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Research Article

Cloning and expression analysis of the chitinase gene *Ifu-chit2* from *Isaria fumosorosea*

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Abstract

Entomopathogenic fungi can produce a series of chitinases, some of which function synergistically with proteases and other hydrolytic enzymes to degrade the insect cuticle. In the present study, the chitinase gene *Ifu-chit2* from *Isaria fumosorosea* was investigated. The *Ifu*-chit2 gene is 1,435-bp long, interrupted by three short introns, and encodes a predicted protein of 423 amino acids with a 22 residue signal peptide. The predicted Ifu-Chit2 protein is highly homologous to *Beauveria bassiana* chitinase Bbchit2 and belongs to the glycohydrolase family 18. Ifu-Chit2 was expressed in *Escherichia coli* to verify chitinase activity, and the recombinant enzyme exhibited activity with a colloidal chitin substrate. Furthermore, the expression profiles of *Ifu-chit2* were analyzed at different induction times under *in vivo* conditions. Quantitative real-time PCR analysis revealed that *Ifu-chit2* expression peaked at two days post-induction. The expression of chitinase *Ifu-chit2* in *vivo* suggests that the chitinase may play a role in the early stage of pathogenesis.

Keywords: *Isaria fumosorosea*, chitinase, prokaryotic expression, quantitative real-time PCR.

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Introduction

Entomopathogenic fungi are widely distributed throughout the fungal kingdom and are an important group of microorganisms that have been used as biological controls against insect pests in many agroecosystems (Hajek and Delalibera Jr, 2010; Mishra *et al.*, 2013a,b). *Isaria fumosorosea* is a cosmopolitan entomopathogenic fungus parasitizing diverse insect species, including the important agricultural pests diamondback moth and whitefly. *I. fumosorosea* is being used increasingly as a biological control agent for several insect pests (Ali *et al.*, 2010a,b).

Chitinases (EC.3.2.1.14) catalyze the hydrolysis of chitin, and these enzymes have diverse functions ranging from nutritional roles in bacteria and archaea, defensive roles in plants, developmental roles in insects and morphogenetic, nutritional and invasive functions in fungi (Li, 2006; Hartl *et al.*, 2012). During fungal penetration through the host cuticle, entomopathogenic fungi produce hydrolytic enzymes such as proteases, chitinases and lipases, that degrade the insect cuticle and can initiate the infection process (Charnley and St Leger, 1991; Charnley, 2003). Entomopathogenic fungal chitinases are attractive candidates for the biological control of insect pests in agroforestry (Zhu *et al.*, 2008).

Chitinases are grouped into glycoside hydrolase families 18 (GH18) and 19 (GH19) by amino acid sequence similarity of their catalytic domains (Henrissat, 1991). Enzymes in these families differ in their primary sequence, three-dimensional (3D) structure, and molecular mechanisms of catalysis (Henrissat and Bairoch, 1996; Henrissat and Davies, 1997). GH family18 are evolutionarily diverse and represent an ancient chitinase family widely distributed in a variety of organisms including bacteria, fungi, animals, and some plant species, while family 19 chitinases are found only in higher plants and some Gram-positive bacteria such as *Streptomyces griseus* (Ohno *et al.*, 1996; Lee *et al.*, 2009; Ahmed *et al.*, 2012). Filamentous fungi have many different chitinases belonging to GH family 18 (Li, 2006), and all GH family 18 proteins present a common α/β TIM-barrel fold and include a DXXDXDXE sequence motif (Bokma *et al.*, 2002).

Several chitinases have been characterized from *Metarhizium anisopliae* and *Beauveria bassiana in vivo* (Fang *et al.*, 2005; Bhanu Prakash *et al.*, 2012). The Chit1 chitinase from *M. anisopliae* was shown to have little or no effect on virulence, while overproduction of chitinase *Bbchit1* in *B. bassiana* did enhance the biocontrol activity of the fungus against aphids (Screen *et al.*, 2001; Fang *et al.*, 2005). Chitinase *chi2* of *M. anisopliae* has been re-

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ported to be responsible for virulence in a bioassay of transgenic isolates with the chitinase gene overexpressed and silenced (Boldo *et al.*, 2009).

Currently, *I. fumosorosea* produces chitinases that are effective in hydrolyzing and destroying the cuticle of various insects (Ali *et al.*, 2010a,b). The *I. fumosorosea* chitinase gene *Ifu-chit1* was isolated by our group previously and transgenic *B. bassiana* overexpressing this gene showed significantly improved virulence against the moth *Dendrolimus punctatus* compared with the wild-type strain (Tang *et al.*, 2009). Whether there are more chitinase genes in *I. fumosorosea* is currently unknown. In this study, we identified another chitinase gene from *I. fumosorosea* (*Ifu-chit2*) and characterized it using bioinformatics, Rapid Amplification of cDNA Ends (RACE) and heterologous expression in *Escherichia coli*. Furthermore, the expression profiles of *Ifu-chit2* under *in vivo* conditions were investigated.

Materials and Methods

Fungal strains, host insects and growth conditions

The fungal strain used in this study was *I. fumosorosea* strain RCEF3304 (isolated from Diptera pupa from Jiangxi province, China), obtained from the Anhui Provincial Key Laboratory of Microbial Pest Control, Hefei, China. Mycelia were harvested from chitinase induction medium containing ground chitin (1%, w/v) in basal salts medium according to Wang's procedure (Wang *et al.*, 2013).

For *in vivo* experiments, larvae of the greater wax moth (*Galleria mellonella*) obtained from Ruiqing Bait (Jiangsu, China) were used as host insects for fungal development. A conidial suspension (100 μL at 10^8 conidia/mL) was evenly spread onto potato dextrose agar (PDA) and grown for 12 days at 25 °C. Fifty insects were rolled on the conidiating culture, individually placed in sterilised plastic vials and incubated at 25 °C. Different stages of infection were monitored over a pathogenesis period of 4 days without feeding. At each of the observed infection stages, 5-7 insects were ground to a fine powder in liquid nitrogen for total RNA extraction.

Cloning and sequencing of the full length cDNA and genomic DNA of *Ifu-chit2*

Total RNA was extracted from ground, frozen mycelia using TRizol reagent (Invitrogen, CA, USA) and quantified using a spectrophotometer. 1 μg of RNA was reverse transcribed using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, China). Reverse-transcribed cDNA was used as template for PCR amplification with degenerate PCR primers designed based on conserved domains of fungal chitinases to amplify the conserved fragment (Table 1). PCR was carried out following manufacturer's instructions. Based on the acquired core region sequence, two pairs of gene-specific primers were designed for 5'- and 3'-RACE PCR to amplify the 5'- and 3'- ends of *Ifu-chit2*, respectively (Table 1). The 5' and 3' UTR regions were amplified using a SMART RACE cDNA Amplification Kit (Clontech, USA), and products were cloned and sequenced.

DNA was extracted using a slight modification of the CTAB method and resuspended in pre-warmed sterile deionized water. The DNA sequence of *Ifu-chit2* gene was amplified with primers designed based on the *Ifu-chit2* chitinase cDNA using *I. fumosorosea* genomic DNA as template.

Bioinformatics analysis of *Ifu-Chit2*

Protein parameters were calculated using Protparam at Expasy (Gasteiger *et al.*, 2005), and signal peptide pre-

Table 1 - Primers used for gene cloning and expression analysis.

Primer	Sequence (5'-3')	Description
chit2-F1	TCCATYGGNGGNTGGACNTG	Degenerate primer, forward
*chit*2-R1	GCRSWNGCYTCCCARAACAT	Degenerate primer, reverse
Ifu-chit2-1	ATGCTGGGTTTCCTCAGGAAATCAATCGCTACGGTCG	Primer for DNA amplification, forward
Ifu-chit2-2	ACTATTCCTGATATTGTCGAACTTC	Primer for DNA amplification, reverse
Ifu-chit2-3	CAGTCTCAGGATACTCCCAATC	Primer for 5'RACE, outer
Ifu-chit2-4	TCAGAGCTGGCGACAACGGCAAAGT	Primer for 5'RACE, inner
Ifu-chit2-5	TCTGAAGGATTGGGGTCTTGACGGT	Primer for 3'RACE, outer
Ifu-chit2-6	GATCCGCGATGAGCTCGACTCCTAC	Primer for 3'RACE, inner
Ifu-chit2-7	AGGATTGGGGTCTTGACG	Primer for qRT-PCR[b] (*in vivo*), forward
Ifu-chit2-8	GGCAATAGAAAGCAGGAAGT	Primer for qRT-PCR(*in vivo*), reverse
TEF-1[a]	ATCGGTGGTATCGGAACG	Control primer for qRT-PCR(*in vivo*), forward
TEF-2	TGGAAGGAGCAAAGGTGAC	Control primer for qRT-PCR(*in vivo*), reverse

[a]: *TEF*: translation elongation factor.
[b]: qRT-PCR: quantitative real-time PCR.

diction was carried out using the SignalP 4.0 server (Petersen *et al.*, 2011). Sequences of homologs from other species were obtained using the BLASTP tool, and homologous sequences were used for multiple sequence alignment and generation of a phylogenetic tree by applying the neighbor-joining (NJ) methods in MEGA version 4.1 with ClustalW. Confidence limits were estimated from 1000 bootstrapping replicates.

Expression and purification of recombinant Ifu-chit2 protein

Primers 5-CCGGAATTCATGCTGGGTTTCCTCA GGAAAT-3 and 5-ATAAGAATGCGGCCGCCTA **GTGGTGATGGTGATGGTG**AGCCATGCTATTCCT GATATTG-3 (restriction enzyme sites are underlined; the 6His-tag sequence is in bold) were designed for PCR amplification based on the encoding region of the *Ifu-chit2* gene. The PCR product was cloned into the pET-28a (+) vector (Novagen), resulting in recombinant expression vector pET-28a-*Ifu-chit2*. This was transformed into *E. coli* strain BL21 (DE3) chemically competent cells (TransGen, China) and grown at 37 °C in Luria-Bertani (LB) medium containing 50 μg/mL kanamycin. Ifu-Chit2 expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and cultivation was continued for an additional 6 h at 28 °C. Cells were collected at 1-6 h by centrifugation (4,000 rpm for 10 min), analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Cells induced for 5 h were harvested and loaded onto a His-Bind Ni-agarose column (Cwbio, China) and the target protein was purified according to the manufacturer's instructions.

Western blot analysis

For Western blot analysis, 12% SDS-PAGE was carried out to separate proteins prior to transfer onto a nitrocellulose membrane (Pall) at 100 mA for 1 h using a Semi-Dry Trans-Blot Cell (Bio-Rad). Non-specific protein-protein interactions were blocked using 5% non-fat dry milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 h. Membranes were incubated overnight at 4 °C with mouse anti-His antibody (Abmart, Shanghai) diluted 1:5,000 in blocking buffer, and washed three times for 5 min each time in TBST buffer. The second anti-body, AP-conjugated goat anti-mouse IgG (Promega) was diluted 1:7,500 in TBST buffer, incubated with the membrane for 1 h, and washed three times for 5 min each time in TBST buffer, followed by visualization with Western blue stabilized substrate for alkaline phosphatase (Promega).

Chitinase activity assay

Chitinase activity was measured as described previously with modifications (Mauch *et al.*, 1984). Incubations consisted of 350 μL 100 mM sodium acetate buffer (pH 6.5), 60 μg of suitably diluted enzyme and 200 μL of 1% colloidal chitin. After 1 h incubation at 37 °C, reactions were stopped by centrifugation at 10,000 rpm for 5 min. Supernatants (300 μL) were boiled with 100 μL of potassium tetraborate buffer for 3 min, and 2.5 mL of DMAB reagent (10% (w/v) 4-(dimethyl amino) benzaldehyde in glacial acetic acid: 11.5 M HCl (87.5:12.5, v/v) was added to the reactions and then incubated at 37 °C for 20 min. The change in absorbance at 420 nm of the supernatants (suitably diluted enzyme boiled for 30 min as a control) was recorded using a Spectra Max M2 (Molecular Devices, USA). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of GlcNAc per mL in 1 h.

Quantitative real-time PCR analysis of *Ifu-chit2* gene expression under *in vivo* conditions

For *Ifu-chit2* expression studies, infected insects were collected for real-time PCR after 1d (1 day after inoculation), 2d (2 days after inoculation), 3d (melanization of the infected insect) and 4d (death insect), respectively. Total RNA was isolated from harvested infected insects using Trizol (Invitrogen, USA). Expression of *Ifu-chit2* was quantified using a quantitative real-time PCR assay performed in a 7500 Real-Time PCR System (Applied Biosystems, USA), using the SYBR Green kit (TaKaRa, China) following the manufacturer's instructions. Specific primers for *Ifu-chit2* and *translation elongation factor* (*TEF*) were designed for real-time PCR amplification (Table 1). Synthesis of cDNA and quantitative real-time PCR was performed in triplicate for each gene. The expression levels of *Ifu-chit2 in vivo* at 1 day after induction were given a value of one, and relative expression levels were calculated using the formula $2^{-\Delta\Delta CT}$ with *TEF* as the internal control for each sample (Livak and Schmittgen, 2001).

Results

Cloning and sequence analysis of *Ifu-chit2*

Degenerate PCR primers corresponding to conserved domains of entomopathogenic fungal chitinases were designed as described above, and a 766-bp fragment was amplified using primers *chit2*-F1 and *chit2*-R1. A BLAST search of the sequenced PCR product revealed high homology to chitinase genes from *Cordyceps confragosa* (72%), *Isaria farinosa* (72%), *Aphanocladium album* (71%) and *B. bassiana* (70%), which indicated that the fragment was a partial sequence of the chitinase *Ifu-chit2* from *I. fumosorosea*.

The 5' and 3' ends of the partial sequence were extended from gene-specific primers using the RACE approach. The 5'-UTR, ORF, and 3'-UTR were 510 bp, 1272 bp, and 202 bp, respectively (Figure 1). The deduced protein was 423 aa with a predicted molecular mass of 46.57 kDa (Figure 1), a predicted isoelectric point (pI) of

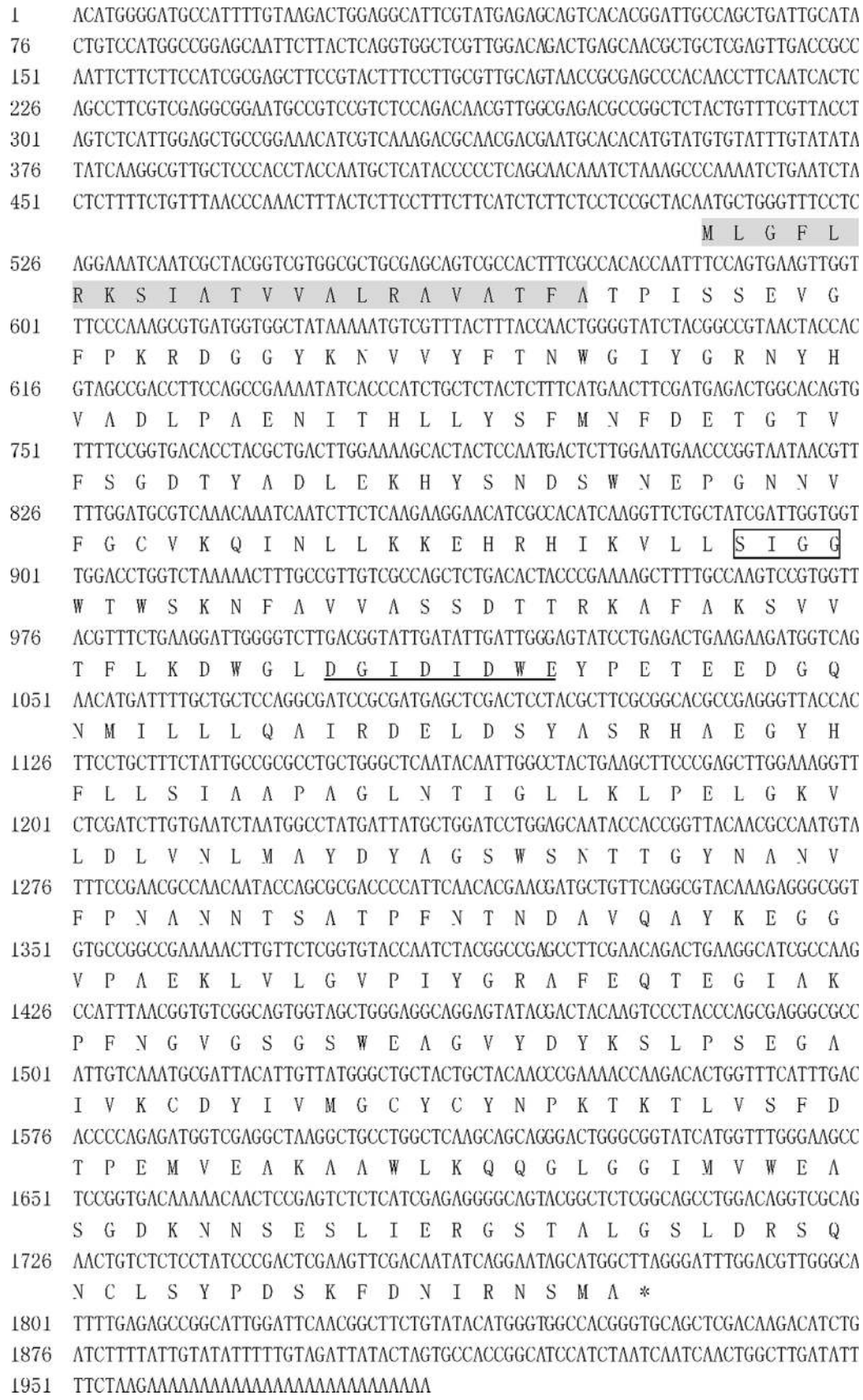

Figure 1 - Full-length cDNA and deduced amino acid sequence of *Ifu-chit2* from *Isaria fumosorosea*. Numbers to the left indicate the nucleotide and amino acid positions. The conserved sequences SIGG and DGIDIDWE are boxed and underlined, respectively. An asterisk indicates the end of the protein sequence, and the signal peptide is shaded.

5.22, and a 22-aa signal peptide at the N-terminus (shaded, Figure 1). Comparison of the predicted Ifu-Chit2 chitinase with fungal orthologs revealed two highly conserved regions of the catalytic domain (SIGG and DGIDIDWE) that correspond to a substrate-binding site and catalytic residues, respectively. This confirmed that Ifu-Chit2 was a member of glycosyl hydrolase family 18 (Figure 2). A phylogenetic tree showed that Ifu-Chit2 is more closely re-

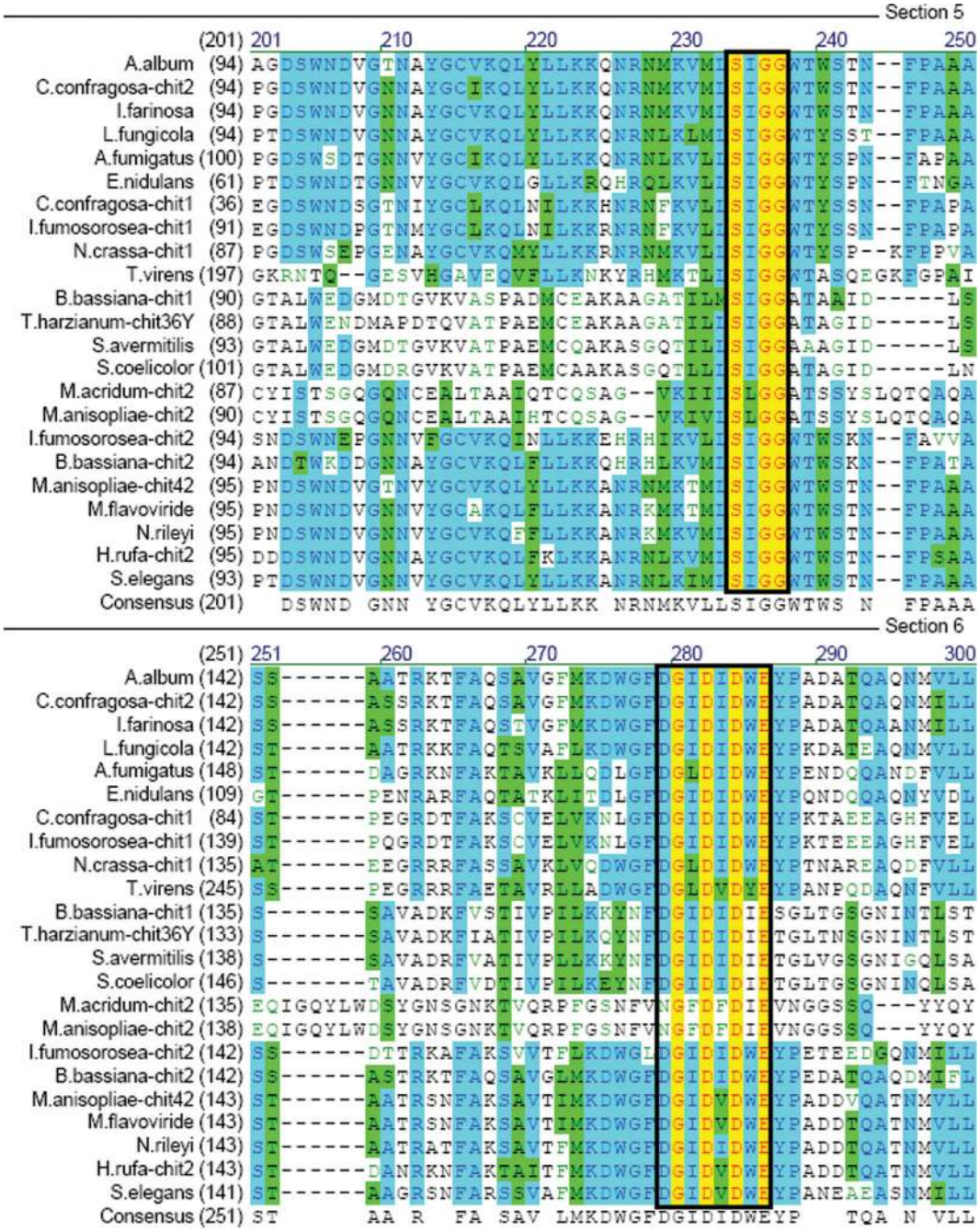

Figure 2 - Multiple sequence alignment of the core region of the catalytic domains of Ifu-Chit2 and related chitinases. Identical amino acids are highlighted in yellow, and similar residues are shown in green or light-green. The consensus (average) sequence is shown below the aligned sequences. Highly conserved motifs SxGG and DxxDxDxE are boxed. GenBank accession numbers for are listed in parentheses: *A. album* CHIT (CAA45468), *C. confragosa* CHIT2 (AAV98692), *I. farinosa* CHIT (ABD64606), *Lecanicillium fungicola* CHIT (AAP45631), *Aspergillus fumigatus* CHIT (AAO61686), *Emericella nidulans* CHIT (BAA35140), *C. confragosa* CHIT1 (AAX56960), *I. fumosorosea* CHIT1 (FJ377733), *Neurospora crassa* CHIT1 (EAA36073), *Trichoderma virens* CHIT (AAL84697), *B. bassiana* CHIT1 (AY145440), *T. harzianum* CHIT36Y (AAL01372), *Streptomyces avermitilis* CHIT (NP_826813), *S. coelicolor* CHIT (NP_626743), *Metarhizium acridum* CHIT2 (AJ293217), *M. anisopliae* CHIT2 (AAY34347), *B. bassiana* CHIT2 (AY147011), *M. anisopliae* CHIT42 (AAB81998), *M. flavoviride* CHIT (CAB44709), *Nomuraea rileyi* CHIT (AY264288), *Hypocrea rufa* CHIT (AAF19617), *Stachybotrys elegans* CHIT (AAM70478).

lated to *B. bassiana chit2* than to chitinases of other species (Figure 3).

Moreover, a knowledge-based protein model tool from the SWISS-MODEL program was used to predict the 3D structure of the Ifu-Chit2 protein based on the tertiary structures of related chitinases in the database. The model exhibited the expected α/β-barrel consisting of eight α-helices and eight parallel β-strands that alternate along the peptide backbone, which is consistent with *Coccidioides immitis* CiX1 and *Aspergillus fumigatus* ChiB1 (Figure 4) (Li, 2006).

The genomic sequence of *Ifu-chit2* was 1435 bp due to the presence of three introns at 121-176, 277-331 and 380-431 (data not shown). The exon/intron splice sites (5'GT-AG3') were representative of gap junctions reported for other fungi chitinase genes isolated from *C. confragosa* (ABD77096), *A. album* (X64104) and *Aspergillus nidulans* (D87063).

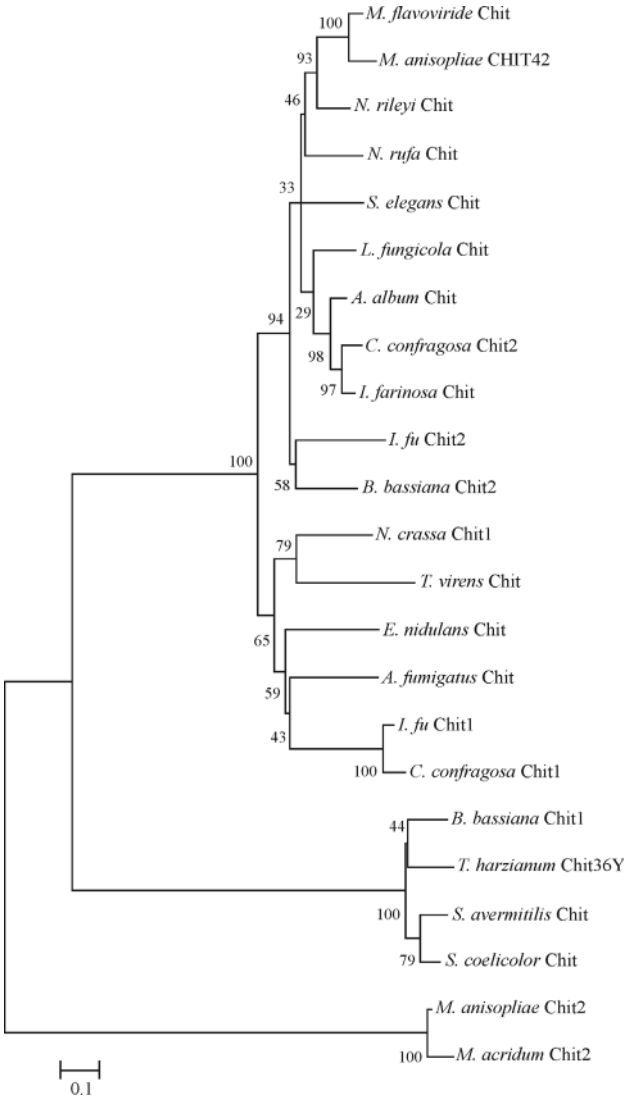

Figure 3 - Phylogenetic analysis of chitinase sequences from different pathogenic fungi. The phylogenetic tree was created by the neighbor-joining method in Mega version 4.1 using default settings based on the multiple sequence alignment. A bootstrap value is attached to each branch. GenBank accession numbers are referenced in the legend of Figure 2.

Expression of recombinant Ifu-Chit2 in *E. coli*

The *Ifu-chit2* encoding sequences were successfully inserted into pET-28a (+) vectors and transformed into E. coli BL21 (DE3), and SDS-PAGE analysis confirmed that the Ifu-Chit2 protein was expressed following induction. A protein with a molecular mass of approximately 50 kDa was clearly visible, which was slightly larger than the predicted size (Figure 5). The maximum expression was achieved 5 h after induction at 28 °C. Western blot analysis with anti-His antibody confirmed that the overexpressed protein included a His-tag (Figure 5), and the protein was purified using a His-Band Ni-agarose column (Figure.5). Purified recombinant Ifu-Chit2 exhibited an enzyme activity of 32.7 U/mL, which was much higher than that of Vlchit1 protein from *Verticillium lecanii* (Zhu *et al.*, 2008). The result indicated that Ifu-Chit2 was successfully expressed in active forms at a higher level.

Expression profiles of *Ifu-chit2* under *in vivo* conditions

The expression profiles of *Ifu-chit2* under *in vivo* conditions were investigated using quantitative real-time PCR (Figure 6). A single product-specific melting curve was obtained using for *Ifu-chit2*, indicating that the primers amplified efficiently and were specific for the gene of interest. *Ifu-chit2* gene was found to be expressed constitutively during all stages of insect infection examined (1-4 days).

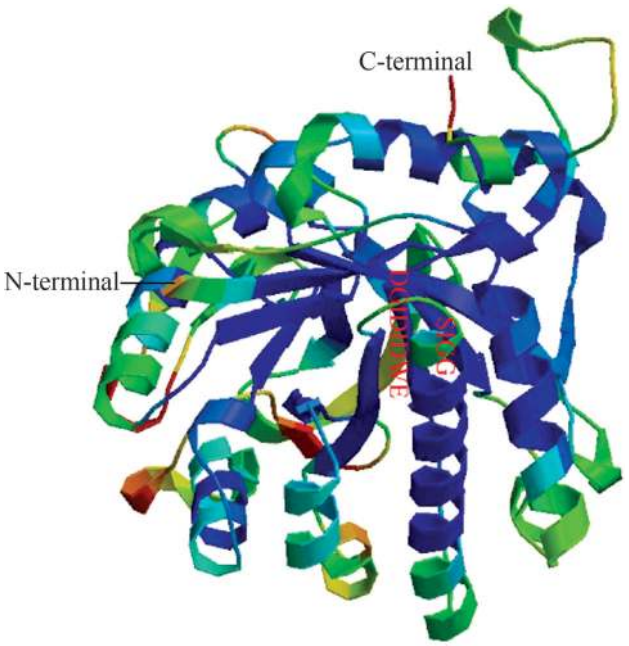

Figure 4 - Predicted structure of *Isaria fumosorosea* Ifu-Chit2. The top view of the TIM barrel structure of the superimposed model is shown. Red letters indicate the conserved sequences SIGG and DGIDIDWE.

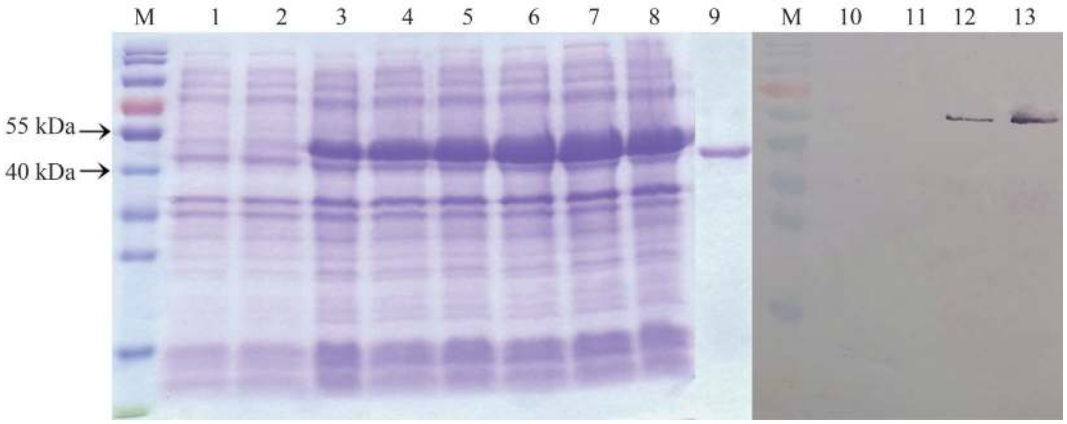

Figure 5 - SDS-PAGE and Western blot analysis of the expression of recombinant Ifu-Chit2 in *E. coli*. Lane M: pre-stained protein marker; Lane 1: total protein of cells containing empty vector pET-28a with IPTG induction; Lane 2: total protein of cells containing expression vector pET-*28a-Ifu-chit2* without IPTG induction; Lanes 3-8: total protein of cells containing expression vector pET-*28a-Ifu-chit2* induced by IPTG at 28 °C for 1, 2, 3, 4, 5 and 6 h, respectively; Lane 9: purified recombinant Ifu-Chit2 (50 kDa). Lane 10: total protein of cells containing empty vector pET-28a with IPTG induction; Lane 11: total protein of cells containing expression vector pET-*28a-Ifu-chit2* without IPTG induction; Lane 12: total protein of cells containing expression vector pET-*28a-Ifu-chit2* induced by IPTG at 28 °C for 4 h; Lane13: Purified recombinant Ifu-Chit2.

The data normalized to endogenous reference gene were presented as the fold-change in gene expression during different stages of infection and relative to the levels of expression observed at 1 day after infection. *Ifu-chit2* expression levels peaked at 2 days after infection (Figure 6). Neither chitinase gene was expressed in uninoculated insects.

Discussion

Like most fungal pathogens, *I. fumosorosea* may use a combination of chitinases, proteases and lipases to penetrate the insect cuticle and access the host hemocoel, and extracellular chitinases may therefore be important for virulence. In the present study, we successfully isolated a gene from *I. fumosorosea* that encodes for a chitinase (Ifu-Chit2). Ifu-Chit2 was successfully expressed in *E.* coli BL21 (DE3) with the product of recombinant protein Ifu-Chit2 (50 kDa).

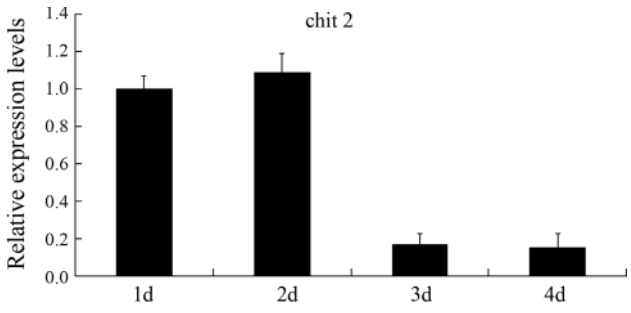

Figure 6 - Expression analysis of *Ifu-chit2* at different times under *in vivo* conditions. mRNA transcripts were measured at 1, 2, 3 and 4 days after inoculation using qRT-PCR. Relative expression levels were calculated using the comparative ΔΔCt method. The constitutively expressed *translation elongation factor* gene served as endogenous control, and expression levels are shown as relative to those at 1 day after inoculation, which were given a value of 1. Data are expressed as the mean ± SE (standard error) of three independent experiments. Student's t-test was used to determine the statistical significance of differences between groups. Differences are considered significant for $p < 0.05$.

Most chitinase genes cloned and characterized to date have been classified into two families of glycosyl hydrolases (family 18 and 19), based on amino acid sequence similarity (Henrissat, 1991; Henrissat and Bairoch, 1993). Our results indicated that Ifu-Chit2 belonged to family 18, as it included the characteristic substrate binding and catalytic motifs (SXGG and DXXDXDXE) (Lu, *et al.*, 2005; Baratto, *et al.*, 2006). Residues that are essential for chitinase activity, particularly Asp164, Asp167, Asp169 and Glu171, were found to be present in the Ifu-Chit2 catalytic domain, indicating a similar catalytic activity and structure to previously characterized enzymes of this family (Liu, *et al.*, 2008). The chitin-binding domain (ChBD) plays an important role in permitting chitinases to bind specifically to insoluble chitin (Limón, *et al.*, 2004). Most bacterial chitinases in family 18 are characterized by the presence of a signal peptide, a catalytic domain, and a ChBD (Wu, *et al.*, 2001). However, Ifu-Chit2 lacks the ChBD domain, as observed previously for some fungal chitinases (Li, 2006). Ifu-Chit2 does include a signal sequence at the N-terminus, suggesting that it is secreted. Moreover, teleomorph of *I. fumosorosea* and *B. bassiana* belong to the genus *Cordyceps* sensu suto according to phylogenetic studies (Sung *et* al., 2007). Interestingly, to some extent, the relationship between Ifu-Chit2 and Bb-chit2 in the phylogenetic tree was consistent with the phylogenetic relationship between *I. fumosorosea* and *B. bassiana*.

It has been reported that the efficient up-regulation of chitinase expression during insect infection may be responsible for virulence in various fungi (Hartl *et al.*, 2012). Recently, Bhanu Prakash *et al.* (2012) reported that the four *M. anisopliae* chitinase genes *chi*, *chi 1*, *chi 2* and *chi 3* iso-

lated from the insect hosts *Spodoptera litura* and *Helicoverpa armigera* during six developmental stages of the pathogen, were up-regulated in *S. litura* during mycosed and conidiated condition, whereas in *H. armigera* they were only expressed 48 h after incubation (Bhanu Prakash *et al.*, 2012). In the present study, most insects were dead four days after induction. Quantitative real-time PCR showed that *Ifu-chit2* was up-regulated at two days after inoculation and then decreased over the duration of the experiment. We speculate that the expression of *Ifu-chit2* acts against *G. mellonella* in the early stage of the infection process in order to solubilize the host cuticle.

Many filamentous fungi have been found to produce more than one kind of chitinase. Research has demonstrated that these chitinases have a mutually synergistic and complementary effect among them. Previous reports indicated that both the chitinase CHI2 and CHIT30 of *M. anisopliae* analyzed by molecular genetic tools play important roles for pathogenicity in the infection process (Boldo *et al.*, 2009; Staats *et al.*, 2013). It has been reported previously that *Trichoderma harzianum* chitinases CHIT33 and CHIT37 are able to enhance CHIT42 activity of degrading phytopathogenic cell walls (de la Cruz *et al.*, 1992).

Although little is known about the roles of chitinases in the infection process of entomopathogenic fungi, chitinases are thought to be key enzymes during the early stages of the infection process. Furthermore, the other hydrolytic enzymes like proteases, lipase and chitosanase secreted during the infection process might compensate for the penetration functions. Taken together, our data demonstrate that expression of *Ifu-chit2* is part of a series of responses of *I. fumosorosea* triggered by the presence of insect host, and may play a role in the early stage of pathogenesis. However, further overexpression and gene knockout experiments for *Ifu-chit2* gene are needed to determine its exact roles in pathogenicity.

In conclusion, we characterized the *I. fumosorosea* chitinase gene *Ifu-chit2* and expressed the recombinant protein in *E. coli.* Expression profiles of *Ifu-chit*2 under *in vivo* conditions were also determined. Whether there are any other chitinases in *I. fumosorosea* needs to be studied further.

Acknowledgments

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Internet Resources

Expasy, http://web.expasy.org/protparam/ (July 19, 2014).

SignalP 4.0 server, http://www.cbs.dtu.dk/services/SignalP/ (July 19, 2014).

BLASTP tool, http://www.ncbi.nlm.nih.gov/BLAST.

ClustalW, http://www.genome.jp/tools/clustalw/.

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