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## Proteomic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*

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**Abstract** *Trichoderma atroviride* has a natural ability to parasitise phytopathogenic fungi such as *Rhizoctonia solani* and *Botrytis cinerea*, therefore providing an environmentally sound alternative to chemical fungicides in the management of these pathogens. Two-dimensional electrophoresis was used to display cellular protein patterns of *T. atroviride* (*T. harzianum* P1) grown on media containing either glucose or *R. solani* cell walls. Protein profiles were compared to identify *T. atroviride* proteins up-regulated in the presence of the *R. solani* cell walls. Twenty-four protein spots were identified using matrix-assisted laser desorption ionisation mass spectrometry, liquid chromatography mass spectrometry and N-terminal sequencing. Identified up-regulated proteins include known fungal cell wall-degrading enzymes such as *N*-acetyl- $\beta$ -D-glucosaminidase and 42-kDa endochitinase. Three novel proteases of *T. atroviride* were identified, containing sequence similarity to vacuolar serine protease, vacuolar protease A and a trypsin-like protease from known fungal proteins. Eukaryotic initiation factor 4a, superoxide dismutase and a hypothetical protein from *Neurospora crassa* were also up-regulated as a response to *R. solani* cell walls. Several cell wall-degrading enzymes were identified from the *T. atroviride* culture supernatant, providing further evidence that a cellular response indicative of biological control had occurred.

**Keywords** *Trichoderma atroviride* · Biological control · Filamentous fungi proteomics · Differential display

### Introduction

*Trichoderma atroviride* ATCC 74058 (formerly *T. harzianum* P1) is a filamentous fungus commonly found in soil that displays biocontrol capabilities against a range of phytopathogenic fungi including *Rhizoctonia solani* and *Botrytis cinerea* (Chet 1987; Papavizas 1985). These fungi are known pests of hundreds of plant crops, including tomatoes, beans, cucumber, strawberries, cotton and grapes (Prins et al. 2000). The mycoparasitic properties of *T. atroviride* enable it to protect plant crops by releasing, for example, cell wall-degrading enzymes (CWDE; Chet et al. 1998; De la Cruz et al. 1992; Geremia et al. 1993; Harman et al. 1993; Lora et al. 1995; Lorito et al. 1994a) and antibiotics (Lorito et al. 1996; Schirmbock et al. 1994) when challenged with pathogenic species of fungi. The cell wall of the majority of filamentous fungi consists primarily of chitin,  $\beta$ -glucans and proteins (Gooday 1995).

The exact mechanism for how biocontrol species of *Trichoderma* recognise and attack phytopathogenic fungi is unknown, although some determinants in this mechanism have been identified. An unknown diffusible substance (less than 90 kDa in size) triggers the expression of the *ech42* gene of *T. harzianum* P1 (Kullnig et al. 2000). The *ech42* gene encodes a chitinase that is expressed before *Trichoderma* comes into physical contact with *R. solani*. The CWDE secreted during this response include chitinases, proteases and  $\beta$ -glucanases, which are understood to directly attack the cell wall of the phytopathogenic fungi, causing cell lysis and death (Goldman et al. 1994).

There are two possibilities for the use of *T. atroviride* as a biological control agent. First, *T. atroviride* can be applied to crops and/or soil during planting, as CWDE are known inhibitors of spore germination in

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phytopathogenic fungi (Lorito et al. 1994b). The application of *T. harzianum* to fruiting clusters of grape crops provided significant protection from *Botrytis* grape rot; and significant protection was obtained when mixtures of the chemical fungicide iprodione and *T. harzianum* conidia were applied together (Harman et al. 1996). Second, genes coding for proteins contributing to biological control can be cloned into crops, providing them with an inbuilt genetic resistance to a particular pathogenic fungus. Towards this end, the *T. harzianum ech42* gene was inserted into tobacco and potato plants; and expression of the fungal *ech42* in different parts of the plant provided protection from foliar and soil-borne fungal pathogens (Lorito et al. 1998). Both approaches provide environmentally friendly alternatives to the use of existing chemical fungicides.

Two-dimensional electrophoresis (2DE) is a reliable and reproducible technique used to separate thousands of proteins on a single SDS-PAGE gel, in order to generate a protein array. The synthesis of new zwitterionic detergents has provided greater solubilising power of proteins, therefore facilitating the display of membrane proteins on 2D gels (Chevallet et al. 1998). Differential displays on 2D gels have been successfully used to compare protein maps and expression levels in an organism treated in two or more different ways (Cordwell et al. 2001; Somiari et al. 2003).

The aim of this work was to display the whole mycelial protein content of *T. atroviride* using 2DE when grown on glucose and *R. solani* cell walls implicated in the induction of the biocontrol response of *T. atroviride* (Vasseur et al. 1995). Differences in protein expression profiles indicate putative mycoparasitism-related proteins. Since there is some information available on the role of secreted proteins in biological control and the process clearly involves several gene functions, our main aim in this work was to contribute to a wider picture by looking into cellular proteins extracted from whole *T. atroviride* mycelia exposed to *R. solani* cell walls, with some reference to secreted proteins.

## Materials and methods

### *R. solani* cell wall preparation

*R. solani* DAR 67330 (NSW Agriculture, Orange, NSW, Australia) was grown on potato dextrose agar (PDA; Difco Laboratories, Ann Arbor, Mich., USA) plates at 28°C for 72 h. An area of 2–3 cm<sup>2</sup> of mycelial growth was scraped from the PDA plate and used to inoculate 50 ml of potato dextrose broth (Difco Laboratories) in a 250-ml Erlenmeyer flask. The mycelia were then grown at 28°C on a shaker at 250 rpm for 7 days. Fungal and yeast protease inhibitor cocktail (0.05% v/v, product P8215; Sigma, St. Louis, Mo., USA) was added to the culture and allowed to incubate at room temperature (RT) for 20 min. The mycelia were filtered through

3 MM paper (Whatman, Maidstone, UK) and washed with 150–200 ml of Milli-Q (18.2 MΩ) water. After lyophilisation, the mycelium was ground to a fine powder in liquid nitrogen. The cell walls were prepared as described by Inglis and Kawchuk (2002).

### *T. atroviride* cultivation

*T. atroviride* ATCC 74058 (formerly *T. harzianum* P1) was maintained on PDA. Conidia were collected using 5 ml of 0.9% (w/v) sodium chloride, 0.01% (v/v) Tween 80. The amount of  $9 \times 10^8$  conidia was used to inoculate 50 ml of minimal medium (MM; 110 mM potassium phosphate, 38 mM ammonium sulphate, 2.4 mM magnesium sulphate, 4.1 mM calcium chloride, 2.9 mM manganese sulphate, 7.2 mM iron sulphate, 0.35 mM zinc sulphate, 0.71 mM cobalt sulphate, pH 5.5; Penttila et al. (1987) with 2% (w/v) glucose in a 250-ml Erlenmeyer flask, similar to a method used by Vasseur et al. (1995). Cultures were grown at 28°C on a shaker at 250 rpm for 48 h (preculture). The mycelia from each flask were collected, washed three times with 50 ml of Milli-Q water by inversion and centrifugation at 4,000 g for 10 min at 15°C and reinoculated in fresh MM with either 2% (w/v) glucose or 0.1% (w/v) *R. solani* cell walls. Cultures were grown for a further 48 h on a shaker at 250 rpm at 28°C. This experiment was set up in triplicate, but materials from only two cultivations were used. Fungal and yeast protease inhibitor cocktail (0.05% v/v) was added to the culture and allowed to incubate at RT for 20 min. Culture supernatants containing protease inhibitors were collected by centrifugation at 4,000 g for 10 min at 15°C and stored at –20°C until required. The mycelia were washed three times with 50 ml of Milli-Q water by inversion and centrifugation at 4,000 g for 10 min at 15°C, collected and stored at –20°C until use.

### Whole-cell protein isolation

Mycelia grown in MM with either 0.1% (w/v) *R. solani* cell wall or 2% (w/v) glucose as a carbon source were resuspended in 10 ml of extraction solution [7 M urea, 2 M thiourea, 1% (w/v) C7Bz0 (detergent C-0856; Sigma), 80 mM citric acid, 5 mM tributylphosphine, 1 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) Protease inhibitor cocktail]. Proteins were extracted from the mycelia using a method described by Grinyer et al. (2004).

### Precipitation of proteins from the culture supernatant

The *T. atroviride* culture supernatant was thawed and 13 ml were taken and spun at 21,000 g for 15 min at 10°C. Ammonium sulphate was added to the supernatant to give an 80% saturated solution that was stirred overnight at 4°C to allow protein precipitation. The

precipitated proteins were pelleted by centrifugation at 21,000 *g* for 15 min at 4°C. Precipitated proteins were resuspended in 1 ml of resuspension solution [7 M urea, 2 M thiourea, 4% (w/v) CHAPS (detergent C-3023; Sigma), 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide] and incubated at RT for 90 min to allow complete reduction and alkylation of proteins. The reduction and alkylation reactions were quenched with 10 mM dithiothreitol before insoluble material was removed by spinning at 21,000 *g* for 10 min. Samples were buffer-exchanged with 7 M urea, 2 M thiourea, 4% (w/v) CHAPS in Ultrafree 5-kDa centrifugal concentrators (UFV4BCC00; Millipore, USA) until conductivity was below 300  $\mu$ S. They were then concentrated to a final volume of 500  $\mu$ l.

### Protein assay

Proteins from both the whole-cell protein preparation and the culture supernatants were assayed using the Bradford reagent (B6916, Sigma), following the manufacturer's instructions (Bradford 1976). Absorbances were read on a Versamax microplate reader (Molecular Devices, USA) and analysed using Softmax PRO ver. 3.1.2 software.

### Isoelectric focusing and 2D-PAGE

The whole-cell protein samples were used to rehydrate immobilised pH gradient (IPG) strips (pH 3–10, pH 4–7, 11 cm; Amersham Biosciences, Sweden) by applying 180  $\mu$ l of each sample (500  $\mu$ g of protein) per IPG strip. Protein (250  $\mu$ g) from the culture supernatant of *T. atroviride* when grown on *R. solani* cell walls was rehydrated onto pH 4–7 IPG strips. Proteins were separated by 2D-PAGE, using a method described by Grinyer et al. (2004). Each protein extract was run on quadruplicate gels for the pH 4–7 range.

### Image analysis of 2DE gels

The 2DE gel images were analysed using ImageIQ (Proteome Systems, Australia). Spot detection of the whole-cell protein samples was conducted on all 16 gels in the pH 4–7 range, using the default spot detection parameter settings. Spot editing was limited to deleting spots from the edges of the gel images; and gel images were matched in two groups of eight (grown on glucose vs grown on *R. solani* cell walls). The composite image generated from each match was subsequently matched to identify differentially displayed protein spots. Protein spots showing at least two-fold up-regulation as determined by ImageIQ, or those unique to growth on *R. solani* cell walls were considered relevant for further characterisation.

### Matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry

Protein spots were prepared for matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry (MALDI-TOF-MS) as described by Grinyer et al. (2004). Peptide mass fingerprints (PMF) were searched against proteins from all fungal species using the Mascot PMF, where a modified MOWSE scoring algorithm was used to rank results ([http://www.matrixscience.com/help/scoring\\_help.html](http://www.matrixscience.com/help/scoring_help.html); Pappin et al. 1993).

### Liquid chromatography–mass spectrometry

Trypsin digest solutions of up-regulated proteins were analysed by nanoflow liquid chromatography–mass spectrometry (LC-MS; Grinyer et al. 2004). Proteins were identified using TurboSequest (ThermoFinnigan), making use of an in-house database created from Swiss-Prot/TrEMBL and NCBI protein entries. The in-house database was comprised of all available sequence information from *Saccharomyces* sp., *Schizosaccharomyces* sp., *Trichoderma* sp., *Neurospora* sp., *Aspergillus* sp. and *Candida* sp. When protein identifications needed confirmation, an all-species search of Swiss-Prot/TrEMBL was conducted. Peptides were identified from MS/MS spectra, in which more than half of the experimental fragment ions matched theoretical ion values and gave cross-correlation, delta correlation and preliminary score values greater than 2.0, 0.2 and 400, respectively.

### N-terminal sequencing

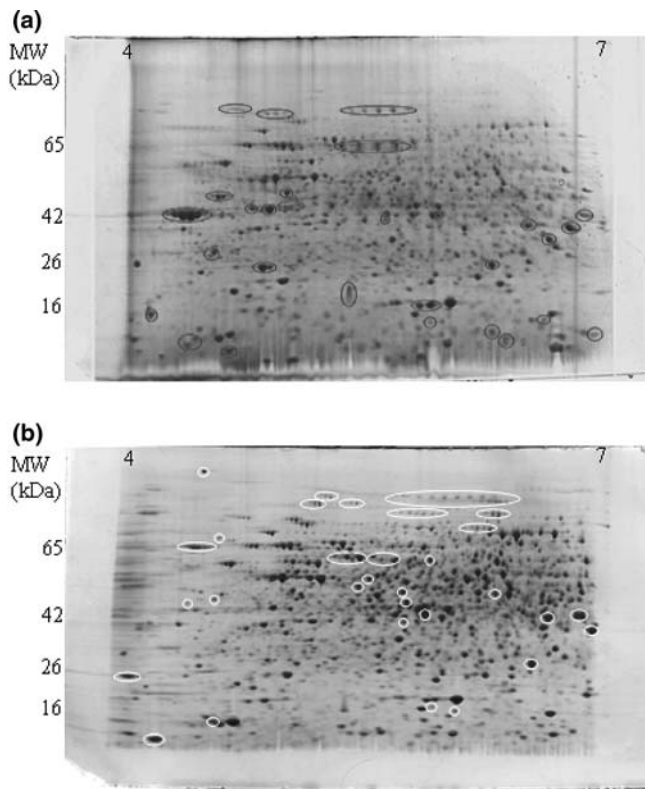
Spots were excised from SDS-PAGE gels and sent for N-terminal sequencing to the Biomolecular Research Facility at Newcastle University, Australia.

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## Results

Proteins extracted from *T. atroviride* were separated on 2D gels over a pH 4–7 gradient, shown in Fig. 1. Proteins separated in this pH range were spread evenly across the pH gradient, which made it easier to detect up-regulated and down-regulated proteins when compared with the more compact pH 3–10 range (data not shown). The protein pattern observed when *T. atroviride* was grown on glucose differed from the protein pattern observed when grown on *R. solani* cell walls. From the pH 4–7 range, 56 protein spots were observed to be up-regulated when *T. atroviride* was grown on the cell walls of *R. solani*. Approximately 67 protein spots from *T. atroviride* were up-regulated when grown on glucose, as seen in the pH 4–7 region. The differentially expressed proteins are circled in Fig. 1 for both conditions. For the purpose of this paper, the up-regulated proteins highlighting putative mycoparasitic-related proteins



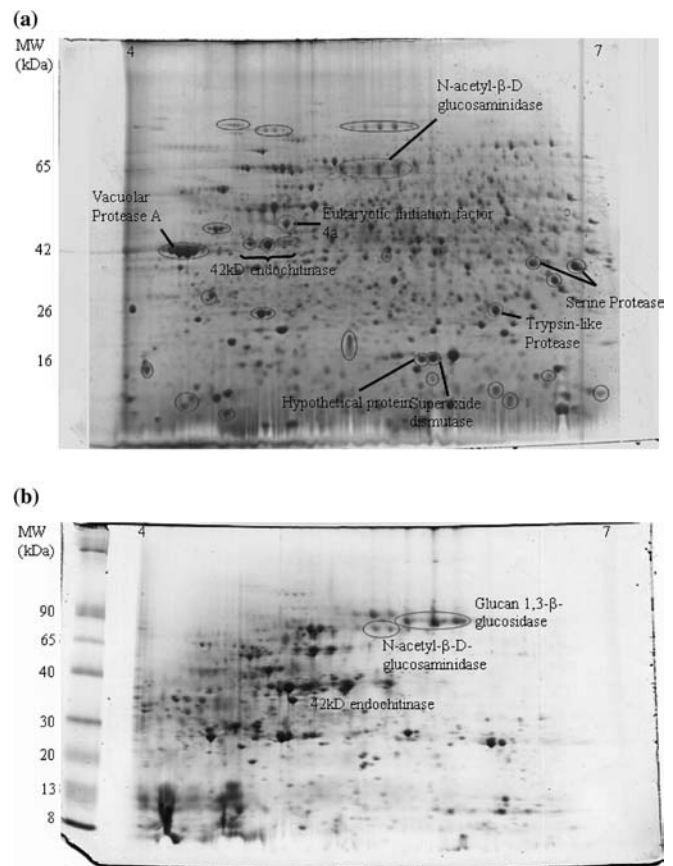


**Fig. 1** Differentially expressed proteins are circled on pH 4–7 protein maps of *T. atroviride*: **a** up-regulated proteins during the biocontrol response, **b** proteins differentially regulated when grown on glucose

when *T. atroviride* was grown on the cell wall of *R. solani* were investigated further.

Protein identifications were made using a combination of MALDI-TOF-MS, LC-MS and N-terminal sequencing. The use of cross-species identification (Wilkins and Williams 1997) increased the size of the protein database searched to include proteins from all fungal species, or selected closely related species. Of the 56 protein spots up-regulated in cultures incubated with *R. solani* cell walls (Fig. 2a), 24 protein spots representing eight genes were identified (Table 1). Identified proteins included *N*-acetyl- $\beta$ -D-glucosaminidase (eight spots), 42-kDa endochitinase (four spots), vacuolar protease A (six spots), eukaryotic initiation factor 4a (one spot), hypothetical protein (one spot), superoxide dismutase (one spot), trypsin-like protease (one spot) and serine protease (two spots), as shown in the annotated map in Fig. 2a. All up-regulated protein spots were observed across at least three of the four replicate gels in the pH 4–7 range from duplicate protein extractions.

Proteins secreted into the culture medium by *T. harzianum* hyphae grown on cell walls of *R. solani* added to the preculture grown on glucose were separated by 2DE to ensure the presence of secreted enzymes previously implicated with biological control, therefore confirming the relevance of the experimental arrangement. Proteins were displayed on a 2D gel across a pH 4–7 range, as shown in Fig. 2b. From this experiment, a 42-kDa



**Fig. 2** Partially annotated 2D SDS-PAGE map of: **a** up-regulated proteins from *T. atroviride* when the biocontrol response is induced with cell walls from *R. solani*, **b** secreted proteins from the culture supernatant of *T. atroviride* when grown on the cell walls of *R. solani*

endochitinase, glucan 1,3-beta-glucosidase (GLUC78) and *N*-acetyl- $\beta$ -D-glucosaminidase from *T. atroviride* were identified. These identified proteins are circled in Fig. 2b and information regarding the proteins is given in Table 1. The secretion of these three known mycoparasitic-related proteins indicates that a biocontrol-like cellular response is likely to occur when *T. atroviride* is grown on the cell wall of *R. solani*. Moreover, these secreted proteins, which are subjected to catabolite repression (Mach et al. 1999; Donzelli et al. 2001), were not observed in the culture supernatants when *T. atroviride* was grown on glucose (data not shown) but were present in the cultures with cell walls added, indicating that potential glucose repression was not an issue at the time of harvesting the culture.

## Discussion

The proteomic comparison of *T. atroviride* grown on *R. solani* cell walls or glucose has highlighted many differentially expressed proteins. Proteins up-regulated in the presence of *R. solani* cell walls range from proteins previously linked with the biological control response of *T. atroviride* to proteins never indicated before in

**Table 1** Identified proteins from the mycelium and/or culture supernatant of *T. atroviride*

Protein	Gene identification number	Theoretical pI	Theoretical MW (Da)	Amino acid coverage or sequence	Species	Identification method
Eukaryotic initiation factor 4a <sup>a</sup>	gi 19115766	4.86	44,408	28%	<i>Schizosaccharomyces pombe</i>	MALDI/LC-MS/MS
42-kDa endochitinase <sup>a,b</sup>	gi 6630946, gi 6630940	5.02–5.11	45,199–45,237	23–33%	<i>Hypocrea rufa</i> , <i>T. atroviride</i>	MALDI/LC-MS/MS
<i>N</i> -acetyl- $\beta$ -D-glucosaminidase <sup>a,b</sup>	gi 7493949	5.58	64,493	20–43%	<i>T. harzianum</i>	MALDI/LC-MS/MS
Superoxide dismutase [Cu-Zn] <sup>a</sup>	gi 101875	5.74	15,875	TVVHAGTDDLK	<i>N. crassa</i>	MALDI/LC-MS/MS
Hypothetical protein <sup>a</sup>	gi 28925968	6.75	16,443	KLAESYQNMQK LAESYQNMQK SDGNVIHFAAPK LAESYQNM <sup>c</sup> QK	<i>N. crassa</i>	MALDI/LC-MS/MS
Vacuolar serine protease/ hypothetical protein <sup>a</sup>	gi 2143220, gi 28927626, gi 14215732	6.02	53,258	GFKGSVANM <sup>c</sup> SLGGGK RKGFKGSVANM <sup>c</sup> SLGGGK GSVANMSLGGGK AYFSNYGK SNGSGTMSDVVK	<i>A. fumigatus</i> , <i>N. crassa</i> , <i>P. chrysogenum</i>	LC-MS/MS
Homology to vacuolar protease A <sup>a</sup>	gi 28921374	5.09	42,933	GLHPVPVTFMNAQ	<i>N. crassa</i>	N-terminal sequencing
Homology to trypsin-like protease /protease P27 <sup>a</sup>	gi 18076138, gi 3661612, gi 1072114	5.48	26,296	IVGGTTAAAG	<i>P. nodorum</i> , <i>T. harzianum</i> , <i>Cochliobolus carbonum</i>	N-terminal sequencing
Glucan 1,3- $\beta$ -glucosidase <sup>b</sup>	gi 8886891	5.39	78,102	18–21%	<i>T. atroviride</i>	MALDI

<sup>a</sup> Mycelium<sup>b</sup> Culture supernatant<sup>c</sup> Oxidated methionine

biological control. Of the previously recognised proteins, 42-kDa endochitinase and *N*-acetyl- $\beta$ -D-glucosaminidase have been found to play a critical role in fungal biocontrol. Both enzymes are secreted from *T. atroviride* and act to hydrolyse the cell wall of the pathogenic fungus (Mach et al. 1999).

*N*-acetyl- $\beta$ -D-glucosaminidase is a secreted protein induced by pathogen cell walls or chitin-degradation products (Peterbauer et al. 1996). The appearance of *N*-acetyl- $\beta$ -D-glucosaminidase in the secreted proteome (Fig. 2b) of *T. atroviride* suggests it is produced to degrade *R. solani* cell walls and its cellular expression continues after 48 h. This enzyme seems to occur in eight protein isoforms, which has not been reported previously.

Secretion of 42-kDa endochitinase is induced by carbon depletion and metabolic stress (Mach et al. 1999) and is up-regulated before *T. atroviride* makes physical contact with *R. solani* (Kullnig et al. 2000). Nitrogen levels in the medium also influence the transcription of the *ech42* endochitinase gene (Donzelli and Harman 2001). Notwithstanding the reason for induction of the 42-kDa endochitinase, the appearance of this protein in the culture supernatant indicates that this endochitinase was secreted by the mycelia to degrade the chitin structure of the cell walls of *R. solani*.

During the biocontrol response, the synthesis and release of proteases is expected (Geremia et al. 1993). Three up-regulated proteases were identified from whole-cell proteins when *T. atroviride* was cultured on *R. solani* cell walls. These include vacuolar serine protease/hypothetical protein, vacuolar protease A and a protein showing sequence similarity to trypsin-like protease/protease P27. Vacuolar serine protease/hypothetical protein has been identified from three different fungal species: *A. fumigatus*, *N. crassa* and *Penicillium chrysogenum*. The amino acid sequence of vacuolar serine protease from *N. crassa* contains 66.6% sequence similarity to a hypothetical protein from *P. chrysogenum* (SIM alignment tool; <http://us.expasy.org/cgi-bin/sim.pl?prot>). The serine protease identified from our 2D gel differs from the known serine protease of *T. harzianum* (encoded by the *prb1* gene), as it has a different observed pI (6.7) instead of 9.2 (Geremia et al. 1993). It is unlikely that such a large pI difference is observed between predicted and observed positions on 2D gels, unless the protein is heavily modified by post-translational modifications. Only one potential N-linked glycosylation site exists in the amino acid sequence of Prb1. It should also be noted that PMF data for all up-regulated proteins were searched against known *Trichoderma* proteins (including Prb1) without success, showing that the serine protease identified here is different from the previously characterised form and is therefore a novel *Trichoderma* protein.

Vacuolar protease A was identified from a 14-amino-acid tag determined through N-terminal sequencing. The amino acid tag was searched against known proteins using BlastP (Altschul et al. 1990) and found to

align best with vacuolar protease A from *N. crassa*, having 85% sequence identity and 92% sequence similarities. The position of vacuolar protease A on the 2D gel aligns well with its predicted position, as determined by theoretical molecular weight and pI. By deduction, all six isoforms were classified as vacuolar protease A, as identical MALDI spectra from each isoform were produced. This protein has also been defined as a novel *Trichoderma* protein, as it has never been detected in *Trichoderma* protein databases.

Trypsin-like protease/protease P27 was identified from a N-terminal sequence tag by amino acid similarity to *Phaeosphaeria nodorum* and *T. harzianum*, respectively. These two proteins contained over 60% sequence identity when their amino acid sequences were aligned. The PMF data from this protein spot was searched against known *Trichoderma* proteins (including the previously described Protease P27) without success, showing that the protease identified here is sufficiently different from the form characterised earlier and is therefore novel to *Trichoderma*. A *T. harzianum* trypsin-like protease has been linked to the degradation of the cell wall of the commercial mushroom *Agaricus bisporus* by enzyme activity assays (Williams et al. 2003), further validating the action of this protease in biocontrol.

Members of the  $\beta$ -glucanase family of proteins have previously been reported to be secreted during biocontrol (Papavizas 1985). These proteins are secreted from the mycelia and hydrolyse the fungal cell wall by breaking down  $\beta$ -1,3-glucans,  $\beta$ -1,4-glucans and  $\beta$ -1,6-glucans (Lora et al. 1995). We did not identify a  $\beta$ -glucanase up-regulation from the whole-cell protein content of *T. atroviride* during growth on the cell walls of *R. solani* but did identify glucan 1,3-beta-glucosidase (GLUC78) by MS from the secreted proteome of *T. atroviride* when grown on *R. solani* cell walls (Fig. 2b). This indicates that the expression of glucan 1,3-beta-glucosidase is switched on for a shorter period of time than that of the chitinases and the enzyme is completely secreted from the fungal hyphae by 48 h of growth. The 78-kDa glucan 1,3-beta-glucosidase is secreted when *T. atroviride* is grown on fungal structures or fungal cell walls and is strongly antigenic against many fungal species (Lorito et al. 1994a). The secretion of a beta-glucanase, an endochitinase and a glucosaminidase, into the culture supernatant provides further support to the experimental setting applied in order to provoke a biological control-like response in *T. atroviride*.

Other cellular proteins showing up-regulation when *T. atroviride* is grown on the cell walls of *R. solani* include a protein matching a hypothetical protein from *N. crassa*, eukaryotic initiation factor 4a and superoxide dismutase [Cu-Zn] (SOD). The amino acid sequence of the hypothetical protein from *N. crassa* was blasted against known proteins and found to contain a predicted nascent polypeptide-associated complex (NAC) domain. NAC domains are associated with ribosomes and protect nascent polypeptides from associating with cytosolic proteins prematurely. NAC subunits may also interact

with DNA in a transcriptional regulation role (Franke et al. 2001). Eukaryotic initiation factor 4a functions during translation initiation to unwind the secondary structure of mRNA to facilitate ribosome binding. As protein secretion is promoted during the biocontrol response, transcriptional and translation initiation factors must be up-regulated to ensure the cell can produce enough proteins for environmental demand.

SOD is a stress-response protein whose function is to destroy toxic superoxide radicals produced in a cell (Petersohn et al. 2001). The appearance of SOD could be indicative of the cellular stresses created when *T. atroviride* mycelia are transferred from an abundant glucose carbon source to a medium containing *R. solani* cell walls. Some level of cellular stress, or adaptational stress is expected when protein secretion is induced and/or when a change of nutrients is introduced, as was the case when growing *T. atroviride* in a similar way to Vasseur et al. (1995). To eliminate an inconclusive proteomic response caused by starvation, we reduced the initial preculture conditions (2% glucose with minimal medium) used by Vasseur et al. (1995), from 3 days to 48 h.

Currently, the genome data of *T. atroviride* or any other mycoparasitic *Trichoderma* sp. are not publicly available. To increase the size of the protein database available for PMF searching, we included all known and deduced proteins across the kingdom fungi. In this work, cross-species identification (Wilkins and Williams 1997), linked with MS and/or N-terminal sequence tags, made it possible to confidently identify six of the eight gene products expressed as 24 protein spots that were up-regulated during the biocontrol response of *T. atroviride*. These six proteins have not previously been recognised from *T. atroviride* and are therefore novel to this species.

In this work, three novel proteases were identified and information about broader cellular protein responses putatively related to biocontrol was obtained. We are currently in the process of isolating the genes encoding the three novel proteases, in order to establish their involvement in the biocontrol response of *T. atroviride*.

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