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Molecular Cloning, Characterization, and Expression of a Chitinase from the Entomopathogenic Fungus *Paecilomyces javanicus*

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Abstract. *Paecilomyces javanicus* is an entomopathogenic fungus of coleopteran and lepidopteran insects. Here we report on cloning, characterization, and expression patterns of a chitinase from *P. javanicus*. A strong chitinase activity was detected in *P. javanicus* cultures added to chitin. The full-length cDNA, designated *PjChi-1*, was cloned from mycelia by using both degenerate primer/reverse transcription polymerase chain reaction (RT-PCR) amplification and 5′-/3′-RACE extension. The 1.18-kb cDNA gene contains a 1035-bp open reading frame and encodes a 345-amino acid polypeptide with a deduced molecular mass of 37 kDa. A conserved motif for chitinase activity -F82DGIDIDWE90- was present in deduced amino acid sequence. Both RT-PCR and Northern analysis revealed that the expression of the *PjChi* gene was constitutive at low level, but enhanced to high level when chitin was the substrate. Fungal inhibitory assay showed that PjChi-1 inhibited the growth of phytopathogenic fungi such as *Sclerotium rolfsii, Colletotrichum gloeosporioides, Aspergillus nidulans*, and *Rhizoctonia solani.*

Mycoparasites including entomopathogens are important regulatory factors in insect populations, and organisms used for microbial control of insects include viruses, bacteria, fungi, protozoa, and nematodes [7]. Chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, which is a β-(1,4)-linked polymer of *N*-acetyl-D-glucosamine and one of the important structural components of insect cuticle and fungal cell wall. Chitinases are produced by a large number of organisms including plants, fungi, and bacteria, and play an important role in the defense mechanism of plants against pathogens and in the mycoparasitic process of fungi. They also play an important role in nutrition, development, and morphogenesis of fungi. The characterization of chitinase genes and enzymes is important to understand the chitinolytic system in entomopathogenic fungi [2, 14]. To date, a few reports have been published on the isolation and characterization of chitinase genes from entomopathogenic fungi such as *Metarhizium anisopliae* [1, 2, 15, 16] and *M. flavoviride* [15]. The genus *Paecilomyces* is a cosmopolitan filamentous fungus that belongs to class Euascomycetes of phylum Ascomycota. Of 31 species of *Paecilomyces*, 14 species are known pathogens of various arthropods and nematode hosts found on plants and in soil [12]. *Paecilomyces javanicus* is an entomopathogenic fungus parasitic to various coleopteran and lepidopteran insects [12]. *P. javanicus* is considered to be a good candidate for microbial control of insect pests because of its potential to cause epizootics naturally. In this study, the chitinase activity was detected from the crude protein extract of *P. javanicus* mycelia by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. A high level of chitinase gene expression was observed in *P. javanicus* grown in medium supplemented with chitin, which in turn led us to isolate a chitinase cDNA from *P. javanicus* for further molecular characterization. Based on this, cloning, characterization, and expression patterns of a chitinase cDNA, *PjChi-1* from *P. javanicus*, and the inhibitory effect of the chitinase on the growth of *Sclerotium rolfsii*, *Colletotrichum gloeosporioides, Aspergillus nidulans*, and *Rhizoctonia solani* were undertaken in the present investigation.

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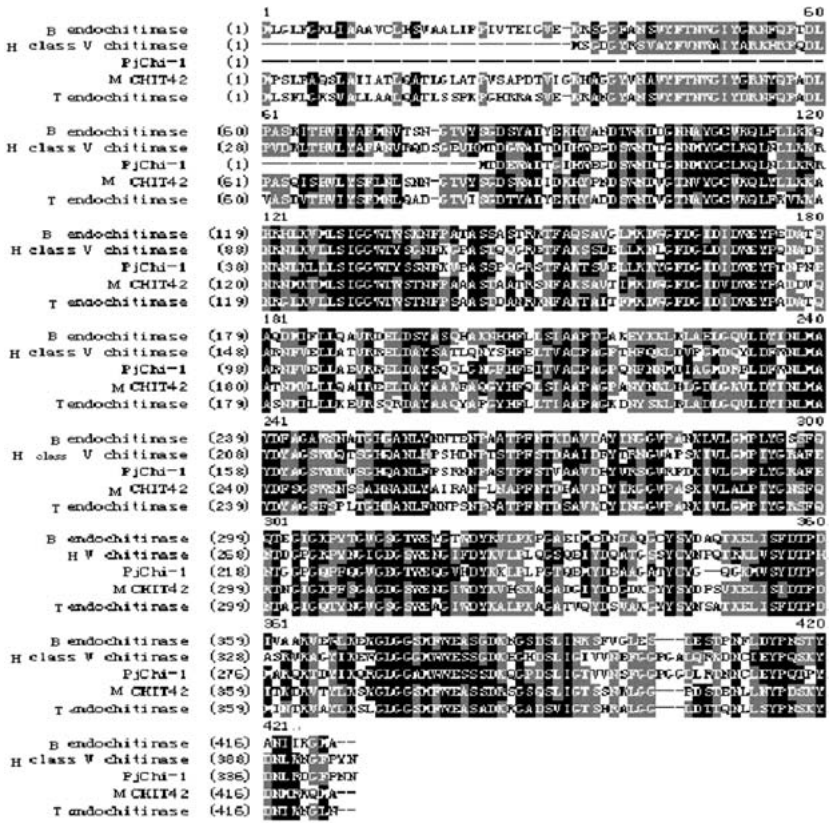

Fig. 1. Alignment of deduced amino acid sequence of *Paecilomyces javanicus PjChi-1* with B-*Beauveria bassiana* CHIT2 (AY147010), H-*Hypocrea virens ECH2* (AF397021), M-*Metarhizium anisopliae* CHIT42 (AAB81999), and T-*Trichoderma harzianum* CHIT42 (AAB34355). Identical amino acids are highlighted on black background and similar amino acids are shown on gray background. The alignment was performed with VectorNTI.

Materials and Methods

Organism and Culture Conditions. *P. javanicus* was collected from infected pupae of *Casuarina* (*Lymantria xylina*) and grown on potato dextrose agar (PDA, Difco Laboratories) at 28°C. For DNA, RNA, and protein extractions, the mycelia were cultured in 250 mL Erlenmeyer flasks containing 50 mL potato dextrose broth (PDB, Difco Laboratories) with or without chitin (1.0 g l^{-1}, Sigma). The flasks were incubated at 26°C for 7 days with continuous shaking (110 rpm).

Extraction of RNA and Protein. Total RNA was isolated from mycelia by the method of Morissette et al. [9]. The protein was extracted from mycelia in phosphate-buffered saline (PBS) by centrifugation for 15 min at 7378 *g*.

Chitinase Activity Assay. SDS-PAGE was performed using the Tris-Tricine system as described by Schagger and von Jagow [13] with modification. For chitinase activity, ethylene glycol chitin (0.01%) was added to the Tricine-SDS-polyacrylamide separating gel. Protein sample was mixed with sample buffer and boiled for 5 min before loading onto the gel. After electrophoresis, the gel was incubated at 37°C for 2 h in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 with shaking (50 rpm). For chitinolytic zymograph assay was performed according to the method described by Trudel and Asselin [17]. After gel electrophoresis, the separating gel was incubated for 4 h (37°C) in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100. After incubation, the gel was stained with 50 mM Tris–HCl buffer (pH 8.9) containing 0.01% Calcofluor White M2R (Sigma). Chitinolytic zones in the Calcofluor-stained gel were visualized under an ultraviolet transilluminator.

Cloning of *PjChi-1*. *P. javanicus* cDNA library was synthesized from total RNA using SMART cDNA library construction kit (Clontech, BD). Forward (5′-GA (C/T) GA (A/G) TGG GC (A/T/C/G) GA (C/T) AC (A/T/C/G) GG-3′) and reverse (5′-GG (G/A)TA (C/T)TC CCA (A/G)TC (A/T/C/G)A(C/T) (A/G)TC-3′) degenerate primers were designed based on conserved regions of chitinase genes and a cDNA fragment (*PjChi*) was amplified by PCR using *P. javanicus* cDNA library as template. Based on *PjChi-1* fragment sequence, 5′-RACE (5′-CGA CGC TCG TCT TGG CGA AGG TAG A-3′) and 3′-RACE (5′-GAC TCG TGG AAT GAC ACC GGC AAC A-3′) primers were designed. The full-length cDNA of *PjChi-1* was obtained by RACE-PCR using SMART RACE cDNA amplification kit (Clontech, BD).

Sequencing and Analysis of the *PjChi-1* Gene. Sequence analysis of cDNA was carried out using NCBI with Blastx and Blastn algorithms (www.ncbi.nlm.nih.gov/blast). Protein translation of the cDNA sequence, and nucleotide and protein sequence analysis were done with ExPaSy program (http://www.expasy.org). Phylogenetic analysis of *PjChi-1* and chitinase amino acid sequences of orthologs was performed using Vector NTI v.8.0 program.

Expression Analysis of *PjChi-1*. The reverse transcription polymerase chain reaction (RT-PCR) was performed on cDNA templates prepared from total RNA using one-step RT-PCR (Takara BioCo, Kyoto, Japan) following the manufacturer's instructions. Northern blot analysis was carried out according to standard protocols [11].

Construction, Expression and Purification of Recombinant Chitinase. To construct a recombinant expression vector, the PjChi-1

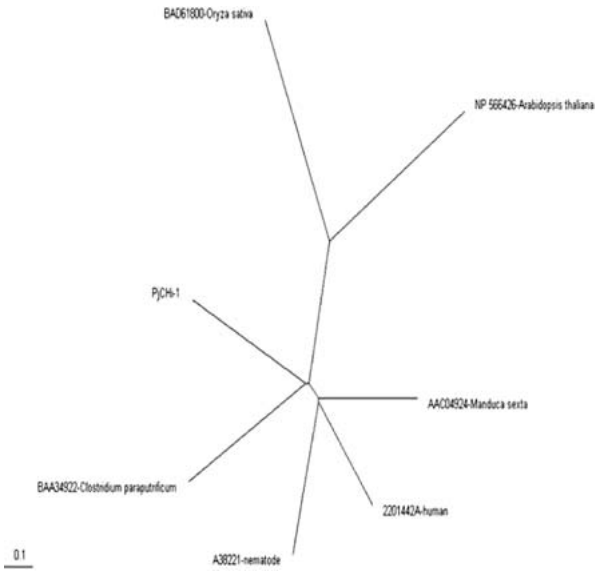

Fig. 2. A Phylogenetic tree showing relationships between *Paecilomyces javanicus* endochitinase (*PjChi-1*) and related chitinase amino acid sequences. The bar represents 0.1 substitutions per site.

chitinase gene was amplified by PCR reaction using two specific primers as previously described. The amplicons were digested with *Xho*I and *Bam*HI, and ligated into *Xho*I and *Bam*HI sites of pGEX-6P-1 vector. The constructs were transformed into *Escherichia coli* BL21 (DE3) and transformants were selected on an LB plate containing 50 μ/mL ampicillin. *E. coli* transformants were grown in 2xYT broth supplemented with ampicillin for 14 h. To induce expression of the recombinant protein, isopropyl-thiogalactopyranoside was added to *E. coli* cultures (final concentration of 1 mM), and incubated at 37°C for 6 h. For protein purification, the *E. coli* cells were collected by centrifugation for 10 min at 4722*g* (4°C), suspended in phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na2HPO4, 2 mM KH2PO4, and 2.7 mM KCl, pH 7.3) containing lysozyme solution (1 mg/mL lysozyme and 0.1 M phenylmethanesulphonyl fluoride (PMSF)) and incubated at 37°C for 30 min. After incubation, the suspended *E. coli* cells were subjected to sonication for 15 min, and then the supernatant was collected by centrifugation 7378*g* for 10 min at 4°C. The filtrate was applied to a Poly-Prep chromatography Columns (Bio-Rad) packed with Glutathione Sepharose 4B agarose beads (Amersham, Biosciences) and then GST-PjChi-1 fusion proteins were eluted with elution buffer (15 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0). To separate the recombinant Pjchi-1 from GST protein, GST-PjChi-1 fused protein was cleaved by preScission protease following the manufacturer's instruction (Amersham Biosciences).

Chitinase Assay Using 4MU-$(GlcNAc)_3$ as Substrate. For the quantification of recombinant chitinase activity, 4-methylumbelliferyl N_I,N_{II},N_{III}-triacetyl-D-chitotrioside [4MU-$(GlcNAc)_3$, Sigma] was used as a fluorogenic substrate, and the endo-chitinase activity was quantified by the detection of fluorescent aglycone released from 4Mu-$(GlcNAc)_3$ [8]. Substrates were prepared as 0.8 mM stock solutions in water, and chitinolytic activity was assayed at pH 5.0 in McIlvaine's buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate). After 30 min of incubation, the reactions were stopped by adding 120 μL of 1 M glycine/NaOH buffer (pH 10.6) and the fluorescence was monitored using a Fluoroskan Ascent FL (Lab Systems, USA) with an excitation at 360 nm and an emission at 460 nm.

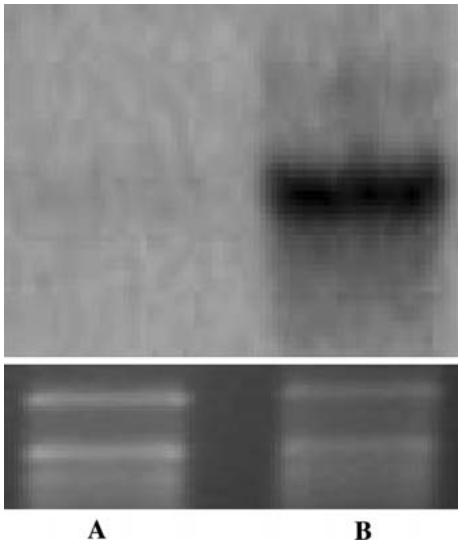

Fig. 3. Analysis of *PjChi-1* expression level under different growth conditions. The expression level of rRNA gene was used as internal control.

Biological Assay of PjChi-1. To assay the biological activity of the purified recombinant PjChi-1, test fungi were cultured on PDA medium and incubated in the dark at 28°C to 3 days for complete growth of mycelium and later stored at 4°C. A small amount of inoculum (3 mm in diameter) was taken from the edge of the mycelium grown in PDA plate and cultured in 50-mL Erlenmeyer flasks containing 10 mL of YEM broth supplemented without or with PjChi-1 protein at 0, 20, or 30 μg/mL, respectively, followed by the method described by Yang et al. [18]. The flasks were incubated at 28°C for 24 h with continuous shaking (200 rpm). The hyphal inhibitory effect was observed under a microscope.

Results and Discussion

Isolation and Characterization of cDNAs Encoding Chitinase PjChi-1. A cDNA fragment of 269 bp was initially amplified from the library and then extended to full length. The complete PjChi-1 cDNA (accession number DQ092417) of 1180 bp has a 1035 bp open reading frames, which potentially encodes for 345 amino acid protein with an estimated MW of 37 kDa. Chitinases of low molecular mass have also been reported in mycoparasitic fungi, such as *M. anisopliae* [10], *Trichoderma harzianum* [3], *T. virens* [4] and *Stachybotrys elegans* [9]. According to ExPasy search, a conserved motif for chitinase activity – F82DGIDIDWE90- was present in the deduced amino acid sequence, and it implies that *PjChi-1* belong to class V of family 18 chitinase.

Phylogenetic Analysis of *PjChi-1*. The amino acid sequence deduced from the nucleotide sequence was compared with the sequence database (Vector NTI).

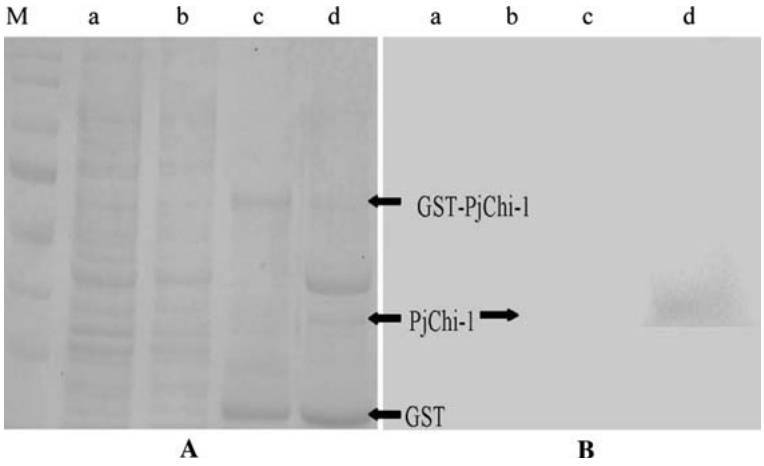

Fig. 4. Expression of GST-PjChi-1 fusion protein in *Escherichia coli* DE3 harboring pGEX-6P-1 was induced with 1 mM isopropyl-thiogalactopyranoside. **(A)** Sample from each step was analyzed by 12% Tris-Tricine (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and **(B)** chitinolytic zymography. Lane a: supernatant of bacterial cell lysate; lane b: Eluate of cell lysate; lane c: column purified GST-PjChi-1; lane d: A cleaved product of GST-PjChi-1; and lane M: molecular marker.

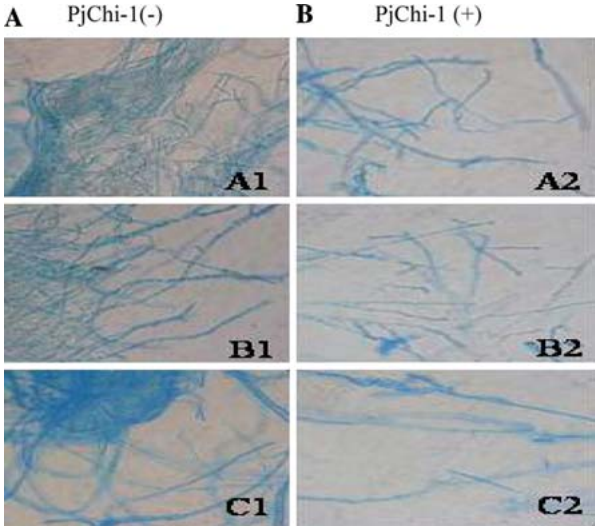

Fig. 5. Effect of recombinant PjChi-1 on the growth of *Colletotrichum gloeosporioides* (A2), *Aspergillus nidulans* (B2), and *Rhizoctonia solani* (C2), respectively. Cultures without recombinant PjChi-1 are shown (A1, B1, and C1). Cultures with 30 μg PjChi-1/mL (PjChi-1(+)) added and no recombinant protein added (PjChi-1(–)).

Based on the sequence alignment, *PjChi-1* shared identity and similarity with chitinases of other fungi, *Beauveria bassiana* (AY147010, 38.6% identity and 50.7% similarity), *Hypocrea virens* (AF397021, 63.7% identity and 71.3% similarity), *M. anisopliae* (AAB81999, 38.5% identity and 52.7% similarity), and *T. harzianum* (AAB34355, 40.4% identity and 53% similarity) (Fig. 1). Apart from this *PjChi-1* also showed identity and similarity with human chitotriosidase (2201442A, 20.7% identity and 30.8% similarity), nematode chitinase precursor (A38221, 16.8% identity and 26.8% similarity), *Manduca sexta* chitinase (AAC04924, 14.3% identity and 24.2% similarity), chitinase of *Clostridium paraputrificum* (BAA34922, 20.7% identity and 33.2% similarity), *Oryza sativa* chitinase (BAD61800, 12.3% identity and 20% similarity) and chitinase of *Arabidopsis thaliana* (NP566426, 12.3% identity and 21.7% similarity).

A phylogenetic tree was computed for the full-length amino acid sequence of the *PjChi-1* and the other five species in order to evaluate the evolutionary relationships among themselves and to classify the predicted chitinase, *PjChi-1*, into bacterial-like or plant-like classes. Based on the dendrogram, the generated tree presented two large clusters, and *PjChi-1* was clustered with chitinase of *Clostridium paraputrificum*, chitotriosidase of human, chitinase precursor of nematode and chitinase of insect (Fig. 2). The second cluster was formed by chitinases of plant origin. Based on the cluster organization, we predicted that the *PjChi-1* is closely related to bacterial type (class V).

Expression Analysis of *PjChi-1*. Differential expression of *PjChi-1* was detected when *P. javanicus* was grown on medium devoid of chitin (noninduced) and medium supplemented with chitin (induced). A basal level of gene expression was noticed when *P. javanicus* was grown under noninduced condition, but the expression level of *PjChi-1* was severalfold higher in induced condition. The results indicated that the presence of an external source of chitin appears to be strongly stimulatory to *PjChi-1* expression. Earlier reports showed that addition of external source of chitin or host extracts also acts as stimulatory to chitinase gene expression in mycoparasitic fungi, *Trichoderma atroviride* [6], and *T. harzianum* [5]. Northern blot analysis revealed that the *PjChi-1* was expressed at low level in mycelia grown under non-induced (medium devoid of chitin) condition. However, high expression of PjChi-1 was noticed in mycelia grown in medium supplemented with chitin (Fig. 3).

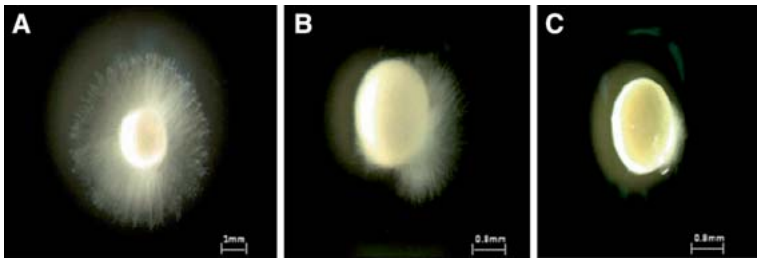

Fig. 6. Effect of recombinant PjChi-1 on the growth of *Sclerotium rolfsii.* **(A)** Profuse growth of *Sclerotium rolfsii* in YEM medium devoid of PjChi-1 (bar = 1 mm). **(B)** Marked inhibition of *S. rolfsii* was observed in YEM supplemented with 20 μg PjChi-1/mL (bar = 0.8 mm). **(C)** Complete inhibition of hyphal growth of *S. rolfsii* in YEM supplemented with 30 μg PjChi-1/mL (bar = 0.8 mm).

Recombinant Chitinase Activity Assays. The recombinant PjChi-1 expressed in *E. coli* was purified by affinity chromatography. The activity of the recombinant protein was assayed by using SDS-PAGE and chitinolytic zymograph assay, and 4MU- $(GlcNAc)_3$ as substrate. The approximate molecular mass of GST-PjChi-1, PjChi-1 and GST was 62, 37, and 25 kDa, respectively (Fig. 4A). The brightly fluorescent areas in Fig. 4B represent the regions without enzyme activity and dark areas indicate the degradation of the substrate due to chitinase activity. This is in agreement with the results of *M. anisopliae* [10], where recombinant protein is active against glycol chitin. The fused forms of PjChi-1 with GST lost its enzyme activity to catalyze ethylene glycol chitin hydrolysis (Fig. 4B). In the presence of 4-Mu-$(GlcNAc)_3$ substrate, the activity of recombinant chitinase increased linearly with the increase in enzyme concentration (data not shown). This suggests that the recombinant protein acts as an endochitinase.

Biological Activity of PjChi-1. The purified recombinant chitinase, PjChi-1, was tested against *C. gloeosporioides, A. nidulans*, *R. solani*, and *S. rolfsii* (Figs. 5 and 6). The culture of *C. gloeosporioides, A. nidulans*, *R. solani* (Fig. 5A1-C1) and *S. rolfsii* (Fig. 6A), grown in YEM broth devoid of PjChi-1 were kept as control. The cultures incubated with PjChi-1 for 24 h at 28°C showed marked inhibition of hyphal growth. The rate of inhibition was calculated according to the concentration of the PjChi-1 used (i.e., medium supplemented with 20 μg/mL) showed a marked level of inhibition in case of *S. rolfsii* (Fig. 6B), and similar results were observed for *C. gloeosporioides, A. nidulans* (data not shown). The culture of *C. gloeosporioides, A. nidulans*, *R. solani*, *and S. rolfsii* containing 30 μg/mL of PjChi-1 showed complete inhibitory effect of hyphal growth observed under the microscope (Fig. 5A2-C2 and Fig. 6C). The average inhibitory effect was significantly different from that of control. This inhibitory activity could be due to hydrolysis of fungal cell wall chitin by PjChi-1, thereby inhibiting the growth of fungi.

In conclusion, we have isolated, characterized, and analyzed the expression pattern of chitinase, *PjChi-1* from the *P. javanicus*. In addition, we have demonstrated that the PjChi-1 inhibits the growth of *S. rolfsii, C. gloeosporioides, A. nidulans*, and *R. solani*.

ACKNOWLEDGMENTS

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