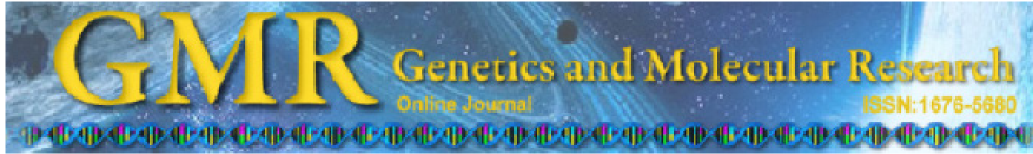

Lipopolysaccharide and β-1,3-glucan binding protein in the hard clam (*Meretrix meretrix*): Molecular characterization and expression analysis

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ABSTRACT. Pattern recognition molecules play an important role in innate immunity by recognizing conserved molecular patterns that are present on the surface of invading microorganisms. In this study, a lipopolysaccharide and β-1,3-glucan binding protein (*LGBP*) gene was cloned from the hard clam *Meretrix meretrix* (designated as *Mm-LGBP*) by the expressed sequence tags and rapid amplification of cDNA ends method. The cDNA was 1827 bp in length, consisting of a 71-bp 5'-terminal untranslated region, a 62-bp 3'UTR, and a 1734-bp open reading frame encoding a 577-amino acid polypeptide with an estimated molecular mass of 60.7 kDa and a theoretical isoelectric point of 5.56. Characteristic potential

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polysaccharide binding, cell adhesion, and glucanase motifs were identified in the *Mm-LGBP*, indicating that *Mm-LGBP* should be a new member of the *LGBP* family. Quantitative real-time polymerase chain reaction was developed to detect the mRNA expression level of *Mm-LGBP* in 6 different tissues. Higher-level mRNA expression of *Mm-LGBP* was detected in the gill and digestive gland tissues. The upregulation of *Mm-LGBP* mRNA after *Vibrio anguillarum* challenge showed that *Mm-LGBP* play a pivotal role in antibacterial immunity.

Key words: *Meretrix meretrix*; Hard clam; Gene cloning; Lipopolysaccharide and β-1,3-glucan binding protein; mRNA expression

INTRODUCTION

The recognition of an invading organism as foreign is the first critical step in any immune response. Unlike vertebrates, invertebrates lack an adaptive immune system, and they rely on innate immune responses against invading organisms (Janeway Jr. and Medzhitov, 2002). Invertebrates possess the ability to recognize conserved pathogen-associated molecular patterns (PAMPs), such as β-1,3-glucan (BG), lipopolysaccharide (LPS), and peptidoglycan (PG), which are present on the surface of bacteria and fungi (Medzhitov and Janeway Jr., 2002). Pattern recognition molecules act as biosensors in the activation of innate immune responses in both vertebrates and invertebrates (Fabrick et al., 2003). Recently, several pattern recognition proteins (PRPs) have been isolated and characterized, including BG recognition protein (BGRP), LPS-binding protein (LBP), PG-binding protein (PGBP), LPS- and BG-binding protein (LGBP), Gram-negative bacteria-binding protein (GNBP), thioester-containing protein, C-type lectin, galactoside-binding lectin (galectin), and fibrinogen-like domain immunolectin (Dziarski, 2004). It has been demonstrated that common motifs, such as bacterial glucanase-like (Kim et al., 2000; Ochiai and Ashida, 2000), bacteriophage lysozyme-like (Yoshida et al., 1996), and immunoglobulin-like (Fearon and Locksley, 1996) motif, exist in part of their primary structure.

Several PRPs have been cloned and characterized in aquatic animals, such as the crayfish *Pacifastacus leniusculus LGBP* (Lee et al., 2000), kuruma shrimp *Marsupenaeus japonicas LGBP* (Lin et al., 2008), Chinese shrimp *Fenneropenaeus chinensis LGBP* (Liu et al., 2009), Zhikong scallop *Chlamys farreri LGBP* (Su et al., 2004), disk abalone *Haliotis discus BGRP* (Nikapitiya et al., 2008), and pearl oyster *Pinctada fucata LGBP* (Zhang et al., 2010). It was demonstrated that *Pacifastacus leniusculus* LGBP played an important role in prophenoloxidase (proPO) activation (Lee et al., 2000). The proPO activating system is an important non-self-recognition system in invertebrates that can be activated by LPS or peptidoglycan from bacteria and β-1-3-glucans from fungi (Hoffmann et al., 1996). The active form of proPO, known as phenoloxidase (PO), can melanize pathogens, sclerotize the cuticle, and heal wounds in invertebrates (Wang et al., 2007).

The hard clam (*Meretrix meretrix*) is a bivalve mollusk of the family Veneridae that is native to the south-north coastal area of China. It is an important aquaculture species in China

and fetches a high price in the Internet trade market. However, over the past decades, the clam aquaculture industry has experienced serious disease and high mortality (Yue et al., 2010). Understanding the immune response mechanisms would be beneficial for disease control in clam aquaculture. To date, many immune-related genes have been cloned and their expression profiles analyzed in the hard clam (Yang et al., 2011; Yue et al., 2011a,b; Li et al., 2012; Gao et al., 2012). However, to our knowledge, there is no information about gene characterization or gene expression of hard clam PRPs. Therefore, to further understand the function of bivalve PRPs in the innate immune response, it is necessary to clone and characterize more PRP genes in the hard clam.

MATERIAL AND METHODS

Clam collection

Adult *M. meretrix* (2 years old, shell length 4.5-5.5 cm) were collected from Guanhe Aquaculture Corporation in Panjin (Bohai Sea, China). Clams were cultured in sand-filtered seawater at 18°-20°C for 10 days before the challenge experiments. The seawater was aerated continuously, and the salinity was set at 28‰. Clams were fed *Chlorella vulgaris* (10 mg dry mass·clam^{-1}·day^{-1}) during the acclimatization period. To investigate the tissue-specific expression pattern of *Mm-LGBP*, 6 tissues including hemocytes, mantle, gill, adductor muscle, digestive gland, and gonad, were collected from 5 unchallenged clams. Hemolymph was collected from adductor muscles using a sterile syringe and then centrifuged at 1000 *g* for 10 min at 4°C. All samples were stored in liquid nitrogen until RNA extraction.

Bacterial challenge

Two hundred clams were used for the bacterial challenge experiment. Clams that were immersed in a high density of *Vibrio anguillarum* with final concentration of 10^9 CFU/mL were used as the challenge group. The untreated clams were used as the control group. Each group was divided into 3 replicates. Hemocytes and gill were sampled at 2, 4, 6, 8, 16, and 32 h post challenge. Three individuals of each replicate were randomly sampled at the same time point and were pooled together as 1 sample.

RNA extraction and cDNA synthesis for gene cloning

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer protocol. Total RNA was incubated with RNase-free DNase I (Roche, USA) to remove any genomic DNA. First-strand cDNA was synthesized from 2 μg total RNA by M-MLV reverse transcriptase using oligo $d(T)_{15}$ (Takara, China) at 37°C for 60 min.

Cloning and sequencing the full-length cDNA of Mm-LGBP

Random sequencing of SMART cDNA libraries generated 3224 successful sequencing reactions (Li et al., 2011). Basic local alignment search tool (BLAST) (National Center for Biotechnology Information, NCBI) analysis of all obtained sequences revealed that an

expressed sequence tag (EST) of 689 bp was homologous to previously identified LGBPs. This EST sequence, Mm-LGBP, was selected for cloning and further investigation. One gene-specific primer, Mm-LGBP-3' (Table 1), was designed to amplify the full-length cDNA of *Mm-LGBP* using the rapid amplification of cDNA ends (RACE) approach. Polymerase chain reaction (PCR) amplification of the 3' end of *Mm-LGBP* was carried out with Mm-LGBP-3' and T7 primers (Table 1). The PCR conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and an additional extension of 72°C for 10 min. The PCR product was cloned into the pMD18-T vector (Takara) and sequenced in both directions.

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Primer information
Mm-LGBP-3'	CTTTGAACGATGGTCCAGATAGC	3'RACE primer
T7	GTAATACGACTCACTATAGGGC	Vector primer
LGBP -RTF	ATGAACGTGGCTGTCGGAG	Real-time LGBP primer
LGBP -RTR	ACGTAGGATACCACTGACCACG	
Actin-RTF	GATCATTGCCCCACCAGAGAG	Real-time β-actin primer
Actin-RTR	CCAGACTCGTCGTATTCTTGTTTAC	

Sequence alignment and phylogenetic tree

The *Mm-LGBP* sequence was analyzed using the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/blast). The amino acid sequence was predicted using the Expert Protein Analysis System (http://www.expasy.org/). The molecular mass and theoretical isoelectric point calculated were predicated by the EMBOSS model of protein isoelectric point program (http://isoelectric.ovh.org/). Multiple protein sequence alignments of *Mm-LGBP* with proteins from other species were performed using the ClustalW program (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree was constructed according to the amino acid sequences of HSPs selected using the neighbor-joining (NJ) method in the MEGA 4.0 program (Tamura et al., 2007). The bootstrap trials were replicated 1000 times to derive the confidence values for the phylogeny analysis.

Analysis of Mm-LGBP mRNA expression using quantitative real-time (qRT)-PCR

The mRNA expression of *Mm-LGBP* in different tissues of healthy clams was measured by qRT-PCR. The gene-specific primers LGBP-RTF and LGBP-RTR (Table 1) were designed to amplify the target gene. A pair of primers for clam β-actin (Actin-RTF and Actin-RTR, Table 1) was used to amplify β-actin as an internal control. Reactions were performed in a total volume of 25 μL: 12.5 μL 2X SYBR *Premix Ex Taq*™, 0.5 μL ROX II, 1.0 μL each primer (10 μM), 2.0 μL diluted cDNA, and 8.0 μL diethylpyrocarbonate water. The thermal profile was set as follows: 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 50s. Dissociation curve analysis was performed at the end of each reaction to confirm that only 1 PCR product was amplified and detected. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to analyze expression levels. All data were given in terms of relative mRNA expressed as the means ± standard deviation (SD). The data were subjected to analysis by one-way analysis of variance (ANOVA), using SPSS 13.0. A P value < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

cDNA cloning and sequence analysis of Mm-LGBP

A 970-bp fragment was amplified by 3'RACE, sequenced, and overlapped with an EST sequence to generate the full-length cDNA sequence of *Mm-LGBP*. The sequence was deposited in GenBank under accession No. KC958578. The complete sequence of *Mm-LGBP* cDNA consisted of a 71-bp 5'-terminal untranslated region (5'-UTR), a 62-bp 3'-UTR with a poly (A) tail, and a 1734-bp open reading frame (ORF) encoding a polypeptide of 577 amino acids with an estimated molecular mass of 60.7 kDa and a theoretical isoelectric point of 5.56. SMART analysis revealed that the amino acid region from 215 to 550 belonged to the glycoside hydrolase family 16. The Mm-LGBP amino acid sequence contained a potential recognition motif for the β-1,3-linkage of polysaccharides, a putative cell adhesion site, a glucanase motif, and a protein kinase C phosphorylation site (Figure 1). An LPS-binding site was also found in the Mm-LGBP sequence (Figure 1). Moreover, 2 putative N-glycosylation sites were identified at positions $N_{138}QT_{140}$ and $N_{249}RT_{251}$ (Figure 1).

```
  1                                                                               M  F  T
  1 ACGGTAGATTGAGACCTACTCCGGTACCTGTGTCGTTCGGCGGAAAGGTAAATAACCATGGCGATCCTGAAATGTTTACT
  8  V  R  G  G  Q  C  V  G  T  G  K  G  Q  P  P  E  T  T  R  A  Q  V  F  C  P  S
 91 CTGTCCGTGGTGGTCAGTGTGTCGGCACAGGCAAAGGTCAGCCTCCTGAAACCACAAGGGCTCAGGTTTTCTGTCCCAGT
 38  L  P  G  V  A  A  G  Q  Y  A  V  D  V  R  S  K  T  G  N  E  W  V  V  E  N  P
181 CGTTACCCGGAGTTGCTGCCGGACAATATGCAGTGGACGTTAGATCAAAAACTGGTAACGAATGGGTGGTAGAGAACCCA
 68  K  P  G  D  T  V  H  Y  W  Y  N  Y  I  Q  N  G  E  G  H  L  V  K  D  Q  T  W
271 TGAAGCCTGGGGACACAGTGCATTACTGGTATAACTATATACAAAACGGTGAAGGTCATTTGGTAAAGGACCAAACCTGG
 98  P  T  S  A  P  T  T  T  T  T  T  R  R  P  T  T  H  A  Q  T  Q  A  P  R  V  T
361 ATCCAACGAGCGCACCTACAACAACCACAACGACAAGGCGACCAACTACGCATGCGCAAACACAAGCACCAAGAGTCACA
128  S  N  M  Q  A  Y  Y  N  S  L  N  Q  T  G  Q  G  T  S  G  S  Q  S  G  S  V  G
451 ACAGCAATATGCAGGCCTATTATAACAGCTTAAATCAAACAGGTCAAGGCACCTCCGGTTCGCAGTCAGGATCTGTTGGC
158  T  G  T  S  G  S  Q  T  G  T  S  G  T  L  N  T  Q  T  G  V  V  S  G  T  S  S
541 GCACGGGAACTTCCGGTTCACAAACAGGCACATCCGGTACACTAAATACCCAAACTGGAGTAGTATCAGGCACTTCCAGC
188  A  S  G  G  S  C  G  C  T  G  K  S  I  R  P  P  G  S  E  L  T  C  T  T  F  P
631 CCGCATCTGGTGGGTCATGTGGATGCACGGGGAAATCAATACGACCACCTGGCTCCGAATTAACCTGCACGACTTTCCCG
218  E  D  N  F  D  S  L  N  L  D  V  W  E  H  E  I  T  A  G  G  G  G  N  W  E  F
721 TTGAAGACAACTTTGATTCCTTGAACCTGGATGTATGGGAGCACGAGATAACAGCTGGAGGCGGGGGTAACTGGGAATTT
248  N  N  R  T  N  S  Y  V  R  D  G  K  L  F  I  K  P  T  L  T  I  E  S  L  N  D
811 CTAACAATCGAACAAATAGCTATGTAAGAGATGGAAAACTTTTTATCAAACCGACTTTGACGATCGAGTCTTTGAACGAT
278  L  T  K  G  A  L  D  I  W  G  S  Q  P  G  D  V  C  T  G  N  N  F  W  G  C  R
901 GCTTAACGAAAGGGGCTCTCGATATTTGGGGCTCCCAGCCTGGAGACGTATGTACGGGCAATAATTTCTGGGGCTGTAGA
308  P  D  H  I  I  N  P  I  Q  S  A  R  L  R  S  S  R  G  F  N  F  K  Y  G  K  V
991 CGCCAGATCATATCATCAACCCAATTCAGTCAGCCCGACTCCGAAGTTCACGTGGCTTTAACTTTAAATACGGCAAGGTT
338  K  M  P  T  G  D  W  I  W  P  A  I  W  M  L  P  L  R  N  A  Y  G  R  W  P  A
081 CCAAAATGCCAACTGGGGATTGGATCTGGCCAGCTATTTGGATGTTGCCGTTGAGAAATGCTTACGGAAGATGGCCAGCA
368  D  I  V  E  A  R  G  N  R  N  Y  H  D  D  K  G  H  S  Q  G  I  D  N  A  G  S
171 TCGACATTGTTGAAGCTAGAGGCAACCGTAACTATCATGATGACAAAGGTCACTCACAAGGAATTGACAACGCCGGAAGT
398  G  S  D  Y  W  H  N  K  W  E  K  A  H  G  E  K  L  A  S  S  G  T  Y  A  D  D
261 TTGGCTCAGACTACTGGCACAACAAGTGGGAGAAGGCACATGGAGAAAAACTGGCATCTTCGGGTACATACGCAGACGAC
428  V  L  I  W  D  E  D  H  I  S  I  T  I  D  D  T  E  V  L  N  V  T  P  Q  T  N
351 ACGTTCTAATTTGGACGAAGATCACATCAGCATTACAATCGACGATACAGAGGTACTTAATGTGACGCCTCAGACGAAC
458  G  G  L  D  K  D  G  V  E  N  P  W  A  A  G  S  K  M  A  P  L  D  Q  E  F  F
441 ATGGCGGACTTGACAAGGATGGTGTTGAAAATCCTTGGGCAGCAGGTTCTAAGATGGCGCCGCTTGACCAAGAGTTCTTC
488  V  A  V  G  G  V  G  F  F  L  D  K  F  V  N  S  P  G  K  P  W  N  D  K  S  D
531 ACGTGGCTGTCGGAGGGGTTGGTTTCTTCCTGGATAAATTTGTTAACTCTCCAGGCAAGCCATGGAATGACAAATCAGAC
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Figure 1. Nucleotide and deduced amino acid sequences of Mm-LGBP. The start codon (ATG) and stop codon are boxed (TAG). A potential recognition motif for β-1,3-linkage of polysaccharides is bold and underlined. A protein kinase C phosphorylation site is shaded and a putative cell adhesive site is boxed. The putative glucanase and polysaccharide binding (PsB) motifs are underlined. Two putative N-glycosylation sites were dotted and underlined. The LPS binding motif is dashed and underlined.

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Alignment and phylogenetic tree of Mm-LGBP

Multiple sequence alignment of Mm-LGBP with other known molluscan LGBP and LGBP amino acid sequences revealed the strong conservation in the polysaccharide binding (PsB) and glucanase motifs (Figure 2) despite the species-to-species variation in the length of the amino acid sequence. The Mm-LGBP amino acid sequence shared significant homology with other known invertebrate PRPs (Table 2). It had the highest identity (77%) to *Tapes literatus* BGRP, and shared 64-25% similarity with other PRP sequences. Using the NJ method, a phylogenetic tree was constructed based on the amino acid sequences of selected invertebrate PRP sequences (Figure 3). Insect BGRPs were grouped into 1 cluster, crustacean BGRP and LGBP were grouped into 1 cluster, and insect GNBP were grouped into 1 cluster. Mm-LGBP was grouped into the PRPs that were identified from other mollusk species, such as clam *Tapes literatus* BGRP, snail *Biomphalaria glabrata* BGRP, Zhikong scallop *Chlamys farreri* LGBP, pearl oyster *Pinctada fucata* LGBP, Pacific oyster *Crassostrea gigas* BGRP, and disk abalone *Haliotis discus* BGRP. It is noteworthy that mollusk *B. glabrata* GNBP was not positioned within the same mollusk clade and was grouped with annelids coelomic cytolytic factor-like sequences.

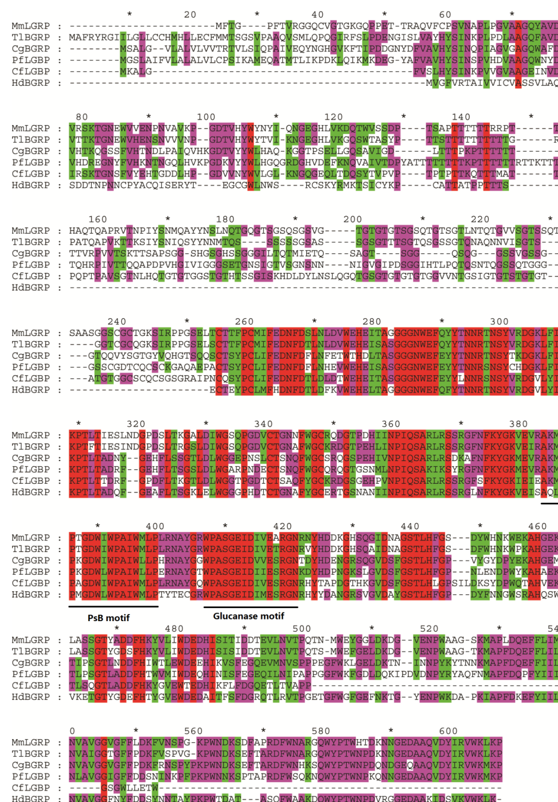

Figure 2. Multiple sequence alignments of Mm-BGRP with other known molluscan BGRPs and LGBPs. The red shading indicates identical amino acids, and the pink and green shading indicates conservative replacements. Putative polysaccharide binding (PsB) and glucanase motifs are underlined. The numbers above the sequence indicate the amino acid position in the corresponding amino acid species.

Table 2. Homology analysis of Mm-BGRP amino acid sequence with other known PRP amino acid sequences.

Species	GenBank No.	Abbreviation-type	Amino acid size (aa)	Similarity (%)
Meretrix meretrix	KC958578	Mm-LGRP		
Tapes literata	AEE89455	Tl-BGRP	571	77
Biomphalaria glabrata	ABL63380	Bg-BGRP	393	64
Biomphalaria glabrata	ABO40828	Bg-GNBP	435	34
Haliotis discus	ABO26613	Hd-BGRP	420	50
Crassostrea gigas	BAG82629	Cg-BGRP	555	52
Chlamys farreri	AAP82240	Cf-LGBP	440	52
Pinctada fucata	ACN76701	Pf-LGBP	585	49
Fenneropenaeus chinensis	AAX63902	Fc-LGBP	366	50
Litopenaeus vannamei	ABU92557	Lv-LGBP	367	51
Marsupenaeus japonicus	ABY89089	Mj-LGBP	354	49
Penaeus monodon	AAM21213	Pm-BGRP	366	52
Homarus gammarus	CAE47485	Hg-BGRP	367	49
Anopheles merus	ABU80009	Am-GNBP	395	44
Anopheles quadriannulatus	ABU80018	Aq-GNBP	395	44
Anopheles gambiae	ABU80037	Ag-GNBP	395	44
Aedes aegypti	EAT38985	Aa-GNBP	386	45
Culex quinquefasciatus	XP_001845963	Cq-GNBP	286	47
Aporrectodea rosea	AAY85744	Ar-CCF-like	385	43
Lumbricus rubellus	AAY85746	Lr-CCF-like	385	42
Dendrobaena veneta	AAY85745	Dv-CCF-like	384	42
Bombyx mori	NP_001036840	Bm-BGRP	495	27
Galleria mellonella	CAK22401	Gm-BGRP	490	28
Manduca sexta	AAN10151	Ms-BGRP	482	25
Plodia interpunctella	AAM95970	Pi-BGRP	488	26

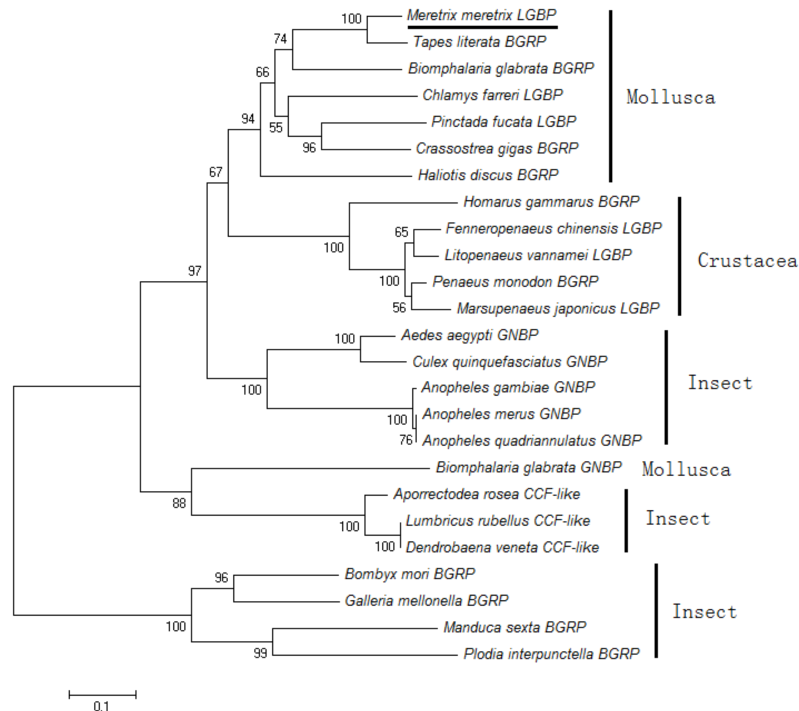

Figure 3. Consensus neighbor-joining tree based on the amino acid sequences of different types of pattern recognition proteins (PRPs). The numbers at the forks indicated the bootstrap.

Tissue distribution of Mm-LGBP mRNA

The expression level of *Mm-LGBP* mRNA in 6 tissues tested was quantified by qRT-PCR with β-actin as an internal control. For both *Mm-LGBP* and β-actin genes, there was only 1 peak at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR amplifications were specific. The tissue distribution of *Mm-LGBP* mRNA is shown in Figure 4. The *Mm-LGBP* transcript was broadly expressed in all tissues tested, with predominant detection in the gill, digestive gland, and mantle tissues, and less expression in the adductor muscle, gonad, and hemocytes tissues (Figure 4).

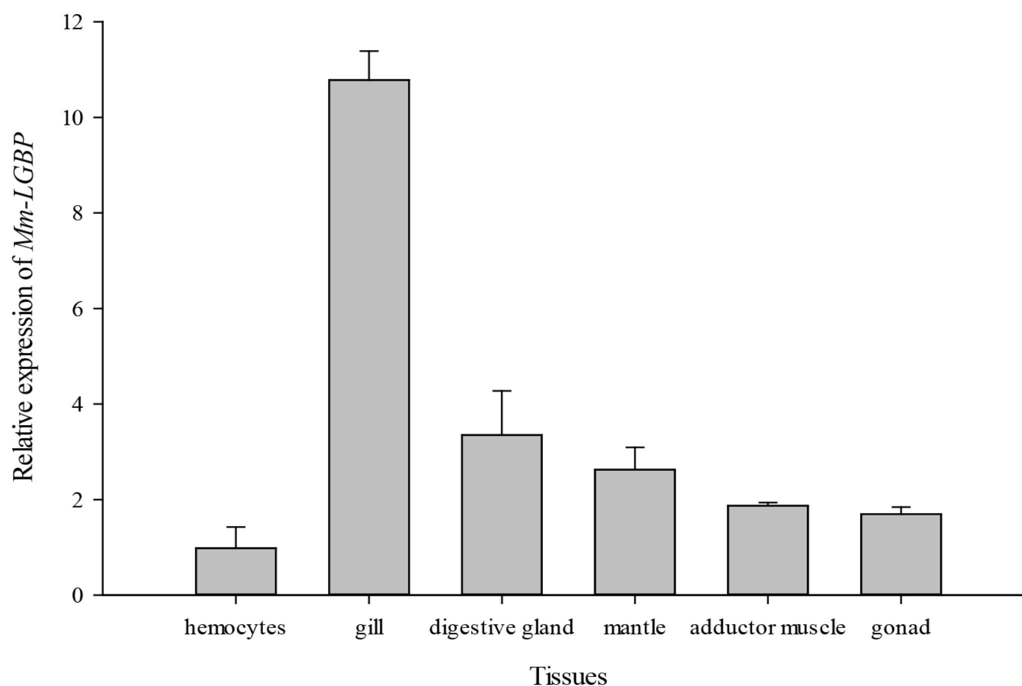

Figure 4. Expression level of the *Mm-LGBP* mRNA in different tissues. Quantitative real-time RT-PCR was carried out with RNA samples from hemocytes, gill, digestive gland, mantle, adductor muscle and gonad of the adult tissues of hard clam. The hard clam β-actin gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represented the means ± SD (N = 5).

Invertebrate PRPs have been demonstrated to be expressed in tissue-specific patterns. The kuruma shrimp *LGBP* was expressed specifically in hemocyte tissue, but it was not detected in gills, hepatopancreas, muscle, eyestalk, and intestine (Lin et al., 2008). The fleshy prawn *LGBP* was mainly expressed in hemocyte tissue, and very low levels of transcript also was detected in hepatopancreas and gills, but the transcript was not detected in heart, stomach, and intestine (Cheng et al., 2005). In this study, the *Mm-LGBP* mRNA was mainly expressed in the gill, which was similar to the BGRP expression profile in disk abalone (Nikapitiya et al., 2008). The gill, which is constructed of only a single layer of fragile cells and covered with a thin layer of protective mucus, has extensive contact with the environment. High expression of *Mm-LGBP* in gill suggests that the gill may be the first line of defense in hard clam because of its frequent exposure to the environment and to defend against invading foreign materials.

Temporal expression of *Mm-LGBP after bacterial challenge*

The temporal expression of *Mm-LGBP* mRNA in gill and hemocytes after *Vibrio anguillarum* challenge was investigated by qRT-PCR (Figure 5). In the gill, the expression level of *Mm-LGBP* mRNA remained at a low level during the first 6 h. At 2 h, the level of *Mm-LGBP* transcript significantly decreased ($P < 0.05$). However, 8 h after bacterial challenge, the *Mm-LGBP* mRNA level was significantly upregulated ($P < 0.01$) and reached a peak that was 10.7-fold higher than the level in the control. In hemocytes, at 6 h after bacterial challenge, the expression level of *Mm-LGBP* increased sharply to 3.2-fold higher than that in the control group. After 6 h, the *Mm-LGBP* mRNA expression dropped gradually, and it returned to the original level at 16 h.

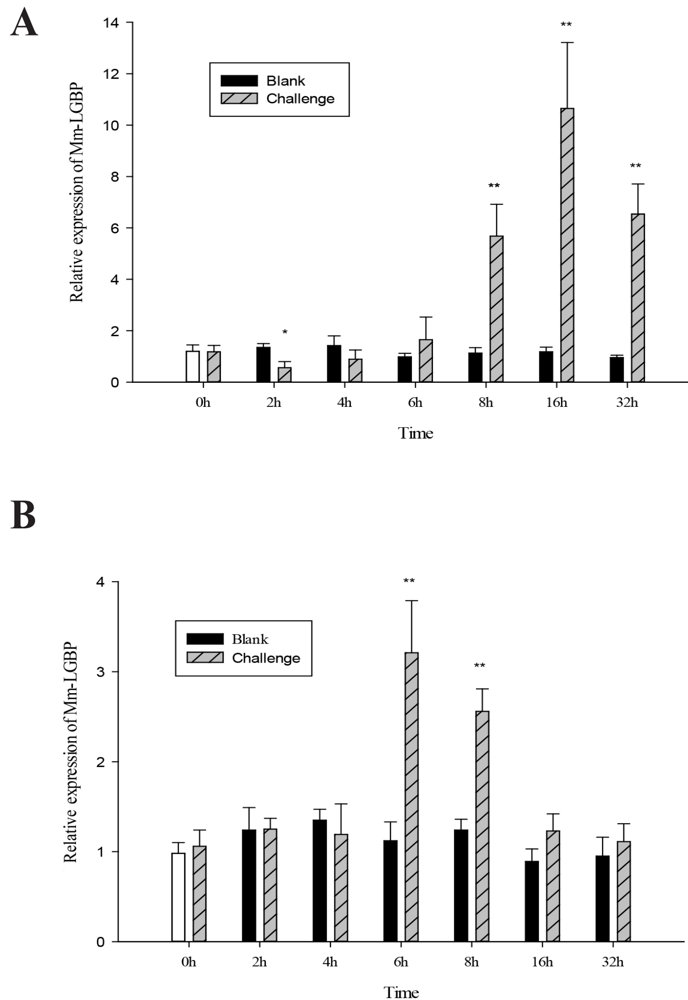

Figure 5. Temporal expression Mm-LGBP mRNA in gill (**A**) and hemocytes (**B**) after *Vibrio anguillarum* challenge measured by real-time PCR. The Mm-LGBP mRNA expression was normalized to blank group, and β-actin was used as an internal control to calibrate the cDNA template for all the samples. Each bar represents the mean value from three replicates with standard error. Three individuals sampled at the same time point were pooled together as one replicate.

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The temporal expression patterns of *Mm-LGBP* mRNA in gill and hemocytes after *Vibrio anguillarum* challenge was recorded to further investigate its possible biological function. The gill represents the main interface between aquatic organisms and the external environment. Bivalve mollusk gills are one of the first lines of defense against bacterial infection; it has become increasingly evident that the hemocytes play a central role in the internal defense of bivalves (Pipe, 1990; Bachere et al., 1991; Wang et al., 2012). The upregulation of *Mm-LGBP* mRNA after *Vibrio anguillarum* challenge showed that Mm-LGBP plays a pivotal role in antibacterial immunity, further confirming the presence of the LPS recognition and binding site in Mm-LGBP. As the main structure component of outer membrane of most gram-negative bacteria and a potent stimulator of proinflammatory cytokines, LPS significantly upregulated the mRNA level of LGBPs in several marine invertebrates, including kuruma shrimp (Lin et al., 2008), crayfish (Lee et al., 2000), and disk abalone (Nikapitiya et al., 2008). The data in this study revealed that when a foreign object enters the body, the *Mm-LGBP* gene shows an increasingly higher level of transcription. This suggests that Mm-LGBP functions by recognizing different PAMPs and may activate different immune genes to defend against these pathogens.

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