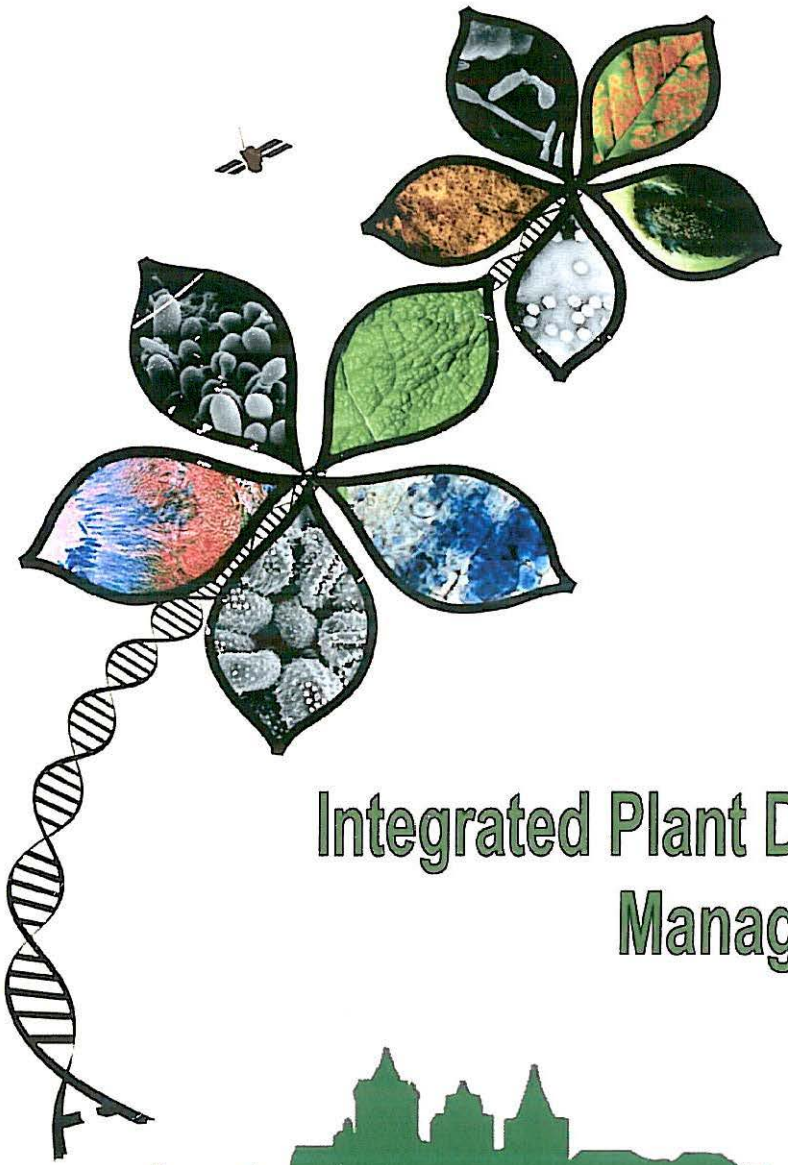
Book of Abstracts

9th Conference of the European Foundation for Plant Pathology

6th Congress of the Sociedade Portuguesa de Fitopatologia



Integrated Plant Disease Management

Évora, Portugal Nov 15-18, 2010

9TH CONFERENCE OF THE EUROPEAN FOUNDATION FOR PLANT PATHOLOGY AND 6TH CONGRESS OF THE SOCIEDADE PORTUGUESA DE FITOPATOLOGIA

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P9.9 Expression analysis by RT-PCR of *GIP* gene from *Phytophthora cinnamomi*

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Species of the genus *Phytophthora* secrete glucanase inhibitor proteins (*GIPs*) to inhibit the activity of enzymes involved in plant defense responses, including during plant infection process of *Castanea sativa* by *Phytophthora cinnamomi.* GIPs show structural homology to the chymotrypsin class of serine proteases (SP) but lack proteolytic activity due to the absence of an intact catalytic triad and, thus, belong to a broader class of proteins called serine protease homologs (SPH), nonfunctional because one or more residues of the essential catalytic triad is absent (His-Asp-Ser). GIPs show high homology to the S1A subfamily of SP, however questions remain about the expression patterns and potential roles of different GIPs during pathogenesis and their possible interaction with host EGases in the plant apoplast. ORF of *GIP* gene from *P. cinnamomi* encodes a 269 aa protein. In order to understand its function, we proceeded to the heterologous expression in *Pichia pastoris*. The expression was studied during growth in different carbon sources and a time course of glucanase inhibitor protein production by RT-PCR was also performed. The major expression levels occurred at the medium with glucose as carbon source.

Keywords: *Castanea sativa* Mill, glucanase inhibitor proteins.

Expression analysis by RT-PCR of *GIP* gene from *Phytophthora cinnamomi*

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INTRODUTION

The oomycetes form one of several lineages within the eukaryotes that independently evolved a parasitic lifestyle and consequently are thought to have developed alternative mechanisms of pathogenicity. *Phytophthora cinnamomi* is one among the most destructive species of *Phytophthora* associated to the decline of forestry, ornamental and fruit species. Associated with this oomycete is the ink disease of *Castanea sativa* Mill. This species secrete glucanase inhibitor proteins (*GIPs*) to inhibit the activity of enzymes (endo-β-1,3 glucanase) involved in plant defense responses, including during plant infection process of *Castanea sativa* Mill by *Phytophthora cinnamomi.* GIPs show structural homology to the chymotrypsin class of serine proteases (SP) but lack proteolytic activity due to the absence of an intact catalytic triad and, thus, belong to a broader class of proteins called serine protease homologs (SPH) nonfunctional because one or more residues of the essential catalytic triad is absent (His-Asp-Ser). GIPs show high homology to the S1A subfamily of SP, however questions remain about the expression patterns and potential roles of different GIPs during pathogenesis and their possible interaction with host EGases in the plant apoplast.

AIMS

Characterize at molecular level of the *GIP* gene by the cloning on pET-28a (+) vector and evaluation of his expression by RT-qPCR and SDS-PAGE.

MATERIAL AND METHODS

Total genomic DNA was isolated from strain *Phytophthora cinnamomi* Pr120 to proceed a TAIL-PCR. We obtained a small sequence of 308bp by amplification using degenerated primers designed based on homology in the open reading frames of other GIPs (*Phytophthora sojae*).

The sequences obtained from TAIL-PCR were cloned in pGEMT vector in order to accomplish the assembly sequences using software ClustalW, BioEdit and ESyPred3D to predict the corresponding structure of *Phytophthora cinnamomi GIP*.

In order to determine protein expression, the ORF of the *GIP* gene was cloned in vector pET-28a (+). The expression was induced for 16h with 100 mM IPTG in LB medium and expression was assessed by SDS-PAGE and RT-qPCR during growth in different carbon sources and a time course of glucanase inhibitor protein production.

Genomic DNA was isolated from *Phytophthora cinnamomi* Pr120

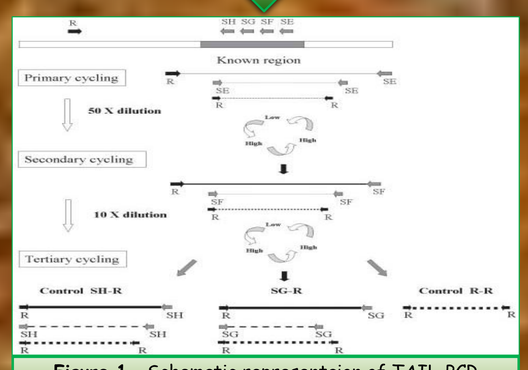

Figure 1- Schematic representaion of TAIL-PCR amplification

Cloning on pGEMT

Sequencing

Assemble sequence
(software ClustalW, Bioedit and ESyPred3D)

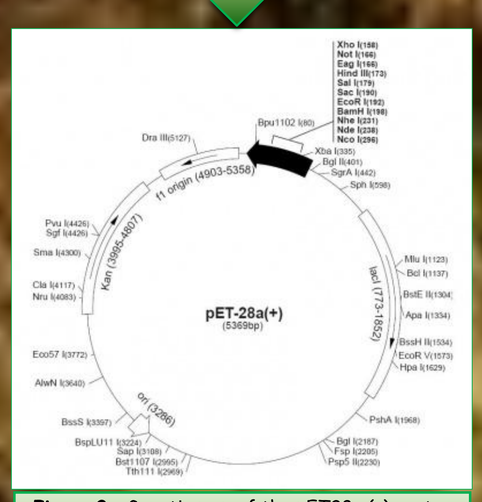

Figure 2- Genetic map of the pET28a (+) vector where we cloned the ORF of GIP gene between SacI and HindIII

Expression by SDS-PAGE and RT-qPCR

Project workflow

RESULTS

The GIP gene ORF was isolated by TAIL-PCR and was obtained the full length gene sequence (1171bp) by flanking the known sequence by asymmetric PCR and assemble sequence using Clustal W, BioEdit and ESyPred3D.

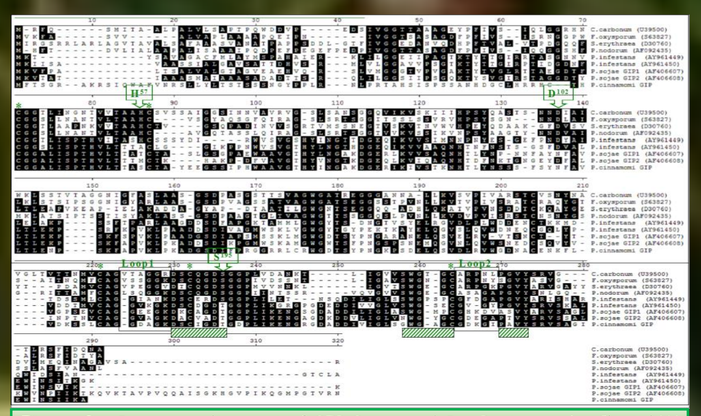

Figure 3- Sequence alignment of GIP Genes and Ser Proteases by Clustal W

The N-terminal signal sequence is indicated by a horizontal bar. Conserved Cys residues involved in disulfide bond formation are indicated by asterisks. The position of the His57, Asp102, and Ser195 residues of the catalytic triad are indicated with arrows. Amino acids predicted to form surface loops 1 and 2 are boxed, and residues forming the walls of the S1 substrate binding pocket are underlined with cross-hatched boxes

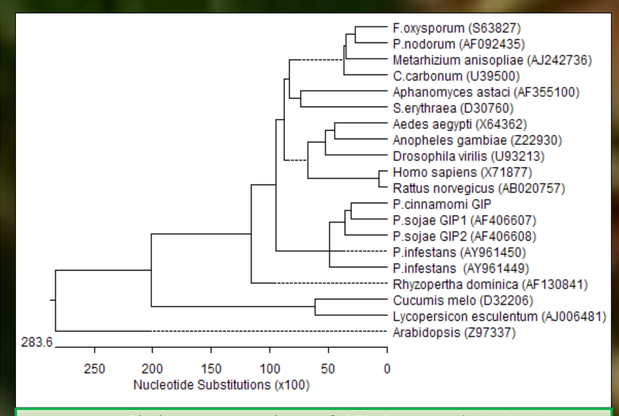

Figure 4- Phylogenetic Analysis of GIP Genes and Ser Proteases

A phylogenetic analysis of the GIP sequences aligned with other SA clan Ser proteases from a number of evolutionarily diverse organisms revealed that the GIPs form a distinct group

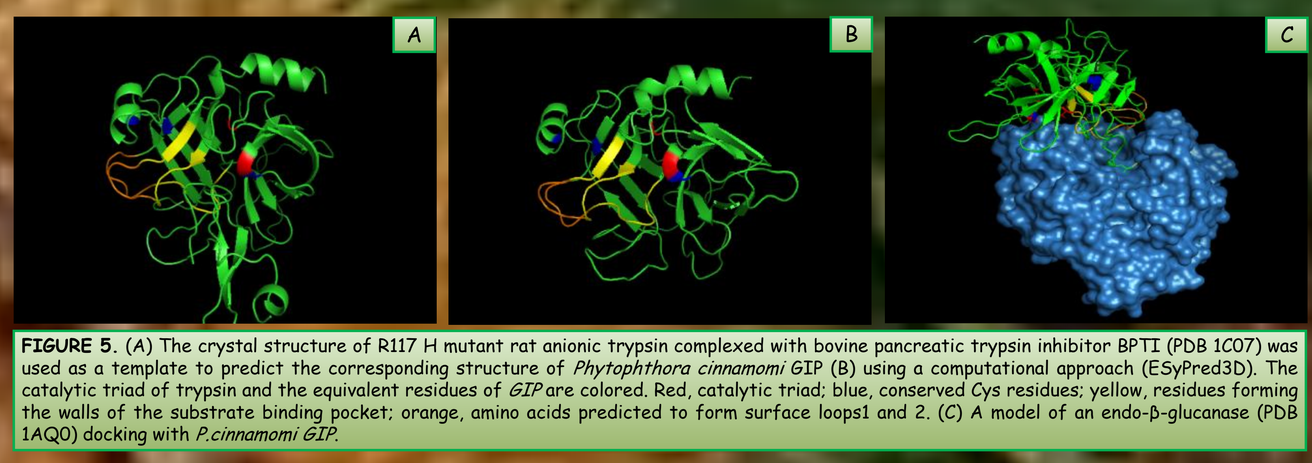

FIGURE 5. (A) The crystal structure of R117 H mutant rat anionic trypsin complexed with bovine pancreatic trypsin inhibitor BPTI (PDB 1C07) was used as a template to predict the corresponding structure of *Phytophthora cinnamomi* GIP (B) using a computational approach (ESyPred3D). The catalytic triad of trypsin and the equivalent residues of *GIP* are colored. Red, catalytic triad; blue, conserved Cys residues; yellow, residues forming the walls of the substrate binding pocket; orange, amino acids predicted to form surface loops1 and 2. (C) A model of an endo-β-glucanase (PDB 1AQ0) docking with *P.cinnamomi GIP*.

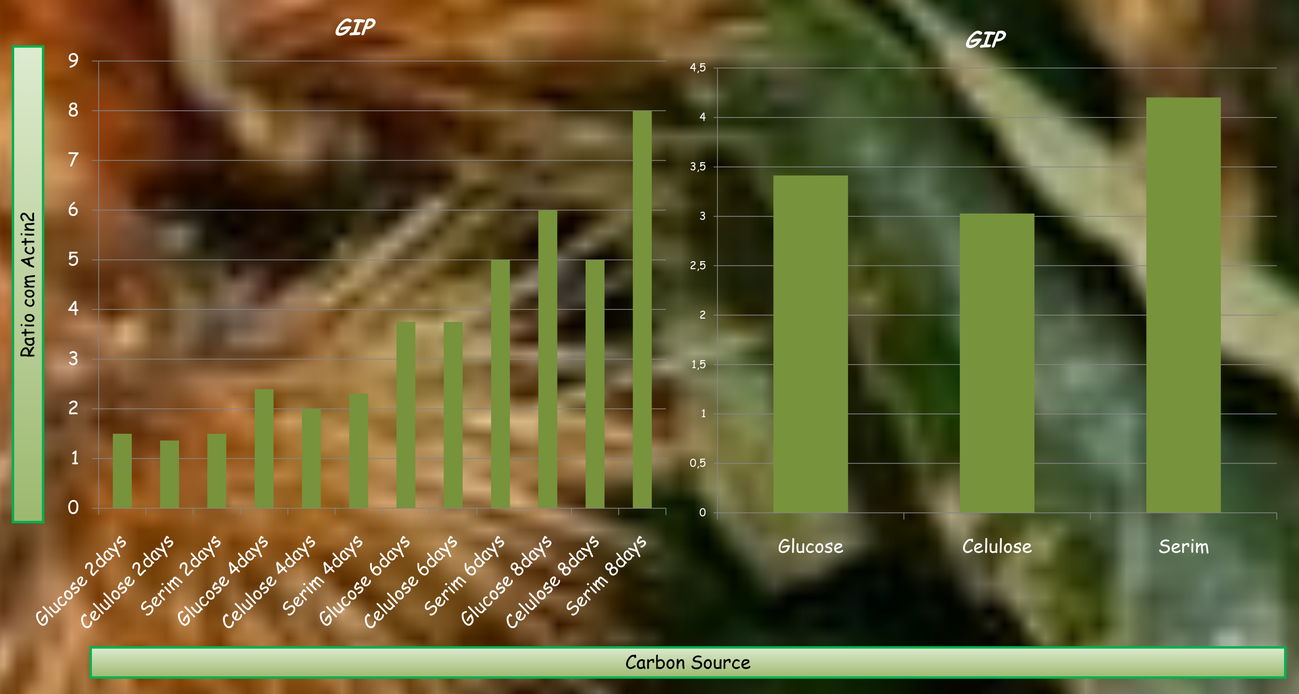

FIGURE 6. Expression of Gip gene during growth in different carbon sources.

CONCLUSIONS

- HE-TAIL PCR is an efficient method to amplify unknown genomic DNA sequences adjacent to short known regions .
- *Phytophthora cinnamomi GIP* gene showed sequence homology with Ser proteases, but doesn't have the catalytic triad charge relay system, referred to as His-57, Asp-102, and Ser-195 that are essential for the proteolytic function.
- The *GIP* gene highest expression is found in the medium with serim chestnut since it is a medium with properties similar that we found "*in vivo*".

Many questions also remain at the molecular level, such as the identity of the domains and key residues of the inhibitor proteins that contribute to the recognition specificity and high actvidity binding for endo-β-1,3 glucanase .

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