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NOVEL MUTATIONS IN β-TUBULIN GENE IN *Trichoderma harzianum* MUTANTS RESISTANT TO METHYL BENZIMIDAZOL-2-YL CARBAMATE

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Twelve low resistant (LR) mutants of *Trichoderma harzianum* with the capability of grow fast at 0.8 μg/mL methyl benzimidazol-2-yl carbamate (MBC) were obtained using UV mutagenesis. MR and HR mutants which could grow fast at 10 and 100 μg/mL MBC, respectively, were isolated by step-up selection protocols in which UV-treated mutants were induced and mycelial sector screening was made in plates with growth medium. Subsequently, β-tubulin genes of 14 mutants were cloned to describe the molecular lesion likely to be responsible for MBC resistance. Comparison of the β-tubulin sequences of the mutant and sensitive strains of *T. harzianum* revealed 2 new MBC-binding sites differed from those in other plant pathogens. A single mutation at amino acid 168, having Phe (TTC) instead of Ser (TCC), was demonstrated for the HR mutant; a double mutation in amino acid 13 resulting in the substitution of Gly (GGC) by Val (GTG) was observed in β-tubulin gene of MR mutant. On the other hand, no substitutions were identified in the β-tubulin gene and its 5'-flanking regions in 12 LR mutants of *T. harzianum*.

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Benomyl and methyl benzimidazol-2-yl carbamate (BBC and MBC), are benzimidazole fungicides widely used in China and worldwide on a large variety of crops to control plant pathogenic fungi [1]. However, many benzimidazole-resistant pathogenic isolates, e.g. *Penicillium* spp. [2], *Colletotrichum gloeosporioides* [3], *Botrytis cinerea* [4], have been detected in field shortly after intensive and exclusive use of these fungicides. In addition, reversion of resistant populations to fungicide sensitive ones has not been observed even after selection pressure has been removed for many years [5]. The appearance of resistance has become an important factor in limiting the efficacy and useful lifetime of fungicides developed at increasingly higher costs. Exploring new chemical fungicides and applying more fungicides in field are needed to control pathogens more efficiently. Stems form a worldwide need to adopt the practice of sustainable agriculture, using strategies that are environment-friendly, less dependent on agricultural chemicals and less damaging to soil and water resources, there has been considerable interest in the utilization of sublethal doses of chemicals with biocontrol strains which are resistant to them, and the information on this kind of integrated control is accumulating more rapidly than on other combinations of control components [6].

To implement integrated control combining biocontrol fungi with chemical fungicides, some biocontrol fungi with fungicide resistant phenotype have been isolated gradually [7]. *Trichoderma* genus has been shown effective as biocontrol agent against a range of economically important aerial and soil-borne fungal plant pathogens [8]. Although mutations of *Trichoderma* spp. resistant to BBC and MBC have already been isolated [9], the obtaining of promising mutants with higher MBC resistant levels than field populations is important for the adoption of these agents with traditional chemical fungicides. Considered from this point, we decided to isolate promising MBC-resistant mutants of *T. harzianum* for integrated control in field with MBC using UV mutagenesis and step-up selection protocols.

Previous biochemical and genetic analyses have identified microtubules of *Saccharomyces cerevisiae* as primary benzimidazole binding target [10]. Benzimidazole functioned by inhibit the polymerization of tubulin monomers into functional microtubules, specifically on β-tubulin subunit. In addition, sequence rearrangement in 5'-flanking region of β-tubulin gene has been also observed in *Epichloe typhina* resistant strain [11]. The aim of the study was to describe molecular lesion likely to be responsible for MBC resistance in *T. harzianum*. The results would enrich the growing body of knowledge of β-tubulins connected with fungicide resistance and to find possible endogenous selectable markers for *T. harzianum*.

MATERIALS AND METHODS

Strains and growth conditions. The isolate *T. harzianum* was obtained from East China Institute of Technology. The growth potato dextrose agar (PDA) medium contained (g/L): potato infusion – 200, dextrose – 20, agar – 20. The strain was cultured in plates of 9 cm diameter at 25°C for 1 week. Fifteen ml distilled water with 0.005% (v/v) Tween 80 was poured onto the plate to prepare spore suspension (10^6 cells per ml). Subsequently, 1 mL of spore suspension was inoculated into 100 mL of liquied potato dextrose medium for chromosomal DNA isolation, incubated at 25°C and 200 rpm on an orbital shaker for 48 h and mycelia were harvested.

Induction of *T. harzianum* MBC-resistant mutant strains. Classical random mutagenesis and selection approach were taken to isolate *T. harzianum* MBC-resistant strains. First, spore suspension (10^6 CFU/mL) of the wild type was overlaid on PDA plates containing 5, 10, 50, 100 or 500 μg/mL MBC. The plates then were exposed to a 15W UV light sources at a distance of 30 cm for 90 sec. After UV exposure, the plates were incubated in the dark for 5–7 days at 25°C. Viable colonies were isolated and subcultured on a series PDA plates supplied with 0, 1, 5, 10, 50, 100, 500 or 1000 μg/mL MBC. Fungicide concentration that results in 50% mycelia growth inhibition (EC_{50}) and MBC sensitivity were calculated. Genetic stabilities were detected by triple subculturing without the fungicide, followed by retesting for MBC resistance.

Step-up selection protocol was designed to achieve higher level of drug resistance in which UV treatment fungal mutants were induced and subjected to form sectors [12]. Colonized agar plugs, 4 mm in diameter, were transferred from the margin of actively growing cultures of the sensitive and UV-induced resistant isolates, mycelium-side down, onto potato dextrose agar plates supplied with 30, 100, 500 or 1000 μg/mL MBC. The MBC concentrations used for selection were previously found to be highly inhibitory for each tested isolate. Plates were sealed with parafilm, incubated at 25°C in darkness and observed at 6 day intervals for colony diameter and sectoring. EC_{50} for growth inhibition mycelium and MBC sensitivity of *T. harzianum* were calculated. Stabilities of the fast growing mycelial sectors were tested as above.

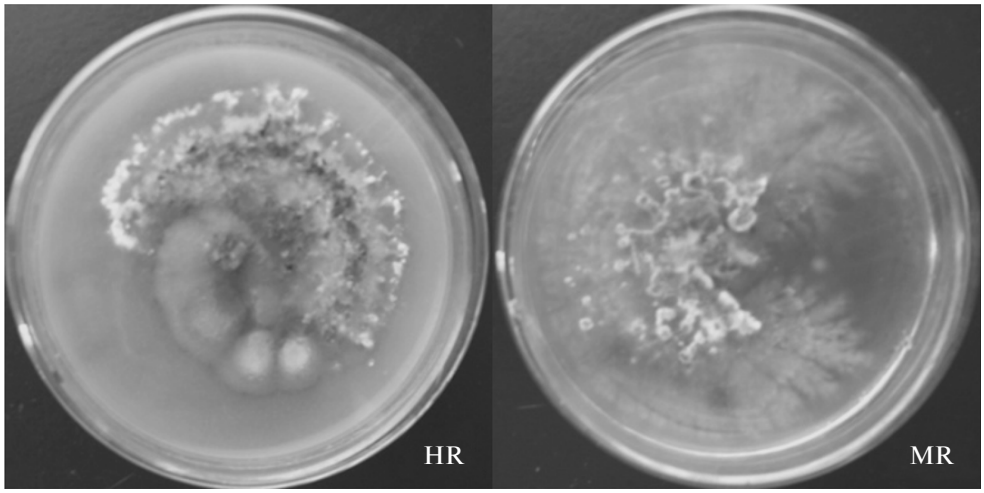
DNA extraction. Mycelium of the sensitive and resistant isolates of *T. harzianum* were ground to powder in liquid nitrogen. About 100 mg powdered mycelium was added to 600 μL extraction buffer (200 mM Tris-HCl with 25 mM EDTA, 250 mM NaCl and 0.5% SDS, pH 8.0) containing 60 μg proteinase K (Sigma, USA). Following gentle homogenization, the sample was placed at 37°C for 30 min, centrifuged 5 min at 11.000 × g. The upper phase was transferred to a new tube and extracted with phenol-chloroform. The clear supernatant was precipitated with 600 μL isopropanol. The nucleic acid pellet was washed with 70% (v/v) ethanol, air-dried, resuspended in 500 μL TE buffer (10 mM Tris-HCl with 1 mM EDTA; pH 8.0) containing 10 μg RNAse (Sigma, USA), incubated 30 min at 37°C, extracted with phenol-chloroform and precipitated with isopropanol as above. The final DNA pellet was washed twice with 70% (v/v) ethanol, air-dried, dissolved in nuclease-free water, and stored at –20°C until use.

Amplification of β-tubulin gene and flanking regions from MBC-resistant mutants. PCR amplifications were designed to generate β-tubulin gene coding region or its 5'-flanking region, using genomic DNA from variant resistant *T. harzianum* mutants as templates. Amplification from the sensitive fungal strain used as a control. Primers were designed based on the β-tubulin gene nucleotide sequences reported previously as follows: 5'-ATGCGTGAGATTGTGAGTTCCC-3' (forward) and 5'-TTACTCCTCCTCGTGCTCAGCA-3' (reverse) for the amplification of 1.7 kb β-tubulin gene coding region, 5'-AAGCTTCTCATCAGCAAGCTCG-3' (forward) and 5'-GATGGCTAGTGATGATGCTGGA-3' (reverse) for 1.5 kb 5'-flanking region. Amplified fragments were examined by agarose gel electrophoresis, followed by cloned and sequenced.

RESULTS AND DISCUSSION

Induction of *T. harzianum* MBC-resistant mutant strains. Four distinct resistance levels were prescribed to classify *T. harzianum* MBC-resistant isolates according to previous studies [13]: sensitive (S), could grow at 0.1 μg/mL of MBC, but was completely inhibited at 0.8 μg/mL; low resistance (LR), could grow fast at 0.8 μg/mL of MBC but completely inhibited at 10 μg/mL; moderate resistance (MR), could grow fast at 10 μg/mL of MBC and slowly at 50 μg/mL, but completely inhibited at 100 μg/mL; high resistance (HR), could grow fast at 100 μg/mL of MBC, partially inhibited at 1000 μg/mL and even could grow slowly at 1500 μg/mL.

A series PDA plates amended with 500, 100, 50, 10 or 5 μg/mL MBC were used for the selection of resistant fungal mutants. UV mutagenesis treatment was performed at 500 μg/mL of MBC and no growth was observed at this top concentration after 7 days of incubation at 25°C. In other experiments MBC concentration was 100, 50, 10 or 5 μg/mL. Twelve colonies were appeared on plates containing 5 μg/mL MBC. The survivors exhibited normal colony morphology and growth rate compared with the sensitive *T. harzianum* strain when cultured on medium without MBC. The UV-treated mutants were able to grow at 0.8 μg/mL of MBC, grew fast at a level of 5 μg/mL, but were completely inhibited at 10 μg/mL. The EC_{50} of the growth inhibition of 12 LR mutants of *T. harzianum* ranged from 3.8 to 6.4 μg/mL of MBC.



Two MBC-resistant mutant sectors (HR and MR) of mycelia growing out by step-up selection protocols in which UV-treated mutants of *T. harzianum*, were used.

UV mutagenesis is the most commonly used technique for microbial strain improvement, but our initial attempts obtained only a high frequency of occurrence of LR mutants. It is difficult to obtain the MR or HR *T. harzianum* mutants only by UV mutagenesis. Sectoring is thought being a strategy to get the target fungi reacted against the toxic effect of fungicides. It has been reported that many fungi e.g. *Fusarium graminearum* and *Alternaria brassicicola* can produced fast growing resistant sectors on agar medium containing fungicides [14, 15]. However, incorporation of fungicides to the growth medium of *Trichoderma* spp. led to sector formation very restricted. In this study, two fast growing sectors were produced by cultivating LR mutants on PDA plates supplied with 1000 or 30 μg/mL of MBC for more than 15 days (Figure). One sector, where mycelium of *T. harzianum* could grow fast at 100 μg/mL of MBC and even be resistant to MBC at concentrations up to 1500 μg/mL, was designated as HR. The other one, with mycelium growing fast at 10 μg/mL of MBC and slowly at 50 μg/mL, was designated as MR. The EC_{50} of the growth inhibition of two mycelial sectors were 628.7 and 38.2 μg/mL of MBC (Table 1). Culturing the sensitive *T. harzianum* strain on MBC-containing medium, we did not observed any sector coming out. From this experiment we can conclude that after exposition to MBC in highly inhibitory concentrations, UV-treated mutants of *T. harzianum* can produce sectors more easily than the sensitive ones.

Considering that the field application concentration of MBC for control plant pathogenic fungi in China changes from 500 to 1000 μg/mL, it is feasible to apply the HR isolate of *T. harzianum* concurrently in the field with MBC to reduce the number of the fungicide applications needed for pathogen. In contrast to some reports that fungal sectors treated with fungicide commonly exhibit abnormal colony type, morphotype or other phenotypic characteristics [16], the two mycelial sectors obtained in this study exhibit-

Table 1. Characteristics of resistance to MBC of the wild and mutant *T. harzianum* strains

Strain	Resistance to MBC (EC_{50}/MIC)* both in μg/mL	Mutationin β_2-tubulin gene	Amino acid of the β_2-tubulin gene	
			codon	substitution
Wild	0.4/0.8	GGC TCC	13 168	Gly Ser
MR**	38.2/100	GTG	13	Val
HR***	628.7/ >1000	TTC	168	Phe

* EC_{50} – concentration of MBC which inhibits the fungal growth to 50%, MIC– minimum inhibitory concentration of MBC which inhibits fungal growth by 100%.

** Fast growing mycelial sector, which was produced by cultivating UV mutants of *T. harzianum* on PDA plates supplied with 30 μg/mL of MBC for more than 15 days.

*** Fast growing mycelial sector, which was produced by cultivating UV mutants of *T. harzianum* on PDA plates supplied with 1000 μg/mL of MBC for more than 15 days.

Table 2. Mutations at the β-tubulins gene causing the resistance of selected filamentous fungi to benzimidazoles

Amino acid of the β-tubulin gene		Organism and reference
codon	substitution	
6	His to Leu	*Aspergillus nidulans* [18]
	His to Tyr	*A. nidulans* [18], *Monilinia fructicola* [19], *Septoria nodorum* [20], *Trichoderma viride* [21]
50	Tyr to Asn	*Fusarium moniliforme* [22]
165	Ala to Val	*A. nidulans* [23]
167	Phe to Tyr	*Cochliobolus heterostrophus* [24], *Neurospora crassa* [25], *Penicillium expansum* [26], *Saccharomyces cerevisiae* [27]
198	Glu to Ala	*Botrytis cinera* [28], *M. fructicola* [19], *Penicillium aurantiogriseum* [29], *P. expansum* [26], *Tapesia yallundae* [30], *Venturia inaequalis* [29]
	Glu to Asp	*A. nidulans* [18]
	Glu to Gln	*A. nidulans* [18], *T. yallundae* [30]
	Glu to Gly	*N. crassa* [31], *T. yallundae* [30]
	Glu to Lys	*A. nidulans* [18], *Colletotrichum.gloeosporioides* [32], *M. fructicola* [19], *P. aurantiogriseum* [29], *P. expansum* [26], *Sclerotinia homoeocarpa* [29], *T. yallundae* [30], *V. inaequalis* [29]
	Glu to Val	*P. expansum* [26]
200	Glu to Lys	*B. cinera* [33]
	Phe to Tyr	*P. aurantiogriseum* [29], *T. yallundae* [30], *V. inaequalis* [29]
240	Leu to Phe	*M. laxa* [34], *T. yallundae* [30]
241	Arg to His	*S. cerevisiae* [35]

ed the same colony morphology with the sensitive strain during growth on MBC-containing and control media. When cultivated on medium without the fungicide, the mycelial sectors could keep growth at the same rates in comparison with the sensitive strain, but reductions were observed with the elevation of MBC concentrations. All 14 resistant mutants (12 LR, MR, HR) of *T. harzianum* maintained genetic stability after triple subculturing without the fungicide, followed by retesting for MBC resistance.

Mutations responsible for MBC resistance in *T. harzianum*. Resistance to benzimidazoles in *S. cerevisiae* has been reported be correlated in most cases with point mutations in β-tubulin gene loci, leading to altered amino acid sequences at the benzimidazole-binding site [10]. This kind of resistance may result from single or multiple β-tubulin gene mutations. Moreover, mutations at different codons and even different substitutions at the same codon may result in different resistance levels to these compounds [17].

PCR amplifications were employed to generate 1.74 kb fragments by using DNA from the sensitive and 14 resistant isolates as template to verify if there exist any mutations in the β-tubulin gene responsible to the *T. harzianum* MBC resistance. Sequence comparisons of the target DNA fragments revealed a single base difference between the sensitive and the HR mutant, a C to T transversion at nucleotide 844, resulting in Ser substitution for Phe at 168 amino acid, which is highly conserved among β-tubulins from other species. The MR mutant presents two consecutive mutations, having Val instead of Gly at the 13 position, thus substituting GGC codon for a GTG codon (Table 1). Previous studies have shown mutations at codon 6, 50, 165, 167, 198, 200, 240, 241 in β-tubulin gene of different fungi could cause benzimidazole resistance (Table 2). However, we have not found any report of mutations at the codons connecting with benzimidazole resistance similar to those found in this research in any field isolates or laboratory-induced mutants from filamentous fungi. Although strict amino acid differences exist in the β-tubulin gene, site-directed mutagenesis and structural analysis of the β-tubulin are also necessary to substantiate the molecular mechanism. The results would enrich the growing body of knowledge of fungal β-tubulins determining fungicide resistance.

No substitution was identified in the β-tubulin gene of all 12 LR mutants of *T. harzianum*. Considering sequence rearrangement in 5'-flanking region of β-tubulin gene observed in *Epichloe typhina* LR strain [12], we cloned the same region of *T. harzianum* LR strain extending about 1.5 kb upstream of ATG initiator codon. No variations altered the expression of the β-tubulin were revealed. It is presumed

that there could be some unrecognized mechanisms responsible for MBC resistance of fungi.

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