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Amino Acid Alterations in the β-Tubulin of *Metarhizium anisopliae* That Confer Benomyl Resistance

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We cloned the *β-tubulin* genes from the wild type strain and two benomyl-resistant mutants of *Metahizium anisopliae* and determined their nucleotide sequences. A β-tubulin encoding 448-residue protein from wild type *M. anisopliae* shows strong homology to other β-tubulins. The coding region is interrupted by four introns. Comparisons of intron position between the *M. anisopliae* gene and other fungal *β-tubulin* genes show considerable positional conservation. The mutations responsible for benomyl resistance were determined in two spontaneous mutants, S-18 and S-19. One mutant S-18 substituted glutamate for asparagine at position 33 and lysine for glutamine at position 134. The other mutant S-19 showed alterations at three positions of β-tubulin; arginine for tryptophan at position 21, lysine for asparagine at position 33, and phenylalanine for leucine at position 240. These data suggest that regions of β-tubulin containing amino acids 21, 33, 134, and 240 interact to form the binding site of benomyl.

Key words: Benomyl, *β-tubulin* gene, Entomopathogenic fungus, *Metahizium anisopliae*

Introduction

M. anisopliae is the most commonly investigated entomopathogenic fungus as a microbial pesticide. *M. anisopliae* can infect eggs of some hosts but generally invades insects during larval, pupal, or adult stages of development (Boucias and Pendland, 1998). *M. anisopliae* var *anisopliae* has a much wider host range, attacking insects from Coleoptera, Lepidoptera, Orthoptera, Hemiptera, and Hymenoptera as well as arachinids. *M. anisopliae* var. *anisopliae* also is used against many insects because of their wide geographic spread and host range as well as their exceptional ability to germinate even at relatively low humidity (Samish and Rehacek, 1999). Success in the development of mycoinsecticides depends on overcoming many problems, including specificity, mass production, storage, speed of action and environmental tolerance. Especially, as an adverse environmental condition, fungicides are problems against the successful use of mycoinsecticides (Moore and Prior, 1993).

Many synthetic fungicides are used as plant protectants and applied prior to the appearance of the fungus and development of symptoms. The benzimidazoles, including benomyl, nocodazole, carbendazim, and thiabendozole, are common fungicides which used successfully to control a large number of plant pathogens (Delp, 1995). Especially, benomyl have been world-widely used to control many fungal pathogens (Deacon, 1997).

The benzimidazole compounds are structurally related derivatives that inhibit microtubules assembly. Davidse (1986) reviewed the modes of action and biological impact of these fungicides. The benzimidazole compounds bind strongly to the extracted β-tubulin of fungi and they prevent this from forming a dimer with á-tubulin. Therefore, they block the self-assembly of microtubules when the tubulin dimers attach end to end (Davidse and Flach, 1977). But they have selective antifungal activity with little or no effect on plant and animal cells despite their mode of action on fungal microtubules (Davidse,

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1986).

However, Davidse and Flach (1977) reported that the benzimidazole-sensitive fungi can develop resistance if the fungicides are used repeatedly. Mutants resistant to benzimidazole, have been isolated in several species of fungi. In each case which the identity of the resistance mutant gene was determined, it was a structural gene for β-tubulin (Sheir-Neiss *et al.*, 1978). The resistance was caused by point mutations at various sites in the *β-tubulin* gene. Up to date, the locations of a single mutation causing benzimidazole resistance have been reported in several fungi. The amino acids were altered at various positions, including amino acid positions 6, 50, 134,165, 167, 198, 200, 241 and 257 depending on fungal species (Thomas *et al.*, 1985; Orbach *et al.*, 1986; Jung and Oakley, 1990; Seip *et al.*, 1990; Jung *et al.*, 1992; Davidse and Ishii, 1995; Mckay *et al.*, 1998). Orbach *et al.* (1986) reported that perhaps these regions of the protein contribute to the binding site for benzimidazole, or alteratively, they may modify the conformation of β-tubulin, thus preventing or decreasing benzimidazole binding at some other site.

One strategy for the biocontrol of insects is to develop strains of fungal entomopathogens resistant to common fungicides. The resistant or transgenic strains can be used to control insect pests at the same time that fungicides are applied to control fungal pathogens of plants. To develop transgenic strains, *M. anisopliae* was transformed to benomyl resistance from *Aspergillus nidulans* (Goettel *et al.*, 1990). Transformants grew at benomyl concentrations up to ten times which inhibited the wild type and were mitotically stable on either selective or non-selective media or insect tissues. The transformants were pathogenic to *Manduca sexta* in the presence of 50 μg/ml of benomyl. This study suggested that transgenic entomopathogenic fungi could potentially be used with fungicides. However, because benomyl have been applied at concentrations of 250 μg/ml or more in fields, strains with higher resistance will have to be selected.

We isolated two benomyl-resistant mutants of *M. anisopliae* by culturing the entomopathogen on fungicide amended agar (Mckay *et al.*, 1998), which can live on plate medium containing 250 μg/ml benomyl. Two mutants were observed no significant differences in conidia formation, fungal morphology, spore viability, and pathogenicity between mutant strains and the wild type. So, in this study we report the nucleotide sequence of the *β-tubulin* gene from *M. anisopliae* and compare it with amino acid sequence of the β-tubulin from other fungi. This is also the first *β-tubulin* gene sequence reported from the entomopathogenic fungus *M. anisopliae* which has benomyl resistance.

Materials and Methods

Fungal strains and culture

M. anisopliae var. *anisopliae* KACC 40029 was obtained from Korean Agricultural Culture Collection, Rural Development Administration, Suwon, Korea. S-18 and S-19 were obtained from KACC 40029 by culturing the entomopathogen on fungicide amended agar and were resistant to 250 μg/ml benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, Aldrich Chemical Co., USA]. Sabouraud dextrose agar (SDAY) or broth (SDB) containing 0.2% yeast extract was used to culture and maintain *M. anisopliae*. Benomyl was added to media from a stock solution in dimethyl sulfoxide.

Bacterial DNA preparation techniques

Escherichia coli XL1-blue was used in this study. Bacterial culture, transformations and DNA preparations were done by standard molecular methods (Sambrook *et al.*, 1989).

DNA extraction

DNA was isolated from mycelia grown in a shake culture for 5 days at 28°C in SDB. Mycelia of the strains from liquid cultures were filtered, washed with distilled water, gently blotted dry, and frozen with liquid nitrogen. The frozen mycelia were ground using precooled mortar and pestle. The powder was suspended in 700 μl of sterile lysis buffer (3% SDS, 50 mM EDTA, 50 mM Tris-HCl, 1% mercaptoethanol), and was heated for 1 h at 65°C. The lysate was extracted with phenol, Phenol-$CHCl_3$, and $CHCl_3$ before ethanol precipitation. DNA was suspended in distilled water and treated with RNAse for 3 hrs at 37°C. The solution was extracted with Phenol-$CHCl_3$, and $CHCl_3$, and the DNA was precipitated by addition of ethanol.

Polymerase chain reaction

For the initial analysis of *β-tubulin* gene, two degenerate primers, stub-F and stub-R (Table 1), based on sequences of conserved regions, were designed (Park *et al.*, 1997). The reaction was performed in a DNA thermal cycler (Bio-Rad, USA) with 35 repetitions of the following temperature cycle: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. After the PCR products were cloned in pGEM-T Easy Vector (Promega, Co., USA), the inserts were sequenced and analysed.

Fungal total RNA isolation

Total RNA of *M. anisopliae* was isolated by the phenol extraction. Two hundred μg of dried mycelia were ground, in liquid nitrogen, with precooled mortar and pes-

Table 1. The nucleotide sequences of PCR and sequencing primers used in DNA sequence analysis of *M. anisopliae β-tubulin* gene

Primer	Sequence (5'-3')	Location (5'-3') bp
Stub-F	CGGAATTCTGGGCNAARGGNCAYTA	660-683
Matub-F1	TGGGCCAAGGGTCACTAC	667-683
Matub-F2	CACTCTCTCGGTGGTGGTACCGG	775-797
Matub-F3	CCGTTACGGATCGTGAAGCTCTGCC	-96 - -72
Matub-F4	GGTGCTGCTTTCTGGCAGACCAT	315-338
Matub-F5	CTTTCCGCGCTGTCAGCGTACCT	1204-1227
Stub-R	CGGGATCCYBRTAYTGYTGRTAYTC	1766-1790
Matub-R1	AGCTTGTCGTAGAGTCTCTTACTCC	1766-1790
Matub-R2	GGCAGACGAGATAGTTCAGG	1038-1059
Matub-R3	CATGCAGATGTCGTACAGAGCCTC	979-1001
Matub-R4	GACAACGGTGTCGGAAACCTTGG	883-906
Matub-R5	CTTCATAGCAACCTTGCCACGGC	1375-1396

tle. The powder was suspended in 550 μl of RNA extraction buffer (0.2 M Tris·HCl, 0.4 M LiCl, 25 mM EDTA, 1% SDS), and added with 550 μl acidic phenol. The mixture was vortexed 10 sec, placed on ice and centrifuged at 13,000 g for 5 min. The supernatant was extracted once more with 550 μl phenol and then with 550 μl chloroform. The aqueous phase harvested into a new tube, added with 1/3 volume of DEPC-treated 8 M LiCl, left on ice for at least 2 hrs, and centrifuged at 13,000 g for 20 min at 4°C. The pellet was resuspended in 300 μl DEPC-treated water, added with 30 μl 3 M Na-acetate and 600 μl ethanol, and centrifuged at 13,000 g for 20 min at 4°C. The total RNA was dried with speed vac and resuspended in 50 μl DEPC-treated water.

Cloning and sequencing of *β-tubulin* gene

To sequence the full-length genomic *β-tubulin* gene, 5'/3' RACE kit (Roche Co., Germany) was used. 5' and 3' analysis of *M. anisopliae β-tubulin* gene were carried out according to the method recommended by the supplier. For 5' RACE analysis three antisense-specific primers were designed (specific primers: SP1; Matub-R2, SP2; Matub-R3 and SP3; Matub-R4) (Table 1). And to confirm 3' region of *M. anisopliae β-tubulin* gene, a specific forward primer was designed (SP4; Matub-F2) (Table 1). The amplified products were purified from agarose gel, cloned into pGEM-T Easy Vector (Promega Co., USA), characterized and completely sequenced.

The DNA sequences were amplified by the dye termination method in ABI 377 Automated DNA Sequencer with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Co., USA) as specified by manufacturer. The sequencing primers were universal M13 forward and reverse primers and *β-tubulin* gene specific primers.

Results

Partial *M. anisopliae* genomic DNA (1001 bp) was cloned by using two degenerate primers, completely sequenced, and compared with the full sequence of other fungal *β-tubulin* genes. The unknown sequences at 5' and 3' end of the *β-tubulin* gene were amplified by using 5' and 3' RACE Kit and fully sequenced. On the basis of the 5 sequence data, new primers were designed to detect the full-length genomic DNA and cDNA of *M. anisopliae β-tubulin* gene. Each isolate were fully sequenced in both directions using the primers, and the DNA sequence submitted to Genbank (AY995134).

Finally, 1,944 bp of the wild type *M. anisopliae* including a full-length *β-tubulin* gene were sequenced (Fig. 1). The coding region and amino acid sequence of â-tubulin from *M. anisopliae* were determined by using the published nucleotide and amino acid sequence of the *benA* gene of *Neurospora crassa*. An open reading frame was identified with 94 ~ 95% amino acid homology to the *benA* gene of *A. nidulans*, *A. flavus*, and *N. crassa* (Orbach *et al.*, 1986) (Fig. 2). Four introns (IVS 1, 2, 5, 6) ranging in size from 60 to 194 bp were predicted in the *M. anisopliae* gene. The positions of these introns were identical to those in other fungal *β-tubulin* gene (Fig. 3); however, introns 3, 4 and 7 were absent in *M. anisopliae*. Three introns (IVS 1, 2, 5) were concentrated in the 5end of the coding region, and one (IVS 6) was located at 3end. The sequence of 5', internal, and 3' splice regions were in agreement with the established consensus sequence of other fungi (Langford *et al.*, 1984; Orbach *et al.*, 1986; May *et al.*, 1987). But, differently from codon usage in other fungal *β-tubulin* genes (Seip *et al.*, 1990), that in the *M. anisopliae β-tubulin* gene was not biased with using 49 of 61 triplets.

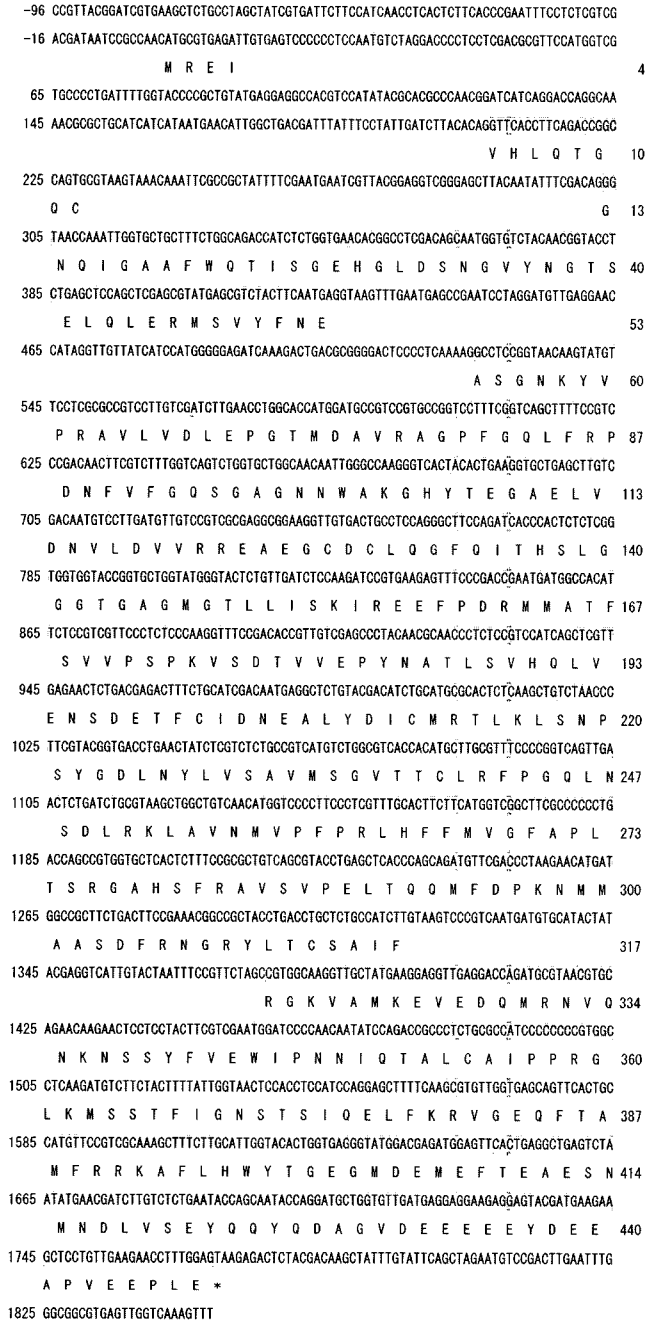

Fig. 1. Nucleotide and deduced amino acid sequence of β-tubulin of *M. anisopliae*. Numbers in the left-hand margin indicate the position of the first nucleotide in each line; numbers in the right-hand margin indicate the position of the last amino acid in each line.

The genomic *β-tubulin* gene in two mutants, S-18 and S-19, were cloned and sequenced. The nucleotide and duduced amino acid sequences altered in two benomyl-resistant mutants were summarized in Table 2. The mutant S-18 substituted glutamate for asparagine at the sequence No. 33, and lysine for glutamine at the No. 134. And

M. anisopliae	EYQQYQDAGVDE—EEEEYDEEAPVE-EPLE	448	Homology
A. nidulans benA	********SIS*—G****A**EIM*—GE*	447	94%
A. nidulans tubC	*******E*T*SD—G*GA**A*EGEAY*QE*	448	83%
A. flavus	********SIS*—G****L**EEPL-*HE*	448	94%
N. crassa	***********—*****E****L*—GE*	448	95%
Pig brain	********TA**QG*F**EG**DEA	445	82%

Fig. 2. Comparison of β-tubulin amino acid sequences. The predicted amino acid of the β-tubulin protein of the *M. anisopliae* is given on the top line. For *A. nidulans benA* (Genbank accession number M17519) and *tubC* (M17520), *A flavus* (M38265), *N. crassa* (M13630), and pig brain sequences (P02554), only amino acids different from those in the *M. anisopliae* protein are listed.

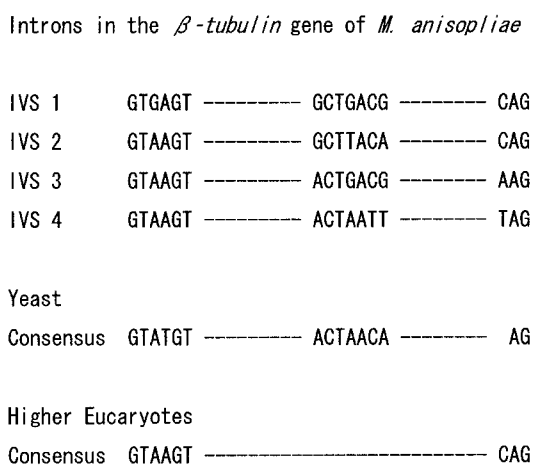

Fig. 3. Comparison of the β-tubulin introns with the consensus sequence of introns from other organisms. The first and last nucleotides listed are the first and last nucleotides of each intron.

Table 2. Changes in bases and amino acids of *β-tubulin* gene and protein in benomyl-resistant strains of *M. anisopliae*

Isolate	ED_{50}* (μg/ml)	Bases	Amino acids
S-18	> 250	AAT→GAG	33 Asn Glu
		CAG→AAG	134 Gln Lys
S-19	> 250	TGG→CGG	21 Trp Arg
		AAT→AAA	33 Asn Lys
		TTG→TTT	240 Leu Phe

*ED_{50} is the concentration of benomyl required for 50% inhibition of growth as judged by colony diameter.

mutant S-19 showed alteration at three positions of β-tubulin; arginine for tryptophan at the No. 21, lysine for asparagine at the No. 33, and phenylalanine for leucine at the No. 240.

Discussion

The *β-tubulin* gene of *M. anisopliae* and its spontaneous mutants S-18 and S-19 related to the benomyl resistance was firstly cloned and sequenced. The amino acid sequence was highly conserved in comparison with other fungal β-tubulin (Orbach *et al.*, 1986; May *et al.*, 1987; Seip *et al.*, 1990). To date, it has been reported that one point mutation of fungal *β-tubulin* gene is enough for benomyl resistance. In laboratory benzimidazoles-resistant mutants, there are point mutations at amino acid codons 6, 50, 134, 165, 167, 198, 200, 241 and 257, whilst in field-resistant isolates mutations have been limited to codons 50, 198, 200 and 241 (Davidse and Ishii, 1995; Butters *et al.*, 2003). Davidse and Ishii (1995) reported that the most obvious conclusion from the comparison is that the regions 4-8, 48-52, 132-136, 163-169 and 196-202 are highly conserved in benzimidazole-sensitive filamentous fungi. However, *Candida albicans*, although having identical sequences in these regions, is highly resistant to benomyl (Smith *et al.*, 1988). It indicates that the other regions of the β-tubulin also are important in determining binding affinity.

To know the molecular reasons why the mutants of *M. anisopliae* show strong benomyl-resistance in this study, β-tubulin sequences of two spontaneous mutants, S-18 and S-19, were compared with that of the wild-type strain *M. anisopliae*. As reported previously, they might be expected to carry a single-site mutation. However, surprisingly, two or three amino acids in two β-tubulin mutants were altered in various positions and the new substitutions at positions 21, 33 and 240 caused resistance. Mutant S-18 substituted glutamate for asparagine at the No. 33, lysine for glutamine at the No. 134 and amino acid alterations of S-19 β-tubulin took place at 3 different sites. But their amino acid alterations were not similar to those of above fungi, excepting the No. 134 alteration in S-18 β-tubulin.

Two mutants S-18 and S-19 grew better on medium containing 250 μg/ml benomyl and more than 250 μg benomyl of ED_{50} when compared to other fungi (Jung *et al.*, 1992). In interpreting such a strongly benomyl-resistant mutation, as yet no data are available on how the resistance mutations affect benomyl, and there is no direct evidence as to whether the mutations in the amino acid 21, 33 and 240 regions affect benomyl binding.

However, as compared and deduced with three-dimensional structure of the pig brain β-tubulin (Nogales *et al.*, 1998), amino acid alterations published previously in the fungi mainly affected β-helix or β-sheet structure of the β-tubulin. The regions 4-8, 132-136, 163-169 and 196-202 at fungal β-tubulin correlated with the sensitivity of the organisms to bezimidazole were a part of β-sheet of the first, fourth, fifth or sixth poison. The region 257 was a part of the eighth β-helix. The regions 48-52 and 239-243 were a connection part between the first β-helix and the second β-sheet or between the seventh and the eighth β-helix. However, amino acid alterations of the mutants S-18 and S-19 at positions 21, 33, and 240 were very different from those of other fungi: the No. 21 altered in β-tubulin was a part of the first α-helix structure and the No. 33 and 240 were connection parts.

From the results reported in this paper, resistance mechanism in the mutants of *M. anisopliae* having strong benomyl-resistance could be due to multiple mutations in

the *β-tubulin* gene. Finally, as discussed previously (Jung *et al.*, 1992; Davidse and Ishii, 1995), the reason that the mutant of *M. anisopliae* are strongly resistant to benomyl appears to be because the benomyl binding site was altered by the physical interaction of the mutated regions of β-tubulin.

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