (EC₅₀) for 10 sensitive and 41 resistant isolates identified above was determined by assessing radial growth on PDA plates amended with carbendazim at 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 mg a.i. liter⁻¹. There were three replicates for each isolate at each concentration. After the plates were incubated at 25°C for 3 days in the dark, radial growth (in millimeters) of each isolate was measured as described above. The experiment was performed three times. For each plate, the average colony diameter of each isolate at each fungicide concentration was used for calculation of the EC₅₀ value with a data processing system (Hangzhou Reifeng Information Technology Ltd., Hangzhou, China).

Analysis of DNA sequences of *Tub2* from carbendazim-resistant isolates of *G. zeae*. Six highly resistant (HR) isolates (HA29, HA36, HA70, HN40, HY11, and GJ33), two moderately resistant (MR) isolates (HA34 and XS19), and one sensitive isolate (PH-1) were used for DNA

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sequence analysis of *Tub2*. Fungal DNA was extracted by using a previously published protocol (16). Briefly, approximately 100 mg of mycelia of each isolate were harvested from PDA plates, transferred to a 1.5-ml microtube, and ground with a motor-driven glass pestle in 1 ml of lysis buffer (200 mM Tris-HCl, 50 mM ethylenediaminetetraacetic acid, 200 mM NaCl, and 1% (wt/vol) n-lauroylsarcosine sodium salt, pH 8.0). The mixture was

incubated for 10 min at room temperature before being centrifuged at $18,000 \times g$ for 5 min at 4°C. The supernatant (800 ml) was transferred to a clean microtube and mixed with an equal volume of isopropanol. The resulting DNA pellet was precipitated by centrifugation at $18,000 \times g$ for 2 min at 4°C. After a wash with 70% ethanol, the DNA pellet was air dried and dissolved in 100 ml of 100 mM Tris-HCl buffer (pH 8.0).

The primer pair Tub2-seq-F + Tub2-seq-R (Table 2) was designed to amplify a partial sequence of *Tub2*. Polymerase chain reaction (PCR) was performed with the following parameters: preheating for 3 min at 95°C and 35 cycles of denaturation at 94°C for 40 s, 55°C for 40 s, 72°C for 1.5 min, and 72°C for 5 min. Using primer pair Tub2-seq-F + Tub2-seq-R, a 1,306-bp PCR fragment was amplified from each sequenced isolate. PCR products were purified, ligated into the pMD18-T Vector (TaKaRa Biotech. Co., Dalian, China), and then sequenced by Invitrogen Biotech. Co. Ltd. (Shanghai, China). PCR amplifications were performed three times independently for each isolate to avoid mismatch sequences during PCR amplification and sequencing. The sequences were submitted to GenBank under accession numbers GU338401 to GU338408. The DNA sequence of putative exons of the *Tub2* from each isolate was identified based on the exon sequence of *Tub2* which was submitted to the *G. zeae* genome database under accession number FGSG 06611. The nucleotide sequence of exons was translated into amino acid sequences using Edit-Seq 5.01 (DNASTAR Inc., Madison, WI). The deduced amino acid sequences were aligned using the program ClustalW1.82 (<http://www.ebi.ac.uk/clustalw/>; European Bioinformatics Institute, Cambridge, UK).

Design of allele-specific PCR primers for the rapid detection of point mutations in *Tub2* mediating carbendazim resistance. Three pairs of allele-specific primers were designed for detection of the point mutations at codons 167, 198, or 200. Based on the single point mutation from T to A at codon position 167, a nucleotide T at the 3' end of the reverse primer 167-RB was designed to match the A at this codon position in the HR isolates. To improve the specificity of traditional allele-specific PCR amplification, we introduced an artificial mismatch base T at the second nucleotide at the 3' end of the

Table 1. Survey of the sensitivity to carbendazim in *Gibberella zeae* isolates collected from different locations in China

Origin (city, province)	No. of isolates	Year of isolation	No. of carbendazim-resistant isolates
Anqing, Anhui	24	2009	0
Bengbu, Anhui	1	2008	0
Bengbu, Anhui	66	2009	0
Chaohu, Anhui	90	2009	0
Chuzhou, Anhui	81	2009	0
Fuyang, Anhui	1	2008	0
Fuyang, Anhui	160	2009	1
Hefei, Anhui	3	2008	0
Huainan, Anhui	2	2008	0
Huainan, Anhui	26	2009	0
Liu'an, Anhui	20	2008	0
Liu'an, Anhui	21	2009	0
Ningguo, Anhui	3	2008	0
Ningguo, Anhui	19	2009	0
Tongcheng, Anhui	15	2009	0
Wuhu, Anhui	3	2008	0
Xuanzhou, Anhui	3	2008	0
Hai'an, Jiangsu	80	2008	4
Hai'an, Jiangsu	170	2009	12
Jingjiang, Jiangsu	42	2008	2
Ruancheng, Hebei	30	2009	0
Shenzhou, Hebei	50	2009	0
Xinji, Hebei	28	2009	0
Jiaozuo, Henan	22	2009	0
Luohe, Henan	52	2009	0
Luoyang, Henan	2	2009	0
Tai'an, Shandong	102	2009	0
Haining, Zhejiang	74	2009	2
Haining, Zhejiang	10	2008	4
Haiyan, Zhejiang	9	2008	0
Haiyan, Zhejiang	238	2009	12
Hangzhou, Zhejiang	20	2009	0
Jiaxing, Zhejiang	12	2008	3
Xiaoshan, Zhejiang	50	2008	1

Table 2. List of primers used in this study

Name	Sequence (5'-3')	Description ^a
Tub2-seq-F	ACAAACGCACACACACACA	PCR primers for amplification of the part of <i>Tub2</i>
Tub2-seq-R	AAGATGGCGGAACAAGCGA	
Tub2-F	TTGTAATTGCAAATTTCAACGTG	PCR primers for specifically detection MBC-resistant isolates
167-RB	GCGAGGGCATAACGGATT	
198-RB	CGTTATCGATACAGAAGGTCAG	
200-RB	GCCTCGTTATCGATACATT	
Tub1-up-F	CTCAGTCTGGTACTGCCAACCCACT	PCR primers for amplification of upstream of <i>Tub1</i> gene with exogenous enzyme
Tub1-up-R	GTCGAGTGCCGTTGTAACACCATTTG	
Tub1-down-F	AAGCTTTTCATGTCCGGCGTCACTA	PCR primers for amplification of downstream of <i>Tub1</i> gene with exogenous enzyme
Tub1-down-R	GGATCCTCGTACTCCTCTTCTTCCTCG	
Tub1-id-F	ATCTGCTCTTCCATCTCGTCG	PCR primers for identification of <i>Tub1</i> deletion mutants
Tub1-id-R	TCAAGGGAGCGAATCCGA	
Tub1-RT-F	TTTCTGGCAGACCATCTCTG	Reverse-transcription PCR primers for analysis of expression of <i>Tub1</i>
Tub1-RT-R	AGTTGTCTGGGTCGGAAAA	
Tub2-RT-F	CGCAACATGAAGACTTTCCA	Real-time PCR primers for analysis of expression of <i>Tub2</i>
Tub2-RT-R	ACGGAAGTTTGGACGTTGTT	
F-actin-F	ATCCACGTCACCACTTTCAA	PCR primers for amplification of the reference gene actin in real-time PCR
F-actin-R	TGCTTGGAGATCCACATTTG	

^a PCR = polymerase chain reaction, *Tub2* = β 2-tubulin gene, MBC = methyl benzimidazole carbamate fungicides, and *Tub1* = β 1-tubulin gene.

primer (Table 2) (5). A G at the 3' end of the reverse primer 198-RB was designed to match the C at codon 198 in the MR isolate, and an artificial mismatch base A at the second nucleotide at the 3' end of the primer was introduced. The nucleotide T at the 3' end of the reverse primer 200-RB was designed to match the A at codon 200 in the HR isolate, and an artificial mismatch base T at the second nucleotide at the 3' end of this primer was introduced. The forward primer Tub2-F was designed based on the sequences of the fifth intron of the *Tub2* from *G. zeae*. This forward primer was expected to be specific for *G. zeae* because we observed that the fifth intron sequence of *Tub2* in *G. zeae* was different from those in other closely related fungal species such as *Fusarium oxysporum* and *F. verticillioides* when the sequences were aligned using the program ClustalW1.82.

To determine the specificity of the allele-specific PCR primers, fungal DNA was extracted from each of the carbendazim-resistant isolates: an additional 459 isolates of *G. zeae*, *F. oxysporum*, *F. verticillioides*, and *F. culmorum* isolated from infected wheat heads. The PCR was performed as described above except for the annealing temperature, which was 58°C.

Construction of β 1-tubulin gene deletion mutants. A *Tub1* deletion vector, pCA-tub1-Del, was constructed by inserting two flanking sequences of the *Tub1* gene into two sides of the hygromycin resistance (*hph*) gene in the pBS-HPH1 vector (10) (Fig. 1A). Briefly, a 481-bp upstream flanking sequence fragment of *Tub1* amplified from genomic DNA of HR isolate GJ33 using the primers Tub1-up-F + Tub1-up-R (Table 2) was inserted into *Xho*I-*Sal*I sites of the pBS-HPH1 vector to generate the plasmid pBS-tub1-up. Subsequently, a 619-bp downstream flanking sequence fragment of *Tub1* amplified from the GJ33 genomic DNA using the primers Tub1-down-F + Tub1-down-R (Table 2) was inserted into *Hind*III-*Bam*HI sites of the pBS-tub1-up vector to generate the plasmid pBS-tub1-UD. Finally, the 2,603-bp fragment containing the *Tub1*-upstream-*hph*-*Tub1*-downstream cassette was obtained by digestion of the plasmid pBS-tub1-UD with *Xho*I and *Bam*HI and ligated into the *Xho*I-*Bam*HI sites of pCambia 1300 (Cambia, Canberra, Australia). The resultant *Tub1* deletion vector pCA-tub1-Del (Fig. 1A) was transformed into *Agrobacterium tumefaciens* strain C58C1. *A. tumefaciens*-mediated fungal transformation was performed for the HR isolate GJ33 using a previously described protocol (14). Briefly, *A. tumefaciens* strain C58C1 containing an appropriate binary vector was grown at 28°C for 2 days in minimal medium (MM) supplemented with kanamycin (100 mg liter⁻¹). The *A. tumefaciens* cells were diluted to an optical density of 600 nm = 0.15 in induc-

tion medium (IM) containing 200 μ M acetosyringone (AS). The cells were grown for an additional 6 h before mixing with an equal volume of a fresh *F. graminearum* conidial suspension (1×10^6 conidia/ml). A 200- μ l sample of the mixture was sprayed onto a nylon membrane (3 by 3 cm) (Millipore Co., Bedford, MA) and plated on IM amended with 200 μ M AS. After incubation at 20°C for 2 days in the dark, the membrane was cut into small pieces (3 by 0.1 cm), and transferred upside down onto PDA plates supplemented with hygromycin B (100 mg liter⁻¹) and cefotaxime (200 μ M). After 5 to 7 days, individual transformants were transferred onto fresh PDA plates amended with hygromycin B at 100 mg liter⁻¹.

Tub1 deletion mutants were identified by primer pair Tub1-id-F+Tub1-id-R and further by southern hybridization analysis using a standard protocol (17) with the probe indicated in Figure 1A.

Analysis of expression of *Tub2* gene in *Tub1* deletion mutants. Expression of *Tub2* was assessed for the *Tub1* deletion mutants and the parent isolate. Five mycelial plugs (5 mm) of each isolate were inoculated in 100 ml of potato dextrose broth medium and incubated at 25°C for 4 days. Before mycelia were harvested for RNA extraction, each mutant was treated with carbendazim at 500 mg a.i. liter⁻¹ for 6 h. RNA from fungal mycelia was extracted using a TaKaRa RNAiso Reagent (TaKaRa Biotech. Co.) according to the

manufacturer's instructions, and cDNA was synthesized by using a RevertAid H Minus First-Strand cDNA Synthesis kit (Fermentas Life Sciences, Burlington, Canada) with the oligo(dT)₁₈ primer. The expression levels of *Tub2* were analyzed with real-time PCR assays using the primers Tub2-RT-F + Tub2-RT-R and the SYBR Green I fluorescent dye in a DNA Engine Opticons 4 System (MJ Research, Waltham, MA). For each sample, PCR amplifications with the primer pair F-actin-F + F-actin-R (Table 2) for the quantification of expression of actin gene were performed as a reference. The experiment was repeated three times. The expression of *Tub2* in the *Tub1* deletion mutant relative to that of the parent isolate was calculated using the 2^{- $\Delta\Delta C_t$} method (9). The analysis of variance procedure of SAS (version 8.0; SAS Institute, Cary, NC) was used to determine significant differences in expression of *Tub2* among the parent isolate and the *Tub1* deletion mutants.

RESULTS

Sensitivity of *G. zeae* to carbendazim.

Among 1,529 isolates, 31 were able to grow slowly (1- to 3-mm colony diameter) on PDA amended with carbendazim at 500 mg a.i. liter⁻¹. The EC₅₀ values for these 31 isolates ranged from 10.35 to 30.26 mg a.i. liter⁻¹, with a mean of 21.04 mg a.i. liter⁻¹ (Table 3). These isolates were designated as HR isolates. Ten MR isolates were able

Fig. 1. Generation and identification of β 1-tubulin (*Tub1*) gene deletion mutants of *Gibberella zeae*. **A**, Gene replacement strategy for *Tub1*. The hygromycin resistance cassette (Hph) is denoted by the large gray arrow. Primer binding sites are indicated by arrows. **B**, Southern blot analysis of transformants. Genomic DNA preparations of the putative deletion mutants Δ *Tub1*-1, Δ *Tub1*-3, and Δ *Tub1*-5 and their wild-type parent (GJ33) were digested with *Apa*LI, separated in an agarose gel, blotted on a nitrocellulose membrane, and hybridized to the 3' flank of the *Tub1* gene. **C**, Reverse-transcription polymerase chain reaction (PCR) analyzed expression of *Tub1* in the *Tub1* deletion mutants Δ *Tub1*-1, Δ *Tub1*-3, and Δ *Tub1*-5 and the parent isolate GJ33. NCK is a negative control without template DNA in the PCR amplification.

to grow slowly (3- to 5-mm colony diameter) on PDA amended with carbendazim at 100 mg a.i. liter⁻¹ but not at 500 mg a.i. liter⁻¹. The EC₅₀ values for these 10 MR isolates ranged from 4.50 to 7.28 mg a.i. liter⁻¹, with a mean of 5.80 mg a.i. liter⁻¹ (Table 3). The remaining 1,488 isolates of *G. zeae* were unable to grow with carbendazim at 2 mg a.i. liter⁻¹ and they were considered sensitive (S) to the fungicide. EC₅₀ values for 10 randomly selected S isolates ranged from 0.56 to 0.64 mg a.i. liter⁻¹, with a mean of 0.59 mg a.i. liter⁻¹.

Point mutations in *Tub2* from HR and MR isolates. Analysis of DNA sequences

of *Tub2* showed that the codon TTT at position 167 was replaced by the codon TAT (F167Y) in three HR isolates (GJ33, HA29, and HN40). The codon TTC at the position 200 was replaced by TAC (F200Y) in three other HR isolates (HA36, HA70, and HY11). In the two MR isolates (HA34 and XS19), a point mutation (E198Q) was detected. The codon GAG for glutamic acid at the position 198 was changed to CAG for glutamine in the MR isolate (Fig. 2). Apart from these mutations at positions 167, 198, and 200, all nine isolates analyzed had identical deduced amino acid sequences of *Tub2*.

Allele-specific PCR primers for the detection of point mutations in *Tub2*. Using the primer pairs Tub2-F + 167-RB and Tub2-F + 200-RB, the expected 398- or 498-bp fragments were amplified only from HR isolates containing changes at codon positions 167 and 200, respectively, but not from any MR or S isolates of *G. zeae* or any isolates of *F. oxysporum*, *F. verticilloides*, or *F. culmorum*. Using the primer pair Tub2-F + 167-RB, a 398-bp fragment was obtained from 28 of 31 HR isolates, and a 498-bp fragment was amplified by the primer pair Tub2-F + 200-RB from the remaining 3 HR isolates. The results indicated that the HR isolates contained a point mutation at either codon 167 or 200. The primer pair Tub2-F + 198-RB amplified a 494-bp fragment only from the MR isolates but not from any HR or S isolates of *G. zeae* or any isolates of *F. oxysporum*, *F. verticilloides*, or *F. culmorum*. Using this pair of primers, the 494-bp fragment was amplified from each of 10 MR isolates (Table 3), suggesting that these isolates had a point mutation at the amino acid position 198 of the *Tub2*.

Sensitivity of *Tub1* deletion mutants to carbendazim. Southern hybridization patterns confirmed that the *Tub1* deletion mutants ($\Delta Tub1$ -1, $\Delta Tub1$ -3, and $\Delta Tub1$ -5) were the result of a single homologous recombination event at the *Tub1* locus (Fig. 1B). Reverse-transcription PCR with primer pair Tub1-RT-F + Tub1-RT-R showed that the *Tub1* gene was not transcribed in the *Tub1* deletion mutants (Fig. 1C).

The *Tub1* deletion mutants were more resistant to carbendazim than their parent isolate GJ33, although they were morphologically indistinguishable from the parent isolate on PDA plates (Fig. 3). The EC₅₀ for the parent isolate GJ33 was 20.6 mg a.i. liter⁻¹, and those for the *Tub1* deletion mutants $\Delta Tub1$ -1, $\Delta Tub1$ -3, and $\Delta Tub1$ -5 were 108.6, 100.7, and 113.6 mg a.i. liter⁻¹, respectively. Results were observed from *Tub1* deletion mutants similar to those obtained from the parent isolate XS9 having a point mutation at codon 198 (*data not shown*).

Expression of *Tub2* in the *Tub1* deletion mutants. Real-time PCR assays showed that the expression levels of *Tub2* in $\Delta Tub1$ -1, $\Delta Tub1$ -3, and $\Delta Tub1$ -5 were 76.0, 45.5, and 11.8%, respectively, higher than that in the parent isolate GJ33 (Fig. 4).

DISCUSSION

In this study, we tested 1,529 isolates and found that 41 were resistant to carbendazim. Among the 41 isolates, 40 were obtained from Zhejiang and Jiangsu Provinces (Table 1), and the frequencies of resistant isolates in the sampled populations ranged from 2 to 40%. Because the carbendazim resistance in *G. zeae* has been fairly common in these regions, growers

Table 3. Point mutation in the *Tub2* from carbendazim-resistant isolates of *Gibberella zeae* and their EC₅₀ values to carbendazim^a

Isolates	Origin (city, province)	EC ₅₀ (mg a.i. liter ⁻¹)	Resistance level ^b	Mutation in <i>Tub2</i>
HN27	Haining, Zhejiang	0.62 ± 0.06	S	No mutation
HB61	Zhaoxian, Hebei	0.63 ± 0.15	S	No mutation
AH41	Ningguo, Anhui	0.63 ± 0.07	S	No mutation
AH64	Liu'an, Anhui	0.64 ± 0.03	S	No mutation
GJ37	Hai'an, Jiangsu	0.62 ± 0.16	S	No mutation
HN18	Haining, Zhejiang	0.61 ± 0.07	S	No mutation
HUBEI121	Jingzhou, Hubei	0.60 ± 0.09	S	No mutation
HZ0921	Hangzhou, Zhejiang	0.56 ± 0.12	S	No mutation
LJ2092	Fuyang, Anhui	0.57 ± 0.01	S	No mutation
TA86	Tai'an, Shandong	0.59 ± 0.09	S	No mutation
GHD19	Hai'an, Jiangsu	6.22 ± 1.41	MR	E198Q
GHD27	Hai'an, Jiangsu	6.30 ± 1.06	MR	E198Q
HA12	Hai'an, Jiangsu	5.32 ± 1.41	MR	E198Q
HA120	Hai'an, Jiangsu	6.14 ± 0.13	MR	E198Q
HA123	Hai'an, Jiangsu	6.26 ± 0.13	MR	E198Q
HA134	Hai'an, Jiangsu	4.82 ± 0.01	MR	E198Q
HA163	Hai'an, Jiangsu	4.50 ± 0.19	MR	E198Q
HA168	Hai'an, Jiangsu	7.18 ± 0.14	MR	E198Q
HA34	Hai'an, Jiangsu	5.79 ± 0.15	MR	E198Q
XS9	Xiaoshan, Zhejiang	7.28 ± 0.10	MR	E198Q
HA70	Hai'an, Jiangsu	27.86 ± 2.11	HR	F200Y
HA36	Hai'an, Jiangsu	30.26 ± 1.06	HR	F200Y
HY11	Haiyan, Zhejiang	20.98 ± 1.29	HR	F200Y
HY215	Haiyan, Zhejiang	18.64 ± 4.24	HR	F167Y
GHD29	Hai'an, Jiangsu	20.53 ± 4.43	HR	F167Y
GHG34	Hai'an, Jiangsu	16.23 ± 1.41	HR	F167Y
GJ23	Jingjiang, Jiangsu	15.84 ± 0.39	HR	F167Y
GJ33	Jingjiang, Jiangsu	20.55 ± 1.03	HR	F167Y
HA29	Hai'an, Jiangsu	17.37 ± 1.06	HR	F167Y
HA131	Hai'an, Jiangsu	28.23 ± 4.40	HR	F167Y
HA166	Hai'an, Jiangsu	28.03 ± 1.73	HR	F167Y
HY213	Haiyan, Zhejiang	23.04 ± 2.14	HR	F167Y
HY23	Haiyan, Zhejiang	10.35 ± 0.39	HR	F167Y
HY310	Haiyan, Zhejiang	19.67 ± 0.71	HR	F167Y
HY412	Haiyan, Zhejiang	17.91 ± 3.06	HR	F167Y
HY414	Haiyan, Zhejiang	26.49 ± 4.24	HR	F167Y
HY524	Haiyan, Zhejiang	19.48 ± 3.53	HR	F167Y
HY535	Haiyan, Zhejiang	19.12 ± 0.37	HR	F167Y
YS0935	Fuyang, Anhui	19.94 ± 0.36	HR	F167Y
ZJ127	Haining, Zhejiang	19.25 ± 1.43	HR	F167Y
ZJ218	Haiyan, Zhejiang	27.40 ± 0.87	HR	F167Y
ZJ312	Haiyan, Zhejiang	17.43 ± 0.45	HR	F167Y
ZJ322	Haiyan, Zhejiang	17.93 ± 0.02	HR	F167Y
ZJ52	Jiaxing, Zhejiang	18.45 ± 0.47	HR	F167Y
ZJ57	Jiaxing, Zhejiang	21.86 ± 4.14	HR	F167Y
ZJ59	Jiaxing, Zhejiang	19.49 ± 5.02	HR	F167Y
ZJHN10	Haining, Zhejiang	23.22 ± 5.05	HR	F167Y
ZJHN4	Haining, Zhejiang	16.62 ± 1.14	HR	F167Y
HN40	Haining, Zhejiang	20.91 ± 1.70	HR	F167Y
ZJHN49	Haining, Zhejiang	26.03 ± 2.79	HR	F167Y
ZJHN5	Haining, Zhejiang	23.26 ± 3.03	HR	F167Y

^a EC₅₀ = fungicide concentration that results in 50% inhibition of mycelial growth.

^b S, MR, and HR indicate that the isolates are sensitive, moderately resistant, and highly resistant to carbendazim, respectively.

have started applying tebuconazole, not carbendazim, for the control of FHB. In Anhui Province, no resistant isolates were found in 2008 and one was detected in 2009 (Table 1); however, five resistant isolates have been obtained in 2010 (J. Jiang and Z. Ma, *unpublished data*), which indicated that the resistance has started developing. Because the resistant populations could develop rapidly under the se-

lection pressure of carbendazim, we have suggested that growers apply a mixture of carbendazim and triadimefon for the control of FHB in this region and other provinces, including Hebei, Henan, and Shandong.

Resistance to benzimidazole fungicides has been detected in many fungal species. In most cases, resistance was correlated with single-point mutations in the β -

tubulin gene that encodes the target at which the fungicide binds (12). In this study, we detected three single-point mutations (F167Y, E198Q, and F200Y) in the *Tub2* from 41 carbendazim-resistant isolates of *G. zeae*. The isolates with F167Y and E198Q mutations accounted for 92.6% of the resistant population, which indicated that these genotypes were predominant in the collected samples.

In this study, we also observed that single-point mutations at codon 167 or 200 of *Tub2* caused a high level of resistance, and the point mutation at codon 198 was associated with a moderate level of resistance to carbendazim in *G. zeae*. In a previous study, double-point mutants at codons 73 and 198 were detected in HR isolate JT04. These results indicate that mutations at different codons and different substitutions at the same codon in the β -tubulin gene may result in different levels of resistance to carbendazim in *G. zeae*, as has been shown with other fungal species (12).

Many fungi have only a single β -tubulin gene in their genomes. The presence of two homologous β -tubulin genes was reported in *Aspergillus nidulans*, *Trichoderma* spp., and *Colletotrichum* spp. (2,13,15). In these species, mutation in one of the β -tubulin genes was found to be associated with MBC resistance (6,13,15) but the functions of the other β -tubulin gene have not been documented. This present study is the first to report the contributions of both β -tubulin genes in sensitivity to MBC fungicide. Targeted disruption experiments showed that the *Tub1* deletion mutants were morphologically indistinguishable from the parent isolate on PDA (Fig. 3). Interestingly, tests for sensitivity to carbendazim showed that the three *Tub1* deletion mutants were more resistant to carbendazim. This phenomenon could be partially explained by a higher expression level of *Tub2* in the *Tub1* deletion mutants. Because there is a point mutant in *Tub2* associated with MBC resistance, a higher level of expression of *Tub2* in the *Tub1* deletion mutants may lead to a higher level of MBC resistance. These results indicate that *Tub1* may interact with *Tub2* in *G. zeae* but the exact molecular mechanisms for the interactions remain unclear.

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PH-1 (S)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HA34 (MR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
XS19 (MR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
GJ33 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HA29 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HN40 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HA36 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HA70 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY
HY11 (HR)	DRMMATFSVMPSPKVS	SDTVVEPYNATLSLNQLVENSEIETFCIDNEALY

Fig. 2. Alignment of the deduced amino acid sequence (positions 161 to 208) of the β 2-tubulin (*Tub2*) gene from carbendazim-sensitive (S), moderately resistant (MR), and highly resistant (HR) isolates of *Gibberella zeae*. Vertical box indicates the changes in amino acid positions 167, 198, and 200 that were correlated with resistance to carbendazim.

Fig. 3. Comparisons in sensitivity to carbendazim between the β 1-tubulin (*Tub1*) gene deletion mutants (Δ *Tub1*-1, Δ *Tub1*-3, and Δ *Tub1*-5) and parent isolate HR GJ33 of *Gibberella zeae*.

Fig. 4. Comparisons in expression level of β 2-tubulin (*Tub2*) between the β 1-tubulin (*Tub1*) gene deletion mutants (Δ *Tub1*-1, Δ *Tub1*-3, and Δ *Tub1*-5) and their parent isolate GJ33 of *Gibberella zeae*. Relative expression of *Tub2* is the relative amount of *Tub2* mRNA compared with the mRNA level from the parent isolate GJ33. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

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