The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 939–946
DOI 10.1007/s12275-012-2199-x

Characterization, Cloning, and Heterologous Expression of a Subtilisin-Like Serine Protease Gene *VlPr1* from *Verticillium lecanii*

Gang Yu, Jin-Liang Liu, Li-Qin Xie, Xue-Liang Wang, Shi-Hong Zhang, and Hong-Yu Pan*

College of Plant Sciences, Jilin University, Changchun, Jilin 130062, P. R. China

(Received April 16, 2012 / Accepted July 14, 2012)

The entomopathogenic fungus *Verticillium lecanii* is a well-known biocontrol agent. *V. lecanii* produces subtilisin-like serine protease (Pr1), which is important in the biological control activity of some insect pests by degrading insect cuticles. In this study, a subtilisin-like serine protease gene *VlPr1* was cloned from the fungus and the VlPr1 protein was expressed in *Escherichia coli*. The *VlPr1* gene contains an open reading frame (ORF) interrupted by three short introns, and encodes a protein of 379 amino acids. Protein sequence analysis revealed high homology with subtilisin serine proteases. The molecular mass of the protease was 38 kDa, and the serine protease exhibited its maximal activity at 40°C and pH 9.0. Protease activity was also affected by Mg^{2+} and Ca^{2+} concentration. The protease showed inhibitory activity against several plant pathogens, especially towards *Fusarium moniliforme*.

Keywords: serine protease, *Verticillium lecanii*, cloning, characterization

Introduction

The mitosporic fungus *Verticillium lecanii* (Zimmermann) Viegas is a well-known entomopathogen with a broad range of insect hosts, including insects: *Coleoptera*, *Orthopteta*, *Homoptera*, and *Lepidoptera* (Xu *et al*., 2011). The insecticidal property of this fungus has been commercially developed into biopesticides (Goettel *et al*., 2008). However, it was recently discovered that this entomopathogenic fungus also exhibits activity against numerous phytopathogenic fungi including *Sphaerotheca macularis* (Miller *et al*., 2004), *Puccinia coronata* (Leinhos and Buchenauer, 1992), *Penicillium digitatum* (Benhamou and Brodeur, 2000), and *Pythium ultimum* (Benhamou and Brodeur, 2001), which suggests that this entomopathogen also has potential to be developed as biopesticides (Faria and Wraight, 2007). Fungi that may control plant pathogenic fungi can act through two mechanisms, antibiosis and mycoparasitism (Kiss, 2003). Antibiosis is the production of antimicrobial compounds, while mycoparasitism is the feeding on a fungus by another organism. Some *Lecanicillium* isolates act as mycoparasites, attaching to powdery mildew mycelia and conidia, producing enzymes such as chitinase, which allow penetration of the mildew spores and hyphae, ultimately killing the pathogen (Askary *et al*., 1997). In addition to mycoparasitism, this mode of action was linked to colonization of host plant tissues, triggering a plant defense reaction (Benhamou and Brodeur, 2001). In mycoparasitism, cell wall-degrading enzyme preparations which play a significant role in cell wall lysis are a mixture of several enzymes, but virtually all of them contain some proteases (Yan and Qian, 2009). Fungal proteases may be significantly involved in antagonistic activity, not only in the breakdown of the host cell wall (composed of chitin and glucan polymers embedded in, and covalently linked to, a protein matrix) (Kapteyn *et al*., 1996), but also by acting as proteolytic inactivators of pathogen enzymes involved in the plant infection process (Suarez *et al*., 2004). By understanding the biocontrol mechanisms of action and regulation of proteases, the development of approaches for detecting and increasing the biocontrol activity of beneficial fungi may be achieved (Yan and Qian, 2009).

Extracellular subtilisin-like serine protease Pr1 (EC 3.4.21) is the most important factor for the insecticidal activity of entomopathogenic fungi (Joshi *et al*., 1995). Previously, the *Pr1* genes from *Metarhizium anisopliae* and *Beauveria bassiana*, which are the biocontrol agents of some insect species, were isolated and analyzed. The former gene was overexpressed in *M. anisoplia*, which greatly enhanced the strain's virulence (St Leger *et al*., 1996), and the subtilisin-like protease CDEP in *B. bassiana* could improve insecticidal activity towards to *H. armigera* larvae when it was co-fused with the Cry1Ac protein and expressed in *Bacillus thuringiensis* (Xia *et al*., 2009). However, the role of *Pr1* gene in *V. lecanii* remains unclear. *V. lecanii* has a wide host range, therefore we explored the molecular mechanism of *V. lecanii* infection at the molecular level. In this study, we cloned and analyzed the *Pr1* gene from *V. lecanii*. The VlPr1 protein was expressed in *E. coli*, and purified from *V. lecanii*, then further characterized as part of our plan for improving *V. lecanii* by genetic engineering.

Materials and Methods

Fungal and bacterial strains and growth conditions

V. lecanii strain CA-12 was originally isolated from a whitefly *Trialeurodes vaporariorum* Changchun urban, Jilin Province,

*For correspondence. E-mail: panhongyu@jlu.edu.cn; Tel.: +86-431-87836251; Fax: +86-431-86758762

China, in 2003. A single-spore isolate of CA-12 was stored in 20% glycerol at -80°C. Cultures were grown on potato dextrose agar (PDA) at 25°C with a daily cycle consisting of 16 h light and 8 h dark. *E. coli* DH5α and BL21 (DE3) were employed for DNA manipulation. Plasmid pMD18T was obtained from TaKaRa (Japan). The expression vector pET28a was purchased from Clontech (Japan). To induce the expression of the protease gene, actively growing mycelium of *V. lecanii* was transferred from a PDA plate to the inducing SMCS medium containing the following: 680 mg of KH_2PO_4, 870 mg of K_2HPO_4, 200 mg of KCl, 1 g of NH_4NO_3, 200 mg of $MgSO_4{\cdot}7H_2O$, 200 mg of $CaCl_2$, 2 mg of $ZnSO_4$, 2 mg of $FeSO_4$, 2 mg of $MnSO_4$, 10 ml of 2% casein, 5 g of sucrose in 1 L of distilled water, final pH 6.5.

Extraction of subtilisin-like serine protease

To obtain mycelia of *V. lecanii* CA12, an aliquot of 100 µl of conidial suspension (1×10^7 conidia/ml) of *V. lecanii* was inoculated into the SMCS medium with shaking (200 rpm) at 25°C for 120 h. For enzyme purification, the mycelia were filtered from of the cultures after 5 days of incubation. The filtrate was centrifuged at 10,000 rpm for 30 min at 4°C. The resultant supernatant was used as crude enzyme solution. All procedures of the protease purification were carried out at 4°C. The concentration of total proteins was measured by the Bradford protein assay (Bradford, 1976), with bovine serum albumin (BSA) used as the standard. The protein samples were then stored at -20°C for further use.

The extracted crude enzyme solution was slowly mixed with solid ammonium sulfate to 90% saturation. After deposition for 12 h, the precipitate was collected by centrifugation at 10,000 rpm for 15 min, dissolved in 50 mM HAc-NaAc and dialyzed overnight against three changes of the 50 mM phosphate buffer (pH 6.0). The dialyzed sample was clarified by centrifugation and then loaded onto a Phenyl-Sepharose column (1×20 cm) equilibrated with 50 mM HAc-NaAc. The proteins were eluted with a gradient of ammonium sulfate from 0% to 50% saturation in 50 mM HAc-NaAc buffer (pH 6.0). Fractions with protease activity were collected and dialyzed overnight against 50 mM HAc-NaAc buffer (pH 6.0). Dialyzed fractions were loaded onto a DEAE-cellulose column (1×20 cm) pre-equilibrated with 50 mM HAc-NaAc buffer (pH 6.0). The unbound proteins were washed out with 50 mM HAc-NaAc buffer (pH 6.0) then bound proteins were eluted with 30 ml of a 0.2–0.4 M NaCl gradient in 50 mM HAc-NaAc buffer (pH 6.0). Fractions with protease activity were dialyzed against 50 mM Tris-HCl buffer (pH 8.0). Dialyzed samples were chromatographed on a CM-Sepharose Fast Flow column (3 cm^3 bed volume) adjusted with 50 mM Tris-HCl buffer (pH 8.0). The unbound proteins were washed out with 50 mM Tris-HCl buffer (pH 8.0) then bound proteins were eluted with 60 ml of the 0.2–0.5 M NaCl gradient in 50 mM Tris-HCl buffer (pH 8.0). Fractions with protease activity were pooled and concentrated to 2 ml using PEG according to the manufacturer's instructions and stored at -20°C. Enzyme purity and molecular mass were determined by SDS-PAGE (Laemmli, 1970). Gels (12%) were stained with silver for protein visualization.

Protease activity assay

Protease activity was examined using a colorimetric assay. The reaction mixture contained 1 ml of 2% casein and 1 ml enzyme sample. After incubation at 37°C for 30 min, 2 ml of 0.4 M Trichloroacetic acid (TCA) were added to reaction mixture. 5 ml of 0.4 M Na_2CO_3 were added to 1 ml the reaction mixture long with 1 ml Folin reagent and then incubated at 40°C for 20 min. It was centrifuged at 4,000 rpm for 5 min. The amount of tyrosine released in the supernatant was determined by the method described by Shimada *et al.* (1994). Tyrosine was used as the standard. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per min under the assay conditions. Protein concentrations were determined using the method of Lowry (1951), using BSA as the standard.

Characterization of protease

The pH activity profile of protease was determined by measuring the enzyme activity at 37°C (Richards, 1970) in a pH range of 3.0–11.0, 50 mM CH_3COOH-CH_3COONa (pH 3–6); NaH_2PO_4-Na_2HPO_4 (pH 6.0–7.0), Tris-HCl (pH 7.0–10.0), and Na_2HPO_4-NaOH (pH 10.0-11.0). The effect of pH on protease stability was investigated by incubating the enzyme with casein in buffers having various pH values (3.0–11.0) at 37°C for 30 min before assaying at pH 9.0, then adjusting the pH to 9.0, and the residual protease activities were tested under standard conditions. Optimal temperature of protease was determined by incubating with casein and 50 mM Tris-Cl (pH 6.0) for 30 min at various temperatures ranging from 20°C–80°C. Activity was estimated as a percentage of the maximum. In addition, thermal stability of the enzyme was determined by pre-incubation at various temperatures (40°C–70°C) in 50 mM Tris-HCl (pH 6.0) for 30 min. The effects of chemicals (50 mM of $CaCl_2$, KCl, $CaCl_2$, $MgCl_2$) and inhibitors (1 mM of Ethylenediaminetetraacetic acid (EDTA), Phenylmethylsulfonyl fluoride (PMSF)) on enzyme stability were investigated by pre-incubating the enzyme with each of the chemicals and inhibitors in 50 mM Tris-HCl (pH 6.0) at 40°C for 30 min.

Antifungal activity

To ascertain whether the protease had antifungal activity, several agronomically important plant pathogens were selected: *Penicillium italicum*, *Fusarium moniliforme*, *Magnaporthe oryzae,* and *Exserohilum turcicum*. In order to obtain sufficient spores, all four pathogens were isolated from newly infected organisms and grown on PDA. After sporulation, spores were collected, washed three times in sterile distilled water, and resuspended in 0.1% (m/v) glucose. Spores were used directly to examine the effects of the protease on spore germination. Conidial suspensions (100 µl) were then mixed in the presence or absence of serially diluted quantities of purified proteasein the wells of sterile depression slides, and incubated at 25°C for 10 h. The results were recorded by light microscopy (Olympus BHS313).

Extraction of DNA and RNA

V. lecanii strain CA-12 was used for cloning *VlPr1* in this

Table 1. Purification of extracellular protease from *V. lecanii* CA12

Purification step	Volume (ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
Crude enzyme	945	800.13	63.45	50765.4	100	1.00
90% $(NH_4)_2SO_4$	31	130.35	176.6	23014.2	45.3	2.78
Phenyl-sepharose	29	80.23	262.7	21078.9	41.5	4.14
DEAE-sepharose	9	32.12	515.1	16543.7	32.6	8.12
CM-sepharose	5	18.65	692.4	12912.6	25.4	10.91

study. Mycelia of *V. lecanii* in the flask after 5 days of incubation were filtered and ground in liquid nitrogen to fine powders with a mortar and pestle. Genomic DNA (gDNA) was extracted from mycelial powders of *V. lecanii* using a cetyltrimethylammonium bromide (CTAB) method. Total RNA was extracted from the mycelial powders of *V. lecanii* with RNAiso Plus Reagent (TaKaRa) following the manufacturer's instructions. DNA and RNA samples of *V. lecanii* were stored at -20°C and -80°C, respectively until use.

VlPr1 gene cloning

The degenerate primers Forward: 5′-ATGCGTCTATCAA TMATYGCWGCCG-3′, and Reverse: 5′-TTARRTGSCGC CGTTAAATGCCAG-3′ were designed on the basis of the conserved region of the aligned amino acid and nucleotide sequences deduced from *Pr1* gene sequences in *B. bassiana* (GenBank Acc. ACZ28128), *Lecanicillium psalliotae* (GenBank Acc. AAW65381), *Fusarium* sp. (GenBank Acc. JC2142), and *M. anisopliae* (GenBank Acc. CAC95042), *M. lepidiotae* (GenBank Acc. ACV71862). A PCR amplification mixture was set up in a 0.25 ml tube containing 25 µl reaction mixture consisting of 1 µl of each primer solution (10 µM), 1 µl of dNTP mix solution (2.5 mM), 1 µl genomic DNA solution (about 50 ng in each reaction mixture), 0.5 µl *pfu* DNA polymerase (2.5 U in each reaction mixture; Fermentas Co., Ltd., USA), 2.5 µl (with Mg^{2+}) of PCR reaction buffer and 18 µl of double-distilled (dd) H_2O. The PCR was performed in a Bio-Rad S1000TM Thermal Cycler (Bio-Rad, USA) programmed as: 94°C for 5 min (1 cycle); followed by 94°C for 30 sec 55°C for 30 sec and 72°C for 2 min 30 sec (30 cycles); finally 72°C for 10 min (1 cycle). The PCR product was separated by 1% (w/v) agarose gel electrophoresis. The target DNA band was purified from the gel with SanPrep™ DNA Gel Extraction Kit (Sangon Scientific, Inc, China) and ligated into the pMD-18T vector (TaKaRa), which was then transformed into competent cells of *Escherichia coli* DH5α. Positive *E. coli* clones grown on Luria–Bertani agar medium containing ampicillin (50 µg/ml) were selected and individually tested for size of the DNA insert. Three positive clones containing the inserted DNA were individually sequenced at Invitrogen. The DNA sequence was used for a homology searched in the NBCI database (http://blast.ncbi.nlm.nih.gov/Blast. cgi) using the BLASTx program.

For phylogenetic analysis, amino acid sequences of proteases were collected from GenBank and aligned by CLUSTALX software (version 1.83) using Gonnet series matrices as multiple alignment parameters, and gap opening and extension parameters as default values. A phylogenetic tree was inferred using the MEGA software (version 4.0.2) neighbor-joining method using 1000 bootstrap replicates to ascertain the reliability of a given branch pattern in the NJ tree.

DNA manipulation and expression of VlPr1 in *E. coli* BL21 (DE3)

The open reading frame (ORF) of the gene encoding *VlPr1* was amplified by PCR using primers *VlPr1*-F (5′-CGCGGATCCATGCGTCTATCAATAATTGCAGCCGC-3′) [*Bam*HI site underlined], and *VlPr1*-R (5′-CCCAAGCTTTTAAATGCCGCCGTTAAATGCCAGG-3′) [*Hin*dIII site underlined] and cDNA of *V. lecanii* as template, with the following thermal cycling conditions: 95°C for 5 min; 30

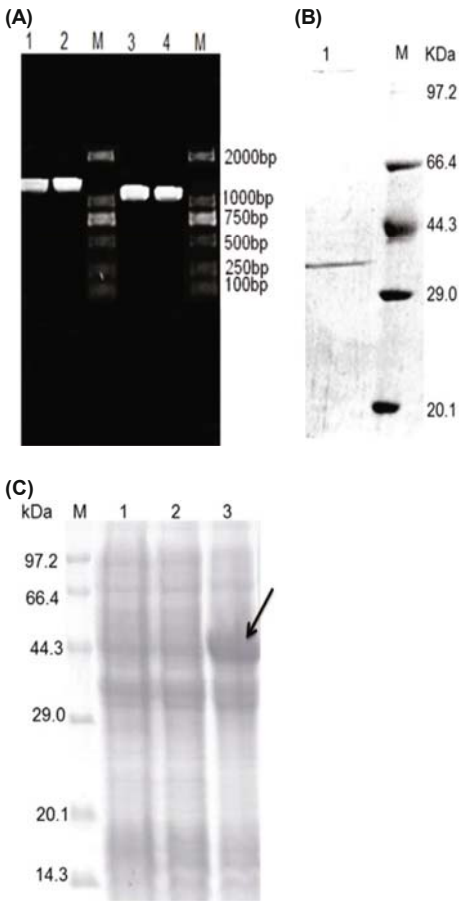

Fig. 1. *VlPr1* gene cloning and protein purification. (A) DNA products of VlPr1. Lanes: 1, 2, Genomic DNA products of *VlPr1*; M, Marker DL2000; 3, 4, cDNA products of VlPr1. (B) SDS-PAGE of purified protease from *V. lecanii* CA12. Lanes: M, standard protein molecular weight marker; 1, Protein samples from CM-Sepharose. (C) SDS-PAGE of recombinant pET28a-*VlPr1*-$(His)_6$ expressed in *E. coli* BL21 (DE3). Lanes: M, standard protein molecular weight markers; 1, 2, and 3 proteins of recombinant *E. coli* BL21 (DE3) harboring pET28a, non-induction control and recombinant pET-*VlPr1*-His, respectively.

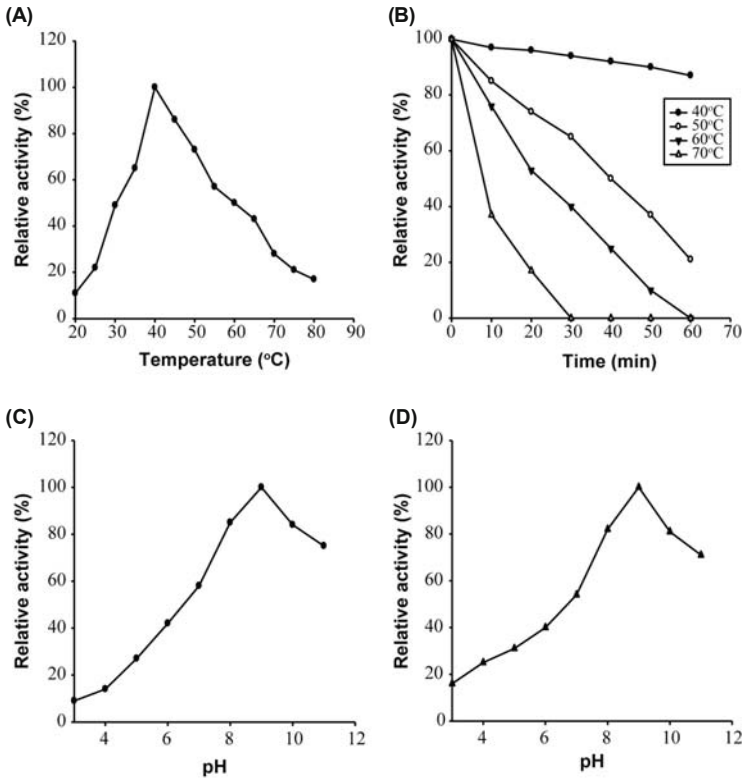

Fig. 2. Activity and stability of the purified protease. (A) The optimum reaction temperature of the purified protease. (B) The stability of the purified protease at different temperatures. (C) The activity of the purified protease at different pHs. (D)The stability of the purified protease at different pHs.

cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 2 min 30 sec and 72°C for 10 min. The amplicon was digested with *Bam*HI and *Hin*dIII, and then ligated into the corresponding restriction sites of pET28a(+), resulting in recombinant pET28a-*VlPr1*-$(His)_6$. The prokaryotic expression construct pET28a-*VlPr1*-$(His)_6$ was transformed into *E. coli* BL21 (DE3) with a $CaCl_2$ chemical transformation method. The transformants having pET28a-*VlPr1*-$(His)_6$ were selected and verified by digestion analyses with restriction enzyme *Bam*HI and *Hin*dIII as well as DNA sequencing, and positive transformants were cultured in Luria-Bertani medium supplemented with 50 μg/ml of kanamycin at 37°C with shaking (200 rpm). When absorbance of 0.5 (measured at 600 nm) was reached, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (1 mM final concentration) and the culture was incubated at 28°C until the OD_{600} reached 2.1–3.0. Cells were harvested and analyzed for protein content by 12% SDS-PAGE.

Results

Characterization of VlPr1 protease

The VlPr1 protease was purified 10.9-fold with a final yield of 25.4% and specific activity of 692.4 U/mg. Purification of extracellular protease from *V. lecanii* CA12 is shown in Table 1. Enzyme purity and molecular mass were determined by SDS-PAGE and silver staining, showing a single polypeptide chain of 38.0 kDa (Fig. 1B).

The effect of temperature and pH on the enzyme's activity and stability are shown in Fig. 2. Optimum enzyme activity was at 40°C (Fig. 2A) and 20% activity was retained after 60 min at 50°C (Fig. 2B). The protease was active in the pH range of 7.0–11.0, with an optimum at pH 9.0. The relative activity at pH 10.0 and 37°C was 80% (Fig. 2C). The protease was stable in a pH range of 6.0–11.0. Enzyme activity was measured in the presence of different enzyme inhibitors, in

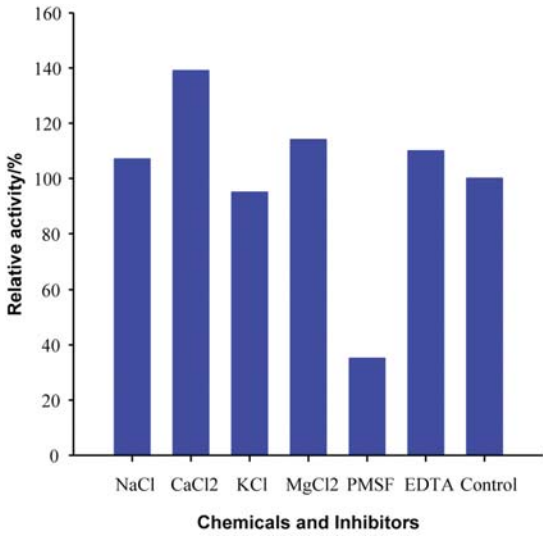

Fig. 3. Activities of protease from *V. lecanii* CA12 in the presence of chemicals (NaCl, $CaCl_2$, KCl, $MgCl_2$) and inhibitors (PMSF, EDTA).

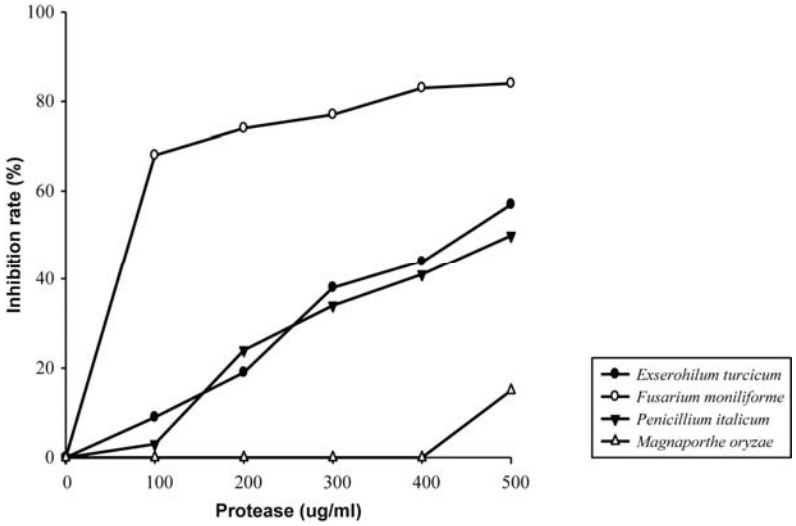

Fig. 4. ***In vitro*** **antifungal activity (%) of the purified protease (VlPr1) at concentrations from 0 to 500 μg/cm^3.** Approximately 1000 fungal spores were cultured in the agar plate with or without the protease at various concentrations, at 25°C for 10 h. Inhibition of spore germination was examined under a light microscope.

order to determine the nature of the protease. The protease was found to be inhibited by PMSF, but not by EDTA, indicating that the protease gene was a serine protease (Manavalan *et al*., 2007). The protease activity was strongly activated by 1 mM Ca^{2+}, and partially (to varying extents) by other ions tested (Fig. 3).

Antifungal activity

To detect *in vitro* antifungal activity of the isolated protease, 100 μl of each fungal spore stock were cultured in the presence or absence of serial dilutions of the purified protein (0, 100, 200, 300, 400, and 500 μg/ml) at 25°C for 10 h. In these experiments, spore germination was inhibited to different

```
1     ATG CGT CTA TCA ATA ATT GCA GCC GCT CTT CCC CTG GCC ATT GCG GCT CCG GTC GTT GAG
1      M  R  L  S  I  I  A  A  A  L  P  L  A  I  A  A  P  V  V  E
61    CCT GCT CCT CTC ATC GAG GCC CGC GGG CAG ACC ATT GCC GGC AAG TAC ATT GTC AAGCTC
21     P  A  P  L  I  E  A  R  G  Q  T  I  A  G  K  Y  I  V  K  L
121   AAG GAC ACT GCG ACC ATT GGT ATC ATG GAT GCT GCG TCC AAG GTG CCC AAC ACC GAG CAC
41     K  D  T  A  T  I  G  I  M  D  A  A  S  K  V  P  N  T  E  H
181   GTC TAT GAA AAT GTC CTC AAG GGA TTC TCG GCC ACT CTT AAC CAG GAA CAA CTT GAC CGT
61     V  Y  E  N  V  L  K  G  F  S  A  T  L  N  Q  E  Q  L  D  R
241   CTC CGC CAC GAT CCT GAT GTCAGTGCCTGTTCTCCTCGGACTTTTCTGTGACTTTATTAC
81     L  R  H  D  P  D
301   TAATGAAATCGTGCCCCACGCGAATAGGGTC GAG TCC ATC GAG CAG GAT GCC ATT GTT AG
87                                   V  E  S  I  E  Q  D  A  I  V  S
361   C ATC AAC GCC GTT GTC CGG CAA GCC GGA GCT CCC TGG GGT CTA GGT CGC ATC TCG CAC AG
98     I  N  A  V  V  R  Q  A  G  A  P  W  G  L  G  R  I  S  H  R
421   G GCA CGA GGC GCG ACC ACG TAT GAC TAC GAC TCG AGC GCC GGC GCG GGT ACA TGC GTA TA
118    A  R  G  A  T  T  Y  D  Y  D  S  S  A  G  A  G  T  C  V  Y
481   T GTC ATT GAC ACT GGC GTC TAT GAC TCT CAC CCT GTAAGCCTACACATACTTGCGGTGCT
138    V  I  D  T  G  V  Y  D  S  H  P
541   CTCCACGAGAAAGCCACTCATTGAGGCATCAATAGGAT TTC GAA GGA CGT GCC AAG CAA A
149                                     D  F  E  G  R  A  K  Q
601   TC AAA TCC TTT GTC TCT GGC ACT TCA GAT GGT CAC GGC CAC GGC ACA CAC TGC GCC GGA A
157    I  K  S  F  V  S  G  T  S  D  G  H  G  H  G  T  H  C  A  G
661   CT ATT GGC TCC AAG ACT TAC GGC GTA GCC AAG AAG GCG TCC ATT TTT GGC GTC AAG GTG C
177    T  I  G  S  K  T  Y  G  V  A  K  K  A  S  I  F  G  V  K  V
721   TC GAG GAC AGT GGC TCG GGT TCG CTC ACC GGC GTC ATT GCC GGA ATG GAC TTT GTC GCT A
197    L  E  D  S  G  S  G  S  L  S  G  V  I  A  G  M  D  F  V  A
781   CG GAC CGG AAA TCC CGT TCA TGC AGC AAA GGC ACC GTC GCC AGC ATG TCC CTT GGC GGT G
217    T  D  R  K  S  R  S  C  S  K  G  T  V  A  S  M  S  L  G  G
841   GC TAC TCG GCC ACC GTG AAC CAG GCC GCC GCG CGT CTG CAG GCT TCG GGC GTT TTT GTC G
237    G  Y  S  A  T  V  N  Q  A  A  A  R  L  Q  A  S  G  V  F  V
901   CC GTC GCC GCC GGC AAC GAC AAT AGG GAT GCC GCC CAG ACC TCG CCC GCC TCG GAG CCG T
257    A  V  A  A  G  N  D  N  R  D  A  A  Q  T  S  P  A  S  E  P
961   CC GTC TGC ACC GTC GGA GCT ACC GAC TCG TCT GAC CGC CGC TCC AGC TTC TCC AAC TTT G
277    S  V  C  T  V  G  A  T  D  S  S  D  R  R  S  S  F  S  N  F
1021  GA AAA GCT GTC GAC ATT TTC GCA CCT GGC ACT GGC ATT CTG TCG ACC TGG AAT AAC GGC G
297    G  K  A  V  D  I  F  A  P  G  T  G  I  L  S  T  W  N  N  G
1081  GC ACT GTGAGTTTCTGTTTTGCATCACGCTGTTCAAATAAAACCTCGAAGCTGACTTTTT
317    G  T
1141  TTTCCATCCTAGAAT ACC ATC TCG GGC ACT TCG ATG GCC ACT CCT CAC ATT GCC GGT CTC
319                    N  T  I  S  G  T  S  M  A  T  P  H  I  A  G  L
1201  GGT GCC TAC CTT TTG GCT CTC GGC AAA GGC ACT GCC GGC AAC CTC TGC CAA ACT ATC CAG
335    G  A  Y  L  L  A  L  G  K  G  T  A  G  N  L  C  Q  T  I  Q
1261  ACT CTC TCC ACC AAG AAT GTC CTT ACT GGC GTT CCT TCA GGC ACC GTC AAC TAC CTG GCA
355    T  L  S  T  K  N  V  L  T  G  V  P  S  G  T  V  N  Y  L  A
1321  TTT AAC GGC GGC ATTTAA
375    F  N  G  G  I  *
```

Fig. 5. Nucleotide and deduced amino acid sequence of protease gene *VlPr1* (GenBank accession no. JF776159). The nucleotide and/or amino acid sequence is numbered on the left. The upper lines show the nucleotide sequence with three introns (one line underlined). The putative signal peptide double underlined, the conserved 3-amino acidcatalytic triad (aspartic acid, histidine, and serine) are indicated in boldface, respectively. The stop codon is indicated with an asterisk (*).

degrees by the experimental treatment as compared with the 50 mM Tris-HCl (pH 6.0) control (Fig. 4). For example, at an enzyme concentration of 100 μg/ml, the inhibition rate of *F. moniliforme* spore germination was almost 70%, which was the maximum inhibition observed.

Cloning and sequence analysis of *VlPr1* gene

Taking the *V. lecanii* total cDNA and genomic DNA as a template, the full-length cDNA ORF was generated. It consisted of 1137 bp that predicted a protein of 379 amino acids. The protease-encoding sequence in the genomic DNA was also amplified and sequenced (Fig. 1A). The gene-encoding sequence on the genomic DNA spans 1337 bp with three introns of 69 bp, 61 bp and 67 bp, respectively (Fig. 5). Each intron contains a representative GT...AG boundary. Many extracellular subtilisin family proteases are translated as the pre-protein form, containing a signal sequence (Wei *et al.*, 2008). The protein VlPr1 contains a hydrophobic N-terminal, and the Signal P 3.0 program analysis revealed that it contained a putative signal peptide of 15 amino acids MRL SIIAAALPLAIA for excreted proteins. The calculated molecular mass of the protease precursor is 38,863 Da, and its *p*I is 7.91. It contained a catalytic triad center containing Asp140, His168, and Ser325, which is characteristic of the subtilisin-like serine proteases. The *VlPr1* cDNA and gDNA sequences were deposited in GenBank (accessions numbers are HQ840790 for cDNA and JF776159 for gDNA). A GenBank database search showed that the protein can be assigned to the serine protease family S8. The nucleotide and amino acid sequence of VlPr1 shares extensive similarities with members of the subtilisin family of serine proteases. VlPr1 exhibited 99% homology with a cuticle-degrading protease from *B. bassiana*, and showed 61–98% sequence identity with subtilisins produced by other entomopathogenic fungi. A multiple alignment analysis was performed with CLUSTAL X. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in MEGA4, the parameters were set to "multiple alignment gap opening penalty was 10, gap extension penalty was 0.2, delay divergent cutoff was 30%" (Fig. 6). The results of this analysis demonstrated that the protease gene obtained in this study belonged to the serine proteases of the subtilisin family.

Expression of VlPr1 in *E. coli* BL21 (DE3)

For heterologous expression in *E. coli*, the coding region of *VlPr1* was amplified using specific primers *VlPr1*-F and *VlPr1*-R, and then ligated into pET28a(+), yielding recombinant pET28a-*VlPr1*-$(His)_6$ encoding VlPr1 fused to a hexahistidine tag at the N-terminus. pET28a-*VlPr1*-$(His)_6$ was used to transform *E. coli* BL21 (DE3), and the gene encoding VlPr1 expression was induced by IPTG. A large amount of $(His)_6$-VlPr1 (molecular weight approximately 46.0 kDa) was produced (Arrow, Fig. 1C).

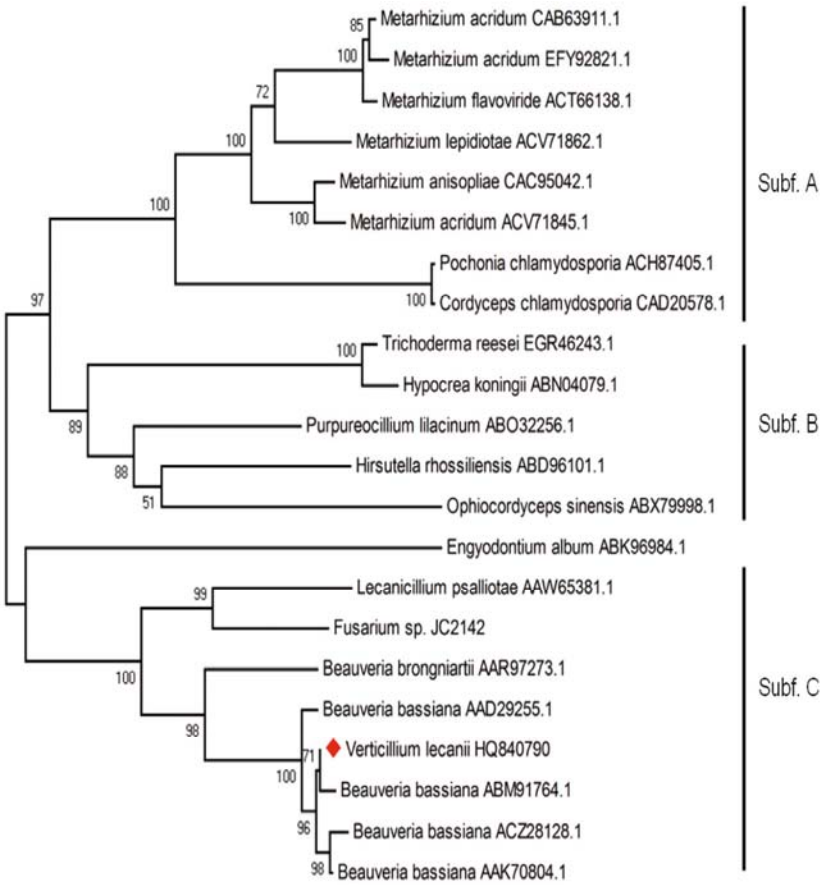

Fig. 6. Phylogenetic analysis of the serine protease gene product of *V. lecanii* and serine proteases characterized in entomopathogenic, mycoparasitic fungi *Metarhizium* sp. and *Beauveria bassiana*. The GenBank Acc. No. for each serine protease protein is followed by the Latin name of each fungus. The unrooted Neighbor-Joining tree was inferred using the MEGA4.0 software. Bootstrap values higher than 50% (1,000 replicates) are given at nodes. Branch length in the tree is proportional to the numbers of nucleotide substitutions as measured by the scale bar (0.5% sequence divergence). The VlPr1 protease produced by *V. lecanii* described in this study is boldfaced.

Discussion

In this study, The VlPr1 protease was purified using 90% $(NH_4)_2SO_4$, Phenyl-Sepharose chromatography, DEAE-cellulose chromatography, and CM-Sepharose Fast Flow chromatography, and the purified subtilisin-like serine protease (VlPr1) had a molecular mass of 38.0 kDa as determined by SDS-PAGE. Purified VlPr1 showed the highest activity at 40°C. It showed differences in the activation profiles of ions for K^+, Ca^{2+}, Na^+, and Mg^{2+}. The protease was found to be inhibited by PMSF, but not by EDTA, indicating that the protease was a serine protease (Manavalan *et al*., 2007). The protease is active in the pH range of 7.0–11.0, with an optimum at pH 9.0. Characterization of the proteases would provide understanding of the biochemical and structural basis of the enzyme's molecular stability for *in vitro* use (Li *et al*., 2011). Heterologous expression of the *VlPr1* gene encoding a subtilisin-like protease in *E. coli* was investigated. The recombinant VlPr1 fused to a histidine tag at the N-terminus gave a high yield of the enzyme and SDS-PAGE analysis revealed a specific band under inducing conditions.

In this study, we have cloned and described the subtilisin-like protease gene, *VlPr1*, of *V. lecanii* CA12. The deduced amino acid sequences of VlPr1 shared identity with the subtilisin-like protease genes from *B. bassiana*, *L. psalliotae*, and *M. acridum* at 99%, 79%, and 65%, respectively. The protein VlPr1 contains a hydrophobic N-terminal, and a putative signal peptide for excreted proteins. It was also reported that the mature extracellular alkaline protease was formed after N-glycosylation, signal peptide cleavage and other modifications of the proper protein (Ni *et al*., 2008). Analysis of the VlPr1 protein sequence showed that it had a high probability of being a serine protease belonging to the subtilisin-like superfamily (or subtilase) (Siezen and Leunissen, 1991; Rawlings and Barrett, 1995). Sequence comparisons using the programs CLUSTAL X and MEGA4 supported the fact that VlPr1 was a subtilase and showed that VlPr1 was most homologous to subtilisin-like proteases of this superfamily.

In order to evaluate the antagonistic activity of subtilisin-like protease *in vitro* against pathogenic fungi, the spore germination inhibition of subtilisin-like protease against four pathogenic fungi was studied. During the *in vitro* experiments, the subtilisin-like protease showed a broad-spectrum antifungal activity toward fungal pathogens that devastate plants, particularly *F. moniliforme*, *P. italicum*, *M. oryzae*, and *E. turcicum*. Our results broaden the understanding of entomopathogenic fungi in phytopathogenic fungal biocontrol. Other examples of proteases from entomopathogenic fungi exhibiting activity against phytopathogenic fungi include *S. macularis* (Miller *et al*., 2004), *P. coronata* (Leinhos and Buchenauer, 1992), *P. digitatum* (Benhamou and Brodeur, 2000), and *P. ultimum* (Benhamou and Brodeur, 2001). It would be of interest in future experiments to determine the efficacy of VlPr1 against these fungi as well.

This report is the first example of successful purification and characterization a subtilisin-like protease from the biocontrol fungus *V. lecanii*. Our report on VlPr1 describes the protease gene product from *V. lecanii* showing its direct activity against phytopathogens. So it will be possible to apply protease *in vitro* to control plant phytopathogens after some required modifications (Yan and Qian, 2009). Proteases may be useful in their own right as an attractive alternative for control of fungi that infect plants, avoiding chemical fungicide applications. Suitable fungal sources for desired enzymes need to be identified. Enzymes that are secreted in the growth media have been studied more frequently than cell-associated enzymes, since culture filtrates can be obtained in substantial quantities (Maheshwari *et al*., 2000). On the other hand, investigations are examining the possibility of introducing *Pr1* into insect viruses and plants, which is expected to improve the capacity of insect virus to kill insects or increase the level of plant disease resistance (St Leger and Roberts, 1997). Such entomopathogens have potential to be developed as biopesticides with multiple roles (Faria and Wraight, 2007). However, to our knowledge, no *Lecanicillium* spp. has been developed for control of phytopathogens.

Acknowledgements

This study is supported by grants from the Ministry of Agriculture Key Projects of GM Cultivation of New Varieties (2009ZX08009-062B to H. Pan), P. R. China, the Ministry of Agriculture Public Benefit and Industry Plan Project (201103016 to H. Pan), P. R. China, and the International Cooperation Projects in Changchun City and Jilin Province (10GH26 and 20100723 to H. Pan). We thank Dr. Jeffrey A. Rollins for critical reading of the manuscript.

References

Askary, H., Benhamou, N., and Brodeur, J. 1997. Ultrastructural and cytochemical investigations of the antagonistic effect of *Verticillium lecanii* on cucumber powdery mildew. *Phytopathology* **87**, 359–368.

Benhamou, N. and Brodeur, J. 2000. Evidence for antibiosis and induced host defense reactions in the interaction between *Verticillium lecanii* and *Penecillium digitatum*, the causal agent of green mold. *Phytopathology* **90**, 932–943.

Benhamou, N. and Brodeur, J. 2001. Pre-inoculation of Ri T-DNA transformed cucumber roots with the mycoparasite, *Verticillium lecanii*, induces host defense reactions against *Pythium ultimum* infection. *Physiol. Mol. Plant Pathol.* **58**, 133–146.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 1105–1112.

Faria, M. and Wraight, S.P. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biol. Control* **43**, 237–256.

Goettel, M.S., Koike, M., Kim, J.J., Aiuchi, D., Shinya, R., and Brodeur, J. 2008. Potential of *Lecanicillium* spp. for management of insects, nematodes and plant diseases. *J. Invertebr. Pathol.* **98**, 256–261.

Joshi, L., St Leger, R.J., and Bidochka, M.J. 1995. Cloning of a cuticle degrading protease from the entomopathogenic fungus, *Beauveria bassiana*. *FEMS Microbiol. Lett.* **125**, 211–218.

Kapteyn, J.C., Montijn, R.C., Cruz, J., Llobell, A., Douwes, J.E., Shimoi, H., Lipke, P.N., and Klis, F.M. 1996. Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-

linked β-1, 3/β-1,6-glucan heteropolymer. *Glycobiology* **3**, 337–345.

Kiss, L. 2003. A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. *Pest Manag. Sci.* **59**, 475–483.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680–685.

Leinhos, G.M.E. and Buchenauer, H. 1992. Hyperparasitism of selected fungi on rust fungi of cereal. *Z. Pflanzenkr Pflanzen* **99**, 482–498.

Li, A.N., Chen, X., Zhang, J., and Zhang, J. 2011. Cloning, expression and characterization of serine protease from thermophilic fungus *Thermoascus aurantiacus* var. *levisporus*. *J. Microbiol.* **49**, 121–129.

Lowry, O.H., Rosebrough, N.J., and Farr, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Maheshwari, R., Bharadwaj, G., and Bhat, M.K. 2000. Thermophilic fungi: their physiology and enzymes. *Microbiol. Mol. Biol. Rev.* **3**, 461–488.

Manavalan, A., Kalaichelvan, A., Kalyanasundaram, V., Perumal, A., and Manavalan, A. 2007. Purification and partial characterization of serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK1. *World J. Microbiol. Biotechnol.* **23**, 475–481.

Miller, T.C., Gubler, W.D., Laemmlen, F.F., Geng, S., and Rizzo, D.M. 2004. Potential for using *Lecanicillium lecanii* for suppression of strawberry powdery mildew. *Biocontrol. Sci. Technol.* **14**, 215–220.

Ni., X., Chi, Z., Ma, C., and Madzak, C. 2008. Cloning, characterization, and expression of the gene encoding alkaline protease in the marine yeast *Aureobasidium pullulans* 10. *Mar. Biotechnol.* **10**, 319–327.

Rawlings, N.G. and Barrett, A.J. 1995. Families of aspartic peptidases, and those of unknown catalytic mechanism. *Method Enzymol.* **248**, 105–146.

Richards, S.A. 1970. The role of hypothalamic temperature in the control of panting in the chicken exposed to heat. *J. Physiol.* **211**, 341–358.

Shimada, K., Ohnishi, Y., Horinouchi, S., and Beppu, T. 1994. Extracellular transport of pseudoazurin of *Alcaligenes faecalis* in *Escherichia coli* using the COOH-terminal domain of *Serratia marcescens* serine protease. *J. Biochem.* **116**, 327–334.

Siezen, R.J. and Leunissen, J.A. 1991. Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci.* **6**, 501–523.

St Leger, R.J., Joshi, L., Bidochka, M.J., and Roberts, D.W. 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc. Natl. Acad. Sci. USA* **93**, 6349–6354.

St Leger, R.J. and Roberts, D.W. 1997. Engineering improved mycoinsecticides. *Trends Biotechnol.* **15**, 83–85.

Suarez, B., Rey, M., Castillo, P., Monte, E., and Llobell, A. 2004. Solation 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.

Wei, Z., Cao, Y., and Xia, Y.X. 2008. Cloning of the subtilisin *Pr1A* gene from a strain of locust specific fungus, *Metarhizium anisopliae*, and functional expression of the protein in *Pichia pastoris*. *World J. Microbiol. Biotechnol.* **24**, 2481–2488.

Xia, L.Q., Zeng, Z., Ding, X.Z., and Huang, F. 2009. The expression of a recombinant *cry1Ac* gene with subtilisin-like protease CDEP2 gene in a crystalliferous *Bacillus thuringiensis* by Red/ET homologous recombination. *Curr. Microbiol.* **59**, 386–392.

Xu, X.Q., Yu, Y.F., and Shi, Y.J. 2011. Evaluation of inert and organic carriers for *Verticillium lecanii* spore production in solid-state fermentation. *Biotechnol. Lett.* **33**, 763–768.

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