Biological Control 61 (2012) 134–140

Contents lists available at SciVerse ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Trichoderma harzianum expressed sequence tags for identification of genes with putative roles in mycoparasitism against *Fusarium solani*

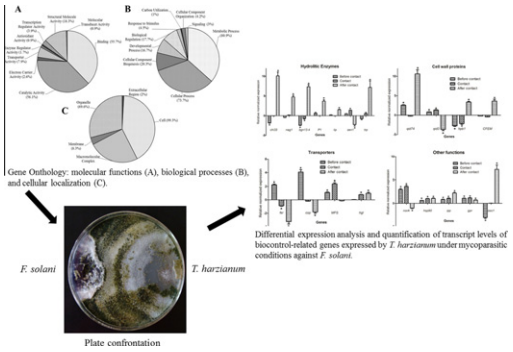
Andrei Stecca Steindorff [a], Roberto do Nascimento Silva [b], Alexandre Siqueira Guedes Coelho [c], Tatsuya Nagata [d], Eliane Ferreira Noronha [d], Cirano José Ulhoa [a,*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Goiás (ICB), Goiânia, GO 74001-970, Brazil
[b] Department of Biochemistry and Immunology, School of Medicine, University of São Paulo, 14049-900 Ribeirão Preto, SP., Brazil
[c] Department of Genetics and Plant Breeding, School of Agronomy, Federal University of Goiás, Goiânia, GO 74001-970, Brazil
[d] Department of Cellular Biology, Brasília University (IB), 70910-900 DF, Brazil

HIGHLIGHTS

- Analysis of a cDNA library of *Trichoderma harzianum* grown in *Fusarium solani* cell wall.
- Functional annotation showing a high diversity of categories.
- The expression of this gene set was up or down-regulated depending of the phase of interaction.
- Some genes with no association with biocontrol were found and further studies must be done.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 August 2011
Accepted 24 January 2012
Available online 2 February 2012

Keywords:
cDNA library
Trichoderma harzianum
Mycoparasitism
Fusarium solani
RT-qPCR

ABSTRACT

The plant pathogen *Fusarium solani* causes a disease root rot of common bean (*Phaseolus vulgaris*) resulting in great losses of yield in irrigated areas of the Southeast and Midwest regions of Brazil. Species of the genus *Trichoderma* have been used in the biological control of this pathogen as an alternative to chemical control. To gain new insights into the biocontrol mechanism used by *Trichoderma harzianum* against the phytopathogenic fungus, *Fusarium solani*, we performed a transcriptome analysis using expressed sequence tags (ESTs) and quantitative real-time PCR (RT-qPCR) approaches. A cDNA library from *T. harzianum* mycelium (isolate ALL42) grown on cell walls of *F. solani* (CWFS) was constructed and analyzed. A total of 2927 high quality sequences were selected from 3845 and 37.7% were identified as unique genes. The Gene Ontology analysis revealed that the majority of the annotated genes are involved in metabolic processes (80.9%), followed by cellular process (73.7%). We tested twenty genes that encode proteins with potential role in biological control. RT-qPCR analysis showed that none of these genes were expressed when *T. harzianum* was challenged with itself. These genes showed different patterns of expression during *in vitro* interaction between *T. harzianum* and *F. solani*.

1. Introduction

The diseases caused by soil fungi are responsible for great losses in common bean (*Phaseolus vulgaris* L.) productivity in irrigated areas of the Southeast and Midwest regions of Brazil (Tolêdo-Souza et al., 2009). The root rots most commonly found in these regions are caused by *Rhizoctonia solani* Kühn and *Fusarium solani* (Mart.) Sacc. *f sp. phaseoli* (Carvalho et al., 2011). The genus *Fusarium* comprises a wide and heterogeneous group of fungi important for the food and drug industry, medicine and agriculture. *F. solani* is a phytopathogenic fungus classified into the Martiella section and it is

* Corresponding author. Fax: +55 62 35211473.
E-mail address: ulhoa@icb.ufg.br (C.J. Ulhoa).

1049-9644/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2012.01.014

the causal agent of several crop diseases (Zaccardelli et al., 2008). The main effects of the bean root rot caused by *F. solani* include reduction of stand and seedling vigor and losses in crop productivity. *F. solani* occurs in practically all of the bean-producing regions of Brazil and has been controlled through the use of chemical fungicides (Tolêdo-Souza et al., 2009).

With the aim of finding an alternative to chemical treatment we isolated species of *Trichoderma* from diverse agro-ecosystems of Brazil and assessed their potential for biocontrol of *Fusarium* spp. Tests included evaluation of antagonistic capacity, production of cell wall-degrading enzymes and production of volatile antibiotics (Páez and Albarracín, 2007). Our results so far have suggested that a specific isolate of *T. harzianum* Rifai (anamorph) ALL42 shows great potential for inhibiting the growth of *F. solani in vitro* (Almeida et al., 2007; Monteiro et al., 2010 and unpublished data). Biological control is a complex process that includes recognition of the host by *Trichoderma*, followed by hydrolytic enzymes and antibiotics production triggered by the direct attachment of the mycoparasite to the host fungi. This contact is mediated by lectins and proteins harboring cellulose binding modules from hyphae of the host and mycoparasitic fungi, respectively, thereby eliciting a signaling cascade comprising G-proteins and MAPKs that can modulate the *Trichoderma's* protein expression pattern. However, the molecular mechanisms, such as identification of new genes as well the global gene regulation expression involved in this process, needs further study.

To gain further insight into this process, we constructed a cDNA library from mycelium of one isolate of *T. harzianum* (isolate ALL42) grown on cell walls of *F. solani* (CWFS), and searched for expressed sequence tags (EST). The evaluation of EST libraries is an effective way to discover novel genes, to explore gene expression patterns and to indentify differentially regulated genes (Bailey et al., 2006). Significant EST resources have been developed for many *Trichoderma* species involved in biocontrol including *Trichoderma hamatum* Bon. (Bain.) (Carpenter et al., 2005), *Trichoderma harzianum* (Liu and Yang, 2005; Scherm et al., 2009), *Trichoderma asperellum* (Liu et al., 2010), *Trichoderma atroviride* P. Karsten (Anamorph) (Seidl et al., 2009) and *Trichoderma* spp. (Vizcaíno et al., 2006, 2007). Knowledge generated by these investigations is contributing to the discovery of new genes involved in the interactions between *Trichoderma* and certain pathogenic fungi (Lorito et al., 2010).

In the present study, we report for the first time the analysis of 3845 ESTs obtained from a library composed of mRNA isolated from the mycelium of *T. harzianum* (ALL42) grown on CWFS. Quantitative real-time PCR (RT-qPCR) studies revealed that six genes encoding hydrolytic enzymes, especially fungal cell wall degrading enzymes (chitinase, β-1,3-glucanase, β-1,6-glucanase, β-1,4-nacetilglucosaminidase and a tripeptidyl protease), three encoding cell wall proteins, one encoding a glucose transporter and three encoding genes with other functions (a chaperone, an inorganic phosphatase and one gene previously described for *Trichoderma reesei* after growth on cellulose), were especially expressed when *T. harzianum* (ALL42) is grown in the presence of *F. solani f sp. phaseoli*.

2. Material and methods

2.1. Organisms and culture conditions

Isolate ALL42 of *T. harzianum* (Enzymology group collection, UFG-ICB) and *F. solani* (EMBRAPA- CNPAF collection) were used in this study. Both fungi were grown on MYG medium containing 0.5% malt extract, 0.25% yeast extract, 1% glucose and 2% agar. Spores from *T. harzianum* were collected in sterile water, centrifuged at 2000*g*, washed twice and used as inoculum (10^7 spores mL^{-1}) in minimal medium containing KH_2PO_4 (2 g l^{-1}), $(NH_4)_2SO_4$ (1.4 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.3 g l^{-1}), $CaCl_2{\cdot}2H_2O$ (0.3 g l^{-1}) supplemented with 0.5% of *F. solani* inactivated (autoclaved at 120 °C for 20 min) cell-wall material. The cultures were grown in conical flasks with constant shaking at 28 °C for 12, 24, 36 and 48 h. Mycelia were harvested, washed twice with sterile water, frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

2.2. Isolation of RNA and library construction

RNA was isolated from the mycelia by grinding with a mortar and pestle under liquid nitrogen, followed by extraction using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The cDNA library was constructed following the protocols of the Superscript plasmid system with Gateway technology for cDNA synthesis and cloning (Invitrogen, USA). Double-stranded cDNA was directionally ligated into the pSPORT1 vector. The sample was mixed with EletroMAX DH5α (Invitrogen, USA), and the mixture was electroporated using a MicroPulser (Bio-Rad). The cDNA library was plated in LB medium with ampicillin (Sigma) and X-gal (USB) to approximately 200 colonies per plate (150 mm Petri dish). The colonies were randomly selected and transferred to a 96-well polypropylene plate containing LB medium with ampicillin (Sigma) and grown overnight at 37 °C. Plasmid DNA was isolated and purified by a modified alkaline lysis protocol (Sambrook and Russel, 2001). The cDNA inserts were sequenced from the 5′ end by employing a standard fluorescence labeling DYEnamic ET dye terminator kit (GE Healthcare) with the T7 flanking vector primer. Automated sequence analysis was performed in an ABI Prism 3100 (Applied Biosystems) with 36 cm capillary system.

2.3. EST analysis and annotation

EST sequences were pre-processed using Phred (Ewing and Green, 1998). Only sequences with at least 100 nucleotides and a Phred quality greater than or equal to 20 were considered for further analysis. Expressed sequence tags (ESTs) were screened for vector sequences using the CrossMatch program. The resulting sequences were assembled into contiguous sequences (contigs) using the CAP3 assembly program (Huang and Madan, 1999). The filtered sequences were compared against the GenBank non-redundant (nr) database using the BLASTX algorithm from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The database sequence matches were considered significant at *E* values $\leqslant 10^{-5}$. Transcripts were annotated using Gene Ontology (GO) terms and hierarchical structure (http://www.geneontology.org). The Blast2GO program (Götz et al., 2008), which assigns the GO terms based on the BLAST definitions, was applied with an *E* value $<10^{-5}$. Unigene results were submitted to InterProScan analysis (Zdobnov and Apweiler, 2001) in order to search for protein motifs. Prediction of signal peptide cleavage sites was carried out by both the Hidden Markov Model and neural network modules of SignalP 3.0 (Bendtsen et al., 2004). Redundancy of the collections of ESTs was calculated as [1 − (Number of unique sequences/Number of sequenced ESTs)] × 100 (Vizcaíno et al., 2006). For this purpose, we only considered those sequenced ESTs with high-quality.

2.4. Expression analysis of biocontrol genes under confrontation

RT-qPCR was used to evaluate gene expression in *T. harzianum* during confrontation by the fungal pathogen *F. solani*. Twenty genes potentially involved in biocontrol were selected for expression analysis (Table 1). Plate challenges against *T. harzianum* by *F. solani* were conducted as described in Scherm et al., 2008. Circular plaques of 5 mm diameter were cut from mycelium of 7-day-old cultures of *T. harzianum* ALL42 and of *F. solani* grown on MYG plates.