INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17F–149/2018/20–4–864–870
DOI: 10.17957/IJAB/15.0588
http://www.fspublishers.org

Full Length Article

Genetically Modified *Beauveria bassiana* Strains Harboring the *Isaria fumosorosea* Protease Gene Exhibit Increased Virulence against *Dendrolimus punctatus*

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Abstract

In this study, we cloned the complete full-length cDNA of *Ifupr1* from the biocontrol agent *Isaria fumosorosea* using SMART rapid amplification of cDNA ends reverse transcription polymerase chain reaction. Our results showed that the *Ifpr1* gene contained an open reading frame (ORF) with 1287 bp encoding 428 amino acids; the N-terminal 16 amino acid residues displayed the characteristics of signal peptide. The mature protease had a molecular mass of 46.3 kDa and a calculated isoelectric point of 7.37. The protein sequence contained two highly conserved regions, a putative enzymatic active site, and a potential chitin-binding domain. The *Ifupr1* gene from *I. fumosorosea* was engineered into *Beauveria bassiana* by blastospore transformation. Compared with the wild-type strain Bb13, the protease activity was increased by 2.65-fold after induction for 36 h. In bioassays against the larvae of Masson's pine caterpillar *Dendrolimus punctatus*, the median lethal time of the transgenic strain was reduced by 33.7% ($P< 0.05$), and mortality rates were increased by 53.3% ($P< 0.05$) as compared with the wild-type at a concentration of 1×10^7 spores/mL. The median lethal concentrations of the wild-type and transgenic isolates were 4.55×10^6 and 0.869×10^6 spores/mL, respectively. Taken together, these findings showed that the transgenic strain exhibited significantly improved virulence against *D. punctatus*. © 2018 Friends Science Publishers

Keywords: *Isaria fumosorosea*; Entomopathogenic fungi; Genetic engineering; Bioassay; Virulence; *Dendrolimus punctatus*

Introduction

Isaria fumosorosea, an entomopathogenic fungus of phylum Ascomycota, is found worldwide and parasitizes over 25 different insect species, including some important agricultural pests, such as *Plutella xylostella*, *Diuraphis noxia*, and *Bemisia tabaci*, making it a promising commercially available biological control agent (Kouassi *et al*., 2003; Shah and Pell, 2003; Shinohara *et al*., 2013; Kim *et al*., 2014). *I. fumosorosea* invades the host by secreting protease, chitinase, and lipase, which facilitate penetration of the host's body (Pu and Li, 1996). *Beauveria bassiana*, another commercially available entomopathogenic fungus (Fernandes *et al.*, 2012; Cafarchia *et al*., 2015), has been used in the management of *Dendrolimus punctatus* on 2 million hectares of forests per year in China in the world's largest and most successful pest biocontrol projects, thus highlighting the important role of this organism in the biological control of forest pests.

Genetic engineering is an effective means for improving the virulence of entomopathogenic fungi by overexpression of virulence genes. Transforming the serine protease *PR1A* gene (a virulence gene of entomogenous fungi) into the genome of *Metarhizium anisopliae* decreased the lethal time by 25% and reduced the amount of pest feeding damage by 40% (St Leger *et al*., 1996). Using agrobacterium-mediated transformation, Fang *et al.* (2005) constructed a *B. bassiana* strain that overproduced chitinase Bbchit1, significantly enhancing its virulence against *Myzus persicae*. They also constructed a *B. bassiana* strain that overproduces *Bbchit1-BmChBD*, reducing the lethal time by 23% compared the wild-type strain and further enhancing the virulence compared with the *Bbchit1* overproducing strain (Fan *et al*., 2007). Wang and Leger (2007) were the first to introduce an exogenous gene into an entomopathogenic fungus. They improved the virulence of *Metarhizium anisopliae* against *Manduca sexta* and *Aedes aegypti* through transformation with a scorpion venom gene from *Androctonus australis*. Lv *et al*. (2008) introduced the scorpion venom gene into *B. bassiana*, thus reducing the spore dosage required for efficacy and the dosage shortening the lethal time.

In the current study, we obtained the protease gene of *I. fumosorosea* and transformed it into *B. bassiana* to improve the virulence of *B. bassiana* against *D. punctatus*.

To cite this paper: Tang, Q., J. Yang, L. Fang, Y. Xu and B. Huang, 2018. Genetically modified *Beauveria bassiana* strains harboring the *Isaria fumosorosea* protease gene exhibit increased virulence against *Dendrolimus punctatus. Int. J. Agric. Biol*. 20: 864−870

Materials and Methods

The plasmids and primers used in this study are listed in Table 1. TRIzol was purchased from Life Technologies (Carlsbad, CA, USA). Taq DNA polymerases, the PrimeScript DNA Synthesis Kit, dNTPs, the Agarose Gel DNA Purification Kit, and restriction endonucleases were purchased from TaKaRa (Dalian, China). A SMART RACE cDNA Amplification Kit was purchased from Clontech. Difco Sabouraud Dextrose Broth was purchased from Becton Dickinson (USA). Other conventional reagents were from Sangon (China).

Strains, Medium, and Culture Conditions

I. fumosorosea strain Ifu 78, which is parasitic in *Diptera* pupae, was derived from Lushan, Jiangxi province, China, and *B. bassiana* strain Bb13, which is parasitic in *D. punctatus*, was derived from Huoqiu, Anhui province, China. Ifu 78 was incubated in SDY liquid medium (w/v: 1% peptone, 1% yeast extract powder, 4% glucose) at 25°C and 200 rpm for 4 h, washed with distilled water, and cultured at 20°C in basic salt medium (w/v: 3% NaCl, 3% $MgSO_4$, 3% KH_2PO_4) supplemented with 1% silkworm chrysalis powder, with shaking at 150 rpm for 12 h. Mycelia were harvested by centrifugation at 12,000 rpm and 4°C and then stored at -80°C (Huang *et al*., 2006). *Escherichia coli* DH5α was cultivated in LB medium at 37°C.

Protease Ifupr1 cDNA Clone

Total RNA was extracted from *I. fumosorosea* using TRIzol, and first-strand cDNA was synthesized using a PrimeScript DNA Synthesis Kit. We then obtained a conservative sequence by comparing the homology between the sequences of proteases from *Lecanicillium lecanii*, *I. farinosa*, *B. bassiana*, and *Metarhizium anisopliae* in GenBank using MegAlign software (DNAStar), and a pair of degenerate primers (PrA and PrB in Table 1) was designed based on this conservative sequence. The polymerase chain reaction (PCR) protocol was as follows: 95°C for 5 min; 40 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min. Gel electrophoresis of all amplification products was carried out on 1.0% agarose gels stained with ethidium bromide (EB). The amplified fragment was recovered and cloned into the pMD18-T vector, and the vector was then transformed into competent cell *E. coli* DH5α for amplification and sequencing (Invitrogen, Shanghai, China).

3′-Rapid amplification of cDNA ends (RACE) reaction and 5'-RACE reaction were performed using the SMART RACE cDNA Amplification Kit. The nested primers were designed using Primer Premier version 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA; NGSP1, NGSP2, NGSP3 and NGSP4 in Table 1). In the 3′-RACE amplification reaction, UPM and NGSP1 were used in the first amplification reaction, and NUP and NGSP2 were used in the second amplification reaction; in the 5′-RACE amplification reaction, UPM and NGSP3 were used in the first amplification reaction, and NUP and NGSP4 were used in the second amplification reaction. Three sequences from reverse transcription PCR (RT-PCR), 5′-RACE, and 3′-RACE were matched to obtain the full-length sequence of the chitinase gene of *I. fumosorosea*, which was named *Ifupr1*.

Sequence Analyses of the Ifupr1 Gene and Protein

The full-length cDNA sequence was obtained using CLUSTAL W sequence analysis software (BioEdit 7.0) (Thompson *et al*., 1994). An online similarity search of the cDNA sequence and the predicted sequence of amino acids was performed using the BLAST algorithm. The structure of the protein was analyzed using DNAMAN and PROSITE (Bairoch *et al*., 1997), and the signal peptides were predicted using the SigalP 3.0 Server (ExPASy Proteomics tools).

Construction of Plasmids

The pBARGPE1 vector (Table 1) containing the *gpdA* promoter (from *Aspergillus nidulans*), which can control the expression of heterologous genes in a variety of fungi, was used to clone and express the *Ifupr1*cDNA. A full-length cDNA clone of *Ifupr1* was amplified by PCR with the forward primer Ifupr1A (Table 1; containing an *EcoR* Ⅰ site) and the reverse primer Ifupr1B (Table 1; containing an *Xho* Ⅰ site). The primer pairs with restriction enzyme sites were designed according to the insertion site sequence of the expression plasmid pBARGPE1. PCR was carried out as follows: 94°C for 5 min; 45 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and a final extension step of 10 min at 72°C. Gel-recovered PCR products were cloned into the vector pBARGPE1 (Wang and Leger, 2007). Plasmid amplification was performed in *E. coli* DH5α. Sequence analysis was carried out by sequencing (Invitrogen).

Preparation of *B. bassiana* blastospores and Transformation of Plasmid DNA

Preparation of blastospores was carried out as described by Ying and Feng (2006). Transformation of plasmid DNA to the blastospore was performed as follows. First, 50 µL of blastospores was added to 240 µL PEG4000 (50%), 36 µL LiAc (l M), 25 µL denatured salmon sperm DNA (4 g/L), 10 µL linear plasmid (0.1 µg/µL), and 35 µL dithiothreitol (1 M). The miscible liquids were incubated on ice for 30 min, heat shocked for 20 min at 42°C and centrifuged for 5 min at 6000 × *g* and 4°C. The supernatant solution was then removed, and the sediment was diluted in 0.5 mL ddH_2O.

Table 1: Plasmids and primers used for cloning

Plasmids and primers	Description	Source
Plasmids		
pMD18-T	Clone vector; Amp^r	Takara
pBARGPE1	Expression vector; carrying a *gpdA* and a *Bar*; Amp^r	Chengshu Wang project team*
Cloning primers		
PrA	5′-CAAGTACATTGTCAAGTTCAAGGA-3′	
PrB	5′-AGTGGCCATGGAAGTACCAGAGAT-3′	
NGSP1	5′-CCGATCCAGGTGGACTGAATGT-3′	
NGSP2	5′-ACAGCCAAGTCAAGCAGGGTAGTCT-3′	
NGSP3	5′-ATTGACACTGGCACCAACATCG-3′	
NGSP4	5′-CAATGGGGTTACACTGCTCC-3′	
Ifupr1A	5′-CCGGAATTCTCCTTCAAGATTGGAACCATCCACC-3′	
Ifupr1B	5′-CCGCTCGAG TTACATATTTAAATCAAGGTTGACA-3′	
BarF	5′-TCGTCAACCACTACATCGAGAC-3′	
BarR	5′-GAAGTCCAGCTGCCAGAAAC-3′	

Notes: *GpdA*, a promoter of the glyceraldehyde 3-phosphoric acid dehydrogenase gene from *Aspergillusnidulans*, was used to control the efficient expression of heterologous genes in a variety of fungi. *Bar* was a gene having resistance to the herbicide PPT (phosphinothricin). Restriction enzyme sites are underlined. Amp^r, ampicillin resistant

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The basal culture medium (containing 200 μg/mL phosphinothricin [PPT]) was incubated at 25°C for 1 week.

The positive fungus transformants were identified by amplifying the sequence of the *Bar* gene, a selectable marker gene in the vector pBARGPE1, which has resistance to the herbicide PPT. Nine transformations were inoculated onto the selective medium for 4–7 days. Single colonies were inoculated into SDB liquid medium (including Difco Sabouraud Dextrose Broth, 30 g/L) and incubated at 150 rpm for 2 days. The genomic DNA of these transformants was extracted for PCR. Primer pairs BarF and BarR (Table 1) were used in the amplified reaction, and PCR was carried out as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension step of 10 min at 72°C. Gel electrophoresis of all amplification products was carried out on 1.0% agarose gels stained with EB.

Protease Activity Determination

Based on the method in St Leger *et al*. (1996), 20 μL enzyme and 10 μL synthesized chromogenic substrate (Suc-(Ala)2-Pro-Phe-NA, 1 mg/mL) were added into 70 μL of 0.05 M Tris-HCl buffer (pH 8.0), and the solutions were then mixed uniformly and incubated at 25°C for 15 min. Relative enzyme activity was then measured by determining the absorbance at 405 nm. A unit of enzyme activity with Suc-(Ala) 2-Pro-Phe-NA as the substrate in Tris-HCl buffer (0.04 M, pH 8.0) was defined as the amount of enzyme required to increase the light absorption value by 0.1 OD within 15 min at 25°C.

Biological Assays

Positive transformation strains of the spore suspension identified on the PDA plates were cultured for 8 days at 25 ± 1°C. The conidial powder from strains with appropriate characteristics was then scraped, and conidial suspensions were prepared using 0.05% Tween-80, with final concentrations of 1×10^6, 5×10^6, 1×10^7, and 5×10^7 spores/mL. *D. punctatus* used in this study were health larva from Chuzhou Forest Farm, Anhui, China.

Using the impregnation method, worms were exposed to different concentrations of spore suspensions for 20–30 s. Control worms were impregnated in 0.05% Tween-80. Each group included 10 worms, and the experiments were repeated three times. The worms were cultivated at 25 ± 1°C, with the humidity maintained at more than 90% for 48 h and about 80% after 48 h. Deaths were recorded beginning on day 2. Dead bugs were removed to glass slides in Petri dishes of wet water paper and incubated under moisturizing culture for 3 days at 25°C. Contamination of the dead body was observed, and *B. bassiana* infection was determined by observation under a microscope for 10 days (Liu *et al*., 2008).

To test the effects of expressing Pr1A on the conidiation of Bb, we cultivated positive transformation strains on growth medium and insect cadavers 14 days. Five samples were then taken from each group (5 mm in diameter), and spore suspensions were prepared using 20 mL Tween-80 solution (0.05%). Blood counts were determined by counting spores under the microscope.

Data Analysis

The following equations were used for data calculations:

Cumulative mortality rate (%) = (total number of dead insect samples/total number of insect samples) × 100%

Cumulative corrected mortality (%) = ([mortality rate of the treatment group – mortality rate of the control group]/[1 – mortality rate of the control group]) × 100%

The method of probability was used to calculate the LC_{50} and LT_{50}, and SPSS software (version 17.0) was used to process and analyze the experimental data.

Results

Full-length cDNA Cloning and Sequence Analysis of the Protease Gene Ifupr1 from *I. fumosorosea*

Using total RNA as a template for RT-PCR, we obtained a fragment of 1100 bp based on the known sequences searched from NCBI. Specific primers were then designed according to the fragment sequence, and 5′-RACE and 3′-RACE were used to amplified unknown sequences (Fig. 1). The three sequences from RT-PCR, 5′-RACE, and 3′-RACE were matched, and a sequence of 1704 bp was obtained (GenBank accession number: FJ423001).

Analysis of the cDNA and Protein Sequences of Protease Gene Ifupr1 from *I. fumosorosea*

The length of the cDNA sequence was 1704 bp, and the code reading frame consisted of 1287 nucleotides encoding 428 amino acids. The 5′-untranslated region (UTR) and 3′-UTR contained 233 and 198 nucleotides, respectively, with a predicted signal peptide of 16 amino acids (Fig. 2). Theoretical molecular weight of the mature protein was 46.3 kDa, and the theoretical isoelectric point was 7.37. Of the total of 428 amino acids, 50 were basic amino acids (K and R), 52 were strongly acidic amino acids (D and E), 146 were hydrophobic amino acids (A, I, L, F, W and V), and 105 were polar amino acids (N, C, Q, S, T and Y), accounting for 11.8%, 12.2%, 34.3% and 24.7%, respectively.

Statistical analysis showed that the coding sequence of the *I. fumosorosea* protease gene contained 44.8% C, 26.7% T, 22.5% G and 6% A, respectively. These data indicated a preference for certain bases, e.g., frequent Cs and rare As.

Comparison of the Homology of the Ifupr1 Gene from *I. fumosorosea*

By comparing the mature protein sequence of the protease Ifupr1 with sequences from some other fungi listed in GenBank (Fig. 3), we found that this protease belonged to the Subtilisin-like protease family; homologies with *I. farinosa* (AAY87460), *Hypocrea lixii* (ABI84117), *Trichoderma atroviride* (ABG57252), and *Metarhizium anisopliae var. Anisopliae* (CAD13274) were 88%, 71%, 71% and 70%, respectivly.

Screening and Verification of Fungal Transformation

Nine transformations were randomly chosen after the exogenous gene was transformed into the blastospores. Positive fungal transformants were analyzed by amplifying the sequence of the selectable marker gene *Bar* with primers barF/barR. The results are shown in Fig. 3 and demonstrated that the exogenous gene was successfully transformed into the blastospores.

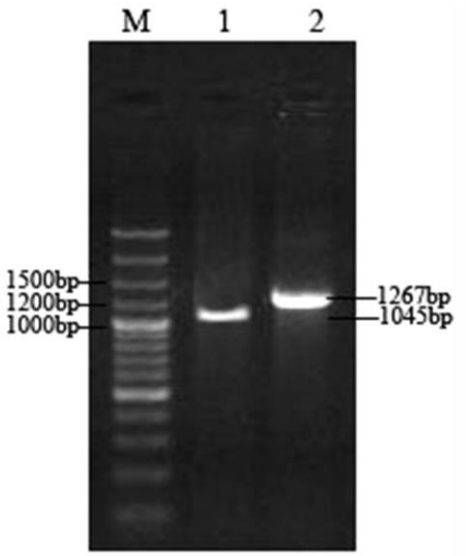

Fig. 1: Amplification of Ifuchi1 3′- and 5′-end cDNA by 3′- and 5′-RACE. Lane 1: 5′-RACE product; lane 2: 3′-RACE product

Determination of Protease Activity

Based on the traits of these positive transformants cultured in solid medium, we selected one transgenic strain to study in greater detail owing to its increased production of high-quality spores. Through variance analysis (Fig. 4), we found that the protease enzyme activity of this modified strain was significantly higher than that of the wild-type strain. Compared with the highest enzyme activity, the protease enzyme activity of the transgenic strain was 2.65-fold higher than that of the wild-type strain ($P< 0.05$). This result suggested that the protease enzyme gene of *I. fumosorosea* achieved high expression in *B. bassiana*.

Effects of Expressing Pr1A on the Conidiation of *B. bassiana*

To test the effects of expressing Pr1A on the conidiation of *B. bassiana*, we collected samples from growth medium and insect cadavers and then counted the spores under a microscope. The quantities of sporulation were 1.3×10^7 conidia/cm^2 in growth medium and 1.1×10^7 conidia/cm^{2}in insect cadavers; these two quantities were not significantly different.

The mortality rates, infection rates, and LT_{50} can reflect the pathogenicity for a target pest. The mortality and infection rates are positively correlated with pathogenicity, and the LT_{50} is negatively correlated with pathogenicity. As

Table 2: Pathogenicity of the tested *B. bassiana* strains against *D. punctatus* (*Ifupr1*)

Strains	Number of larvae	Larval mortality (days) (%) 4 5 6 7 10	Mortality (%)	Corrected mortality (%)	Infection Ratio (%)	LT_{50} (days)
Bb13	30	6.7 13.3 16.7 20 53.3	53.3 ± 0.4	48.1 ± 0.4	46.7 ± 0.2	9.8
Bb13-*Ifupr1*	30	16.7 20 30 60 86.7	86.7 ± 0.5	85.2 ± 0.7	86.7 ± 1.2	6.5
CK	30	0.0 0.0 3.3 6.7 10	10 ± 0.6	/	0.0	0.00

Table 3: Median lethal concentration (LC_{50}) of different strains against *D. punctatus* larvae

Strain	Regression equation	LC_{50} (conidia/mL)	95% confidence interval	χ^2	*P*
Bb13	Y = -6.203 + 0.932X	4.55×10^6	2.13×10^6–8.32×10^6	7.390	0.056
Bb13-*Ifupr1*	Y = -5.81 + 0.978X	0.869×10^6	0.14×10^6–1.85×10^6	8.024	0.061

Notes: The results fit best using the Probit model equation and LC_{50} with Pearson model goodness-of-fit test

```
1    CATTCCTCTCTTCCCTCAACACAACCTCCGTCTTCCTTCATCATCTTCCATCTCTGCTCTTCATCTCGCTGGCCC
76   TTTCCGACATCCAGCACTCCCCTTCCTCTTCTTCCTCTTCCTCGTCTTGATTTTCTCGCTTCTTTCGAAACACCA
151  CCCATCGCAGGCCGGCCCGCGTCATCCACTCGTCTGCACTCGTGAGCGCGGCCTACCTACATCATGAAGTCCGTC
51   M K S V
226  GTCGCCGCCCTCTCCGTGGTCGCCATCGCCCAGGCATCGTCCTTCAAGATTGGAACCATCCACCATGACTCTGCC
76   V A A L S V V A I A Q A S S F K I G T I H H D S A
301
CCGATCCTCGATGCCACCAACGCCAACACTATTCCTGGCGCCTACATCATCAAGTTCAAGGACCACGTCGATGAG
101  P I L D A T N A N T I P G A Y I I K F K D H V D E
376
CCCAAGGCCAAGTCCCACCATGCCTGGGTCGCGGATATCCACAGCGATGGCGAGCAGCAGCGTCTTGAGCTCCGC
126  P K A K S H H A W V A D I H S D G E Q Q R L E L R
451  AAGCGCGGTCTCTTCGACTCCGTCGAGGATGCTTTTGCCGGTATGAAGCACAGCTTTGGTCTTGGCTCTGGCTTC
151  K R G L F D S V E D A F A G M K H S F G L G S G F
526  CGTGGTTATTCTGGCCACTTTGACGACGCCGTCATTGAGCAGATTCGCAACCACCCCGACGTTGAGGCCATTGAA
176  R G Y S G H F D D A V I E Q I R N H P D V E A I E
601
CGGGACTCCGTTGTCCACACCACCGTCCCCGTCACCACCAAGCATGCTGTCACTGATGACACCTGCAAGCATGAG
201  R D S V V H T T V P V T T K H A V T D D T C K H E
676  GAGGAAAAGCAGGCTCCTTGGGGCCTTGCTCGTATCTCTCACCGCGATACTCTGGGTTTCAGTACCTTCAACAAG
226  E E K Q A P W G L A R I S H R D T L G F S T F N K
751  TACCTGTACGCCGAGGAGGGTGGTCAGGGTGTTGATGCCTATGTTATTGACACTGGCACCAACATCGATCACGTC
251  Y L Y A E E G G Q G V D A Y V I D T G T N I D H V
826
GACTTCGAGGGCCGTGCCCAATGGGGTTACACTGCTCCTGACGGCGATGCGGATGAGGACGGCAACGGCCACGGC
276  D F E G R A Q W G Y T A P D G D A D E D G N G H G
901
ACTCACTGCTCTGGCACTATCGCCGGTAAGAAGTACGGTGTTGCCAAGAAGGCCAACGTGTACGCCGTCAAGGTC
301  T H C S G T I A G K K Y G V A K K A N V Y A V K V
976
CTTCGCTCTAACGGCTCGGGCACTATGTCGGATGTCATCAAGGGAGTTGACTGGGCTGCCAAGAAGCACGAGGAG
326  L R S N G S G T M S D V I K G V D W A A K K H E E
1051
CAGGTACAACTGGCCAAGGATGGTAAGCGCAAGGGCTTCAAGGGCTCCGTCGCCAACATGTCTCTCGGTGGTGGC
351  Q V Q L A K D G K R K G F K G S V A N M S L G GG
1126 AAGACTACCCTGCTTGACTTGGCTGTTGATGCTGCCGTCGACAAGGGGCTTCACTTTGCCGTCGCTGCCGGTAAC
376  K T T L L D L A V D A A V D K G L H F A V A A G N
1201
GATAACGCGGACGCCTGCAACTACTCCCCTGCTGCCGCTAAGAAGGCCGTCACTGTTGGGGCCTCGACCATTGAT
401  D N A D A C N Y S P A AA K K A V T V G A S T I D
1276
GACAGCCGTGCCTACTTCTCCAACAGCACGAGTTCGAGGTCGTCAGTGGCGAGATTACCAAGTCCGCCAAGACCT
426  D S R A Y F S N S T S S R S S V A R L P S P P R P
1351
TCGGCAGCAAGGCTGAGAAGTTCGCCAAGAAGATCCAGCACATTGTCGATGCTGAGGTCGAGGATTTCCTCTCCC
451  S A A R L R S S P R R S S T L S M L R S R I S S P
1426 ACCTGGAGTAAATTCCTCATCATACACGAATATCCTATCATCCTGAATGAGAATACTGTCAACCTTGATTTAAAT
476  T W S K F L I I H E Y P I I L N E N T V N L D L N
1501 ATGTAAACTGCAAGTCATCTGGTGTTTTTCTTAGGTTTTGTTTTTTGATTGATTTGAATTACAATACGGGATAGA
501 M *
1576 TACTTGACTCCCACGGGGGAATGCATGGATGAGGATACTAGCTCAGATGAAATACATTTCTTTTTATTTTTAACA
1651 AAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 2: Nucleotide and deduced amino acid sequence of I. fumosorosea protease. Numbers to the right are the nucleotide and amino acid positions. The signal peptide is shaded, and the propeptide is underlined. The potential glycosylation sites are boxed. The active site aspartic acid, histidine, and serine residues are marked with italics and overstriking

shown in Fig. 5 and Table 2, the virulence of the transgenic strain was much higher than that of the wild-type strain for *D. punctatus*; when the infection rate of the control group CK was 0, the LT_{50} of the transgenic strain group was reduced by 33% compared with that of the wild-type group (6.52 days versus 9.83 days, $P < 0.05$) after culture for 10 days; the mortality of the transgenic strain group was increased by 62.6% compared with that of the wild-type group (86.67% versus 53.3%, $P < 0.05$). The transgenic strain significantly increased the insecticidal efficiency of *B. bassiana* strain Bb13.

Analysis of LC_{50} Values

Next, we evaluated the virulence of the wild-type strain Bb13 and the transgenic strain Bb13-Ifupr1 with four different concentrations for *D. punctatus*. We found that the dosage of spores was reduced because the virulence

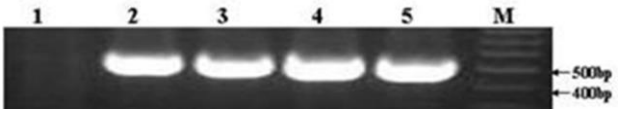

Fig. 3: Examination of the transgenic Bb13-Ifupr1 by PCR (barF/barR). Lane 1: negative control, Bb13; lanes 2–5: four random transformants

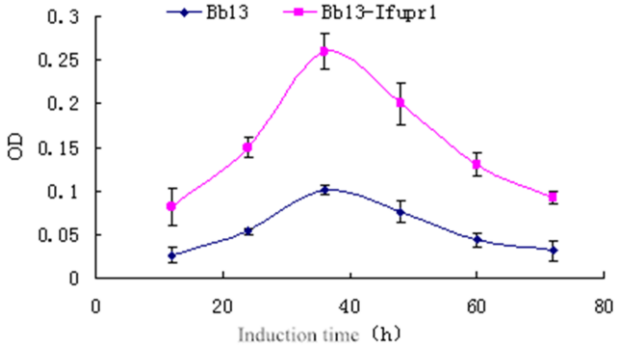

Fig. 4: Examination of pr1 activities

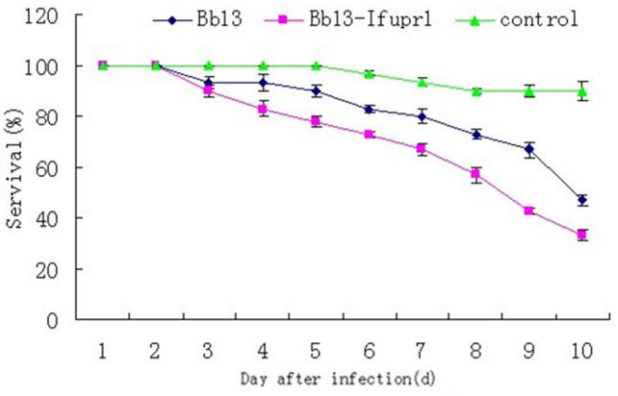

Fig. 5: Survival of *D. punctatus* larvae infected with different isolates

of the transgenic strain was greatly improved (Fig. 5 and Table 3). The LC_{50} of the wild-type strain (4.55×10^6 spores/mL) was 4.2 times that of the transgenic strain (0.869×10^6 spores/mL). Thus, the use of this transgenic strain may greatly reduce the economic cost of biological control.

Discussion

In this study, we cloned the functional gene of *I. fumosorosea*, *Ifupr1*, which is involved in the infection process. We discovered that this gene encoded a precursor peptide that contained a signal peptide and a propeptide. Generally, the presence of nine hydrophobic amino acids in the core of the hydrophobic region is thought to indicate that the peptide can cross the membrane to transmit the signal; the identified signal peptide from the *Ifupr1* gene conformed to this characteristic, suggesting that this protein would be an extracellular secreted protein and may assist the fungus in the infection process by decomposing proteins within the insect body wall. Leger cloned the cuticle-degrading protease gene of *Metarhizium anisopliae*, *Pr1A*, and found that the protein encoded by this gene contains only eight hydrophobic amino acids in the core of the hydrophobic region of the signal peptide. This is slightly different from the results of our study. Moreover, proteases function to cleave proteins into smaller peptides, and the function of the identified protease may involve protecting the activity of protein decomposition during the transfer process. However, the homology of this region with precursor peptides of PR1H from other entomogenous fungi is not high, and Leger's research also concluded that the sequence of the core of the hydrophobic region is not conserved and is evolving rapidly (Wang and Leger, 2006).

Preferential codon usage is universally observed for all highly expressed genes of filamentous fungi, and T/C is preferred in the third base of the codon. Leger's study showed that the frequencies of utilization of bases in the third of codon are 43% for T, 36% for C, 16% for G, and 4% for A (Screen *et al*., 2001). Thus, our results were slightly different, with a higher percentage of C bases in the protease gene of *I. fumosorosea* and a better balance for T and G.

In most fungal genes, the first initiation codon of the cDNA is ATG, and the third site upstream of the initiation codon is often A; 83% of fungal genes are consistent with this characteristic. The gene in this study was also consistent with these parameters.

Conclusion

We obtained a *B. bassiana* engineering strain containing the protease gene of *I. fumosorosea* and found that the virulence of this engineering strain for *D. punctatus* was improved significantly. These resulted supported that insecticidal effects may be enhanced by transferring protease genes into entomogenous fungi. The mechanism mediating the observed effects may involve increased ability of Ifupr1 to degrade the insect body wall, thereby speeding up the penetration through the body wall and improving the insecticidal effects of the strain (Lv *et al*., 2008). Accordingly, our findings will provide an important basis for constructing more efficient insecticidal engineering strains and further study of its insecticidal mechanism.

Acknowledgements

The first author acknowledges the supported by the Key Project for Natural Science Research of Anhui Provincial Higher School (Grant No. KJ2016A761), the Key Project for Natural Science Research of Anhui Provincial Higher

School (Grant No. KJ2017A556), the Education and training program for excellent skills talents in Anhui quality engineering and pharmaceutical production technology (Grant No. 2016zjjh073), and the Excellent Youth Talent Support Program project of Anhui Provincial Higher School (2014).

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(Received 18 December 2017; Accepted 02 January 2018)