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Proteomic analysis of secreted proteins from *Trichoderma harzianum* Identification of a fungal cell wall-induced aspartic protease

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Abstract

Trichoderma mycoparasitic activity depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall. We have analysed the extracellular proteome secreted by *T. harzianum* CECT 2413 in the presence of different fungal cell walls. Significant differences were detected in 2DE maps, depending on the use of specific cell walls or chitin. A combination of MALDI-TOF and liquid chromatography mass spectrometry allowed the identification of a novel aspartic protease (P6281: MW 33 and p*I* 4.3) highly induced by fungal cell walls. A broad EST library from *T. harzianum* CECT 2413 was used to obtain the full-length sequence. The protein showed 44% identity with the polyporopepsin (EC 3.4.23.29) from the basidiomycete *Irpex lacteus*. Lower identity percentages were found with other pepsin-like proteases from filamentous fungi (<31%) and animals (<29%). Northern blot and promoter sequence analyses support the implication of the protease P6281 in mycoparasitism.

Keywords: Aspartic protease; Biocontrol; Expressed sequence tags; Filamentous fungi proteomics; Gene expression; LC/MS–MS; MALDI-TOF; Mycoparasitism; *Trichoderma harzianum*

1. Introduction

The fungal genus *Trichoderma* includes a wide spectrum of biotypes that range from very effective soil colonizers to non-strict plant symbionts that reside in the rhizosphere and colonize the plant epidermis (Harman et al., 2004). Many strains of *Trichoderma* spp. are able to antagonize plant pathogens, using competition for the substrate, antibiosis, and/or parasitism as main antagonistic strategies (Howell, 2003). Efficient biocontrol of plant-parasitic nematodes has also been reported (Sharon et al., 2001). Furthermore, *Trichoderma* strains can also exert positive effects on plants by promoting plant growth and plant defensive systems against different pathogens (Harman et al., 2004; Yedidia et al., 2003). All of these activities, single or combined, are assumed to be responsible for the success of *Trichoderma* strains as biocontrol agents against a wide set of phytopathogen fungi (Harman and Björkman, 1998).

The strong biodegradation and substrate colonization performances of *Trichoderma* strains are the result of an amazing metabolic versatility and a high secretory potential that leads to the production of diversified sets of hydrolytic enzymes. Similarly, the direct attack of *Trichoderma* spp. to phytopathogenic fungi (mycoparasitism) is based on the secretion of complex cocktails of enzymes involved in the degradation and further penetration of the fungal host cell wall. A principal function in this process has been attributed to chitinolytic and glucanolytic enzymes, many of which have been isolated and characterized (see Benítez et al., 1998, 2004; and Lorito, 1998 for reviews). In comparison to these enzymatic activities, little is known about the

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proteolytic system secreted by *Trichoderma* strains, despite the fact that it can also play a significant role in the biocontrol ability. In fact, recently it has been found that *T. harzianum* mutants (obtained by UV-irradiation) showing improved antagonistic properties had higher secretion of proteolytic enzymes (Szekeres et al., 2004). *Trichoderma* spp. proteases can take part in the host cell wall breakdown (composed of chitin and glucan polymers embedded in a protein matrix; Kapteyn et al., 1996), or act as proteolytic inactivators of pathogen enzymes involved in the plant infection process (Elad and Kapat, 1999). Elicitation of plant defense response by a 18 kDa-protein from *T. virens* with similarity to serine proteases has also been recently described (Hanson and Howell, 2004).

The study of the components of the proteolytic system of *Trichoderma* spp. and their contribution to biocontrol has been receiving increased attention. Different serine (subtilisin-like, chymotrypsin/elastase-like and trypsin-like activities) and aspartic proteases have been detected and/or purified from several *Trichoderma* species (Delgado-Jarana et al., 2000; De Marco and Felix, 2002; Dunaevsky et al., 2000; Geremia et al., 1993; Haab et al., 1990; Suárez et al., 2004; Uchikoba et al., 2001; Williams et al., 2003), and some of the corresponding genes have been cloned. The gene *prb*1 encoding a subtilisin-like protease involved in mycoparasitism has been isolated and characterized from *T. atroviride* IMI 206040 (Cortés et al., 1998; Flores et al., 1997 Geremia et al., 1993; Olmedo-Monfil et al., 2002). A homologous gene, *tvsp*1, has also been studied in *T. virens* Gv29.8 (Pozo et al., 2004). Genes corresponding to one aspartic protease (PAPA), detected in casein supplemented media, and one trypsin-like protease (PRA1), expressed on fungal cell walls or chitin, have been cloned from *T. harzianum* CECT 2413 (Delgado-Jarana et al., 2002; Suárez et al., 2004). Finally, *papA* and a vacuolar aspartic protease encoding gene (*papB*) have been isolated from *T. asperellum* T-203 and related to mycoparasitic and plant root colonization activities (Viterbo et al., 2004).

An initial mitochondrial and intracellular proteome map for *T. atroviride* IMI 206040 has been described (Grinyer et al., 2004a,b) and, during the preparation of this manuscript, a report on the proteomics analysis of the response of *T. atroviride* P1 (ATCC 74058) to the presence of *Rhizoctonia solani* cell walls has been published (Grinyer et al., 2005). In this last report, some proteins have been identified as identical or related to cell wall degrading enzymes involved in biocontrol activities (De la Cruz et al., 1995; Suárez et al., 2004; Ulhoa and Peberdy, 1991, 1992), but the full amino acid sequence and expression profiles were not provided. The lack of genome sequence data for antagonistic *Trichoderma* strains clearly limits the potential of proteomics approaches when used alone.

In the present work, we have carried out a proteomic approach by two-dimensional electrophoresis (2DE) to display extracellular proteins from *T. harzianum* CECT 2413 that are secreted in the presence of three different fungal cell wall systems. The proteomic study was integrated as a part of a wider functional genomics initiative where a broad expressed sequence tags (ESTs) library and database from *Trichoderma* spp. is also being built (TrichoEST-QLK3-2002-02032, Rey et al., 2004). This allowed us to overcome the problems associated with the lack of genome sequence data for the identification of non-conserved *Trichoderma* spp. proteins. Optimized 2DE protein profiles were compared to that obtained on chitin and the most abundant protein induced by fungal cell walls was identified as the novel pepsin-like aspartic protease P6281 by a combination of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF), liquid chromatography mass spectrometry (LC–MS/MS) and in silico analysis of the available EST library. Analysis of the complete deduced P6281 sequence, promoter cloning and analysis of expression of *p*6281 gene are also reported.

2. Materials and methods

2.1. Fungal strains and culture conditions

T. harzianum CECT 2413 and *R. solani* CECT 2815 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *Botrytis cinerea* 98 was isolated from diseased strawberry plants in Huelva (Spain). *Pythium ultimum* isolate was kindly provided by Dr. Matteo Lorito (University of Naples, Italy). All fungi were maintained at −80 °C in a 20% glycerol solution.

Production of extracellular proteins was carried out in synthetic medium [SM: 680 mg KH_2PO_4, 870 mg K_2HPO_4, 1.7 g $(NH_4)_2SO_4$, 200 mg KCl, 200 mg $CaCl_2$, 200 mg $MgSO_4 \cdot 7\,H_2O$, 2 mg $FeSO_4$, 2 mg $MnSO_4$ and 2 mg $ZnSO_4$ in 1 L of distilled water, adjusted to pH 6.0] under two-steps culture conditions to reduce the dependence on total growth. First, a conidial suspension of *T. harziamun* (1×10^6 conidia/mL) was used to inoculate 1-L Erlenmeyer flasks containing 300 mL of SM supplemented with 1% glucose as carbon source. After incubation on a rotatory shaker at 150 rpm and 25 °C for 20 h, the mycelium was filtered, thoroughly washed with 2% magnesium chloride and water, and transferred to a new 1-L Erlenmeyer flask containing 300 mL of SM and a variable carbon source: 1% glucose, 1% chitin (Sigma), 1% *B. cinerea* cell walls (BcCW), 1% *P. ultimum* cell walls (PuCW), or 1% *R. solani* cell walls (RsCW). Nitrogen starvation condition was 10% of the nitrogen concentration in SM and 1% glucose as carbon source. Fungal cell walls were prepared as previously reported (Fleet and Phaff, 1974), proving they were contaminant free of intracellular proteins after protein extraction. After 20 or 48 h, *T. harzianum* culture supernatants were collected by filtration through a 0.45 μm filter. Protein concentration of culture filtrates was determined by Bradford assay (Bradford, 1976) with bovine serum albumin (Sigma) as a protein standard. Two independent cultures were done for every growing condition.