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Characterization of the β-Tubulin Genes from *Stemphylium* Species

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The β-tubulin genes from three *Stemphylium* species which are insensitive to high concentration of carbendazim were amplified with two pairs of primers and sequenced. The lengths of three genes are 1431bp for *Stemphylium solani*, 1431bp for *Stemphylium lycopersici* and 1424bp for *Stemphylium spinaciae* respectively. All genes have three introns and encode proteins with 398 amino acids. The protein sequences of β-tubulins of three *Stemphylium* species are highly homologous to those of other plant pathogenic fungi that are sensitive to carbendazim. The main difference is that the 167th amino acid of β-tubulins in *Stemphylium* is the residue Tyr instead of Phe, which is commonly found at the same position of β-tubulins in other fungi. These results suggest that carbendazim insensitive of the three *Stemphylium* species probably attributes to Tyr167.

Key words: β-Tubulin, Amino acid, Insensitive, *Stemphylium*.

Characterization of the β-tubulin genes from three carbendazim insensitive Stemphylium species

Benzimidazole fungicides were among the early systemic fungicides developed and used for comntrolling a wide variety of plant disease. Even though they were initially proved very effective, their life was often cut short by the apperance and spread benzimidazole resistant strains[1]. These fungicides were known as a specific inhibtor of microtubule assembly by binding to the β-subunit of β-tubulin and interfering with microtubule formation during mitosis of cell division[2,3]. Resistance to benzimidazole fungicides has been detected in many fungal species. In most cases, resistance was correlated with point mutations in the β-tubulin gene, which result in altered amino acid sequences at the benzimidazole-binding site. Results from numerous studies have shown changes at codon 6, 50, 134, 165, 167, 198, 200, and 257 in the β-tubulin gene could cause benzimidazole resistance in pathogenic fungi[3,4].

Benzimidazole fungicides were broad spectrum chemical fungicides, they were very effective to ascomycotina, most deuteromycotina and basidiomycotina, but oomycetes, zygomycotina and some deuteromycotina , such as *Phytophthora* spp., *Alternaria* spp. and *Stemphylium* spp. are inherently insensitive to them[5,6,7,8]. Since the microtubule protein β-tubulin has been identified as the benzimidazole target in fungi, it would be of primary importance to investigate the β-tubulin gene in a naturally occuring benzimidazole-resistant fungus. The objective of this study was to elucidate the β-tubulin gene in Stemphylium spp. and the relationship between benzimidazole insensitivity and the predicted amino acid sequence of this fungus.

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MATERIALS AND METHODS

Fungicides and isolates

Technical-grade carbendazim (98.0% active ingredient; YangNong Chemicals, Jiangsu, China) was dissolved in acetone to provide stock solutions containing 10,000 μg/ml. The fungicides were stored at 4°C in the dark to maintain and reserve fungicide activity.

Stemphylium solani, Stemphylium lycopersici, Stemphylium spinaciae and *Botrytis cinerea*, all of them are wild-type isolates .

Sensitivity of three *Stemphylium* species and *Botrytis cinerea* to carbendazim

The sensitivity test was performed by transferring a 4mm diameter disc of a colony grown on a PDA plate medium amended with 0, 0.1, 1, 10, 100 μg/ml carbendazim. After incubation at 25°C in the dark for 7 days, the diameter of the mycelial colony was measured. For each isolate, three replicates per concentration were used and the experiments were performed twice.

Polymerase chain reaction (PCR) and DNA sequencing

PCR was used for gene amplification using fungal genomic DNA as a template. Fungal genomic DNA isolation was carried out according to Grasso *et al*.[9] and utilizing two primer pairs Q1/Q2 and Z1/Z2 (Table 1). Amplified PCR productions were purified by electrophoresis and cloned into the pMD 20-T Vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) and sequenced by Shanghai Sangon Biological Engineering Technology And Service Co., Ltd in both directions.

Sequence analysis

DNA and amino acid sequences were edited with DNAman software and compared with previously characterized β-tubulin genes of other fungi obtained from GenBank database using the computer program ClustalW1.82 (http://www.ebi.ac. uk./clustal/).

RESULTS

Sensitivity of three *Stemphylium* species to carbendazim

Three Stemphylium species *Stemphylium solani, Stemphylium lycopersici* and *Stemphylium spinaciae* were all highly resistant to carbendazim with MIC (minimal inhibitory concentration) higher than 100 μg/ml, while *Botrytis cinerea*, the carbendazim-sensitivite fungus, with MIC less than 1 μg/ml (Table 2).

Cloning and sequencing of the three *Stemphylium* species β-tubulin genes

Two β-tubulin genes fragment were amplied with two pairs Q1/Q2 and Z1/Z2. The length were approximate 650bp and 1100bp repectively (Fig. 1 & Fig. 2). Two β-tubulin genes fragment were assembled and the lengths of three genes were 1431 for *Stemphylium solani*, 1431bp for *Stemphylium lycopersici* and 1424 for *Stemphylium spinaciae* respectively. All genes have three introns and encode proteins with 398 amino acids. The amino acid sequences of β-tubulin of three *Stemphylium* species are highly homologous to those of other plant pathogenic fungi that are sensitive to carbendazim (Table 3).

DISCUSSION

In fungus, Most β-tubulin mutations affect benzimidazoles fungicides sensitivity were seen to cluster within a small volume of the molecular structure: residues 6, 50, 134, 165, 167, 198, 200, and 257 form a cluster within a compact region of the folded protein10. By comparing the deduced β-tubulin amino acid sequence of three *Stemphylium* species with that of *S. nodorum's*, which is sensitive to carbendazim, it was found that 56, 107 and 167 were different between three *Stemphylium* species and *S. nodorum* ((Fig.3). The difference of amino acid residue at position 56 and

Table 1. Primers used for PCR amplification

Primer	Sequence	Direction
Q1	5'ACCCACAACCGCCAACATGCGTGA-3'	+
Q2	5'-GGTGATCTGGAAACCCTGGAGGCA-3'	-
Z1	5'-CAGCTCGAGCGTATGAACGTCT-3'	+
Z2	5'-TGTACCAATGCAAGAAAGCCTT-3'	-

107 were not unique to three *Stemphylium* species, these amino acid were found in other fungi known to lack resistance to carbendazim. However, the amino acid at position 167 is unusual. In β-tubulin amino acid sequence of three *Stemphylium* species, it was tyrosine, whereas this amino acid is phenylalanine in all of the β-tubulin amino acid sequences of species known to be sensitive to *carbendazim*. The Tyr167 has been demonstrated to be responsible for benomyl resistance in *N.crassa*[11]. The other benzimidazole implicated residues in β-tubulin amino acid sequences of three Stemphylium species, His6, Tyr50, Gln134, Ala165, Glu198, Phe200, Thr237, Arg241, Leu250 and Met257 coincided with those of other fungi known to be sensitive to benzimidazole fungicides. These results suggest that carbendazim insensitive in three Stemphylium species may be associated with residue Tyr167 of β-tubulin amino acid sequences. The only other organism reported to contain Tyr167 in its β-tubulin is the protozoan *Trichomonas vaginalis*, which is not affected by the benzimidazole derivatives[12, 13].

Table 2. Inhibition rate of three *Stemphylium* species by carbendazim

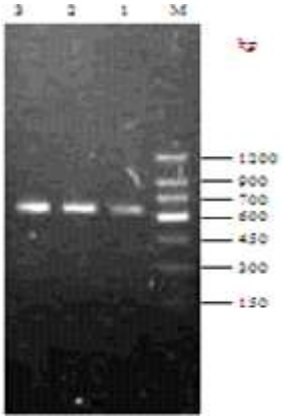
Species	Inhibition (%)			
	1μg/mL	10μg/mL	50μg/mL	100μg/mL
S.solani	6.21b	7.84 c	14.38bc	20.92bc
S.lycopersici	3.22c	5.16 c	10.31c	17.57c
S.spinaciae	8.21b	15.80b	21.28b	25.55b
B. cinerea	100.00a	100.00a	100.00a	100.00a

Note: Values in a column followed by different letters are significantly different according to Duncan's test (P=0. 05)

The mutation from Phe to Tyr in position 167 of β-tubulin amino acid is known to be a

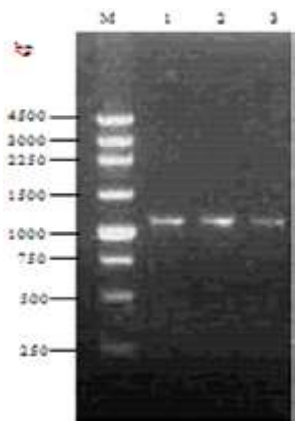
Table 3. Comparison of β-tubulin proteins of three *Stemphylium* species with those of other common plant pathogentic fungi that are sensitive to carbendazim

Fungal species	Accession	Homology of amino acid (%)		
		S.solani	*S.lycopersici*	*S.spinaciae*
Septoria nodorum	AAB25800	98.24	97.99	98.24
Neurospora crassa	AAA33617	96.23	95.98	96.23
Botryotinia fuckeliana	CAA93254	98.24	97.99	98.24
Cercospora beticola	AAX52522	96.73	96.48	96.73
Rhynchosporium secalis	CAA56936	97.74	97.49	97.74
Gibberella zeae	AAP68979	95.23	94.97	95.23
Venturia inaequalis	AAA34230	98.74	98.49	98.74



Product of Q1/Q2

Fig. 1. PCR product of Q1/Q2 primers. M:150bp Marker; 1: *Stemphylium solani*; 2: *Stemphylium lycopersici*; 3: *Stemphylium spinaciae.*



Product of Z1/Z2

Fig. 2. PCR product of Z1/Z2 primers. M:250bp Marker; 1: *Stemphylium solani*; 2: *Stemphylium lycopersici*; 3: *Stemphylium spinaciae*.

Fig. 3. Comparison of three *Stemphylium* species β-tubulin amino acid sequences with that of *Septoria nodorum.*

mutation that confers moderate resistant phenotype in *N. crassa*. The minimal inhibitory concentration of Tyr167 mutant of *N. crassa* on carbendazim is 100 µg/ml, and one of a wild type is 0.1 µg/ml14. However, the MIC of three Stemphylium species on carbendazim is higher than 100 µg/ml, the level of resistance caused by Tyr167 resistance is unclear. Contrasting argument that the Tyr167 is not the only requirement for high resistance to carbendazim may be made. Additional mechanism, for example, an active transport system reported in *Candida albicans*[15], might also be involved in the nature of insensitivity to benzimidazole in three *Stemphylium* species. Construction of the transformant whose *Stemphylium* spp. β-tubulins gene is replaced by the mutated one with Phe167 will promote a better insight into the mechanism of insensitivity to benzimidazole in *Stemphylium* spp.

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