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β-1,3-Glucan recognition protein 3 activates the prophenoloxidase system in response to bacterial infection in *Ostrinia furnacalis* Guenée

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ABSTRACT

Pattern recognition receptors (PRRs) are biosensor proteins that bind to non-self pathogen associated molecular patterns (PAMPs). β-1,3-glucan recognition proteins (βGRPs) play an essential role in immune recognition and signaling pathway of insect innate immunity. Here, we report the cloning and characterization of cDNA of *OfβGRP3* from *Ostrinia furnacalis* larvae. The *OfβGRP3* contains 1455 bp open reading frame, encoding a predicted 484 amino acid residue protein. In hemocytes, the expression levels of *OfβGRP3* in *Escherichia coli*-challenged group were higher than those of *Bacillus subtilis*-challenged group at 2, 4, 8, 10 and 12 h post injection (HPI). In fat body, *OfβGRP3* expression in both *B. subtilis* and *E. coli*-challenged group was significantly higher than that in untreated group from 4 to 10 HPI, and then the expression continuously dropped from 12 to 36 HPI. The *OfβGRP3* expression in laminarin-injected group was higher than that in lipopolysaccharides (LPS)-injected group in various test tissues from 4 to 24 HPI. The LT_{50} of *E. coli*-infected *OfβGRP3*-RNAi larvae (1.0 days) was significantly lower compared with that of *E. coli* infected wild-type larvae (3.0 days) ($p < 0.01$). Only 10.2% Sephadex G50 beads (degree 3) were completely melanized in the larvae inoculated with *OfβGRP3* dsRNA, as compared to 48.8% in control larvae ($p < 0.01$). A notable reduction in the PO activity and IEARase activity in hemolymph was also detected in the *OfβGRP3* knockdown larvae. Our study demonstrates that *OfβGRP3* is one of PRR members involved the PPO-activating system in *O. furnacalis* larvae.

1. Introduction

Due to lack of adaptive immune system, insects like many invertebrates rely on their innate immune system to resist the invading pathogens and parasitoids (Lemaitre and Hoffmann, 2007). Broadly defined, the innate immunity system is encoded by three major functional categories of genes that are involved in (1) recognition of invading microbes, (2) immune-signal amplification and transduction, and (3) effector mechanisms that mediate the killing and clearance of infectious micro-organisms (Das et al., 2009). Once the recognition has taken place, it may trigger a protective response involving cellular and humoral immunity, including phagocytosis, encapsulation and nodule formation, clotting cascade, the synthesis of a wide array of antimicrobial peptides, the prophenoloxidase (PPO) activating system (Wang et al., 2011). The PPO activating system (PPO-AS) involves the activation of serine proteinases, which is triggered by pathogen associated molecular patterns (PAMPs) to enhances the PPO cascade (Sivakamavalli and Vaseeharan, 2014). This includes the sequential activation of a yet unknown number of proteinases and cofactors, leading to the limited proteolysis of a PPO-activating protease (PAP) (Jiang et al., 2003; Wang and Jiang, 2006; Chu et al., 2017). This enzyme catalyzes the proteolytic cleavage of the inactive PPO precursor into the active phenoloxidase (PO). Furthermore, insects and crustaceans contain serine protease inhibitors (serpins) in hemolymph to prevent unwanted activation of this complex system (Chu et al., 2015; Zhang et al., 2016). PO catalyzes the oxidation of tyrosine-derived phenols to quinones, which are speculated to kill invading microorganisms (Franssens et al., 2008).

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Pattern recognition receptors (PRRs) are biosensor proteins that bind to non-self PAMPs (Fabrick et al., 2004a). PAMPs are molecules present on the surface of microorganisms, typically as structure components and are not found within host organism (Medzhitov and Janeway, 1997). PAMPs such as lipopolysaccharides (LPS), peptidoglycans (PGs) and β-1,3-glucans (BGs) are first detected by PRRs. Several PRRs have been reported in insects and other arthropods including β-1,3-glucan recognition proteins (βGRPs), also named β-1,3-glucan binding proteins (Sritunyalucksana et al., 2002; Jiang et al., 2004), peptidoglycan recognition proteins (PGRPs) (Werner et al., 2000), galactoside-binding lectins (galectins), fibrinogen-related proteins, thioester-containing proteins (Christophides et al., 2004; Wang et al., 2011.), C-type lectins (Yu and Kanost, 2000), a scavenger receptor protein (Pearson et al., 1995) and hemolin (Yu et al., 2002). Once invading microbes are recognized through specific interaction between PRRs and PAMPs, a variety of defense reactions can be activated. The autoactivation of an initiating serine protease upon binding of pattern recognition proteins to pathogen surfaces is an essential step in triggering insect immune responses such as the activation of Toll and PPO activating pathways (Takahashi et al., 2015). So far, β-1,3-glucan recognition protein has been purified and cloned in many invertebrate groups. For example, Söderhäll et al. (1988) and Ochiai and Ashida (1988) were the first to purify these proteins from the plasma of insects, *Blaberus craniifer* and *Bombyx mori*, respectively. βGRPs have been cloned from *Manduca sexta* (Ma and Kanost, 2000), *Tenebrio molitor* (Zhang et al., 2003), *Plutella xylostella* (Huang et al., 2015), shrimp *Penaeus monodon* (Sritunyalucksana et al., 2002), *Penaeus vannamei* (Jiménez-Vega et al., 2002) and mussel *Perna viridis* (Jayaraj et al., 2008). Additionally, a 52 kDa β-1,3-glucan binding protein isolated form *M. sexta* was shown to aggregate bacteria and fungi and to stimulate PPO activation (Jiang et al., 2004). Although some of these molecules have been characterized at the molecular level, the constituents, initiation, and regulation of the proteinase pathway are still poorly understood (Takahashi et al., 2015).

The Asian corn borer, *Ostrinia furnacalis* Guenée, is an important component of the lepidoptera pest complex of corn and cotton in China, and has been used as a model species for investigating the physiological interaction between this insect and foreign microbes and parasitoids. Recently, many efforts have been contributed to the study of biological control of *O. furnacalis* with parasitic wasps (Feng et al., 2011) and fungus *Beauveria bassiana* (Liu et al., 2014). However, the knowledge on immune recognition and signaling pathway of PRRs in its innate immune response is limited. The identification and isolation of βGRP genes may improve our understanding of lepidopteran immunity and lead to the development of new safer pesticides as potential environment friendly pest control agents.

In the present study, one of βGRP genes, *OfβGRP3* was cloned and characterized from *O. furnacalis* larvae. We reported the characterization and functional analysis *OfβGRP3* from *O. furnacalis*, and investigated its tissue distribution and temporal expression profiles after bacterial challenge. *OfβGRP3*-silenced *O. furnacalis* larvae, generated by dsRNA inhibition of transcript, exhibited a higher mortality than the wild-type, and the activities of PO and IEARase in hemolymph were down regulated by the RNAi of *OfβGRP3* gene. Our study indicated that OfβGRP3 may be a molecule which can respond to pathogenic bacterial infection and activate the PPO system in *O. furnacalis*.

2. Material and methods

2.1. Insect rearing

O. furnacalis Guenée (Lepidoptera: Pyralidae) larvae were reared at 25 ± 1 °C, RH>80% and a photoperiod of 14 L: 10 D. The larvae were fed on an artificial diet as described by Zhou et al. (1980) and the larval instars were determined by measuring the head-width.

2.2. cDNA cloning of OfβGRP3 and sequence analysis

Total RNA was isolated from the 5th instar larvae of *O. furnacalis* using Trizol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using the PrimeScript™ 1st Strand cDNA Synthesis Kit (cat. no. D6110A; TaKaRa, Dalian, China). cDNA quality was checked on an 1% agarose gel and quantified by spectrophotometry at A_{260}. The resulting cDNA solution was stored at −20 °C till further analysis.

Multiple alignments of βGRP amino acid sequences from other lepidopterans were performed using the Clustal omega multiple sequence alignments program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Based on sequences of the two highly conserved amino acid regions in βGRPs, a pair of primers, *m*-OfβGRP1-F and *m*-OfβGRP1-R, was designed (Table 1). The PCR condition was as following: 3 min initial denaturation at 94 °C, then 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 50 S, followed by a 10 min final extension at 72 °C and cooling to 4 °C. Reaction products were purified on agarose gels. A fragment of 439 bp was cloned into the T vector (Takara, Tokyo, Japan) and then sequenced using an ABI 3730 sequencer (SeqGen Inc., Torrance, CA).

The full-length *OfβGRP3* cDNA was covered by 3′-RACE and 5′-RACE systems based on the obtained partial cDNA sequences of *OfβGRP3*. The 3′-RACE PCR amplification was performed with 3′-RACE-ready cDNA. Gene-specific primers (3′GSP1, 3′GSP2) and 3′CDS (Table 1) were designed for 3′-RACE. The PCR was carried out according to the program of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 50 S, and then an extension of 72 °C for 10 min. For 5′-RACE PCR, nested PCR strategy was employed to increase specificity using Takara 5′-Full RACE Kit (TaKaRa Code: D315). The 5′-RACE PCR amplification was performed with 5′-RACE-ready cDNA. The outer primer, inner primer and the gene-specific primer 5′GSP1 and 5′GSP2 (Table 1) were designed for the 5′-RACE. The PCR conditions were 94 °C for 3 min, 36 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 50 S, and then an extension of 72 °C for 10 min. The full length cDNA sequence of *OfβGRP3* was verified by PCR amplification and nucleotide sequencing with the primers of *βGRP3*-F and *βGRP3*-R (Table 1).

The βGRP amino acid sequences of other species were downloaded from GenBank in FASTA format. The amino acid sequence of *OfβGRP3* was aligned with other insects' βGRPs previously identified by using the Clustal Omega program. Protein motif features were predicted using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The calculated molecular mass and isoelectric point of the mature protein by ExPASy tools (http://web.expasy.org/compute_pi/). The domain analysis of protein family was performed by pfam tool (http://pfam.xfam.org/). SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Phylogenetic trees were constructed on the basis of the amino acid differences (p-distance) of βGRPs by the neighbor-joining method, using the MEGA5 software. The *Pacifastacus leniusculus* lipopolysaccharide and beta-1,3-glucan binding protein (GenBank accession number: CAB65353.1) was used as an out-group to root the phylogeny. For construction of the phylogenetic tree, the reliability of the branching was assessed by a bootstrap re-sampling method, using 1000 bootstrap replications.

2.3. Transcriptional analysis

For the tissue distribution analysis of *OfβGRP3* transcript in 5th

Table 1
Nucleotide sequences of the PCR primers.

Name	Purpose	Sequence (5′-3′)
m-OfβGRP3-F	middle fragment cloning	GCGAAATTACGCATCAGG
m-OfβGRP3-R		GTTCCAGAAGTTGAGCATGGC
3′GSP1	3′-RACE PCR	GACCTGACGAGTATGGGAACA
3′GSP2		CAGTGGATGGCGAGCAA
3′CDS		ATTCTAGAGGCCGAGGCGGCCGACATG-d (T) 30VN
5′GSP1	5′-RACE PCR	CGCCCAGAGTTAGGTAGAAGTG
5′GSP2		CCCATTGCTCGCCATCCACT
5′Outer primer		CATGGCTACATGCTGACAGCCTA
5′Inner primer		CGCGGATCCACAGCCTACTGATGATCAGTCGATG
βGRP3-F	Full length cDNA identification	GAAACAGTGACAAGATGGC
βGRP3-R		CACTTTTACAATTATATTATTAA
e-βGRP3-F	βGRP3 qPCR	GACGATTTCCACGAATACTCCA
e-βGRP3-R		CATTGCTCGCCATCCACT
RPL8-F		ACGGAGGTGGTAACCATCAACA
RPL8-R		ACGCCTCCTTCTTGGTGTCG
	Gene silencing	
dsβGRP3(T7)-F		GGATCCTAATACGACTCACTATAGGATGGCCGTCGCACTGGTCAGAC
dsβGRP3(T7)-R		GGATCCTAATACGACTCACTATAGGGCCAGAGATCGTCCTTGAAG
dsGFP(T7)-F		GGATCCTAATACGACTCACTATAGGGTAAACGGCCACAAGTTCAG
dsGFP(T7)-R		GGATCCTAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTC

instar day 1 larvae, the quantitative real-time polymerase chain reaction (qPCR) was applied using the *OfβGRP*3 specific primers: e-βGRP3-F and e-βGRP3-R (Table 1). The larval tissues, such as hemocyte, fat body, midgut, and integument were collected, homogenized in Trizol (Invitrogen, Shanghai, China) and total RNA isolated. The total RNA preparations were treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. First-strand cDNAs were synthesized from 1.0 μg of total RNA samples using the Takara PrimeScript™ RT reagent Kit (TaKaRa, code: DDR037A) according to the manufacturer's protocol. The *O. furnacalis* ribosome protein L8 (RPL8) gene was used as an internal control in all experiments as previously described by Feng et al. (2011) (primers in Table 1). qPCR was carried out using a Bio-RAD CFX96 Real Time Detection System (Bio-RAD, USA) in 20 μL reaction volume containing 1 μL of cDNA from each tissue, 10 μL of SSOfast SYBR-Green Mix, 1.0 μL of each primer (20 pmoL/μL) and 7 μL ddH_2O. The qPCR reaction conditions were an initial denaturing step for 3 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 59.1 °C for 10 s and 72 °C for 15 s, followed by 95 °C for 1 min and 55 °C for 1 min. The same qPCR cycle profile was used for the internal control gene. All samples were analyzed in triplicate in three independent experiments performed using different tissues from *O. furnacalis* larvae. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression level of *OfβGRP*3.

For the expression analysis of temporal and spatial expression pattern of *OfβGRP*3 in larvae after pathogens challenge, the 5th instar day 1 larvae were divided into four groups: *Escherichia coli*-challenged, *Bacillus subtilis*-challenged, untreated group and physiological saline-treated (0.65% NaCl, 0.025% KCl, 0.03% $CaCl_2$, 0.025% $NaHCO_3$, pH6.8). To infect the larvae with *E. coli* or *B. subtilis*, 4 μL of 3×10^6 CFU of sterilized *E. coli* BL21 (DE3) or *B. subtilis* in 0.85% (w/v) NaCl were injected into larvae. The control larvae were injected with 4 μL physiological saline solution or without injection. At 0, 2, 4, 6, 8, 10, 12, 24 and 36 h post-pathogen injection, hemocytes, fat body, midgut and integument from ten individual larvae were collected using CAC buffer (100 mmoL/L $CaCl_2$, 10 mmoL/L Na_2CAC, pH 6.5) as an anticoagulant. The procedure of qPCR for different tissues has been described as above. The data are expressed as the mean ± standard deviation from three independent experiments.

2.4. *OfβGRP3* expression in LPS-injected and laminarin-injected larvae

The 5th instar day 1 larvae were injected with LPS and laminarin as the treatments respectively, and with physiological saline as the control group. LPS dissolved in 0.85% NaCl solution to 0.1 mg/mL, and 10 mmoL/L laminarin, a soluble β-1,3-glucan, were used as the test solution. The larva was injected intra-abdominally with the LPS and laminarin solution at 4 μL per larvae, respectively. The control group was injected with 4 μL of physiological saline. At the each time point (0, 2, 4, 6, 8, 10, 12, 24 and 36 h after injection), hemocytes, fat body, midgut and integument were collected and stored in liquid nitrogen for RNA extraction using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The procedure of qPCR for different tissues has been described as above. The data are expressed as the mean ± standard deviation from three independent experiments.

2.5. RNAi

Double-stranded RNAs for *OfβGRP*3 and Green fluorescent protein (GFP) were prepared following the protocol of the Promega RiboMax™ T7 system (Promega, USA). A template for *in vitro* transcription reactions was prepared by PCR amplification of a clone containing the *OfβGRP*3 ORF using gene-specific primers (Table 1) with the T7 polymerase promoter sequence at the 5′ end. After phenol-chloroform extraction and heat treatment, the dsRNA was diluted with nuclease-free water to a final concentration of 5 μg/μL for injection into the 4th instar larvae. In the experimental group, GFP dsRNA and nuclease-free DEPC water served as negative controls. Fourth instar larvae were injected intra-abdominally with 20 μg of *OfβGRP*3 dsRNA (RNAi group). The second group (GFP group) received a 4 μL injection with 20 μg of GFP dsRNA. The third group of larvae received a 4 μL injection of nuclease-free DEPC water to control for handling effects. Sixty 4th instar larvae were treated in three experiments, respectively. The puncture was immediately sealed with liquid paraffin. Larvae were removed from the slide after injection and raised at 25 °C until analysis. qPCR were used to detect the efficiency of the RNAi. The procedure of qPCR has been described as above. All reactions were completed in triplicate

using independently extracted RNA samples.

2.6. Assessment of efficiency of RNAi and cumulative mortality after RNAi

The procedures for dsRNA production and larvae injection were carried out according the method described above. Following injection of *OfβGRP*3 dsRNA, 20 larvae were inoculated with 3×10^6 CFU of *E. coli* BL21(DE3) intra-abdominally (RNAi-infected). Twenty larvae were treated with RNAi without bacterial infection (RNAi) and 20 larvae were inoculated with 3×10^6 CFU of *E. coli* BL21(DE3) only (infected). The control group received a 4 μL injection of nuclease-free DEPC water. The efficiency of RNAi in the larvae hemocytes and fat body was detected by qPCR after 3 days post injection (DPI) of *OfβGRP*3 dsRNA. Experiments were conducted in triplicate.

The hemolymph was collected at 1, 2, 3, 4, 5, 6, 7 and 8 DPI (at least 20 larvae per time-point for experimental samples and controls). Hemolymph retrieved by capillary suction from cutting prolegs of larval abdomen was used for the detection of activities of PO and IEARase subsequently. The data are expressed as the mean ± standard deviation from three independent experiments.

For the cumulative mortality rate analysis, the numbers of dead larvae were recorded for each group over the 8 days period post-injection, and the cumulative mortality was calculated from three independent experiments. Mortality observations were made daily for 2 h (10: 30–11: 30 and 14: 30–15: 30).

2.7. Detection of the ratio of melanotic encapsulation after RNAi

For the detection of variation of the ratio of melanotic encapsulation in the larvae after *OfβGRP*3 dsRNA treatment, 4th instar larvae were chilled on ice for several minutes, then surface sterilized with 70% ethanol before injection of 3 μL physiological saline that contained 10 to 15 Sephadex G50 beads. The beads were injected into the body cavity of larvae following *OfβGRP*3 dsRNA injection at day 3, and the puncture was immediately sealed with liquid paraffin. Briefly, each test insect was placed in an individual container with fresh diet and the injected larvae (n = 10) were dissected 24 h later after receiving Sephadex G50 beads, and beads were then observed and recorded by phase contrast microscopy and scored for encapsulation. In order to compare the extent of melanotic encapsulation of the injected beads, capsules of the beads were classified into four grades according to the thickness of the capsule as follows: degree 0, unencapsulated; degree 1, a few hemocytes (less than 10) attached to the beads; degree 2, more than ten cells up to one layer covering the beads; degree 3, hemocytes more than one layer but less than the thickness of the bead's radius.

2.8. Activities of PO and IEARase from hemolymph of larvae after OfβGRP3 RNAi

To investigate the involvement of *OfβGRP*3 in larvae PPO system, the activity of PO and IEARase from 100 larvae hemolymph after *OfβGRP*3 RNAi at different time-points post-injection was assayed. A preparation of hemolymph proteins of the larvae was produced as described above. Briefly, hemolymph from cutting prolegs of fifth instar larvae was collected after *OfβGRP*3 RNAi (approximately 30 μL/larva) into chilled polypropylene tubes. Hemocytes were pelleted by centrifugation at 1000 × g for 10 min. Aliquots (10 μL) of fresh plasma (cell free hemolymph) were added to 150 μL 2 mmoL/L dopamine in 50 mM sodium phosphate, pH 6.5, placing them on microtiter plate and mixing them to measure PO activity. One unit was defined as 0.001 absorbance increases at 470 nm per min. Enzymatic reactions were carried out in triplicate.

The IEARase activity in hemolymph was assayed using chromogenic substrate acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide, IEARpNa (A0180; Sigma) as a chromogenic substrate according to the method modified from Jiang et al. (2003). The reaction mixture contained 30 μL fresh plasma (cell free hemolymph), 200 μL 100 mmoL/L sodium phosphate buffer, pH 8.0, 50 μL 50 μM IEARpNa. The reaction was carried out at 30 °C for 5 min and the release of *p*-nitroanilide was measured spectrophotometrically at 405 nm. One unit was defined as 0.001 absorbance increase at 405 nm/min. Enzymatic reactions were carried out in triplicate.

2.9. Data analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD test and Duncan's multiple comparison tests, and the Kaplan-Meier survival analysis was conducted with Statistical Package for the Social Sciences software (SPSS version 23.0). Differences with a *P*-value less than 0.05 were considered statistically significant for all treatments.

3. Results

3.1. Molecular characteristics of full-length OfβGRP3 cDNA

Full-length cDNA representing a novel *O. furnacalis* β-1,3-glucan recognition protein, named *OfβGRP*3, was obtained based on RACE. The complete cDNA of *OfβGRP*3 consists of 1570 bp, containing a 1455 bp open reading frame (ORF), a 14 bp 5′-untranslated region, and a 104 bp 3′-untranslated region with a putative polyadenylation signal (AATAA) (Fig. S1). The ORF begins at nucleotide 15 and ends at nucleotide 1469. Based on the deduced polypeptide sequence, Pfam analysis showed that the ORF of *OfβGRP*3 consists of 484 amino acids and a *N*-terminal signal peptide spanning residues 1–25. The calculated molecular mass of the OfβGRP3 protein is 53.37 kDa, with an estimated pI of 6.46. The *OfβGRP*3 cDNA sequence and deduced amino acid sequence have been deposited in GenBank under accession no. KF425324. Multiple alignment of *OfβGRP*3 with βGRPs of different insects showed conserved motifs, including a protein kinase C phosphorylation site at positions S_{313} to R_{315}, a modified cell adhesive site (integrin-binding motifs) at positions Q_{282} to D_{284}. The pfam analysis showed the *N*-terminal domain is very likely a member of the carbohydrate binding domain family 32. Additionally, one glycosyl hydrolase family 16 domain was identified from positions Leu_{351} to Gln_{398} (Fig. 1).

The deduced amino acid sequence of the peptide encoded by the *OfβGRP*3 cDNA has high level identity with those of *Antheraea pernyi* GNBP (AHD25001, identity = 62%), *M. sexta* GNBP-like protein (ADT82662.1, identity = 57%), *Helicoverpa armigera* beta-1,3-glucan recognition protein 3 (ACI32828, identity = 60%), *Papilio xuthus* beta-1,3-glucan binding protein-like (NP_001299245.1, identity = 58%) and *Danaus plexippus* Beta-1,3-glucan-binding protein (EHJ65186, identity = 53%). The high homology of *OfβGRP*3 sequence to pattern recognition proteins (PRPs) of other insect species indicates an important role in the recognition of pathogen.

Using representative insect βGRP sequences, the phylogenetic tree was reconstructed using the neighbor-joining method of MEGA5 based on the multiple alignment built with Clustal Omega. *P. leniusculus* lipopolysaccharide and beta-1,3-glucan binding protein amino acid sequences were applied as out-group, and the constructed phylogenetic tree was composed of three main distinct branches with strong bootstrap, thus suggesting that these βGRPs share a common ancestor. *OfβGRP*3, *H. armigera βGRP*3, *B. mori βGRP*2, *M. sexta βGRP*3, *D. plexippus βGRP* and *βGRP*3, *Pieris rapae βGRP*3, and *Delias nigrina βGRP*3 are clustered to a branch, while the

Fig. 1. Multiple sequence alignment of four lepidopteran insect βGRPs. The amino acid sequences of *O. furnacalis* βGRP3 (OfβGRP3, KF425324), *H. armigera* βGRP3 (HaβGRP3, EU770384), *D. plexippus* βGRP3 (DpβGRP3, EHJ65188), *P. rapae* βGRP3 (PraβGRP3, ACI32823) are aligned. Asterisk, identical; dot, conservative substitution. The sequence of the signal peptide is underlined. The putative cell adhesive site (QGD) regions are shaded green. The amino acid sequences of potential kinase C phosphorylation site (SAR) are shaded yellow; The carbohydrate binding domain (family32) regions of *O. furnacalis* βGRP3 are shaded pink, and glycosyl hydrolases family16 domains of *O. furnacalis* βGRP3 are shaded gray, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

others are clustered to βGRP1 and βGRP2, respectively (Fig. 2). These results indicate that there are at least three groups of βGRPs in invertebrate, and their amino acids share low level of identity with each other.

3.2. Tissue-specific expression profiles of OfβGRP3

The qPCR was employed to investigate the tissue-specific distribution of the *OfβGRP*3 mRNA in larvae, and the RPL8 gene was applied as an internal control in tissues. In both *OfβGRP*3 and *RPL*8 genes, there was only one peak at the corresponding melting temperature in the dissociation curve, indicating that the qPCR was specifically amplified. The mRNA of *OfβGRP*3 was detected in hemocytes, fat body, midgut and integument with different expression levels, and the highest expression level of *OfβGRP*3 was in fat body and the lowest in midgut (Fig. 3).

3.3. Temporal and spatial expression pattern of OfβGRP3 in response to pathogenic infection

To detect the function of *OfβGRP*3 in the immune response in *O. furnacalis* larvae, the expression of *OfβGRP*3 in hemocytes, fat body, midgut and integument after bacterial challenge was measured using qPCR method with *RPL*8 as an internal control. Hemocytes were chosen for the analysis because they were directly involved in defense mechanism. Fat body, midgut and integument are also the major tissues involved in both humoral and cellular immune responses in insects (Feng et al., 2011). In hemocytes, the expression levels of *OfβGRP*3 in *E. coli*-challenged group were higher than those of *B. subtilis*-challenged group at 2, 4, 8, 10 and 12 HPI (Fig. 4A). The results showed that the expression level of *OfβGRP*3 in two challenged groups from 2 to 24 h post-injection (HPI) was higher than that in untreated group. The peak of

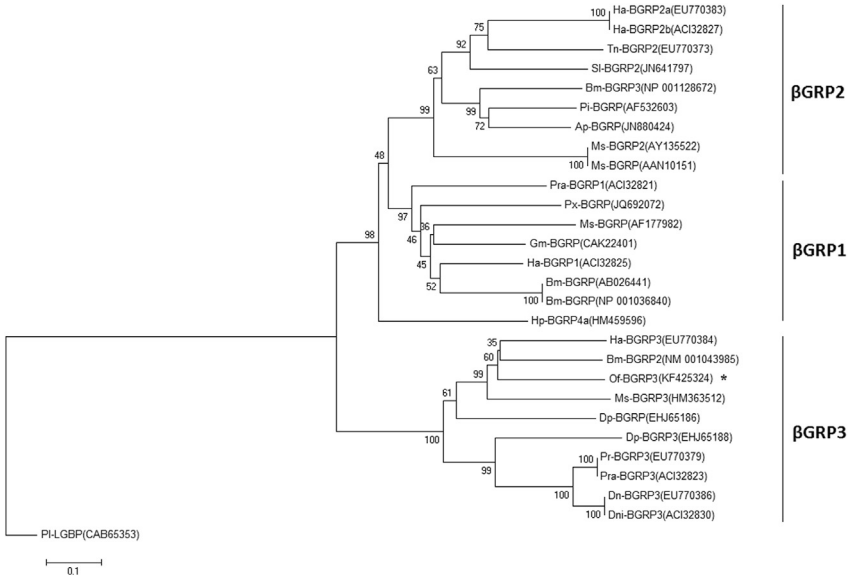

Fig. 2. Phylogenetic analysis of 28 members of the βGRPs including the OfβGRP3 from various insect. e.g. *O. furnacalis* (Of), *H. armigera* (Ha), *Trichoplusia ni* (Tn), *Spodoptera litura* (Sl), *B. mori* (Bm), *Plodia interpunctella* (Pi), *Antheraea pernyi* (Ap), *M. sexta* (Ms), *Hepialus pui* (Hp), *P. rapae* (Pr and Pra), *P. xylostella* (Px), *Galleria mellonella* (Gm), *D. plexippus* (Dp), *D. nigrina* (Dn and Dni) and crayfish *Pacifastacus leniusculus* (Pl). The numbers are neighbor-joining distances. Asterisks indicate the OfβGRP3. Vertical lines indicate three groups of βGRPs in the phylogenetic tree.

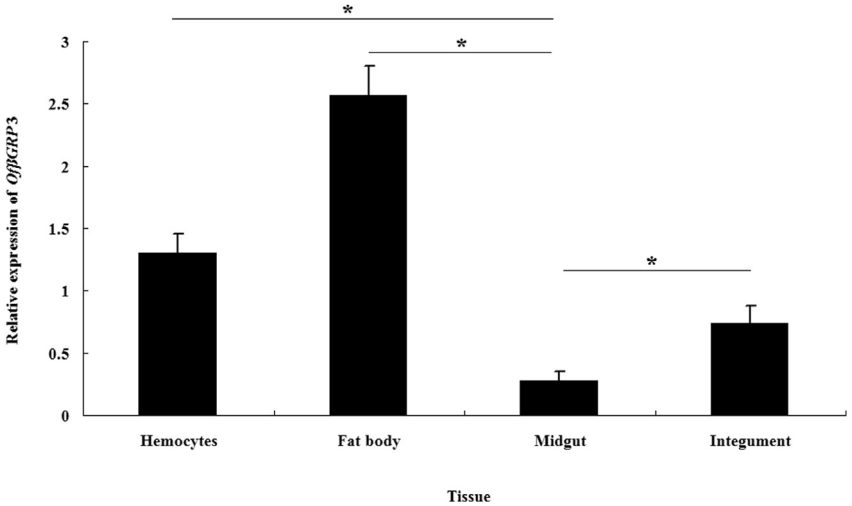

Fig. 3. Tissue-specific expression of the *OfβGRP*3 mRNA in larvae measured by the SYBR Green qPCR. The tissues include hemocytes, fat body, midgut and integument collected from the 5th instar day 1 *O. furnacalis* larvae. Values are shown as mean ± SD (n = 3).

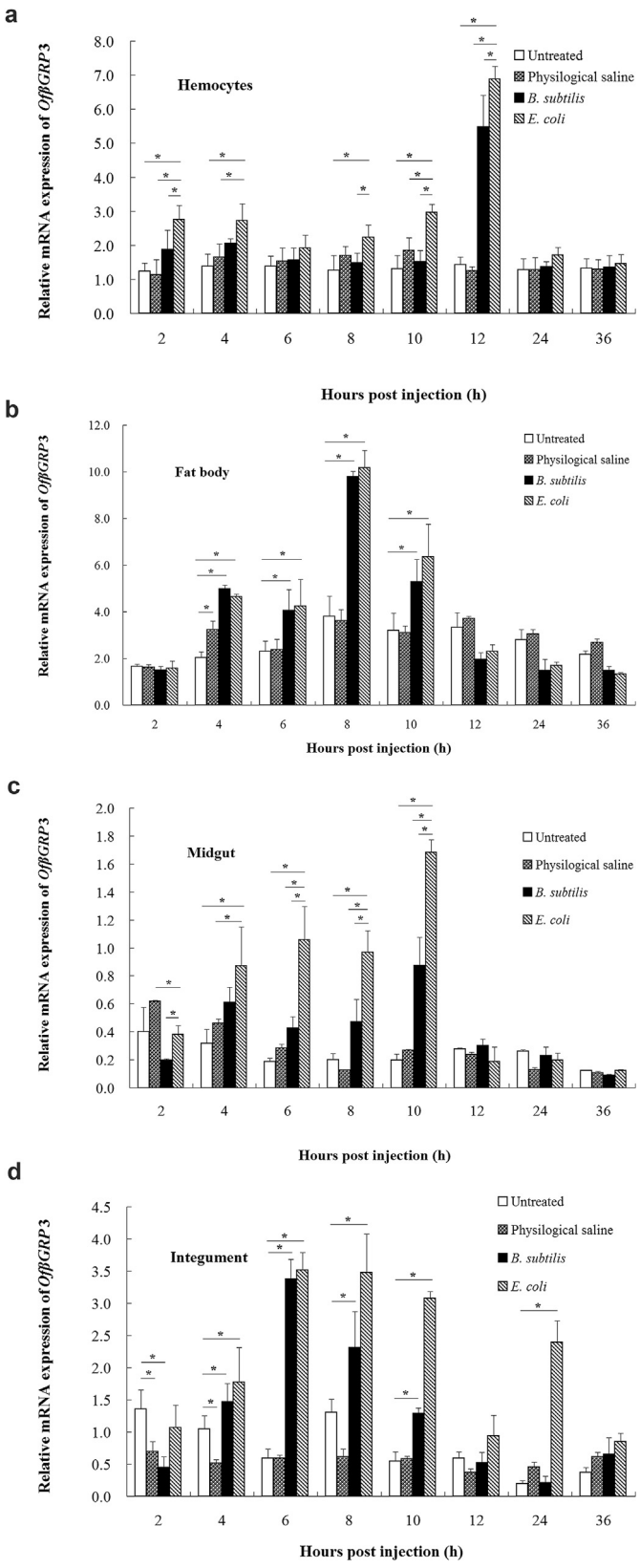

Fig. 4. Expression profiles of *OfβGRP3* mRNA analyzed by qPCR in hemocytes (A), fat body (B), midgut (C) and integument (D) of the 5th instar *O. furnacalis* naïve larvae or larvae after *B. subtilis* and *E. coli* challenge for 2, 4, 6, 8, 10, 12, 24 and 36 h. The *O. furnacalis RPL8* gene was used as an internal control. Vertical bar represented the mean ± SD (n = 3).

OfβGRP3 mRNA transcripts was observed at 12 HPI, which was 10.58-fold to that in untreated group for *B. subtilis*-challenge group and 13.87-fold for *E. coli*-challenge group ($p < 0.05$) (Fig. 4A). In fat body, the results revealed that *OfβGRP3* expression in both *B. subtilis* and *E. coli*-challenged group was significantly higher than that in untreated group from 4 to 10 HPI, and then the expression continuously dropped from 12 to 36 HPI (Fig. 4B). In midgut, the expression of *OfβGRP3* in *E. coli*-challenged group was notably higher than those in untreated group and physiological saline injection group from 4 to 10 HPI, with the peak level detected at 10 HPI (Fig. 4C). Interestingly, the expression patterns of *OfβGRP3* in response to *E. coli and B. subtilis* challenges were similar, with the dramatic increase of expression at early stage and then decline thereafter.

3.4. Temporal expression of OfβGRP3 after LPS and laminarin stimulation

The *OfβGRP3* expression in laminarin-injected group was higher than that in LPS-injected group in various test tissues from 2 to 24 HPI. The results also showed that the *OfβGRP3* expression was the highest in fat body and the lowest in the midgut.

In fat body, the *OfβGRP3* expression in the larvae injected with laminarin was gradually up-regulated from 2 to 12 HPI, and had a peak expression at 12 HPI. However, the expression level of *OfβGRP3* transcript became down-regulated and returned to the similar expression level in the control group at 36 HPI (Fig. 5).

3.5. Effect of OfβGRP3 RNAi on resistance against bacterial infection

The effects of knockdown in *OfβGRP3* transcription in larvae were investigated by injection of *OfβGRP3* dsRNA. To perform the RNAi in the healthy 4th instar larvae of *O. furnacalis,* 4 μL *OfβGRP3* dsRNA (20 μg/larvae), 4 μL GFP dsRNA (20 μg/larvae) and DEPC water (20 μg/larvae) were injected into 60 larvae for each treatment. As compared to DEPC-H_2O group, transcription of *OfβGRP3* in hemocytes was reduced to approximately 44.7% at 3 DPI of *OfβGRP3* dsRNA and 22.4% at 4 DPI. In fat body, transcription of *OfβGRP3* was reduced to approximately 46.0% at 3 DPI of *OfβGRP3* dsRNA and 21.4% at 5 DPI, thus demonstrating effective dsRNA-mediated knockdown in *OfβGRP3* transcription (Fig. 6A and B).

The role of *OfβGRP3* in resistance against bacterial pathogens was investigated by measuring survival rates after RNAi-mediated silencing of *OfβGRP3* in *O. furnacalis* larvae. In this study, the Kaplan-Meier survival analysis showed that the median lethal time (LT_{50}) of *E. coli* infected wild-type larvae was 3.0 days, which was significantly higher than the that of *E. coli* infected *OfβGRP3*-RNAi larvae (1.0 days) ($p < 0.01$) (Fig. 6C). These data suggest that *OfβGRP3* is required for resistance against infection by bacterial pathogens.

3.6. Effect of RNAi on melanotic encapsulation of Sephadex G50 beads

To address whether *OfβGRP3* gene silencing had any effects on melanotic encapsulation, the Sephadex G50 beads were injected into *O. furnacalis* larvae following *OfβGRP3* dsRNA injection at day 3, and then 24 h later the larvae were dissected and the degree of melanotic encapsulation recorded on the 0–3 scale (Fig. 7). Since Sephadex G50 beads does not have charge, therefore they do not interfere the cellular encapsulation experiment with the electrostatic binding *in vivo*. As shown in Table 2, 56.3% of beads in *OfβGRP3* knockdown larvae were not encapsulated (degree 0), but only 13.4% of beads in control larvae were not encapsulated. Completely melanized beads (degree 3) in larvae injected with

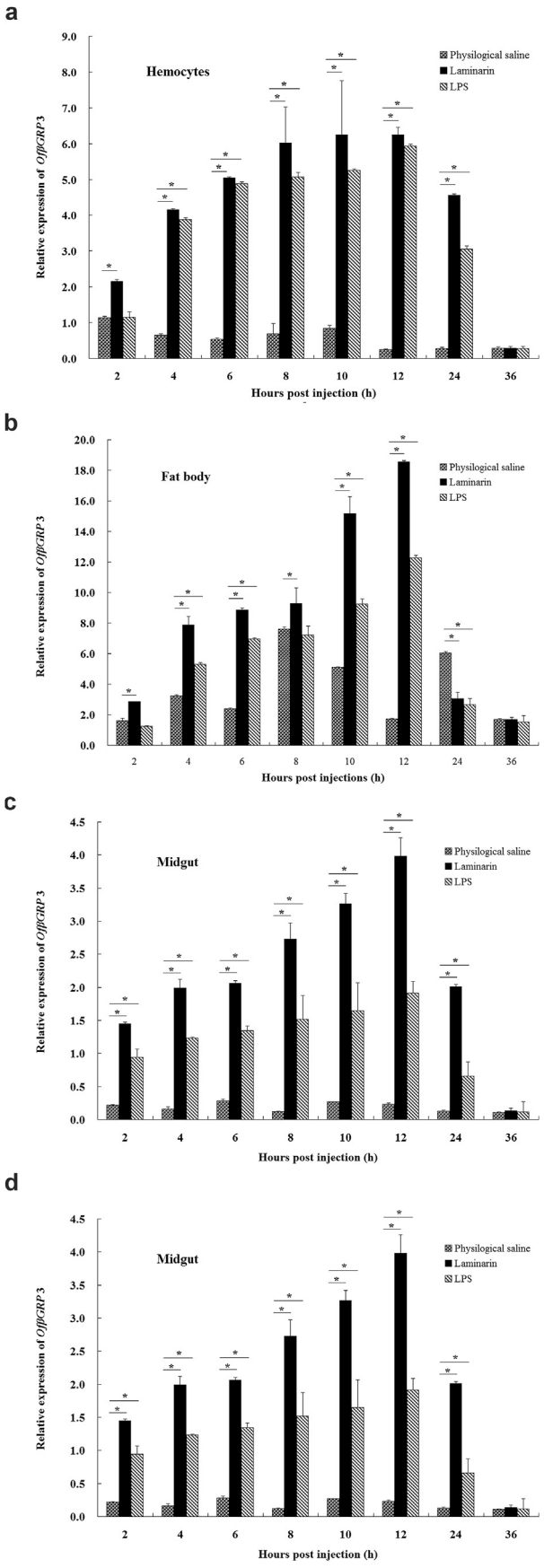

Fig. 5. qPCR analysis of *OfβGRP3* expression level after LPS and laminarin stimulation in different tissues of *O. furnacalis* larvae. The *RPL8* gene was used as an internal control. Vertical bar represented the mean ± SD (n = 3).

OfβGRP3 dsRNA was 10.2% of the total larvae, as compared to 48.8% in control larvae ($p < 0.01$) (Table 2).

3.7. The PO activity and IEARase activity in hemolymph after *OfβGRP3* RNAi

To determine the importance of any functions of *OfβGRP3 in O. furnacalis* larvae PPO activating system, hemolymph from *OfβGRP3* knockdown larvae was subjected to PO and IEARase activity assays, respectively. A notable reduction in the PO activity, down to 53% of that of the control group (DEPC-H_2O injection), was detected in the *OfβGRP3* knockdown larvae at 96 HPI, whereas no significant change in the PO activity was observed between the *GFP* dsRNA-injected and DEPC-H_2O group larvae (Fig. 8A). The results of IEARase activity showed that the decrease of IEARase activity in *OfβGRP3* dsRNA-injected group was statistically significant ($p < 0.05$) when compared with that in control groups (*GFP* dsRNA-injected and DEPC-H_2O) (Fig. 8B). Thus, *OfβGRP3* as one of PRR members is an essential factor molecule involved in the PPO activating system in *O. furnacalis* larvae.

4. Discussion

Invertebrates lack the adaptive immune system and depend on PRRs to detect exogenous microorganisms as non-self through binding to PAMPs (Lemaitre and Hoffmann, 2007). One candidate for such immediate recognition and defense mechanisms is the prophenoloxidase activating system that is often analogous to the alternate pathway of complement activation (Wang et al., 2011).

We isolated a cDNA clone encoding OfβGRP3 from *O. furnacalis* larvae, and found that OfβGRP3 contained the typical and predicted βGRP domains and motifs. Like other insect GRPs, OfβGRP3 contains conserved motifs such as two N-linked glycosylation sites, two O-linked glycosylation sites, the putative cell adhesive site (QGD), a potential kinase C phosphorylation site (SAR) and a glycosyl hydrolase family 16 domain. The *N*-terminal domain of OfβGRP1 (a.a. 1–25) contains a signal peptide sequence (Fig. 1). Two N-linked glycosylation sites were observed in LGBPs from fleshy prawn *Fenneropenaeus chinensis* (Du et al., 2007) and white shrimp *Litopenaeus vannamei* (Cheng et al., 2005) as well as BGBP from tiger shrimp *Penaeus monodon* (Sritunyalucksana et al., 2002). In the present study, *OfβGRP3* from *O. furnacalis* possessed a QGD motif, indicating OfβGRP3 to induce a series of immune responses such as PPO through the QGD site. In *Plutella xylostella*, the QGD cell adhesion motif was also found to serve as a ligand for cell surface integrins and to mediate blood cell adhesion and cellular immunity (Huang et al., 2015). A RGD motif usually observed in insects such as *β*GBP2 of *M. sexta* (Jiang et al., 2004) is occasionally found in the crustacean LGBP and bGBP (Valli and Vaseeharan, 2012). So it can be speculated that GRP, a receptor protein present in plasma, could bind to hemocyte membrane through QRD or RGD site and induce a series of immune reactions. However, scallop *Chlamys farreri* LGBP contains no RGD motif, but a potential transmembrane domain (Su et al., 2004). Due to the presence of these conserved motifs, OfβGRP3 was postulated to recognize invading microorganisms like other insect *β*GRPs. A phylogenetic tree was generated from the analysis of amino acid sequences of the *β*GRPs from lepidopteran insects and crayfish to verify the evolutionary relationship of βGRPs. The tree could be separated into three groups. Insect βGRP of *O. furnacalis* and *B. mori* were in group 3 while *P. leniusculus* βGBP

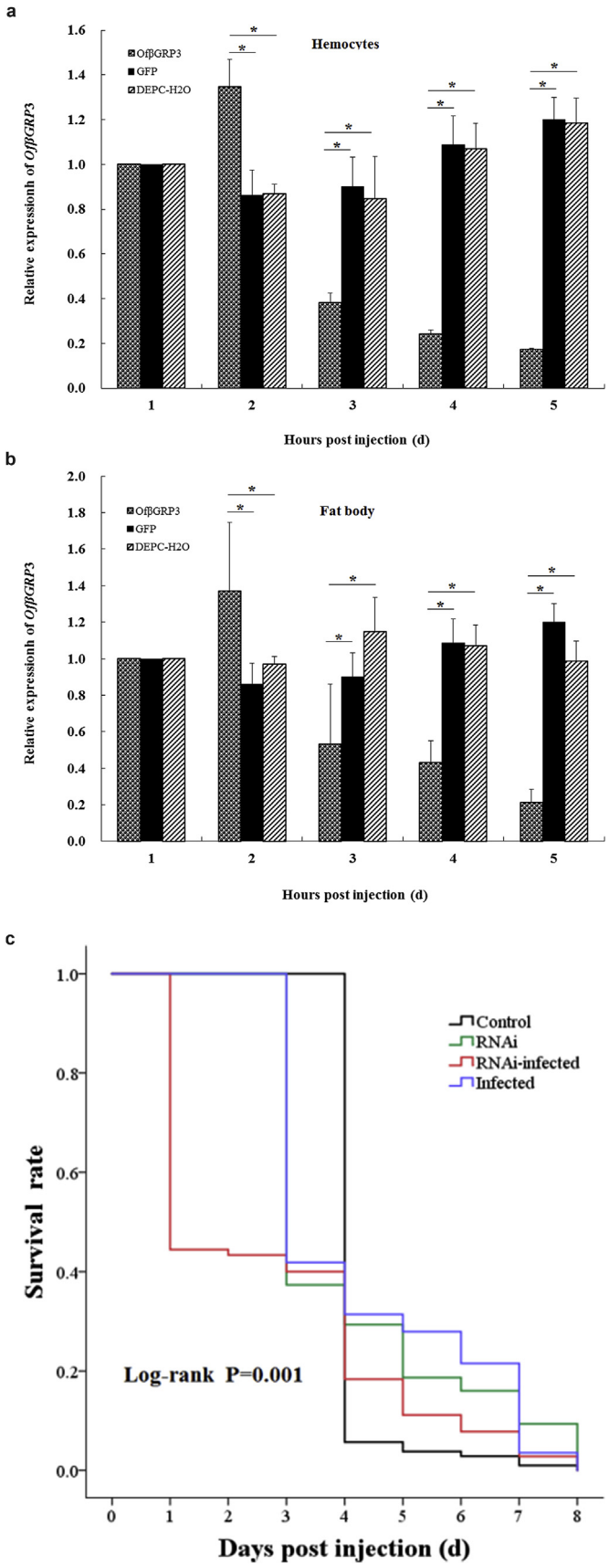

Fig. 6. Bioassay of *OfβGRP3* interfered by dsRNA in *O. furnacalis* larvae. Relative expression of *OfβGRP3* was analyzed by qPCR. (A) Effective dsRNA-mediated interference in *OfβGRP3* transcription in hemocytes. (B) Effective dsRNA-mediated interference in *OfβGRP3* transcription in fat body. (C) Survival of wild-type and *OfβGRP3* dsRNA treated *O. furnacalis* larvae. Following injection of *OfβGRP3* dsRNA, 20 larvae were inoculated with *E. coli* intra-abdominally (RNAi-infected). Twenty larvae were treated with RNAi without bacterial infection (RNAi) and 20 larvae were inoculated with *E. coli* only (infected). The number of dead larvae was counted daily. Larvae injected with dsRNA specific to green fluorescent protein (GFP dsRNA) were used as controls. Results are expressed as the mean ± standard deviation of three independent experiments.

was as out-group. OfβGRP3 shares a closer relationship with *H. armigera* βGBP3 and *B. mori* βGBP2 from primary sequence alignment and phylogenetic analysis.

It was extremely important to detect the tissue-specific expression pattern of *βGRP* gene in order to explore its functions. The qPCR analysis showed *OfβGRP3* mRNA appeared in fat body and hemocyte, suggesting that fat body and hemocyte are the main sites for synthesizing OfβGRP3. Previous studies found that GRPs in *B. mori* (Ochiai and Ashida, 2000), *M. sexta* (Ma and Kanost, 2000) and *P. interpunctella* (Fabrick et al., 2003) are mainly transcribed in fat body and, following expression, are secreted into hemolymph. In mosquitoes, fat body and hemocytes are the primary tissues that generate immune-related proteins (Wang et al., 2005). In *Spodoptera exigua*, *βGRP* transcripts were expressed in brain, hemocytes, and cuticle (Bang et al., 2013). The kuruma shrimp *LGBP* was expressed specifically in hemocyte, but not detected in gills, hepatopancreas, muscle, eyestalk and intestine (Lin et al., 2008). The fleshy prawn *LGBP* was mainly expressed in hemocyte, and very low transcripts also was detected in hepatopancreas and gills, but not detected in heart, stomach and intestine (Cheng et al., 2005).

After *E. coli* stimulation, the expression level of the *OfβGRP3* mRNA was also significantly up-regulated and was 13.87-fold higher than the untreated group at 12 HPI. Although the gene is constitutively expressed, synthesis of *OfβGRP3* is enhanced by infection with bacteria, suggesting that OfβGRP3 is an acute-phase protein. Similarlly, the *M. sexta* βGRP2 and βGRP3 are also acute-phase proteins like *O. furnacalis* GRP3 (Jiang et al., 2004; Rao et al., 2014). It is likely that the induced expression of *OfβGRP3* is required to maintain a high level of OfβGRP3 for rapid pathogen recognition in host hemolymph. The mRNA level of *PxβGRP3* was also induced after injection of *S. aureus*, *Bacillus thuringiensis*, *E. coli*, and *Isaria fumosorosea*, respectively, indicating *PxβGRP3* gene is a strongly inducible gene (Huang et al., 2015), while *M. sexta* GRP is constitutively expressed and not induced as an acute phase response protein (Ma and Kanost, 2000). In *S. exigua*, *SeβGRP* mRNA transcriptional levels were elevated following immune challenge with UV killed *F. oxysporum* but not *E. coli* DH5α (Bang et al., 2013). These results suggest that the *β*GRP family proteins are differentially regulated by developmental stage and immune challenge.

In our study, *OfβGRP3* expression in response to infection with two PAMPs, such as LPS and laminarin, was investigated. The *OfβGRP3* mRNA expression was analyzed in all different test tissues after larminarin stimulation. In fat body, the *OfβGRP3* mRNA expression was gradually up-regulated, the expression level was significantly different between the physiological saline group and larminarin stimulation groups, and was 10.91-fold higher than that of the physiological saline group at 12 HPI. LPS and laminarin significantly induced *OfβGRP3* expression, which indicated the participation of OfβGRP3 in innate immunity and its specific function in defense against Gram-negative bacterial and Gram-positive bacterial infection. This result suggests that OfβGRP3 plays a critical role in *O. furnacalis* infection during the early stage. LPS could significantly up-regulated the mRNA level of *β*GRPs in several marine invertebrates, including kuruma shrimp (Han et al., 1999), crayfish (Beschin et al., 1998) and disk abalone (Mikes and Man, 2003). A LGBP (36 kDa) isolated from the hemocytes of

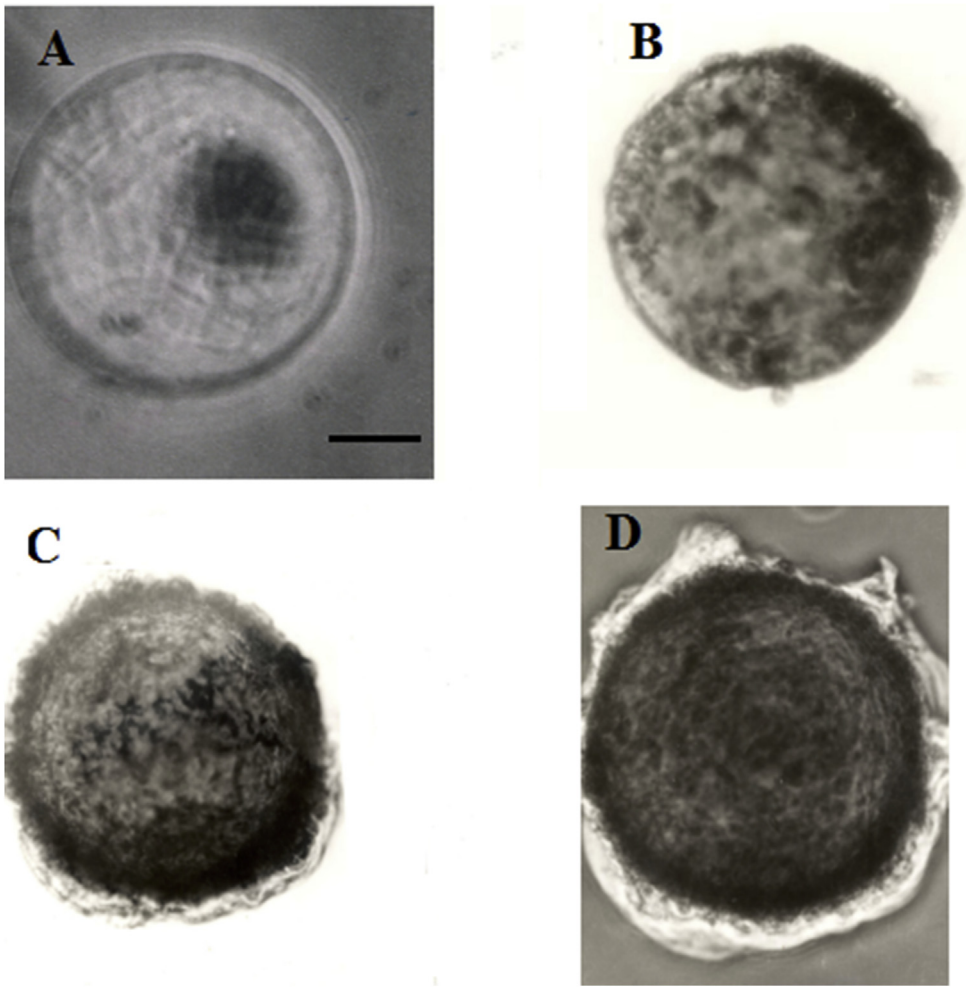

Fig. 7. Melanotic encapsulation of Sephadex G50 beads in normal and RNAi-mediated silencing of *OfβGRP3* gene in *O. furnacalis* larvae. A, degree 0; B, degree 1; C, degree 2; D, degree 3 (See "Material and Methods" 2.7 for details). Scale bar = 50 μm. All figures are with the same magnification as indicated in A.

Table 2
The percentage of different degrees of melanotic encapsulation of Sephadex G50 beads in *O. furnacalis* larvae cavity (mean ± SD, n = 10).

Scale	CK (%)	RNAi-Beads (%)
Degree 0	13.4 ± 1.3 Aa	56.3 ± 5.4 Bc
Degree 1	16.2 ± 1.4 ABab	18.4 ± 2.3 Ab
Degree 2	21.6 ± 3.6 Bb	15.1 ± 2.4 Aab
Degree 3	48.8 ± 3.4 *Cc*	10.2 ± 1.3 Aa

Values represent the mean percentage of different degrees of melanotic encapsulation of Sephadex G50 beads in *O. furnacalis* larvae cavity. Values within a column are followed by different capital and small letters differed significantly at $p < 0.01$ and $p < 0.05$, respectively by Duncan's multiple range test.

crayfish *P. leniucsulus* has binding activity to LPS as well as β-1,3-glucans such as curdlan and laminarin, but not to peptidoglycans (Di Luzio et al., 1976). In tiger shrimp *P. monodon*, A BGBP (31 kDa) isolated and purified from hemocytes can bind only to β-1,3-glucans such as curdlan and zymosan, but not to LPS, which indicated that its binding is specific for β-1,3-glucans (Han et al., 1999). In *P. leniusculus*, a LGBP has binding ability to LPS as well as to β-1,3-glucans such as laminarin and curdlan (Vargas-Albores et al., 1997). For *P. monodon* BGBP, the injection of curdan or heat-killed bacterial cell *V. harveyi* could not modify its mRNA expression within 12 h post-injection (Cerenius et al., 1994). In the present study, the remarkable up-regulation in fat body suggested that the *OfβGRP3* could be effectively induced by LPS or bacteria and strong transcripts of the *OfβGRP3* took place to recruit the immune related proteins in response to infection. It demonstrated that *OfβGRP3* gene was not only a constitutively expressed gene, but also an inducible acute-phase expression gene, which could play an essential role in bacterial pathogenesis. Except for being receptor of LPS or BG and transducing signals, abundant OfβGRP3 may be needed to enhance the activity of PPO and play other unknown roles.

RNAi has been successfully used to investigate the βGRP function in *P. interpunctella* (Fabrick et al., 2004b), *Locusta migratoria* manilensis (Zheng and Xia, 2012) and subterranean termites (Hamilton and Bulmer, 2012). In our study, RNAi-mediated knockdown experiments were performed to evaluate the essential role of *OfβGRP3* in immunological defense. *O. furnacalis* larvae were injected with *OfβGRP3* dsRNA in the presence or absence bacterial infection, and significantly reduced *OfβGRP3* expression was detected. Our results also showed that silencing of *OfβGRP3* in larvae increased mortality rates after challenge with *E. coli*. These results suggest that *OfβGRP3* positively regulates larvae survival via suppression of bacterial growth by the critical roles of PO in the process. RNAi-mediated silencing of *OfβGRP3* resulted in a lower LT_{50} following bacterial infection compared with wild-type larvae, thus confirming the requirement for *OfβGRP3* in defense against bacterial infection. Volkmann (1991) observed that the

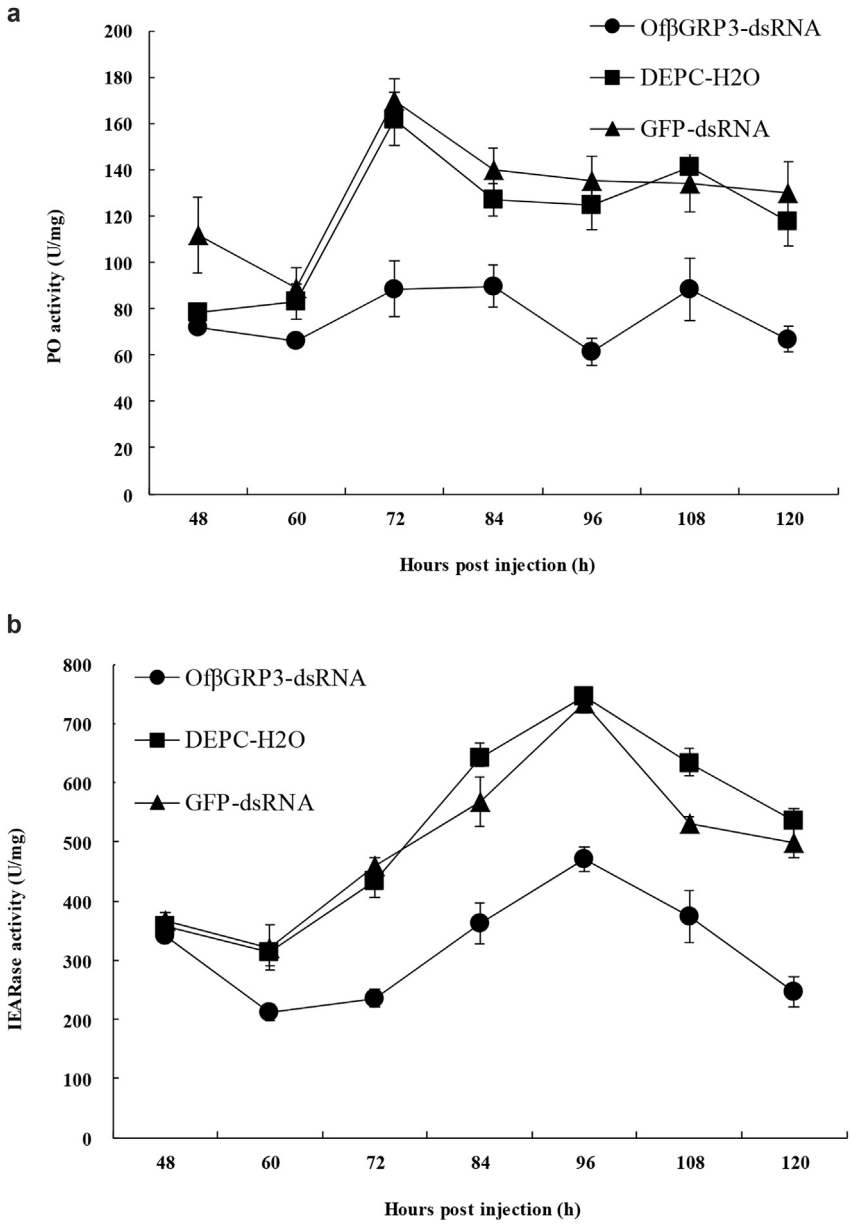

Fig. 8. The activity profiles of PO and IEARase from the hemolymph of *O. furnacalis* larvae after the RNAi-mediated silencing of *OfβGRP*3 gene (n = 3).

melanogenic enzyme PO was histochemically localized in midgut and hemocytes in cockroaches infected with *Moniliformis moniliformis*. Therefore, it can be speculated the lethal effects of RNAi-mediated *OfβGRP*3-silencing in larvae are mediated by alteration in the capacity of hemocytes to defend against constant challenge from the bacterial community. In our research, melanotic encapsulation response against Sephadex G50 beads became weaker after RNAi. These results suggested that *O. furnacalis* bodies might protect themselves against host defense in part by inhibiting the expression of the recognition proteins. Melanization of pathogens and damaged tissues is a major innate defense system in invertebrates. In our study, the high-level inducible expression of *OfβGRP*3 by *E. coli* in larvae suggested the possible physiological role of βGRPs in host defense against bacterial infection. In *M. sexta*, when βGRP preincubated with the soluble β-1,3-glucan, laminarin, was added to diluted plasma, the PPO activation pathway, which involves a serine proteinase cascade, was triggered, and active phenoloxidase accumulated in the plasma (Ma and Kanost, 2000). Recognition of PAMPs by PRPs is an essential step for activation of the PPO cascade. This result suggests that a complex of βGRP with laminarin stimulated activation of the PO cascade. The mussel βGBP specifically binds to only laminarin, as revealed by inhibition of yeast agglutinating activity by various carbohydrates, and this property was also supported by a strong yeast agglutinating activity of purified βGBP. Moreover, the PPO defense pathway was also triggered by binding of PGRP to β-1,3-glucan or peptidoglycan only when the concentration of the recognition protein was sufficiently high (Jayaraj et al., 2008).

In our study, about 10.2% of the injected Sephadex G50 beads were melanized in the RNAi-beads group, indicating the function of *OfβGRP*3 in promoting melanization. Based on the critical role of the PPO cascade in melanization, we hypothesized that *OfβGRP*3 may enhance melanization via PPO cascade activation. Some serine proteinases with clip domain which have IEARase activities are

involved in PO activation, and thus an inhibitor of serine proteinases can be used to suppress the production of PO and melanization. The decreased PO and IEARase activities in hemolymph were observed in the group of *OfβGRP*3 dsRNA injection following the injection of *E. coli*, which indicated that *OfβGRP*3 positively regulates PO activity.

Several immune mechanisms are known to be involved in the response to invaders in insects. Collectively, these data suggest a critical role for OfβGRP3 in PPO cascade regulation. However, it is unclear whether OfβGRP3 has functions other than defense against invading organisms in insects. This should be a focus of future investigations. Our study demonstrates that OfβGRP3 may be a molecule capable of recognizing selective microorganisms and activating plasma PPO system in *O. furnacalis*. These results also provided the foundation for developing novel chemicals and microbial pesticides targeting PRRs for improvement of the function of microbial pesticides against entomopathogenic bacteria and fungi.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2017.10.004.

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