ISOLATION AND SEQUENCE ANALYSIS OF ALPHA-TUBULIN GENE FROM *PHYTOPHTHORA CINNAMOMI*

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INTRODUCTION

Phytophthora diseases cause widespread economic and environmental losses worldwide. Thousands of plant species are susceptible. In Portugal, *Phytophthora cinnamomi* is responsible for chestnut ink disease. Despite the differences there are a number of key steps common to most infection strategies, including adhesion to the plant surface, plant penetration through the secretion of a diverse range of cell wall-degrading enzymes and hyphal growth. The cell cytoskeleton plays a critical role in these processes. Microtubules are a major constituent of the cell cytoskeleton. They participate in a wide range of cellular functions, such as motility, division, maintenance of cell shape, and intracellular transport. However, microtubule role is variable depending on the organism, cell type and other factors. Tubulin is the major constituent of microtubules and is composed of a heterodimer of two closely related proteins, alpha and beta tubulin. In *S. cerevisiae* cells, the essential *TUB1* gene is the major gene, while the nonessential gene *TUB3* is a minor gene, encoding α-tubulin. The β-tubulin subunit is encoded by the *TUB2* gene. In *Magnaporthe grisea* both α-and β-tubulins are found as single-copy genes. The Oomycetes are, however, phylogenetically quite distinct from the fungi. Analysis of structural, biochemical and molecular characteristics have led to the Oomycetes being grouped with the chromophyte algae. In order to elucidated the role of cytoskeleton in pathogenicity mechanisms of *Phytophthora cinnamomi*, was cloned a gene encoding alpha-tubulin from *P. cinnamomi.*

MATERIAL AND METHODS

DNA preparation

The *P. cinnamomi* (strain PR 120, genotype WT, was provided By Eugénia Gouveia, Escola Superior Agrária de Bragança) was cultivated for 4-6 days at 28°C in Petri dishes using PDA-Cellophane medium. Each fungal pellet was submitted to a DNA extraction protocol as described by Raeder and Broda (1985)

Sequencing and DNA analysis.

The amplified fragments obtained by PCR using specific were sequenced using an ABI-3100 automated DNA sequencing system (University Salamanca) . The software **DNASTAR** was used to check the quality of the sequences produced. Sequences were submitted to and compared at the GenBank database by using the *BLASTN* tool (Basic Local Alignment Search Tools Nucleotide), a program from the National Center for Biotechnology Information. The alignments were built using the software *Clustal W* . Both programs are network served.

Amplification of α-tubulin *Coding Regions.*

PCR primers were designed based on the homology sequences of α-tubulin Of *P. palmivora* and *S. cerevisiae*, available online at National Center for Biotechnology Information (NCBI)

Tub1: 5`GGY AAT GCS TGT TGG GAA YTM TAT 3` ; Tub2 : 5`CAT MCC YTC WCC SAC RTA CCA GTG3`

PCR amplification was performed in a 25 µl reaction mix containing: 0,8 µg total DNA, 200 µM dNTPs , 1,5 mM $MgCl_2$, 20 µM of each primer, , 10 mM tris-HCl (pH 9), 50 mM KCl, 2,5 U *Taq* DNA polymerase, reaction buffer and distilled water. Samples were amplified as follows: 5 min at 95°C; 30 cycles of: 30 s at 95°C, annealing temperature of 61ºC for 30 s, and 90 s at 72°C; and finally 7 min at 72°C for extension. The PCR products were separated on a 0,7% agarose gel and cloned into plasmid vector pGEM-T (Promega).

Amplification of α-tubulin *Upstream and Downstream Regions.*

It was used a HE-TAIL PCR as described by Michiels *et al.*(2003) . Degenerated 16bp primer R2 (5'- GTN CGA SWC ANA WGT T -3') as described by Michiels *et al.*(2003) was used. Gene-specific primers (TubA2.1p 5`-GCGCCAGTCTCGGAGAAGAAGGTG-3`; TubA2.2p 5´-GCGTCCTCCTTGCCCGAGATGAT -3´; TubA2.3p 5´-GCGTTGAACACCAGGAAACCCTG-3´) were designed in the incomplete ORF of the tubulin gene, in order to amplifie the promotor region. Gene-specific primers TubA2.1t 5´-GCAGCGTGCCGTGTGCATGATCT -3´; TubA2.2t5´-GGCCGTGGCCACCATCAAAACGAA-3´; TubA2.3t 5´-CGAGATCACCAACAGCGCCTTCGA-3´), were designed to amplifie the terminator region. The primers position are schematic in Figure 4. Genomic DNA was used as template. Three rounds of PCR were performed on a Thermal Cycler Gene Amp PCR System 2400" (Perkin Elmer) , using the product of the previous PCR as a template for the next. In primary and secondary PCR reactions, a single-step annealing-extension at 62ºC-66ºC was used. A detailed cycler program and conditions are given in Table1. The PCR products were separated on a 0,7% agarose gel and cloned into plasmid vector pGEM-T (Promega).

TABLE 1 - HE-TAIL PCR cycle settings and conditions*

Reaction	Number of cycles	Thermal settings
Primary	1	93ºC (1min); 95ºC (5min)
	5	94ºC (0,5min); 62ºC (1min) ; 72ºC (2,5min)
	1	94ºC (0,5min); 25ºC ramping at 72ºC en 3min; 72ºC (2,5min)
	15	94ºC (20s) ; 66ºC (3,5min); 94ºC (20s) ; 66ºC (3,5min); 94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC. Hold.
Secondary	12	94ºC (20s) ; 65ºC (3,5min); 94ºC (20s) ; 65ºC (3,5min); 94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC. Hold
Tertiary	30	94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC Hold.

*The primary PCR was performed in a 50µl volume containing 1,665 ug of genomic DNA; 10 µM of the 1st specific primer; 20 µM of a random primer R2, 0,2 mM of each dNTP, 50 mM MgCl2. The secondary PCR was performed with the 2nd specific primer (10 µM) and the same random primer R (20 µM) as used in the primary reaction. 4µl of 1/50 dilution of the primary PCR was used as a template. The tertiary reaction was carried out with 4 µl of 1/10 dilution of the secondary reaction, 10 µM of 3rd specific primer, 20 µM of random primer R2, 0.2 mM of each dNTP. All PCR reactions were performed with 1U Taq DNA polymerase (Promega) and 10X amplification buffer supplied with the enzyme. To exclude nonspecific amplification, a tertiary control reaction R-R was set up without adding gene-specific primers.

RESULTS AND DISCUSSION

As the tubulin gene family is composed of highly conserved proteins which are the principle structural and functional components of eukaryo tic microtubules, sequence of α-tubulin gene of *S. cerevisiae* and *P. palmivora* , were chosen and alignment. Primers were designed in highly homoly regions and using to amplify α-tubulin gene of *P. Cinnamomi.* The position where the primers were designed is shown in Figure 1.

Figure 1- Schematic diagram showing the position where the primers were designed to amplified the tubulin gene of *P. cinnamomi* from genomic DNA.

The PCR reaction as generated one fragment of ± 1200 bp. The amplified product was cloned in pGEM-T vector. To confirm the size of the insert, the plasmids isolated from the tranformed colonies were digested with N*ot* I and N*co I.* The expect fragment of ± 1200 bp was obtained(Figure 2).

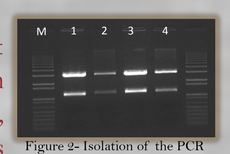

Figure 2- Isolation of the PCR fragment cloned into PGEM_T. *Lane M- 1Kb ladder;* lane 1,2,3,4, pGem-T digested with N*ot* I and N*co I.*

The fragments was sequenced and analysed by DNASTAR. The nucleotide sequence determined in this study was aligned and edited using the BioEdit Sequence Alignment Editor, deposited in the GenBank database and compared with available sequences. The α-tubulin gene of *P. cinnamomi* cloned revelead a 99% homology with α-tubulin gene of *P. palmivora* and 98% homology with *Phythium graminicola.*

However, the ORF of the gene was not complete, the beginning of the ORF and the promoter region, as well the stop codon, was not cloned .

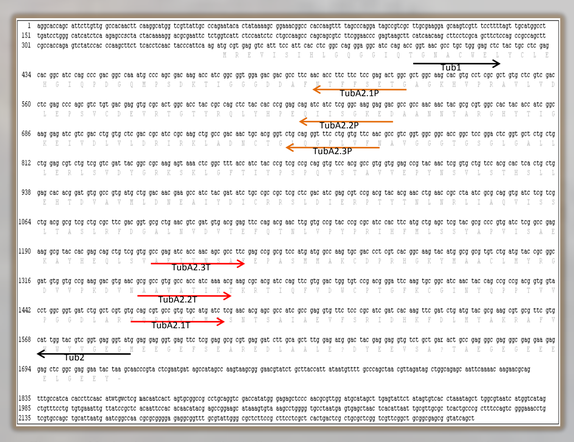

Figure 3- Nucleotide sequence of tubulin gene of *P. cinnamomi, showing the primers used to amplified the complete sequence. Primers to amplified the sequence from the genomic DNA (Black arrows); primers to amplified the Upstream sequence(orange arrows); primers to amplified the Downstream sequence* (red arrows)

. To identify the flanking regions from the gene was used the HE-TAIL PCR (High-Efficiency Thermal Asymetric Interlaced PCR). Gene-specific primers, were designed in the incomplete ORF of the tubulin gene at positions referred in Figure 3. The PCR reaction with the specific primers designed to amplified the upstream region of the gene was generated two fragments, one with ± 1300 bp and other with ± 550 bp. The ampfication of the downstream region was generated one fragment with ±400bp.

The PCR products were sequenced. The sequences were aligned and edited using the SeqMan program (Figure 4). The HE-TAIL PCR was elucidated the complete gene nucleotide sequence of *TubA2*, including 352 bp of the promoter region and 549 bp of the terminator region (Figure 3). The complete ORF was sequenced and submitted in EMBL databases (Accession number AM412177.1). Based on the computational analysis through BioEdit software, *TUB1* has a 1362 bp ORF and encodes a 453 a.a protein with a molecular weight of 49,911kDa. Phylogenetic analysis of deduced amino acid sequence using FASTA programs from EMBL databases revealed that Tub*1* revealed 99.6% identity with alpha-tubulin of *P. palmivora* and 98.9% identity with *P. capsici*, but only 68,1 % with alpha-tubulin of *S. Cerevisiae (figure 5).*

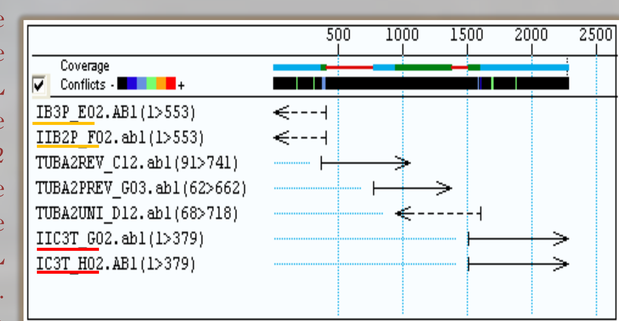

Figure 4- Schematic diagram showing the alignment of fragment obtained by HE-TAIL PCR. IB3P and IIB2P fragments of upstream region. IIC3T and IC3T fragments of downstream region.

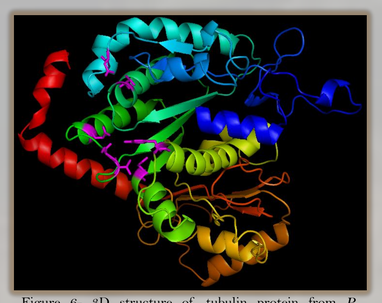

Figure 6- 3D structure of tubulin protein from *P. cinnamomi.*

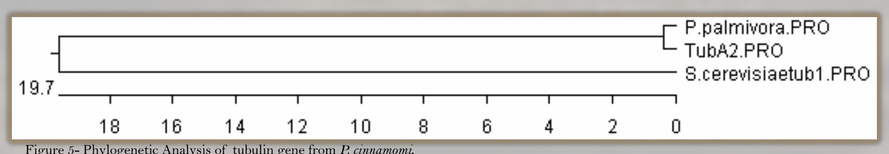

Figure 5- Phylogenetic Analysis of tubulin gene from *P. cinnamomi.*

As the amino-acid sequence of tubulin protein shared 76,3% identity to crystallized structure of 1Z2B protein deposited in PDP, was selected as the model template. The terciary stucture of tubulin protein contained four subdomains (Figure 6).

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Michiels, A., Tucker, M., Van Den Ende, W. & Van Laere, A., 2003. Chromosomal Walking of Flanking Regions From Short Known Sequences in GC-Rich Plant Genomic DNA. *Plant Molecular Biology Reporter*, 21:295-302.

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