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Molecular analysis and characterization of the *Cochliobolus heterostrophus* β-tubulin gene and its possible role in conferring resistance to benomyl

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Cochliobolus heterostrophus Tub1 **described here is the first β-tubulin gene characterized from a naturally occurring benomyl-resistant ascomycete plant pathogen. The gene encodes a protein of 447 amino acids. The coding region of *Tub1* is interrupted by three introns, of 116, 55, and 56 nt, situated after codons 4, 12, and 53, respectively. As a result of the preference for pyrimidines in the third position of the codons when a choice exists between purines and pyrimidines, codon usage in the *Tub1* gene is biased. *Tub1* shows high homology with β-tubulin genes of other ascomycete species. However, *Tub1* is exceptional in having Tyr^{167}, compared with Phe^{167}, possessed by β-tubulin genes of other ascomycetes sequenced thus far. The Tyr^{167} residue has been associated with benomyl resistance in other organisms. In contrast, all other benomyl-implicated residues of *Tub1* correspond to sensitivity. Based on these results, we suggest that benomyl resistance in the fungus probably is attributed to Tyr^{167}.**

Key Words——benomyl; benzimidazole; beta-tubulin; *Bipolaris maydis*; fungicide resistance

Benzimidazole compounds were among the early systemic fungicides developed and used for controlling several important plant diseases. Even though they were initially proved very effective, their life was often cut short by the appearance and spread of benzimidazole resistant strains (Davidse, 1986). The activity of benzimidazoles as microtubule inhibitors was first indicated in fungi (Clemons and Sisler, 1971; Davidse, 1973; Hammerschlag and Sisler, 1973). The characterization of mutant strains in several fungal species established that resistance to benzimidazoles resulted from mutations in the β-tubulin gene that alter the deduced amino acid (aa) sequence and subsequently destabilize the β-tubulin protein (Li et al., 1996) or reduce its affinity to these fungicides (Cooley et al., 1991; Davidse and Flach, 1977). These investigations have developed to the point where resistance has been related to certain amino acid substitutions at one or more specific regions within the β-tubulin molecule (Cooley and Caten, 1993; Fujimura et al., 1992a, b, 1994; Jung and Oakley, 1990; Jung et al., 1992; Koenraadt and Jones, 1993; Koenraadt et al., 1992; Orbach et al., 1986; Thomas et al., 1985; Yarden and Katan, 1993). Since the microtubule protein β-tubulin had been identified as the benomyl target in fungi, it would be of primary importance to investigate the β-tubulin gene in a naturally occurring benomyl-resistant fungus. The objective of this study was to elucidate the β-tubulin gene in *Cochliobolus heterostrophus* (Drechsler) Drechsler [anamorph: *Bipolaris maydis* (Nisikado & Miyake) Shoemaker], a major pathogen of maize, and the relationship between benzimidazole insensitivity and the predicted aa sequence of this fungus. This fungus is inherently insensitive to benzimidazole, including benomyl and thiabendazole (Greenaway, 1973; Tanaka et al., unpublished observation), despite the sensitivity of most fungi belonging to the ascomycetes (Bollen and Fuchs, 1970). We report in this paper the molecular characterization of the *C. heterostrophus* β-tubulin gene, including a determination of its sequence and its possible role in conferring resistance to benzimidazole. This is the first investigation of a naturally occurring benzimidazole-resistant ascomycete.

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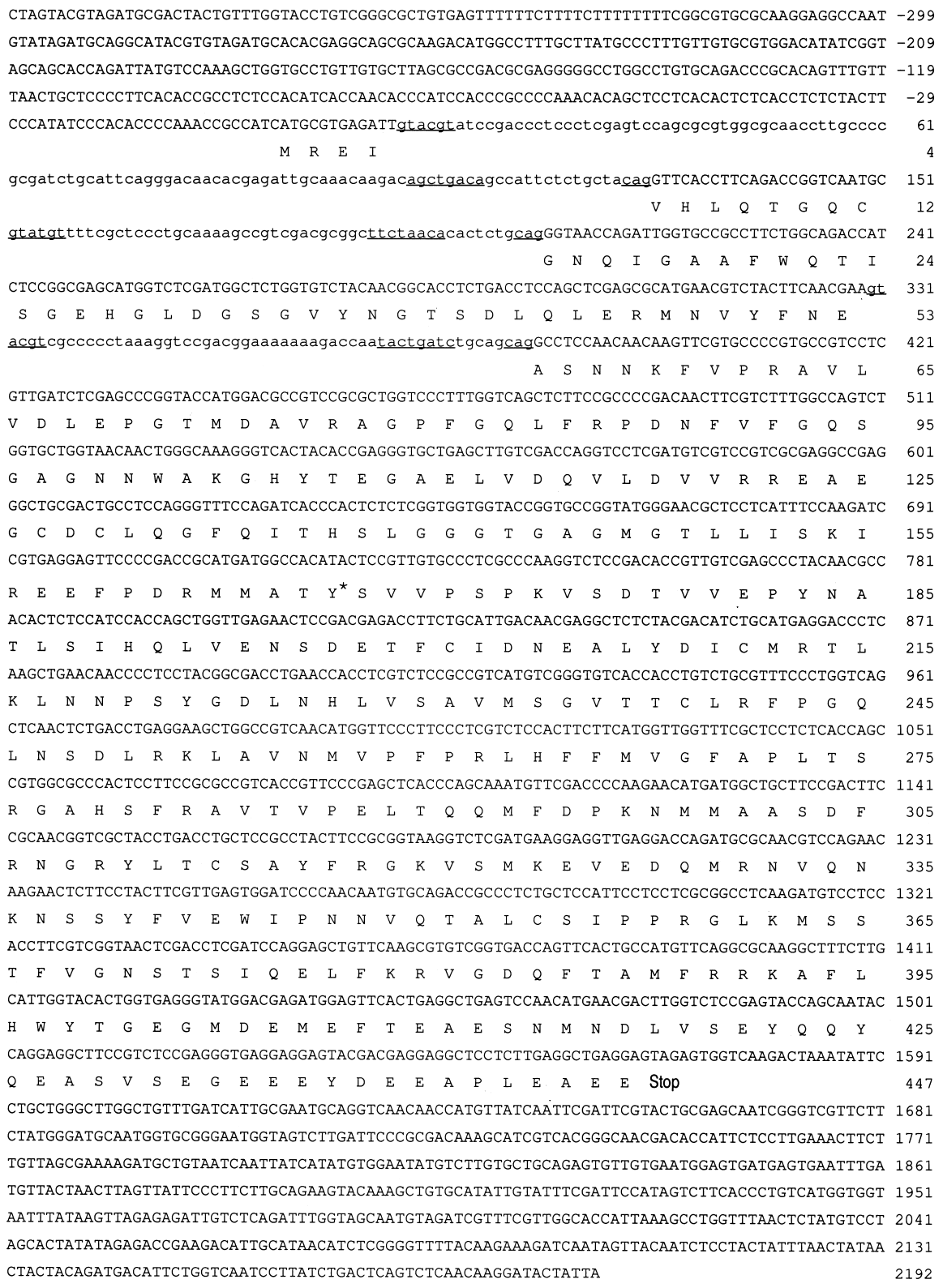

Fig. 1. Nucleotide and predicted amino acid (aa) sequences of the *Cochliobolus heterostrophus Tub1* gene.

The amino acid sequence was deduced from the DNA sequence and is indicated by the single letter aa code below the nucleotides. Numbers to the right side of nucleotide and aa sequences refer to the number of nucleotides and aa, respectively, starting at the ATG initiation codon. The three introns are indicated by lowercase letters, and their 5′ and 3′ sites and internal consensus sequences are underlined. The Tyr^{167} residue (Y) is marked by an asterisk. These sequence data appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Data Libraries under the accession number AB009971.

Materials and Methods

Strain and plasmid. *Escherichia coli* strain DH10B (Life Technologies Inc., Rockville, MD, USA) was used for plasmid propagation. This strain was also used as the host for maintaining the genomic library of *C. heterostrophus* constructed in cosmid vector sCOS1 (Evans et al., 1989). Plasmid pSV50 (Vollmer and Yanofsky, 1986) was used as a probe to screen for the β-tubulin gene from a genomic DNA library of *C. heterostrophus* strain HITO7711 (Tanaka et al., 1991).

Genomic library screening. Construction of the genomic DNA library of *C. heterostrophus* was described previously (Shimizu et al., 1997). DH10B cells carrying the cosmids were plated on LB agar medium containing 50 ppm (w/v) kanamycin and screened by colony hybridization (Sambrook et al., 1989). Colonies were blotted onto Hybond-N^+ membrane (Amersham International plc., Amersham, UK) according to the manufacturer's recommendations. To screen for the *C. heterostrophus* β-tubulin gene, a 2.6 kbp *Sal* I fragment from pSV50 encoding the *Neurospora crassa Bml* β-tubulin gene was radiolabeled with [α-^{32}P]dCTP by using a Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim, BRD) as the manufacturer directed. Hybridization and washing were conducted at 58°C, but other conditions followed the manufacturer's protocol (Boehringer Mannheim GmbH). To elucidate the existence of the β-tubulin gene of *C. heterostrophus* in the cosmid identified by colony hybridization, Southern hybridization was performed. Cosmid DNA was digested with appropriate restriction endonucleases, electrophoresed in a 0.7% agarose gel, and transferred onto a Hybond-N^+ membrane. The same DNA fragment from pSV50 was used as a probe, but it was labeled with DIG-dUTP and detected by ELISA with a DIG-DNA Labeling and Detection Kit (Boehringer Mannheim GmbH). Hybridization and washing were as described for colony hybridization.

DNA sequencing and gene analysis. DNA sequencing of the β-tubulin gene was performed by the dideoxy chain termination method (Sanger et al., 1977) with the ALFred DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden), using Thermo Sequenase™ (Amersham International plc), as the manufacturer recommended. Sequencing primers included the fluorescent-dye (Cy5)-labeled M13 forward, M13 reverse (Pharmacia Biotech), and 2 synthetic oligonucleotide primers derived from established sequences. The locations of exons and introns were deduced from interruptions in aa sequence homology with the β-tubulin gene of *Septoria nodorum* (Cooley and Caten, 1993) and confirmed by sequencing PCR products amplified from cDNA. Template cDNA was synthesized previously (Shimizu et al., 1997). Two primers, 5′-ACACCCCAAACCGCCATCATGC-3′ and 5′-TGGAAACCCTGGAGGCAGTCGC-3′, were used in the PCR amplification for mapping introns. PCR products were cloned into pT7Blue T vector (Novagen Inc., Madison, WI, USA). DNA and aa sequences were edited with DNASIS for Mac software (Hitachi Software Co., Ltd., Yokohama, Japan) and compared with previously characterized β-tubulin genes of other fungi obtained from the GenBank database using the CLUSTAL W multiple alignment program (Thompson et al., 1994).

Results and Discussion

Cloning and sequencing of the C. heterostrophus β-tubulin gene

Following Southern hybridization, one out of about 5,000 cosmid clones showed a positive reaction to the *N. crassa Bml* β-tubulin gene probe. The cosmid of this clone (designated pCOS/TB18) was recovered and subjected to restriction enzyme analysis. The preliminary Southern blot analysis of total *C. heterostrophus* DNA using the *Bml* gene as a probe implied that the β-tubulin gene was a single gene in the genome

Table 1. Some characteristics of and degree of homology between the *Cochliobolus heterostrophus Tub1* gene and β-tubulins of several fungi.

Source	GC content (%)[a]	Number of codons used	Nucleic acid homology (%)[a,b]	Amino acid homology (%)[b]	Reference
Aspergillus nidulans benA	56.8	40	86.1	93.0	May et al., 1987
Aspergillus nidulans tubC	53.4	58	69.5	80.9	May et al., 1987
Colletotrichum graminicola TUB 1	57.3	56	65.3	72.8	Panaccione and Hanau, 1990
Colletotrichum graminicola TUB 2	57.6	41	72.0	93.0	Panaccione and Hanau, 1990
Erysiphe graminis tub-2	46.0	60	76.3	96.4	Sherwood and Somerville, 1990
Neurospora crassa Bml	59.0	42	89.3	94.4	Orbach et al., 1986
Septoria nodorum tubA	59.1	48	92.3	98.2	Cooley and Caten, 1993
Cochliobolus heterostrophus Tub1	58.7	46	—	—	Present study

[a] Only the coding regions were used.
[b] Sequences are aligned for best fit.

and located in a ca. 6.4 kbp *Eco* RI fragment. The restriction map and results of the Southern hybridization experiment also suggested that the homologous region to the probe is in the ca. 6.4 kbp *Eco* RI fragment of pCOS/TB18 (data not shown). Further analyses indicated that the β-tubulin gene was included in a ca. 2.6 kbp *Spe* I fragment. This fragment was cloned into the *Xba* I site of pBluescriptIISK (Stratagene Co., Ltd., La Jolla, CA, USA) to produce pTUB16spe26, then sequenced. The complete nucleotide sequence of the *C. heterostrophus* β-tubulin gene, designated as *Tub1*, is presented in Fig. 1. The *Tub1* gene contained an expected product of 1,570 bp and, by deduction, encoded a protein of 447 aa.

Comparison with β-tubulin of some other fungal species

The *Tub1* gene displayed extensive homology with other β-tubulin genes (Table 1) at the nucleotide and polypeptide levels. The highest degree (98.2%), however, was detected with the *S. nodorum* β-tubulin protein. We deduced the coding region of *Tub1* by comparing the predicted amino acid sequence with published sequences of other β-tubulin genes and by sequencing PCR products amplified from cDNA. The GC content of the coding region (excluding introns) was 58.7% (Table 1). The high GC content of the coding region was due to the predominance of codons with G or C at the third position (78.1%), an indication that codon usage in *Tub1* is biased (Table 2). This bias in codon usage was also evidenced by the absence in *Tub1* of 15 of the possible 61 aa codons. This figure was similar to those reported for β-tubulin genes of *S. nodorum* and some other fungal species, except *Erysiphe graminis* (Table 1).

Position, structure, and analysis of introns

The coding sequence of *Tub1* was interrupted by 3 introns, of 116, 55, and 56 nucleotides, occurring after amino acid numbers 4, 12, and 53, respectively. All the introns contained the filamentous fungal consensus GTANGT and YAG at 5′ and 3′ splice junctions, respectively (Gurr et al., 1987). Sequences similar to the internal lariat signal YGCTAACN characteristic of the 3′ end of introns in filamentous fungi (Gurr et al., 1987) were also found (Fig. 1). A comparison of intron positions between *Tub1* and *tubA* of *S. nodorum* revealed extensive positional conservation (Table 3) and was correlated with the relative sequence homologies (Table 1).

The number of introns in the fungal β-tubulin gene sequenced thus far ranged from 0 in *Saccharomyces cerevisiae* (Neff et al., 1983) to 8 in *Aspergillus nidulans* (May et al., 1987). Although the number of introns in *Tub1* was not the same as those of most as-

Table 2. Codon usage in the *Cochliobolus heterostrophus Tub1* gene.

Amino acid	Codon	Number of occurrences
Phenylalanine	UUU	2
	UUC	24
Leucine	UUA	0
	UUG	2
	CUU	3
	CUC	21
	CUA	0
	CUG	8
Isoleucine	AUU	5
	AUC	7
	AUA	0
Methionine	AUG	20
Valine	GUU	10
	GUC	22
	GUA	0
	GUG	3
Serine	UCU	6
	UCC	20
	UCA	0
	UCG	5
Proline	CCU	6
	CCC	13
	CCA	0
	CCG	0
Threonine	ACU	3
	ACC	19
	ACA	2
	ACG	1
Alanine	GCU	12
	GCC	16
	GCA	1
	GCG	0
Tyrosine	UAU	0
	UAC	14
Histidine	CAU	2
	CAC	7
Glutamine	CAA	3
	CAG	19
Asparagine	AAU	1
	AAC	26
Lysine	AAA	0
	AAG	13
Aspartic acid	GAU	3
	GAC	19
Glutamic acid	GAA	1
	GAG	35
Cysteine	UGU	1
	UGC	7
Tryptophan	UGG	4
Arginine	CGU	8
	CGC	12
	CGA	0
	CGG	0
Serine	AGU	0
	AGC	1
Arginine	AGA	0
	AGG	3
Glycine	GGU	28
	GGC	8
	GGA	1
	GGG	0

comycetes, their positions were characteristic of the taxonomic class. Orbach et al. (1986) suggested that taxa may be characterized in part by the pattern of intron position in present-day tubulin genes, which, according to Liaud et al. (1992), reflects the organization of their common ancestor. Byrd et al. (1990) noted that introns within codons 21 and 35 were characteristic of diverse fungi. Given that *C. heterostrophus* is phylogenically closer to *S. nodorum* than to other fungal species whose β-tubulin genes have been sequenced (Table 3), these results suggest that the loss of introns 21 and 35 may have occurred independently within the fungi, since taxa that lack these introns such as Pleosporales (*Cochliobolus* and *Septoria*) and Hypocreales (*Epichloë*) are not closely related (Berbee, 1996).

Correlation between the C. heterostrophus β-tubulin gene and benomyl insensitive

Because most benzimidazole resistance in various fungi has been mapped to β-tubulin loci, characterizations of their genes have been made, revealing several mutations at codon for aa 6, 50, 134, 165, 167, 198, 200, 237, 241, 250, and 257 (Table 4). By comparing the deduced aa sequence of the *C. heterostrophus Tub1* gene with that of *S. nodorum*, which is benomyl sensitive, it was found that aa residues, 56, 124, 167, 356, 365, 381, and 430 were different between these two fungi. The differences of aa residue at positions, 56, 124, 356, 365, 381, and 430 were not unique to *C. heterostrophus*; these aa were found in other fungi known to lack resistance to benomyl. However, the aa residue at position 167 is unusual. In *Tub1*, it was tyrosine, whereas this aa is phenylalanine in all of the β-tubulin amino acid sequences of

Table 3. Number and position of introns in several fungal β-tubulin genes.

Source	Number of introns	Position of intron[a]								Reference
		4/5	12/13	21	35	53/54	205/206	317	437/438	
Aspergillus nidulans benA	8	+	+	+	+	+	+	+	+	May et al., 1987
Aspergillus nidulans tubC	5	+	+	+	+	−	−	+	−	May et al., 1987
Colletotrichum graminicola TUB 1	6	+	+	+	+	+	−	+	−	Panaccione and Hanau, 1990
Colletotrichum graminicola TUB 2	6	+	+	+	+	+	−	+	−	Panaccione and Hanau, 1990
Epichloë typhina tub2	4	+	+	−	−	+	−	+	−	Byrd et al., 1990
Erysiphe graminis tub-2	6	+	+	+	+	+	−	+	−	Sherwood and Somerville, 1990
Neurospora crassa Bml	6	+	+	+	+	+	−	+	−	Orbach et al., 1986
Septoria nodorum tubA	3	+	+	−	−	+	−	−	−	Cooley and Caten, 1993
Cochliobolus heterostrophus Tub1	3	+	+	−	−	+	−	−	−	Present study

[a] +, intron is present; −, intron is absent.

Table 4. Deduced amino acid substitution in the β-tubulins of some selected fungi with resistance to benzimidazole.

Amino acid		Organism	Reference	Predicted amino acid in *Tub1*
Position	Substitution			
6	His to Tyr	*Trichoderma viride*	Goldman et al., 1993	His
50	Tyr to Asn	*Aspergillus nidulans*	Koenraadt et al., 1992	Tyr
	Tyr to Ser	*Aspergillus nidulans*	Koenraadt et al., 1992	
134	Gln to Lys	*Aspergillus nidulans*	Koenraadt et al., 1992	Gln
165	Ala to Val	*Aspergillus nidulans*	Jung and Oakley, 1990	Ala
167	Phe to Tyr	*Neurospora crassa*	Orbach et al., 1986	Tyr
198	Glu to Ala	*Botrytis cinerea*	Yarden and Katan, 1993	Glu
	Glu to Asp	*Aspergillus nidulans*	Jung et al., 1992	
	Glu to Gln	*Aspergillus nidulans*	Jung et al., 1992	
	Glu to Gly	*Neurospora crassa*	Fujimura et al., 1992a	
	Glu to Lys	*Venturia inaequalis*	Koenraadt et al., 1992	
	Glu to Val	*Penicillium expansum*	Koenraadt et al., 1992	
200	Phe to Tyr	*Botrytis cinerea*	Yarden and Katan, 1993	Phe
237	Thr to Ala	*Neurospora crassa*	Fujimura et al., 1994	Thr
241	Arg to His	*Saccharomyces cerevisiae*	Thomas et al., 1985	Arg
250	Leu to Phe	*Neurospora crassa*	Fujimura et al., 1994	Leu
257	Met to Leu	*Aspergillus nidulans*	B. R. Oakley[a]	Met

[a] Personal communication cited in Koenraadt et al., 1992.

species known to be sensitive to benomyl. The Tyr^{167} has been demonstrated by Orbach et al. (1986) to be responsible for benomyl resistance in *N. crassa*. The other benzimidazole-implicated residues in *Tub1*, His^{6}, Tyr^{50}, Gln^{134}, Ala^{165}, Glu^{198}, Phe^{200}, Thr^{237}, Arg^{241}, Leu^{250}, and Met^{257} coincided with those of other fungi known to be sensitive to benzimidazole fungicide (Table 4). These results suggest that benomyl insensitive in *C. heterostrophus* may be associated with residue Tyr^{167} of *Tub1*. The only other organism reported to contain Tyr^{167} in its β-tubulin is the protozoan *Trichomonas vaginalis* (Katiyar and Edlind, 1994; Katiyar et al., 1994), which is not affected by the benzimidazole derivatives.

The Phe→Tyr change at aa position 167 is known to be a mutation that confers moderate resistant phenotype in *N. crassa*. The minimal inhibitory concentration (MIC) of Tyr^{167} mutant of *N. crassa* on carbendazim is 100 μg/ml, and one of a wild type is 0.1 μg/ml, (Fujimura et al., 1992b). However, the MIC of *C. heterostrophus* on benomyl is $>$400 μg/ml, and the median effective concentration (EC_{50}) on colonial growth is 30 μg/ml (data not shown). In the absence of the strain that is sensitive to benzimidazole in *C. heterostrophus*, the level of resistance caused by Tyr^{167} residue remains unclear. Contrasting argument that the Tyr^{167} residue is not the only requirement for high resistance to benomyl may be made. Additional mechanism, e. g., an active transport system reported in *Candida albicans* (Ben-Yaacov et al., 1994), might also be involved in the nature of insensitivity to benzimidazole in *C. heterostrophus*. Construction of the transformant whose *Tub1* gene is replaced by the mutated one with Phe^{167} will promote a better insight into the mechanism of insensitivity to benzimidazole in *C. heterostrophus*.

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