RESEARCH LETTER

Cloning and heterologous expression of aspartic protease SA76 related to biocontrol in *Trichoderma harzianum*

Yan Liu & Qian Yang

Department of Life Science and Engineering, Harbin Institute of Technology, Harbin, China

Correspondence: Qian Yang, Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China. Tel.: +86 0451 86402652; fax: +86 0451 864 12952; e-mail: liuyanyan626@163.com

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Abstract

Trichoderma harzianum is a soil-borne filamentous fungus that exhibits biological control properties because it parasitizes a large variety of phytopathogenic fungi. The production of hydrolytic enzymes appears to be a key element in the parasitic process. Among the enzymes released by *Trichoderma*, the aspartic proteases play a major role. A gene (SA76) encoding an aspartic protease was cloned by 3′ rapid amplification of cDNA ends from *T. harzianum* T88. The coding region of the gene is 1593 bp long, encoding a polypeptide of 530 amino acids with a predicted molecular mass 55 kDa and a pI of 4.5. The catalytic aspartic residues characteristic of aspartic proteases are conserved with an active-site motif (DSG); however, the DSG in the N-terminal lobe is unusual in that Ser replaced Thr. Northern blot analysis indicated that SA76 was induced in response to different fungal cell walls. Aspartic protease SA76 was expressed in *Saccharomyces cerevisiae* under control of the GAL1 promoter. The enzyme activity culminates (10.5 U mL^{-1}) 72 h after induction with galactose. The temperature optimum of the enzyme was 45 °C and its pH optimum was 3.5. The culture supernatant of the *S. cerevisiae* strain that expressed the aspartic protease SA76 was able to inhibit the growth of five phytopathogenic fungi. The inhibition of mycelial growth varied between 7% and 38%.

Introduction

Biological control of soil-borne plant pathogens by antagonistic microorganisms is a potential nonchemical means of plant disease control. *Trichoderma harzianum* is an active mycoparasite and can be used as a biocontrol agent. This filamentous fungus is ubiquitous in the soil environment and parasitizes a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Botritis cinerea*. It appears that the main mechanism involved in the antagonism to pathogenic fungi by *T. harzianum* is the release of lytic enzymes, mainly chitinases, glucanases, and proteases, released in the presence of a suitable host (Chet & Chernin, 2002).

In comparison with chitinases and glucanases, little is known about the proteases secreted by *Trichoderma* strains, despite the fact that it also plays a significant role in the biocontrol ability. A basic protease of 31 kDa PBR1 from *T. harzianum* (Geremia *et al.*, 1993), probably equivalent to the basic protease detected in *T. harzianum* CECT 2413 (Delgado-Jarana *et al.*, 2000), was identified as a subtilisin that was induced by fungal cell walls and played a fundamental role in mycoparasitism against phytopathogenic fungi and biocontrol of root-knot nematodes (Sharon *et al.*, 2001).

The study of the components of the proteolytic system of *Trichoderma* spp. and their contribution to biocontrol has been receiving increasing attention. Recently, it has been found that *T. harzianum* mutants showing improved antagonistic properties had higher secretion of proteolytic enzymes (Szekeres *et al.*, 2004). Elicitation of plant defense response by an 18 kDa -protein from *Trichoderma virens* with similarity to serine proteases has also been described recently (Hanson & Howell, 2004). The gene prb1 encoding a subtilisin-like protease involved in mycoparasitism has been isolated and characterized from *Trichoderma atroviride* IMI 206040 (Flores *et al.*, 1997; Cortes *et al.*, 1998; Olmedo-Monfil *et al.*, 2002). A homologous gene, tvsp1, has also been studied in *T. virens* Gv29.8 (Pozo *et al.*, 2004).

Aspartic proteases are one of the four main classes of proteinases, the others being serine, cysteine, and metallo

proteinases (Barrett, 1998). Their role in the biocontrol activity of diverse fungal agents has also been demonstrated. Different aspartic proteases had been detected and/or purified from several *Trichoderma* species (Delgado-Jarana *et al.*, 2000; Dunaevsky *et al.*, 2000; De Marco & Felix, 2002), and some of the corresponding genes had been cloned. Genes corresponding to one aspartic protease (PAPA), detected in casein-supplemented media, have been cloned from *T. harzianum* CECT 2413 (Delgado-Jarana *et al.*, 2002). PapA and a vacuolar aspartic protease-encoding gene (papB) have been isolated from *Trichoderma asperellum* T-203 and related to mycoparasitic and plant root colonization activities (Viterbo *et al.*, 2004). Three novel proteases related to the biocontrol response in *T. atroviride* growing on the cell walls of *R. solani* have been recently identified by a proteomic approach (Grinyer *et al.*, 2005). Finally, a novel aspartic protease has been identified as a cell wall-degrading enzyme involved in biocontrol activities (Suárez *et al.*, 2005).

In this paper, the cloning, sequence analyses and heterologous expression are described of the aspartic protease gene of *T. harzianum* in a laboratory strain of *Saccharomyces cerevisiae*. The temperature stability and pH dependence for activity are among the features explored in the study of this protease. The expression pattern of SA76 in *T. harzianum* was analyzed in the presence of different fungal cell walls. The antifungal activity of this protease was assessed *in vitro* against five phytopathogenic fungi. This is, to the authors' knowledge, the first time that *T. harzianum* aspartic protease was functionally expressed in a heterologous host, and it was important for fungal development and biocontrol applications. This study is one of the many attempts to search for more environmentally and toxicologically safe efficacious fungicides.

Materials and methods

Cultivation conditions, strains, and plasmids

Trichoderma harzianum strain T88 was used. The phytopathogenic fungi tested included *Phytophthora sojae, Fusarium oxysporum, R. solani, Sclerotinia sclerotiorum* and *Valsa sordida Nit*. Fungal strains were routinely maintained on potato dextrose agar (PDA; Difco, Detroit, MI) at 28 °C. An area of 2–3 cm^2 of mycelial growth of *T. harzianum* 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 r.p.m. *Saccharomyces cerevisiae* H158 was used as the host and was grown in YPD broth medium (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, glucose). For expression studies, it was grown on SC-U (0.67% YNB, 0.01% adenine, arginine, lyseine, leucine, threonine, tryptophan, 0.005% aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, valine, tyrosine) (Adams *et al.*, 1998). The pYES2 vector (Novagen) was used for the expression of protease in *Saccharomyces cerevisiae*.

Isolation of full-length SA76 cDNA using 3′ rapid amplification of cDNA ends (RACE)

For total RNA isolation, mycelia of *T. harzianum* were collected by filtration, thoroughly washed with sterile water, lyophilized, and maintained kept at −80 °C until RNA extraction. Total RNA was isolated using a Yeast RNA mini kit (Watson Biotechnologies, China). The unidirectional cDNA library from *T. harzianum* mycelium and 3298 expressed sequence tag (EST) were acquired after sequencing (Pi-gang & Qian, 2005). Sequences were aligned using the basic local alignment search tool (BLAST), and the final sequences were searched against the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were compared with the nonredundant protein database using BLASTX and default parameters. Sequences with no significant similarity with sequences in the protein database were compared with the nucleotide database using BLASTN.

A total of 3′ RACE were performed using this library to obtain the sequence of the full-length SA76 cDNA. The 3′ ends of the transcripts were amplified by the BD SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, TaKaRa.). The design of the gene specific primers for 3′ RACE was based on the sequence of aspartic protease EST. The 5′ gene-specific primer for 3′ RACE was a 27-mer with the sequence 5′-CCAACCGTACAGCTTGCTGCTCAACAC-3′. Following 3′ RACE with the 5′ gene-specific primer and the CDS adaptor primer, the complete 3′ ends of the SA76 cDNA were generated. Nucleotide sequencing of these products was performed.

From the sequence information, the forward primer, 5′-*CCCAAGCTTTCTTCCGTCTCTTTGTTCCTCTTTC*-3′, and the reverse primer, 5′-*CCGGAATTCATGGTCCTCTCG CTCGTCAATATAT*-3′, were designed and used to PCR amplify a continuous clone representing the entire coding region of the SA76 cDNA. The underlined regions signify HindIII and EcoRI restriction sites, respectively. 'Touchdown' PCR cycling conditions were 94 °C for 30 s, 72 °C for 3 min, six cycles; 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min, six cycles; and 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min, 25 cycles.

Protease expression

To study the expression of SA76 in submerged cultures, mineral medium (MM) (Fleet & Phaff, 1974) with 2% glucose and 0.5% ammonium sulfate was inoculated with

1×10^6 conidia mL^{-1} and incubated in a rotary shaker at 200 r.p.m. for 48 h at 28 °C. Mycelium was collected, washed with distilled water and 2% $MgCl_2$, and transferred to fresh MM and a variable carbon source: 1% glucose, 1% chitin (Sigma), 1% *P. sojae* cell walls, 1% *F. oxysporum* cell walls, 1% *R. solani* cell walls, 1% *Sclerotinia sclerotiorum* cell walls, or 1% *V. sordida Nit.* cell walls. Nitrogen starvation condition was 10% of the nitrogen concentration in MM and 1% glucose as the carbon source. Fungal cell walls used as a carbon source for protease-inducing conditions were prepared according to Fleet and Phaff (Penttilä *et al.*, 1987).

Transformation of *Saccharomyces cerevisiae* H158 with the SA76 gene

In order to express the SA76 gene in *Saccharomyces cerevisiae*, its coding region was amplified by PCR. The amplified fragment was digested with HindIII and EcoRI, and then ligated into HindIII and EcoRI sites downstream of the GAL1 promoter of pYES2, using *Escherichia coli* DH5α-competent cells. The resulting plasmid (pYES2/SA76) was characterized by restriction analysis. The pYES2/SA76 plasmid and control (pYES2) were transformed into *Saccharomyces cerevisiae* H158 by the lithium acetate method, as described by Krautwurst *et al.* (1998).

Expression of recombinant *T. harzianum* SA76 gene

The transformed *Saccharomyces cerevisiae* H158 cells harboring the expression plasmid pYES2/SA76 were grown on minimal medium SC-U. In *Saccharomyces cerevisiae*, the expression of pYES2/SA76 was induced by the addition of 2% galactose and repressed by glucose. Isolated colonies were used to inoculate 200 mL minimal medium plus 10 mg L^{-1} adenine and 2% raffinose and were grown for 24 h at 30 °C. These cells were then used to inoculate 50 mL SC-U medium containing 2% galactose and were grown for 108 h at 30 °C. The suspension collected every 12 h by centrifugation was used for identifying enzyme activity.

Northern blot analysis

Total RNA was extracted from mycelia of *T. harzianum* T88 cultured in MM with different carbon sources or starvation conditions. Mycelia were harvested at 4, 12, and 24 h. For expression study of SA76 in transformants, the transformants were cultured in SC-U medium containing 2% galactose for 12, 36, 60, and 72 h, respectively. Total RNA was extracted using a Yeast RNA mini kit (Watson Biotechnologies, China). The total RNA (20 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde and blotted onto a Nylon membrane. Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals, Germany) were used for the preparation of the probe and detection of the transcripts of the SA76 gene. The SA76 gene digested by HindIII and EcoRI from plasmid (pYES2/SA76) was labeled with digoxigenin as a probe. Probes for hybridization were prepared by the random primer extension method. The blotted membrane was incubated at 45 °C in a digoxigenin Easy Hyb solution, and then reincubated in the same solution supplemented with a digoxigenin-labeled SA76 probe overnight. The membrane was washed with 2 × SSC and 0.1% sodium dodecylsulphate (SDS) at room temperature, followed by 0.1 × SSC and 0.1% SDS at 68 °C After washing the membrane with a solution containing 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, the hybridized bands were detected using a chemiluminescent substrate, CDP-STAR, in the detection kit (Roche) according to the manufacturer's instruction.

Measurement of enzyme activity

The *Saccharomyces cerevisiae* cell culture was centrifuged at 3100 ***g*** and 4 °C for 10 min. The supernatant (1.0 mL) was mixed with 1.0 mL of 1% casein solution in lactic acid buffer (pH 1.0–7.0), preincubated at 40 °C for 5 min. The mixture was incubated at 40 °C for 10 min, and 2 mL of 0.4 mol L^{-1} trichloroacetic acid solution was added to the mixture immediately to stop the reaction. The reaction mixture was centrifuged at 9500 ***g*** and 4 °C for 10 min. The supernatant (1.0 mL) was mixed with 5 mL of 0.4 mol L^{-1} sodium carbonate and 1 mL Folin–phenol reagent. The mixture was incubated at different temperatures (20–65 °C) for 20 min. The tyrosine content in the supernatant was determined colorimetrically at 650 nm using Folin–phenol reagent (Lowry *et al.*, 1951). Empty pYES2 and transformant cultured in repression medium (containing 2% glucose) served as control, respectively. One unit of protease is defined as the amount of enzyme that catalyzes the release of 1 μg of L-tyrosine per minute under the above assay conditions.

Assay for antagonistic activity

The experiment was carried out in 90 mm × 15 mm petri plates containing 15 mL PDA. Five milliliters of culture supernatant of transformant SA76 (cultured in SC-U medium containing 2% galactose for 72 h) was added to 45 mL PDA at 45 °C, mixed rapidly, and poured into petri dishes. A 4 mm diameter plug of the actively growing mycelium of phytopathogen was placed at the center of the PDA plate and incubated at 28 °C. The discs of phytopathogen on PDA with 5 mL of supernatant obtained from the control yeasts (empty vector) served as a control. Four plates were prepared as replications. The colonies,

radius of phytopathogen growth was measured daily after inoculation. The percentage growth inhibition was calculated using the following formula:

$$\%\,\text{Inhibition} = \left[\frac{(R-r)}{R} \times 100\right]$$

where R is the phytopathogenic colony radius of control and r is the radius of phytopathogenic colony with treatment.

Results

Amplification of the 3′ ends

By screening *c.* 3300 clones of a cDNA library, an EST (hzm-002076), encoding a protein as aspartic protease, was identified by BLASTX analysis of ESTs from the *T. harzianum* mycelium cDNA library against the GenBank nonredundant protein database. The EST was an internal segment of the gene encoding aspartic protease that the 3′ stop codon, and poly A tail was missing. In the homology study using BLAST program, database search showed that the sequence of the cDNA fragment has a high homology of 61%, 37%, and 33% with cDNA encoding *Gibberella zeae*, *Neurospora crassa*, and *Chaetomium globosum* aspartic proteinase, respectively. Based on this cDNA sequence, a primer was designed to perform RACE-PCR of 3′ end to amplify a full-length cDNA. From 3′ RACE, a 1.2-kb PCR product was produced. This sequence overlapped the 3′ end of the previously internal and encoded 344 amino acids before a TAA stop triplet was encountered. Following the stop triplet, there was a 201-bp UTR that included a 29-bp poly A tail. Sequencing of these PCR products resulted in a final continuous cDNA sequence that was 2019 bases in length.

Amino acid sequence analysis

By joining the 3′ end sequence with the known SA76 EST from cDNA library of *T. harzianum*, a full-length cDNA sequence was acquired. The results showed that the ORF of the SA76 gene consisted of 1593 bp, where G+C content was 57%. The sequence was submitted to GenBank under accession number EF063645. Based on similarity to other fungal aspartic proteases, the mature protease was expected to contain 530 residues, with a molecular weight (MW) of 55 kDa and an isoelectric point (IP) of 4.5.

Analysis of the amino acid sequence by SignalP v3.0 identified a cleavage signal sequence site between positions A18 and V19. The putative signal peptide corresponding to the first 18 amino acids shows typical features of signal peptides, such as a highly hydrophobic region and alanine residues at -3 and -1 position (relative to the cleavage site) (Nielsen *et al.*, 1997). Based on the presence of an N-terminal signal sequence and on the results obtained by the *k*-NM Prediction algorithm (http://us.expasy.org/tools/), SA76 encoding aspartic protease could be an extracellular protein. Three potential N-glycosylation sites (NXS/T) were also located at positions Asn77, Asn260, and Asn422 by the NetNGlyc v1.0, and 18 possible O-glycosylation sites were predicted by NetOGlyc v3.1.

Alignment of the SA76 sequence with the closest proteases allowed identification of residues necessary for a functionally active aspartic protease (Fig. 1). In aspartic proteases, the catalytic Asp residues occur within the motif Asp–Xaa–Gly in which Xaa can be Ser or Thr (Rawlings & Barrett, 1994; Simões & Faro, 2004). In fungal aspartic proteases, the catalytic Asp residues are contained in a DTGS and a DTGT motif in the N- and C-terminal lobes of the enzyme, respectively. In SA76, the region around the active-site residue Asp286 corresponded to the conserved motif DTGS while the active-site residue Asp83 occurs within the motif DSGT. This change distinguished SA76 from most of the fungal aspartic proteases, including PAPA from *T. harzianum* CECT 2413 (Delgado-Jarana *et al.*, 2002), *T. asperellum* (Viterbo *et al.*, 2004), and P6281 from *T. harzianum* CECT 2413 (Suárez *et al.*, 2005), which retained a D residue at this position. Hydrophobic motifs (XXG) responsible for substrate specificity were also preserved in SA76 at positions SSG496. Other residues involved in substrate specificity were present at position$Y^{133}G^{134}D135$ (Fig. 1).

Expression pattern of SA76

The expression pattern of SA76 in *T. harzianum* was analyzed under different starvation conditions or with diverse carbon sources. No SA76 transcript was detected in mycelium cultivated with glucose (2%) and ammonium ($5\,g\,L^{-1}$) as carbon and nitrogen sources, respectively, at any of the times considered (Fig. 2a, Glc). However, a weak SA76 signal was observed under carbon or nitrogen starvation at 4 h (after this time, the transcript level decreased) (Fig. 2b, MM-C and Fig. 2c, MM-N, respectively). For conditions of simulated parasitism, fungal cell walls were added to the medium as the sole carbon source. All five cell walls used strongly induced the expression of SA76, indicating that the presence of transcripts on fungal cell walls was not simply due to the lack of glucose as a carbon source and that induction occurs in this condition.

As can be observed in Fig. 2a (VS, RS), in the presence of fungal cell walls (*V. sordida Nit, R. solani*) SA76 mRNA reached the highest levels detected at 4 h (with a strong decay after 12 h). When *F. oxysporum or Sclerotinia sclerotiorum* cell walls were used as the only carbon source, maximal accumulation of mRNA was observed, with a strong decay of the signal after 4 h (Fig. 2b, FO and Fig. 2c, SS, respectively). The *P. sojae* cell walls induced expression at

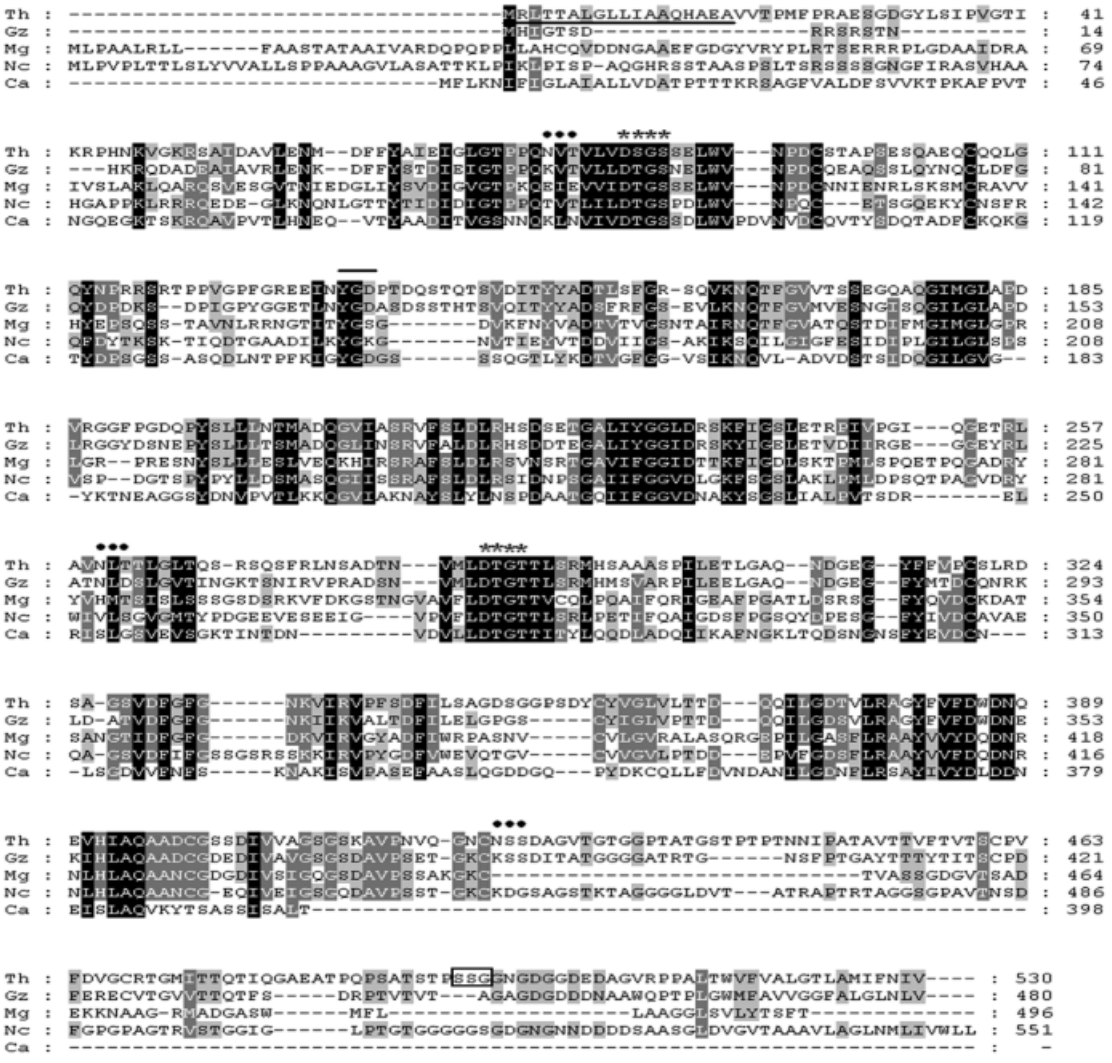

Fig. 1. Comparison of predicted amino acid sequence for aspartic protease identified from *Trichoderma harzianum* T88 withaspartic proteases from Gz, *Gibberella zeae* (GenBank accession no. XM389018); Ma, *Magnaporthe grisea* (GenBank accession no. XM01406914); Nc, *Neurospora crassa* (GenBank accession no. XM955235); and Ca, *Candida albicans* (GenBank accession no. XAF481100). Identical amino acids in all proteins are shaded in black. Those that are present in nearly all the proteins are shaded in gray. The putative signal peptide is underlined. Asterisks indicate the catalytic motif. N-Glycosylation sites are marked by black dotted lines. Hydrophobic motifs are marked by a black line. Residues involved in substrate specificity in members of the aspartic proteases family A1 are boxed. Alignments were performed by the CLUSTALX method using the MEGALIGN program of the informatic package DNASTAR (Lasergene).

4 h, with the maximum expression occurring at 12 h, and beyond this time the signal decreased (Fig. 2c, PS). From mycelium cultivated with chitin, SA76 transcript levels were similar to those obtained with fungal cell walls (Fig. 2b, Chi).

Heterologous expression of aspartic protease gene in *Saccharomyces cerevisiae*

To demonstrate the presence of the RNA transcript during expression of the SA76 gene, transformants in which SA76 gene expression was inhibited by glucose and induced by galactose were analyzed by Northern blotting (Fig. 3). Using the SA76 gene as a probe, no hybridization signals were observed from transformant RNA inhibited by glucose, but a single band was observed from transformant RNA induced by galactose. The mRNA level of SA76 detected 12 h after inoculation remained low and constant during the first 36 h, increased rapidly between 36 and 72 h, and then decreased. The maximum expression of SA76 was observed at 72 h, and it was approximately five times more than the expression levels obtained at 12 h.

Expression plasmid pYES2/SA76, together with empty pYES2 as a control, was transformed into *Saccharomyces cerevisiae*. Positive recombinants were identified by both

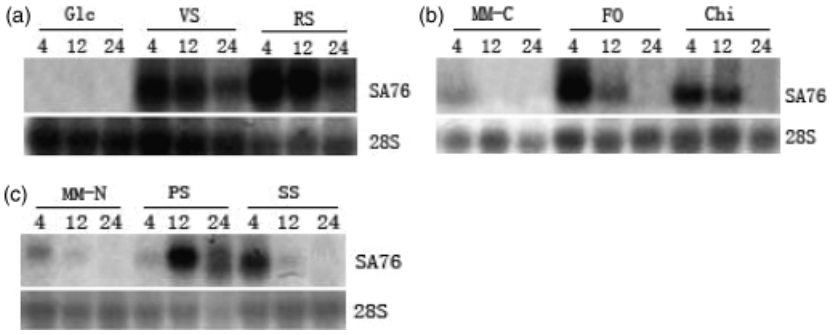

Fig. 2. Northern analysis of SA76 expression. Listed at the top are hours after transferring to the various media. Total RNA (20 μg) was extracted from mycelia of *Trichoderm harzianum* cultured in MM with different carbon sources or starvation conditions. Glc: 1% glucose; VS: 1% *Valsa sordida Nit* cell walls; RS: 1% *Rhizoctonia solani* cell walls; MM-C: absence of a carbon source; FO: 1% *Fusarium oxysporum* cell walls; Chi: 1% chitin; MM-N: 1/10 of a nitrogen- source with 1% glucose as a carbon source; PS: 1% *Phytophthora sojae* cell walls; SS: 1% *Sclerotinia sclerotiorum* cell walls. Mycelia were harvested at 4, 12, and 24 h. The hybridizations were carried out with the SA76 and ribosomal 28S DNA probes.

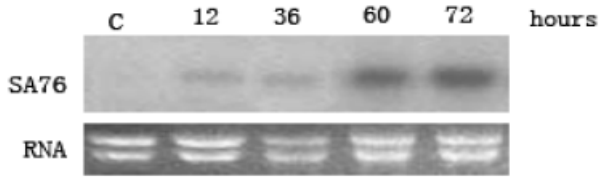

Fig. 3. Northern blotting analysis of total RNA from the SA76 yeast transformant. Total RNA was isolated from cultures grown in glucose (Lane C) or galactose (lanes 2–5). Listed at the top are hours after transferring to the galactose media (lanes 2–5).

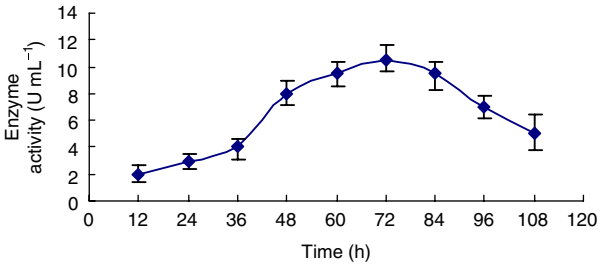

Fig. 4. Effect of culture time on pYES2-SA76 DNA transformant's enzymatic activity.

PCR and enzyme digestion. The transformed yeast cells were cultured in minimal medium with 2% raffinose as a carbon source and then used to inoculate rich medium containing 2% galactose, where induction of the gene took place (Fig. 4). From these data, it is apparent that aspartic protease activity was present in cells grown in raffinose, and a substantial increase was produced after a 12-h growth using galactose as the carbon source. At 72 h, the enzyme activity approached a peak (10.50 U mL^{-1}). The optimal enzyme reaction temperature was 45 °C and optimal pH was 3.5 (data not shown). No enzyme activity was detected in control. The results were in agreement with the Northern analysis.

Assay for antagonistic activity

In order to evaluate the antagonistic activity of aspartic protease against pathogenic fungi, *in vitro* inhibition of mycelial growth of five pathogenic fungi by the culture supernatant of aspartic protease was performed. During the preliminary *in vitro* experiments, aspartic protease was observed to possess obvious antagonistic activity against *F. oxysporum* at 38% of mycelial growth inhibition, whereas weak growth inhibition occurred against *P. sojae* (9%) and *Sclerotinia sclerotiorum* (7%). The mycelia of control *F. oxysporum* covered the petri at day 8, while the radius of *F. oxysporum* with treatment was 27.90 mm. In contrast, the radius of *Sclerotinia sclerotiorum* with treatment was 41.66 mm when control mycelia covered the petri at day 5. It indicated that aspartic protease did not or very slightly inhibited the mycelial growth of *Sclerotinia sclerotiorum*. Aspartic protease showed moderate growth inhibitions against *R. solani* (21%) and *V. sordida Nit* (19%), respectively (Table 1).

Discussion

Aspartic proteases have been detected and isolated from many different *Trichoderma* strains but there are no reports about their expression in *Saccharomyces cerevisiae*. This is the first time that *T. harzianum* aspartic protease was functionally expressed in *Saccharomyces cerevisiae*.

In this study, the cloning of a novel gene coding a 55 kDa aspartic protease from *T. harzianum* was reported. BLASTP analysis against the GenBank nonredundant protein database revealed that SA76 has a consistent degree of similarity

Table 1. Inhibition of mycelial radial growth by aspartic protease against different phytopathogens

Fungi	Radius of colony (mm)*	% Of inhibition of colony	Days†
Fusarium oxysporum	27	38	8
Rhizoctonia solani	35	21	5
Valsa sordida Nit	36	19	6
Phytophthora sojae	40	9	4
Sclerotinia sclerotiorum	41	7	5

*Radius of fungal colony on the media containing the SA76, when the control colony covered the used Petri dish.

†Time elapsed to the control colony covered the used Petri dish.

to aspartic proteases (EC 3.4.23.) belonging to the pepsin family, catalogued as family A1 (http://merops.sanger.ac.uk). The highest identity in sequence was found with the aspartic protease from the fungus *G. zeae* (53%). Lower identity percentages were found with other aspartic proteases from different filamentous fungi (<38%) or animals (<31%). The amino acid sequence data for SA76 and its biochemical properties clearly differentiated this aspartic protease from *Trichoderma* spp. aspartic proteases. Identity with the aspartic protease P6281 from *T. harzianum*, PAPA from *T. harzianum* CECT 2413 or its homologue in *T. asperellum* was 31% and 29%, respectively. Phylogenetic analysis of SA76 demonstrated that it is a novel protein substantially different from other fungi aspartic proteases.

Compared with the aspartic protease from the other fungi, SA76 presents several specific features. SA76 contains 18 O-glycosylation sites, which is unusual for fungal enzymes. Characteristically, eukaryotic aspartic proteases consist of two conserved motif DTGS/T, each of which provides a catalytic aspartic residue to the active site (Rawlings & Barrett, 1994). In SA76, the active-site motif DSGT in the N-terminal lobe is unusual in that Ser is present instead of the Thr used in all other fungal aspartic proteases. SA76 contains three putative N-linked glycosylation consensus sites NXS/T, while this motif is not found in other proteases.

The majority of extracellular proteases related to biocontrol processes in *Trichoderma* spp. have been characterized as serine proteases (Flores *et al.*, 1997; De Marco & Felix, 2002; Hanson & Howell, 2004; Pozo *et al.*, 2004; Suárez *et al.*, 2004; Grinyer *et al.*, 2005), whereas aspartic proteases have been mostly associated with problems in protein production due to proteolytic degradation (Haab *et al.*, 1990; Delgado-Jarana *et al.*, 2000). However, a homologous gene to the aspartic protease PAPA of *T. harzianum* has been recently cloned in *T. asperellum*, and expression analysis by RT-PCR has shown that it is induced during colonization of cucumber roots and mycoparasitic interaction with *R. solani* (Viterbo *et al.*, 2004). The regulation studies by Northern analysis show that aspartic protease P6281 from *T. harzianum* is related to antagonistic activities (Suárez *et al.*, 2005).

The analysis of SA76 expression confirmed that aspartic protease activity is induced in simulated parasitism by the presence of fungal cell walls. This indicated that this protein corresponds to an enzyme (an aspartic protease) whose target could be fungal cell wall-containing proteins. The increase in the activity is due to induction at the transcription level, because the transcripts accumulated abundantly shortly after induction (4 h). This increase seems to correspond to the inductor stimulus, and not to starvation conditions, as incubation in a medium lacking carbon or nitrogen did not result in a significant increase in transcription. Similar to prb1 and tvsp1 (Cortes *et al.*, 1998; Pozo *et al.*, 2004), the highest expression of SA76 takes place at a very short time (4 h), suggesting that it might participate in early stages of the mycoparasitic process. The present results show that protease mRNAs accumulate in fungi grown in the presence of chitin, reaching a maximum at 4 h. This fact indicated that chitin is a true inducer of protease expression.

In order to obtain enough aspartic protease to analyzes its function, *Saccharomyces cerevisiae* was used as a powerful and versatile heterologous expression system. In this study, the expression of genes coding for an aspartic protease was investigated. The protease activity culminates (10.5 U mL^{-1}) when cultured for 72 h, in accordance with Northern blot analysis. The optimal enzyme reaction temperature is 45 °C and the optimal pH is 3.5. It is to other similar explanations that have been described in the past (Togni *et al.*, 1991; Reichard *et al.*, 1995).

Owing to the complexity of the *in vitro* interaction between the mycoparasitic fungus *T. harzianum* and plant pathogenic fungi, we have studied the fungicidal activity of the culture supernatant of the *Saccharomyces cerevisiae* strain has been studied, which expressed the aspartic protease against five pathogenic fungi. Aspartic protease was observed to possess different fungicidal activity against five pathogenic fungi. The mycelial growth of *F. oxysporum* was most sensitive to aspartic protease. Mycelial growth of *Sclerotinia sclerotiorum* was less inhibited by aspartic protease. The mechanism by which aspartic protease showed different degrees of inhibition of mycelial growth against pathogenic fungi is unknown but some hypothesis can be offered. In *T. harzianum*, the basic protease plays a fundamental role in mycoparasitism towards different pathogens. The regulation seems to depend on cell wall components of the host (Pozo *et al.*, 2004; Suárez *et al.*, 2005; Viterbo *et al.*, 2004). It could be that the proteases degrade a suppressor of defense or release an active elicitor by degradation of a host protein. Similar explanations have been invoked to account for elicitor activity of bacterial Avr determinants that have protease activity (Shao *et al.*, 2002). In *T. virens*, a protease

with elicitor activity has been isolated recently (Hanson & Howell, 2004). In contrast, the aspartic protease was encoded by SA76, which appears to be extracellular; this was confirmed by the fact that culture supernatants of the *Saccharomyces cerevisiae* transformant expressing the aspartic protease SA76 were able to inhibit growth of phytopathogenic fungi. Meanwhile, this implied that the heterologous protein was secreted by the *Saccharomyces cerevisiae*. Analysis of the amino acid sequence identified a cleavage signal sequence site between positions A18 and V19. The putative signal peptide corresponding to the first 18 amino acids shows typical features of signal peptides. It can be speculated that this signal sequence might be recognized by the signal recognition particle (SRP) of *Saccharomyces cerevisiae*, and it led the protease to pass through the membrane. This protein might facilitate the penetration into the host tissue by degrading the protein linkages in the host's external layers and/or the utilization of the host proteins for nutrition (Pozo *et al.*, 2004).

The functional expression in *Saccharomyces cerevisiae* of SA76 from *T. harzianum* has been obtained. It is the first example of the successful expression of a functional aspartic protease in a heterologous host. The utility of expressing the SA76 in yeast is twofold: it is enzymatically highly active, and it is more readily recoverable during purification. This report on SA76 describes the protease gene product from *T. harzianum* showing direct activity by itself against phytopathogens. It has been demonstrated to have effective biological control competence with respect to phytopathogens.

The purpose of this study was to clone and express excessively this protease and to evaluate its antifungal potential against major phytopathogenic fungi. Finally, it should be possible to modify pYES2 to enable application of alternative purification schemes, for example by addition of a glutathione-*S*-transferase fusion tag, or a polyhistidine tag. Such schemes have been widely used to purify heterologous proteins expressed in yeast, and would eliminate the need for affinity purification with pYES2, which is not available commercially, thus providing distinct advantages in terms of economy and convenience.

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