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An N-acetyl-β-D-glucosaminidase gene, *cr-nag1*, from the biocontrol agent *Clonostachys rosea* is up-regulated in antagonistic interactions with *Fusarium culmorum*

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ABSTRACT

Clonostachys rosea is a widely distributed fungus that often acts as a parasite on other soil fungi. This fungus has also been reported as a potential parasite against nematodes and insects. The antagonistic activity is thought to be correlated with the secretion of cell wall-degrading enzymes, including chitinases. In this work, we identified and characterized an N-acetyl-β-D-glucosaminidase-encoding gene, *cr-nag1*, belonging to glycosyl hydrolase family 20, from the *C. rosea* strain IK726 using a degenerated primer strategy designed from conserved motifs. The complete gene, including its promoter region, was obtained by genomic walking. Southern analysis showed that *cr-nag1* is present as a single copy gene in *C. rosea*. Phylogenetically, *cr-nag1* showed the highest similarity to N-acetyl-β-D-glucosaminidase genes from other mycoparasitic fungi. Enzymatic assays and RT-PCR showed that the NAGase activity of *C. rosea* is specifically repressed in medium containing a high glucose content and is expressed in media containing chitin or *Fusarium culmorum* cell walls as sole carbon sources. Macroscopic and microscopic observations indicated that the mycelial growth of *F. culmorum* and *Pythium ultimum* were inhibited during interactions with *C. rosea*. High expression of *cr-nag1* was found in interactions between *C. rosea* and *F. culmorum*, whereas the expression of *cr-nag1* in interactions between *C. rosea* and *P. ultimum* was similar to the control. This indicates that although *C. rosea* secretes chitin-hydrolysing agents in order to target the cell wall of *F. culmorum*, it seems to use another strategy for controlling the development of the oomycete *P. ultimum*.

Introduction

The mycoparasitic fungus *Clonostachys rosea* (syn. *Gliocladium roseum*; teleomorph: *Bionectria ochroleuca*) belongs to the family *Bionectriaceae* and the order *Hypocreales* from ascomycetous fungi (Schroers *et al.* 1999). *C. rosea* is ubiquitous in the soil worldwide and a growing number of reports show that is a potentially useful biocontrol agent against several economically important plant pathogens (Sutton *et al.* 1997; Jensen *et al.* 2002, 2004; Dugan *et al.* 2005; Luongo *et al.* 2005). This fungus

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has been frequently associated with cysts of *Heterodera* spp., *Globodera* spp. and other nematodes in soil (Sutton *et al.* 1997). Recently, the first record of *C. rosea* as an entomopathogenic fungus of leafhoppers has been reported (Toledo *et al.* 2006). A strain of *C. rosea*, IK726, was isolated from barley roots infected with *Fusarium culmorum* and evaluated in an *in vivo* screening program for the selection of biological control organisms (Knudsen *et al.* 1995, 1997). The strain has proven highly efficient against seed-borne diseases of cereals under field conditions (Knudsen *et al.* 1995; Jensen *et al.* 2000) and has also been shown to control *Alternaria radicina* and *A. dauci* on carrot seeds (Jensen *et al.* 2004), *Ciboria* in acorns (Knudsen *et al.* 2004), and diseases caused by soil-borne *Pythium* spp. in agricultural and horticultural crops (Møller *et al.* 2003). The modes of action of *C. rosea* as a mycoparasite and as a biological control agent are not well understood. Enzymatic activity, substrate competition, induced resistance, and the production of secondary metabolites all have been suggested to play a role (Pachenari & Dix 1980; Sutton *et al.* 1997; Roberti *et al.* 2002; Lahoz *et al.* 2004). Recent studies have shown that *C. rosea* produces cell wall-degrading enzymes, including chitin, glucan, and cellulose-degrading enzymes (Davila *et al.* 1999; Lübeck *et al.* 2002; Roberti *et al.* 2002; Inglis & Kawchuk 2002; Goedegebuur *et al.* 2002).

Chitin, together with β-(1, 3)-glucan, are the major constituents of the cell walls of asco- and basidiomycetes (Latgé 2007), as well as insect skeletons. The enzymatic hydrolysis of chitin is catalysed by the action of three types of chitin-hydrolysing enzymes belonging to glycosyl hydrolase families 18, 19 and 20. Exo- and endo-acting enzymes (EC 3.2.1.14) can belong to family 18 or 19, whereas the exo-acting β-hexosaminidases (EC 3.2.1.52) belong to family 20. Both endo- and exo-chitinases have been found to be involved in the interaction between mycoparasitic *Trichoderma* strains and their hosts (Zeilinger *et al.* 1999). Furthermore, the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* produce several chitinases. Some of these chitinases are important cuticle-degrading enzymes and act synergistically with proteases to hydrolyse the insect cuticle (Fang *et al.* 2005). Several chitinolytic genes have been characterized from *Trichoderma* species (Lorito 1998; Kim *et al.* 2002; Ramot *et al.* 2004; Seidl *et al.* 2005), and recently three endochitinase genes were identified in *C. rosea* (Mamarabadi *et al.* 2008). As a first step towards elucidating the role of chitinolytic enzymes in biocontrol, the aim of this work was to identify and characterize a gene coding an N-acetyl-β-D-glucosaminidase of *C. rosea* IK726 and study the expression analysis of this gene in different carbon sources and during interactions with plant pathogenic fungi using RT-PCR.

Materials and methods

Fungal strains and culture conditions for enzyme production

Clonostachys rosea strain IK726 (IBT 9371) was maintained in 10 % glycerol at −80 °C and cultivated on PDA (potato–dextrose agar; Scharlau, Barcelona, Spain) for general growth. *C. rosea* strain IK726 has been deposited in the IBT Culture Collection with the number: IBT 9371. For enzyme production, *C. rosea* IK726 was precultivated in 100 ml synthetic medium (SM, pH 6.7) containing: 0.2 $g\,l^{-1}$ $MgSO_4$. $7H_2O$, 0.87 $g\,l^{-1}$ K_2HPO_4, 0.68 $g\,l^{-1}$ KH_2PO_4, 0.2 $g\,l^{-1}$ KCl, 1 $g\,l^{-1}$ NH_4NO_3, 0.2 $g\,l^{-1}$ $CaCl_2$ (trace elements: 0.002 $g\,l^{-1}$ $FeSO_4$, 0.002 $g\,l^{-1}$ $MnSO_4$, 0.002 $g\,l^{-1}$ $ZnSO_4$) with 1 % glucose as carbon source. The medium was inoculated with 5×10^6 conidia per ml and incubated on a rotary shaker at 150 rev min^{-1} at 22 ± 2 °C. After 72 h, the mycelium was harvested by filtration through sterile polyester cloth, washed with sterile water, and transferred to 250 ml Erlenmeyer flasks containing 100 ml of five different fresh media: (1) SM with 1 % glucose (control), (2) V8 (vegetable juice, Campbell Soup Company, Camden, NJ) containing 850 ml Czapek Dox (Scharlau, Barcelona, Spain), 150 ml V8 and 4 g $CaCO_3$, (3) SM with 0.1 % glucose, (4) SM with 0.2 % colloidal chitin (Sigma, St Louis, MO), and (5) SM with 0.1 % glucose and 0.1 % *Fusarium culmorum* cell walls. Each treatment was repeated three times. The culture filtrate was used as crude protein extract by taking 1 ml from each flask after 24, 48 and 72 h incubation, respectively. For production of *F. culmorum* cell walls, *F. culmorum* IK5, isolated from barley seed was grown in potato–dextrose broth (PDB) for 10 d at 25 °C. The mycelium then was harvested through a polyester membrane (42 mm), washed with sterile water, lyophilized, and crushed into powder in liquid nitrogen using a mortar and pestle. To remove residual cytoplasmic components, the powder was suspended in sterile water, sonicated for 1 min on ice, and washed by centrifugation. This process was repeated four times, followed by lyophilization of the cell wall preparation. The cell walls were stored at −80 °C until use.

PCR screening of Clonostachys rosea *genomic DNA for N-acetyl-β-D-glucosaminidase-encoding gene(s)*

The homologous blocks among ten fungal N-acetyl-β-D-glucosaminidases were identified using the ClustalW multiple alignment program (Higgins *et al.* 1994). The homologous blocks were subsequently used for design of degenerated primers using the CODEHOP program (Consensus Degenerate Hybrid Oligonucleotide Primers) (Rose *et al.* 1998) and eventually the primers with lowest amount of degeneracy were selected. A forward primer, NDF = 5′-GCGGGTGATCCCCGAG RTNGAYATGCC with a degeneracy of 16, was used in combination with a reverse primer, NDR = 5′-CGCTGCCAGTTCTTG TAGGGNBSRCACCA with a degeneracy of 48.

Total genomic DNA was extracted using Nucleon PhytoPure kit (Amersham, Buckinghamshire). PCR was performed on a Mastercycler® personal (Eppendorf AG, Hamburg) using DynaZyme version 2.0 DNA polymerase (Finnzymes OY, Espoo) with the following program: 4 min at 95 °C, then 35 cycles of 30 s at 92 °C, 30 s at 59 °C and 30 s at 72 °C, followed by a final extension for 5 min at 72 °C. PCR fragments of the expected size were cloned into the pGEM® Easy Vector System (Promega, Madison) according to the manufactures instructions and sequenced using the sequencing service from MWG-Biotech, Ebersberg, Germany. The sequences were analysed using the BLAST service at the National Center for Biotechnology Information (NCBI) for gene identification. The Blastx was used in this study which compares translated sequence *versus* protein database (Altschul *et al.* 1990).