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 family 18 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-
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 Ruqiing Bait (Jiangsu, China) were used as host insects for fungal development. A conidial suspension (100 μL at 10⁸ 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-

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

| Primer     | Sequence (5’-3’)                              | Description                                      |
|------------|-----------------------------------------------|-------------------------------------------------|
| chit2-F1   | TCCATYGGNNGNTGGACNTG                          | Degenerate primer, forward                       |
| chit2-R1   | GCRSWNGCYTCCCARAACAT                         | Degenerate primer, reverse                      |
| Ifu-chit2-1| ATGCTGGGTTCCTCAGGAAATCAATCGCTACGTCG         | Primer for DNA amplification, forward           |
| Ifu-chit2-2| ACTATTCCTGTATTTGTCGACCTTC                    | Primer for DNA amplification, reverse           |
| Ifu-chit2-3| CAGTCTCAGGATACTCCCAATC                      | Primer for 5’RACE, outer                        |
| Ifu-chit2-4| TCAGAGCTAGGACAAACGCAAGGCTGTCGTAGCCG         | Primer for 5’RACE, inner                        |
| Ifu-chit2-5| TCTGAAAGATGGGTTCGTGACGTCG                    | Primer for 3’RACE, outer                        |
| Ifu-chit2-6| GATCCCGATGAGCTCGACTCCTACTTC                 | Primer for 3’RACE, inner                        |
| Ifu-chit2-7| AGGATTTGGGCTTIGAC                           | Primer for qRT-PCR<sup>b</sup> (*in vivo*), forward |
| Ifu-chit2-8| GGCATATAGAAAGGCAAGGCAAGGT                  | Primer for qRT-PCR<sup>b</sup> (*in vivo*), reverse |
| TEF-1      | ATCGGTGGATGACTCCGACGGTCG                    | Control primer for qRT-PCR<sup>b</sup> (*in vivo*), forward |
| TEF-2      | TGGAAGGCCAAAGGTGAC                          | Control primer for qRT-PCR<sup>b</sup> (*in vivo*), reverse |

<sup>a</sup>: TEF: translation elongation factor.

<sup>b</sup>: 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-CCGGAATTCTAGCTGTTGTTCCCTCA GGAAAT-3 and 5-ATAAGAATATCCGCGCCTA GTGGTGATGGTGATGGTGAGCCATGCTATTCTC 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, 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^{-ΔΔCT} 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%), Aphanoocladium 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-
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 $\alpha/\beta$-barrel consisting of eight $\alpha$-helices and eight parallel $\beta$-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).

**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).

Expression of 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).
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).

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 lato 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 conidiation 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 these chitinases have a mutually synergistic and important roles for pathogenicity in the infection process. Furthermore, the other hydrolytic enzymes like proteases, lipase and chitosanase secreted during the infection process 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-chit2* under in vivo conditions were also determined. Whether there are any other chitinases in *I. fumosorosea* needs to be studied further.

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**Internet Resources**

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

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

BLASTP tool, [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/).

ClustalW, [http://www.genome.jp/tools/clustalw/](http://www.genome.jp/tools/clustalw/).

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