Isolation and Characterization by Asymmetric PCR of the *ENDO1* Gene for Glucan endo-1,3-β-D-glucosidase in *Phytophthora cinnamomi* Associated with the ink Disease of *Castanea sativa* Mill.

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ABSTRACT

Ink disease is one of the most destructive diseases in Castanea sativa. *The most common symptoms are root necrosies and a reduction in root growth, which invariably lead to the death of the trees.* Phytophthora cinnamomi *is an* oomycete *associated with this disease whose life cycle develops integrally in the soil. In the present work, was a fragment with 1231bp of the glucan endo-1,3-β-D-glucosidase gene obtained by amplification, using conserved primers and the full-length gene sequence by flanking this known sequence by asymmetric PCR. This fragment was obtained from genomic DNA of* Phytophthora cinnamomi *isolated in the European Regions of Castilla-Leon (Spain) and Trás-os-Montes (Portugal) and associated with the ink disease of* Castanea sativa Mill.

Key words: *Phytophthora cinnamomi*; glucan endo-1,3-β-D-glucosidase; asymmetric PCR

INTRODUCTION

The genus *Phytophthora* belongs to the oomycetes in the eukaryotic stramenopile lineage and comprises over 65 species, all of which are plant pathogens for a wide range of dicotyledons. *P. cinnamomi* is a widespread soil-borne pathogen that infects woody plant hosts. It requires moist soil conditions and warm temperatures to be active, but the damage caused by the disease occurs most often in summer, when plants are drought stressed. *P. cinnamomi* is one of the most destructive species of *Phytophthora*, and it has been associated with the decline of several forestry, ornamental, and fruit species.

Glucan endo-1,3-β-D-glucosidase (EC 3.2.1.39) catalyzes the hydrolysis of 1,3-β-D-glucoside linkages in callose, laminarin, and several carbohydrates found in the cell wall of plants and fungi. 1,3-β-glucanases are included among the family of pathogenesis-related proteins because many forms are rapidly induced during the fungal invasion. It is generally thought that glucanases play a role in plant defense by digesting wall components of the fungal pathogen. In yeast, 1,3-β-glucanases have been studied for their role in germination, sporulation, mating and cell growth, since they are regulated in cell cycle dependent manner, and are differentially expressed during the vegetative growth, mating and the late stages of

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sporulating diploids. In plants, 1,3-β-glucanases have been characterized for their major role in plant defense, as well as for their involvement in germination, microsporogenesis and embryogenesis. In oomycetes, glucanases have been studied at biochemical level for their possible role in hyphal tip growth and branching, where there is thought to be a delicate balance between the cell wall synthesis and hydrolysies. They have also been studied for their role in Ca^{2+}- induced sporulation and their role in the host pathogen interaction (McLeod, A., 2002).

In the present work, was a fragment with 1231bp of the endo-1,3-β-glucanase gene obtained by standard PCR, using conserved primers, and the whole genomic sequence, with 2586 bp, by amplifiying the previous sequence by asymmetric PCR.

MATERIALS AND METHODS

Oomycete growth conditions

P. cinnamomi mycelia for genomic DNA extraction were obtained by growth in cellophane-PDA media. The C-PDA plates were inoculated by placing a plug of mycelium (0.5 cm in diameter) on new plates and incubating in the dark for 4-6 days to AT 22-25ºC.

PCR Amplification

The polymerase chain reaction (PCR) was used to amplify a 1231bp fragment of the glucan endo-1,3-β-D-glucosidase gene. The conserved primers Gendo1F and Gendo1R were applied (McLeod et al., 2003). PCR was performed with 10X amplification buffer (Promega, Madison, Wis.); 100ng DNA template; 0.2 mM dATP, dCTP, dGTP, and dTTP (each); 0.2μM each primer; 1.5mM MgCl2; and 1U *Taq* DNA polymerase (Promega), in a final reaction volume of 50 μl. Amplification consisted of: one cycle of 1 min at 94ºC, and 36 cycles of denaturing for 1 min at 94ºC; annealing for 1 min at 60ºC, and extension of 30 s at 72ºC. A final extension step of 5 min at 72ºC was performed for one cycle.

TAIL-PCR Procedure

Gene-specific primers SP1 (5'– GGT ACA CGG CCT CGC TGC CAA CG – 3'), SP2 (5' – ACG AGT CCA CCC ACA TGC CGA GC – 3'), SP3 (5' – CTC GGT GGC AAT CTG CGA GGC GG – 3') were used for flanking the 5' region and SP4 (5' – ACG GTG GCG TAC TTC GGC CT – 3'), SP5 (5' – CAC CCA TCA CTG CCG TCG GCG CG – 3'), SP6 (5' – TCG GCC GAC GCC ACT TCC ACG GA – 3') were used for flanking the 3' region. Degenerated primer R3 (5'-WGT GNA GWA NCA NAG A-3') was applied (Fig. 1). Three rounds of PCR were performed on a MyCyclerTM Thermal Cycler (Bio-Rad) using the product of the previous PCR as the template for the next one. A detailed cycler program is given in Table1.

Table 1 - TAIL-PCR cycle setting for genomic DNA to walk from a short known sequence to isolate upstream or downstream regions.

Reaction	Number of Cycles	Termal Settings
Primary	1	93°C-1 min, 95°C-5 min
[R3-SP1]	5	94°C-30 s, 62°C-1 min, 72°C-2,5 min
or	1	94°C-30 s, 25°C-1 ramping to 72°C in 3 min
[R3-SP4]		72°C-2,5 min
	15	94°C-20 s, 68°C-3,5 min
		94°C-20 s, 68°C-3,5 min
		94°C-30 s, 42°C-1 min, 72°C-2,5 min
	1	72°C-5 min, 4°C hold
Secondary	12	94°C-20 s, 65°C-3,5 min
[R3-SP2]		94°C-20 s, 65°C-3,5 min
or		94°C-30 s, 42°C-1 min, 72°C-2,5 min
[R3-SP5]	1	72°C-5 min, 4°C hold
Tertiary	30	94°C-30 s, 42°C-1 min, 72°C-2,5 min
[R3-SP3] or [R3-SP6]	1	72°C-5 min, 4°C hold

The primary PCR was performed in 50μl containing 80ng of genomic DNA; 0.2 μM of primer SP1; 2 μM of primer R3; 0.2mM of each dNTP; 1U *Taq* DNA polymerase (Promega) and 10X amplification buffer supplied with the enzyme. The secondary PCR was performed with primer SP2 (0.2 μM) and the same random primer R3 (2 μM) as used in the primary reaction. One 1μl of 1/50 a dilution of the primary PCR was used as a template. Single-step annealing-extension PCR consisting of a combined annealing and extension step at 65ºC or 68ºC was used in the primary and secondary PCR reactions. The tertiary reaction was carried out with 1 μl of a 1/10 dilution of the secondary reaction, 0.2 μM of primer SP3, 0.2 μM of random primer R3, 0.2 mM of each dNTP, 1U DNA *Taq* polymerase (Promega) and 10X amplification buffer. In the tertiary reaction was included the control SP3-SP3, SP6-SP6 and R3-R3, to exclude further non-specific amplification.

The "DNA and Gel Band Purification kit" (GE Healthere) protocol was used to purify the fragments interest.

DNA Sequencing
DNA fragments were sequenced in an automatic capillary sequencing ABI Prism 377™ at the University of Salamanca.

Sequences Analysis
The "European Bioinformatics Institute" was accessed to extract the sequences from the databases (EMBL - www.ebi.ac.uk), and from the "National Center of Biotechnology Information" (NCBI - www.ncbi.nlm). These servers allowed the comparison of DNA sequences and of proteins by several programs: Clustal, Fasta, and Blast.
The BioEdit, Sequencher and DNASTAR™ (SeqMan and EditSeq) programs were used to analyze the sequences.
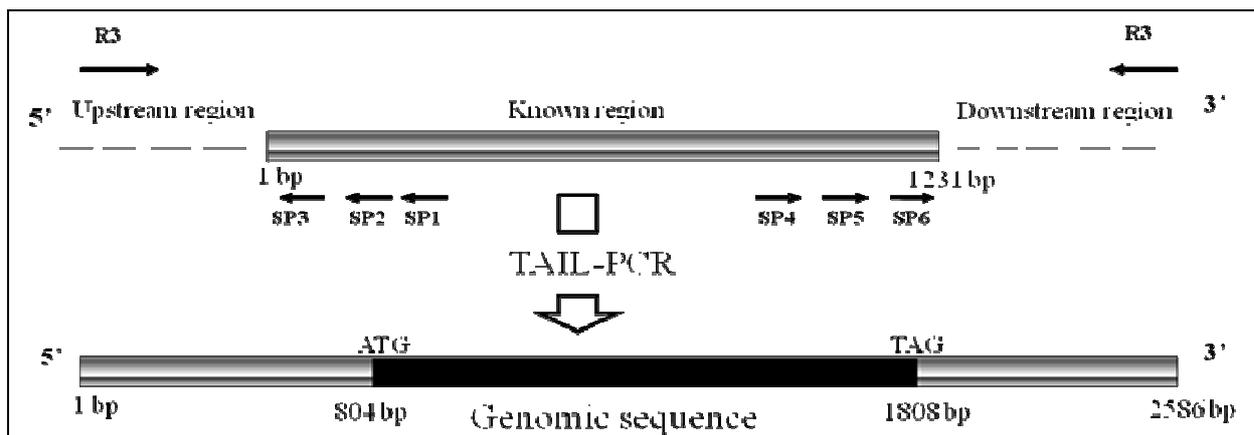

Figure 1 - Schematic representation of TAIL-PCR amplification.

RESULTS AND DISCUSSION

The polymerase chain reaction (PCR) was used to amplify a 1231 bp sequence of the glucan endo-1,3-D-glucosidase gene. For total amplification of the gene, the Thermal Asymmetric InterLaced (TAIL)-PCR method was used, which allowed the amplification of DNA fragments adjacent to a known sequence. Tail-PCR uses three nested specific primers in successive reactions together with a shorter arbitrary degenerate (R3) primer so that the relative amplification efficiencies of

specific and non-specific products can be thermally controlled.
Fig. 2, showed the products of the tertiary TAIL-PCR, together with the control, on a 0.7% agarose/EtBr gel. Controls were included to distinguish between the specific and non-specific amplification the tertiary control reactions. During tertiary cycling, three types of products were formed: (a) primed by gene-specific primers alone (SP3-SP3 and SP6-SP6); (b) primed by random primers alone (R3-R3), and (c) primed by specific and random primers (SP3-R3 and SP6-R3). The fragments amplified by TAIL-PCR were isolated

and sequenced. In the upstream region, a fragment of approximately 800 bp was obtained, while in the downstream region, one of approximately 600 bp was collected.
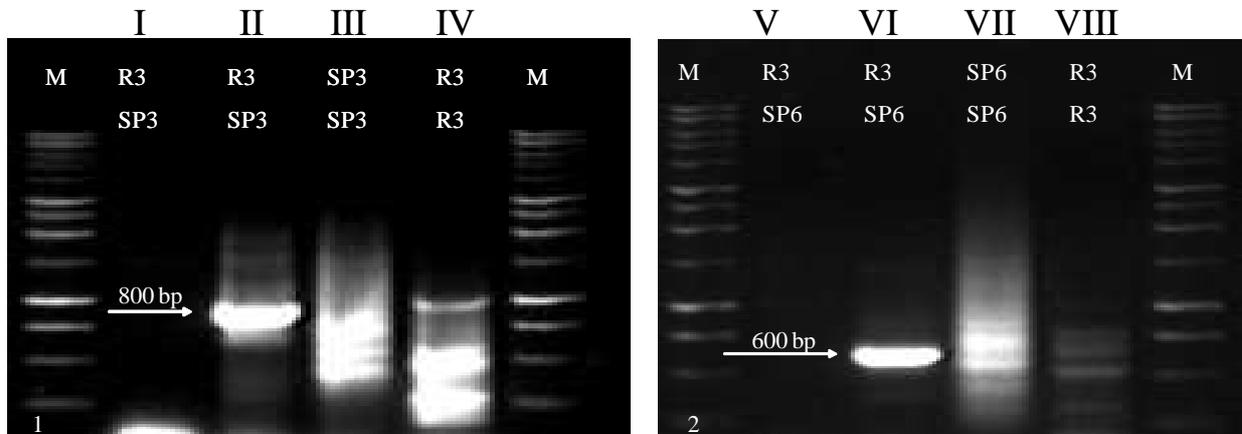

Figure 2 - Products of the tertiary PCR with controls. [1] Agarose gel analysis of tertiary TAIL-PCR products using degenerated primer R3 to isolate the upstream region of the *endo1* gene of *P. cinnamomi*. The gene-specific primer used was SP3. [2] Agarose gel analysis of tertiary TAIL-PCR products using degenerated primer R3 to isolate the downstream region of the *endo1* gene of *P. cinnamomi*. The gene-specific primer used was SP6. M is a Promega 1kb DNA Ladder. Lanes I and V - reaction control without DNA. Lanes II and VI - amplification product. Lanes III, IV, VII and VIII - tertiary reaction control.

The complete sequence of the glucan endo-1,3-D-glucosidase gene was compared with the sequences deposited in the NCBI and EMBL databases. According to the BLASTX search program, the glucan endo-1,3-D-glucosidase gene of *P. cinnamomi* had the homology with a putative endo-1,3-beta-glucanase of *P. infestans* (E-value = 8e^{-135}), an endo-1,3-beta-glucanase of *Saprolegnia parasitica* (E-value = 7e^{-56}), an endo-beta-1,3-glucanase of *Tetrapisispora phaffii* (E-value = 8e^{-25}) and an endo-beta-1,3-glucanase of *Saccharomyces cerevisiae* (E-value = 1e^{-24}). This sequence presented the conserved domain Exo-beta-1,3-glucanase an carbohydrate transport and metabolism(COG5309), and homology with a glycoside hidrolase family 17 (InterProSan – EMBL).

Alignment of glucan endo-1,3-D-glucosidase gene with several endo-1,3-β-glucanases from GH family 17 revealed that the conserved domain of this family contained ([LIVMKS] -X-[LIVMFYWA](3)- [STAG] -E- [STACVI] -G-[WY] -P- [STN] -X- [SAGQ]), (Fig. 3). The dendrogram obtained by phylogenetic analyzes (Fig. 4), showed that the glucan endo-1,3-D-glucosidase of *P. cinnamomi* was very similar to the endo-1,3-beta-glucanase of *P. infestans*.

The promoters from non-oomycete species did not function in *Phytophthora*, suggesting the presence of a unique transcriptional machinery. Canonical eukaryotic elements, such as the TATA box, were not always detected in oomycete promoters, as occasionally reported for fungal been determined and are typically only 50 to 100 bp upstream from the star codon. This suggested that oomycetes had highly compact transcripts, with some of the shortest untranslated regions noted for eukaryotes. A conserved 16-bp sequence motif, GCTCATTYBNCAWTTY, surrounded the transcriptional start site of many oomycete genes (Kamoun, 2003). This motif facilitated the prediction of transcribed regions from genomic sequences. The conserved sequence motif was present in the 5' flanking regions of the Pcendo1 gene, 68 to 83 nt upstream from the ATG start codon (Fig. 5).
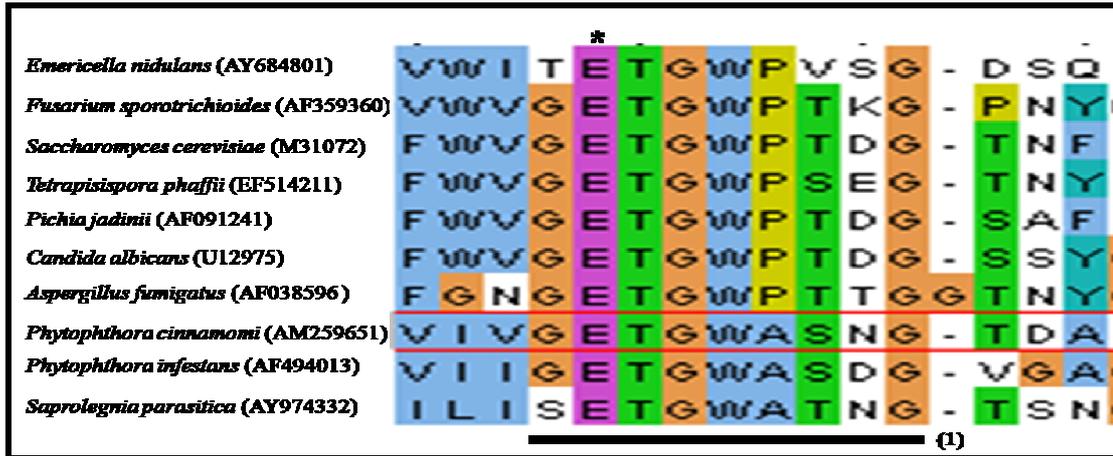
Figure 3 - Alignment of the amino acid sequences of glycoside hydrolase family 17 fungal endo-1,3-β-glucanases and plant endo-1,3-β- and 1,3; 1,4-β-glucanses, as well as an endo-1,3-β-glucanases of *P. infestans* and *P. cinnamomi*. (∗) Is an active site residue. (1) Characteristic domain of GH family 17 ([LIVMKS]-X-[LIVMFYWA](3)-[STAG]-E-[STACVI]-G-[WY]-P-[STN]-X-[SAGQ]).
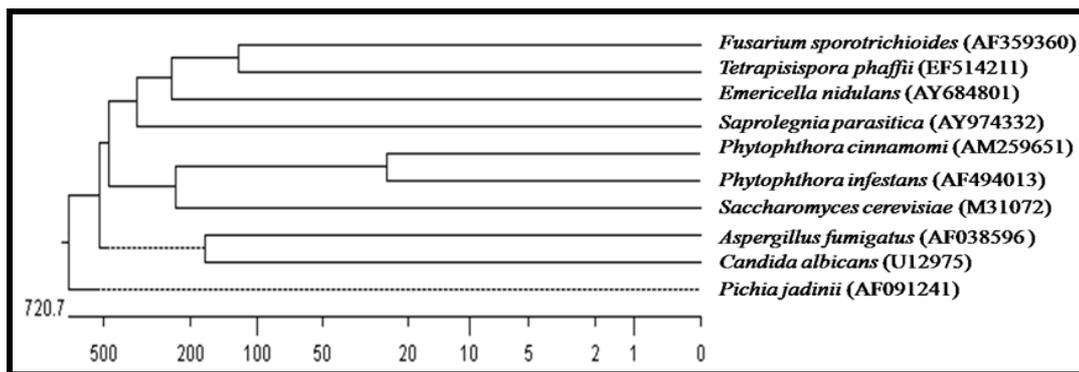

Figure 4 - Phylogram of glycoside hydrolase family 17 fungal endo-1,3-β-glucanase and oomycete endo-1,3-β-glucanase.

Figure 5 - Alignment of the 5' upstream region of *Phytophthora* glucanases genes to a conserved sequence GCTCATTYYNCAWTT found in a promoter region of oomycete genes. [a] *Piendo1* (AF494013), *Piendo2* (AF494017), *Pcendo1* (AM259651). [b] The promoter region of each glucanase gene was searched upstream from the ATG for highest similarity with the consensus sequence GCTCATTYYNCAWTT (where Y=C or T; N= and nucleotide; W= A or T). [c] Nucleotides that are similar to the consensus sequence are shaded.

CONCLUSIONS

The *P. cinnamomi* glucan 1,3-beta-D-glucosidase gene (PcENDO1) was closely related to glycoside hydrolases of family 17 because it also contained the conserved domain of this family (Fig. 3 and 4). A putative conserved 16 nt core sequence (GCTCATTYYNCAWTT) possibly used as an initiation of transcription point in oomycetes has been also identified in *Pcendo1* (Fig. 5).

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RESUMO

Doença da tinta é um das doenças mais destrutivas em *Castanea sativa.* Os sintomas mais comuns são necroses e uma redução em crescimento da raiz que invariavelmente leva à morte das plantas. *Phytophthora cinnamomi* é o oomycete associado a esta doença cujo ciclo de vida acontece integralmente no solo.
Foi obtido um fragmento com 1231pb do gene *glucan endo-1,3-β-D-glucosidase* por amplificação usando oligonucleotidos conservados e a sequência completa do gene foi obtido flanqueando esta sequência conhecida por PCR assimétrico. Este fragmento foi obtido de ADN genómico de *Phytophthora cinnamomi* isolado por nós nas Regiões Europeias de Castilla-Léon (Espanha) e Trás-os-Montes (Portugal) e associado à doença da tinta da Castanea sativa Mill.

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