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Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*

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ABSTRACT

As a notable biocontrol agent, *Trichoderma harzianum* can antagonize a diverse array of phytopathogenic fungi, including *Botrytis cinerea*, *Rhizoctonia solani* and *Fusarium oxysporum*. Elucidating the biocontrol mechanism of *T. harzianum* in response to the pathogens enables it to be exploited in the control of plant diseases. Two-dimensional gel electrophoresis (2-DE) was performed to obtain secreted protein patterns of *T. harzianum* ETS 323, grown in media that contained glucose, a mixture of glucose and deactivated *B. cinerea* mycelia, deactivated *B. cinerea* mycelia or deactivated *T. harzianum* mycelia. Selected protein spots were identified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Ninety one out of 100 excised protein spots were analyzed and some proteins were sequence identified. Of these, one L-amino acid oxidase (LAAO) and two endochitinases were uniquely induced in the media that contained deactivated *B. cinerea* mycelia as the sole carbon source. Activities of the cell wall-degrading enzymes (CWDEs), including β-1,3-glucanases, β-1,6-glucanases, chitinases, proteases and xylanases, were significantly higher in media with deactivated *B. cinerea* mycelia than in other media. This finding suggests that the cell wall of *B. cinerea* is indeed the primary target of *T. harzianum* ETS 323 in the biocontrol mechanism. The possible roles of LAAO and xylanase were also discussed.

Introduction

Biocontrol refers to the use of natural organisms, or genetically modified genes or gene products, to eliminate the effects of undesirable organisms in favor of organisms that are beneficial to humans, including crops, trees, animals and beneficial microorganisms (Deng *et al.* 2007; Viterbo *et al.* 2002). Biocontrol represents an environmentally friendly approach to the management of plant diseases. Accordingly, understanding the biocontrol mechanisms is critical to obtaining the most effective as well as commercially acceptable biocontrol agents.

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Trichoderma spp. have attracted for the biocontrol of phytopathogens such as *Botrytis cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Papavizas 1985; Chet 1987). The biocontrol mechanisms of *Trichoderma* spp. are myriad and varied; they include mycoparasitism, antibiosis, competition for resources and space, and promotion of plant growth (Ghisalberti & Sivasithamparam 1991). However, most studies have focused on the identification, purification, characterization and cloning of single protein – mostly cell wall-degrading enzymes (CWDEs) (Lorito *et al.* 1996; Benitez *et al.* 1998). Of these, cellulases (Benko *et al.* 2007), chitinases (Carsolio *et al.* 1994; Mach *et al.* 1999; El-Katatny *et al.* 2001; Nguyen *et al.* 2008), β-1,3-glucanases (El-Katatny *et al.* 2001) β-1,6-glucanases (Montero *et al.* 2005) and proteases (Geremia *et al.* 1993; Pozo *et al.* 2004; Grinyer *et al.* 2007) have been described as important components of the multi-enzymatic system and the antifungal ability of *Trichoderma* spp. *Trichoderma harzianum* has been demonstrated to be reliable and practical in more than ten commercial products that are currently on the market and were formulated based on strains of *T. harzianum* (Burges 1998; Butt & Copping 2000). The proteomic study of the response of *T. harzianum* to the phytopathogenic pathogen, *B. cinerea*, the causal agent of gray mould disease over 200 species, is a rapid and direct method for identifying the key proteins that are involved in the biocontrol mechanism. Comparative analysis of proteins from *T. harzianum* grown in media with or without deactivated mycelia of *B. cinerea* represents a new means of identifying both novel and known proteins from the globally expressed proteins.

This work attempts to identify (1) the differentially induced proteins of *T. harzianum* ETS 323 that is grown in media that contain glucose, a mixture of glucose and deactivated *B. cinerea* mycelia, deactivated *B. cinerea* mycelia, and deactivated *T. harzianum* mycelia, by 2-DE and LC–MS/MS analysis (2) and the CWDEs that are secreted by *T. harzianum* ETS 323 against deactivated *B. cinerea*.

Materials and methods

Preparation of deactivated B. cinerea *mycelia*

Botrytis cinerea (DYU-201), isolated in Taiwan, was grown on PDA (Difco, Detroit, USA) plates at 22 °C for 10 d. The mycelia were scraped from the PDA plates and inoculated in 250 ml PDB in a 500 ml Erlenmeyer flask. The mycelia were further grown at 22 °C on a shaking incubator at 180 rpm for 7 d, and then filtered through cheese cloth and washed three times in sterile Milli-Q (18.2 MΩ) water. Following centrifugation at 3000*g* at room temperature for 15 min, the mycelia were collected and deactivated in a water bath at 100 °C for 30 min and cooled to room temperature; the preceding step was then repeated once. The deactivated *B. cinerea* mycelia were either used immediately or stored at −20 °C until use.

Preparation of deactivated T. harzianum *ETS 323 mycelia*

Trichoderma harzianum ETS 323 (DYU-102) was grown on PDA plates at 22 °C for 5 d (Liu *et al.* 2007). The green conidia were flushed from the PDA plates in sterile water: about 10^6 conidia ml^{-1} was inoculated in 250 ml of PDB in a 500 ml Erlenmeyer flask. Conidia germinated upon further cultivation at 22 °C in a shaking incubator at 180 rpm for 72 h; the mycelia were then filtered through cheese cloth and washed three times in sterile Milli-Q water. Following centrifugation at 3000*g* at room temperature for 15 min, the mycelia were collected and deactivated in a water bath at 100 °C for 30 min and then cooled to room temperature; the prior step was then repeated once. The deactivated *T. harzianum* ETS 323 mycelia were either used immediately or stored at −20 °C until use.

Cultivation of T. harzianum *ETS 323*

Trichoderma harzianum ETS 323 was grown on PDA plates at 22 °C for 7 d. Two ml of sterile water was added to flush each plate of *T. harzianum* ETS 323. Conidia (about 10^6 ml^{-1}) were collected and used to inoculate 250 ml of minimal medium, which contained (in $g\,l^{-1}$) $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4.7H_2O$, 0.3; peptone, 1.0; urea, 0.3; the pH was 5.0, and 1 % (w/v) glucose was added to this minimal medium in a 500 ml Erlenmeyer flask. Cultures were grown at 22 °C on a shaking incubator at 180 rpm for 72 h. The mycelia from each flask were then collected by filtration through cheese cloth and washed three times with sterile Milli-Q water and inoculated in fresh minimal medium that contained four carbon sources – medium 1 % G: 1 % (w/v) glucose; medium 0.5 % G + 0.5 % dBc: 0.5 % (w/v) glucose plus 0.5 % (w/v) deactivated *Botrytis cinerea* mycelia; medium 1 % dBc: 1 % (w/v) deactivated *B. cinerea* mycelia, and medium 1 % dTh: 1 % (w/v) deactivated *T. harzianum* ETS 323 mycelia. Cultures were grown for another 72 h in a shaking incubator at 180 rpm at 22 °C. The culture media were collected by filtration using cheese cloth following by Advantec No. 1 filter paper.

Protein precipitation

Ammonium sulfate (Riedel-de Haën, Seelze, Germany) was added slowly to the culture media to yield an 80 % saturated solution and stirred overnight. The precipitated proteins were pelleted by centrifugation at 17 000*g* for 1 h. The protein pellets were resuspended in a minimum possible volume of prechilled sterile Milli-Q water. One percent (v/v) fungal and yeast protease inhibitor cocktail (P8215, Sigma, St. Louis, USA) was added to each protein sample. The protein samples were desalted by dialysis against ice-cold Mili-Q water in a dialysis membrane (CelluSep, MWCO = 6000–8000, Seguin) for 12 h; the water was changed for every 2 h. All steps were performed at 4 °C. The protein samples were either used immediately or stored at −20 °C until use.

SDS-PAGE

The secreted protein samples of *Trichoderma harzianum* ETS 323 were subjected to 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), following Laemmli's procedure (Laemmli 1970). Proteins were visualized by placing gels directly into the colloidal Coomassie Blue G250 staining solution (Neuhoff *et al.* 1988).