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ThPTR2, a di/tri-peptide transporter gene from *Trichoderma harzianum*

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Abstract

The generation of a wide ESTs library and database from *Trichoderma harzianum* CECT 2413 was the base for identifying the gene *ThPTR2*, coding for a PTR family di/tri-peptide transporter. The deduced protein sequence of the *ThPTR2* gene showed the conserved motifs and also the 12 transmembrane domains typical of the PTR transporters. The highest level of *ThPTR2* expression was found when the fungus was grown in chitin as sole carbon source. We also found that *ThPTR2* expression was increased when *Trichoderma* interacted directly in solid medium with the plant-pathogenic fungus *Botrytis cinerea*, showing that *ThPTR2* is involved in the mycoparasitic process. Additionally, its expression was triggered by nitrogen starvation and a higher level of expression was also found when *Trichoderma* was grown in secondary nitrogen sources like allantoin, yeast extract, and urea. However, no difference was found when *Trichoderma* was grown in presence or absence of glucose as carbon source. Strain T34-15, a transformant that overexpressed the *ThPTR2* gene, showed about a 2-fold increase in the uptake of the dipeptide Leu–Leu. Additionally, two transformants from the strain *Trichoderma longibrachiatum* T52 that overexpressed *ThPTR2* were also studied, confirming the role of this gene in peptide transport. Other homologous genes to *ThPTR2* were identified in other *Trichoderma* strains. *ThPTR2* is the first experimentally confirmed PTR family transporter gene from filamentous fungi.

keywords: Functional genomics; Biocontrol; Membrane transport; Mycoparasitism; EST

1. Introduction

The wide use of strains of the genus *Trichoderma* is mainly based on their ability to degrade plant polymers, such as cellulose (Kubicek and Penttilä, 1998) and to antagonize other fungi (Hjeljord and Tronsmo, 1998). For this reason, different *Trichoderma* species have been commonly used as biocontrol agents of important plant-pathogenic fungi (Elad, 2000). However, the genome of *Trichoderma* had been poorly surveyed compared to other microorganisms. This was due to the large diversity of species (Hermosa et al., 2004), the absence of optimized systems for its exploration, and the great variety of genes expressed under a wide range of ambient conditions (Rey et al., 2004). The functional genomic EU-funded project "TrichoEST" (www.trichoderma.org) was undertaken by an international consortium comprised of academic institutions and enterprises. The aims were to identify genes and gene products from 12 strains with biotechnological value from seven *Trichoderma* species (Rey et al., 2004). In this project, *Trichoderma harzianum* CECT 2413 was selected as the strain representing the *T. harzianum*

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biotype. mRNA populations from *Trichoderma* spp., transcribed under mycoparasitic and nutrient stress conditions, and cloned as cDNAs, were the origin of expressed sequence tags (ESTs).[1] A structural genomic project, carried out under the auspices of the US Department of Energy Joint Genome Institute (http://gsphere.lanl.gov/trire1/trire1.home.html), has just provided the first version of the full genome sequence of the *Trichoderma reesei* strain QM 9414.

Peptide transport is a universally observed physiological phenomenon in both prokaryotes and eukaryotes. This process is characterized by the ability of cells to transport peptides across membranes in an energy-dependent manner. Internalized peptides are rapidly hydrolyzed by peptidases and the resulting amino acids are used for protein synthesis or as alternative sources of nitrogen and carbon (Stacey et al., 2002). There are three families of proteins involved in this process: (i) the ATP-binding cassette (ABC-type) transporters (Detmers et al., 2001), that use ATP hydrolysis for transport; (ii) the peptide transporter (PTR) family (Steiner et al., 1995), that use the proton-motive force (PMF) to translocate di- and tripeptides. For this reason, these proteins have also been termed "proton oligopeptide transporters" (POT); and (iii) the oligopeptide transporter (OPT) family (Hauser et al., 2000, 2001; Lubkowitz et al., 1998), which mediates pH-dependent transport of tetra- and pentapeptides. This variation in energy coupling and substrate preference likely reflects unique structural features and suggests different evolutionary pathways for the three families.

The PTR family transporters are found in plants, animals or yeasts but they have not yet been functionally characterized in filamentous fungi. The members of this family have been shown to transport a wide range of nitrogen-containing substrates, including di- and tripeptides, nitrate and peptide-based drugs (Herrera-Ruiz and Knipp, 2003; Stacey et al., 2002). They contain 12 predicted transmembrane domains, with a majority of the proteins having both the N- and C-termini localized intracellularly (Herrera-Ruiz and Knipp, 2003). Several PTR transporters have been cloned and characterized in different organisms, including mammals (Fei et al., 1994, 2000; Liang et al., 1995), plants (Song et al., 1996; West et al., 1998), bacteria (Hagting et al., 1994) and the nematode *Caenorhabditis elegans* (Fei et al., 1998). The *PTR2* and *CaPTR2* transporters from *Saccharomyces cerevisiae* (Perry et al., 1994) and *Candida albicans* (Basrai et al., 1995) are the only fungal *PTR* genes functionally characterized so far. Both were cloned by functional complementation of a peptide transport deficient mutant of *S. cerevisiae*. Another peptide transporter gene, *fPTR2* (originally named *AtPTR2A*), was also isolated by functional complementation of a yeast peptide transport mutant using an *Arabidopsis* cDNA library (Steiner et al., 1994), but was later found not to be an *Arabidopsis* gene, but probably derived from a fungal contaminant. The substrate specificity and kinetic properties of fPTR2 have been recently studied (Chiang et al., 2004).

Using a genomic approach, in the present work we report on the cloning and characterization of a gene, *ThPTR2*, which encodes a PTR protein of the biocontrol fungus *T. harzianum* CECT 2413. We also report the identification of homologous genes to *ThPTR2* in other *Trichoderma* species. When overexpressed, *ThPTR2* results in a significant enhancement of the dipeptide Leu–Leu transport, a fact that makes *ThPTR2* the first experimentally documented PTR transporter from a filamentous fungus.

2. Material and methods

2.1. Fungal isolates

T. harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) and *Trichoderma longibrachiatum* T52 (NewBioTechnic, Seville, Spain, NBT52) were used in this study. *Colletotrichum acutatum* IMI (International Mycological Institute, Egham, UK) 364856 was used as a source to obtain fungal cell walls. *Botrytis cinerea* B98 was isolated in our lab from infected strawberries. They were maintained on potato dextrose agar (PDA, Difco Becton Dickinson, Sparks, MD).

2.2. cDNA library construction

To make a cDNA library (named L03), the biomass was obtained following a two-step liquid culture procedure. First, *T. harzianum* CECT 2413 was grown in potato dextrose broth (Difco Becton Dickinson), in baffled flasks at 25 °C and 160 rpm in an orbital incubator for two days. Biomass was harvested, rinsed twice with sterile distilled water and transferred to minimal medium (Penttila et al., 1987) (MM: 15 g/l NaH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 600 mg/l $CaCl_2 \cdot 2H_2O$, 600 mg/l $MgSO_4 \cdot 7H_2O$, 5 mg/l $FeSO_4$, 2 mg/l $CoCl_2$, 1.6 mg/l $MnSO_4$, 1.4 mg/l $ZnSO_4$) under the following conditions in separate cultures: (i) 0.1% glucose, (ii) 1.5% chitin (Sigma, St. Louis, MO), (iii) 100-fold increase in the concentration of metals or (iv) 1% of a 1:1 mixture of fungal cell walls from *Penicillium digitatum* and *B. cinerea*. The cultures were incubated under these conditions for 8 and 12 h. Total RNA was extracted using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After total RNA extraction, an equal amount of RNA from the different growth conditions was mixed and mRNA purified using Dynabeads (Dynal, Oslo, Norway). The cDNA library was constructed using the Uni-ZAP XR Vector System (Stratagene, La Jolla, CA) following the manufacturer's instructions.

[1] *Abbreviations used:* ABC, ATP-binding cassette; DTT, dithiothreitol; EST, expressed sequence tag; MM, minimal medium; NCR, nitrogen catabolite repression; OPT, oligopeptide transporter; PDA, potato dextrose agar; PTR, peptide transporter; RT-PCR, retrotranscriptase polymerase chain reaction; TM, transmembrane.