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Overexpression of a cuticle-degrading protease Ver112 increases the nematicidal activity of *Paecilomyces lilacinus*

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Abstract Due to their ability to degrade the proteins in nematode cuticle, serine proteases play an important role in the pathogenicity of nematophagous fungi against nematodes. The serine protease Ver112 was identified from the nematophagous fungus *Lecanicillium psalliotae* capable of degrading the nematode cuticle and killing nematodes effectively. In this study, the gene *ver112* was introduced into the commercial biocontrol fungal agent *Paecilomyces lilacinus* by the restriction enzyme-mediated integration transformation. Compared to the wild strain, the transformant *P. lilacinus* 112 showed significantly greater protease activity, with nematicidal activities increased by 79% and 96% to *Panagrellus redivivus* and *Caenorhabditis elegans* at the second day, respectively. The crude protein extract isolated from the culture filtrate of *P. lilacinus* 112 also showed 20–25% higher nematicidal activity than that of the wild-type strain. Reverse transcription PCR results showed that the expression of gene *ver112* in *P. lilacinus* 112 was correlated to protease activity of the culture filtrate. Our results demonstrated the first successful transfer of a virulence gene from one nematophagous fungus to another nematophagous fungus, and improved the pathogenicity of the recipient fungus against pest nematodes.

Keywords Nematophagous fungi · Serine protease · *Paecilomyces lilacinus* · Nematicidal activity · REMI transformation

Introduction

Plant-parasitic nematodes cause significant damages on a host of world crops every year (Siddiqui and Mahmood 1996). In recent years, due to their unique ability to infect and kill the nematodes, nematophagous fungi have been proposed as potentially effective biological agents to control the harmful nematodes (Siddiqui and Mahmood 1996; Nordbring-Hertz et al. 2006). This strategy is attracting increasing attention due to increasing resistance of these nematodes to the currently available synthetic chemical pesticides and the pollution these chemical agents have caused to the environment. At present, the detailed molecular pathogenic mechanisms against nematodes by nematophagous fungi have not yet been fully elucidated. However, increasing evidences show that extracellular hydrolytic enzymes including proteases, collagenases, and chitinases may be involved in nematode-cuticle penetration and host cell digestion (e.g., Åhman et al. 2002; Morton et al. 2004; Yang et al. 2007). Although nematophagous fungi have a great potential for controlling nematodes, low nematicidal activity and instability in field conditions have hindered the application of this group of fungi.

Paecilomyces lilacinus is an opportunistic fungal pathogen that can infect both nematodes and insects. It has been widely studied and successfully implemented for controlling plant-parasitic nematodes (e.g., Basualdo et al. 2000; Brand et al. 2004; Kalele et al. 2007). This fungus can infect eggs and cyst nematodes using its secreted hydrolyzing enzymes (Khan et al. 2003, 2004). In 1995, a basic serine protease

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pSP-3 was identified from *P. lilacinus* culture filtrate by affinity chromatography (Bonants et al. 1995), which could be inhibited by phenylmethanesulfonyl fluoride, and this enzyme shared a high degree of sequence similarity to subtilisin-like serine proteases. The fungus *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) is an effective parasite of plant-parasitic nematodes (Zare et al. 2000). In our previous report, a cuticle-degrading protease Ver112 was purified and cloned from *L. psalliotae* (Yang et al. 2005a, b) and this enzyme showed a strong activity to hydrolyze the proteins in nematode cuticles.

One way to improve the pathogenicity of pathogenic fungi is to increase the copy number of virulence genes or to introduce exogenous virulence genes. This strategy has been successfully used in entomopathogenic fungi and other pathogenic fungi (Åhman et al. 2002; St Leger and Wang 2010). Recently, Wang and St Leger (2007) improved the fungal virulence of *Metarhizium anisopliae* by expressing an insect-specific neurotoxin from the scorpion *Androctonus australis*. Lately, Qin et al. (2010) constructed a transgenic *Beauveria bassiana* strain by expressing an insecticidal protein (Vip3A) from *Bacillus thuringiensis*. The engineered *B. bassiana* strain showed enhanced fungal virulence to the *Spodoptera litura* larvae. A few similar studies have also been reported for nematophagous fungi. In 1994, a cuticle-degrading protease PII was isolated from *Arthrobotrys oligospora* (Tunlid et al. 1994). Subsequently, an *A. oligospora* mutant with additional copies of the *PII* gene was constructed, which developed a higher number of infection devices and showed an increase in the speed of capturing and killing nematodes when compared to the wild-type strain (Åhman et al. 2002).

In this study, we describe the transformation and expression of the cuticle-degrading serine protease gene *ver112* from *L. psalliotae* in *P. lilacinus*. We analyzed and compared the proteolytic and nematicidal activities of the wild-type and engineering strains. In addition, the expression of protease genes *ver112* and *pSP-3* were analyzed by reverse transcription polymerase chain reaction (RT-PCR) method.

Materials and methods

Microorganisms, nematodes, and culture conditions

The fungus *L. psalliotae* was isolated from soil samples in Yunnan Province and deposited as the strain code CGMCC1312 in the China General Microbiological Culture Collection Center (CGMCC). *P. lilacinus* used in this study was isolated from nematode eggs and deposited in CGMCC as the strain code CGMCC3422. It was incubated on potato dextrose agar (PDA) medium at 28 °C. The PL-4 liquid medium for protease production was described in our previous work (Yang et al. 2005a). *Escherichia coli* strain DH5α was used in all DNA manipulations, and this strain was typically grown in Luria-Bertani medium containing (per liter): 10 g tryptone, 10 g NaCl, 5 g yeast extract, and 16 g agar.

The free-living nematodes *Panagrellus redivivus* and *Caenorhabditis elegans* were maintained on oatmeal medium (Dong et al. 2007). Nematodes were separated and washed thoroughly with 50 mM sodium phosphate (pH 7.0) before being used in the assays.

Plasmid construction

The complementary DNA (cDNA) sequence of cuticle-degrading protease Ver112 was amplified from the plasmid pGEM-112 constructed in our previous work (Yang et al. 2005b) using primer pairs #1 (112BamN and 112BamC, Table 1) containing the restriction site (*Bam*HI, underlined) as designed based on the original nucleotide sequence of *ver112* (GenBank accession no. AY692148). The PCR product was digested with *Bam*HI and inserted into the *Bam*HI site of the fungal expression vector pAN52-1N (GenBank accession no. Z32697; provided by Dr. PJ Punt). The orientation of the inserted DNA fragment was confirmed by PCR amplification using primers of 112BamN and trpC1 (Table 1). The constructed plasmid, pAN52-112, contained the gpdA promoter, the *ver112* gene, and the terminator sequence of the trpC gene. The plasmid pBS/Sk-hph harboring the hygromycin-resistant gene *hph* (provided by Dr. YJ Zhang) was also used. Both the pAN52-112 and pBS/Sk-hph plasmids were linearized with *Hin*dIII individually and used for subsequent transformation.

Protoplast preparation and transformation

The protoplast of *P. lilacinus* was prepared according to our previous report (Zhang et al. 2008). For transformation, 100 μL protoplasts (circa 8.0×10^7 mL^{-1}) were mixed with 60 U *Hin*dIII and 10 μg linear vector DNA (pAN52-112 and pBS/Sk-hph; linearized by 20 U *Hin*dIII in 150 μL of 1× restriction enzyme buffer just before transformation) in a 1.5-mL centrifuge tube. After 30 min of incubation on ice, 600 μL of PTC (50% polyethylene glycol 6,000, 20 mM L^{-1} Tris-HCl, pH 7.5, 50 mM L^{-1} $CaCl_2$) was added into the mixture and mixed gently. After incubation at 28 °C for 30 min, regeneration for 3 h, the putatively transformed protoplasts were plated with 10 mL of the PDAS medium (PDA supplemented with 10 gL^{-1} molasses, 0.6 ML^{-1} saccharose, 0.3 gL^{-1} yeast extract, 0.3 gL^{-1} tryptone, and 0.3 gL^{-1} casein peptone) containing 1,000 μg L^{-1} of hygromycin B (Roche Corporation, Germany). The

Table 1 List of primers used in this study

Primers		Sequence (5-3)	Expected size (bp)	Roles
1	112BamN	CGCGGATCCATGCGTCTGTCGATCATCGC	1,149	Amplified the gene *ver112*
	112BamC	CGCGGATCCTTACGTGGCGCCGTTGAAG		
2	112BamN	CGCGGATCCATGCGTCTGTCGATCATCGC	1,535	Amplified the gene *ver112* and partial sequence of *trpC*
	trpC1	TGGAGCCAAGAGCGGATTC		
3	gpdN1	ACACAAGCTGGCAGTCGAC	1,915	Amplified the gene *ver112* and partial sequences of *gpdA* and *trpC*
	trpC1	TGGAGCCAAGAGCGGATTC		
4	hphP	GTCTCCGACCTGATGCAGCT	865	Amplified the gene *hph*
	hphR	CTTCTGCGGGCGATTTGTGTA		
5	pSP3-1	CCACTTGGCTCCATCTCAAT	1,469	Amplified the gene *pSP-3*
	pSP3-2	GGCCATGGAAGTACCAGAGA		
6	Tub1	CGTGATGCCTAAACGGACAA	167	RT-PCR primers for β-Tubulin gene
	Tub2	AGACTGGCCGAAGACGAAGT		
7	Ver112-1	CAGAGCTCCGGTGTCTTTGT	80	RT-PCR primers for gene *ver112*
	Ver112-2	GGCCGTAGTTGGAGAGGTC		
8	Psp-1	TCTTCTGATGCGCGATCTTC	158	RT-PCR primers for gene *pSP-3*
	Psp-2	AGGTAGGCACCGAGACCAAC		

The restricted sites are underlined

transformation colonies were selected after incubation at 28 °C for 6–7 days.

Validation and stability analysis of transformants

After single spore isolation, the putative transformants were transferred onto PDA containing 1,000 μg mL^{-1} of hygromycin B and incubated for 5 days at 28 °C. The mycelia were collected by filtration in a sterilized filter funnel and ground in liquid nitrogen. Genomic DNA was extracted according to the method of Zhang et al. (1996). The insertion of gene *ver112* in *P. lilacinus* was verified by PCR using primers gpdN1 and trpC1 (Table 1).

The stability of transformants was tested in accordance with the methods described by Tunlid et al. (1999). The wild-type and transformant strains were respectively transferred to corn meal agar (CMA) plates and incubated for 7 days at 28 °C. The growth rate and morphological features of mycelia were examined.

Protease activity and nematode bioassays

The transformed and wild-type *P. lilacinus* strains were incubated in PL-4 medium for 10 days, at 28 °C. Samples (100 μL) were taken every 24 h to determine the proteolytic activity. Quantitative analysis of protease activity was determined by a caseinolytic method described by Wang et al. (2006).

In order to examine the nematicidal activities of the wild-type and engineered strains, the wild-type and transformant of *P. lilacinus* were incubated on CMA at 28 °C for 6 days. Then, 50 nematodes were added into the plate, and the infection was observed under a light microscope (Olympus, Japan) after incubating at 28 °C for another 1–3 days. The effect of culture filtrate and crude protease on nematode was investigated according to our previous report (Yang et al. 2005a). There were three replicates for each treatment.

Total RNA isolation and expression analysis

To verify the expression of genes *ver112* and *pSP-3*, the transformants were incubated in PL-4 medium. The mycelia were collected after culturing at 28 °C from 2 to 8 days. The mycelia were frozen with liquid nitrogen and ground to a fine powder using a sterile mortar and pestle. Approximately 100 mg of ground mycelium powder was transferred into sterile 1.5-mL microcentrifuge tubes. Total RNA was extracted with RNeasy mini kit (TianGen, China) according to the manufacturer's protocol. Extracted RNA was eluted in 40 mL of RNAse-free water and stored at −80 °C. First-strand cDNA was synthesized using the quantitative reverse transcription kit (TianGen, KP104) according to the manufacturer's instructions.

Three pairs of primers (Table 1) were designed for amplification of genes *Psp-3* (EF094858), *ver112* (AY692148), and β-Tubulin gene (*Tub*) (AY624228) based on their sequences deposited in the GenBank. To assess PCR efficiency, serial dilutions of standard cDNA preparation were used to generate the standard curve for each

primer set. The PCR conditions were: one cycle at 94 °C for 5 min, 25–33 cycles (at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min), and one cycle at 72 °C for 10 min.

Results

Plasmid construction

Plasmid pAN52-112 was constructed by inserting the gene *ver112* into pAN52-1N (Fig. 1a). Cloning of the cuticle-degrading protease Ver112 under the conditions described above, a 1,149-bp PCR product (Fig. 1b) was successfully amplified and cloned into vector pAN52-1N after digested with *Bam*HI. In order to ensure the expression of *ver112*, the orientation of gene *ver112* was verified by two methods. Firstly, the orientation of gene *ver112* in plasmid pAN52-112 was confirmed by PCR using primer pairs #2 (112BamN and trpC1, Table 1), a fragment of about 1,535 bp $(1,149+387=1,535\text{bp})$ (Fig. 1c) was amplified, while no product should be amplified if the gene ver112 was inserted in pAN52-112 in a reverse orientation. Secondly, the plasmid pAN52-112 was digested using *Sal*I. The *Sal*I restriction site was localized at the 1,944 position in plasmid pAN52 and at the 907 position in gene *ver112*. If the insertion direction was correct, a fragment about 1,272 bp $(2,310-1,944+907=1,272)$ (Fig. 1d) would be produced after digestion by *Sal*I. In contrast, if the orientation was reversed, the fragment of approximately

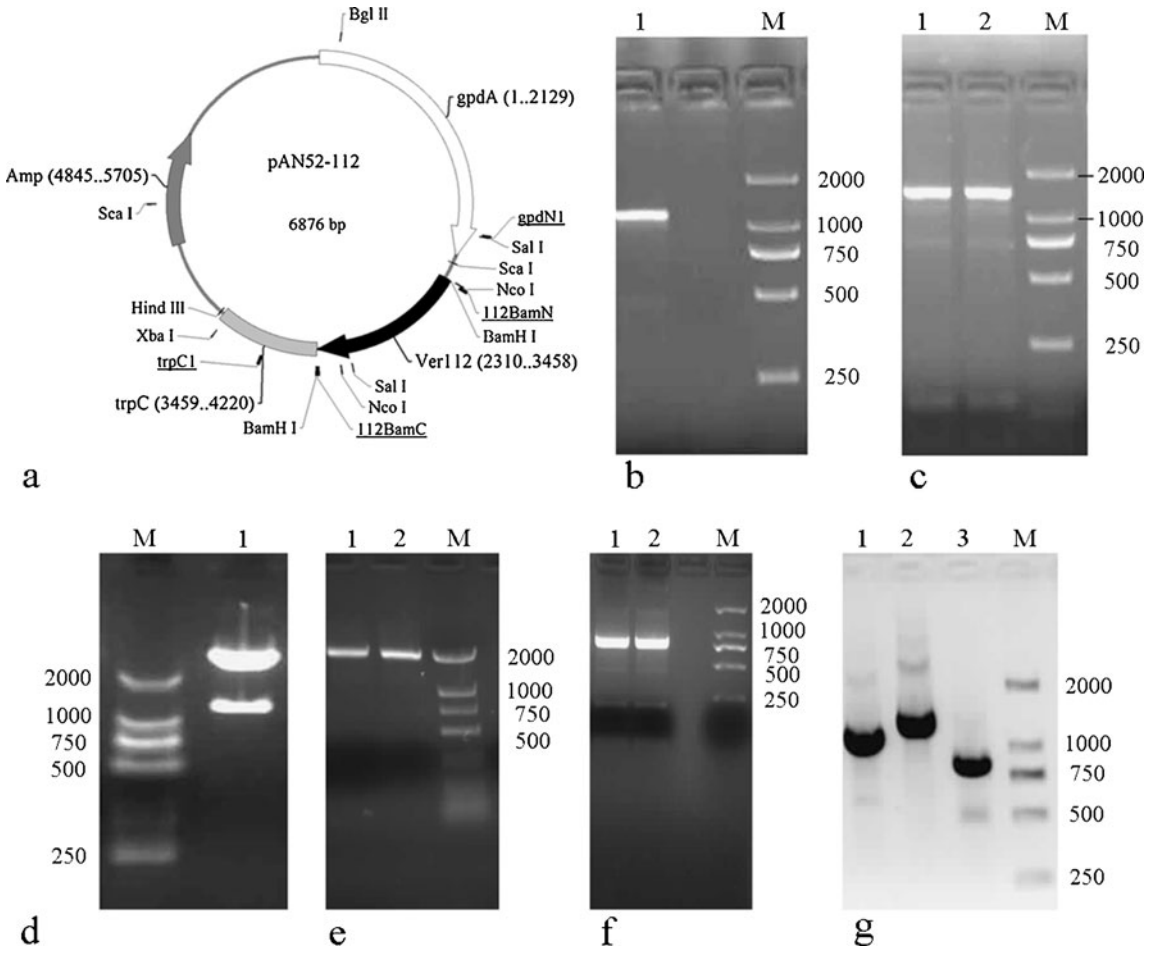

Fig. 1 Construction of plasmid pAN52-112 and validation of transformant. **a** Construction and map of plasmid pAN52-112. The primers were *underlined*. **b** PCR amplification of gene *ver112* using primers 112BamN and 112BamC. *Lane 1* was the PCR product. *M* was the DNA marker (DL 2000), following is the same. **c** PCR amplification of gene *ver112* and partial sequence of *trpC* using primers 112BamN and trpC1. *Lanes 1* and *2* were the PCR products. **d** Results of restriction enzyme digestion by *Sal*I. *Lane 1* was the sample. **e** PCR amplification of gene *ver112* and partial sequences of gpdA and *trpC* using primers gpdN1 and trpC1. *Lanes 1* and *2* were the PCR products. **f** PCR amplification of gene *hph* from *P. lilacinus* 112 using primers hphP and hphR. *Lanes 1* and *2* were the PCR products. **g** PCR amplification of genes *ver112*, *pSP-3*, and *hph* from *P. lilacinus* 112. *Lane 1* was the PCR result of gene *ver112*. *Lane 2* was the PCR result of gene *pSP-3*. *Lane 3* was the PCR result of gene *hph*

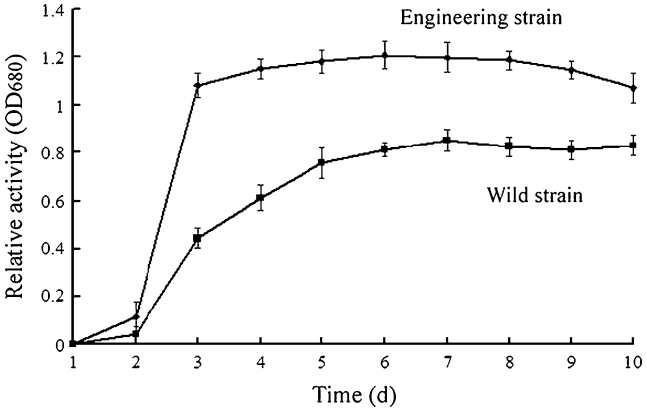

Fig. 2 Activity of extracellular protease in culture filtrates from the wild strain and *P. lilacinus* 112 at various time points. The maximum protease activity was at day 6 during cultivation. Each *point* represents the average of triplicate assays, and the *bars* represent the standard deviation values

608 bp $(2,310-1,944+1,149-907=608)$ would be produced. Our results demonstrated that the gene *ver112* was cloned into the plasmid pAN52-1N in the correct orientation.

Transformation and validation of transformant

The protoplasts of *P. lilacinus* ($1.0–1.9\times10^7$ mL^{-1}) were transformed with the linearized plasmids pAN52-112 and pBS/Sk-hph. On PDAS media, 1,000 μg mL^{-1} of hygromycin B can completely inhibit the regeneration of protoplasts and the growth of the wild-type strain. The resistant colonies were selected and isolated after incubation for 7 days at 28 °C.

The putative transformants were verified by PCR to check for the integration of genes *ver112* and *hph* in the genome. The genomic DNA of transformant was isolated, and PCR was carried out using primer pairs #3 (gpdN1 and trpC1) and #4 (hphP & hphR; Table 1) for amplification of genes *ver112* and *hph*, respectively. Fragments of 1,915 bp $(379+1,149+387=1,915)$ (Fig. 1e) and 865 bp (Fig. 1f) were amplified from the transformants, consistent with our expectation of positive transformants. One positive transformant, named as *P. lilacinus* 112, was selected for following analysis.

Mitotic stability and the physiological features of *P. lilacinus* 112

The *P. lilacinus* 112 showed a high degree (100%) of mitotic stability. After incubated continuously on PDA plate for five transfers, genes *ver112*, *pSP3*, and *hph* were all amplified using specific primers (Table 1; Fig. 1g). Compared to the wild-type parental strain, the transformant showed similar morphological properties on non-selective medium. However, the growth rate of *P. lilacinus* 112 was much lower than that of the wild type. After incubation for 7 days at 28 °C, the colony diameter of *P. lilacinus* 112 was 3.5 cm, while that of the wild type was 9.0 cm.

Extracellular proteases production in *P. lilacinus* 112

The production of extracellular proteases by *P. lilacinus* 112 was investigated in liquid medium (PL-4) under constant shaking (200 rpm). Protease activity in the culture filtrate was assayed every 24 h after inoculation. The protease was produced on the second day and reached the highest activity on the sixth day after inoculation and then slowly decreased (Fig. 2). Compared to the wild-type strain, strain *P. lilacinus* 112 showed a higher protease activity (Fig. 2), indicating protease Ver112 was expressed in strain *P. lilacinus* 112.

Nematicidal analysis

The infection of *P. lilacinus* against nematodes was observed under a light microscope. The *P. lilacinus* 112 showed a stronger ability to immobilize, infect, and degrade nematodes than the wild-type strain in CMA plate. Figure 3 shows that both the wild-type and transformant strains could kill the nematodes *P. redivivus* and *C. elegans* effectively, with a higher nematicidal activity against *C. elegans* than *P. redivivus*. In addition, *P. lilacinus* 112 showed a higher nematicidal activity than the wild-type strain. At 12-, 24-, and 36-h time points, the mortality of nematode *P. redivivus* by *P. lilacinus* 112 was enhanced by 50%, 79%, and 45% than the wild strain, while the mortality of nematode *C. elegans* by *P. lilacinus* 112 was enhanced by 82%, 96%, and 43%, respectively.

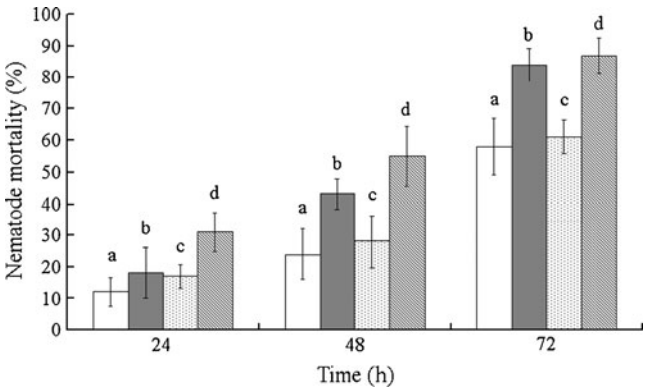

Fig. 3 The nematicidal activity of *P. lilacinus* 112 and wild strain on CMA plate at various time points. **a** Mortality of *P. redivivus* treated by wild strain. **b** Mortality of *P. redivivus* treated by *P. lilacinus* 112. **c** Mortality of *C. elegans* treated by wild strain. **d** Mortality of *C. elegans* treated by *P. lilacinus* 112. The *bars* represent the standard deviation values

The effects of culture filtrate and crude enzyme on nematode *P. redivivus* were further investigated as described below. First, the crude enzyme extract was prepared according to our previous report (Yang et al. 2005a). The culture filtrates of *P. lilacinus* 112 and wild-type strain showed weak activity to nematode, while the crude enzymes showed strong nematicidal activities. After 6- and 12-h incubation with nematodes, 25–30% and 75–80% nematodes were killed by crude enzyme isolated from wild strain, while 45–50% and 100% nematode were killed by crude enzyme isolated from *P. lilacinus* 112, respectively. Meanwhile, nematode cuticle became rough after being treated by crude enzyme for 10–12 h (Fig. 4) and was degraded completely after 24 h. In contrast, the cuticle of nematodes in the negative-control treatment was intact.

Expression analysis of genes *ver112*, *pSP-3*, and *Tub* in *P. lilacinus* 112

The expression of *Ver112* and *pSP-3* were analyzed by RT-PCR in *P. lilacinus* 112 and wild-type strain during different growth periods from 2 to 8 days. In order to remove genomic DNA contamination, the RNA samples were treated by RNase-free DNAase (Takara, Japan) before reverse transcription. In this study, the gene *Tub* was selected as reference gene, and the expression of *Ver112* and *pSP-3* were analyzed by RT-PCR (Fig. 5). The expression of gene *Tub* was stable during different culture periods, while the expression levels of genes *ver112* and *pSP-3* were variable. The expression levels of genes *ver112* and *pSP-3* were weak on the second day, increased at the fourth day, and reached the highest level on the sixth day, while decreased on the eighth day (Fig. 5).

Discussion

Subtilisin-like serine proteases play an important role in the pathogenicity of pathogenic fungi (Yang et al. 2007). By using subtilisin-like serine proteases, pathogenic fungi disrupt the physiological integrity of the hosts during penetration and colonization (Li et al. 2010). In 1992, the first pathogenicity-related serine protease P32 was identified from the nematophagous fungus *Pochonia rubescens* (syn. *Verticillium suchlasporium*) (Lopez-Llorca and Robertson 1992). Subsequently, similar proteases have also been identified from other nematophagous fungi (Yang et al. 2007). The nematode cuticle is a thin and flexible exoskeleton, composed primarily of proteins, including collagens (Cox et al. 1981; Maizels et al. 1993). These

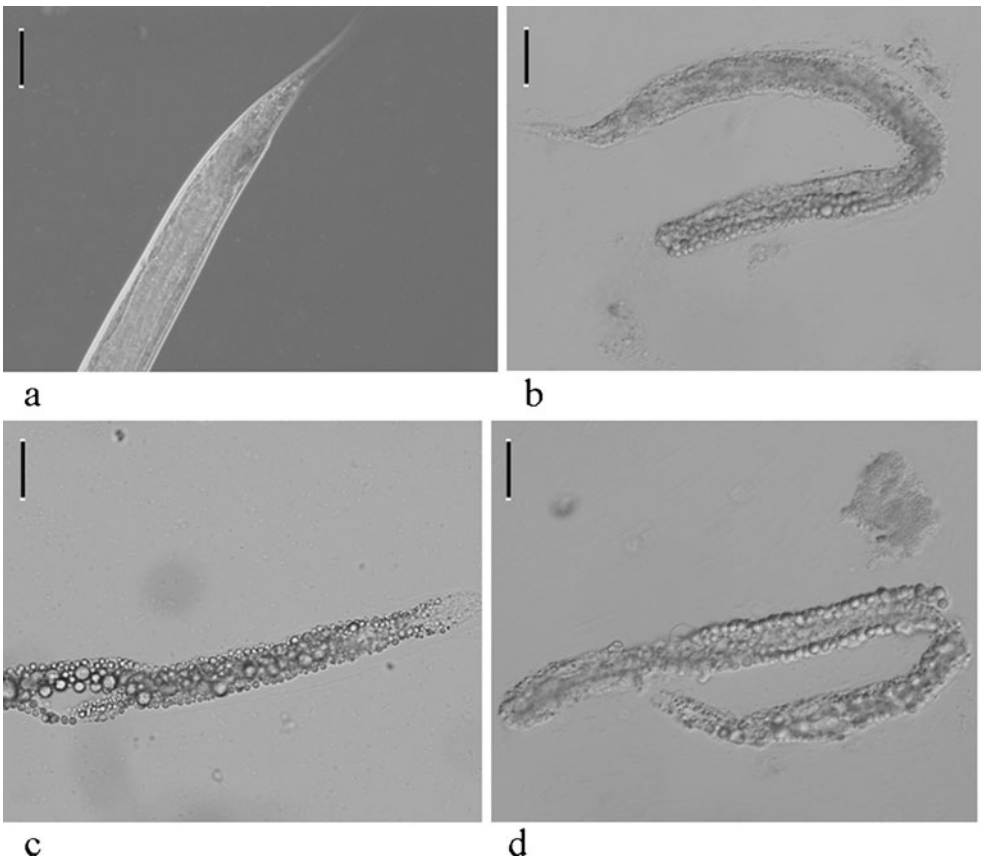

Fig. 4 Nematode *P. redivivus* was treated by crude enzyme from *P. lilacinus* 112. **a** Control nematode. **b**–**d** Nematode treated by crude enzyme for 10–12 h. *Scale bar*, 100 μm

serine proteases secreted by nematophagous fungi can degrade protein constituents of nematode cuticle and help the penetration process. In our previous studies (Yang et al. 2005a, b), a cuticle-degrading protease Ver112 was identified from *L. psalliotae*, and showed to be capable of degrading nematode cuticle and killing nematode.

The plasmid pAN52-1N contains the promoter region of the gpdA gene encoding glyceraldehyde-3-phosphate dehydrogenase and the terminator region of *Aspergillus nidulans* trpC gene, as well as antibiotic (ampicillin) resistance selectable marker (Punt et al. 1990). These elements make the plasmid pAN52-1N an invaluable expression vector in fungi. Recently, Ying and Feng (2006) developed a novel system for efficient transformation of the fungal biocontrol agent *B. bassiana* using plasmid pAN52-1N, and the green fluorescence protein gene *egfp* was introduced into *B. bassiana* via a blastospore-based transformation. Zhang et al. (2008) also transferred the gene *egfp* into the nematophagous fungus *Clonostachys rosea* through the plasmid pAN52-1N. These results provided an important approach for studying the molecular mechanisms of interaction between pathogenic fungi and their hosts.

Restriction enzyme-mediated integration (REMI), a method for generating nonhomologous integration of transforming DNA into the chromosomes of eukaryotic cells, has been used for insertion mutagenesis and other genetic studies in diverse organisms (Riggle and Kumamoto 1998). Although we successfully obtained transformants by REMI transformation, the efficiency was quite low. However, previous studies have shown that the frequency of transformation is considerably improved by optimizing the following factors: the concentration of PEG, the category and quantity of the added restriction enzymes, and the protoplast/DNA ratio (Xu et al. 2005). This indicates that the REMI method may still hold the potential to increase transformation efficiency under further optimized conditions for nematophagous fungi. Jiang et al. (2007) optimized REMI conditions and enhanced the frequency of *B. bassiana* by 73% by the blastospore transformation.

The *P. lilacinus* 112 showed a high degree of mitotic stability. However, compared to the wild-type strain, its growth rate became slow. The slow growth may be caused by factors such as the abundant expression of the gene *ver112* (Xu et al. 2005). Despite its slow growth, the *P. lilacinus* 112 showed a higher protease activity than the wild-type strain, the protease was produced on the second day and reached the highest activity on the sixth day (Fig. 2), which was consistent with the RT-PCR results (Fig. 5) and our previous report (Yang et al. 2005a). In parallel, *P. lilacinus* 112 showed a stronger nematicidal activity than the wild-type strain (Fig. 3), and the crude enzymes isolated from the culture filtrate of *P. lilacinus* 112 showed a higher nematicidal potential and degraded the nematode cuticle more than that of the wild-type strain (Fig. 4). Taken together, our results suggested that gene *ver112* was inserted successfully into the *P. lilacinus* genome, was overexpressed, and conferred on the fungus a higher nematicidal potential than the wild-type parental strain.

In this study, we established an effective tranformation protocol in *P. lilacinus* and successfully utilized this transformation procedure to introduce a virulence gene from another species into this fungus. Other virulence genes including chitinase, adhesion- and recognition-related genes would be alternative candidates for future fungal engineer-

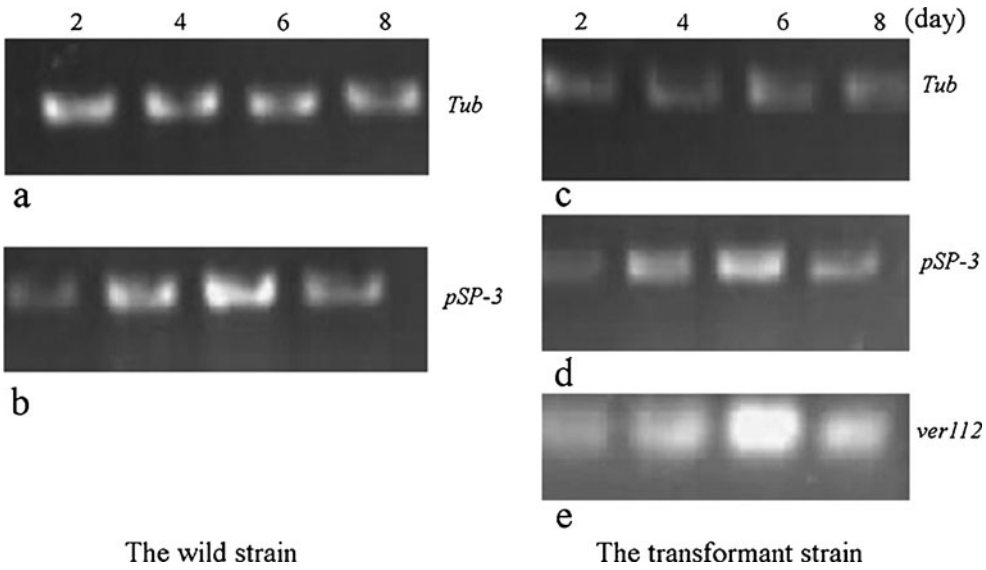

Fig. 5 RT-PCR amplification of genes *ver112*, *pSP-3*, and *Tub* in *P. lilacinus* 112 and the wild strain at different culture periods. **a** The expression of gene *Tub* in wild-type strain. **b** The expression of gene *pSP-3* in wild-type strain. **c** The expression of gene *Tub* in *P. lilacinus* 112. **d** The expression of gene *pSP-3* in *P. lilacinus* 112. **e** The expression of gene *Ver112* in *P. lilacinus* 112

ing with overexpression. Recently, the crystal structures of serine proteases and chitinases from nematophagous fungi were determined (Liang et al. 2010; Yang et al. 2010). These significant works are providing a solid basis for improving the pathogenicity of this group of fungi against their hosts (nematodes) by genetic and protein engineering.

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