Available online at www.sciencedirect.com
SCIENCE DIRECT®
Fungal Genetics and Biology 41 (2004) 336–348
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*

María J. Pozo, Jong-Min Baek, Juan M. García, and Charles M. Kenerley*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA

Received 11 March 2003; accepted 10 November 2003

Abstract

Serine proteases are highly conserved among fungi and considered to play a key role in different aspects of fungal biology. These proteases can be involved in development and have been related to pathogenesis or biocontrol processes. A gene (*tvsp1*) encoding an extracellular serine protease was cloned from *Trichoderma virens*, a biocontrol agent effective against soilborne fungal pathogens. The gene was expressed in *Escherichia coli* and a polyclonal antibody was raised against the recombinant protein. The expression pattern of *tvsp1* was determined and its physiological role was addressed by mutational analysis. Strains of *T. virens* in which *tvsp1* was deleted (PKO) or constitutively overexpressed (POE) were not affected in growth rate, conidiation, extracellular protein accumulation, antibiotic profiles nor in their ability to induce phytoalexins in cotton seedlings. *Tvsp1* overexpression, however, significantly increased the ability of some strains to protect cotton seedlings against *Rhizoctonia solani*. Our data show that Tvsp1 is not necessary for the normal growth or development of *T. virens*, but plays a role in the biocontrol process.

Index descriptors: Biocontrol; Hydrolytic enzymes; Gene deletion; Gene overexpression; Mycoparasitism; Phytoalexins; Serine protease; *Trichoderma virens*

1. Introduction

Serine proteases are endopeptidases that catalyze the hydrolysis of covalent peptidic bonds through the nucleophilic attack of the targeted bond by a serine (Rose et al., 1999). They play an important role in many biological processes of most organisms. There is evidence for their involvement in fungal development and morphology (Reichard et al., 2000), and the importance of fungal subtilisin-like serine proteases in different pathogenic processes has been widely reported. They can act as virulence factors for fungal pathogens of animals (*Aspergillus fumigatus*) (Kolattukudy et al., 1993) and plants (*Magnaporthe poae*) (Sreedhar et al., 1999). Their role in the biocontrol activity of diverse fungal agents has also been demonstrated. Entomopathogenic fungi such as *Metarhizium anisopliae* (St Leger et al., 1996), the nematophagous biocontrol agents *Paecilomyces lilacinus* and *Arthrobotrys oligospora* (Ahman et al., 2002; Bonants et al., 1995), as well as mycoparasitic fungi belonging to the genus *Trichoderma* (Geremia et al., 1993) have been found to secrete proteases in the presence of their hosts. In these cases, the proteases are believed to facilitate the penetration into the host tissue by degrading the protein linkages in the host's external layers (the insect cuticle, nematode eggshell and fungal cell wall, respectively) and/or the utilization of the host proteins for nutrition.

By understanding the basic mechanisms of action and regulation of serine proteases, the development of approaches for detecting and reducing the impact of deleterious fungi or increasing the biocontrol activity of beneficial fungi may be achieved. For example, overproduction of serine proteases in beneficial organisms has lead to an increase in biocontrol activity against several plant pathogens (Ahman et al., 2002; Flores et al., 1997; St Leger et al., 1996).

Among the mycoparasitic fungi, *Trichoderma* spp. are considered highly effective biocontrol agents. These filamentous fungi are ubiquitous in the soil environment and

* Corresponding author. Fax: 1-979-845-6483.
E-mail address: c-kenerley@tamu.edu (C.M. Kenerley).

1087-1845/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2003.11.002

parasitize a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, and *Botritis cinerea*. Their mycoparasitic activity is facilitated by antifungal products or secondary metabolites, including peptide and non-peptide toxins, and a battery of lytic enzymes, mainly chitinases, glucanases, and proteases, released in the presence of a suitable host (Chet and Chernin, 2002). Among these lytic enzymes, an alkaline serine protease, *prb1*, has been described from *Trichoderma harzianum* (Geremia et al., 1993). The expression of this gene was strongly induced in the presence of different fungal hosts, and is regulated by nutrient availability (Cortés et al., 1998). Moreover, the overexpression of the gene resulted in an increase in the biocontrol ability of the fungus against *R. solani* (Flores et al., 1997).

The biocontrol agent *T. virens* is unique among mycoparasitic fungi as it produces several antibiotic compounds, including viridin, gliotoxin, and peptaibols, that inhibit the growth of pathogens in vitro and have a synergistic effect with lytic enzymes (Howell et al., 1993; Wiest et al., 2002). Here we report the cloning and analysis of expression of *tvsp1*, a gene coding for an alkaline serine protease in *T. virens*, and the effects of deleting or constitutively expressing this gene in *T. virens*. This is, to our knowledge, the first report of disruption or deletion of a serine protease in a fungal biocontrol agent, and its impact on fungal development and biocontrol.

2. Materials and methods

2.1. Fungal strains and growth conditions

Two strains of *T. virens* were used in this study: wild-type strain Gv29-8 and an arginine auxotrophic strain, Tv10.4 (Baek and Kenerley, 1998). The strains were routinely maintained on potato dextrose agar (PDA, Difco). For expression studies, Vogel's minimal medium (Vogel, 1956) (VM) supplemented with different carbon sources was used. Isolates of the cotton seedling pathogens, *R. solani* and *P. ultimum*, were kindly provided by Dr. C. Howell, Southern Crops Research Laboratory, College Station, Texas. Fungal cell walls used as a carbon source for lytic enzyme inducing conditions were prepared according to Ren and West (1992). A selective medium (GVSM) used for the isolation of *T. virens* from soil and cotton roots was prepared as reported by Baek et al. (1999).

2.2. Cloning the tvsp1 gene

A genomic clone, containing the *tvsp1* gene, was isolated from a *T. virens* cosmid library (Kenerley, unpublished) using the *prb1* gene of *T. harzianum* (Geremia et al., 1993) as a probe. To isolate the corresponding cDNA, RNA was extracted from mycelia grown for 24 h in medium containing *R. solani* cell walls, as described below. cDNA products were produced by a combination of first-strand cDNA synthesis and subsequent PCR amplification, using gene-specific primers designed from the genomic sequence. For both genomic and cDNA clones, full DNA sequences were determined by a primer-walking strategy. General molecular biology methods were performed as described by Sambrook et al. (1989). Analysis of the deduced protein sequence and post-translational modification predictions was performed using proteomic tools (ScanProsite, TargetP, SignalP, NetNGlyc, YingOYang, and Sulfinator) at the ExPASy proteome server from the Swiss Institute of Bioinformatics (www.expasy.org/tools).

2.3. Northern analysis

For time-course expression analysis, conidia from *T. virens* strains were used to inoculate VMS. After 48 h of growth at 28 °C with shaking at 160 rpm, the mycelia were harvested from the liquid culture and rinsed with sterile H_2O. The mycelia were then transferred to fresh VM without a carbon source (VM-C) or supplemented with different carbon sources: 1.5% glucose (VMG), 1.5% sucrose (VMS), 0.5% fungal cell walls from *R. solani* (VMR), *P. ultimum* (VMP) or *T. virens* (VMT). In some cases, a medium lacking ammonium (VM-N) was used. Mycelia were harvested by filtration after 0, 1, 2, 4, 6, 12, 24, and 48 h incubation in the various media, frozen in liquid N_2, and lyophilized. Total RNA was extracted using Ultraspec II RNA isolation kit (Biotex). Ten micrograms of RNA per sample was electrophoresed in 1.2% agarose–formaldehyde gels and transferred to a Nylon membrane. Probes for hybridization were prepared by random primer extension, and hybridization was carried out overnight at 42 °C using Ultrahyb (Ambion).

2.4. Construction of pPKO and pPOE

For constitutive overproduction of the protease gene, the vector pPOE was constructed by placing the ORF (1.3 kb) of the *tvsp1* cDNA clone between the promoter and terminator regions of the *T. virens gpd* (glyceraldehyde-3-phosphate dehydrogenase) gene. This plasmid includes a 3 kb fragment containing the *arg2* gene as a selectable marker (Baek and Kenerley, 1998) (Fig. 1A). The deletion vector pPKO was constructed by replacement of a 3 kb *Xba*I/*Sma*I DNA fragment containing the complete ORF with the 3 kb *arg2* fragment (Fig. 1B). For transformation pPKO was digested with *Pvu*II, liberating the KO cassette from the vector DNA.

2.5. Transformation and primary selection methods

Preparation of protoplasts and PEG-mediated transformations of *T. virens* were performed using a

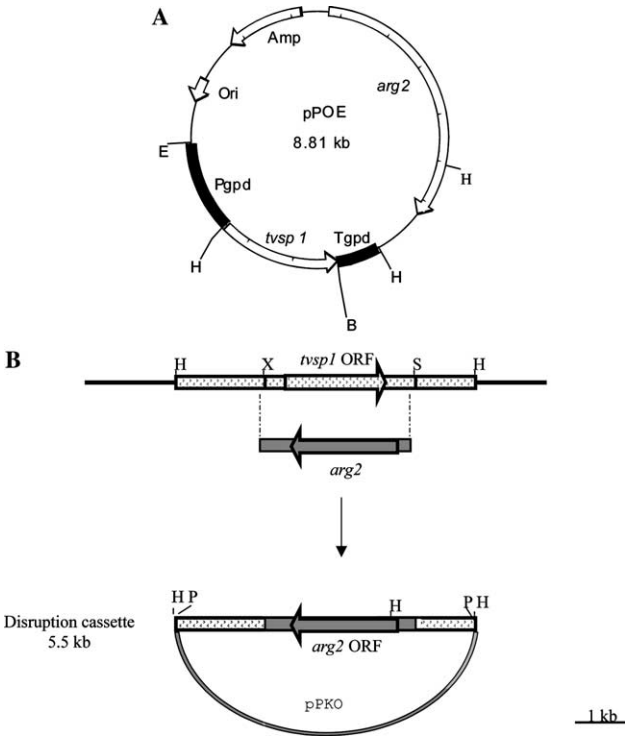

Fig. 1. (A) Structure of pPOE. The 1.3 kb ORF of *tvsp1* was placed under the control of the constitutive *gpd* 1.0 kb promotor (Pgpd) and 0.5 kb terminator (Tgpd). The 3 kb fragment containing the *arg2* gene was inserted as a selectable marker. (B) Construction of pPKO. A 3 kb *Xba*I–*Sma*I fragment containing the *tvsp1* ORF was replaced with the 3 kb fragment containing *arg2* gene. Arrows indicate ORF orientation. Some restriction enzymes sites are represented (H: *Hin*dIII, P: *Pvu*II, X: *Xba*I, S: *Sma*I, E: EcoRI, B: BamHI).

prototrophic complementation system as previously described (Baek and Kenerley, 1998). Stable prototrophic transformants were selected following sequential passage on VMS, PDA, and VMS.

2.6. Screening of transformants

Stable transformants were assayed for constitutive overexpression of the protease gene by placing 10 μl of a conidial suspension (10^8 conidia/ml) on agar plates containing 10% skim milk. After incubation at 28 °C for 48 h, strains with the widest halo in the opaque background were selected for further characterization. The presence of additional copies of the gene in these selected transformants was confirmed by Southern blot analysis.

Screening for deletion transformants (PKO) was initially performed by PCR. The primers (pp2: CACA CCTGGAGTGCCTATG and ppt: GAAAACGTCAA CGCCAGCACC) correspond to sequences in the ORF and were expected to amplify a 0.85 kb region in the wild type. Strains with no PCR product under these conditions were selected for Southern blotting analysis.

2.7. Enzymatic activity assays

General and basic protease activities in the culture filtrates were measured using Hide Azure powder (Calbiochem) and chymotrypsin substrate Succ-Ala-Ala-Pro-Phe-pNA (Sigma) (Flores et al., 1997; Geremia et al., 1993), respectively. One unit of proteolitic activity was defined as the amount of enzyme required to produce an increase of 0.01 absorbance units under standard assay conditions.

β-1,3-Glucanase activity was determined by measuring the liberation of reducing sugars from a laminarin solution after incubation of the culture filtrate for 30 min at 37 °C (Nelson, 1957). Activity of chitinases was determined following procedure of Ren et al. (2000) using the substrates 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (Sigma) for endochitinase and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma) for *n*-acetyl glucosaminidase activity.

2.8. Protein extraction and polyacrylamide gel electrophoresis of proteins

Culture filtrates of *T. virens* strains grown in different conditions were collected by filtration through miracloth. Extracellular proteins in the culture filtrate were precipitated with 80% ammonium sulfate, resuspended in small volumes of 10 mM Tris–HCl buffer, pH 7.3, and dialyzed against the same buffer. Protein contents were determined by the Bio-Rad Bradford microassay using BSA as a standard. Protein extracts were analyzed by discontinuous SDS–PAGE with prestained molecular mass protein standards (Bio-Rad) included for molecular mass determination.

2.9. Growth and sporulation assays

Cultures of selected transformant strains were compared with the wild-type strain for growth rate and sporulation. Agar plugs from actively growing colonies were placed on PDA plates. The plates were incubated at 28 °C, and four radial measurements from four repetitions for each strain were recorded at 24, 48, and 72 h. After 7 days, three plugs (0.5 mm) of each plate were collected, suspended in 10 ml of sterile H_2O and vortexed for 1 min. The number of conidia in the suspension was quantified using a Newbauer chamber. Germination percentage and branching patterns on PDA were determined and linear growth measured in racetubes containing PDA.

2.10. Antibiotic analysis

The culture filtrate of all the strains was analyzed for the presence of viridiol, viridin, gliotoxin, dimethyl-

gliotoxin, and heptelidic acid by HPLC following procedure of Howell et al. (1993).

2.11. Heterologous expression of the protein in Escherichia coli and production of antibodies

The truncated region of the *tvsp1* ORF corresponding to the expected mature protease (MP) was expressed in *E. coli* using the pET-30 LIC vector (Novagen). The sequence corresponding to MP was amplified by PCR using cDNA as template, and the product was directionally cloned into the expression plasmid. After transformation and screening for positive clones the expected nucleotide sequence was confirmed by sequencing and the clone MP-pET30 was selected.

MP-pET30 was expressed in *E. coli* BL21 (DE3) cells (Novagen). Expression was induced with 1.0 mM IPTG for 4 h at 37 °C. Protein extraction was performed under denaturing conditions (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8.0). The proteins were incubated with Ni–NTA agarose (Qiagen), washed, and eluted using the same buffer at decreasing pH (6.3, 5.9, and 4.3). Samples were dialyzed against Tris–HCl (10 mM, pH 7.5) for 2 days. Aliquots of the eluates were analyzed on 10% SDS–PAGE. The presence of the his-tag fusion protein was confirmed by staining the gels with 6× His Protein Tag stain (Pierce). The purity of the recombinant protein was determined by a subsequent staining with Coomassie. To further purify the protein, the corresponding band was excised and the protein electroeluted. Custom antiserum preparation of the purified MP protein from rabbit was performed by Alpha Diagnostic.

2.12. Western blot analysis

Proteins separated by 10% SDS–PAGE were electroblotted to a nitrocellulose membrane (Nitropure, Osmonic). Membranes were blocked with a solution of 7% skimmed milk in TBS–Tween 20 and incubated with the diluted rabbit polyclonal antibody. After washes, blots were incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) as secondary antibody, and developed according to manufacturer's instructions.

2.13. In vitro fungal growth inhibition assays

Wild-type and mutant strains of *T. virens* were confronted in vivo against *R. solani* and *P. ultimum* on PDA plates. Agar plugs from actively growing colonies of *T. virens* and the pathogen were placed approximately 7 cm opposite to each other and incubated at 27 °C until *Trichoderma* had begun to overgrow the pathogen colony. Confrontation areas were observed under microscope for coiling structures. Three agar plugs were harvested from each plate, ground in water, and serially diluted aliquots were plated on GVSM. Colonies of *T. virens* were counted after incubation for 24–36 h at 27 °C.

2.14. Induction of plant phytoalexins

Induction of terpenoids in cotton roots by culture filtrates of the different *T. virens* strains was determined as described by Howell et al. (2000). Briefly, strains were grown in liquid culture for 7 days at 27 °C. The culture filtrates were filter sterilized and then applied to radicles of germinated cotton seeds. After 2 days, terpenoids were extracted with acetone and analyzed by HPLC. The chromatogram was collected at a signal of 235 nm and the UV spectrum obtained for 210–400 nm.

2.15. Root colonization efficiency

Wild-type and transformed strains were compared for their ability to colonize a soilless mix (Metromix 366) and cotton roots. Inoculum of the different *Trichoderma* strains was added at a rate of 10^8 propagules per 1800 ml non-sterile soilless mix and incubated in trays in a growth chamber (EGC) (25 °C, 60% relative humidity, 12 h photoperiod). After 7 days, samples of the mixture were taken from each treatment to estimate colonization. Cotton seeds were then planted in the *Trichoderma* infested mix (10 seeds per 1800 ml soilless mixture) and incubated for another 7 days. Five plants per replicate were harvested, and their root systems washed, weighed, and homogenized in a blender with water for 30 s. Serial dilutions from both Metromix and root samples were assayed for colony forming units on GVSM. Each treatment was replicated five times with the entire experiment repeated twice.

2.16. Biocontrol assay

Two pathogens, *R. solani* and *P. ultimum*, were used to test the ability of *T. virens* strains to protect cotton seedlings from infection. The experimental design was as above except that the pathogen inoculum was added to the soilless mixture simultaneous with the *Trichoderma* inoculum and 20 cotton seeds were planted for each repetition. Healthy surviving seedlings were counted after 10 days. Additionally, the extension of the disease symptoms in the root system was evaluated for pathogen infected plants using an arbitrary scale of 0 (no symptoms) to 5 (entire root system discolored and decayed) with a maximum of 6 for non-germinated/dead seeds. Each treatment was replicated 6 times, and the entire experiment repeated twice. Data were subjected to analysis of variance (ANOVA) and Fisher's LSD test for significance using Statview v 5.0.1 (SAS Institute, Cary, NC). All analyses were performed at $P = 0.05$ level unless otherwise indicated.

3. Results

3.1. Isolation of tvsp1 from T. virens Gv 29-8 and sequence analysis

Using the alkaline protease gene *prb1* from *T. harzianum* as probe, we isolated a genomic DNA clone (cos31D1) from a *T. virens* cosmid library. The *prb1* probe hybridized to a 5.5 kb *Hin*dIII fragment. Sequence analysis confirmed that this fragment contained an alkaline serine protease gene that has been designated *tvsp1* (GenBank Accession No. AY242844). Southern blot hybridization showed that *tvsp1* is a single copy gene in *T. virens* genomic DNA (data not shown). Comparison of the genomic and cDNA sequences confirmed the presence of two introns (61 and 77 bp) interrupting the *tvsp1* coding region.

The *tvsp1* sequence is predicted to encode a polypeptide of 409 aa, with an estimated molecular weight of 42 kDa and an isoelectric point of 6.6. The polypeptide shows high homology (over 65% similarity) with serine proteases of different fungi including the pathogens *A. fumigatus* and *M. poae*, and biocontrol agents *T. harzianum*, *Me. anisopliae*, *Beauveria nivea*, and *Beauveria bassiana*. The highest similarity (91%) was to the *prb1* gene product from *T. harzianum*. Analysis of the protein sequence with SignalP v1.1 and TargetP v1.0 predict that the coded protein is destined to the secretory pathway, directed to the RE by a signal peptide of 20 aa. Two lys–arg (KR) sequences, potential sites for KEX2-like proteases, are found in the sequence at positions 65–66 and 119–120. The protein presents the typical features of the active site of subtilisin-type serine proteinases (catalytic triad formed by the functional residues Asp, His, and Ser at positions 161, 192, and 254, respectively). These characteristics define the *tvsp1* encoded protein, Tvsp1, as a serine-type peptidase included in the subtilase family (catalogued as S8) (Rose et al., 1999).

Several potential post-translational modifications are predicted from the sequence. There are two potential N-glycosylated asparagines at positions 165 and 252. Two possible O-glycosylation sites at serines in positions 140 and 294, two sulfated tyrosines at positions 108 and 111, and multiple potential phosphorylation sites are suggested.

The 5′ untranslated sequence of the *tvsp1* gene was analyzed for the presence of regulatory motifs using NSITE (http://genomic.sanger.ac.uk) and MatInspector v2.2 (Quandt et al., 1995). The putative TATA and CAAT boxes are located at −118 and −168 upstream from the ATG, respectively. A consensus site (5′ SYGGRG 3′) for binding of the carbon catabolite repressor CreA (Sophianopoulou et al., 1993) is located at −883. Two consensus HGATAR motifs, possible binding sites for nitrogen regulator such as AreA in *A. nidulans* (Ravagnani et al., 1997), were found at positions −867 and −904. At positions −209 and −753 there are two well-conserved target sequences for the repressor of *CAR1* expression, controlled by nitrogen catabolic repression (Messenguy et al., 2000). The pentanucleotide CCCCT, consensus sequence for the yeast stress response regulator (STRE) (Marchler et al., 1993), was found at positions −778 and −1900. One copy of the motif GCCARG, the recognition site for the PacC protein mediating pH regulation in *Aspergillus* (Tilburn et al., 1995), was found at −296. Two of the four mycoparasitic response elements (MYREs) described for *ech42* and *prb1* of *Trichoderma atroviride* (Cortés et al., 1998) have been found to be identical in the *tvsp1* promotor, MYC1 (GCTTCA), at position −477 and MYC4 (GGCACTCGGCAT) at position −790. Nearly identical sequences to MYC2 at positions −664 (TGGGCAA), and to MYC 3 (MGGC AC) at −91, −795, and −872 were also detected.

3.2. Pattern of expression of tvsp1: enzymatic activity and mRNA accumulation

The expression pattern of *tvsp1* in *T. virens* was analyzed over time under different starvation conditions or with diverse carbon sources. No transcript was detected for any time point from mycelia incubated in medium containing either glucose or sucrose as a carbon source (data not shown). Carbon starvation resulted in a weak signal 4 h after transferring the mycelia to fresh medium (Fig. 2A, VM-C); whereas, nitrogen starvation did not appear to have any detectable effect on *tvsp1* expression (data not shown). For conditions of simulated parasitism, fungal cell walls were added to the medium as the sole carbon source. All three cell walls employed (*R. solani*, *P. ultimum*, and *T. virens*) strongly induced the expression of *tvsp1* (Fig. 2A, VMR, VMP, and VMT, respectively). The *R. solani* cell wall preparation highly induced expression at 4 hours, with maximum expression occurring at 24 h. The expression of *tvsp1* in VM with *Pythium* cell walls was lower than with *Rhizoctonia* cell walls, but maximum expression was also reached after 24 h of incubation. In *T. virens* cell wall medium, maximum expression was obtained at 12 h, but the level of expression was lower than in the presence of *Rhizoctonia*. The addition of *Rhizoctonia* cell walls to a medium containing sucrose, but lacking nitrogen, resulted in the maximum level of induction occurring earlier compared to other media (6 h) (Fig. 2A, VM-N + S + R).

The levels of serine protease activity in the culture filtrates from the time course experiment were in agreement with the results from the Northern blot analysis (Fig. 2B). No activity was detected in VMS, and only low levels of activity were found in media lacking either carbon (VM-C) or nitrogen (VM-N). A strong increase in the activity was detected at 12 h after transferring to media containing cell walls of *Rhizoctonia* or

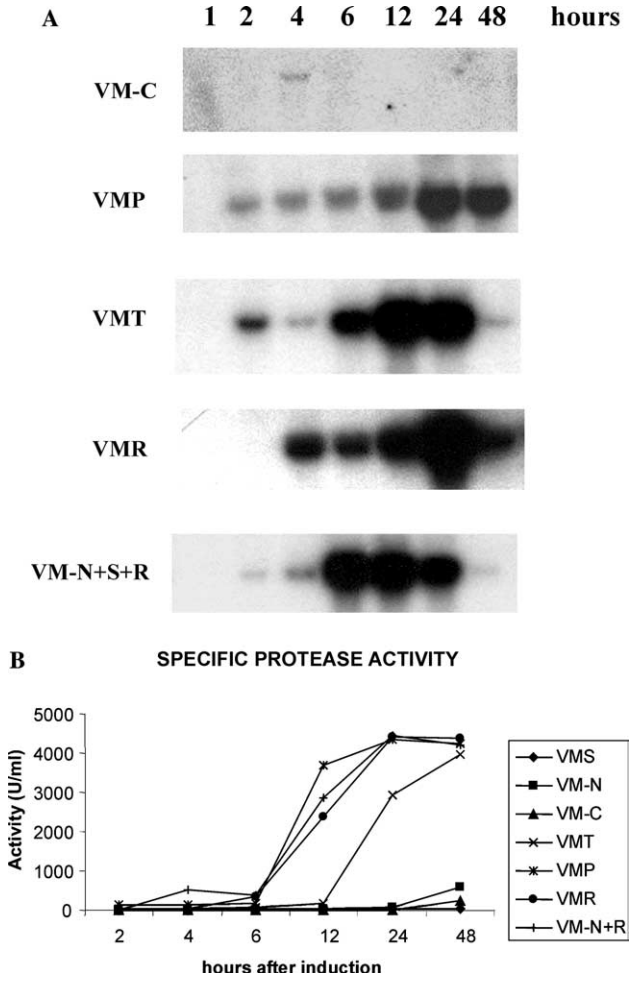

Fig. 2. Expression of the *tvsp1* gene and alkaline protease activities during growth in Vogel's minimal medium (VM) with different carbon sources or under starvation conditions. (VM-C, no carbon source; VMP, VMT, and VMR, *P. ultimum*, *T. virens*, and *R. solani* cell walls as carbon sources, respectively; VM-N + S + R, no nitrogen source but supplemented with sucrose and *Rhizoctonia* cell walls). (A) Northern analysis of *tvsp1* expression. Listed at the top are hours after transferring to the various media. A 285 kb *Nco*I fragment of *tvsp1* coding region was used as probe. Ten micrograms of total RNA was loaded per lane. (B) Alkaline protease activity in culture filtrates. Each point represents the average of three repetitions.

Pythium, with the maximum level of expression observed at 24 h. When using *T. virens* cell walls (VMT), the increase in expression was not observed until 24 h after induction, with maximum expression reached at 48 h. When *Rhizoctonia* cell walls were added to a media lacking a nitrogen source, the increase in enzyme activity appeared as early as 4 h, although the final level of activity seemed to be similar to the other treatments with cell walls (Fig. 2B).

3.3. Heterologous expression of tvsp1

To raise antibodies against Tvsp1, the coding region corresponding to the predicted mature protein (residues 120–409) was expressed in *E. coli*. The purified protein had an apparent molecular weight (MW) of 38 kDa, consistent with the expected MW of the mature protein (29 kDa) plus the two His tags and one S tag (total estimated to be 37.1 kDa) (Fig. 3A). The presence of large quantities of the recombinant protein was confirmed by staining the gel with 6× His Protein Tag stain (Fig. 3B). The protein was then purified to apparent homogeneity (Fig. 3C) and a polyclonal antibody reacting with the purified Tvsp1 was obtained. Enterokinase treatment eliminated the N-terminus tags, and most of the protein was found at 33 kDa, corresponding to the mature protein (29 kDa) plus the additional 32 aminoacids from the introduced C-terminus sequence (Fig. 3D). Even with the enterokinase treatment some protein with the intact tags remained (38 kDa).

3.4. Isolation of tvsp1 transformants

Seven stable prototrophic transformants putatively containing additional copies of *tvsp1* were selected as they demonstrated a wider halo of casein hydrolysis compared to the wild-type colony. Six of the selected

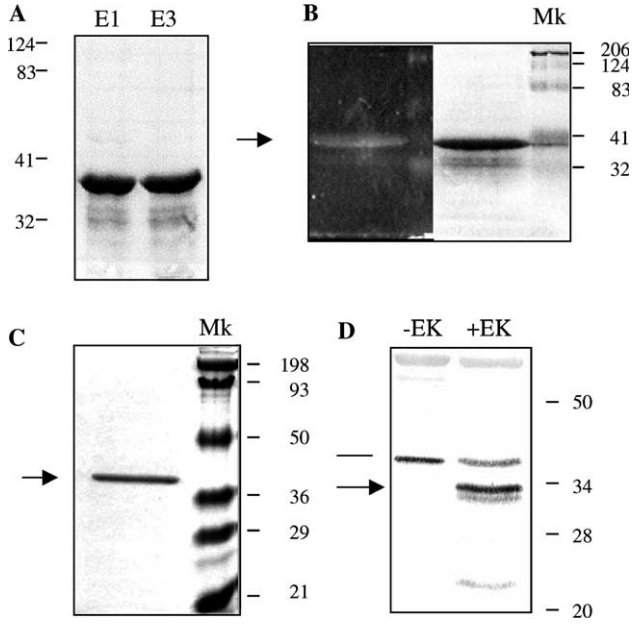

Fig. 3. Purification of the His-tag recombinant protease encoded by *tvsp1* expressed in *E. coli*. (A) SDS–PAGE analysis of the eluted fractions (E1 and E3) during the affinity purification procedure after coomasie staining. Molecular markers are on the left. (B) Confirmation of the presence of the recombinant protein by His-tag specific staining (see Materials and methods). Arrow indicates the recombinant protein. Left panel shows fluorescence of the stained recombinant protein under UV light, right panel shows same gel after Coomassie staining. (C) Coomasie staining of the purified protein (arrow) after separation on 10% SDS–PAGE. (D) Inmunoblot analysis of the *E. coli* protein extract eluted after the affinity chromatography, before (−EK) and after (+EK) enterokinase treatment. Line indicates the full recombinant protein. Arrow indicates the truncated form after enterokinase cleaving. Molecular weights indicated on the right.

transformants had at least one additional copy of the gene, as demonstrated by Southern blotting analysis. One of them (POE3) had three additional bands, indicating different integration events with genomic rearrangements (Fig. 4A). The intensity of hybridization of the 2.1 kb band in some of the transformants, such as POEB, suggests integration of multiple copies of the construct. This was confirmed by probing with a fragment of the *arg2* gene (data not shown). Multiple bands were seen in POEB, while a unique band at 5.9 kb, corresponding to the native *arg2* gene, was demonstrated in the other transformants (data not shown). These results indicate that the majority of the integration events occurred in the *arg2* locus while POEB has copies integrated in different locations within the genome. A similar hybridization pattern was observed when blots were probed with the *gpd* promoter (Pgpd), indicating that the additional copies are under the control of the constitutive promoter (data not shown).

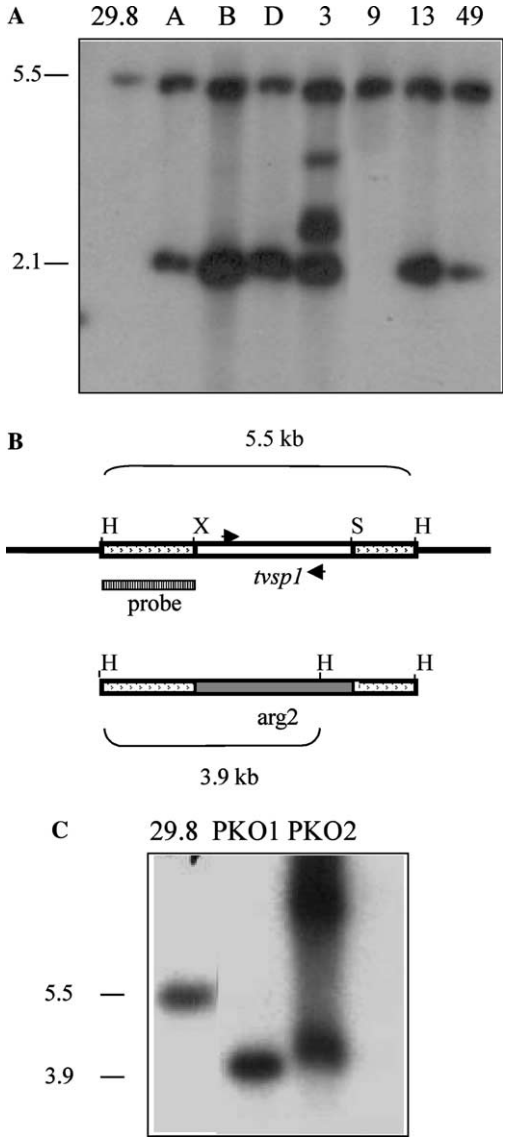

Fig. 4. Southern blot analysis of wild-type strain Gv 29-8 and pPOE transformants (A, B, D, 3, 9, 13, 49) probed with the *tvsp1* coding region. Genomic DNA (10 μg) was digested with *Hin*dIII. (B) Diagram of the gene deletion screening strategy. Arrows indicate the position of the primers used in the PCR screening. The 1.5 kb fragment upstream *tvsp1* ORF used as probe for southern analysis is indicated by a dashed bar. Numbers over brackets indicate expected size in native and deleted sequences. (C) Southern blot analysis of gene deletion transformants (PKO1 and PKO2).

Gene deletion transformants were confirmed by Southern blotting, using a 1.5 kb *Hin*dIII-*Xba*I fragment immediately upstream of the *tvsp1* coding region as probe (Fig. 4B). After digestion of the genomic DNA with *Hin*dIII, the probe was expected to hybridize to the native gene yielding a 5.5 kb band, while in the deletion mutants the expected band should be of 3.9 kb (see diagram in Fig. 4B). Two transformants lacking the native *tvsp1* were found, but PKO2 showed additional copies of the construct integrated in the genome; thus, PKO1 was selected for further analysis (Fig. 4C). Probing with a 0.6 kb fragment from *arg2* gene confirmed a single integration event in PKO1 and multiple integrations in PKO2 (data not shown).

3.5. Tvsp1 expression and protease activity in transformants

Northern analysis of the selected transformants grown in VM supplemented with sucrose (non-inducing conditions, S) or *Rhizoctonia* cell walls (inducing conditions, R) is presented in Fig. 5. As expected, *tvsp1* is expressed in the wild type in the medium with fungal cell walls, but not in sucrose. There are no transcripts of the gene in the PKO1 transformant under any of the conditions, confirming the replacement of the gene. The gene was constitutively expressed in the three selected overexpresors (POEB, POED, and POE3) as transcripts were observed when the strains were grown in VMS.

Total protease activity was measured in the culture filtrate from the different strains after 24 h of fungal growth in fresh media. In VMS, 3.2 U of total protease activity per ml were detected with Gv29-8, while PKO1 displayed 2 U versus 62, 18, and 32 U in POEB, POED, and POE3 culture filtrates, respectively. In VMR, there was a significant increase in activity for the wild type (14 U) and POEB, POED, and POE3 (70, 35, and 45 U, respectively), but no significant change for PKO1 (2 U). The level of serine protease activity detected in the culture filtrates corresponding to the times tissue was analyzed by Northern blotting is illustrated in Figs. 6A and B. Under non-inducing conditions, almost no activity was detected in wild type or deletion mutant, but the three overexpressors accumulated this enzyme in the culture filtrate at levels clearly detectable after 6 h of growth, increasing throughout the sampling period (24 h) (Fig. 6A). The culture filtrates from VMR

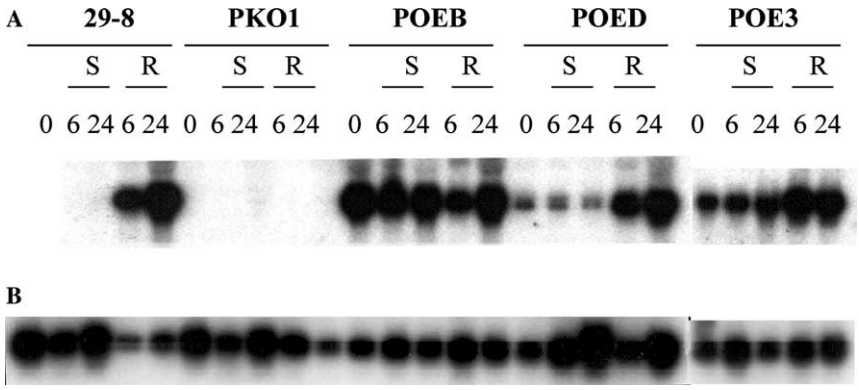

Fig. 5. Northern analysis of *tvsp1* expression in POE and PKO strains. Total RNA was extracted from PKO1, POEB, POED, and POE3 after 6 and 24 h of culture in sucrose (S) or *Rhizoctonia* cell wall (R) supplemented media. Each lane contained 7 μg of RNA. (A) A 285 kb *Nco*I fragment of *tvsp1* was used as probe. (B) Same blot probed with a histone encoding gene fragment as loading control.

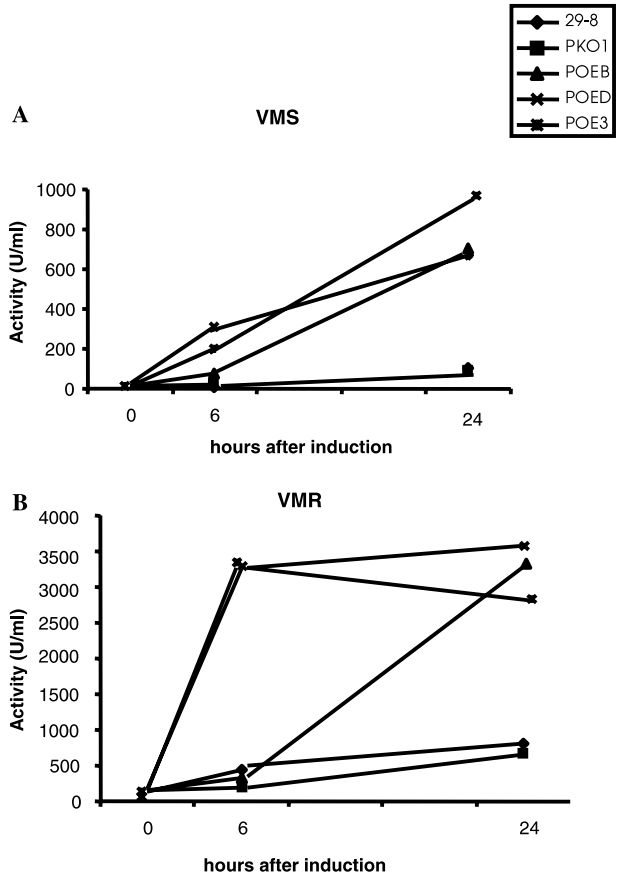

Fig. 6. Serine protease activity in culture filtrates from the different strains during the time course indicated in Fig. 5. (A) Samples from medium supplemented with sucrose (VMS). (B) Samples from medium supplemented with *Rhizoctonia* cell wall (VMR). Each point represents the average of four replicates.

displayed very high levels of protease activity for POED and POE3 at 6 h, while POEB achieved similar levels by 24 h. The wild-type and deletion mutant strains showed only a small increase in activity over the same sampling period (Fig. 6B).

3.6. Protein profile and quantification of other enzymatic activities

To assess the effect of the overexpression or deletion of *tvsp1* on the pattern of secreted proteins and activity levels of extracellular hydrolytic enzymes, the strains were grown in VMS or VMR for 6 days. No apparent alteration was observed in the secreted protein profile for either strain grown in VMS or VMR. Quantification of total protease, serine protease, β-1,3-glucanase and endochitinase activities in the protein extracts are summarized in Table 1. As expected, constitutive expression of the proteases is evident for the POE strains in VMS, and only residual activity is found in PKO1 in VMR. No significant alteration in the activity levels for glucanase or chitinase was detected.

3.7. Western blot analysis

Western blotting was used to verify the overexpression and deletion of *tvsp1* and correlate changes in specific protease activity to levels of the Tvsp1 protein. As shown in Fig. 7A, under non-inducing conditions, only the overexpressor strains accumulated high levels of the protein. The apparent molecular weight of the protein is about 30 kDa, slightly higher than the theoretical MW estimated to be 29 kDa. Under inducing conditions, a band can be observed for all strains except the PKO1 strain (Fig. 7B).

3.8. Growth, sporulation, antibiotic production, and antifungal activity

Phenotypic analysis of the mutant strains compared with the wild type failed to demonstrate any significant differences. Colonies grown on solid medium were phenotypically similar with respect to radial growth, production of aerial hyphae, and pigmentation during sporulation. Quantification of conidia did not show

Table 1
Total protease, serine protease, β-1,3-glucanase, and endochitinase activities in protein extracts from the culture filtrate of wild-type and mutant strains after 6 days of growth on VMS or VMR

	Total protease, U/mg		Serine protease, U/mg		β-1,3-glucanase, U/mg		Endochitinase, % of control	
	VMS	VMR	VMS	VMR	VMS	VMR	VMS	VMR
Gv29-8	0	5180	0	9550	8.4	26.7	0	100
PKO1	0	3200	0	370	18.2	23.7	5	100
POEB	3160	5220	1330	11250	10.6	26.7	10	100
POED	810	4870	5100	8750	3.3	24.5	5	100
POE3	2620	5020	980	5950	24.5	23.0	10	100

Values are an average of four repetitions and expressed per milligram of protein.

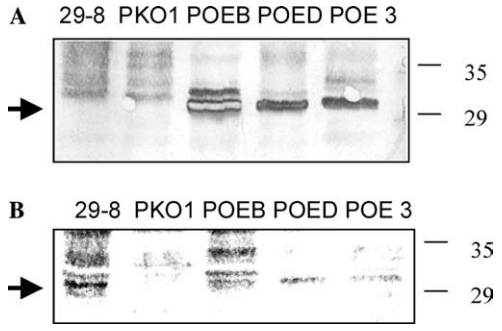

Fig. 7. Immunoblot analysis of crude extracts from *T. virens* wild-type and mutant strains after growth in non-inducing (VMS) (A) or inducing (VMR) (B) conditions. The polyclonal antibody raised against the recombinant Tvsp1 was used. The arrow indicates the signal corresponding to the Tvsp1 protein. Molecular weight markers are indicated in the right.

Table 2
Concentrations of terpenoids (μg compound/g tissue) in roots of cotton seedlings inoculated with the wild-type (Gv 29-8), the overexpression (POEB, POED, and POE3) or deletion (PKO1) strains

	HG	dHG	HGAL	G
Control	13.173a	12.543a	3.073a	117.857a
Gv 29-8	30.640b	23.607b	6.813b	148.033a
PKO1	37.540b	32.760c	7.863b	149.467a
POEB	41.983b	33.450c	6.717b	144.967a
POED	45.623b	35.470c	7.407b	145.767a
POE3	35.440b	21.960b	8.133b	113.900a

Numbers are average of three repetitions. HG, hemigossypol; dHG, desoxyhemigossypol; HGAL and G, gossypol. Values in the same column with a letter in common did not differ significantly according to Fisher's PLSD test at a significance level of 5%.

significant differences among the strains. The rate of spore germination and pattern of hyphal branching were similar for all the strains studied. However, POE3 showed a significantly greater growth rate in racetubes. This overexpressor strain also produced significantly greater biomass in VMR than PKO1 or Gv29-8 for each of the four sampling times (3, 5, 7, and 9 days).

No significant differences were found among the strains (five overexpressors, deletion mutant, and wild type) for production of gliotoxin, dimethylgliotoxin, and heptelidic acid. The response of these same strains to *R. solani* and *P. ultimum in vitro* plate assays was similar.

3.9. Root colonization, induction of plant phytoalexins, and biocontrol assays

The deletion and overexpression mutants did not differ significantly from the wild-type strain in their ability to colonize either Metromix or developing cotton roots. These strains also did not differ in their ability to induce terpenoid synthesis in cotton roots (Table 2). Roots treated with any of the *Trichoderma* strains significantly accumulated higher levels of HG, dHG, and HGAL than control roots (not inoculated with *Trichoderma*). Although the gossypol concentration was slightly higher in *Trichoderma* colonized roots, this increase was not statistically significant.

The biocontrol ability of the strains overexpressing or deleted in *tvsp1* was tested in vivo against *R. solani* and *P. ultimum*. Only 3% of the control cotton plants survived after incubation with *R. solani* for 10 days. Treatment with any *Trichoderma* strain resulted in a significant increase in plant survival. Protection by the deletion mutant PKO1 (45%) was less than the wild-type strain (60%), although this difference was only statistically significant at a significance level of 10%. The percentage of surviving seedlings was significantly higher for plants treated with POED (75%) and POE3 (92%), reaching a number of healthy plants equivalent to controls in the noninfested mixture. However, there was no difference in protection by POEB and wild-type strains (Fig. 8A). The root systems of these plants were further evaluated for disease symptoms (Fig. 8B). The disease index for plants treated with wild-type and overexpressor strains was significantly lower than in control plants, while in plants treated with the deletion strain, PKO1, the average disease index was equivalent to the rating for control seedlings in the *R. solani* infested mixture. The disease index for plants in the mixture inoculated with POED or POE3 was comparable to the noninfested controls. Treatment with all *Trichoderma* strains reduced the biomass loss of pathogen infected plants, but the weights of plants from treatments with wild type or PKO1 were significantly less than those treated with the overexpressor transformants (Fig. 8C). Overexpression

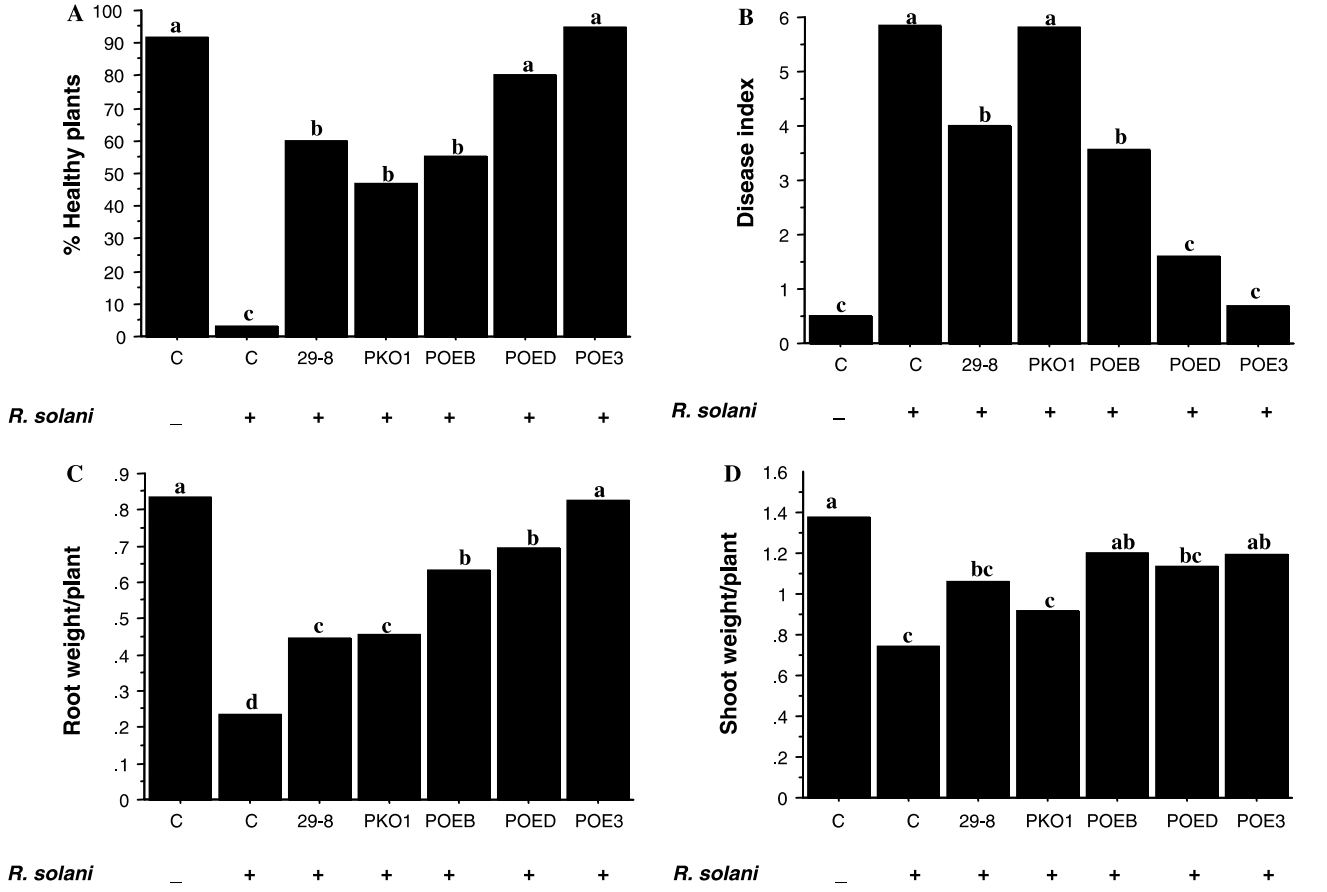

Fig. 8. Biocontrol activity of wild-type strain Gv 29-8, three overexpressor (POEB, POED, and POE3) and the deletion (PKO1) strains against *R. solani*. (A) Percentage of healthy cotton seedlings from the different treatments. (B) Disease index of the root systems. (C) Fresh weight of the root systems. (D) Fresh weight of the shoots. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at a significance level of 5%. (+) or (−) denotes the presence or absence of the pathogen, respectively.

of *tvsp1* resulted in an increase in the average root mass in all treated seeds, but it is noteworthy that POE3 treated plants did not exhibit any loss of root mass (Fig. 8C). Treatments with the overexpressor strains, POEB and POE3, also resulted in shoot weights comparable to those of the control plants grown in the noninfested mixture (Fig. 8D).

When control cotton seeds were planted in soilless mixture infested with *P. ultimum*, no seedlings survived. Treatment with all wild-type and mutant strains resulted in 80–95% survival and very low disease index values, but the differences among them were not significant. The root and shoot weight of plants from POE3 treatment was significantly higher than the other strains (data not shown).

4. Discussion

Tvsp1 gene cloned from *T. virens* encodes an extracellular serine protease. The protein is highly similar to several serine proteases of the subtilisin family from different fungi, among them animal and plant pathogens as well as biocontrol agents. Commonly, these types of proteins are synthesized as zymogens, requiring cleavage by the propeptide to activate the protein after secretion (Markaryan et al., 1996). In the Tvsp1 polypeptide there are two KR sequences, which are substrates for Kex2-like proteins, known to be present in *Trichoderma* (Goller et al., 1998). Based on similarity to other fungal serine proteases, we expected the mature protease to contain 289 residues, with a molecular weight (MW) of 29 kDa and an isoelectric point (IP) of 8.98, resulting from the cleavage after the second KR sequence. This was experimentally confirmed as the polyclonal antibody obtained against the purified recombinant protein reacted with a protein of approximately 30 kDa in fungal culture filtrates. The small difference between the theoretic and experimental molecular weight may reflect post-translational modifications, as several putative modifications can be predicted from the amino acid sequence.

The analysis of *tvsp1* expression confirmed that serine protease activity is induced in simulated parasitism by the presence of fungal cell walls with diverse composition. The increase in the activity is due to

induction at the transcription level, since the transcripts accumulated abundantly shortly after induction (2 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 or enzyme activity. This is in contrast with the presence in the promoter region of potential sites for carbon (CreA) and nitrogen (AreA) regulation. However, involvement of these elements may have been indicated by the weak transcript accumulation observed after 4 h of incubation under carbon starvation and by the earlier increase in response to fungal cell walls in a medium lacking nitrogen. The homologue of *tvsp1* in *T. atroviride*, *prb1*, is subject to nitrogen catabolite repression and induction by cell walls, and is completely dependent on nitrogen derepression conditions (Olmedo-Monfil et al., 2002). The authors also demonstrated the induction of the gene under derepressed conditions by osmotic stress. The presence of stress response elements in the *tvsp1* promoter in *T. virens* suggests that the gene would also respond to different types of stress. Moreover, the presence of a PacC binding site suggests possible control by pH, as pH plays an important role in the regulation of many proteases (St Leger et al., 1998). Four mycoparasitism responsive elements (MYRE) described by Cortés et al., 1998, were found to be almost identical in *tvsp1*. The presence of one or both of the MYRE boxes 1 and 2 were shown to be required for the induction of *prb1* by fungal cell walls (Olmedo-Monfil et al., 2002). Comparison of *prb1* and *tvsp1* expression under equivalent media and growing conditions will help to clarify the role of these regulatory elements in fungal protease gene expression.

To understand the role of serine proteases in *T. virens* biology, we have combined mutagenesis with physiological and biochemical analyses. The deletion of the *tvsp1* gene in *T. virens* by homologous recombination had a drastic effect on the total extracellular protease activity. This result shows that Tvsp1 enzyme is a major extracellular protease in *T. virens*. Very small levels of residual proteolytic activity were detected in the PKO mutant in the presence of cell walls, indicating the existence of other proteases able to degrade the same substrate. The presence of another extracellular serine protease of 73 kDa has been demonstrated in *T. harzianum* (Dunaevsky et al., 2000). A role for serine proteases in developmental processes has been proposed in other fungi. For example, in *Aspergillus fumigatus*, disruption of the serine protease gene *alp2* resulted in slower vegetative growth and more than 80% reduction of sporulation, correlated with a reduction of the diameter of conidiophore vesicles (Reichard et al., 2000). In our study, neither deletion nor overexpression of *tvsp1* affected cell viability, growth, conidiation, protein profile or the activity of other hydrolytic enzymes. Thus, we would not assign a morphogenetic or developmental role to Tvsp1 in *T. virens*.

In several biocontrol agents, serine proteases are reported to be involved in the host-parasite interaction. In *T. atroviride*, *prb1* expression is regulated by the presence of a host or its cell walls, and the overexpression under its native promoter resulted in an increase in plant protection against *R. solani* (Flores et al., 1997). In this study, the overexpressor strain showed a significantly higher increase in biomass compared to the wild-type and deletion strains when grown in a medium with cell walls as sole carbon source (data not shown). The results are consistent with the hypothesis that these proteases are involved in the liberation of nutrients from the host. Moreover, overexpression of *tvsp1* resulted in an increase of the biocontrol activity of *T. virens*, as evidenced by a significant increase in plant survival in *R. solani* infested medium after treatment with two overexpressors, POED and POE3. The survival rate for these treatments reached the level of controls in the non-infested medium. The disease index was greatly reduced, again to levels of non-infected plants for POED and POE3. Loss of root biomass, as result of the disease, was significantly less or totally neutralized for the overexpressors tested. The overexpressor POEB, which showed integration of additional gene copies in multiple locations of the genome and a delay in the accumulation of protease activity compared to POED and POE3, did not show an increased biocontrol ability compared to the wild type.

The effects of *tvsp1* deletion on *T. virens* ability to protect cotton plants against *R. solani* or *P. ultimum* were limited. Treatment with PKO1 resulted in an increased disease index and a small reduction in plant survival compared to the wild-type strain. This limited impact on plant protection is not surprising if considering that biocontrol by *Trichoderma* species is a consequence of complex interactions involving many factors. Mycoparasites antagonize fungal pathogens first by antibiosis leading to cell death, followed by degradation of the cell wall by hydrolytic enzymes (Belanger et al., 1995). As we have confirmed that the antibiotic profile was similar in the deletion mutants and the wild-type strain, any effect these secondary metabolites may have on a host would be the same for both strains. Moreover, other more indirect mechanisms, such as induction of plant defense reactions, are likely to be involved in the biocontrol process. Supporting this hypothesis, we report the increase in terpenoid synthesis in plants treated with all the *Trichoderma* strains tested.

The biocontrol assays show that despite the convergence of different mechanisms in the *Trichoderma*–pathogen interaction, the serine protease encoded by *tvsp1* has an impact on the efficacy of biocontrol.

The role played in mycoparasitism may explain the high conservation of this gene throughout different biocontrol agents, since no other vital functions on fungal morphology and development have been observed. Considering that serine proteases are also effective against oomycetes (Dunne et al., 2000) and nematodes (Bonants et al., 1995), the constitutive expression of the gene is a promising tool toward the improvement of *T. virens* ability to protect plants against a broader range of different pests.

Acknowledgments

We thank Dr. C. Howell for the antibiotics and terpenoids analysis, and Dr. R. Omarov for helpful discussions. This research was supported by a grant from USDA/NRI Research Program, Biologically Based Pest Management (99-35316-7940) to C.M.K.

References

Ahman, J., Johansson, T., Olsson, M., Punt, P.J., van den Hondel, C.A.M.J.J., Tunlid, A., 2002. Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. Appl. Environ. Microbiol. 68, 3408–3415.

Baek, J.M., Howell, C.R., Kenerley, C.M., 1999. The role of an extracellular chitinase from *Trichoderma virens* Gv 29-8 in the biocontrol of *Rhizoctonia solani*. Curr. Genet. 35, 41–50.

Baek, J.M., Kenerley, C.M., 1998. The *arg2* gene of *Trichoderma virens*: cloning and development of a homologous transformation system. Fungal Genet. Biol. 23, 34–44.

Belanger, R.R., Dufour, N., Caron, J., Benhamou, N., 1995. Chronological events associated with the antagonistic properties of *Trichoderma harzianum* against *Botrytis cinera*: indirect evidence for sequencial role of antibiosis and parasitism. Biocont. Sci. Technol. 5, 41–53.

Bonants, P.J., Fitters, P.F., Thijs, H., den Belder, E., Waalwijk, C., Henfling, J.W., 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141, 775–784.

Chet, I., Chernin, L., 2002. Biocontrol, microbial agents in soil. In: Bitton, G. (Ed.), Encyclopedia of Environmental Microbiology, vol. 1. Wiley, NY, pp. 450–465.

Cortés, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet, I., Herrera-Estrella, A., 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol. Gen. Genet. 260, 218–225.

Dunaevsky, Y.E., Gruban, T.N., Beliokova, G.A., Belozersky, M.A., 2000. Enzymes secreted by filamentous fungi: regulation and purification of an extracellular protease of *Trichoderma harzianum*. Biochemistry (Mosc.) 65, 723–727.

Dunne, C., Moenne-Loccoz, Y., de Bruijn, F., O'Gara, F., 2000. Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81. Microbiology 146, 2069–2078.

Flores, A., Chet, I., Herrera-Estrella, A., 1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb1*. Curr. Genet. 31, 30–37.

Geremia, R.A., Goldman, G.H., Jacobs, D., Ardiles, W., Vila, S.B., Van Montagu, M., Herrera-Estrella, A., 1993. Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. 8, 603–613.

Goller, S.P., Schoisswohl, D., Baron, M., Parriche, M., Kubicek, C.P., 1998. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. Appl. Environ. Microbiol. 64, 3202–3208.

Howell, C.R., Hanson, L.E., Stipanovic, R.D., Puckhaber, L.S., 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatments with *Trichoderma virens*. Phytopathology 90, 248–252.

Howell, C.R., Stipanovic, R.D., Lumsden, R.D., 1993. Antibiotic production by strains of *Gliocladium virens*. Biocontrol Sci. Tech. 3, 435–441.

Kolattukudy, P.E., Lee, J.D., Rogers, L.M., Zimmerman, P., Ceselski, S., Fox, B., Stein, B., Copelan, E.A., 1993. Evidence for possible involvement of an elastolytic serine protease in aspergillosis. Infect. Immun. 61, 2357–2368.

Marchler, G., Schuller, C., Adam, G., Ruis, H., 1993. A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 12, 1997–2003.

Markaryan, A., Lee, J.D., Sirakova, T.D., Kolattukudy, P.E., 1996. Specific inhibition of mature fungal serine proteinases and metalloproteinases by their propeptides. J. Bacteriol. 178, 2211–2215.

Messenguy, F., Vierendeels, F., Scherens, B., Dubois, E., 2000. In *Saccharomyces cerevisiae*, expression of arginine catabolic genes *CAR1* and *CAR2* in response to exogenous nitrogen availability is mediated by the Ume6 (CargRI)–Sin3 (CargRII)–Rpd3 (CargRIII) complex. J. Bacteriol. 182, 3158–3164.

Nelson, N.J., 1957. Colorimetric analysis of sugars. Methods Enzymol. 3, 85–86.

Olmedo-Monfil, V., Mendoza-Mendoza, A., Gómez, I., Cortés, C., Herrera-Estrella, A., 2002. Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene *prb1* in *Trichoderma atroviride*. Mol. Genet. Genomics 267, 703–712.

Quandt, K., Frech, K., Karas, H., Wingender, E., Werner, T., 1995. MatInd and MatInspector—new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. 23, 4878–4884.

Ravagnani, A., Gorfinkiel, L., Langdon, T., Diallinas, G., Adjadj, E., Demais, S., Gorton, D., Arst Jr., H.N., Scazzocchio, C., 1997. Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. EMBO J. 16, 3974–3986.

Reichard, U., Cole, G.T., Hill, T.W., Ruchel, R., Monod, M., 2000. Molecular characterization and influence on fungal development of ALP2, a novel serine proteinase from *Aspergillus fumigatus*. Int. J. Med. Microbiol. 290, 549–558.

Ren, Y., West, C.A., 1992. Elicitation of diterpene biosynthesis in rice (*Oryzae sativa* L.) by chitin. Plant Physiol. 99, 1169–1178.

Ren, Y., Wee, K.E., Chang, F.N., 2000. Deficiency of current methods in assaying endochitinase activity. Biochem. Biophys. Res. Commun. 268, 302–305.

Rose, T., Rose, J.M., Di Cera, E., 1999. Serine Protease Web Site. www.biochem.wustl.edu/~protease.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sophianopoulou, V., Suarez, T., Diallinas, G., Scazzocchio, C., 1993. Operator derepressed mutations in the proline utilization gene cluster of *Aspergillus nidulans*. Mol. Gen. Genet. 236, 209–213.

Sreedhar, L., Kobayashi, D.Y., Bunting, T.E., Hillman, B.I., Belanger, F.C., 1999. Fungal proteinase expression in the interaction of the plant pathogen *Magnaporthe poae* with its host. Gene 235, 121–129.

St Leger, R.J., Joshi, L., Bidochka, M.J., Roberts, D.W., 1996. Insecticidal properties of a genetically engineered entomopathogenic fungus constitutively expressing a toxic protease. Proc. Natl. Acad. Sci. USA 93, 6349–6354.

St Leger, R.J., Joshi, L., Roberts, D., 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl. Environ. Microbiol. 64, 709–713.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Penalva, M.A., Arst Jr., H.N., 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 14, 779–790.

Vogel, H.J., 1956. A convenient growth medium for *Neurospora* (Medium N). Microbiol. Genet. Bull. 13, 42–43.

Wiest, A., Grzegorski, D., Xu, B.W., Goulard, C., Rebuffat, S., Ebbole, D.J., Bodo, B., Kenerley, C.M., 2002. Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J. Biol. Chem. 277, 20862–20868.