Fungal Genetics and Biology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization

Luis V. Lopez-Llorca [a,*], Sonia Gómez-Vidal [a], Elena Monfort [a], Eduardo Larriba [a], Juan Casado-Vela [c], Félix Elortza [c], Hans-Börje Jansson [a], Jesús Salinas [a], José Martín-Nieto [b]

[a] *Multidisciplinary Institute for Environmental Studies (MIES) "Ramón Margalef", Department of Marine Sciences and Applied Biology, University of Alicante, Aptdo. 99, 03080 Alicante, Spain*

[b] *Multidisciplinary Institute for Environmental Studies (MIES) "Ramón Margalef", Department of Physiology, Genetics and Microbiology, University of Alicante, Aptdo. 99, 03080 Alicante, Spain*

[c] *Proteomics and Metabolomics Platform, CIC bioGUNE, Technological Park of Bizkaia, Building 801 A, 48160 Derio, Spain*

ARTICLE INFO

Article history:
Received 28 September 2009
Accepted 17 January 2010
Available online xxxx

Keywords:
Barley
Gene expression
Immunolocalization
Mass spectrometry
Nematophagous fungi
Pochonia chlamydosporia
Pochonia rubescens
Serine proteases

ABSTRACT

Nematophagous fungi *Pochonia chlamydosporia* and *P. rubescens* colonize endophytically barley roots. During nematode infection, serine proteases are secreted. We have investigated whether such proteases are also produced during root colonization. Polyclonal antibodies against serine protease P32 of *P. rubescens* cross-reacted with a related protease (VCP1) of *P. chlamydosporia*, but not with barley proteases. These antibodies also detected an unknown ca. 65-kDa protein, labeled hyphae and appressoria of *P. chlamydosporia* and strongly reduced proteolytic activity of extracts from fungus-colonized roots. Mass spectrometry (MS) of 32-kDa protein bands detected peptides homologous to VCP1 only in *Pochonia*-colonized roots. Peptides homologous to barley serine carboxypeptidases were found in 65 kDa bands of all roots. RT-PCR detected expression of VCP1 and a new *P. chlamydosporia* serine carboxypeptidase (SCP1) genes only in fungus-colonized roots. SCP1 shared limited sequence homology with VCP1 and P32. Expression in roots of proteases from nematophagous fungi could be greatly relevant for nematode biocontrol.

1. Introduction

Fungal parasites of nematode eggs have great potential as biocontrol agents, since they also infect females of sedentary nematodes and destroy the eggs they contain (Stirling, 1991). *Pochonia chlamydosporia* (Goddard) Zare & Gams has provided significant control of populations of root-knot and cyst nematodes (de Leij et al., 1993; Kerry et al., 1984; Kerry and Bourne, 1996). De Leij and Kerry (1991) suggested that root colonization by *P. chlamydosporia* is a prerequisite for successful biological control. In order to exploit fully the biocontrol potential of this fungus, it is important to understand its ecology in soil and its interactions with both plant and nematode hosts (Hirsch et al., 2001; Jansson and Lopez-Llorca, 2004). Nematophagous fungi can act as endophytes in both monocots (Lopez-Llorca et al., 2002a) and dicots (Bordallo et al., 2002). This seems to be a general trait for all ecological groups of nematophagous fungi except for endoparasites, which are the most interrelated with their hosts (Lopez-Llorca et al., 2006). When developing endophytically, nematophagous fungi interact with their plant host eliciting defence responses and promoting plant growth (Maciá-Vicente et al., 2009b). Such responses have been recently proven *in vivo* in barley roots using a *P. chlamydosporia* GFP-expressing transformant as well as the lipophylic tracker FM4-64 (Maciá-Vicente et al., 2009a). This study indicated that hyphae of the endophyte are surrounded by the host plant membrane. Often hyphae were dead, perhaps indicating recognition by plant host defences.

An important virulence factor for nematophagous fungi is the production of extracellular proteases. Most of these proteolytic enzymes belong to the family of serine proteases (Lopez-Llorca et al., 2008). Specific polyclonal antibodies against the major extracellular proteases VCP1 and P32 from *P. chlamydosporia* and *P. rubescens*, respectively, have been obtained and shown cross-reactivity toward both proteins (Lopez-Llorca and Robertson, 1992; Segers et al., 1995). Polyclonal antibodies are also suitable for antigen immunolocalization purposes. According to this and considering the close relationship between *P. chlamydosporia* and *P. rubescens*, we have raised in this work a specific polyclonal antiserum to P32. These antibodies are thus expected to recognize the major

* Corresponding author. Fax: +34 96 590 9897.
E-mail address: lv.lopez@ua.es (L.V. Lopez-Llorca).
URL: http://www.ua.es/en/dpto/dcmba/botanica/gefpv_inv.html (L.V. Lopez-Llorca).

1087-1845/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2010.01.004

proteases of these two egg-parasitic fungi. The availability of the VCP1 encoding sequence (Morton et al., 2003) prompted us to use RT-PCR in order to detect the expression of *VCP1* in barley roots (*Hordeum vulgare*) colonized by *P. chlamydosporia*. We also used MS analysis to obtain protein sequence data from bands detected by Western blotting corresponding to fungal proteases. Our main aim was to use these techniques in a complementary fashion for investigating protease production by the two nematophagous fungi colonizing barley roots endophytically.

2. Materials and methods

2.1. Fungi

P. chlamydosporia, isolates 123 (from our laboratory collection) and 144 (kindly provided by B. Kerry, IACR, Rothamsted, UK), and *P. rubescens* CBS 464.88 grown on corn meal agar (CMA) at 25 °C in the dark for 1–4 weeks were used as inoculum.

2.2. Plants

Barley (*H. vulgare* L. var. disticum) seeds were surface-sterilized, plated and grown for 2 days on germinating medium (Bordallo et al., 2002) at 25 °C in the dark. Culture tubes containing water-saturated vermiculite were inoculated with 5-mm diameter disks, placed 10 mm deep, cut from the edge of fungal colonies actively growing on CMA, and one barley seedling was then placed in each tube 5–10 mm below the surface (Maciá-Vicente et al., 2009a). The tubes were then incubated at 25 °C under a 16/8 h (light/dark) photoperiod for up to 3 weeks, and barley roots were sequentially sampled weekly. Control tubes without fungus were carried in parallel.

A different experimental setup was also used, in which sterilized barley seedlings were placed onto CMA Petri dishes pre-inoculated 1–4 weeks in advance with either *P. chlamydosporia* or *P. rubescens*, and after 1, 2, 5 and 7 days of incubation at 25 °C in the dark seedlings were harvested.

2.3. Polyclonal anti-P32 antibodies

P. rubescens was grown at 25 °C stationary in the dark in flasks containing 150 ml of protease induction medium (Lopez-Llorca, 1990) in which gelatin was replaced by 0.05% yeast extract (Sigma, St. Louis, MO, USA). The mycelium was then recovered by filtration through Millipore (Molsheim, France) 0.2 μm pore-size filters, frozen and lyophilized. P32 serine protease was purified as described (Lopez-Llorca, 1990), with slight modifications.

A polyclonal antiserum was produced in albino New Zealand rabbits after immunization with four injections each of 120 μg of purified P32 given in a 2-month time period. A precipitation line was obtained in double diffusion assays (Ouchterlony and Wilson, 1968) between purified P32 and the antiserum, which was not obtained with the pre-immune serum (data not shown). The IgG fraction was affinity-purified from the rabbit antiserum using HiTrap Protein A HP 1-ml columns (GE Healthcare, Uppsala, Sweden) following the manufacturer's instructions, which resulted in a 10-fold increase in antibody specificity.

2.4. Proteolytic activity assays

Roots, either from tubes or Petri dish cultures, were ground at 4 °C in an ice-chilled mortar under liquid N_2 and the resulting powder was resuspended in 50 mM Tris–HCl buffer, pH 7.5. The mixture was kept at 4 °C and shaken for 1 h. The supernatant was recovered after centrifugation at 13,500 rpm for 5 min at 4 °C and kept frozen at −20 °C.

Proteolytic activity was measured according to Lopez-Llorca (1990) using fluorescein thiocarbamyl (FTC)-casein as the substrate. The inhibitory effect of anti-P32 antibodies (7.5 μg) or 10 mM phenylmethylsulphonyl fluoride (PMSF) was tested separately on either purified P32 (150 ng) or root extracts (1.5 μg of protein), to which 0.5 M Tris–HCl buffer, pH 8.5, was added to a volume of 490 μl. After preincubation at 4 °C for 30 min 10 μl of 0.5% FTC-casein solution was added, and fluorescence was measured after incubation at 37 °C for 1 h, following precipitation with trichloroacetic acid. Excitation and emission wavelengths were 485 and 535 nm, respectively.

For zymogram analysis, purified P32 (100 ng) or root extracts (0.5 μg of protein) were subjected to electrophoresis under semi-denaturating conditions on 12% polyacrylamide gels containing 1% SDS and 0.1% gelatin, according to Tikhonov et al. (2002). The gels were then incubated at 35 °C for 1 h in 50 mM Tris–HCl buffer, pH 8.0, containing 2% Triton X-100, and thereafter in the same buffer without detergent for 2 h, and finally stained with Coomassie Blue.

2.5. Western blotting

Root extract proteins were resolved by SDS–PAGE on 12% polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. The blots were blocked at room temperature for 30 min in 5% skimmed milk powder in phosphate buffered saline, pH 7.4 (PBS), containing 0.1% Tween 20 (PBST). This was followed by incubation with anti-P32 IgG at 0.2 μg ml^{-1} for 2 h in PBST, and thereafter with a 1:30,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) for further 2 h. Immunodetection was carried out with 5-bromo-4-chloro-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT).

2.6. Elisa

Root extracts diluted in 0.1 M $NaHCO_3$ buffer, pH 8.6, were applied to 96-well microtiter plates at 20 μg of protein per well, and incubated at 4 °C overnight. Plates were washed twice with distilled H_2O and then blocked with 3% skimmed milk powder in PBST at 37 °C for 1 h. Then, incubation with purified anti-P32 IgG added to each well at 0.4 μg ml^{-1} in blocking solution was carried out at 25 °C for 2 h, and thereafter with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG in PBST for further 2 h. After the addition of BCIP and NBT, absorbance at 405 nm was measured.

2.7. Immunolocalization

One or two week-old barley roots, grown either on tube or Petri dish cultures, were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, for 1.5 h. Roots were then cut into 0.5-cm pieces and embedded in OCT (Leica, Nusslock, Germany). Longitudinal sections (20 μm thick) were obtained using a Leica CM1510 cryostat and deposited on precooled (−20 °C) SuperFrost Plus slides (Menzel-Glaser, Germany). After OCT removal, the sections were blocked with 1% skimmed milk powder in PBS at room temperature for 30 min. Incubation with anti-P32 IgG (0.4 μg ml^{-1}) was then carried out overnight, and thereafter with Alexa Fluor 647-conjugated goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) at a 1:10,000 dilution for 1 h. The sections were mounted with Vectashield (Vector Laboratories, UK) and examined on a Leica TCS SP2 laser-scanning confocal microscopy system.

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

2.8. Mass spectrometry and de novo sequencing

Coomassie Blue-stained bands from SDS–PAGE gels matching those detected by anti-P32 IgG on a duplicate Western blot were manually excised from the gel, reduced and Cys-alkylated, followed by in-gel digestion with sequencing-grade trypsin (Promega, Madison, WI, USA) as described previously (Casado-Vela et al., 2007). The supernatants were transferred to new tubes, speed-vac dried and resuspended in 0.1% formic acid. Peptides were resolved on a Zorbax RP C18 column (75 μm ∅ × 15 cm) from Agilent (Santa Clara, CA, USA) using a 40 min linear gradient of 3–60% acetonitrile in 0.1% formic acid on an Agilent 1200 HPLC system, at a constant flow rate of 300 nl/min. The eluting peptides were scanned and fragmented in an LTQ-Orbitrap XL mass analyzer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Proxeon nanospray source. The four most intense precursor ions, ranging from 400 to 2000 *m/z*, were scanned and measured in the Orbitrap at a 60,000 resolution. The following parameters were set for searches using Sequest (Eng et al., 1994): enzyme, trypsin; fixed modifications, carboxyamidomethyl cysteine; variable modifications, oxidation of methionine; precursor ion tolerance, 10 ppm; fragment ion tolerance, 0.5 Da. All fragmentation spectra were searched against the NCBInr database (ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz), and only those peptides containing >5 amino acids, with X_{corr} values above 1.5, 2.0 and 2.5 for singly, doubly and triply charged species, respectively, and peptide probabilities >0.5 were accepted as positive identifications. The experiment was repeated twice.

For *de novo* sequencing, tryptic peptides generated from purified P32 bands were analyzed by reverse-phase HPLC as described above. Both precursor and fragment ions were accurately measured in the Orbitrap. Fragment ions were generated by high-energy collisional dissociation (Olsen et al., 2007) at 35% collision energy. Fragment spectra were *de novo* sequenced using Peaks Studio v4.5 SP2 (www.bioinformaticssolutions.com). Parental and fragment mass error tolerances were 0.01 Da and 0.02 Da, respectively. Only those sequences with peptide score ⩾50, quality values ⩾0.75 and containing ⩾4 amino acids with confidence ⩾99.5 were considered as valid candidates. Fragment spectra were also manually validated and the proposed sequences searched using the BLASTP software (Altschul et al., 1997).

2.9. DNA and RNA isolation

P. chlamydosporia isolate 123 was inoculated in flasks containing 50 ml of protease induction medium (Lopez-Llorca, 1990) with slight modifications (0.1% gelatin, 0.02% yeast extract) and incubated at 25 °C for 8 days with shaking at 120 rpm. Mycelium was then collected by vacuum filtration, frozen in liquid N_2, lyophilized and stored at −80 °C until use. Control and fungus-inoculated *H. vulgare* roots grown for 2 weeks on culture tubes were washed with 0.5% DEPC-treated H_2O and treated in the same fashion for storage at −80 °C.

A cetyl-trimethyl-ammonium bromide (CTAB)-based extraction method adapted from O'Donnell et al. (1998) was used for genomic DNA isolation from *P. chlamydosporia* lyophilized mycelium. This protocol was modified by adding 2% polyvinylpyrrolidone (Sigma) to the extraction buffer to perform genomic DNA isolation from barley roots. DNA was quantified using the Hoechst 33258 dye (Sigma) according to Ausubel et al. (2002). The RNaqueous Kit from Ambion (Austin, TX, USA) and the RNeasy Plant Mini Kit from Qiagen (Valencia, CA, USA) were used for RNA extraction from mycelia and lyophilized roots, respectively, following the manufacturers' instructions. DNAse treatment was carried out using the Turbo DNA-Free Kit (Ambion), and RNA was quantitated using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes).

2.10. Cloning of the P. chlamydosporia SCP1 gene

Based on available mRNA sequences – most of them partial – encoding serine carboxypeptidases from six filamentous fungi, namely *Neurospora crassa* (GenBank accession number XM_955807), *Magnaporthe grisea* (XM_364693), *Aspergillus fumigatus* (XM_726431), *A. clavatus* (XM_001270418), *A. phoenicis* (D25288) and *A. niger* (XM_001400804), two degenerate primers were designed whose sequences were: forward (240-F), 5′-TBG GHGARTCBTAYGCBGG-3′, and reverse (1271-R), 5′-CCAVGTCATR TTYTGDAT-3′. A segment of 1249 bp was PCR-amplified from *P. chlamydosporia* genomic DNA (100 ng) as indicated below, except that the annealing temperature was 55 °C and PCR was run for 35 cycles. This DNA segment was cloned into the pSC-A vector (Stratagene) and its sequence deposited in GenBank under accession number GQ355960. Its encoded serine carboxypeptidase was designated as SCP1. Comparative sequence analyzes were performed using the BLAST (Altschul et al., 1997) and FASTA (www.ebi.ac.uk/Tools/fasta) algorithms. Sequence alignments were carried out using ClustalW2 and MUSCLE tools (http://www.ebi.ac.uk/Tools/sequence.html) and improved manually. N-glycosylation sites (Asn-Xaa-Ser/Thr sequons) were predicted using the NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc).

2.11. PCR and RT-PCR

Specific primers were designed using Primer3 (Rozen and Skaletsky, 2000) and evaluated with the NetPrimer software (www.premierbiosoft.com) to flank one intron of genes encoding *P. chlamydosporia* VCP1 (GenBank accession number AJ427460), *H. vulgare* CP-MII (Y09602) and *P. chlamydosporia* SCP1 (cloned in this work). These oligonucleotides were used for PCR amplification from both genomic DNA (gDNA) and RNA reverse-transcribed into cDNA. For endogenous controls, primers were also designed from the β-tubulin coding sequences from *P. chlamydosporia* (AJ012713) and *H. vulgare* (Y09741). Primer sequences were as follows: VCP1–1F (5′-CGCTGGCTCTCTCACTAAGG -3′) and VCP1–2R (5′-TGCCAGTG TCAAGGACGTAG -3′) for the *VCP1* gene, CPM-1F (5′-GGTCGCTC TTCTACCTGCTG-3′) and CPM-2R (5′-ACTTGTAGTGCGGGAACCTCT-3′) for the *CP-MII* gene, VSCP1–1F (5′-TGAGCAGCCCATCAACACT-3′) and VSCP1–1R (5′-CGACAATGGAAGGATCGTAAA-3′) for the *SCP1* gene, HvTUB-1F (5′-GCAGTTCTTATGGACCTTGAGC-3′) and HvTUB-1R (5′-CGAGGGAAGGGGATAAGGT-3′) for the barley β-*tubulin* gene, and VsTUB-1F (5′-GTCGCTACCTGACCTGCTCT-3) and Tub2r (Hirsch et al., 2001) for the fungus β-*tubulin* gene.

Total RNA (2 μg) isolated from barley roots or mycelium was reverse-transcribed into cDNA using the RETROscript kit (Ambion), following the manufacturer's instructions. PCR reactions contained 20–50 ng of template (gDNA or cDNA), forward and reverse primers at 0.5 mM each, the four dNTPs at 0.2 mM each, 2 mM $MgCl_2$, 1 × Green Flexi buffer and 1.25 U of Go*Taq* DNA polymerase (Promega). After an initial denaturation step at 95 °C for 5 min, amplification was performed for 30 cycles each of 57 °C for 1 min, 72 °C for 2 min and 95 °C for 1 min, followed by a final step at 57 °C for 1 min and 72 °C for 5 min. PCR products were electrophoresed on 2% agarose gels stained with SYBR Green I (Sigma), and were verified by automated DNA sequencing in all cases.

3. Results

3.1. Proteolytic activity of barley roots inoculated with nematophagous fungi

Proteolytic activity assayed in root extracts from plants inoculated with nematophagous fungi in Petri dishes was higher than

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

in control, uninoculated plants at 1, 5 and 7 days post-inoculation (Fig. 1a). A strong increase in proteolytic activity was found in inoculated roots at 7 days after inoculation, especially for *P. chlamydosporia* isolates 123 and 144 (Fig. 1a). On the other hand, although proteolytic activity was much higher when plants were grown in culture tubes (albeit with higher variance), no significant differences were found between fungus-inoculated roots and controls (Fig. 1b). Proteolytic activity under these conditions was also found to decrease with time (Fig. 1b).

Zymograms showed qualitative differences between control and fungus-inoculated roots in both Petri dish (data not shown) and culture tube experiments (Fig. 1c). All the root extracts from plants grown in tubes shared a single band of substrate (gelatin) degradation at a molecular weight of ca. 65 kDa (R_f = 0.21; Fig. 1c, lanes 2–5), which was the only protease activity detected in control, uninoculated plants (lane 2). In plants inoculated with nematophagous fungi other bands of proteolytic activity were also visualized (Fig. 1c, lanes 3–5), including a faint 32 kDa band (R_f = 0.37) which migrated at the same position as purified *P. rubescens* serine protease P32 (lane 1).

3.2. Inhibition of root proteolytic activity by anti-P32 IgG

A rabbit polyclonal antiserum was obtained against P32 purified from *P. rubescens*, from which the IgG fraction was purified by protein A-affinity chromatography. These antibodies were able to recognize on immunoblots of fungal culture filtrates, in addition to P32 from *P. rubescens*, the major extracellular protease from *P. chlamydosporia*, named VCP1 (Segers et al., 1996). On immunoblots of *P. chlamydosporia* isolates 123 and 144 and *P. rubescens* these antibodies yielded a band with a molecular weight of 32 kDa, corresponding to VCP1 and P32, respectively (data not shown).

A proteolytic activity assay performed after preincubation of purified P32 protease with the anti-P32 antibodies resulted in a 60% decrease of its enzyme activity. This degree of inhibition was similar to that caused by the serine protease inhibitor, PMSF (data not shown). The proteolytic activity measured in root extracts of barley grown on culture tubes 2 weeks after inoculation with the nematophagous fungi *P. chlamydosporia* 123 or 144, or *P. rubescens*, was decreased when the extracts were pre-incubated with either anti-P32 IgG or PMSF (Fig. 1d). Conversely, inhibition of proteolytic activity of uninoculated roots was only attained upon PMSF treatment (Fig. 1d).

3.3. Protease immunolocalization in root tissue

Sections of barley roots colonized by *P. chlamydosporia* 123 incubated with secondary antibody only showed a weak autofluorescence in both plant cell walls and fungal hyphae (Fig. 2a). This was also true for sections of uninoculated roots and for mycelium incubated without any antibodies (data not shown). When the anti-P32 IgG was used, a strong fluorescence was found in both hyphae (Fig. 2b–d) and appressoria (Fig. 2d), confirming the production of P32-related protease(s) by *P. chlamydosporia* upon colonization of barley root tissues.

3.4. Western blotting and mass spectrometry analyzes

When assayed on root extracts of plants inoculated with *P. chlamydosporia* or *P. rubescens*, anti-P32 antibodies yielded on Wes-

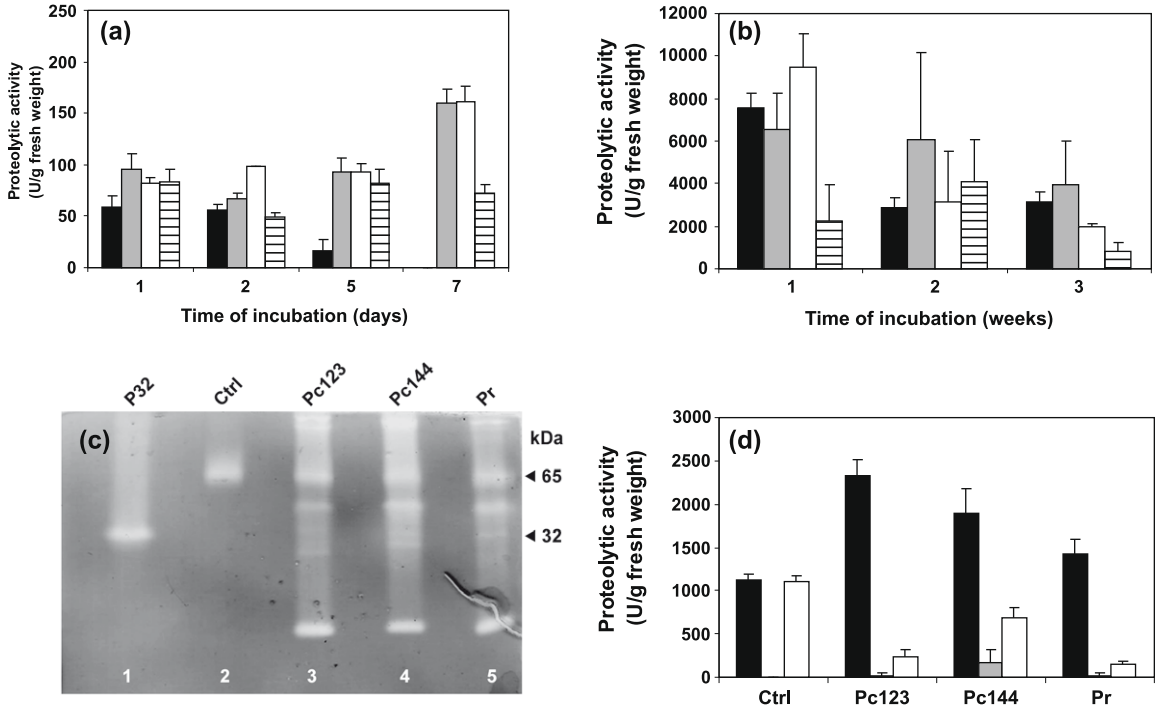

Fig. 1. Proteolytic activity of fungus-colonized barley root extracts. (a) Proteolytic activity of plants grown in Petri dishes for the indicated times post-inoculation (up to 7 days) with the nematophagous fungi *P. chlamydosporia* isolates 123 (grey bars) or 144 (white bars), or *P. rubescens* (stripped bars). Results from uninoculated plants are shown in black bars. (b) As in (a), except that experiments were carried out in culture tubes for up to 3 weeks after inoculation. (c) Zymographic analysis of proteolytic activity 2 weeks post-inoculation in culture tubes. Lanes contained: 1, purified *P. rubescens* P32 (100 ng); 2, uninoculated root extract (0.5 μg of protein); 3–5, root extracts from plants inoculated with *P. chlamydosporia* isolates 123 or 144, or *P. rubescens*, respectively (0.5 μg of protein). Arrowheads point to 65 and 32 kDa proteolytic activity bands. (d) Proteolytic activity of barley roots grown on culture tubes for 2 weeks after inoculation with the indicated fungi, measured in root extracts before (black bars) and after incubation with PMSF (grey bars) or anti-P32 IgG (white bars). Abbreviations: Ctrl, control; Pc123, *P. chlamydosporia* 123; Pc144, *P. chlamydosporia* 144; Pr, *P. rubescens*. Experiments in Fig. 1a and b were carried out in triplicate and experiment in Fig. 1d in duplicate. Bars represent standard deviation values.

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

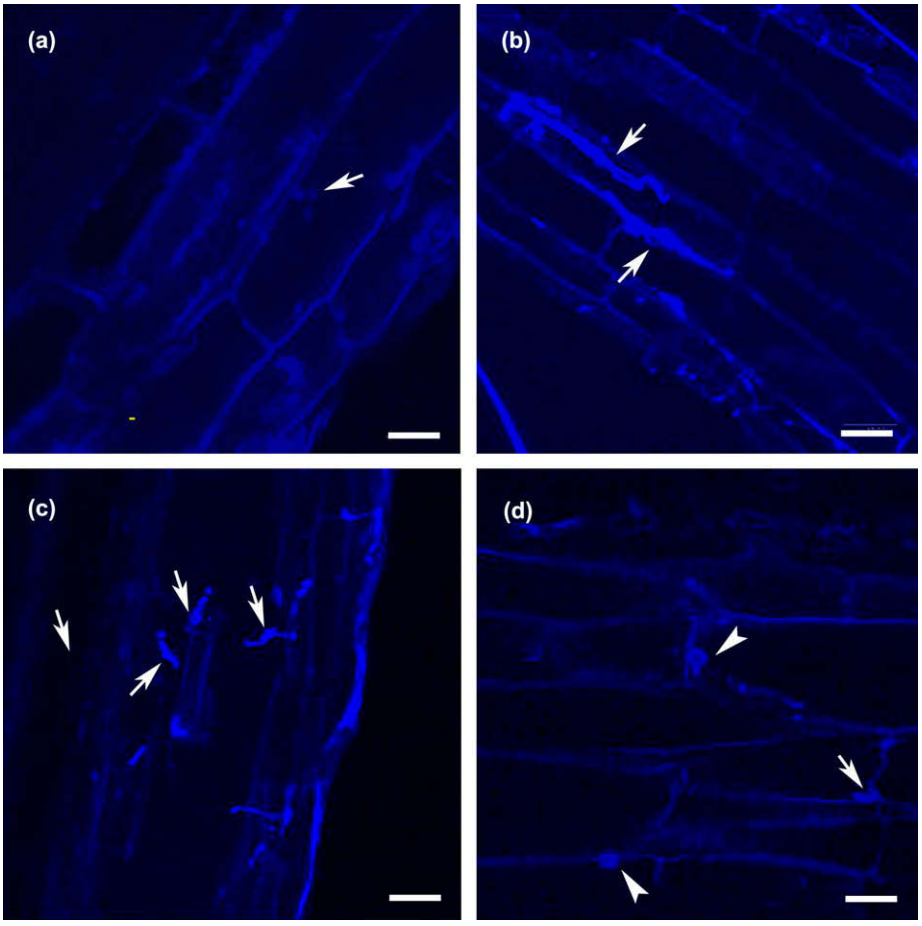

Fig. 2. Fungal protease immunolocalization in barley roots colonized by *P. chlamydosporia*. (a) Root section incubated with secondary antibody only. Note the low autofluorescence from hyphae (arrow) and plant cell walls. (b–d) Root sections were incubated with primary anti-P32 IgG plus secondary antibodies. Sections were obtained after fungus inoculation in Petri dishes (a–c) or culture tubes (d). Intensely-immunolabeled hyphae are pointed by arrows (in b–d), and appressoria by arrowheads (in d). Scale bar, 10 μm.

tern blots a faint protein band with a molecular weight of ca. 32 kDa (Fig. 3, lanes 3–5 and 7) which was absent from uninoculated plants (lanes 2 and 6). This protein migrated at the same position as purified *P. rubescens* P32 (Fig. 3, lane 1). However, an additional, more intense band was detected in the immunoblots corresponding to a protein with an approximate molecular weight of 65 kDa, which was as well only present in fungus-inoculated roots (Fig. 3, lanes 3–5 and 7). In coherence with these results, no absorbance was detected by ELISA in wells containing control root extracts, although a positive signal was obtained when testing fungus-inoculated roots obtained under the same conditions as for Western blotting analysis (data not shown). In these cases, the amount of fungal protease (P32 or VCP1) quantitated by ELISA in inoculated roots ranged between 1.5 and 4.5 μg mg^{-1} total root protein, with the highest values for *P. rubescens*.

The protein bands of 32 and 65 kDa detected by anti-P32 IgG on Western blots were excised from a Coomassie Blue-stained SDS–PAGE gel run in parallel, and after tryptic digestion the resulting peptides were analyzed by MS. A gel slice at the migration position corresponding to 65 kDa from uninoculated roots was also excised and analyzed in the same fashion. The 32 kDa band from roots inoculated with *P. chlamydosporia* 123 yielded upon MS analysis two double-charged peptides, of 14 and 21 amino acids. Upon BLASTP search, these two peptides were found with 100% identity within the 281 amino acid sequence of mature alkaline serine protease (VCP1) from *P. chlamydosporia* (teleomorph *Cordyceps chlamydosporia*), corresponding to amino acid stretches 1–14 and 201–221, respectively, of this protein (Table 1). The N-terminal sequence found is in agreement with post-translational removal of

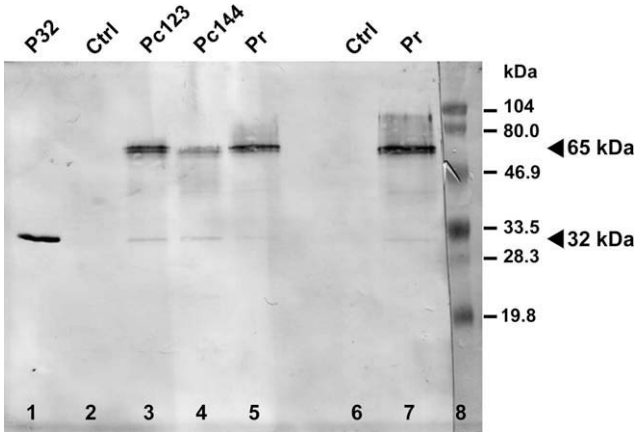

Fig. 3. Western blotting analysis of fungus-colonized barley root proteins with anti-P32 IgG. Lanes contained: 1, purified *P. rubescens* P32 (150 ng); 2 and 6, uninoculated root extract (20 and 40 μg of protein, respectively); 3–5, root extracts from plants inoculated with *P. chlamydosporia* isolates 123 or 144, or *P. rubescens* (20 μg), as indicated; 7, idem *P. rubescens* (40 μg); 8, molecular weight markers. Protein extracts were obtained 2 weeks after inoculation on culture tubes. Arrowheads point to 65 and 32 kDa immunoreactive bands. Abbreviations: Ctrl, control; Pc123, *P. chlamydosporia* 123; Pc144, *P. chlamydosporia* 144; Pr, *P. rubescens*.

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

Table 1
Identification of anti-P32-reactive proteins by HPLC–MS/MS analysis after gel excision and tryptic digestion.

Inoculated fungus[a]	Protein band	Peptide sequence[b]	Identified protein			X_{corr}[c]	z[d]	m/z	Δ mass[e] (ppm)	Retention time (min)
			Name	Species	GenBank accession No.					
Pc123	32 kDa	1-AIVEQQGAPWGLGR-14	Alkaline serine protease	*Cordyceps chlamydosporia*	CAD20581	3.79	+2	741.3964	0.2	27.23
		201-LVDIFAPGSAILSTWINGGTR-221				4.22	+2	1094.5929	1.1	37.35
Pr	32 kDa	27-YDDSAGNGACVYVLDTGIETTHPEFEGR-54	Alkaline serine protease	*Cordyceps chlamydosporia*	CAD20581	5.45	+3	1025.1210	7.1	28.39
		172-YSPASEPSACTVGATTSTDAR-192				3.81	+2	1064.9735	7.6	22.00
		201-LVDIFAPGSAILSTWINGGTR-221				5.91	+2	1094.5907	−0.9	37.91
Pc123	65 kDa	494-ALVLVQNFLAGK-505	Serine carboxy-peptidase II-3	*Hordeum vulgare*	P52711	2.94	+2	636.8868	−0.9	34.21
Pr	65 kDa	382-IWVFSGDTDAVVPLTATR-399	Serine carboxy-peptidase II	*Hordeum vulgare*	P08818	3.70	+2	974.5125	0.1	31.62
None	65 kDa	382-IWVFSGDTDAVVPLTATR-399	Serine carboxy-peptidase II	*Hordeum vulgare*	P08818	3.47	+2	974.5115	−0.9	30.93

[a] Abbreviations: Pc123, *P. chlamydosporia* 123; Pr, *P. rubescens*.
[b] The position of the first and last amino acids of each peptide within the identified protein sequence are indicated.
[c] Probability-based score using the SEQUEST engine for database searches.
[d] Charged state of each peptide.
[e] Peptide mass differences between the measured and theoretical values are indicated in parts per million (ppm).

the first 106 amino acids to yield the secreted form of VCP1, with a molecular weight of 32 kDa (Morton et al., 2003). In roots inoculated with *P. rubescens* the 32 kDa band yielded upon MS three different peptides, which also matched the sequence of *P. chlamydosporia* alkaline serine protease (Table 1). The 65 kDa band from *P. rubescens*-inoculated as well as from uninoculated roots yielded one double-charged peptide that matched the sequence of serine carboxy-peptidase II from *H. vulgare*, named CP-MII (Dal Degan et al., 1994). Likewise, the analogous band from *P. chlamydosporia* 123-inoculated roots yielded a peptide that turned out to correspond to a related barley serine carboxypeptidase, named CP-MII.3 (Table 1).

3.5. Expression of serine protease genes in *P. chlamydosporia*-inoculated barley roots

The expression of the *P. chlamydosporia* extracellular alkaline serine protease VCP1 and of *H. vulgare* serine carboxypeptidase CP-MII was analyzed at the mRNA level by means of RT-PCR using specific primers for their encoding genes. Fig. 4 shows their expression pattern in *H. vulgare* uninoculated roots, colonized by *P. chlamydosporia* 123 and cultured mycelium from this fungus. Using *VCP1* primers, we detected expression of this gene as a 230 bp RT-PCR-amplified band from RNA extracted from *P. chlamydosporia*-colonized roots and mycelium (Fig. 4a). These primers yielded a 290 bp band when used to PCR-amplify gDNA isolated from inoculated roots or mycelium. This difference in size between gDNA and RNA (50 bp) ruled out contamination from gDNA in our RNA samples and corresponded to the absence of the first intron in the *VCP1* mRNA. Since no band was obtained in PCR reactions containing gDNA or reverse-transcribed RNA from uninoculated roots, it was concluded that the signal detected in inoculated barley roots corresponded to the fungus *VCP1* gene. In order to assess the expression of 65 kDa serine carboxypeptidase from *H. vulgare* identified by MS in both *Pochonia*-colonized and control plants (Table 1), we used primers that flanked the first intron (60 bp) of the *CP-MII* gene. PCR amplification yielded the expected 400 bp band from gDNA isolated from inoculated and control roots, but not from fungal DNA (Fig. 4b). Consistently, a 340 bp band was obtained in RT-PCR amplifications from root RNA, but not from mycelium RNA (Fig. 4b), which corroborated that *CP-MII* is a plant-specific gene.

3.6. Cloning and expression of the *P. chlamydosporia* serine carboxypeptidase *SCP1* gene in colonized barley roots

No recognition of the 65 kDa band by anti-P32 antibodies was attained in Western blots from barley uncolonized roots. Thus, we hypothesized that such protein could be of fungal origin. In this light, the identification by MS of serine carboxypeptidases from plant (barley) origin in fungus-containing and control extracts (Table 1) could be due to their overrepresentation in our samples and in protein databases. Conversely, the misrepresentation of *Pochonia* spp. sequences, for which to our knowledge no proteomics, EST or massive sequencing data are available, should hinder their identification by MS. Therefore, we sought to clone a gene encoding a putative ca. 65 kDa serine carboxypeptidase from *P. chlamydosporia* expressed in root extracts from barley inoculated with the fungus. A 1249 bp PCR product was amplified from this fungus gDNA by using a pair of degenerate primers whose sequence was derived from the serine carboxypeptidase-encoding mRNAs from six distinct filamentous fungi, with molecular weights in the range of 53–66 kDa, available at GenBank (see Section 2). Its sequencing and further comparative analysis revealed that the *P. chlamydosporia* sequence exhibited 77% and 74% homologies with *N. crassa* (*cpdS*) and *Chaetomium globosum* mRNAs encoding serine carboxypeptidases (XM_955807 and XM_001224845), respectively, and was submitted to GenBank under accession number GQ355960. This gene sequence, termed *SCP1* (from Serine Carboxy Peptidase-1), encompassed a predicted partial ORF and two introns (bases 132–214 and 490–549, both starting with GT and ending with AG) intervening the ORF. This encoded a polypeptide sequence of 368 amino acids which displayed a 64.2% identity (84.6% similarity) with *N. crassa* CpdS, a 60.4% identity (83.8% similarity) with *C. globosum* serine carboxypeptidase, and a 56.5% identity (81.4% similarity) with *M. grisea* serine carboxypeptidase. The *P. chlamydosporia* SCP1 partial sequence (Fig. 5) contained a serine residue in position 133 as part of the stretch GESYAG, which has been defined as exclusive of the serine carboxypeptidase S10 family of peptidases (Rawlings et al.,

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

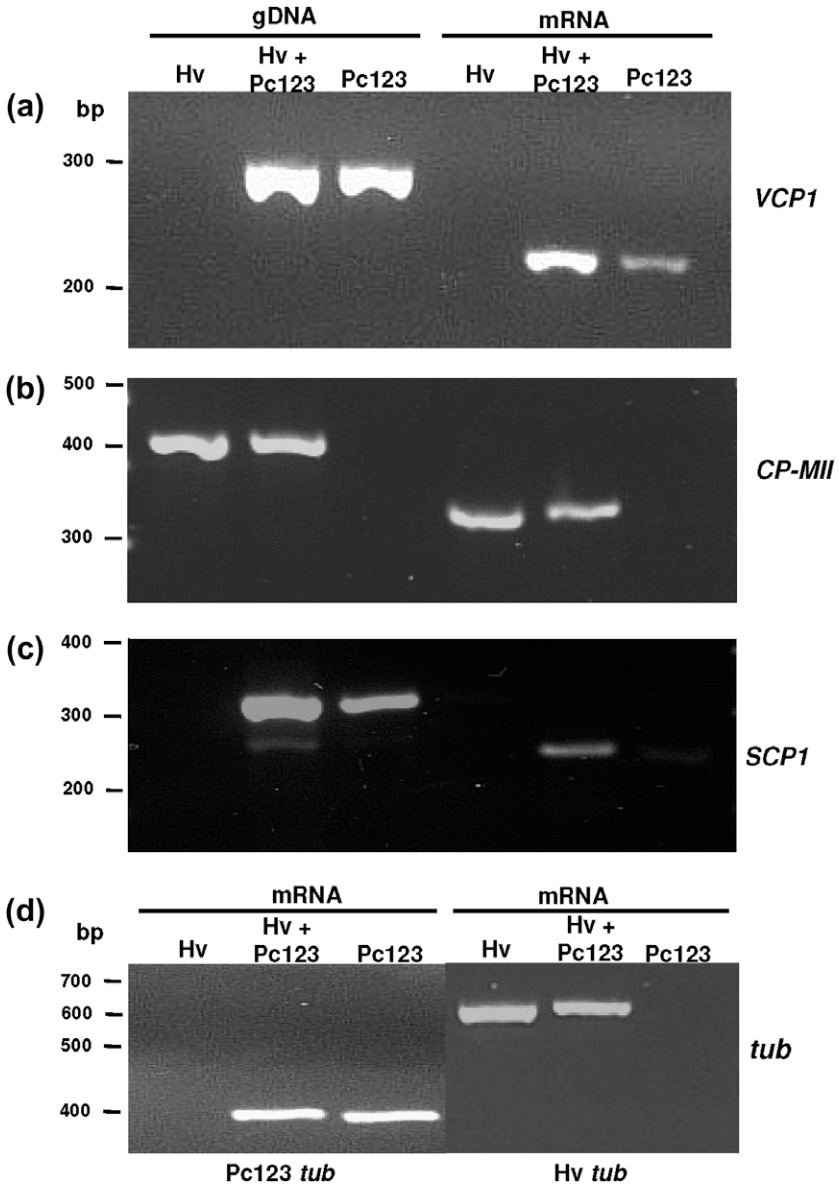

Fig. 4. PCR detection of serine carboxypeptidase genes. PCR amplifications were carried out from genomic DNA (gDNA) or RNA reverse-transcribed into cDNA (mRNA) isolated from uninoculated barley roots (Hv), *P. chlamydosporia* 123-colonized barley roots for 2 weeks in growth tubes (Hv + Pc123), or *P. chlamydosporia* 123 mycelium grown for 8 days in liquid protease induction medium (Pc123). (a) Amplification of the Pc123 *VCP1* gene or mRNA. (b) Amplification of the Hv *CP-MII* gene or mRNA. (c) Amplification of the Pc123 *SCP1* gene or mRNA. (d) Control amplification of the *tub* (β-tubulin) mRNA using primers specific for the Pc123 or the Hv gene.

2008). Such serine residue together with an aspartic acid and a histidine residue conform the catalytic triad of proteins belonging to such family. The aspartic residue was found in the *P. chlamydosporia* SCP1 segment in position 351 (Fig. 5), while the histidine residue was not present in our partial gene-derived sequence. Four predicted N-glycosylation sites were also found in this polypeptide (Fig. 5).

We then analyzed the expression of the *P. chlamydosporia SCP1* gene using primers designed from the newly cloned sequence. As shown in Fig. 4c, bands of 300 and 240 bp were obtained when using as template gDNA and reverse-transcribed RNA, respectively, for PCR amplification. This difference in size corresponded to the 60-bp intron intervening the *SCP1* ORF, as expected. These bands were specific for the fungus, and their detection in inoculated roots but not in control plants indicated that, as it was the case for *VCP1*, they pertained to the root-colonizing *P. chlamydosporia*.

3.7. Sequence homology among proteases from Pochonia spp

Since the gene sequence encoding the P32 serine protease from *P. rubescens* is not yet available, *de novo* sequencing of the purified protein by MS was performed. Eight different peptides were sequenced and identified, accounting for a total of 136 amino acids of protein sequence (Table 2). In order to further validate the sequences bioinformatically obtained using the Peaks algorithm, the spectra were also manually validated and sequences searched against the NCBInr database using the BLASTP software. The sequences of these peptides were concluded to match homologous fungal peptidases from two species of the *Clavicipitaceae* family, namely *Metarhizium anisopliae* and *P. chlamydosporia* (Table 2).

A sequence alignment of the serine proteases from *Pochonia* spp. expressed in barley roots is shown in Fig. 5. From this alignment, data from *de novo* sequencing of P32 when compared to

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

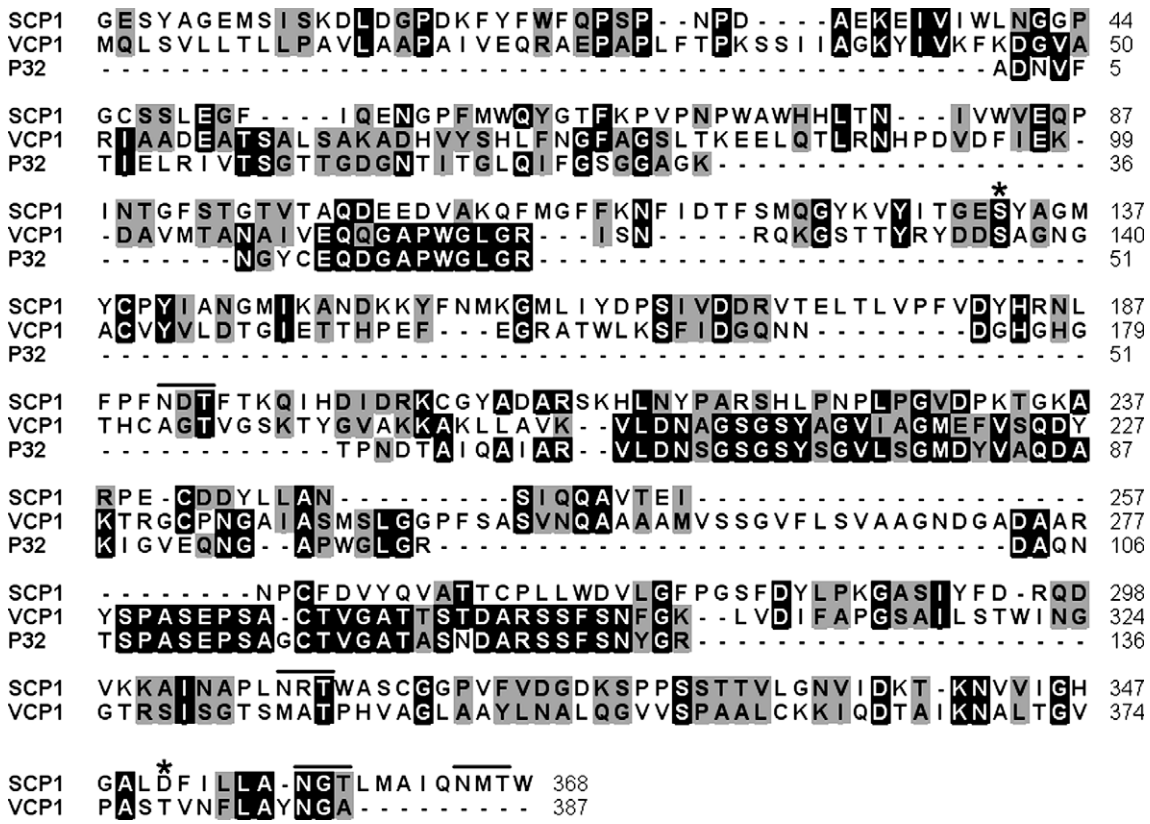

Fig. 5. Alignment of amino acid sequences of *P. chlamydosporia* SCP1 (encoding gene cloned in this work), *P. chlamydosporia* VCP1 (GenBank CAD20584) and *P. rubescens* P32 (peptides derived from *de novo* sequencing in this work). Residues conserved in at least two of the three sequences are highlighted in black, and conservative amino acid differences (PAM250 matrix) in gray. Catalytic serine and histidine residues in the SCP1 sequence (inferred from alignment with *N. crassa* CpdS) are asterisked. Predicted N-glycosylation sites in SCP1 are overlined.

Table 2
De novo sequencing of tryptic peptides from *P. rubescens* serine protease P32.

No.	Peptide sequence[a]	*m/z*	*z*	Peaks score[b]	Fungus[c]			
					Metarhizium anisopliae		*Pochonia chlamydosporia*	
					BLASTP score	*E* value	BLASTP score	*E* value
1	V(L/I)DNSGSGSYSGVLSGMDYVAQDAK	1260.5809	+2	100	66.4	3×10^{-10}	54.1	2×10^{-6}
2	NGYCEQDGAPWG(L/I)GR	541.5711	+3	98	36.3	0.38	34.6	1.2
3	ADNVFT(L/I)ELR	589.3141	+2	94	35.4	0.70	n.f.	n.f.
4	SSFSNYGR	549.2094	+2	93	28.2	82	n.f.	n.f.
5	DAQNTSPASEPSAGCTVGATASNDAR	1239.5441	+2	90	67.2	2×10^{-10}	50.7	2×10^{-5}
6	(L/I)GVEQNGAPWG(L/I)GR	727.3813	+2	83	38.8	0.066	37.1	0.21
7	(L/I)VTSGTTGDGNT(L/I)TG(L/I)Q(L/I)FGSGGAGK	1205.1177	+2	81	59.6	3×10^{-8}	n.f.	n.f.
8	TPNDTA(L/I)QA(L/I)AR	635.8410	+2	74	30.3	24	n.f.	n.f.

[a] Peptide sequences obtained by *de novo* sequencing after the analysis of tryptic peptides by HPLC–MS/MS, ordered according to their Peaks scores. The impossibility to distinguish between leucine (L) and isoleucine (I) isobaric residues is indicated as (L/I).
[b] Probabilistic scores using the Peaks *de novo* sequencing algorithm. The default peaks score for acceptance as confident peptide sequence was $\geqslant 65$.
[c] BLASTP scores and *E* values for each peptide from comparison with serine protease PR1A or carboxypeptidase from *M. anisopliae*, and with VCP1 from *P. chlamydosporia*. n.f., not found.

the VCP1 sequence deposited in GenBank revealed a 48.6% identity (66.7% similarity). Comparison between partial sequence data available for P32 and SCP1 revealed a 10.9% identity (26.1% similarity). A similar analysis between sequence data generated in this work for SCP1 and complete VCP1 revealed a 13.3% identity (34.8% similarity). Despite this low homology, three cysteine residues (at positions 139, 241 and 260) were found to be conserved between SCP1 and VCP1, one of them (260) also matching the partial P32 sequence (Fig. 5).

4. Discussion

This is the first report on the detection of proteases from nematophagous fungi in roots colonized endophytically by these organisms. In fact, the main proteases of the fungi used in this study are putative pathogenic determinants, since they are synthesized in appressoria of these pathogens upon nematode egg infection (Lopez-Llorca and Robertson, 1992). Alone (Segers et al., 1996), or in combination with chitinases (Tikhonov et al., 2002),

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

they damage the nematode egg shell. P32, the main serine protease of the nematophagous fungus *P. rubescens*, also degrades nematode egg shell proteins (Lopez-Llorca, 1990). In this investigation we have developed and purified polyclonal antibodies towards P32. These antibodies had catalytic activity, i.e. reduced by more than 50% the proteolytic activity of purified P32. Consequently, anti-P32 antibodies largely (60–90%) inhibited the proteolytic activity present in extracts from fungus-inoculated roots. Conversely, they had no effect on proteolytic activity of control (uninoculated) barley roots. This strongly suggests that our anti-P32 antibodies are fungus-specific. A further proof for their fungal specificity is that they did not recognize plant proteins in immunoblots of extracts from uninoculated roots. This would in turn confirm root colonization by these nematophagous fungi upon their inoculation. This has previously shown microscopically (Bordallo et al., 2002; Lopez-Llorca et al., 2002a; Maciá-Vicente et al., 2009a), by culturing (Bourne et al., 1996; Maciá-Vicente et al., 2009b; Monfort et al., 2005) and by using qPCR (Maciá-Vicente et al., 2009a). The antibodies revealed, in extracts of roots inoculated with either *P. chlamydosporia* or *P. rubescens*, a protein band of similar molecular weight (ca. 32 kDa) to that of VCP1 or P32, the main and similar serine proteases of these nematophagous fungi. MS also detected peptides with homology to VCP1, an alkaline serine protease from *P. chlamydosporia*, in the zone (ca. 32 kDa) recognized by the antibody in electrophoresis gels containing extracts from roots colonized with either *P. chlamydosporia* or *P. rubescens*. This confirms the presence of both fungal proteases in the roots colonized by either of the two fungi.

Anti-P32 antibodies also revealed, yielding a strong signal on immunoblots, a fungal protein band of higher molecular weight (ca. 65 kDa) in extracts of roots inoculated with either fungus. Zymograms from colonized roots showed proteolytic activity at this molecular weight, indicating that the ca. 65 kDa protein is a protease. However, zymograms also revealed that extracts from uninoculated roots (control) contained as well a ca. 65 kDa protease. Nevertheless, the extracts from fungus-colonized roots did contain a 65 kDa protease of fungal origin, since its labeling with the anti-P32 specific antibody was never found in extracts from uninoculated control roots. However, this protease happened to colocalize in the gels with a plant protease of similar (ca. 65 kDa) molecular weight. This is perhaps the reason why MS only detected barley proteases (namely serine carboxypeptidases CP-MII and CP-MII.3) (Dal Degan et al., 1994) in extracts from both fungus-inoculated and uninoculated roots. Besides, the abundance in databases of sequences from proteases of plant origin would make difficult the MS detection of peptides with homology to fungal proteases. We therefore tested the presence of a serine carboxypeptidase of putative fungal origin. A bioinformatic search revealed the availability of serine carboxypeptidases from six fungus species with a molecular weight close to 65 kDa. Primers based on a consensus sequence were thus designed which allowed the amplification by PCR from *P. chlamydosporia* gDNA of a partial gene sequence showing (upon translation) homology with fungal serine carboxypeptidases. Furthermore, RT-PCR with the designed primers detected expression of this novel putative carboxypeptidase from *P. chlamydosporia*, named SCP1, only in extracts from roots inoculated with the fungus and not in uninoculated (control) roots. Sequence comparison was performed between VCP1, the new serine carboxypeptidase SCP1 and P32. Since for the latter no gene sequence was available, *de novo* sequencing of the purified protein by MS was carried out, this yielding eight peptides spanning approximately 35–40% of the total protein sequence (Fig. 5). It turned out that P32 and VCP1 were significantly homologous, as expected from the fact they belong to the same, S8 family of serine endopeptidases (subtilisin family) defined by Rawlings et al. (2008). As for comparison between SCP1 and P32 or VCP1, despite low homology encountered due to SCP1 belonging to the peptidase family S10 (carboxypeptidases), common epitopes could be formally found among the three proteases. If the ca. 65 kDa protein from extracts of inoculated roots was SCP1, this would explain the fact that anti-P32 antibodies would recognize *Pochonia* spp. serine carboxypeptidases. Also, out of the four cysteine residues conserved in VCP1 (Morton et al., 2003), three were found to be present at analogous positions in SCP1.

Summarizing, we have shown by proteolytic activity assays, Western blotting, MS peptide identification, RT-PCR and ELISA that nematophagous fungi colonizing endophytically roots express some of the proteases they produce on their nematode hosts. Therefore, nematophagous fungi express in roots important pathogenicity determinants even in the absence of their nematode hosts. This would imply that plants colonized endophytically by *Pochonia* spp. may be, to some extent, protected from nematode attack. Experiments pursuing confirmation of this hypothesis are in progress in our laboratory.

We have so far no data on the possible relationship between fungus root colonization and fungal proteolytic activity. This would require further studies. The detection of a fungal protease (or its gene expression) involved in pathogenicity to nematodes in order to monitor nematophagous fungi in roots has the advantage of scoring mycelia metabolically active. This has been difficult to achieve with other detection methods based on nucleic acid probes (Maciá-Vicente et al., 2009a; Mauchline et al., 2002).

The anti-P32 antibody labeling showed that P32-related proteases are localized not only in the appressoria of the fungi, but also along the hyphae. This is similar to what was found for P32 in *P. rubescens* infecting plant-parasitic nematode eggs (Lopez-Llorca and Robertson, 1992). The fact that the nematode host is not needed for the synthesis of these fungal proteases agrees with the fact that constitutive basal production of several proteases (including P32) by *Pochonia* spp. takes place during pre-penetration events (Lopez-Llorca et al., 2002b).

We do not know the effect of the proteases from nematophagous fungi on root cells and on plant proteases such as the ones detected in this work. To this respect, plant proteases are known to be involved both in plant development (Schaller, 2004) and defence (Liu et al., 2008). From previous work we have evidence of induction of plant defences, at both morphological (papillae and vacuoles) and histochemical levels in roots colonized by *P. chlamydosporia* (Bordallo et al., 2002). These responses could account for localized hyphal death of the fungus growing endophytically in barley roots (Maciá-Vicente et al., 2009a). The presence of proteases, and perhaps chitinases, from nematophagous fungi in the root could also have an important role in plant defence against nematodes and other root pathogens. These important aspects deserve further investigation.

Acknowledgments

The authors wish to thank Dr. Vladimir Tikhonov for the purification of *P. rubescens* protease P32, Ms. Gema C. Martínez-Navarrete for help with confocal microscopy and Ms. Nuria Escudero for technical support. This research was funded by the Spanish Ministry of Science and Innovation Grant AGL2008-00716/AGR.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Grapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), 2002. Short Protocols in Molecular Biology. John Wiley and Sons, New York, pp. 21–28.

Bordallo, J.J., Lopez-Llorca, L.V., Jansson, H.B., Salinas, J., Persmark, L., Asensio, L., 2002. Colonization of plant roots by egg-parasitic and nematode-trapping fungi. New Phytol. 154, 491–499.

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004

Bourne, J.M., Kerry, B.R., De Leij, F.A.A.M., 1996. The importance of the host plant on the interaction between root-knot nematodes *Meloidogyne* spp. and the nematophagous fungus, *Verticillium chlamydosporium* Goddard. Biocontrol Sci. Technol. 6, 539–548.

Casado-Vela, J., Ruiz, E.J., Nebreda, A., Casal, I., 2007. A combination of neutral loss and targeted product ion scanning with two enzymatic digestions facilitates the comprehensive mapping of phosphorylation sites. Proteomics 7, 2522–2529.

Dal Degan, F., Rocher, A., Cameron-Mills, V., von Wettstein, D., 1994. The expression of serine carboxypeptidases during maturation and germination of the barley grain. Proc. Natl. Acad. Sci. USA 91, 8209–8213.

De Leij, F.A.A.M., Kerry, B.R., 1991. The nematophagous fungus *Verticillium chlamydosporium* Goddard, as a potential biological control agent for *Meloidogyne arenaria* (Neal) Chitwood. Revue de Nématologie 14, 157–164.

De Leij, F.A.A.M., Kerry, B.R., Dennehy, J.A., 1993. *Verticillium chlamydosporium* as a biocontrol agent for *Meloidogyne incognita* and *M. hapla* in pot and microplot tests. Nematologica 39, 115–126.

Eng, J.K., McCormack, A.L., Yates, J.R.I.I.I., 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spec. 5, 976–989.

Hirsch, P.R., Atkins, S.D., Mauchline, T.H., Morton, C.O., Davies, K.G., Kerry, B.R., 2001. Methods for studying the nematophagous fungus *Verticillium chlamydosporium* in the root environment. Plant Soil 232, 21–30.

Jansson, H.-B., Lopez-Llorca, L.V., 2004. Control of nematodes by fungi. In: Arora, D.P. (Ed.), Fungal Biotechnology in Agricultural, Food, and Environmental Applications. Marcel Dekker, New York, pp. 205–215.

Kerry, B.R., Bourne, J.M., 1996. Importance of rhizosphere interactions in the biological control of plant-parasitic nematodes – a case of study using *Verticillium chlamydosporium*. Pest. Sci. 47, 69–75.

Kerry, B.R., Simon, A., Rovira, D., 1984. Observations on the introduction of *Verticillium chlamydosporium* and other parasitic fungi into soil for control of the cereal cyst-nematode *Heterodera avenae*. Annu. Appl. Biol. 105, 509–516.

Liu, H., Wang, X., Zhang, H., Yang, Y., Ge, X., Song, F., 2008. A rice serine carboxypeptidase-like gene *OsBISCPL1* is involved in regulation of defense responses against biotic and oxidative stress. Gene 420, 57–65.

Lopez-Llorca, L.V., 1990. Purification and properties of extracellular proteases produced by the nematophagous fungus *Verticillium suchlasporium*. Can. J. Microbiol. 36, 530–537.

Lopez-Llorca, L.V., Robertson, W.M., 1992. Immunocytochemical localization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. Exp. Mycol. 16, 261–267.

Lopez-Llorca, L.V., Bordallo, J.J., Salinas, J., Monfort, E., López-Serna, M.L., 2002a. Use of light and scanning electron microscopy to examine colonisation of barley rhizosphere by the nematophagous fungus *Verticillium chlamydosporium*. Micron 33, 61–67.

Lopez-Llorca, L.V., Olivares-Bernabeu, C.M., Salinas, J., Jansson, H.-B., Kolattukudy, P.E., 2002b. Pre-penetration events in fungal parasitism of nematode eggs. Mycol. Res. 106, 499–506.

Lopez-Llorca, L.V., Jansson, H.-B., Vicente, J.G.M., Salinas, J., 2006. Nematophagous fungi as root endophytes. In: Schulz, B., Boyle, C., Sieber, T. (Eds.), Microbial Root Endophytes. Springer-Verlag, Berlin, pp. 191–206.

Lopez-Llorca, L.V., Maciá-Vicente, J.G., Jansson, H.-B., 2008. Mode of action and interactions of nematophagous fungi. In: Ciancio, A., Mukerji, K.G. (Eds.), Integrated Management and Biocontrol of Vegetable and Grain Crops Nematodes. Springer, Dordrecht, pp. 51–76.

Maciá-Vicente, J.G., Jansson, H.-B., Talbot, N.J., Lopez-Llorca, L.V., 2009a. Real-time PCR quantification and live-cell imaging of endophytic colonization of barley (*Hordeum vulgare*) roots by *Fusarium equiseti* and *Pochonia chlamydosporia*. New Phytol. 182, 213–228.

Maciá-Vicente, J.G., Rosso, L.C., Ciancio, A., Jansson, H.-B., Lopez-Llorca, L.V., 2009b. Colonisation of barley roots by endophytic *Fusarium equiseti* and *Pochonia chlamydosporia*: effects on plant growth and disease. Annu. Appl. Biol. 155, 391–401.

Mauchline, T.H., Kerry, B.R., Hirsch, P.R., 2002. Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. Appl. Environ. Microbiol. 68, 1846–1853.

Monfort, E., Lopez-Llorca, L.V., Jansson, H.-B., Salinas, J., Park, J.-O., Sivasithamparam, K., 2005. Colonisation of seminal roots of wheat and barley by egg-parasitic nematophagous fungi and their effects on *Gaeumannomyces graminis* var. tritici and development of root-rot. Soil Biol. Biochem. 37, 1229–1235.

Morton, C.O., Hirsch, P.R., Peberdy, J.F., Kerry, B.R., 2003. Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. Mycol. Res. 107, 38–46.

O'Donnell, K., Cigelnik, E., Nirenberg, H., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.

Olsen, J.V., Macek, B., Lange, O., Makarov, A., Horning, S., Mann, M., 2007. Higher-energy C-trap dissociation for peptide modification analysis. Nat. Methods 4, 709–712.

Ouchterlony, O., Wilson, L.A., 1968, second ed.. In: Weir, D.M. (Ed.), Handbook of Experimental Immunology, vol. I Blackwell Science, Oxford, pp. 19.1–19.39.

Rawlings, N.D., Morton, F.R., Kok, C.Y., Kong, J., Barrett, A.J., 2008. MEROPS: the peptidase database. Nucleic Acids Res., 36. Database issue D320–D325.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Misener, S., Krawetz, S.A. (Eds.), Bioinformatics Methods and Protocols. Humana Press, Totowa, pp. 365–386.

Schaller, A., 2004. A cut above the rest: the regulatory function of plant proteases. Planta 220, 183–197.

Segers, R., Butt, T.M., Keen, J.N., Kerry, B.R., Peberdy, J.F., 1995. The subtilisins of the invertebrate mycopathogens *Verticillium chlamydosporium* and *Metarhizium anisopliae* are serologically and functionally related. FEMS Microbiol. Lett. 126, 227–231.

Segers, R., Butt, T.M., Keen, J.N., Kerry, B.R., Beckett, A., Peberdy, J.F., 1996. The role of the proteinase VcP1 produced by the nematophagous fungus *Verticillium chlamydosporium* in the infection process of nematode eggs. Mycol. Res. 100, 421–428.

Stirling, G.R., 1991. Biological Control of Plant Parasitic Nematodes. CAB International, Wallinford.

Tikhonov, V.E., Lopez-Llorca, L.V., Salinas, J., Jansson, H.-B., 2002. Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fung. Genet. Biol. 35, 67–78.

Please cite this article in press as: Lopez-Llorca, L.V., et al. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genet. Biol. (2010), doi:10.1016/j.fgb.2010.01.004