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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.
INTRODUCTION

The oomycete Phytophthora cinnamomi is one among the most destructive species of oomycetes associated to the decline of forestry, ornamental and fruit species. Associated with this oomycete is the ink disease of Castanea sativa Mill. This species secretes glucanase inhibitor proteins (GIPs) to inhibit the activity of enzymes (endo-1,3-glucanases) 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 SIA subfamily of SP, however questions remain about the expression patterns and potential roles of different GIPs during pathogenesis and their possible interaction with host defense in the plant apoplast.

AIMS

Characterize at molecular level the GIP gene by the cloning as 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 bioagent). 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 100mM IPTG in LB medium and expression was assayed by SDS-PAGE and RT-qPCR during growth in different carbon sources and a time course of glucanase inhibitor protein production.

Experimental Design

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

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RESULTS

The GIP gene ORF was isolated by TAIL-PCR and was obtained the full length gene sequence (1172bp) by flanking the known sequence by asymmetric PCR and assembly sequencing using Clustal W. BioEdit and ESyPred3D.

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

CONCLUSIONS

The GIP gene shows sequence homology with Ser proteases, but don’t have the catalytic triad charge relay system, referred to as His-Asp-102; and Ser-199 that are essential for the proteolytic function.

The GIP gene highest expression is found in the medium with cornstarch 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 activity binding for endo-1,3-glucanases.

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Figure 1 - Schematic representation of TAIL-PCR amplification

Figure 2 - Genes map of the pET-28a (+) vector where we cloned the ORF of GIP genes between SacII and HindIII

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

Figure 4 - Phylogenetic Analysis of GIP genes and Ser Proteases

Figure 5 - (A) The crystal structure of BPTI in complex with natural tetrameric trypsin inhibitor (1U1T, PDB 308) 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 S1 substrate binding pocket are underlined with cross-hatched boxes.

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