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Cloning of β-Tubulin Gene and Effect of Pencycuron on Tubulin Assembly in *Rhizoctonia solani*

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To illustrate the action mechanism of pencycuron on *Rhizoctonia solani*, two experiments were conducted including the comparison of amino acids of β-tubulin between R-C (sensitive isolate) and Rh-131 (non-sensitive isolate), and the inhibitory effect of pencycuron on tubulin assembly *in vitro*. Both β–tubulin genes of R-C and Rh-131 proved to have 1,582 nucleotides encoding a protein of 445 amino acids, showing 98% homology in amino acid sequences between them. It was found that codons at 103, 236, and 267 for lysine (AGG), valine (GTC) and isoleucine (ATT) in R-C were replaced by codons for methionine (ATG), isoleucine (ATT) and methionine (ATG) in Rh-131, respectively. No inhibitory effect of pencycuron on the tubulin assembly was observed. It suggests that pencycuron may have no direct inhibitory effects on the assembly of tubulin at least *in vitro*.

Keywords **:** pencycuron, *Rhizoctonia solani*, β-tubulin gene, tubulin assembly

Pencycuron, 1-(4-chlorobenzyl)-1-cyclopentyl-3-phenylurea, has been used to control plant diseases such as rice sheath blight and potato black scurf caused by *Rhizoctonia solani* worldwide since its development in 1985 (Yamada et al., 1985). However, the action mechanism of pencycuron still largely remains obscure, though it was reported that the destruction of microtubules which are an integral part of the cytoskeleton in hyphal tips of *R. solani* (Ueyama et al., 1990) was caused by the chemical. As for its effect on functions of plasma membrane, we showed that the application of pencycuron brought the change of the osmotic stability and the fluidity of plasma membrane in pencycuron sensitive *R. solani* (Kim et al., 1996; Kim and Yamaguchi, 1996).

In order to illustrate mode of action of pencycuron against *R. solani,* it should be preferentially manifested whether it directly affects the assembly of tubulins or not. As is already well known, benzimidazole fungicides inhibit the assembly of tubulins into microtubules. In particular, the amino acid at position 198 of the β-tubulin in *Neurospora crassa* has been revealed to the an important site for binding of benzimidazoles, leading to the inhibition of tubulin assembly (Fujimura et al., 1992). Furthermore, the change of a single amino acid in β-tubulin proved to cause resistance against benzimidazoles not only in *N. crassa* but also in *Aspergillus nidulans* and *Venturia inaequalis* (Koenraadt et al., 1992; Jung et al., 1992). If the action mechanism of pencycuron is closely related to the inhibition of tubulin assembly in *R. solani*, the isolates non-sensitive to pencycuron might have the change of amino acids in β-tubulin protein. It may explain the very narrow active spectrum of pencycuron which can be seen even in the same anastomosis group of *R. solani.*

In order to reveal the relationship between the tubulin assembly and the action mechanism of pencycuron, two experiments were performed in this study. The β-tubulin genes were cloned from pencycuron-sensitive and non-sensitive isolates of *R.solani* and their deduced amino acid sequences were compared. The inhibitory effect of pencycuron on the tubulin assembly was also investigated *in vitro* by using the tubulins prepared from the mycelia of *R. solani.*

Materials and Methods

Isolates of *R. solani.* Two isolates of *R. solani* in anastomosis group 4 (AG4) were used in this study; R-C, an isolate sensitive to pencycuron, and Rh-131, a non-sensitive one. They were incubated in potato dextrose broth (PD broth) at 25°C for 3 days without shaking. After homogenizing the mycelial mats in sterilized PD broth, 1 ml of mycelial suspension was inoculated into 100 ml of fresh PD broth. To extract mRNA of *R. solani*, mycelia were harvested by filtration 1 day after incubation at 25°C and washed with sterilized distilled water twice. Tubulin proteins were pre-

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pared from 3-day-old mycelia cultured on a reciprocal shaker at 25°C.

Cloning the β-tubulin genes from *R. solani* isolates. cDNAs were synthesized from mRNAs of isolates R-C and Rh-131 using the TimeSaver™ cDNA synthesis kit (Pharmacia Biotech) and cloned into the unique *Eco*RI site of λZAP II (STRATAGENE®). Following the construction of cDNA libraries, the β-tubulin genes of *R. solani* were isolated by using the β-tubulin gene of *N. crassa* F914 as a probe; plaques were transferred to Hybond™-M⁺ membrane (Amersham) and hybridized with the *Hind*III and *Eco*RI fragment of pEF50 (Fujimura et al., 1992). The resulting hybridized phage DNAs were excised *in vivo* using the ExAssist/SOLR system (Stratagene). Then, southern analysis was carried out according to the standard procedure (Sambrook et al., 1989). In the case of R-C, the 5'-terminal region of β-tubulin cDNA was amplified with Marathon™ cDNA Amplification Kit (CLONTECH) and cloned with pCRII TA-cloning vector (Invitrogen).

Sequencing of the β-tubulin genes from *R. solani*. A series of deletion of DNA region for the β-tubulin was constructed using the Deletion Kit for Kilo-Sequencer (TaKaRa). Sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with the A.L.F. DNA sequencer (Pharmacia).

Preparation of tubulin. Tubulins from mycelia of *R. solani* were isolated as described by Kilmartin (Kilmartin, 1981). Mycelia of *R. solani* were homogenized with a blender in the isolation buffer containing 0.1 M PIPES (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM GTP (Na salt), and 1/100 solution P (87 mg of ρ-methylphenylsulfonyl fluoride and 1.5 mg of pepstain A in 5 ml of ethanol). The homogenate was centrifuged at 10,000×g for 30 min and subsequently at 100,000×g for 60 min. After concentration of the supernatant with Amicon 400 (PM-10 membrane), the sample was applied onto DEAE-Sephadex A-50 column equilibrated with extraction buffer [0.1 M PIPES (pH 6.9), 0.2 mM $MgCl_2$, 0.1 mM GTP, and 1/1000 solution P]. The column was eluted with stepwise gradients of KCl; 0.2 M, 0.5 M, and 1.0 M. Proteins in the 0.5 M KCl fraction were precipitated by $(NH_4)_2SO_4$ precipitation at 85% of saturation. The precipitate was dissolved in the assembly buffer 0.1 M PIPES (pH 6.9), 0.1 mM $MgCl_2$, 1 mM GTP, and 1/100 solution P and subsequantly dialyzed against the same buffer. For further purification of the tubulin proteins, the concentrated protein fraction was subjected to the assembly-disassembly procedures which were repeatly conducted at 37°C and 4°C in turns.

The effects of fungicides on the assembly of tubulin. The purified tubulins were incubated with fungicides at 4°C for 30 min. Subsequently the samples were kept at 37°C for 50 min to make tubulins assemble to microtubules. After centrifugation at 120,000×g for 60 min, the quantity of protein was measured by Bradford's method (Bradford, 1976).

Results and Discussion

The cDNA libraries of *R. solani* R-C and Rh-131 containing 2×10^8 and 1.5×10^9 plaques, respectively, were screened with the DIG-labeled β-tubulin DNA from *N. crassa* F914 strain as a probe. Two recombinant plasmids named as pTuRC8005 and pTuRh305 (from R-C and Rh-131, respectively) hybridized strongly with the probe by southern analysis. They contained 1.47 kb (pTuRC8005) and 1.58 kb (pTuRh305) inserts and their nucleotide sequence analyses revealed that both of them had 1,582 nucleotides encoding a protein of 445 amino acids (Fig. 1, 2). The deduced amino acid sequences for the β-tubulin from *R. solani* R-C had high homology with those of β-tubulins from *Schizophyllum commune, Neurospora crassa* (Orbach et al., 1986), *Pneumocystis carini, Trichoderma viride,* and *Aspergillus nidulans* (Jung et al., 1992), with 90.6, 83.4, 82.1, 80.2, and 76.5% homology, respectively (Fig. 3). The comparison of the amino acid sequences of β-tubulin from R-C to those from Rh-131 showed 98% in identity. The codons at 103, 236, and 267 for lycine (AGG), valine (GTC) and isoleucine (ATT) in *R. solani* R-C were replaced by codons for methionine (ATG), isoleucine (ATT) and methionine (ATG) in *R. solani* Rh-131, respectively.

It is known that a single codon change in β-tubulin gene causes the emergence of resistance to benzimidazoles in a

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        10        20        30        40        50        60        70        80        90
tgacaccaaccactacactctccagtttaattcacgacctaaaaaccatgcgcgagatcgttcaccttcagactggccagtgcggcaac
                                                 M  R  E  I  V  H  L  Q  T  G  Q  C  G  N

       100       110       120       130       140       150       160       170       180
cagattggtgccaagttctgggaggtcgtttccgatgagcacggaattgccggtgacggaaagtaccagggcaataatgacctccaactc
 Q  I  G  A  K  F  W  E  V  V  S  D  E  H  G  I  A  G  D  G  K  Y  Q  G  N  N  D  L  Q  L

       190       200       210       220       230       240       250       260       270
gagtgtatctcggtctactacaacaccgtaggagacaacaagtacgtgcctcgtgcggtccttgtcgatctcgagcctggtacgatggac
 E  C  I  S  V  Y  Y  N  T  V  G  D  N  K  Y  V  P  R  A  V  L  V  D  L  E  P  G  T  M  D

       280       290       300       310       320       330       340       350       360
tctgttcgctcgggtcctctcggtggtctcttccgccccgataacttcgtgttcggccagagcggtgctggtaacaactgggccaagggc
 S  V  R  S  G  P  L  G  G  L  F  R  P  D  N  F  V  F  G  Q  S  G  A  G  N  N  W  A  K  G

       370       380       390       400       410       420       430       440       450
cattataccgagggtgctgagcttgtcgaccaagtgctcgatgtgacgcgcaaagaagctgaaggatgcgactgcttgcagggcttccag
 H  Y  T  E  G  A  E  L  V  D  Q  V  L  D  V  T  R  K  E  A  E  G  C  D  C  L  Q  G  F  Q

       460       470       480       490       500       510       520       530       540
attacccactcgctcggtggaggaactggtgctggtatgggtacccttttgatctccaagatccgtgaagagtaccccgaccgcatgatg
 I  T  H  S  L  G  G  G  T  G  A  G  M  G  T  L  L  I  S  K  I  R  E  E  Y  P  D  R  M  M

       550       560       570       580       590       600       610       620       630
tgcacgttctcggtcgttccttctcccaaggtctctgataccgtcgtcgagccctacaatgccaccctctcggtccatcagcttgttgag
 C  T  F  S  V  V  P  S  P  K  V  S  D  T  V  V  E  P  Y  N  A  T  L  S  V  H  Q  L  V  E

       640       650       660       670       680       690       700       710       720
aactcagacgagaccttctgcattgacaacgaggccttgtacgatatctgcttccgtaccctcaagctctctacccccacgtatggtgac
 N  S  D  E  T  F  C  I  D  N  E  A  L  Y  D  I  C  F  R  T  L  K  L  S  T  P  T  Y  G  D

       730       740       750       760       770       780       790       800       810
ctcaaccacctcgtgtccattgtcatgtctggtgtcaccacctgcttgcgtttccctggtcagctcaactctgatcttcgcaaacttgct
 L  N  H  L  V  S  I  V  M  S  G  V  T  T  C  L  R  F  P  G  Q  L  N  S  D  L  R  K  L  A

       820       830       840       850       860       870       880       890       900
gtcaacatggttcctttccctcgtttgcacttcttcattgtcggctttgcgcccttgaccgctcgtggctctgttcaataccgtgccgtc
 V  N  M  V  P  F  P  R  L  H  F  F  I  V  G  F  A  P  L  T  A  R  G  S  V  Q  Y  R  A  V

       910       920       930       940       950       960       970       980       990
tcggtacccgagcttacccaacaaatgttcgatgccaagaacatgatggctgcttccgaccctcgccacggccgctacctcaccgttgcc
 S  V  P  E  L  T  Q  Q  M  F  D  A  K  N  M  M  A  A  S  D  P  R  H  G  R  Y  L  T  V  A

      1000      1010      1020      1030      1040      1050      1060      1070      1080
gccttcttccgtggcaaggtctcgatgaaggaagtcgaggagcaaatgcagaatgttcagaacaagaactcggcctactttgtcgagtgg
 A  F  F  R  G  K  V  S  M  K  E  V  E  E  Q  M  Q  N  V  Q  N  K  N  S  A  Y  F  V  E  W

      1090      1100      1110      1120      1130      1140      1150      1160      1170
atcccgaacaacgttctcaccgcgcagtgcgacatccctcctcgtggcatgaagatggccgccacctttatcggcaactcgactgccatc
 I  P  N  N  V  L  T  A  Q  C  D  I  P  P  R  G  M  K  M  A  A  T  F  I  G  N  S  T  A  I

      1180      1190      1200      1210      1220      1230      1240      1250      1260
caagagttgttcaagcgtgtcagcgagcagttctcggccatgttcaagcgcaaggccttcttgcattggtacactcaagagggtatggac
 Q  E  L  F  K  R  V  S  E  Q  F  S  A  M  F  K  R  K  A  F  L  H  W  Y  T  Q  E  G  M  D

      1270      1280      1290      1300      1310      1320      1330      1340      1350
gagatggagttcaccgaggccgagtcgaacatgcaagacttggttgccgagtaccagcaataccaggacgcgtctgtcgaggatacggag
 E  M  E  F  T  E  A  E  S  N  M  Q  D  L  V  A  E  Y  Q  Q  Y  Q  D  A  S  V  E  D  T  E

      1360      1370      1380      1390      1400      1410      1420      1430      1440
gagtacgaagagggtgtccacgaggaggaccagtaaacccgtcgttgtcttgttccgctttctctctggcctagttctcgcatagtattc
 E  Y  E  E  G  V  H  E  E  D  Q  *

      1450      1460      1470      1480      1490      1500      1510      1520      1530
ttttagttctcatgggtctcttttcaacttgtctactaccccacgattctttttaacgactacgatactctaactactcgcgagcgaatt

      1540      1550      1560      1570      1580      1590
caacttcgtttagagcttgtctctcgttaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. Nucleotide sequence of β-tubulin gene from *Rhizoctonia solani* R-C. The deduced amino acid sequence is indicated by the one-letter amino acid codes below the nucleotides.

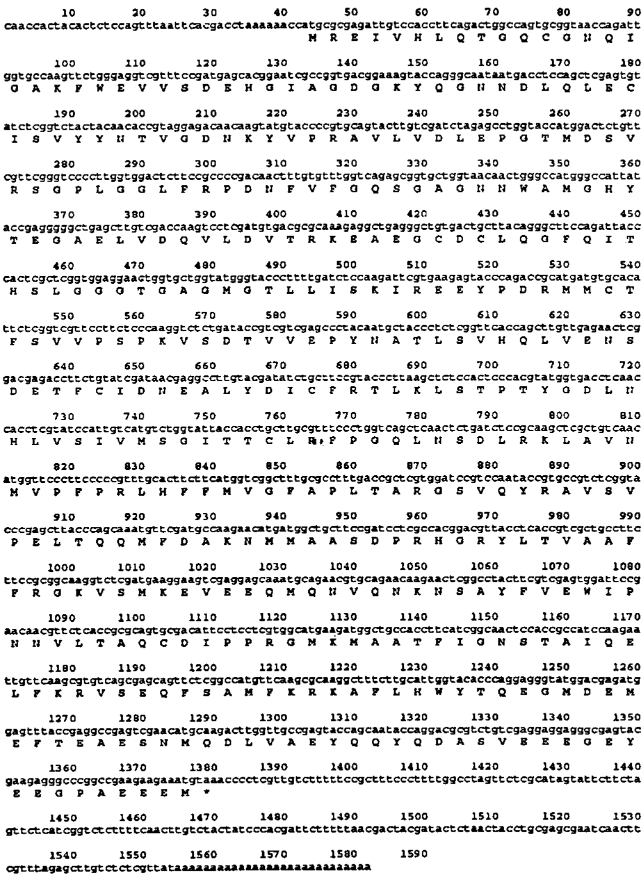

Fig. 2. Nucleotide sequence of β-tubulin gene from *Rhizoctonia solani* Rh-131.

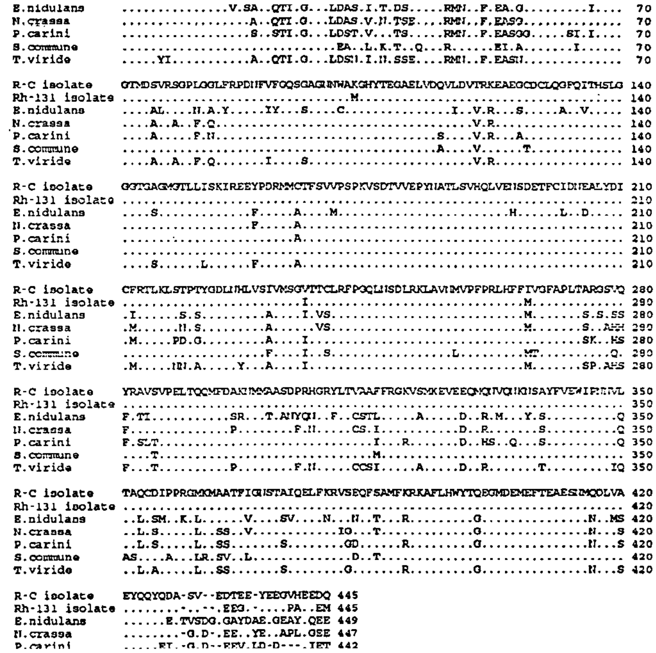

Fig. 3. Comparison of β-tubulin amino acid sequences. The predicted amino acid sequence of the β-tubulin protein of *Rhizoctonia solani* R-C sensitive to pencycuron is given on the top line. For *R. solani* Rh-131, *A. nidulans, P. carini, S. commune,* and *T. viride* sequence, only amino acids different from those of *R. solani* R-C are listed.

couple of filamentous fungi (Fujimura et al., 1992; Jung et al., 1992; Thomas et al., 1985). In particular, the change of codon 198 was suggested to be very important for the pathogens to acquire the resistance to benzimidazoles in *N. crassa* and *V. inaequalis* (Fujimura et al., 1992; Koenraadt et al., 1992). Codon 198 for glutamic acid in the sensitive strain was replaced by a codon for alanine in the benzimidazole-resistant strain of *V. inaequalis* and by glycine in *N. crassa.* In this experiment, 3 major codon changes were detected in *R. solani* R-C and Rh-131 as described above, but they were not consistent with codon sites responsible for resistance to benzimidazoles in β-tubulin genes for other pathogens reported, *e.g.*, both *R. solani* R-C and Rh-131 had a codon 198 for glutamic acid in the β-tubulin gene.

Although the substitution pattern of codons in the β-tubulin genes of *R. solani* R-C and Rh-131 is not consistent with that of other fungi, we can not rule out the possibility that there are some correlations between resistance to pencycuron and the inhibition of tubulin assembly. Thus, in order to examine the direct effect of pencycuron on the assembly of tubulin, the *in vitro* assay for the microtubule formation was carried out using the tubulin prepared from the mycelia

Table 1. Effects of pencycuron on the assembly of tubulin extracted from *Rhizoctonia solani* R-C, a sensitive isolate[a]

Chemicals	Concentration (μg/ml)	Amount of tubulin (μg/ml)
Pencycuron	10.0	41.2 ± 6.0
	1.0	40.0 ± 0.9
	0.1	42.5 ± 3.4
Carbendazim	19.1	20.8 ± 4.1
Untreated control		45.4 ± 3.1

[a]Effects of chemicals on the assembly of tubulins were estimated by the amount of precipitated proteins by centrifuging at 120,000×g for 1 hr after the assembly of tubulins at 37 for 1 hr.

of *R. solani.* The assembly-disassembly process of tubulins by the fluctuation of temperature is a dynamic equilibrium (Shelanski et al., 1973; Gaskin et al., 1974; Davidse and Flach, 1977); tubulins assemble at 37°C and disassemble by cooling to 4°C. In fact, it was confirmed by SDS electrophoresis that precipitated proteins at 37°C were mostly consisted of tubulins. In this study, the distinct characteristics of microtubule formation were examined to investigate the inhibitory effect of fungicides on the assembly of tubulins. The application of carbendazim to this assay system resulted in 54.2% inhibition of the tubulin assembly; however, pencycuron did not inhibit the assembly of tubulin (Table 1). This result suggests that the pencycuron has no direct inhibitory effect on the assembly of tubulin at least *in* *vitro*. Therefore, it can be concluded that the abnormal features of cytoskeletal microtubules observed in hyphal tips of *R. solani* is a secondary effect brought by the deterioration of plasma membrane caused by pencycuron.

References

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Davidse, L. C. and Flach, W. 1977. Differential binding of methyl benzimidazole-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strain of *Aspergillus nidulans. J. Cell Biol.* 72:174-193.

Fujimura, M., Kamakura, T., Inoue, H., Inoue, S. and Yamaguchi, I. 1992. Sensitivity of *Neurospora crassa* to benzimidazole and *N*-phenylcarbamate: Effect of amino acid substitution at position 198 in β-tubulin. *Pestic. Biochem. Physiol.* 44:165-173.

Gaskin, F., Cantor, C. R. and Shelanski, M. L. 1974. Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* 89: 737-758.

Jung, M. K., Wilder, I. B. and Oakley, B. R. 1992. Amino acid alterations in the *benA*, (beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. *Cell Motil. Cytoskeleton* 22:170-174.

Kilmartin, J. V. 1981. Purification of yeast tubulin by self-assembly *in vitro. Biochemistry* 20:3629-3633.

Kim, H. T., Kamakura, T. and Yamaguchi, I. 1996. Effect of pencycuron on the osmotic stability of protoplast of *Rhizoctonia solani. J. Pesticide Sci.* 21: 159-163.

Kim, H. T. and Yamaguchi, I. 1996. Effect of pencycuron on fluidity of lipid membrane of *Rhizoctonia solani. J. Pesticide Sci.* 21:323-328.

Koenraadt, H., Somerville, S. C. and Jones, A. L. 1992. Characterization of mutations in the β-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology* 82:1348-1354.

Orbach, M. J., Porro, E. B. and Yanofsky, C. 1986. Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Mol. Cell Biol.* 6:2452-2461.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Shelanski, M. L., Gaskin, F. and Cantor, C. R. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Nat. Acad. Sci. USA* 70:765-768.

Thomas, J. H., Neff, N. F. and Botstain, D. 1985. Isolation and characterization of mutant in the β-tubulin gene of *Saccharomyces cerevisiae. Genetics* 112:715-734.

Ueyama, I., Araki, Y., Kurogochi, S., Yoneyama, K. and Yamaguchi, I. 1990. Mode of action of the phenylurea fungicide Pencycuron in *Rhizoctonia solani. Pestic. Sci.* 30:363-365.

Yamada, Y., Saito, J. and Takase, I. 1988. Development of new fungicide, Pencycuron *J. Pesticide Sci.* 13:375-387.