Abstracts

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IDENTIFICATION OF A *Phytophthora cinnamomi* GLUCANASE INHIBITOR PROTEIN: A MOLECULAR FACTOR ASSOCIATED TO INFECTION MECHANISM

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INTRODUCTION

P. cinnamomi is the causal agent of *Castanea sativa* ink disease, one of the most destructive species of *Phytophthora* genus, and has been associated with the decline of several forest, ornamental and fruit trees and shrubs, causing enormous economic losses worldwide.

Plant endo-β-1,3-glucanases and chitinases inhibit the growth of some fungi and generate elicitor-active oligosaccharides while depolymerizing polysaccharides of mycelial walls. *Phytophthora* cell walls are composed of glucans and have no chitin. Production of glucanase inhibitor proteins (GIPs) by *Phytophthora* species are thought to provide them a counter-defense against plant hosts β-1,3-glucanases (Rose *et al*., 2002), that otherwise would degrade these pathogen cell walls.

We report the identification of a gene encoding the first known *P. cinnamomi* GIP, presumably involved in the pathogen infection mechanism.

MATERIALS AND METHODS

Genomic DNA was obtained from strain *P. cinnamomi* Pr120 as described by Cooke and Duncan (1997). Polymerase chain reaction was used to amplify a 308bp fragment of the GIP gene, using degenerate oligonucleotide primers - M1 (5'-GAGAAGCCCAGCAAGTTCSCC-3') and M3 (5'-GCSGACACGCGMGMGTACACA-3'), which were designed based on homology of previous published *Phytophthora sp.* GIP's sequences from EMBL databases. PCR was performed with 10X amplification buffer; 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 5 min at 94ºC, and 36 cycles of denaturing for 1 min at 94ºC; annealing for 1 min at 63ºC; and extension for 30 s at 72ºC. A final extension step of 5 min at 72ºC was done for one cycle. Full gene sequence length elucidation (1171bp) was achieved by flanking the previous known sequence with asymmetric HE-TAIL PCR using the degenerated primers R1, R2, R3 and R4 and the methodology described by Michiels *et al.*(2003), and the gene-specific primers M1, M3, M2 (5'-GCCGTTYTCCTTGATCAGCGG-3'), S1 (5'-AGGCGTTGTCGCCCCAGACC-3´), S2 (5'-CGGCCGCGGTGACGCTGACG-3') and S4 (5'-GGTCTGGGGCGACAACGCCT-3') (Figure 1),. Three rounds of PCR were performed on a MyCycler Thermal Cycler (BIORAD), using the product of the previous PCR as a template for the next. A detailed cycler program and conditions are given in Table1.

DNA sequencing was performed using an ABI 373 automated sequencer. The open reading frame (ORF) of *P. cinnamomi* GIP was identified by BioEdit program and submitted to EMBL databases (Accession number CAJ90742.1); Nucleotide and amino acid sequences were analyzed using FASTA programs from EMBL databases. ClustalW2 (Larkin *et al*., 2007) was used to align the *Phytophthora* genus GIP sequences.

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

Reaction	Number of Cycles	Thermal Settings
Primary		
	1	93°C (1min); 95°C (5min)
	5	94°C (30seg), 62°C (1min), 72°C (2min30s)
	1	94°C (30seg), 25°c ramping 72°C (3min); 72°C (2min30s)
	15	94°C (20s), 65°C (3min30s); 94°C (20s), 65°C (3min30s); 94°C (30s), 42°C (1min), 72°C (2min30s)
	1	72°C (5min), 4°C Hold
Secondary		
	12	94°C (20s); 65°C (3min30s); 94°C (20s); 65°C (3min30s); 94°C (30s); 42°C (1min); 72°C (2min30s)
	1	72°C (5min), 4°C Hold
Tertiary		
	30	94°C (30s); 42°C (1min); 72°C (2min30s)
	1	72°C (5min); 4°C Hold

*The primary PCR was performed in a 50μl volume containing 80ng of genomic DNA; 0.2 μM of primers M1 or M3; 2 μM of a random primer (R1,R2,R3,R4); 0,2mM of each dNTP; 1U Taq DNA polymerase (Promega) and 10X amplification buffer supplied with the enzyme. The secondary PCR was performed with primers M2 or S4 (0.2 μM) and the same random primer R (2 μM) as used in the primary reaction. One microliter of 1/50 dilution of the primary PCR was used as a template. Single-step annealing-extension PCR consisting of a combined annealing and extension step at 62ºC or 65ºC was used in primary and secondary PCR reactions. The tertiary reaction was carried out with 1 μl of 1/10 dilution of the secondary reaction, 0.2 μM of primers S1 and S2, 0.2 μM of random primer R (the same as used in the previous cycles), 0.2 mM of each dNTP, 1U DNA Taq polymerase (Promega) and 10X amplification buffer. To exclude nonspecific amplification, a tertiary control reaction R-R was set up without adding gene-specific primers.

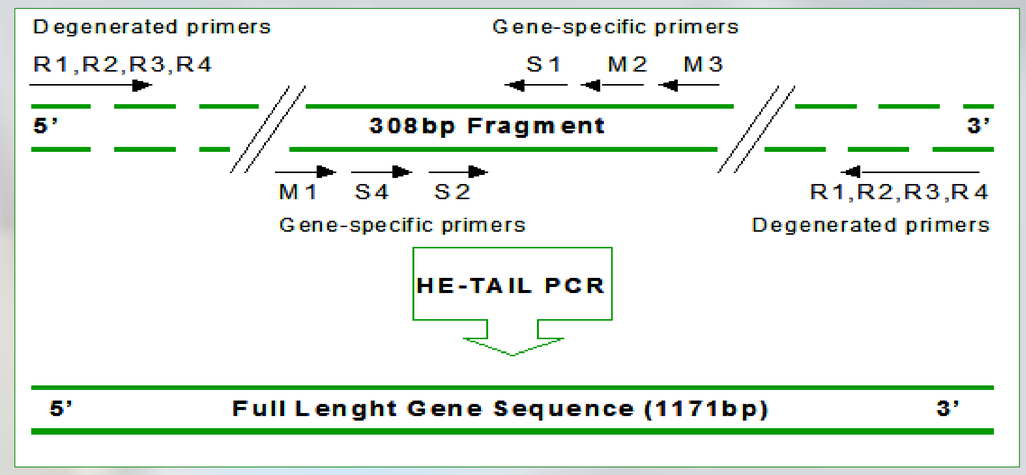

FIGURE 1 - Schematic representation of TAIL-PCR amplification.

RESULTS AND DISCUSSION

The translated ORF of *P. cinnamomi* GIP codifies a 269aa protein, with a predict Mw of 28,8KDa and a theoretical iso-electric point of 8,54. Scanning against protein search databases revealed that *P. cinnamomi* GIP are a serine protease, with a trypsin domain profile. A characteristic feature of Ser proteases is to have a catalytic triad charge relay system, with residues of H, D and S in that order along the sequence, essential for the proteolytic function.

In Figure 2 are shown the multiple alignment of various sequences who showed great homology with *P. cinnamomi* GIP, including another GIPs of *Phytophthora* genus, and a serine protease and a trypsin protease from *P. infestans.* GIPs have in common the fact that none of them have an intact catalytic triad, like other serine proteases, although they share with them several stretches of amino acids and motifs that are highly conserved.

Thus, in all *Phytophthora* GIPs, there are substitutions in residues of the catalytic triad: H-79→A,S,T,I,M-79 (in *P. cinnamomi:* S-79), D-128→N-128 (only in *P. cinnamomi* and *P. sojae* GIP2, and Ser-217→T-217, in all *Phytophthora* GIPs.

Therefore, GIPs are proteolytically inactive, referred as serine protease homologs, and presumably function as host-enzyme inhibitors. It can be hypothesized that a major role for GIP is to suppress the release of glucan elicitors during *Phytophthora sp.* infections, thereby reducing the effectiveness of the plant host's surveillance system (Rose *et al*., 2002).

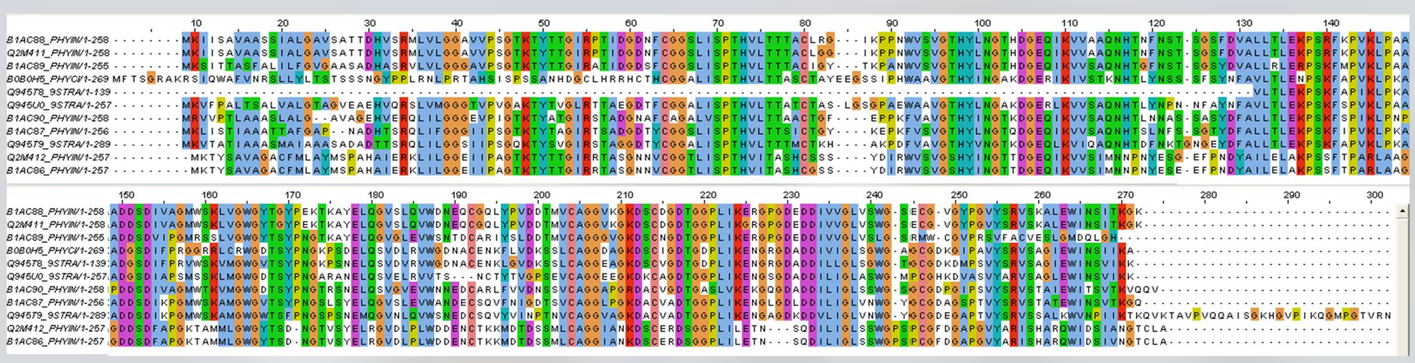

FIGURE 2 – Multiple sequence alignment of GIP and GIP-like genes from *Phytophthora* sp.

B0B0H5_PHYCIN – putative GIP from *P. cinnamomi*; Q945U0_9STRA – GIP1 from *P.sojae*; B1AC90_PHYIN – GIP1 from *P.infestans*; Q945T9_9STRA – GIP2 from *P.sojae*; B1AC87_PHYIN – GIP2 from *P.infestans*; Q945T8_9STRA - GIP2 from *P.sojae*; B1AC88_PHYIN – GIP3 from *P.infestans*; Q2M411_PHYIN – trypsin protease GIP-like; B1AC89_ PHYIN – GIP4 from *P.infestans;* B1AC86_ PHYIN – serine protease from *P.infestans*; Q2M412_PHYIN- trypsin protease GIP-like.

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