ISOLATION AND PHYLOGENETIC ANALYSIS OF TWO ACTIN GENES FROM *Phytophthora cinnamomi*

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INTRODUCTION

Actins, as the essential component of cellular microfilament, are ubiquitous and highly conserved proteins that play key roles in several basic functions of organism such as cytoskeleton morphology, cell division, cell motility, cellular signal transduction, cellular interaction and organelle movements, as well as locomotion, phagocytosis, endocytosis and exocytosis . Actins are highly conserved structural proteins, found in all eukaryotes. So, actin gene sequences are used as tools in scientific research, for example, for phylogenetic analysis.
Actin in *Phytophthora infestans* is encoded by at least two genes, in contrast to unicellular and filamentous fungi (*Candida albicans, Saccharomyces cerevisiae, Schizosaccharomyces pombe*, *Kluyveromyces lactis* and *Filobasidiella neoformans)* where there is a single gene. These genes (designated actA and actB) have been isolated from a genomic library of *P. infestans*. *Phytophthora cinnamomi* is a host-nonspecific, soilborne, fungal pathogen of many plant species. In Portugal it is most important as a pathogen of *chestnut trees.*
The purpose of this study was to clone and determine the phylogenetic relationships evidenced by *Phytophthora cinnamomi* actins.

MATERIAL AND METHODS

DNA preparation
P. cinnamomi strain PR 120, genotype WT, 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. DNA extraction was made by the protocol described by Raeder & Broda (1985)
Sequencing and DNA analysis.
The amplified fragments obtained by PCR using specific primers were sequenced using an ABI-3100 automated DNA sequencing system (University of Salamanca). 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 (NCBI). The alignments were built using the software *Clustal W*. Both programs are network served.
Amplification of actin A and actin B coding sequences
As the actin gene family is composed of highly conserved proteins, sequence of actin genes of *P.hythophora spp.* were choosen and aligned. Primers were designed in highly homologous regions and used to amplify actin gene of *P. cinnamomi*. The position where the primers were designed is shown in Figure 1.
Act1: 5'- GYM ATG GAS GAC GAY ATT CAR GC-3', Act2 : 5'-GYM GYC TTA GAA GCA CTT GCG RTG, were used to amplify the Actin B gene. Act3: 5'- CAW TCA AGA TGG CTG ACG AWG AYG **-3**'; Act4: 5'-CAR CTT AGA AGC ACT TGC GGT GC **-**3', were used to amplify the Actin A gene. PCR amplification was performed in a 25 µl reaction mix containing: 80ng 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 Actin A and B Upstream and Downstream Regions.
It was used a HE-TAIL PCR as described by Michiels *et al.*(2003). Degenerated 16bp primer R2 (5'-GTNCGASWCANAWGTT-3') and gene-specific primers ActA2.1p (5'-TGCTTGGGCATGCCTACGATGGAG-3') ActA2.2p: 5'-TCGTCCCAGTTGGTCACGATGCCG-3' and ActA2.3p: 5'- TGATCTGCGTCATGCGCTCGCGG-3' were designed in the incomplete ORF of the actin A gene, in order to amplify the promotor region; ActA2.1t: 5'-GCGCAAGTACTCCGTATGGATTGG-3´; ActA2.2t: 5'-CAACAATGTCGTGCTCTCCGGTGG-3'; ActA2.3t: 5'-CAACGAGCGCTTTCGCACACCGGA-3; were designed to amplify the terminator region; ActB2.1p: 5'-GCCCACCATGATGCCCAGGTGCTT-3´; ActB2.2p:5´-GTGCCAGATCTTCTCCATGTCGTC-3'; ActB2.3p:5'- CACGTACATGGCGGGCACGTTGAA-3' were designed in the incomplete ORF of the act B gene to amplify the promotor region, and ActB2.1t: 5'-AAGTACTCGGTCTGGATCGGTGGC-3'; ActB2.2t: 5´-TACTGCAACATCGTGCTGTCGGGC-3' and ActB2.3t. 5'-ATCGGCAACGAGCGCTTCCGTACC-3´ were designed to amplify the terminator region.
The primers position are schematized in Figure 2 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).

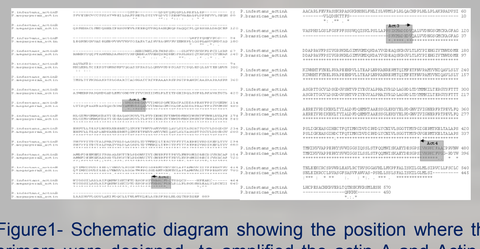

Figure1- Schematic diagram showing the position where the primers were designed to amplified the actin A and Actin B gene of *P. cinnamomi* from genomic DNA.

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

Reaction	Number of cycles	Thermal settings
	1	93ºC (1min); 95ºC (5min)
	5	94ºC (0,5min); 62ºC (1min) ; 72ºC (2,5min)
Primary	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 80ng 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.

RESULTS AND DISCUSSION

The PCR reaction has generated one fragment when the primers act1/act2 were used, as well as with the primers act3/act4. The amplified product were 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 expected fragment of ± 1200 bp was obtained(Figure 2).
The fragments were 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. ActA2 revealed 98% homology with *P. megasperma*, 96% with *P. infestans* and 86% with *P. brassicae*. ActB2 revealed 98% homology with actin gene of *Pythium splendens.*

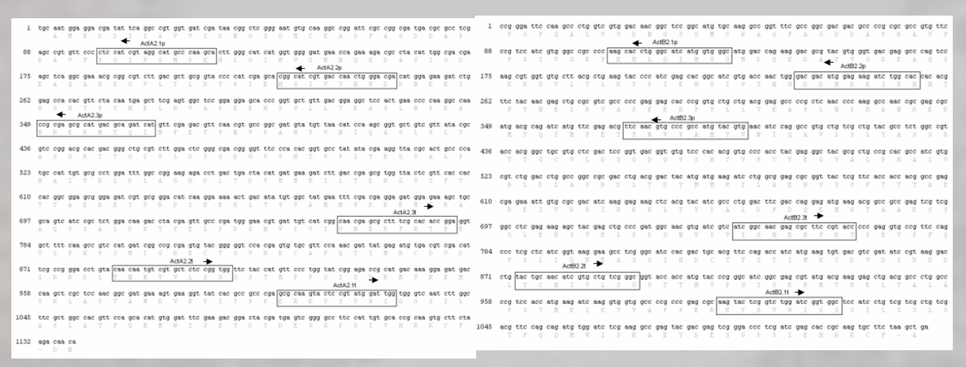

Figure 2- Nucleotide sequence of actin gene of *P. cinnamomi,* showing the primers used to amplified the complete sequence. Primers to amplified the sequence of actin A from the genomic DNA (left side); primers to amplify the complete sequence of actin B gene (right side).

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 actin gene at positions referred in Figure 2. The HE-TAIL PCR only amplify the flanking region of actin A gene. The PCR reaction with the specific primers designed to amplified the upstream region of the gene has generated two fragments, one with ± 550 bp and another with ± 250 bp. The amplification of the downstream region has generated one fragment with ±700bp.

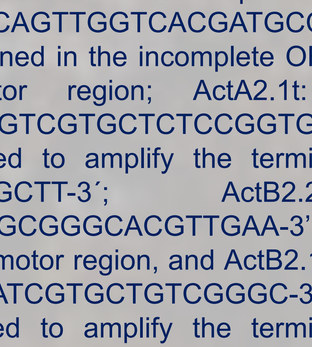

Figure 3- PCR amplification of actin gene. *Lane M- 1Kb ladder;* lane 1 amplification of Actin B. lane 2 amplification of actin A..

These PCR products were sequenced. The sequences were aligned and edited using the SeqMan program. HE-TAIL PCR has elucidated the complete gene nucleotide sequence of *ActA*, including 113 bp of the promoter region and 640 bp of the terminator region. Complete open reading frames (ORFs) of *act1* and *act2* genes were achieved by HE-TAIL PCR, and submitted to EMBL databases (Accession numbers AM412175.1 and AM412176.1).
A*ct1* has an 1128bp ORF, encoding a deduced protein of 375aa and 41,972kDa. *Act2* ORF has 1083bp and encodes a deduced protein of 360aa and 40,237kDa.
Deduced amino acid sequences were analyzed using FASTA programs from EMBL databases. *Act1* showed a 98.9% identity with *P. melonis* actB, 94.4% with *P. megasperma* actin and 96.0% with *P. infestans* actin2. *Act2* showed a 98.9% identity with *Pythium splendens* actin and 98.6% with *P. brassicae* actinA

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