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Cloning of the gene *Lecanicillium psalliotae* chitinase *Lpchi1* and identification of its potential role in the biocontrol of root-knot nematode *Meloidogyne incognita*

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Abstract The nematophagous fungus *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) is a well-known biocontrol agent. In this study, a chitinase gene *Lpchi1* was isolated for the first time from *L. psalliotae* using degenerate primers and DNA-walking technique. The cloned gene *Lpchi1* encoding 423 amino acid residues shares a high degree of homology with other pathogenicity-related chitinases from entomopathogenic and mycoparasitic fungi. The complementary DNA sequence of the mature chitinase was amplified via reverse transcription polymerase chain reaction and expressed well in *Pichia pastoris* GS115. Through gel filtration, the recombinant chitinase was purified as a protein of ca. 45 kDa with an optimal activity at pH 7.0 and 37.6°C. The purified chitinase LPCHI1 was found degrading chitinous components of eggs of the root-knot nematode *Meloidogyne incognita* and significantly influence its development. Moreover, our results also demonstrate that the protease Ver112 and the chitinase LPCHI1 from the same fungus interacted on the egg infection.

Keywords Chitinase · Cloning · Expression · *Lecanicillium psalliotae* · *Meloidogyne incognita* · *Pichia pastoris*

Introduction

Nematophagous fungi are an important group of soil microorganisms that can suppress the populations of plant-parasitic nematodes. Their pathogenesis is a complex process including adherence, penetration, and digestion. To infect the nematodes, the nematophagous fungi must first contact and penetrate host cuticle, a barrier to prevent fungal infection (Tunlid and Jansson 1991). The cuticle of nematodes or the shells of eggs play an important role in preventing infection by nematophagous fungi. The penetration of the nematode cuticle or the eggshell has been assumed to result from the actions of mechanical forces and hydrolytic enzymes including proteases, chitinases, and collagenases (Kim et al. 1992; Meyer and Wergin 1998; Yang et al. 2007). Recently, several pathogenicity-related proteases and chitinases have been identified from different nematophagous fungi (e.g., Åhman et al. 2002; Bonants et al. 1995; Khan et al. 2003; Tikhonov et al. 2002; Yang et al. 2005a).

Chitin is a linear polymer of β-1,4-linked *N*-acetylglucosamine residues and is a major structural component of the cell walls of many pathogenic fungi and of insect cuticle (Bartnick-Garcia 1968). The nematode eggshell also contains chitin fibrils embedded in a protein matrix, and the chitin complex is probably the major barrier against fungal infection (Wharton 1980). Chitinases (EC3.2.1.14) is a type of inducible enzymes to degrade chitins and can be produced by a wide variety of organisms. Their potential role in fungal infection towards nematode eggs was first suggested by Wharton (1980). Recently, the first chitinase CHI43 serving as a nematicidal factor was identified from two nematophagous fungi *Verticillium chlamydosporium* (syn. *Pochonia chlamydosporia*) and *Verticillium suchlasporium* (syn. *Pochonia rubescens*; Tikhonov et al. 2002). After treatment of nematode eggshells with CHI43, scars appeared on the surface of eggshells. Moreover, studies on extracellular enzymes of *Paecilomyces lilacinus* also revealed that its protease and chitinase could drastically

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alter the eggshell structures when applied individually or in combination (Khan et al. 2003). However, little is known about the encoding genes of chitinases from nematophagous fungi.

The filamentous fungus *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) is known as an effective parasite of plant-parasitic nematodes (Zare et al. 2000). In our previous report, a cuticle-degrading protease Ver112 was identified from *L. psalliotae*, and this enzyme was shown to be involved in infection against nematode *Panagrellus redivivus* (Yang et al. 2005a). In this study, this fungus was found infecting the eggs of the root-knot nematode *Meloidogyne incognita* and harboring another pathogenic factor, a chitinase gene *Lpchi1*. This novel gene was cloned for the first time from *L. psalliotae* and its potential role in fungal infection against *M. incognita* was explored.

Materials and methods

Strains, plasmids, and growth conditions

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. The fungal strain was grown on Potato Dextrose Agar (PDA) medium at 28°C. *Escherichia coli* DH5á, used as the recipient strain for recombinant plasmids, was grown in Luria–Bertani medium at 37°C. *Pichia pastoris* GS115 strain (Invitrogen, USA) was grown in YDP (2% polypeptone, 1% yeast extract, and 2% dextrose) or BMGY [0.1 M potassium phosphate, 2% peptone, 1% yeast extract, 1.34% yeast nitrogen base (YNB), and 2% glycerol]. BMMY (0.1 M potassium phosphate, 2% peptone, 1% yeast extract, 1.34% YNB, and 1% methanol) was used for protein induction. The transformants were grown and selected on MD plates (2% dextrose, 1.34% YNB).

The eggs of *M. incognita* were isolated from egg masses on roots of infected tomato plants (*Lycopersicon esculentum*) as described by Zhang et al. (1993).

The pPIC9K (Invitrogen, USA) vector was used for heterogenous expression of *L. psalliotae* chitinase gene *Lpchi1* in *P. pastoris* GS115. The vector pMD18-T (Takara, Japan) was used for polymerase chain reaction (PCR) cloning.

Amplification of a chitinase gene from the strain *L. psalliotae* CGMCC1312

L. psalliotae was cultured in PL-4 liquid medium (Yang et al. 2005a) for 4 days at 26°C. Mycelium for DNA extraction was collected by filtration in a sterilized filter funnel and was ground to a fine powder in liquid nitrogen. DNA was extracted as described by Zhang et al. (1996).

A pair of degenerate primers (Table 1, primer set 1) was designed as described by Bogo et al. (1998) and used to amplify the conserved sequence of the chitinase gene from *L. psalliotae*. The genomic DNA was used as template, and PCR conditions followed those described in a previous report (Yang et al. 2005b). All the PCR products were purified and sequenced using an ABI PRISM 3730 automated sequencer (Perkin-Elmer, America) with four fluorescent dyes.

To obtain the complete chitinase gene from *L. psalliotae*, two pairs of nested PCR primers (Table 1) were designed according to the conserved sequence using the above method. The 5′ and 3′ terminal fragments of the chitinase gene were amplified using nested PCR primers (2 and 3) and the DNA-Walking Speedup™ Premix Kit (Seegene, Korea), respectively. PCR amplification was performed according to the user's manual.

Sequence analysis

Analysis of the DNA sequence was done using the DNAman software package (Version 5.2.2, Lynnon Biosoft, Canada). Homology searching was performed using the BLAST algorithms against various databases of the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Potential *N*-glycosylation sites were analyzed by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular weight, theoretical p*I* and amino acid composition were analyzed by ProtParam tool (http://us.expasy.org/tools/protparam.html). Amino acid sequences of chitinases from different organisms were aligned using the DNAman software package and a phylogenetic tree was constructed using PAUP version 4.0b10 (Swofford 2002).

Construction of the recombinant plasmid and the expression of *Lpchi1* in *P. pastoris* GS115

Total RNA was extracted from the mycelia of *L. psalliotae* using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Based on the chitinase gene of *L. psalliotae*, a pair of specific primers containing restriction sites (*EcoR I* and *Not I*, respectively; Table 1, primer set 4) was designed to amplify the complementary DNA (cDNA) sequence encoding the mature chitinase. Reverse transcription PCR was performed using the RNA PCR Kit (AMV) version 2.1 (Takara, Japan) according to the manufacturer's protocol. The PCR product was digested with *EcoR I* and *Not I*, and subcloned into the expression vector pPIC9K (Invitrogen, USA) in accordance with the manufacturer's protocol (Invitrogen, USA).

The recombinant vector pPIC9K/*Lpchi1* was linearized through enzymatic digestion by *Bpu*1102I and subsequently electroporated into *P. pastoris* GS115 using a Bio-Rad

Table 1 List of oligonucleotides used in different experiments

	Primers	Sequence (5′–3′)	Location
1	Forward	GC(TC) GT(TC) TA(TC) TT(TC) AC(TC) AA(TC) TGG	425-445
	Reverse	GG(AG) TA(TC) TCC CA(AG) AT(AG) TC(CGAT) AT	975-953
2	Forward TSP1	AAG TTG CGT CCG TAG ATA CC	514-495
	Forward TSP3	TTA GAT CCA ATG ATA CAC CAT GAG	483-460
3	Reverse TSP1	CTG TCG GCT TCC TAA AGG	918-935
	Reverse TSP3	AAG GAC TGG GGC TTT GAC G	932-950
4	Forward	CG*GAATTC*GCAAGTGGTTACGCCAATG	407-425
	Reserve	TAT*GCGGCCGC*TTAGTTCATGCCCTTCTTAATG	1729-1708

Gene Pulser (Bio-Rad Laboratories, USA). One milliliter of ice-cold sorbitol solution (1 M) was added immediately after electroporation. The mixture was spread onto MD plates and cultured at 30°C for 2 days. Then transformants were patched on YPD plates containing G418 (0.5–3 mg ml^{-1}) and cultured at 30°C for 2 days. The expression analysis of the recombinant *P. pastoris* GS115 was conducted according to the manual for expression of recombinant proteins in *P. pastoris* (Invitrogen, USA).

Enzyme purification and analysis

For chitinase purification, the supernatant fluid was brought to 30% (*v*/*v*) saturation by ethanol and stirred for 6 h at 4°C for protein precipitation. The precipitated proteins were collected by centrifugation at 9,000 rpm for 15 min and suspended in 20 mM Tris–HCl buffer (pH 7.0). The recombinant chitinase was purified using gel filtration (Hiload 26/60 Superdex 75pg, Amersham). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify protein purity and determine the molecular mass of the purified enzyme under denaturing conditions using a 12% acrylamide gel.

Chitinase activity was measured colorimetrically using colloidal chitin as substrate. Enzyme activity was determined following the previous method (Miller 1959). An acetylglucosamine glucose curve was carried out as standard. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the colloidal chitin and released 1 μM *N*-acetylglucosamine in 1 min at 37.6°C. Colloidal chitin was prepared as described by Roberts and Selitrennikoff (1988).

Effects of pH and temperature on enzyme activity

The optimum temperature for enzyme activity was investigated at pH 5.4 by incubating purified chitinase and colloidal chitin (0.5%) at 20–60°C for 3 h. The effect of pH on chitinase activity were studied by incubating purified chitinase and colloidal chitin (0.5%) with the Britton Robinson universal buffer system (Yang et al. 2005a) at pH 3–8. Chitinase activities were all quantitatively assayed. Water was used as a control. All assays were repeated three times.

Effects of *L. psalliotae* protease and chitinase on M. incognita eggs

The bioassays were performed in glass plates (3 cm). Root-knot nematode (*M. incognita*) eggs were incubated with the purified chitinase LPCHI1, protease Ver112, a mixture of both LPCHI1 and Ver112, and a negative control (20 mM Tris–HCl pH 7.0), respectively. Approximately 100 eggs at different stages of development were incubated in each plate and incubated on 28°C. All solutions used were filter-sterilized through 0.22-μm filters. All assays were done in triplicate. Chitinase activity was 15 mU ml^{-1} (nmol ml^{-1} min^{-1}), and protease Ver112 was purified as described by Yang et al. (2005a) and its activity was 32 U ml^{-1}. Effects of *L. psalliotae* protease and chitinase on *M. incognita* eggs were detected at different time intervals under a light microscope (Olympus, Japan). Microsoft Excel T-test program was used for statistics analysis. A P value of less than 0.05 was considered statistically significant.

Results

Cloning of the chitinase gene from L. *psalliotae*

A 548 bp PCR product was amplified using degenerate primers (Table 1) and the 5′ and 3′ flank sequences were amplified using the DNA-Walking Speedup™ Premix Kit, respectively. The complete chitinase gene was obtained by assembling sequences from the three PCR fragments (the conserved sequence of the chitinase gene, 5′ flank DNA sequences and 3′ flank DNA sequences) with the DNAman software package. The sequence was verified as a chitinase gene by homologous analysis with other fungal chitinase genes, and the complete nucleotide sequence of *Lpchi1* was submitted to GenBank, gaining the accession number EF203917.

The encoding gene of LPCHI1 consisted of one ORF (open reading frame) and three short introns (49, 51, and 53 bp in length). The splice junctions were determined by comparing the sequence of *Lpchi1* with that of its corresponding cDNA sequence. Each intron began with GT and ended with AG, similar to other fungal introns (Gurr et al. 1987). A putative promoter from position −103 to −152 upstream of the start codon ATG was identified by an online prediction, and a putative TATA box was identified at −142 bp upstream of the start codon. The chitinase gene *Lpchi1* and its corresponding amino acid residues were shown in Fig. 1.

The chitinase gene encoded a polypeptide of 423 amino acid residues. The calculated molecular mass and the p*I* of the putative protein was 46.01 kDa and 5.76, respectively. Comparison of the LPCHI1 amino acid sequences with the known chitinases from other fungi (e.g., *Aphanocladium album*; Blaiseau and Lafay 1992), indicated that LPCHI1 was synthesized as a preproenzyme (Fig. 1). The first 34 amino acids had diverse characteristics of signal peptides and the signal peptidase cleavage site was between Ala_{34} and Ser_{35}. After processing of the 34 N-terminal amino acids, the calculated size of LPCHI1 was 42.49 kDa. Two potential *N*-glycosylation sites (NRS and NPS) were found by online prediction. Comparison of the LPCHI1 amino acid sequence with proteins in the GenBank database revealed that LPCHI1 shared a high degree of similarity with other fungal chitinases. For example, the percentage identity of LPCHI1 from *L. psalliotae* was 88.42% with that from *A. album* (accession no. P32470; Blaiseau and Lafay 1992), 85.82% from *Lecanicillium lecanii* (accession no. AAV98691; Lu et al. 2005), 80.61% from *Verticillium fungicola* (accession no. AY292527). Moreover, the alignment of the most conserved region of glycosyl hydrolase family 18 from different organisms indicated that regions of MLSIGGW and GFDGIDVDWE were highly conserved among those chitinases (Fig. 2). The active site centered around the catalytic residues Asp and Glu are also conserved in all aligned sequences.

Phylogenetic analysis

The phylogenetic tree of chitinases derived from various organisms was constructed using PAUP version 4.0b10 (Swofford 2002). This tree consisted of two main clades corresponding to glycosyl hydrolase families 18 and 19 (Fig. 3). Two chitinases classified to glycosyl hydrolase family 19 from *Brassica juncea* (Zhao and Chye 1999) and *Zea diploperennis* (Tiffin 2004) were clustered together. Other chitinases belonged to glycosyl hydrolase family 18. In family 18 of the glycosyl hydrolase superfamily, three chitinases from bacteria and virus (*Ha*NPV, *Ld*MNPV, and *Serretia marcescens*) were clustered and formed a subclade, and the majority of chitinases from pathogenic fungi were clustered together. Among the fungal chitinases, two chitinase from human pathogenic fungi *Aspergillus fumigatus* and *Coccidioides posadasii* were clustered and formed a subclade, and three chitinases from mycoparasitic fungi *Trichoderma hamatum*, *Trichoderma viride*, and *Hypocrea vinosa* were clustered together. Based on this phylogenetic tree, chitinase LPCHI1 was clustered together with chitinases from entomopathogenic fungi. Interestingly, chitinases Bbchit1 and CHIT36, identified from *Beauveria bassiana* (Fang et al. 2005) and *Hypocrea lixii* (anamorph *Trichoderma harzianum*; Viterbo et al. 2001), respectively, formed a single subclade, they showed low levels of similarity to other chitinases from entomopathogenic and mycoparasitic fungi.

Expression and purification of recombinant chitinase

More than 100 colonies grew on the selective MD plates. Transformants were selected on YDP plates containing different concentration of G418 from 0.5 to 3 mg ml^{-1}. Six transformants resistant to the highest concentration were selected. After induction with methanol for *Lpchi1* expression, one transformant expressed the chitinase at high level and this chitinase was selected for further experiment.

To determine whether the engineered strain of *P. pastoris* produced chitinase, we tested the recombinant protein from GS115/pPIC9K/*Lpchi1* vector using the method described in "Materials and methods." As expected, the control vector (GS115/pPIC9K) did not have any chitinase activity. In contrast, the transformant containing the recombinant plasmid showed a high-level chitinase activity. The LPCHI1 enzymatic activity was stable between 1 and 5 days after induction and with an activity of up to 12.3 mU ml^{-1} (nmol ml^{-1} min^{-1}) after 4 days of induction. The chitinase LPCHI1 exhibited activity under a broad range of temperatures from 20 to 50°C with highest activity at 37.6° C. In addition, it also exhibited activity under a broad pH range from 4.0 to 8.0 with highest activity at 7.0. SDS-PAGE profile of the GS115/pPIC9K/*Lpchi1* culture supernatant confirmed that the transformant secreted a major protein band at about 45 kDa. The recombinant protein was purified using gel filtration with a buffer containing 50 mM sodium phosphate and 150 mM NaCl. The molecular weight of the purified LPCHI1 was estimated to be 45 kDa by SDS-PAGE (Fig. 4).

Effects of L. psalliotae protease and chitinase on M. incognita eggs

The effects of the purified chitinase LPCHI1 and protease Ver112 on the development of root-knot nematode eggs were studied under a light microscope. Incubation of eggs

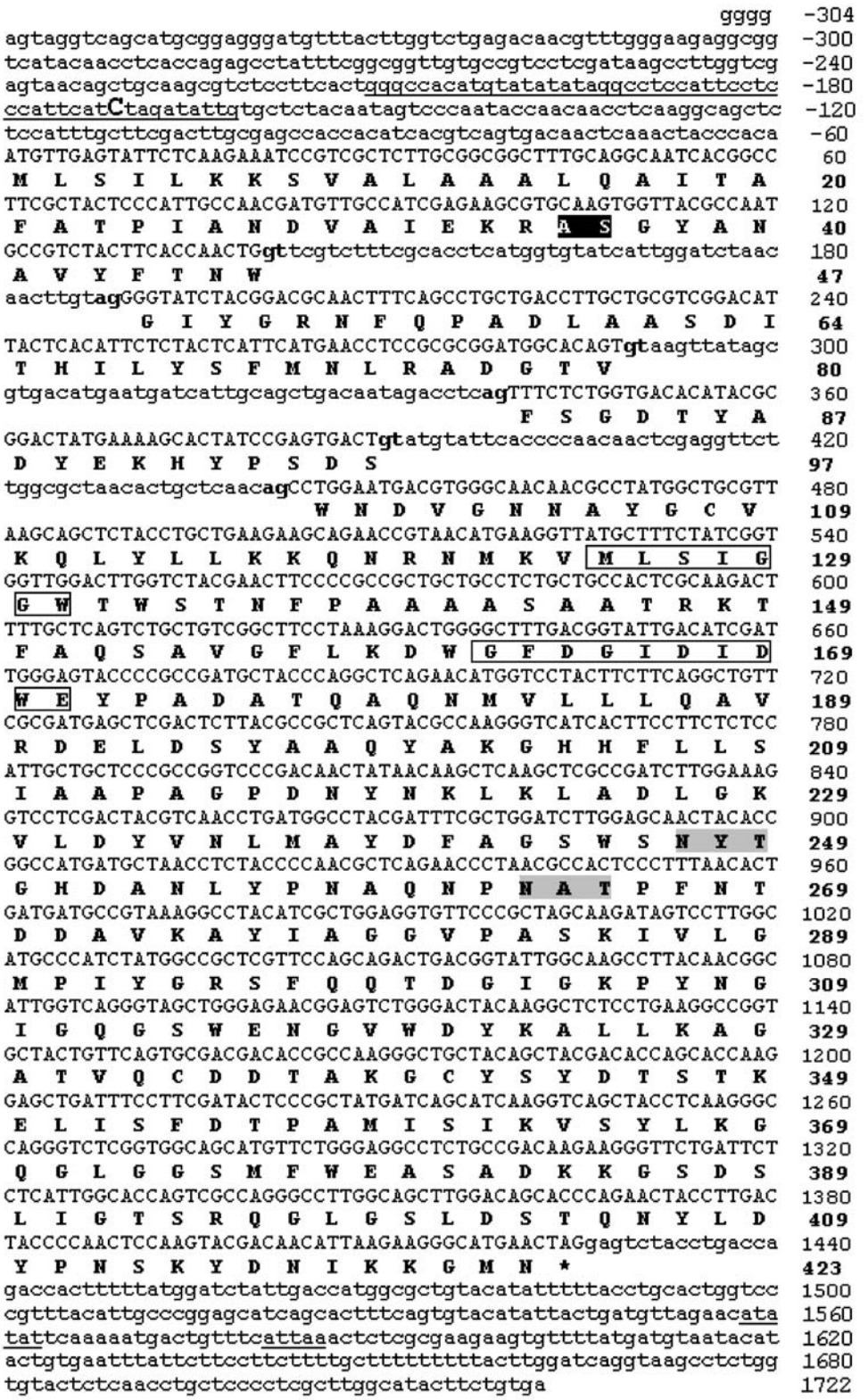

Fig. 1 The nucleotide sequence of chitinase gene *LPchi1* from *L. psalliotae* (GenBank accession no. EF203917). The deduced amino acid sequence is shown in *one-letter code* under the DNA sequence. Putative promoter and polyadenylation sites are *underlined*. The ORF sequence showed in *larger font* and *bold*. Introns are shown in *lowercase letters*, and their conserved 5′ GT, 3′AG are in *bold*. The mature protein is preceded by A and S (*bold*) residues and shaded in *black*. Potential *N*-glycosylation sites are shaded in *grey*. The *bold* and *boxed* region (amino acids 125–131 and 162–171) corresponds to the conserved motif characteristic of glycoside hydrolases

of root-knot nematode (*M. incognita*) in the presence of purified chitinase for 3 days significantly inhibited egg hatching in vitro. After 3 days of treatment, approximately 38.2% of the immature eggs did not develop into eggs or juvenile stage. In contrast, 19.8% failed to develop in the negative control (Fig. 5). Extending the incubation time to 4–6 days did not influence hatching rates of nematode eggs (data not shown). When nematode eggs were treated with

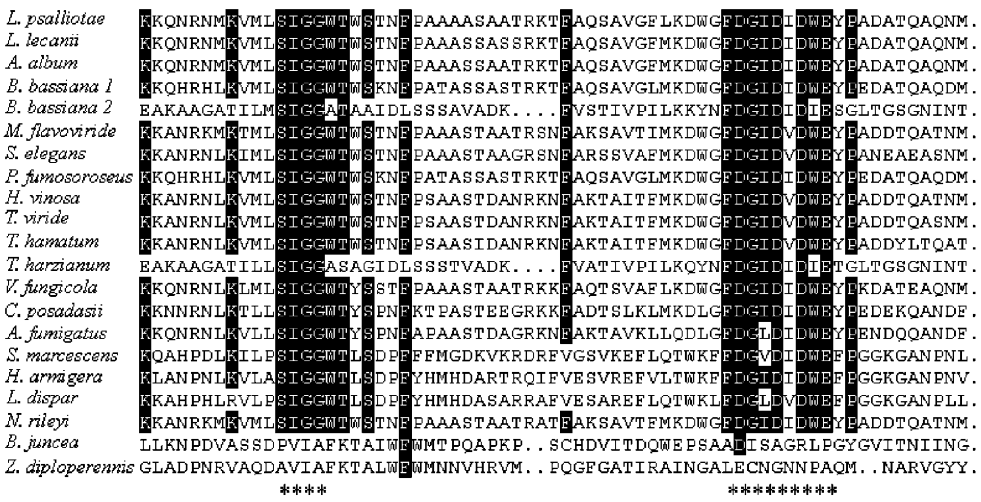

Fig. 2 Alignment of the amino acid sequences of chitinases from different organisms. The GenBank accession numbers of chitinases were described in Fig. 3. Areas shaded in *black* are high degree homology (more than 75% identify) and *unshaded* areas are regions of variability between the chitinases. *Triple asterisk* indicated the conserved substrate binding and catalytic domains (SXGG and DXXDXDXE) of glycosyl hydrolase family 18

the purified protease Ver112, 45.9% of the nematode eggs failed to hatch, with the combined treatment with both the chitinase and protease, the hatching rate reduced by 56.5%.

Structurally and morphologically, after treatment with the purified chitinase, the immature eggs of *M. incognita* were anamorphic, and the enzyme-treated eggs were swollen and the eggshell lost its original structural features as indicated by light microscopy (Fig. 6). There were large vacuoles in the chitin layer of *M. javanica* eggs after treatment with chitinase (Fig. 6b), the eggshell was partially degraded (Fig. 6c), and some eggs deformed (Fig. 6d). The immature eggs did not hatch after they were treated with chitinase (Fig. 6f). However, the eggs from the negative control treatment were intact (Fig. 6a) and could develop into eggs containing juveniles (Fig. 6e).

Discussion

In this study, we cloned an endochitinase gene *Lpchi1* by the DNA-Walking technique. This is the first report from the nematophagous fungus *L. psalliotae*. Our analysis showed that this gene was similar to other intron-containing epiphyte chitinase genes. The predicted amino acid sequence showed it is a typical member of family 18 of the glycosyl hydrolase superfamily. The putative polypeptide included a signal sequence at the N-terminal and a catalytic domain that contained the highly conserved consensus sequence of the active site signature (Figs. 1 and 2). Comparison of LPCHI1 with other chitinases in the GenBank revealed that the sequences MLSIGGW and GFDGIDVDWE were highly conserved (Fig. 2). These two motifs might be the chitinase catalytic center (Henrissat and Bairoch 1993). Among these residues, the locations of active sites Asp and Glu were the most conserved residues. Glu acts as a general acid catalyst which donates a proton to the glycosidic oxygen and contributes to the lowering of the energy barrier of the reaction by stabilizing the transient carbonium ion intermediate electrostatically (Phillips 1967). Chemical modification and site-directed mutagenesis confirmed that Asp/Glu residues were essential for chitinase activity (Mileweski et al. 1992; Watanabe et al. 1993).

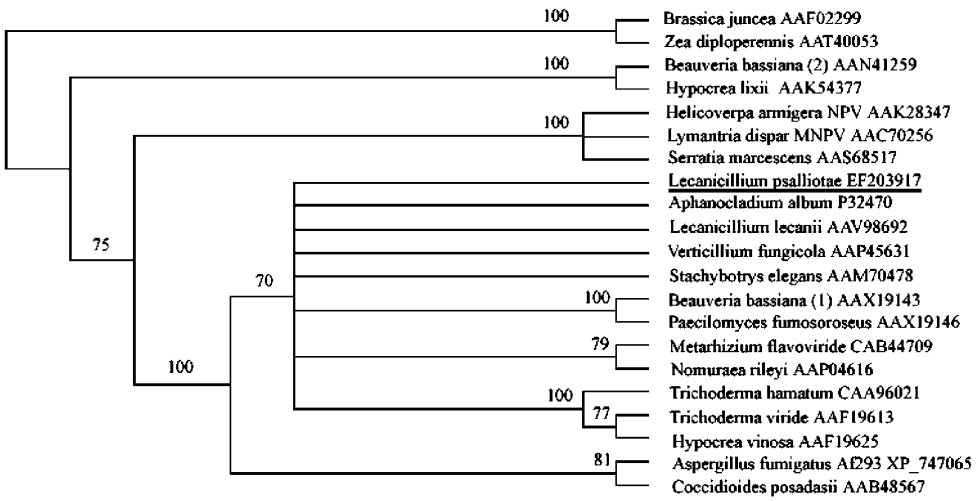

Fig. 3 Phylogenetic tree showing the relationship between LPCHI1 and other chitinases. The tree was constructed with the PAUP version 4.0b10 (Swofford 2002) program packages. The chitinase genes of *Brassica juncea* and *Zea diploperennis* were used as an outgroup in order to root the tree

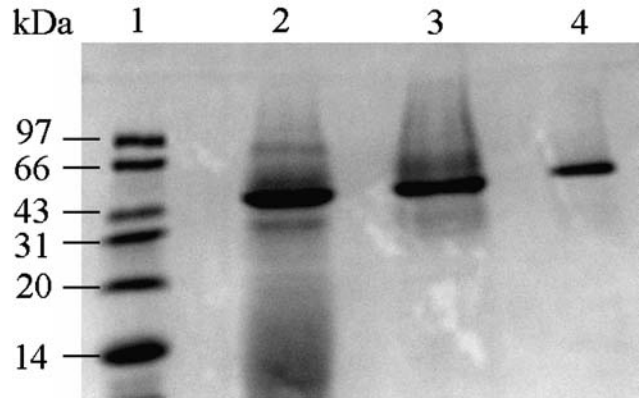

Fig. 4 SDS-PAGE analysis of *Lpchi1* expressed in *P. pastoris* strain GS115. SDS-PAGE was performed on a 12% gel and stained with Coommassie Brilliant Blue R250. *Lane 1*, molecular weight marker of proteins; *lanes 2* and *3*, crude extract; *lanes 4*, purified chitinase

The gene families encoding the chitinases are large and the genes from the same as well as different families have undergone considerable divergence. For example, while *Lpchi1* showed identities between 66.6 and 84.4% to the sequences from insect pathogenic fungi (e.g., *L. lecanii*, *P. fumosoroseus*, and *M. flavoviride*), it showed low levels of identity (12.3–19.3%) to chitinases from human pathogenic fungi *A. fumigatus* and *C. posadasii*. Moreover, chitinases identified from parasitic fungi *B. bassiana* (Bbchit1) and *H. lixii* (CHIT36) showed low identity (16.7–21.4%) to other members of glycosyl hydrolase family 18. Despite these differences, they all shared conserved substrate binding and catalytic domains (SXGG and DXXDXDXE; Fig. 2). The phylogenetic tree (Fig. 3) revealed that chitinases from *B. bassiana* (AAN41259) and *H. lixii* (AAK54377) were clustered and were phylogenetically distant from other insect-pathogenic fungi. The sequence diversities of chitinases from different organisms may reflect their functional differences. Moreover, more than one chitinases have been identified from the same species, e.g., two chitinases (AAN41259 and AAX19143) were identified from *B. bassiana*. Similarly, three endochitinases (CHI1, CHI2, and CHI3) and one exochitinase were found in *Metarhizium anisopliae* (St Leger et al. 1993). These chitinases played different roles during the fungal growth and differentiation (St Leger et al. 1993). In the mycoparasitic *Trichoderma* species, multiple chitinases have been identified, however, only ech42 (Lorito et al. 1998) and chit33 (Limon et al. 1999) were found capable for biological activity enhancement. The multiplicity of the chitinase genes within the same species may reflect their functional differences between related proteins (Orikoshi et al. 2005).

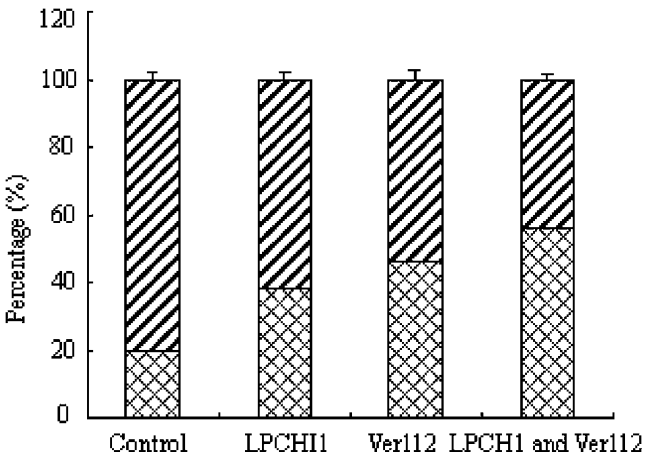

Fig. 5 Effect of chitinase (LPCHI1), protease (Ver112) and control (20 mM Tris–HCl pH 7.0) on eggs of the root-knot nematode *M. incognita*. ▨ indicate the percentage of normal nematode eggs; ▩ indicate the percentage of nematode eggs can not develop into eggs containing a juvenile. Microsoft Excel T-test program was used for statistics analysis ($P<0.03$)

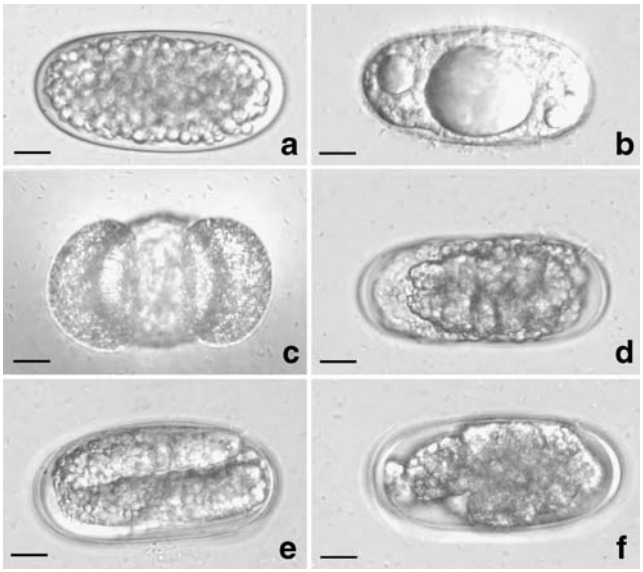

Fig. 6 Eggs of the root-knot nematode *M. incognita* after treatment with the purified chitinase. **a** and **e**, the control; **b**, large vacuole was observed in eggs; **c**, the eggshell was partially degraded; **d**, some eggs deformed; **e**, immature eggs developed into eggs containing a juvenile after treating by control. **f**, immature eggs did not hatched after treating by chitinase. Photographs were taken on day 3. *Scale bar*, 10 μm

Based on their developmental stages, nematode eggs can be classified as either immature eggs (two-cell stage to gastrula stage) or eggs containing a juvenile (lima-bean stage to pretzel stage; Tahseem et al. 1991). From Fig. 5, we can see that the purified chitinase had a significant effect on nematode eggs. The chitinase disturbed the development of root-knot nematode eggs, and immature eggs were highly vulnerable to treatments with chitinase, similar to that observed by Bonants et al. (1995). Mercer et al. (1992) found increased hatching rates after incubation of eggs of the *Meloidogyne hapla* with several plant and

microbial chitinases. However, Galper et al. (1990) found a lower hatching rate of *Meloidogyne* eggs treated with other enzyme solutions. In this study, the egg hatching rates after treatment with chitinase LPCHI1 was not stimulated as compared with the control, which was different from those of others (Galper et al. 1990; Mercer et al. 1992). Our result was more similar to that of Don et al. (1997). They incubated the eggs of the potato cyst nematode *Globodera rostochiensis* using purified chitinase for 2 weeks and found no correlation between the level of egg-hatch inhibition in vitro and the quantity of chitinase used. Therefore, the differences in the ability to inhibit egg hatching maybe related to the origins and types of chitinases and nematode species.

In our previous report, we identified an extracellular cuticle-degrading protease Ver112 from the same nematophagous fungus *L. psalliotae*. We found that this protease played a role in penetrating nematode cuticle (Yang et al. 2005a). Because the cuticle and eggshell are complex structures consisted of proteinous and chitinous components (Cox et al. 1981), the nematophagous fungi must produce multiple enzymes to involve infection of nematodes (Khan et al. 2003; Tikhonov et al. 2002). In this study, we identified the second factor, an endochitinase, from *L. psalliotae* and expressed it in *P. pastoris* GS115. Our result indicated that both the protease Ver112 and the chitinase LPCHI1 identified from *L. psalliotae* play a role in infection against nematode eggs of *M. incognita* in vitro (Fig. 5). This study provided the nucleotide information of a chitinase for the first time from nematophagous fungi. Our study should help elucidate the chitinase genes from other nematophagous fungi and identify the relationships between *Lpchi1* and other virulence factors in their infection of nematodes.

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