Journal of Microbiology (2014) Vol. 52, No. 2, pp. 129–138
DOI 10.1007/s12275-014-3308-9

Functional Analysis of a Subtilisin-like Serine Protease Gene from Biocontrol Fungus *Trichoderma harzianum*

Haijuan Fan, Zhihua Liu*, Rongshu Zhang, Na Wang, Kai Dou, Gulijimila Mijiti, Guiping Diao, and Zhiying Wang*

School of Forestry, Northeast Forestry University, 26 Hexing Road, 150040 Harbin, P. R. China

(Received June 3, 2013 / Revised Oct 4, 2013 /Accepted Oct 14, 2013)

The subtilisin-like serine protease gene *ThSS45* has been cloned from *Trichoderma harzianum* ACCC30371. Its coding region is 1302 bp in length, encoding 433 amino acids, with a predicted protein molecular weight of 44.9 kDa and pI of 5.91. *ThSS45* was shown by RT-qPCR analysis to be differentially transcribed in response to eight different treatments. The transcription of *ThSS45* was up-regulated when grown in mineral medium, under carbon starvation, and nitrogen starvation, and in the presence of 1% root powder, 1% stem powder, and 1% leaf powder derived from *Populus davidiana* × *P. bolleana* (Shanxin poplar) aseptic seedlings. The highest increase in transcription approached 3.5-fold that of the control at 6 h under induction with 1% poplar root powder. The transcription of *ThSS45* was also slightly up-regulated by 1% *Alternaria alternata* cell wall and 5% *A. alternata* fermentation liquid. Moreover, the analyses of coding and promoter regions of *ThSS45* homologs indicated that serine protease may be involved in both mycoparasitism and antibiotic secretion. *ThSS45* was cloned into the pGEX-4T-2 vector and then expressed in *Escherichia coli* BL21. The recombinant protein, with an expected molecular weight of approximately 69 kDa, was then purified. When transformant BL21-ss was induced with 1 mM IPTG for 6 h, the purified protease activity reached a peak of 18.25 U/ml at pH 7.0 and 40°C. In antifungal assays the purified protease obviously inhibited the growth of *A. alternata* mycelia.

Keywords: *Trichoderma harzianum*, subtilisin-like serine protease, RT-qPCR, prokaryotic expression, genome

Introduction

Trichoderma species are used as biological control agents against many fungal phytopathogens, including *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Cytospora chrysosperma*, and *Rhizoctonia solani* (Yang *et al.*, 2013). Proteases, which are secreted by *Trichoderma* species during biocontrol of plant diseases, not only enhance plant resistance to biotic stresses via root colonization by *Trichoderma* species (Hanson and Howell, 2004; Pozo *et al.*, 2004), but also suppress phytopathogenic fungi by hydrolyzing cell wall proteins (De Marco and Felix, 2002; Pozo *et al.*, 2004; Viterbo *et al.*, 2004; Suarez *et al.*, 2005; Liu and Yang, 2007; Liu *et al.*, 2009). Furthermore, oligopeptides released from hydrolysis of phytopathogenic fungal proteins may play important roles in recognition between *Trichoderma* species and fungal phytopathogens (Druzhinina *et al.*, 2011). This recognition can trigger a signaling cascade and enhance the expression of secondary-metabolite-biosynthesis enzymes and cell wall lytic enzymes, and ultimately enhance the biocontrol ability of *Trichoderma* (Druzhinina *et al.*, 2011). Therefore, proteases play significant roles in the interaction of *Trichoderma* with fungal phytopathogens.

The role of proteases in biocontrol has been studied at the levels of both transcription and translation. Transcription of a serine protease gene, *tvsp1*, could be induced in wild-type *Trichoderma virens* and three *tvsp1*-overexpression strains, by the purified cell walls of *R. solani* (Pozo *et al.*, 2004). Overexpression of *tvsp1* in *T. virens* strains significantly increased the survival rate of cotton seedlings infected with *R. solani* by 15–32% over the wild-type strain (Pozo *et al.*, 2004). In addition, the aspartic protease gene *papA* from *Trichoderma asperellum* T-203 is involved in mycoparasitism (Viterbo *et al.*, 2004). Dual plate confrontation assays with the plant pathogen *R. solani* revealed a 4-fold induction in *papA* transcription in the presence of the pathogen prior to physical contact (Viterbo *et al.*, 2004). Aspartic protease P6281 (MW 33 kDa and pI 4.3) from *Trichoderma harzianum* CECT 2413 was also highly induced by cell walls of fungal pathogens *Pythium ultimum*, *Botrytis cinerea*, and *R. solani* (Suarez *et al.*, 2005). Moreover, the purified protease from an 18.8 kDa protease-overproducing *T. harzianum* strain substantially hydrolyzed the cell wall of the phytopathogen *Crinipellis perniciosa* 48 h after contact (De Marco and Felix, 2002). Protease activity has been detected not only in wild-type and protease-overproducing *Trichoderma* species, but also in heterologous overexpression strains. The aspartic protease gene *SA76* (Liu and Yang, 2007), serine protease gene *SL41* (Liu *et al.*, 2009), and subtilisin-like serine protease gene *SS10* (Liu and Yang, 2009) from *T. harzianum* T88 have been expressed in *Saccharomyces cerevisiae*, and the aspartic protease gene *TaAsp* from *T. asperellum* T4 has been expressed in *Pichia pastoris* (Yang *et al.*, 2013). The activity of these four heterologously expressed proteases was analyzed, and they were found to inhibit the growth of five phytopathogenic fungi. For example, recombinant SA76

*For correspondence. (Z. Liu) E-mail: LZHNEFU@126.com; Tel.: +86-451-82191512 / (Z. Wang) E-mail: WZYNEFU@126.com; Tel.: +86-451-8219 1512

inhibited the radial growth of five phytopathogenic fungi (*Alternaria alternata*, *F. oxysporum*, *C. chrysosperma*, *R. solani*, and *S. sclerotiorum*) by 7–38% (Liu and Yang, 2007). Thus, studying proteases from *Trichoderma* species is significant for developing approaches to detect and increase the biocontrol activity of beneficial fungi (Liu and Yang, 2009). The *T. harzianum* ACCC30371 strain is an excellent biocontrol agent against many kinds of phytopathogenic fungi (Ding *et al.*, 2012), but there are no reports on whether proteases are involved in the biocontrol activity of this strain. Thus, further research is required to identify the function of protease genes in the *T. harzianum* ACCC30371 biocontrol process.

In this study, the subtilisin-like serine protease gene *ThSS45* from biocontrol strain *T. harzianum* ACCC30371 was cloned and its sequence was analyzed. The transcription of *ThSS45* was also studied by RT-qPCR in response to eight nutritional conditions (including exposure to Shanxin poplar and its fungal host). Coding and promoter regions of *ThSS45* homologs were analyzed. In addition, *ThSS45* was cloned into the pGEX-4T-2 vector and then expressed in *Escherichia coli* BL21. The recombinant protease was purified and its enzymatic and antifungal activities were assayed to further verify its biocontrol function.

Materials and Methods

Strains, plasmids, and plant material

T. harzianum ACCC30371 was obtained from the Agricultural Culture Collection of China. *E. coli* strain TOP10 and vector pMD18-T were obtained from TakaRa Biotechnology Co. (China). *E. coli* BL21 and prokaryotic expression vector pGEX-4T-2 were from GE Healthcare (UK). Phytopathogenic fungus *A. alternata* (poplar leaf wither) was obtained from the Forestry Protection Laboratory, Northeast Forestry University of China. Aseptic *Populus davidiana* × *Populus bolleana* (Shanxin poplar) seedlings were cultured in 0.5× Murashige and Skoog (MS) differentiation medium (supplemented with 0.05 mg/L α-naphthalene acetic acid (NAA), 0.5 mg/L 6-benzyladenine (6-BA), 20 g/L sucrose, and 6.0 g/L agar at pH 5.8) and liquid rooting medium, woody plant medium (WPM) (supplemented with 0.4 mg/L indole-3-butyric acid (IBA) and 20 g/L sucrose at pH 5.8), at 25°C under a 16/8 h light/dark cycle (Faisal *et al.*, 2012).

Gene cloning and bioinformatic analysis

The full-length cDNA sequence of *ThSS45* was obtained from the transcriptome database of *T. harzianum* ACCC30371, according to a BLASTX search result. Primers for *ThSS45* cloning were ss-1 (5′-TCATCTCGCATCTCCAAGTCCT-3′) and ss-2 (5′-CGGATAAGCAGATGAGATGTCG-3′). The PCR conditions were 94°C for 3 min; 35 cycles of 94°C for 30 sec, 56°C for 40 sec, and 72°C for 80 sec; 72°C for 7 min. The amplified fragment was purified and ligated into the vector pMD18-T according to the manufacturer's instructions and then sequenced (Shanghai Sangon Co., China).

The ThSS45 amino acid sequence was used as a query to search for similar coding sequences with tBLASTN in the genome databases of *Trichoderma atroviride* ATCC74058, *T. virens* Gv29-8, *T. harzianum* CBS 226.95, and *T. asperellum* CBS 433.97 (http://genome.jgi-psf.org/). Bioinformatic analysis was performed according to the methods of Mijiti *et al.* (2012), Tamura *et al.* (2011), and Liu and Yang (2009). Furthermore, the 1,500 bp upstream fragments of these coding regions were obtained as predicted promoter regions. Regulatory motifs in these regions were predicted using the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD/) and the STRING tool (Franceschini *et al.*, 2013).

Transcription analysis of *ThSS45* in *T. harzianum*

The transcription of *ThSS45* in response to eight inducing conditions was studied, namely mineral medium (MM) with 0.5% (w/v) glucose and 0.5% (w/v) ammonium sulfate (Penttilä *et al.*, 1987), carbon starvation (0.5% (w/v) ammonium sulfate in MM without glucose), nitrogen starvation (0.5% (w/v) glucose in MM without ammonium sulfate), and variable carbon source in MM (0.5% (w/v) ammonium sulfate) as follows: 1% (w/v) root powder, 1% (w/v) stem powder, or 1% (w/v) leaf powder from Shanxin poplar, 1% (w/v) powdered cell walls of *A. alternate*, and 5% (v/v) fermentation supernatant of *A. alternata* grown in 0.25× potato dextrose (PD) (Difco, USA) medium for 10 days.

Root, stem, and leaf powders for studying induction of *ThSS45* transcription were prepared by grinding the respective parts of 30-day-old aseptic poplar seedlings with a height of 10 cm and a root length of 3.0 cm into powder. To prepare fungal phytopathogen cell walls, *A. alternata* spores were inoculated into 0.25× PD medium and cultured at 25°C and 170 rpm for 36 h. Mycelia were collected by gauze filtration, then ground into powder, which was washed five times with sterile water and collected by centrifugation at 8000 × *g* for 15 min. Fungal cell walls were prepared according to the description of Fleet and Phaff (1974).

A total of 1×10^6 conidia from *T. harzianum* (6 days of age) were inoculated into 180 ml of 0.25× PD and grown at 28°C in a shaker at 200 rpm for 48 h. Mycelia were filtered, washed, grown in MM for 2 h and then in one of the different inducing media for 72 h. Induction experiments were performed in triplicate (three flasks per inducing medium). Twenty-five milliliters of mycelia per flask were mixed and collected at 0, 2, 6, 12, 24, 48, and 72 h post-induction, and then stored at -80°C. Total RNA was extracted from mycelia using Trizol reagent (Invitrogen, USA), digested by DNaseI (Promega, USA), and reverse-transcribed into cDNA using a PrimeScript

Table 1. Primers for RT-qPCR

Gene name	Primers name	Sequence (5′→3′)	Tm (°C)	Size of product (bp)
ThSS45	Yss-L	CCTCATCAAGAACCTCGCTACC	58	89
	Yss-R	GGTTGGCGTTGTTGGCAATG		
α-Tubulin	atu-L	TGGTATGTCGGTGAGGGCATG	59	93
	atu-R	GGCGGCAACCTCTTCGTAGT		
actin	Act-L	CGACCGACTCAACAATGAGCTG	58	88
	Act-R	GAATCTACGCTCGCTCGACAAG		

RT Kit (TaKaRa) according to the manufacturer's instructions. The transcription level of *ThSS45* in *T. harzianum* was evaluated by RT-qPCR according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008; Liu *et al.*, 2010), using cDNA as a template and *actin* and *α-tubulin* as reference genes. Three RT-qPCR replicates per cDNA sample were performed. All of the above experiments were performed three times. Primer pairs for RT-qPCR (Table 1) were designed using Primer 6.0 software (PREMIER Biosoft Co., USA).

Vector construction and *E. coli* transformation

To express the ThSS45 protein in *E. coli*, the primers ss-3 (5′-TGCGGATCCATGCCTTTCCATAACCGCCACTCG CT-3′, *Bam*HI site underlined) and ss-4 (5′-CATGAATT CCGAGGTTGGCGTTGTTGGCAATGAGG-3′, *Eco*RI site underlined) were used to amplify the region of *ThSS45* that encodes the mature protein. The recombinant vector pGEX-ss and the transformant BL21-ss were obtained using the method of Tu *et al.* (2005).

SDS-polyacrylamide gel electrophoresis (PAGE)

The recombinant transformant BL21-ss and control transformant BL21-pGEX were inoculated into 10 ml of LB broth (containing 50 μg/ml ampicillin) and grown at 37°C and 160 rpm for 12 h. Cultures of BL21-ss and BL21-pGEX in logarithmic phase (OD_{600} = 0.5–0.6) were induced for 5 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C and 160 rpm. The *E. coli* cells were harvested by centrifugation at 12000 × *g* for 5 min after culturing for 1, 2, 3, 4, and 5 h. The induced cells were processed using *E. coli* Protein Extraction Solution (HaiGene, China) following the manufacturer's instructions. After adding 1× loading buffer, the supernatants and the cell pellets were boiled for 5 min, centrifuged for 10 min at 8,000 × *g*, and loaded onto a 12% (v/v) polyacrylamide gel for SDS-PAGE. Recombinant protein ThSS45 was purified using the method of Sun *et al.* (2012).

Measurement of enzyme activity

The purified recombinant protein ThSS45 (0.3 ml) and 0.5% (w/v) casein solution (0.3 ml) in NaH_2PO_4-K_2HPO_4 buffer (pH 6.0–8.0) were pre-incubated at 40°C for 5 min and then mixed. The mixture was incubated at 40°C for 10 min, and then 1.2 ml of 0.4 M trichloroacetic acid was added to the mixture to stop the reaction. The reaction mixture was centrifuged at 13000 × *g* and 4°C for 15 min. The supernatant (0.3 ml) was mixed with 1.5 ml of 0.4 M sodium carbonate and 0.3 ml of Folin phenol reagent. The mixture was incubated at different temperatures (25–55°C) for 20 min. The tyrosine content in the supernatant was determined colorimetrically at 660 nm using the Folin-phenol method (Lowry *et al.*, 1951). The heat-denatured protease ThSS45 served as the control. Four replicates were performed for each experiment. One unit activity of protease was defined as the amount of enzyme that catalyzes the release of 1 mg of L-tyrosine/min under the above conditions (Liu and Yang, 2007).

Anti-fungal activity assay

To prepare agar plates for the anti-fungal activity assay, the purified recombinant protease ThSS45 was added to potato dextrose agar (PDA) (Difco, USA) medium at a final concentration of 10% (v/v). The same volume of heat-denatured protease ThSS45 was added to PDA medium as a control. A disc of *A. alternata* mycelia (5 mm diameter) was inoculated at the center of the PDA agar plate and cultured at 28°C. Ten replicates were performed. Growth diameters of the pathogens were measured 5 d post-inoculation, and the growth inhibition rate was calculated by the method of Liu and Yang (2007).

Results

Sequence analysis of subtilisin-like serine protease ThSS45

The cDNA sequence of the subtilisin-like serine protease gene *ThSS45* is 1,302 bp in length, encoding a protein of 433 aa with a calculated molecular weight of 44.9 kDa and a pI of 5.91. The cDNA sequence was deposited in GenBank under the accession number KC876057. Analysis of the amino acid sequence identified a signal sequence cleavage site between positions Ala21 and Met22. ThSS45 is a member of the "Peptidases_S8_PCSK9_ProteinaseK_like family" (Pf00082) (Fig. 1A), and is a stable extracellular protease. Furthermore, MEROPS BLAST analysis of the amino acid sequence revealed that protease ThSS45 from *T. harzianum* ACCC30371 belongs to the subfamily S8A. The ThSS45 amino acid sequence shares 95.3% (E-value = $1.5e^{-142}$) and 69.4% (E-value = $3.6e^{-100}$) identity with known subfamily S8A unassigned peptidases (ABK64119) from *T. harzianum* T88 and *Neurospora crassa*, respectively (Rawlings *et al.*, 2012). Two potential N-glycosylation sites (Asn41-Met-Thr and Asn211-Asp-Thr) were identified in the ThSS45 amino acid sequence by the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

BLASTP analysis indicated that the amino acid sequence of protease ThSS45 has a peptidase S8 family domain that is common to protease K-like proteins. Protease K is a secreted endopeptidase with a high degree of sequence conservation. It can hydrolyze keratin and other proteins with subtilisin-like specificity (Marchler-Bauer *et al.*, 2013). To further identify the characteristics of the ThSS45 amino acid sequence, a MOTIFS search and a multiple sequence alignment were performed. The amino acid sequence of protease ThSS45 and twelve similar sequences from the BLASTP results were analyzed. The three characteristic sequence domains, seven active site residues, and two calcium binding sites of peptidase S8 were all highly conserved (Fig. 1B). The MOTIFS search showed that the sequence of *T. harzianum* ACCC 30371 ThSS45 contained the sequence domains of peptidase S8 at amino acid residues 183–194 (AYVVDTGIRVTH), 218–228 (HGSHVAGTIGG), and 374–384 (GTSMATPHV AG). Amino acid residues Asp187, His218, and Ser376 of ThSS45 form the catalytic triad of the active site, and the other four active site residues are Leu282, Gly283, Asn310, and Ser373. Calcium binding site 1 is composed of amino acid residues Pro324, Ala326, and Asp350, and calcium

binding site 2 is made up of residues Ile159, Ser162, and Asn163 (Fig. 1B). These results further confirm that the ThSS45 protein of ACCC30371 is a member of the subtilisin-like serine protease family.

Phylogenetic tree

A phylogenetic tree from amino acid sequences of subtilisin-like serine proteases was constructed based on multiple sequence alignment (Fig. 1B). The evolutionary distances ranged from 0–0.35. The phylogenetic analysis revealed that subtilisin-like serine proteases from *Trichoderma* cluster in the same branch (Fig. 2).

Analyses of the coding and promoter regions of sequences similar to ThSS45

Twenty-one sequences that were homologous to *ThSS45* were obtained from the genomes of four biocontrol *Trichoderma* species (Table 2). The genomes of *T. atroviride*, *T. virens*,

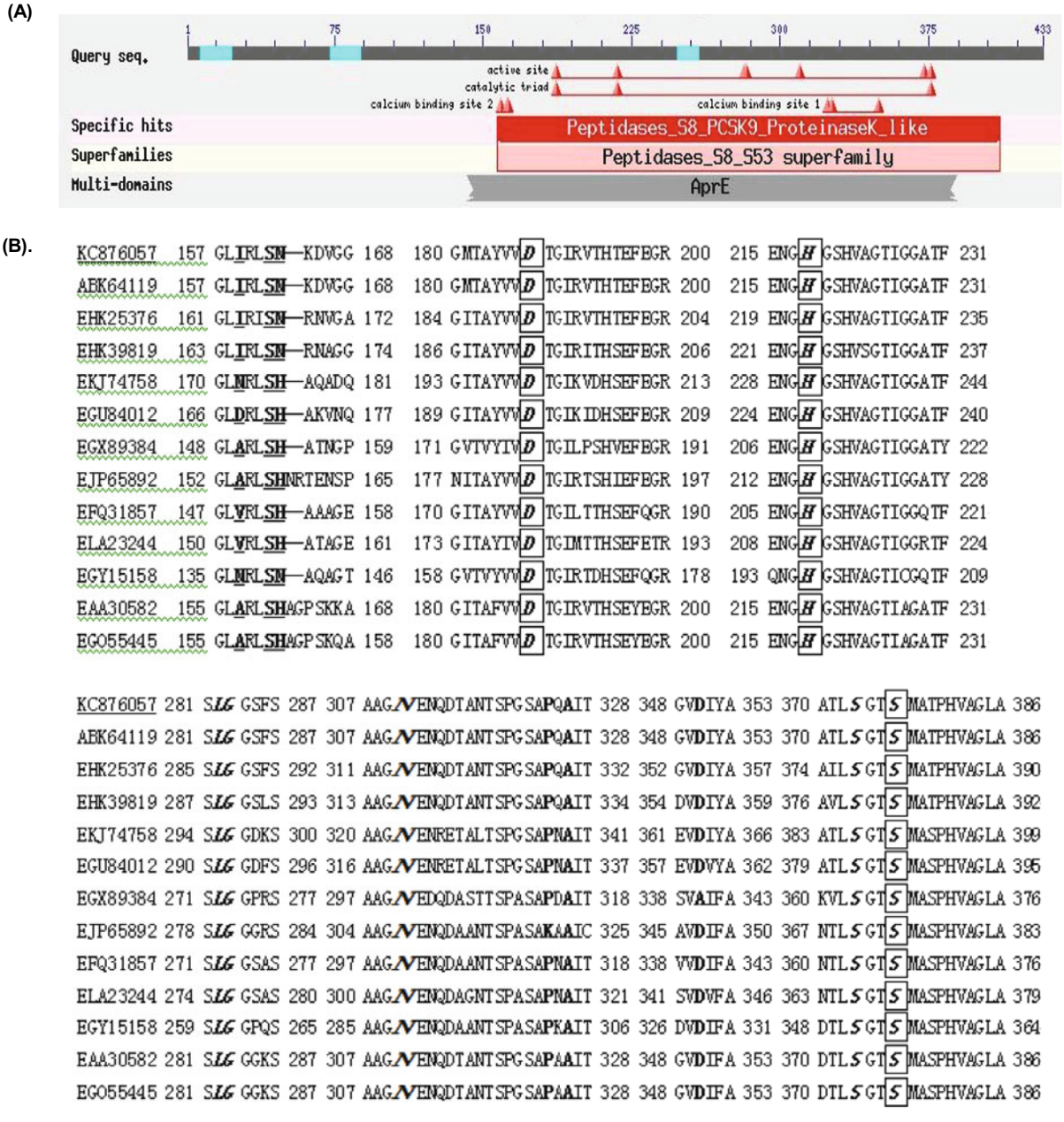

Fig. 1. Multiple sequence alignment of partially conserved domains in different subtilisin-like serine (SS) protease amino acid sequences. (A) Putative conserved domains of the ThSS45 protease from *T. harzianum* ACCC30371. B, Multiple sequence alignment of the partially conserved domain in SS proteases; KC876057 indicates ThSS45 from *T. harzianum* ACCC30371 used in the current study. The catalytic motifs of subtilisin-like serine proteases are shown with gray background. Catalytic residues are boxed. Active site residues are in bold italics. Calcium binding site 1 is marked in bold type, and calcium binding site 2 is marked in bold and underlined.

Table 2. **Coding genes and promoters of *ThSS45*-similar sequences**

Gene and promoter	Location of gene on chromosome	Similarity to amino acid of ThSS45 (%)	Number of exons	Number of introns	AA	pI	MW (kDa)	Cleavage site of signal peptide	CD
SS1-Tat	scaffold_11: 90955–92477(−)	81	4	3	439	5.74	45.19	15//16: AAA–LP	163–416
SS1-TatP	92478–93977(−)								
SS2-Tat	scaffold_1: 86459–88053(−)	43	3	2	409	6.40	42.27	20//21: VLA–AP	130–388
SS2-TatP	88054–89553(−)								
SS3-Tat	scaffold_4: 1879532–1880898(−)	42	3	2	418	6.57	45.60	20//21: ITA–LP	138–398
SS3-TatP	1880899–1882398(−)								
SS4-Tat	scaffold_1: 5552932–5554166(−)	40	3	2	371	5.90	37.89	No	101–325
SS4-TatP	5554167–5555666(−)								
SS5-Tat	scaffold_8: 226251–228890(+)	39	4	3	633	5.88	68.00	No	256–533
SS5-TatP	224751–226250(+)								
SS1-Tvi	scaffold_3: 1001421–1002769(−)	90	4	3	388	5.67	39.96	No	112–366
SS1-TviP	1002770–1004269(−)								
SS2-Tvi	scaffold_18: 661146–662949(+)	46	3	2	409	6.59	42.34	20//21: VLA–AP	130–388
SS2-TviP	659646–661145(+)								
SS3-Tvi	scaffold_9: 355685–357006(+)	45	3	2	402	7.17	41.99	20//21: VLA–AP	126–378
SS3-TviP	354185–355684(+)								
SS4-Tvi	scaffold_10: 1029518–1031340(+)	46	3	2	391	5.98	40.42	15//16: VLA–AP	117–367
SS4-TviP	1028018–1029517(+)								
SS5-Tvi	scaffold_4: 1083966–1085310(+)	39	3	2	407	6.27	43.78	20//21: VTA–YP	134–387
SS5-TviP	1082466–1083965(+)								
SS6-Tvi	scaffold_10: 1166934–1169073(+)	36	2	1	539	5.81	57.31	No	160–437
SS6-TviP	1165434–1166933(+)								
SS1-Tha	scaffold_6: 1492679–1494156(+)	100	4	3	433	5.91	44.91	21//22: AAA–MP	157–411
SS1-ThaP	1491179–1492678(+)								
SS2-Tha	scaffold_3: 3071034–3072619(+)	45	3	2	409	6.40	42.48	20//21: VLA–AP	130–388
SS2-ThaP	3069534–3071033(+)								
SS3-Tha	scaffold_11: 1032060–1033521(+)	47	3	2	380	5.52	39.03	No	106–356
SS3-ThaP	1030560–1032059(+)								
SS4-Tha	scaffold_9: 1349423–1350792(−)	36	3	2	408	6.06	43.65	20//21: VTA–LP	135–386
SS4-ThaP	1350793–1352292(−)								
SS5-Tha	scaffold_11: 1164822–1166952(+)	35	2	1	539	5.43	57.60	16//17: AQA–ST	161–438
SS5-ThaP	1163322–1164821(+)								
SS1-Tas	scaffold_7: 783056–784580(−)	78	4	3	438	5.91	45.20	15//16: AAA–LP	162–415
SS1-TasP	784581–786080(−)								
SS2-Tas	scaffold_5: 2272226–2274070(+)	44	3	2	409	6.80	42.46	20//21: VLA–AP	130–387
SS2-TasP	2270726–2272225(+)								
SS3-Tas	scaffold_1: 3758894–3760129(−)	43	3	2	371	5.60	37.97	No	101–325
SS3-TasP	3760130–3761629(−)								
SS4-Tas	scaffold_10: 880180–881513(+)	43	3	2	402	6.57	43.99	20//21: IAA–LP	128–382
SS4-TasP	878680–880179(+)								
SS5-Tas	scaffold_18: 33698–35800(+)	39	2	1	539	5.43	57.53	No	160–437
SS5-TasP	32198–33697(+)								

The subtilisin-like serine protease genes were named in the order that they were retrieved. SS, subtilisin-like serine protease; Tat, *T. atroviride* ATCC74058; Tvi, *T. virens* Gv29-8; Tha, *T. harzianum* CBS 226.95; Tas, *T. asperellum* CBS 433.97; AA, number of amino acids; pI, isoelectric point; MW, molecular weight of protein; CD, conserved domain; (+), plus strand coding; (−), minus strand coding.

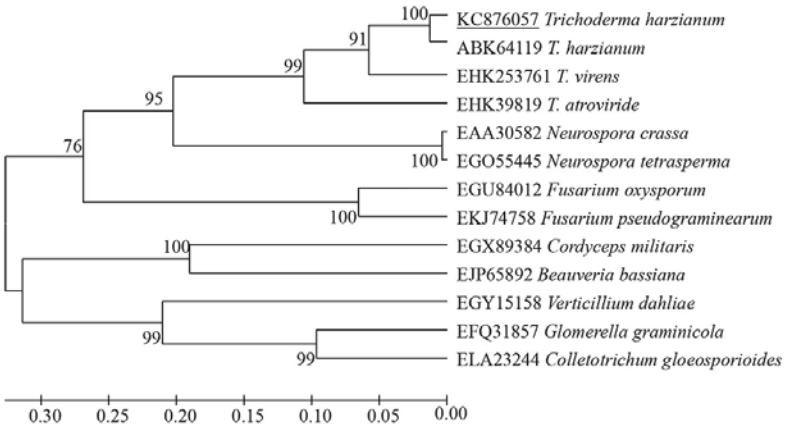

Fig. 2. Phylogenetic analysis of amino acid sequences of subtilisin serine proteases from 13 different fungi. KC876057 indicates ThSS45 from *T. harzianum* ACCC30371 used in this study. The tree was constructed by the maximum likelihood method using the MEGA 5.1 program. The numbers next to the branching points indicate the percentages with which a given branch was supported in 1000 bootstrap replicates. Scale bar: 0.05 represents 5% amino acid difference in protein evolution.

T. harzianum, and *T. asperellum* contained five, six, five, and five *ThSS45*-similar sequences, respectively (Table 2). The similarity of their amino acid sequences to ThSS45 ranged from 35–100%. The numbers of exons in the coding regions of these sequences ranged from two to four, and the number of corresponding introns ranged from one to three. The number of amino acids ranged from 371–539, and predicted molecular weights ranged from 37.9–58.0 kDa. Fourteen sequences contained signal peptide cleavage sites. Among them, nine cleavage sites were between positions 20 and 21. The conserved domains were mostly composed of 250–260 amino acids. Except for protease SS3-Tvi, most serine proteases in the four genomes could be classified as acidic proteases according to their isoelectric point (pI) (Table 2). The calculated pI of 7.17 for SS3-Tvi indicated that this serine protease could hydrolyze protein under alkaline conditions. This means higher functional stability in hydrolyzing phytopathogenic fungal protein during mycoparasitism of *Trichoderma* species.

The names of promoters of *ThSS45*-similar sequences and their locations in the genomes of the four biocontrol *Trichoderma* species are shown in Table 2. The functional descriptions of regulatory motifs in promoter regions are shown in Table 3, and their locations in the promoter regions are shown in Fig. 3. They are involved in stress response, glucose hydrolysis, ethanol metabolism, transcription enhancement, and repression of gene expression. Many motifs were related to the biocontrol of *Trichoderma* species. The presence of these motifs further verified that serine proteases are involved in biocontrol by *Trichoderma* species. For instance, four alcohol dehydrogenase regulator 1 (ADR1) binding motifs in SS1-ThaP may confer on *T. harzianum* the ability to generate high levels of antifungal chemical substances such as acetaldehyde (Fig. 3).

Transcription of ThSS45 in response to eight different induction conditions

To investigate the transcription of *ThSS45* in response to plant and fungal phytopathogens, RT-qPCR was performed. The RT-qPCR analysis indicated that transcription of *ThSS45* was differentially regulated by different culture conditions. The transcription of *ThSS45* was up-regulated in cultures supplemented with 1% (w/v) root, stem, and leaf powders from the Shanxin poplar, with peak transcription increases of 3.47-, 2.28-, and 2.65-fold at 6, 12, and 6 h of culture, respectively (Fig. 4D–4F). The transcription of *ThSS45* was slightly up-regulated in media containing 1% (w/v) *A. alternata* cell wall and 5% (v/v) *A. alternata* fermentation liquid, at 6 h and 12 h post-induction, reaching levels of 1.40 and 1.50 times those of pretreatment, respectively (Fig. 4G and 4H). In addition, the impact of carbon or nitrogen starvation on the transcription of *ThSS45* was studied. The transcription of *ThSS45* was up-regulated in normal, carbon-

Table 3. Number of element-binding motifs in promoters of *ThSS45*-similar sequences and descriptions of the function of elements

Element name	TasP	TatP	ThaP	TviP	Total	Function description[a]
GCN4	27	27	27	33	114	amino acid biosynthesis and multiple stress responses
HSE	6	6	4	5	21	heat stress response
STRE	2	1	4	4	11	multiple stress responses
AP-1	0	0	1	2	3	involved in pleiotropic drug resistance
GCR1	24	20	20	23	87	glycolytic genes transcriptional activator
GAL4	1	1	1	1	4	contributes to converting galactose to glucose
ADR1	7	9	12	20	48	glucose-repressible alcohol dehydrogenase transcriptional activator
ABF1	3	4	4	5	16	transcriptional activator
TBP	2	4	2	2	10	directing the transcription of genes
MIG1	0	0	3	1	4	involved in glucose repression of some gene expression

TatP, TviP, ThaP, TasP: Promoters of *ThSS45*-similar sequences in genomes of *T. atroviride* ATCC74058, *T. virens* Gv29-8, *T. harzianum* CBS 226.95 and *T. asperellum* CBS 433.97, respectively. [a] Functional descriptions were obtained by searching the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/).

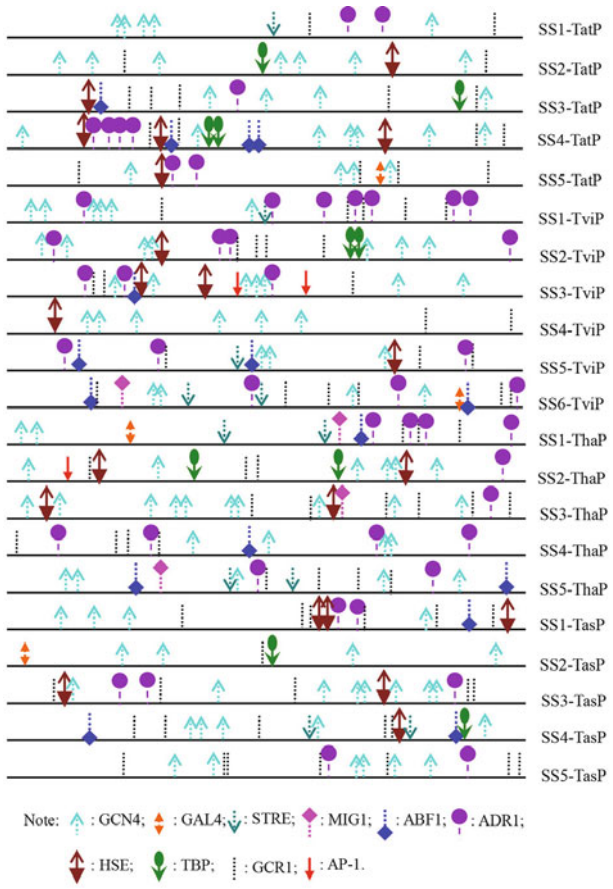

Fig. 3. Regulatory element-binding motifs in promoters of *ThSS45*-similar sequences from four biocontrol *Trichoderma* genomes.

deficient, and nitrogen-deficient MM media, in which the transcription peaks were 3.83, 2.15, and 2.02 times those of the control at 72, 12, and 72 h, respectively (Fig. 4A–4C). In summary, the transcription of *ThSS45* was mainly up-regulated under the eight different conditions. Both plant and plant pathogenic fungi triggered *ThSS45* transcription, and the transcription peak was obtained within 12 h of treatment.

Heterologous expression of *ThSS45* in *E. coli* BL21

Compared to the control transformant BL21-pGEX, the recombinant transformant BL21-ss produced a clear protein band with a molecular weight of approximately 69 kDa on the SDS-PAGE gel (Fig. 5A). This result indicated that protease ThSS45 had been successfully synthesized in the *E. coli* cells. The recombinant protein was also successfully purified (Fig. 5B). The optimal enzyme reaction temperature was 40°C and the optimal pH was 7.0 (data not shown). When transformant BL21-ss was induced with 1 mM IPTG for 6 h, the purified protease activity reached a peak of 18.3 U/ml at pH 7.0 and 40°C (Fig. 6).

Inhibition of purified recombinant protein ThSS45 on the growth of *A. alternata* mycelia

Purified recombinant protein ThSS45 clearly inhibited the growth of *A. alternata* mycelia on PDA plates (Fig. 7). When *A. alternata* mycelia were cultured for 5 d on PDA plates with 10% (v/v) purified protease ThSS45, the inhibition rate reached 12%.

Discussion

In this study, the subtilisin-like serine protease gene *ThSS45* was cloned from *T. harzianum* ACCC30371. The coding and promoter regions of *ThSS45*-similar sequences in the genomes of four biocontrol *Trichoderma* species were also analyzed.

Analysis of the coding regions of sequences homologous to *ThSS45* from four biocontrol *Trichoderma* species showed there were at least five *ThSS45*-similar sequences in each genome (Table 2), and that these serine proteases may complement each other in the process of *Trichoderma* mycoparasitism. Furthermore, bioinformatic analysis revealed that many regulatory motifs in promoter regions of *ThSS45*-similar sequences were closely related to the biocontrol role of *Trichoderma*. Binding motifs of stress response elements GCN4, HSE, STRE, and AP-1, which are involved in response to heat, oxidative and osmotic stress, and drugs, were detected in these promoters (Table 3 and Fig. 3). The STRE binding motif has also been found in the aspartyl protease (*papA*), basic proteinase (*prb1*), and endochitinase (*ech42*) gene promoter sequences in *T. harzianum* (Cortés *et al.*, 1998; Delgado-Jarana *et al.*, 2002). The presence of stress response element binding motifs indicated the serine proteases possess obvious roles in the biocontrol by *Trichoderma* species. Several recognition sites for GCR1 and GAL4, which are related to glycolysis and conversion of galactose to glucose, respectively, were also detected in the promoters (Table 3 and Fig. 3). The presence of these motifs may confer *Trichoderma* species with high glucose hydrolytic ability, suggesting that serine proteases have synergistic effects with glucanase on the degradation of phytopathogenic fungal cell walls, similar to the phenomenon described by Elad and Kapat (1999). ADR1 binding motifs were also found in the promoters (Table 3 and Fig. 3). These motifs may contribute to the conversion of ethanol to acetaldehyde, which is an antifungal secondary metabolite released by *Trichoderma* species (Sivasithamparam and Ghisalberti, 1998). Finally, binding sites for TATA-binding protein TBP and transcription activator ABF1 were identified in promoters (Table 3 and Fig. 3). These may help activate the transcription of serine protease genes and increase the biocontrol activity of *Trichoderma* species. In summary, the above results revealed that serine proteases play key roles in the interaction of *Trichoderma* species with phytopathogenic fungi.

Many biocontrol proteases in *Trichoderma* species have been identified and their transcription patterns have been studied under stimulation by phytopathogenic fungi, plants, and different carbon or nitrogen sources. In these studies, the transcription of protease genes was induced by cell walls of phytopathogenic fungi. For example, in the presence of cell walls from fungi (*C. chrysosperma*, *R. solani* or *F. oxysporum*), *SS10* mRNA levels peaked at 4 h, with a strong decay of the signal after 12 h (Liu and Yang, 2009). In this study,

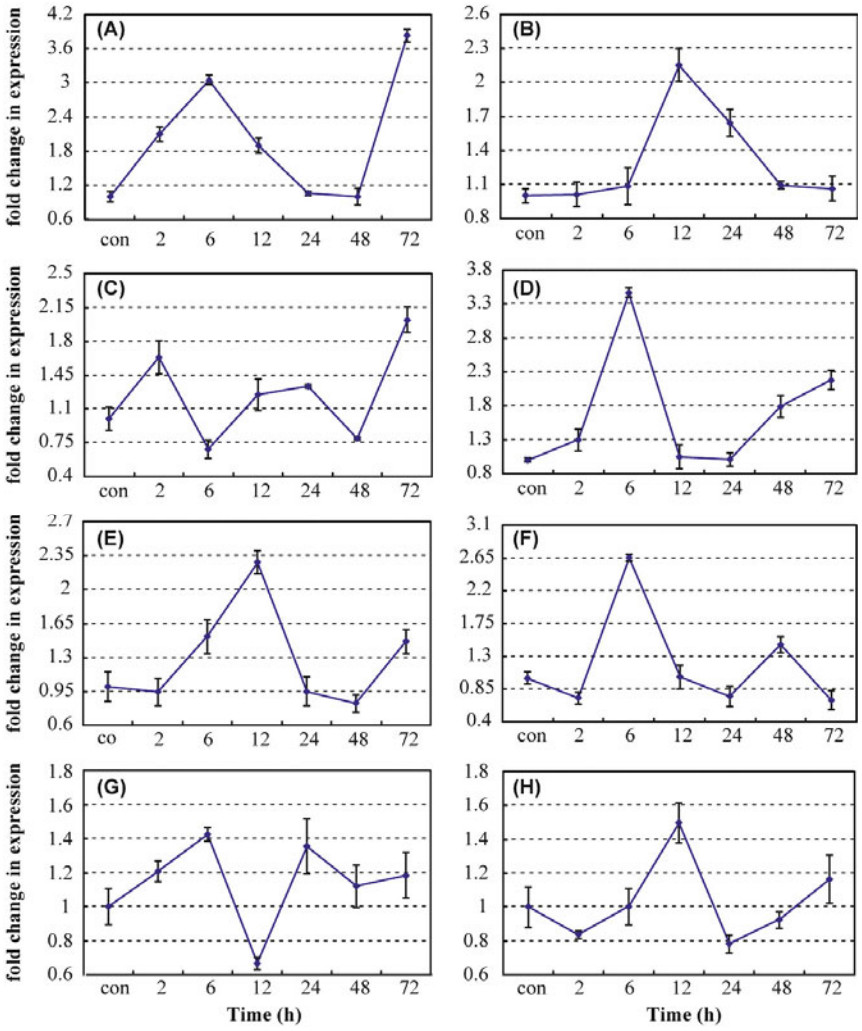

Fig. 4. Expression of serine protease gene *ThSS45* in *T. harzianum* in response to eight different culture conditions. X-axis, time points; Y-axis, fold change in expression, i.e. expression level of treatment/expression level at 0 h. Con, control gene expression level at 0 h. Expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method. Values represent the mean of three independent experiments ± standard deviation. (A) MM with 0.5% glucose and 0.5% ammonium sulfate; (B) carbon starvation; (C) nitrogen starvation; (D) 1% Shanxin poplar root powder; (E) 1% Shanxin poplar stem powder; (F) 1% Shanxin poplar leaf powder; (G) 1% *A. alternate* cell wall; (H) 5% fermentation supernatant from *A. alternata* grown in 0.25× PD for 10 d.

transcription of the serine protease gene *ThSS45* from *T. harzianum* ACCC30371 was also slightly up-regulated in the presence of 1% cell wall or 5% fermentation supernatant from *A. alternata*, as determined by RT-qPCR analysis (Fig. 4G and 4H). These results indicate that exposure to phytopathogenic fungi could trigger *ThSS45* gene transcription, resulting in increased biocontrol activity of *Trichoderma* species against fungal phytopathogens.

The transcription of protease genes from *Trichoderma* species is also up-regulated by plant roots. For example, transcription levels of aspartic protease and subtilisin-like serine protease were significantly up-regulated by 2-fold after 20 h in a *T. harzianum* T34-tomato roots interaction model (Rubio *et al.*, 2012). In this study, the highest level of induction approached 3.5 times the control at 6 h, under induction with 1% poplar root powder. The transcription of *ThSS45* could also be induced by poplar stem or leaf powders (Fig. 4D–4F). This induction of *ThSS45* gene transcription shows plants can stimulate the biocontrol activity of *Trichoderma* species.

In addition, the transcription of some biocontrol proteases is subject to nitrogen and carbon repression. For instance, transcription of serine protease gene *SS10* was repressed by MM (1% glucose and 0.5% ammonium) and nitrogen starvation (1% glucose and 0% ammonium), and weakly in-

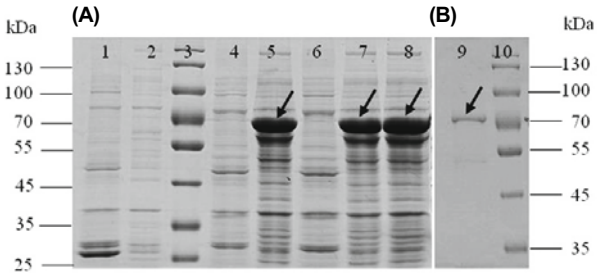

Fig. 5. SDS-PAGE of recombinant protein ThSS45 from transformant BL21-ss. (A) Unpurified recombinant protein; (B) Purified recombinant protein. Arrows indicate the protein ThSS45. Lanes: 1, supernatant of control transformant BL21-pGEX induced for 4 h; 2, Cell lysate of control transformant BL21-pGEX induced for 4 h; 3, Protein marker; 4, Supernatant from transformant BL21-ss induced for 3 h; 5, Cell lysate of transformant BL21-ss induced for 3 h; 6, Supernatant from transformant BL21-ss induced for 4 h; 7, Cell lysate of transformant BL21-ss induced for 4 h; 8, Cell lysate of transformant BL21-ss induced for 5 h; 9, Purified recombinant ThSS45 protein; 10, Protein marker.

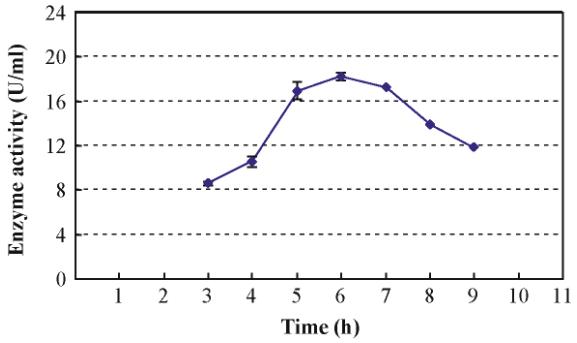

Fig. 6. Effect of inducing time on enzymatic activity of purified protease ThSS45. Transformant BL21-ss was induced with 1 mM IPTG and samples were collected from 3–9 h post-inoculation at 1-h intervals. Protease was purified from the samples and then activity was measured. Activity values represent protease activity per ml supernatant from purified protease ThSS45. The error bars indicate the mean of four replications ± standard deviation. One unit activity of protease is defined as the amount of enzyme that catalyzes the release of 1 mg of L-tyrosine/ min at pH 7.0 and 40°C.

duced by carbon starvation stress (0% glucose and 0.5% ammonium) (Liu and Yang, 2009). Transcription of aspartic protease gene *SA76* (Liu and Yang, 2007) and serine protease gene *SL41* (Liu *et al.*, 2009) was repressed by MM (2% glucose and 0.5% ammonium), and weakly induced under carbon or nitrogen starvation. In the current study, the regulatory motif search revealed the presence of MIG1-binding motifs in promoters SS1-ThaP, SS3-ThaP, and SS5-ThaP (Fig. 3). The regulatory motif for MIG1 is related to glucose repression (Westholm *et al.*, 2008). The MIG1-binding motif is functionally similar to motifs of the carbon catabolite repressor CreA, which was found in the basic protease gene (*prb1*) and aspartic protease gene (*P6281*) promoter sequences of *T. harzianum* (Cortés *et al.*, 1998; Suarez *et al.*, 2005). However, the transcription of *ThSS45* from *T. harzianum* ACCC30371 was not repressed by glucose or nitrogen. The peak transcription level of *ThSS45* in mycelia was 3.83 times that of the control at 72 h in MM culture (0.5% glucose and 0.5% ammonium sulfate). Transcription of *ThSS45* was also up-regulated, to peak at more than 2-fold that of the control, in response to carbon or nitrogen starvation (Fig. 4A–4C). The main difference between the present study and previous studies is that we used glucose concentrations 0.25–0.5 times those of the other studies (Suarez *et al.*, 2004; Viterbo *et al.*, 2004; Liu and Yang, 2007, 2009; Liu *et al.*, 2009). This suggested that glucose repression of protease gene transcription may be heavily dependent on the concentration of glucose. Further investigations will be required to determine how the role of protease ThSS45 in the mycoparasitic process is affected by environmental factors. In summary, the transcription of subtilisin-like serine protease could be induced by both pathogenic fungi and their host plant, including both the herbaceous and woody parts of the plant.

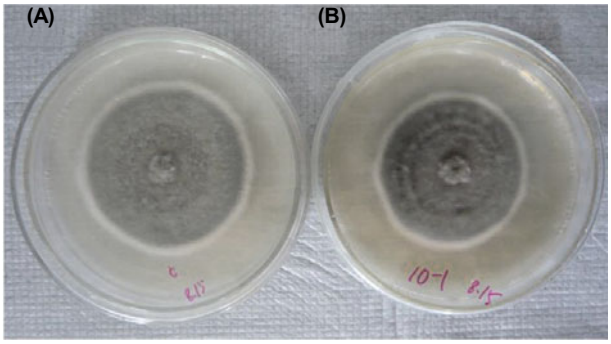

Fig. 7. Antifungal assay of purified protease ThSS45. Five-day-old *A. alternate* mycelia grown on a PDA plate supplemented with 10% (v/v) purified protease ThSS45. (A) Denatured purified protease ThSS45; (B) Purified protease ThSS45.

In this study, the subtilisin-like serine protease gene *ThSS45* was cloned and its characteristics were analyzed by bioinformatic methods. Furthermore, *ThSS45* was expressed in *E. coli*. Enzymatic activity and antifungal ability of the purified recombinant protease ThSS45 were further assayed. These results may provide theoretical support and a practical reference for the development of biological protease fungicides from *T. harzianum*.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NSFC: 31170601), the Chinese National Scientific Research Special Project in Forestry Public Industry (201104069), the National High Technology Research and Development Program of China (863 Program) (2013 AA102701), the Science and Technology Innovation Talents Foundation of Harbin City of China (2011RFQXN051), and the Heilongjiang Province Postdoctoral Science Foundation (LBH-Q10156).

References

Cortés, C., Gutiérrez, A., Olmedo, V., Inbar, J., Chet, I., and 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.

De Marco, J.L. and Felix, C.R. 2002. Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches' broom disease. *BMC Biochem.* **3**, 3.

Delgado-Jarana, J., Rincon, A.M., and Benitez, T. 2002. Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization. *Microbiology* **148**, 1305–1315.

Ding, D., Li, S., Tang, S., and Gu, J. 2012. Analysis of ITS PCR-RFLP and differences in disease resistance diversity of *Trichoderma*. *Biotechnol. Bull.* **2012**, 146–153.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V., and Kubicek, C.P. 2011. *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol.* **9**, 749–759.

Elad, Y. and Kapat, A. 1999. The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. *Eur. J. Plant Pathol.* **105**, 177–189.

Faisal, M., Alatar, A.A., Ahmad, N., Anis, M., and Hegazy, A.K. 2012. An efficient and reproducible method for *in vitro* clonal multiplication of *Rauvolfia tetraphylla* L. and evaluation of ge-

netic stability using DNA-based markers. *Appl. Biochem. Biotechnol.* **168**, 1739–1752.

Fleet, G.H. and Phaff, H.J. 1974. Glucanases in *Schizosaccharomyces* isolation and properties of the cell wall-assiociated β-(1-3)-glucanases. *J. Biol. Chem.* **249**, 1717–1728.

Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., von Mering, C., and *et al.* 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* **41**, 29.

Hanson, L.E. and Howell, C.R. 2004. Elicitors of plant defense responses from biocontrol strains of *Trichoderma viren*. *Phytopathology* **94**, 171–176.

Liu, Y. and Yang, Q. 2007. Cloning and heterologous expression of aspartic protease SA76 related to biocontrol in *Trichoderma harzianum*. *FEMS Microbiol. Lett.* **277**, 173–181.

Liu, Y. and Yang, Q. 2009. Cloning and heterologous expression of SS10, a subtilisin-like protease displaying antifungal activity from *Trichoderma harzianum*. *FEMS Microbiol. Lett.* **290**, 54–61.

Liu, Y., Yang, Q., and Song, J. 2009. A new serine protease gene from *Trichoderma harzianum* is expressed in *Saccharomyces cerevisiae*. *Prikl. Biokhim. Mikrobiol.* **45**, 28–32.

Liu, Z.H., Yang, X.X., Sun, D.M., Song, J.Z., Chen, G., Juba, O., and Yang, Q. 2010. Expressed sequence tags-based identification of genes in a biocontrol strain *Trichoderma asperellum*. *Mol. Biol. Rep.* **37**, 3673–3681.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M.K., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., and *et al.* 2013. CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res.* **41**, D348–352.

Mijiti, G., Fan, H.J., Liu, Z.H., Wang, N., Dou, K., Huang, Y., and Wang, Z.Y. 2012. Cloning and sequence analysis of small molecular hydrophobin protein *hyb2* gene from *Trichoderma asperellum* T4. *Chinese Agricultural Science Bulletin* **28**, 85–91.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., and Knowles, J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* **61**, 155–164.

Pozo, M.J., Baek, J.M., Garcia, J.M., and Kenerley, C.M. 2004. Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. *Fungal Genet. Biol.* **41**, 336–348.

Rawlings, N.D., Barrett, A.J., and Bateman, A. 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* **40**, 15.

Rubio, M.B., Dominguez, S., Monte, E., and Hermosa, R. 2012. Comparative study of *Trichoderma* gene expression in interactions with tomato plants using high-density oligonucleotide microarrays. *Microbiology* **158**, 119–128.

Schmittgen, T.D. and Livak, K.J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **3**, 1101–1108.

Sivasithamparam, K. and Ghisalberti, E. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. *Trichoderma* and *Gliocladium*, vol. 1 ed. *In* Harman, G.E. and Kubicek, C.P. (eds.), pp. 139–191. Taylor and Francis Ltd., London, UK.

Suarez, B., Rey, M., Castillo, P., Monte, E., and Llobell, A. 2004. Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. *Appl. Microbiol. Biotechnol.* **65**, 46–55.

Suarez, M.B., Sanz, L., Chamorro, M.I., Rey, M., Gonzalez, F.J., Llobell, A., and Monte, E. 2005. Proteomic analysis of secreted proteins from *Trichoderma harzianum*. Identification of a fungal cell wall-induced aspartic protease. *Fungal Genet. Biol.* **42**, 924–934.

Sun, H., Yang, H., Yang, L., Li, Z., Du, M., Chen, Y., and Jiang, X. 2012. Construction of a prokaryotic expression vector of human tau multi-epitope peptide and immunogenicity of the expressed product. *J. South Med. Univ.* **32**, 185–188.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Tu, Z., He, G., Li, K.X., Chen, M.J., Chang, J., Chen, L., Yao, Q., Liu, D.P., Ye, H., and Shi, J. 2005. An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains. *Electron. J. Biotechnol.* **8**, 113–120.

Viterbo, A., Harel, M., and Chet, I. 2004. Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. *FEMS Microbiol. Lett.* **238**, 151–158.

Westholm, J.O., Nordberg, N., Muren, E., Ameur, A., Komorowski, J., and Ronne, H. 2008. Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. *BMC Genomics* **9**, 1471–2164.

Yang, X., Cong, H., Song, J., and Zhang, J. 2013. Heterologous expression of an aspartic protease gene from biocontrol fungus *Trichoderma asperellum* in *Pichia pastoris*. *World J. Microbiol. Biotechnol.* **29**, 2087–2094.