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Isolation and Sequencing of the cDNA Encoding β-tubulin from *Pleurotus sajor-caju*

Beom-Gi Kim*, Pyung-Gyun Shin, Mi-Jeong Jeong, Soo-Chul Park, Young-Bok Yoo, Jin-Chang Ryu and Suk-Tae Kwon[1]

Division of Molecular Genetics, National Institute of Agricultural Science and Technology, RDA, Suwon 441-707, Korea

[1]Department of Genetic Engineering, Sung Kyun Kwan University, Suwon, Korea

여름느타리버섯으로부터 β-tubulin cDNA의 분리 및 염기서열 결정

김범기 · 신평균 · 정미정 · 박수철 · 유영복 · 류진창 · 권석태[1]

농업과학기술원 분자유전과, [1]성균관대학교 유전공학과

ABSTRACT: The cDNA encoding β-tubulin of *Pleurotus sajor-caju* was isolated using an internal gene segment probe amplified by polymerase chain reaction (PCR) of genomic DNA and by cDNA library screening. The cDNA was consisted of 1560 nucleotides(nt), including a 5'-untranslation region (UTR) of 27nt, an open reading frame (ORF) of 1341nt, and a 3'-UTR of 191nt. The ORF encoded a protein of 446 amino acids(aa), which shows over 80% homology with β-tubulins of other filamentous fungi. Southern hybridization analysis showed that there were two isotypes of β-tubulin genes in *P. sajor-caju*. Through sequence analysis we found that β-tubulin had a unusual Cys^{165} residue, which might be a significant factor for the insensitivity of fungi to fungicide benomyl.

KEYWORDS: Basidiomycetes, Benomyl resistance, *Pleurotus sajor-caju*, β-tubulin

Microtubules are basic elements of cytoskeleton and they take part in essential cellular functions such as cell division, motility, and transport. Consequently, their protein components are abundant and present in all eukaryotic cells. Tubulin, a heterodimer formed by two different subunits, α and β, is the major constituents of microtubules. Comparison of full length amino acid sequences indicates that β-tubulins are highly conserved polypeptides in all eukaryotic organisms (Hepler and Palevitz, 1974; Cleveland and Sullivan, 1985; Fosket, 1992; Alberts *et al.*, 1994).

In fungi the cloning and characterization of β-tubulin genes have been widely reported in ascomycetes, deuteromycetes and yeasts but no β-tubulin genes have yet been reported in edible mushrooms (Orbach *et al.*, 1986; May *et al.*, 1987; Russo *et al.*, 1992).

Fungicide benomyl interferes with mitosis in fungi by binding to tubulin (Fujimura *et al.*, 1994). Molecular characterization of benomyl resistant mutants in *Aspergillus nidulance, Neurospora crassa* and *Saccharomyces cerevisiae* provides evidences that benomyl resistance is due to point mutations of β-tubulin and common mutation sites are known (Orbach *et al.*, 1986; Koenraadt *et al.*, 1992; Fujimura *et al.*, 1994). In many fungi, benomyl resistance has been used as a dominant selection marker in genetic transformation. The development of genetic transformation system in edible mushroom, *Pleu-*

*Corresponding author

rotus species is necessary for breeding and molecular biology studies (Henson *et al.*, 1988; Bernier *et al.*, 1989; Seip *et al.*, 1990; Cooley *et al.*, 1991; Nowak and Kuck, 1994). We started a program of cloning and sequencing the β-tubulin gene of *P. sajor-caju* in order to develop the benomyl resistant β-tubulin *in vitro*. The benomyl resistant β-tubulin can be used as selection marker for vector.

In the present paper, we report the full sequences of the cDNA encoding β-tubulin in *P. sajor-caju* and comparison with those of other fungi and benomyl resistant β-tubulin.

Materials and Methods

Enzymes and Chemicals

Restriction enzymes and modifying enzymes were obtained from Promega and used as recommended by supplier. [α-^{35}S]dATP and [α-^{32}P]dCTP were purchased from Amersham. The sequencing kit was purchased from USBiochemical, USA. Other chemicals were purchased from the Sigma Chemical Co.

Strains and media

The *P. sajor-caju* (MGL 2084) stocked at National Institute of Agricultural Science and Technology was grown in mushroom complete medium at 30°C (MCM; Bennett and Lasure, 1991). The *E. coli* strain was XL 1Blue MRF'.

cDNA library construction

Total RNA was isolated from submerged-grown mycelia by the method using Cesium Chloride (Ullrich *et al.*, 1977). Polyadenylate-containing [poly(A)+] RNA was isolated from total RNA using poly(A)Quik mRNA isolation kit (Stratagene) and cDNA library was constructed using ZAPII cDNA synthesis kit (Stratagene) and Gigapack II kit (Stratagene). The kits were used as recommanded by supplier.

Polymerase chain reaction

PCR amplification of *P. sajor-caju* genomic DNA was performed with oligo deoxynucleotide primers (upstream primer 5'-GAGGAATTCCCAGACCGTATGATG-3', downstream primer 5'-TACAAGCTGGGTTTCTTGTACTACCGAC-3') used in *Venturina velutipes* for β-tubulin gene cloning. PCR reaction was carried out in a thermal cycler (Perkin Elmer Cetus) for 40 cycles with each cycle consisting of 94°C for 1 min to denature the template, 55°C for 1 min for primer annealing and 72°C for 2 min for polymerization.

Plaque hybridization and Southern hybridization

Pleurotus sajor-caju mycelia cDNA library was screened with a PCR product encoding the β-tubulin conservative region as probe. Approximately 200,000 phages from the cDNA library were screened with the probe labelled with ECL hybridization kit (Amersham). Hybridization and washing were performed as recommended from supplier.

DNA sequencing and homology analysis

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.* with [α-^{35}S]dATP. DNA homology analysis was carried out with Dnasis program (HITACH) and Genbank database (NIH). Amino acid homology analysis was performed with Prosis program (HITACH) and Swiss-plot database.

Southern blot analysis

An insert fragment of Myc7tub clone was labelled by random primer labelling system (promega) with [α-^{32}P]dCTP. The labelled DNA was used for hybridization at 65°C with a blotted DNA fragment on a nylon memebrane by the procedure of Southern (1975).

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1     GCC AAT CCG TCC TCC ATC CTC ATC TAT ATG CGC GAA ATC GTC CAC CTT CAG ACT GGC CAG
                                           M   R   E   I   V   H   L   Q   T   G   Q
61    TGT CGG AAC CAA ATT GGT GCC AAG TTC TGG GAG GTT GTT TCC GAT GAG CAT GGA ATC GAG
       C   R   N   Q   I   G   A   K   F   W   E   V   V   S   D   E   H   G   I   E
121   CCC GAT GGT CTG TAC AAA GGC AAC AAT GAC CTC CAA CTC GAG CGC ATC TCT GTC TAC TAC
       P   D   G   L   Y   K   G   N   N   D   L   Q   L   E   R   I   S   V   Y   Y
181   AAC GAG GTC GGA GCG AAC AAG TAC GTT CCC CGA GCT GTC TTG GTC GAT CTC GAG CCC GGT
       N   E   V   G   A   N   K   Y   V   P   R   A   V   L   V   D   L   E   P   G
241   ACC ATG GAT TCG GTG CGG TCG GGC CCA CTG GGC AGC CTT TTC CGC CCA GAC AAC TTT GTC
       T   M   D   S   V   R   S   G   P   L   G   S   L   F   R   P   D   N   F   V
301   TTT GGT CAG AGT GGA GGC GGT AAT AAC TGG GCG AAA GGG CAT TAT ACG GAG GGC GCT GAA
       F   G   Q   S   G   G   G   N   N   W   A   K   G   H   Y   T   E   G   A   E
361   CTC GTT GAT GCC GTT CTC GAT GTT GTG CGC AAG GAG GCT GAG GGC ACG GAT TGT CTG CAA
       L   V   D   A   V   L   D   V   V   R   K   E   A   E   G   T   D   C   L   Q
421   GGT TTC CAG ATC ACC CAC TCT CTA GGT GGT GGA ACT GGT GCC GGT ATG GGT ACA CTT TTG
       G   F   Q   I   T   H   S   L   G   G   G   T   G   A   G   M   G   T   L   L
481   ATC TCC AAA ATT CGT GAG GAA TAT CCC GAC CGA ATG ATG TGC ACA TAT TCC GTT GTT CCA
       I   S   K   I   R   E   E   Y   P   D   R   M   M   C   T   Y   S   V   V   P
541   AGT CCC AAG GTA TCG GAC ACT GTC GTT GAG CCG TAC AAC GCC ACG CTC TCC GTC CAC CAA
       S   P   K   V   S   D   T   V   V   E   P   Y   N   A   T   L   S   V   H   Q
601   CTT GTC GAG AAT TCC GAC GAG ACT TTC TGT ATT GAT AAC GAG GCT CTC TAC GAC ATC TGT
       L   V   E   N   S   D   E   T   F   C   I   D   N   E   A   L   Y   D   I   C
661   TTC CGC ACT CTC AAA CTC ACG ACA CCC ACG TAC GGC GAT CTC AAC CAT CTC ATC TCA ATT
       F   R   T   L   K   L   T   T   P   T   Y   G   D   L   N   H   L   I   S   I
721   GTC ATG TCA GGC ATT ACA ACT TGT TTA CGA TTC CCT GGT CAA CTC AAC TCG GAT CTG AGA
       V   M   S   G   I   T   T   C   L   R   F   P   G   Q   L   N   S   D   L   R
781   AAG TTG GCC GTC AAC ATG GTT CCT TTC CCC CGT CTC CAT TTC TTC ATG CCT GGC GTT TGC
       K   L   A   V   N   M   V   P   F   P   R   L   H   F   F   M   P   G   V   C
841   CCG CTC ACC GCA CGA GGA AGC CAA CAA TAT CGC GCC GTC ACC GTC CCT GAG TTG ACC CAG
       P   L   T   A   R   G   S   Q   Q   Y   R   A   V   T   V   P   E   L   T   Q
901   CAA ATG TTC GAC GCG AAG AAC ATG ATG GCT GCC TCG GAT CCC CGC CAT GGC CGC TAC CTC
       Q   M   F   D   A   K   N   M   M   A   A   S   D   P   R   H   G   R   Y   L
961   ACT GTT GCT GCC GTT TTC CGT GGC AAA GTA TCG ATG AAG GAA GTC GAA GAG CAA ATG CAG
       T   V   A   A   V   F   R   G   K   V   S   M   K   E   V   E   E   Q   M   Q
1021  AAC GTC CAG AAC AAA AAC TCT GCC TAC TTC GTG GAG TGG ATC CCC AAT AAC GTC CTT ACT
       N   V   Q   N   K   N   S   A   Y   F   V   E   W   I   P   N   N   V   L   T
1081  GCC CAA TGT GAC ATT CCA CCC CGC GGA TGC AAG ATG GCC GTT ACC TTC TTG GGC AAC TCG
       A   Q   C   D   I   P   P   R   G   C   K   M   A   V   T   F   L   G   N   S
1141  ACC GCC ATC CAA GAA CTC TTC AAG CGT GTC AAC GAC CAA TTC GCG GCT ATG TTC AAG CGC
       T   A   I   Q   E   L   F   K   R   V   N   D   Q   F   A   A   M   F   K   R
1201  AAG GCT TTC TTG CAT TGG TAC ACG CAA GAG GGT ATG GAC GAG ATG GAG TTC ACG GAG GCC
       K   A   F   L   H   W   Y   T   Q   E   G   M   D   E   M   E   F   T   E   A
1261  GAA TCA AAC ATG CAG GAT CTT GTC GCT GAA TAC CAA CAA TAC CAG GAT GCT ACT GCC GAT
       E   S   N   M   Q   D   L   V   A   E   Y   Q   Q   Y   Q   D   A   T   A   D
1321  GAG GAA GAG GGT GAA TAC GAA GAG GAA CCC GCT GAA GAA GAA CAG TGA TCA ATG GAT TGT
       E   E   E   G   E   Y   E   E   E   P   A   E   E   E   Q   *
1381  CTG TAT CGC CGA CTG CAC TCG CCG GGT AAA TCC TAC CTA CCT TCT GCA TAT TGC TCT TCA
1441  CCC TCT TCA TTC TTC CCT TTC ATC ATC TCA TCA TCA CTA TTC CGC CCA GTT ACC CTT GAT
1501  GCA TGT TAC GCT CTT TCA TTT CTC TAT GTG GAT GTC TGA ATA AAT TGG CTT TTA TCA GCA
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Fig. 1. Nucleotide sequence and the deduced amino-acid sequence of the *P. sajor-caju* β-tubulin cDNA.
The concensus sequences of poly A signal are underlined. The sequence was determined by following the protocol for sequencing double stranded templates using sequenase from USB, and by using [α-^{35}S] dATP as a label. The sequence is available in the Genbank database under accession no. U64720.

Results and Discussion

PCR amplification of the β-tubulin gene

Optimal annealing temperature in PCR yielded only one band at about 500bp on a gel (data not shown). This PCR product showed the same size estimated in *V. velutipes*.

Isolation of cDNA clones encoding β-tubulin and sequencing analysis

Twenty positive clones were selected in 200, 000 plaques and one of five positive clones that carried suitable insert size was further analyzed and was named My7tub. The cDNA

Table 1. Amino acid sequence homology among Myc7tub clone and β-tubulin isolated from 5 different species

Species	%[a]
Schizophyllum commune	93
Homo sapience	86
Drosophila melanogaster	83
Neurospora crassa	82
Arabidopsis thaliana	80

[a] Sequences were collected from the SWISS-PLOT Databank and comparison was made with PROSIS (Hitachi) software.

was consisted of a 5'-UTR of 27 nt, an ORF of 1341nt, and a 3'-UTR of 191nt (Fig. 1). The 3'-UTR had a consensus AATAA polyadenylation signal 16nt upstream from the polyadenylation site. The deduced aa sequence of the ORF shows over 70% homology with β-tubulins of various organisms such as human, *Drosophila, Neurospora*, and *Arabidopsis* (Table 1). Guanidine moiety of GTP is now known to bind to β-tubulin within the sequence AI(v) LVDLEPGTMDSVR, between aa 63-77 and β-tubulin of *P. sajor-caju* has 100% identity to these aa sequences in the same region (Linse and Mandelkow, 1988). The carboxylterminal region has recently been shown to be involved in the binding of microtubule associated proteins (MAPs). The acidic nature of carboxylterminal region is thought to form the basis for the interaction of MAPs with tubulin. In β-tubulin of *P. sajor-caju* the aa of carboxylterminal region also includes a high number of glutamic acid residues (Vallee *et al.*, 1990).

Benzimidazole fungicides such as carbendazim, thiabendazole, nocodazole and benomyl, interfere mitosis in fungi by binding to β-tubulin. Ala[165] to Val, Phe[167] to Tyr, and Glu[198] to Gly changes in aa sequences commonly confer to resistance to a benomyl sensitivity in many fungi (Orbach *et al.*, 1986; Jung and Oakley., 1990). In contrast, the β-tubulin of *P. sajor-caju* has a Cys[165] residue which is un-

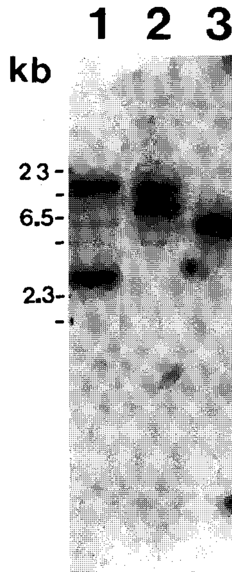

Fig. 2. Southern blot analysis of *Pleurotus sajor-caju* genomic DNA probed with the fragment of the Myc7tub clone digested with *Eco*RI 5g of genomic DNA prepared *Pleurotus sajor-caju* was separated on 0.8% agarose gel and transfered. Genomic DNA was digested with *Eco*RI (Lane 1), *Hin*dIII (Lane 2) and *Eco*RV (Lane 3). The fragment of myc7tub clone was labelled with [α-^{32}P]dCTP (3000Ci/mmol) by the random primer labelling system (Promega). The blot was prehybridized at 65°C in a solution containing 0.1% SDS, 6X SSC, 5X Denharts solution, 50 mM Tris-HCl (pH 7.6), 100 μg/*ml* salmon sperm DNA for at least 1h and then hybridized in the same solution plus the denaturated probe for 16 hr. The blots were washed twice with 2X SSC and 0.1% SDS for 10 min each time at room temperature, twice with a solution containing 1X SSC and 0.1% SDS for 20 min at 65°C and twice with 0.5X SSC and 0.1% SDS for 20 min at 65°C.

usual among β-tubulins of other fungi except for the slime mold and *Schizophyllum commune*. These three fungi which have a Cys^{165} residue in their β-tubuin show benomyl in sensitivity. It suggest that the presence of Cys^{165} residue in β-tubulin results in the insensitivity of *S. commune, P. sajor-caju* and slime molds (Burland *et al.*, 1988; Wenenskoid *et al.*, 1988).

Southern blot analysis

Genomic DNA was isolated from *P. sajor-caju* and digested with *Eco*RI, *Hin*dIII, *Eco*-RV. The total DNA fragments were transferred to nylon membrane and then hybridized with a cDNA fragment of My7tub clone digested with *Eco*RI. Genomic DNA digested with *Eco*RI and *Hin*dIII showed a double signal at about 2.7 kb, 12 kb, 9 kb, and 8 kb, respectively (Fig. 2). This suggest that two different isotypes of β-tubulin are present in *P. sajor-caju*.

적 요

여름느타리버섯 균사체의 cDNA library로부터 β-tubulin 유전자를 분리하여 염기서열을 분석하였다. 분리된 β-tubulin cDNA유전자는 27nt의 5'-untranslation region과 1341nt의 open reading frame, 191nt의 3'-untranslation region으로 구성되어 있었다. ORF는 445개의 아미노산들로 구성되어 있으며, 동물, 식물, 사상균에서 보고된 β-tubulin과 80% 이상의 상동성을 보였다. 분리된 Myc7tub clone을 사용하여 Southern hybridization 한 결과 여름느타리버섯에는 두 가지의 isotype β-tubulin이 존재할 것으로 생각된다.

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