Full length article

Molecular characterization, immune responsive expression and functional analysis of QM, a putative tumor suppressor gene from the Pacific white shrimp, Litopenaeus vannamei

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A B S T R A C T

The QM, firstly identified as a putative tumor suppressor gene from human, has been confirmed to possess varieties of functions in a range of organisms. In the present study, the cDNA that encodes a 220-amino-acid QM protein with calculated molecular mass of 25.5 kDa and isoelectric point of 10.07 was characterized from the Pacific white shrimp Litopenaeus vannamei. Analysis of the deduced amino acid sequence of LvQM revealed that it contained a series of conserved functional motifs. Quantitative real-time PCR (qRT-PCR) results showed that the transcript of LvQM was extensively distributed in the tissues under investigation and most highly expressed in gill. After challenged with Vibrio anguillarum, the LvQM transcripts were significantly increased ($P < 0.05$) both in hepatopancreas and hemocytes in the early experimental phase. When LvQM was knocked down by RNA interference (RNAi), the transcript of prophenoloxidase (proPO) and the phenoloxidase activity (PO) in shrimp hemolymph were dramatically decreased, while the mortality was significantly increased. Furthermore, the recombinant LvQM protein (rLvQM) was successfully expressed in Escherichia coli BL21 (DE3)-pLysS. Injecting the purified rLvQM mixed with V. anguillarum markedly increased the clearance rate of bacteria and PO activity in the shrimp hemolymph. Hence, we conclude that LvQM was involved in the host defense of L. vannamei, probably as a positive regulator to phenoloxidase activity.

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1. Introduction

Disease outbreaks are considered to be a vital restriction to aquaculture industry, affecting both the production increase and economic revenue of the industry worldwide [1]. Similar with other marine organisms, shrimps have been severely threatened by bacterial and viral pathogens [2–4]. Mass mortalities have been reported in farmed shrimp, which may be caused by suppression of immunity associated with increased susceptibility to bacterial and viral pathogens infection [5,6].

At first the use of antibiotics was an effective way to contain the outbreak of diseases [7,8]. However, the misuse and overuse of antibiotics in aquaculture caused an increasing risk and potential threat to aquatic animals, environment and consumers [9,10]. What’s worse, antibiotic residue in animal bodies, water environment and the development of antibiotic resistant strains of bacteria aroused increased difficulty in treating diseases [11,12]. Thus novel approaches, to be effective, safe and non-toxic, are urgently demanded to solve the burning question. Be aware of lacking an adaptive immune system in shrimps, a better understanding of the innate immune defense mechanism will potentially facilitate the development of effective approaches for disease prevention or treatment. For this, important immune-related molecules involving in shrimp innate immune defense competed for researchers’ attention.

As one of the major invertebrate immune responses, the prophenoloxidase activated system (proPO-AS) serves an important role in the shrimp innate immune responses [13]. Through a non-self-recognition system, lipopolysaccharide (LPS), peptidoglycan or β-1, 3-glucan was recognized by non-self-recognition proteins, which led to the activation of the proPO cascade [14–16]. Then, with the involvement of proPO activating enzyme (PPEA), the inactive proPO zymogen is converted to the active phenol oxidase (PO). The produced PO could catalyze diphenols into the toxic quinone, finally, form melanin in cuticular wounds or around the invading microorganisms [17–19]. To date, some

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molecules involved in proPO-AS have been reported. For instance, expression of LvproPO-2 transcript was down-regulated after challenged with WSSV [20]. Silencing of PmPpAE increased susceptibility to Vibrio harveyi infection [21]. Beyond that, LvproPO-b played an important role in defense against WSSV virus [22]. All these investigations revealed the vital function of proPO-AS in shrimp innate immune responses. More interestingly, the PjQM protein in Penaeus japonicus was reported to interact with shrimp hemocyanin. As reported, in arthropod, hemocyanin could defend invaders through converting to catechooxidase and tyrosinase, two enzymes of phenol oxidase. Considering the fact, researchers inferred that QM protein could regulate the proPO activation system via the conversion of hemocyanin to phenol oxidase in shrimp [23].

The QM gene was originally identified from human by subtractive hybridization between a tumorigenic Wilms’ tumor cell line (G401) and non-tumorigenic microcell hybrid of G401 (110.1/ G401.1) [24]. Owing to the higher expression level of QM in non-tumorigenic Wilms’ microcell hybrid cells than in the tumorigenic parental cell line, the QM gene was initially considered to be a tumor suppressor. Thereafter, QM homologues have been discovered in various species and found to be hydrophilic, with molecular weights of 25–26 kDa. Moreover, the deduced amino acid sequences of QMs were remarkably conserved during evolution, which suggested that the QM has fundamental and vital functions across species. In eucaryotic organisms, the QM gene encodes for the ribosomal protein L10 [25–27], and growing numbers of evidences demonstrated that QM proteins are involved in cell growth, differentiation and apoptosis [28–30]. Of note is that the QM gene was also confirmed involving in the immune response. In grass carp, GcQM expression in head kidney was strongly induced by stimulation of Aeromonas hydrophila [31]. In abalone, the QM gene in gill was significantly increased after Viral Hemorrhagic Septicemia Virus (VHSV) infection [32]. Litopenaeus vannamei, one of the three major breeding Penaeidae shrimp in several Asia countries, suffered from the threat of bacteria seriously. Unfortunately, no investigations regarding the bacterial immune response of QM gene have been reported in shrimp up to now.

In the present study, we described the molecular characterization of CDNA sequence of the QM gene from L. vannamei. Then, tissue-specific expression and the immune-regulatory capability of LvQM challenged with Vibrio anguillarum were analyzed. Beyond that, LvQM gene was silenced by injecting double-stranded RNA, and the mRNA expression of proPO in hemocytes and the PO activity in shrimp hemolymph were analyzed. Furthermore, the LvQM was expressed in Escherichia coli, and the purified rLvQM protein was obtained and used for the investigation of bacterial clearance and involvement of QM protein in regulating the PO activity.

2. Materials and methods

2.1. Animals, bacteria challenge and sample collection

Healthy L. vannamei (average body mass of 15–20 g) were obtained from Nanjiang Company in Hainan province, China, and cultured in the laboratory for a week before processing. In this phase, shrimps were fed with commercial feed at 5% of body weight every day. To study the tissue-specific expression of LvQM, samples of gill, hepatopancreas, muscle, ovary, eyestalk, hemolymph, and intestine were isolated from six healthy (untreated) shrimps. To determine the immune response of LvQM, individuals were challenged with live V. anguillarum (4 × 10^6 CFU/ml) suspended in Physiological Saline Solution (PSS), 20 μl of bacterial suspension was injected in the second abdominal segment, and control group was injected with 20 μl PSS separately. Subsequently, hepatopancreas and hemolymph samples were removed from three shrimps of both the bacteria-injected and the control groups at 0, 2, 6, 12, 24 and 48 hpi. The shrimp hemolymph was collected into a sterilized syringe with an equal volume of anticoagulant modified Alsever solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0), and centrifuged (800 × g at 4 °C for 10 min) immediately to collect hemocytes [33]. All of the tissue samples were snap-frozen in liquid nitrogen and used for RNA isolation.

2.2. Production and injection of dsRNA

Double stranded RNA (LvQM dsRNA and LveGFP dsRNA) were generated in vitro using T7 RiboMAX Express (Promega, USA) following the manufacturer's instruction. Briefly, T7 promoter was incorporated to gene specific primers (Table 1) to produce sense and anti-sense strand separately. Then PCR products were purified with Easy TrapTM (Takara, Japan) following the manufacturer's protocol, and the purified products were quantified and transcribed to yield single stranded RNAs. Equal amount of single stranded RNAs were annealed to produce double stranded RNA which were further purified and quantified for the in vivo experiment. Ten microgram (1 μg/g shrimp) of LvQM dsRNA was injected to shrimp, and sample of hemolymph was collected with sterilized syringe containing anticoagulant solution at 0, 24, 36 and 48 hpi for further assay. Both the LveGFP dsRNA (1 μg/g shrimp) and Phosphate Buffer Saline (PBS) were injected to serve as controls.

2.3. Total RNA isolation and first-strand cDNA synthesis

The total RNA was extracted from selected tissues of shrimps using the TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA concentration was determined by Qt-F Real-time RT-PCR. The total RNA was extracted from selected tissues of shrimps using the TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA concentration was determined by RT-PCR. Sequences of primers used in this research.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Objective</th>
<th>Tm (°C)</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
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<tr>
<td>QM-F</td>
<td>ORF</td>
<td>50</td>
<td>CTCGGGGTCGTTTCTGGAGT</td>
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<tr>
<td>QM-R</td>
<td>amplification</td>
<td>52</td>
<td>TTAAGCAGAGCAGAGCT</td>
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<tr>
<td>Q5-F</td>
<td>5’ side</td>
<td>52</td>
<td>CCGGGAGTGATTGCTCCCA</td>
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<tr>
<td>Q5-R</td>
<td>3’ side</td>
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<tr>
<td>QF-F</td>
<td>Protein expression</td>
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<td>T7QM-F</td>
<td>QM-dsRNA</td>
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<tr>
<td>T7QM-R</td>
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<td>49</td>
<td>TACAGTAACGGTAGCATC</td>
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<tr>
<td>T7EGFP-F</td>
<td>EGFP-dsRNA</td>
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<tr>
<td>T7EGFP-R</td>
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<td>EGFP-F</td>
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<td>QF</td>
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<td>QR</td>
<td>Confirming sequence</td>
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measuring the absorbance at 260 nm with a UV-spectrometer (Bio-Rad, USA), and its integrity was examined by electrophoresis on a 1% agarose gel. Prior to cDNA synthesis, RNA from each sample was diluted to the concentration of 1 μg/μl and 1 μg of which was used to synthesize cDNA using the PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan) in accordance with the manufacturer’s instructions. Briefly, RNA was first incubated with 1 μl gDNA Eraser, 2 μl 5 × gDNA Eraser Buffer and with RNase Free dH2O up to 10 μl for 2 min at 42 °C to remove contaminated genomic DNA. Then, 4 μl 5 × PrimeScript® Buffer 2, 1 μl PrimeScript® RT Enzyme Mix I, 1 μl RT Primer Mix and RNase Free dH2O were added (total 20 μl), and incubated for 15 min at 37 °C, 5 s at 85 °C and finally cooled on ice. Synthesized cDNA was diluted 4-fold (total 80 μl) before storing at −80 °C for further use.

2.4. Molecular cloning of LvQM cDNA sequence

Based on the full-length cDNA sequence of PmQM from Penaeus monodon, sequence-specific primers QF and QM-R (Table 1) were designed for the amplification of the cDNA sequence of L. vannamei. Then, the obtained sequence was used as the query to screen the EST database of NCBI. Three ESTs from L. vannamei gill and hemocytes (Accession no. H0761868; H0761919; GE325392) were identified in GenBank dbEST database. Then a contig was obtained through the assembling of the ESTs with software CAP3 [34]. According to the contig, two sequence-specific primers (Q5-F, Q5-R; Q3-F, Q3-R) (Table 1) were designed for the amplification of 5’ and 3’ untranslated regions (UTRs) of L. vannamei. The amplification reaction and PCR temperature profiles were as follows: 1 cycle of 95 °C/5 min; 38 cycles of 95 °C/30 s, annealing temperature/30 s, 72 °C/1 min; 1 cycle of 72 °C/10 min. The PCR products were gel-purified and cloned into the pMD18-T simple vector (TaKaRa, Japan), then transformed into the competent cells of E. coli DH5α. The potentially positive recombinant clones were identified by colony PCR. Then the positive recombinants were picked for sequencing. The cDNA sequence of LvQM was obtained by the overlapping of the three fragments. The acquired cDNA sequence was confirmed by sequencing the PCR product amplified by primers QF and QR (Table 1) within the predicted 5’ and 3’ UTRs, respectively.

2.5. Bioinformatics analyses

Sequence homology was obtained using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Pairwise and Multiple sequence alignments were created using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the sequence manipulation suite programs (http://www.bioinformatics.org/sms/). The protein domains were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The tertiary structure was predicted by the SWISS-MODEL Server and Repository (http://swissmodel.expasy.org/). Signal peptide and nuclear localization signals (NLS) were predicted using signal 3.0 prediction (http://www.cbs.dtu.dk/services/SignalP/) and NLS prediction programs (http://cubic.bioc.columbia.edu/cgi-bin/psort). The phylogenetic analysis was conducted using MEGA version 5.0. The phylogenetic tree was constructed by the neighbor-joining method based on the QM amino acid sequence distances and was tested for reliability using 1000 bootstrap replications.

2.6. Transcriptional analysis of LvQM by qRT-PCR

Quantitative real-time PCR was performed to determine the mRNA expression profiles of LvQM in shrimp tissues mentioned above and the temporal expression patterns of LvQM in hepatopancreas and hemocytes following injection with V. anguil- larum. Sequence-specific primers (Qt-F, Qt-R; Actin-F, Actin-R) (Table 1) were designed to amplify the cDNA fragments of LvQM and β-actin, which were used for quantitative measurement. The qRT-PCR amplifications were carried out in a total volume of 20 μl containing 10 μl SYBR Green Super mix (TaKaRa, Japan), 2 μl of the 1:4 diluted cDNA, 0.4 μl each of primer (10 pmol/ul) and 7.2 μl nuclelease-free water. The cycling protocol was as follows: 95 °C for 10 min, then 95 °C, 15 s; 60°C, 1 min for 40 cycles, followed by dissociation curve analysis to verify the amplification of a single product. The relative expression of target gene was calculated by the 2−ΔΔCT method [35]. All data represent means ± standard deviation and were subjected to a one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test using the SPSS 16.0 program. Differences were considered statistically significant at P < 0.05.
mixed with the V. anguillarum (4 × 10^8 CFU/ml). The hemolymph samples were collected individually at 5 min, 30 min and 2 h after challenged and serially diluted in sterile 0.85% NaCl. The diluted hemolymph samples were then plated onto the 2216E-agar (1.5% agar, 0.5% Tryptone, 0.1% Yeast extract, 0.01% Ferric phosphate, aged seawater, pH 7.8) further grown at 28°C for 24 h. The bacterial colonies were counted and calculated as CFU/ml. All experiment tests were done in triplicate and statistical analysis was performed using t-test.

2.10. Phenol oxidase activity assay

Both the hemolymph collected from LvQM dsRNA injected shrimp and rLvQM injected shrimp were subjected to phenol oxidase activity assay. After centrifugation at 800 × g to remove hemocytes, 10 μl of the serum was mixed with 10 μl of 0.3% L-DOPA (L-β-3, 4-dihydroxyphenylalanine) (Sigma, USA) and 80 μl of 0.9% NaCl. The mixture was incubated at room temperature for 30 min. Subsequently, the absorbance of the mixture was measured at 490 nm using an iMark Microplate Reader (Bio-Rad, USA). The absorbance of the control groups was also measured. One PO activity unit was defined as an increase of 0.001 absorbance value for one milliliter mixture in one minute. The PO activity was the total enzyme activity unit for 10 μl serum.

3. Results

3.1. Cloning and characterization of LvQM cDNA

A 693 bp fragment containing the complete coding sequence (CDS) was amplified with the primers Q5-F and Q5-R (Table 1). By means of assembling of the three L. vannamei ESTs, a contig of 740 bp was acquired. Based on the contig, two fragments were amplified with the primers (Q5-F, Q5-R; Q3-F, Q3-R) (Table 1), respectively. Subsequently, the three overlapping sequences obtained above were then aligned to give the cDNA sequence of 733 bp. To confirm that the resulting 733 bp consensus sequence represented a single mRNA, primers (QF, QR) were designed within the 5’ and 3’ UTRs of the sequence. Using the primers, a single product was amplified from hepatopancreas cDNA.

As shown in Fig. 1, the LvQM cDNA sequence (X880087) contains a 40 bp 5’ UTR, a 663 bp ORF coding for a 220 aa protein and a 3’ UTR of 30 bp with a polyadenylation signal sequence “attaa”. The deduced protein has a calculated molecular mass of 25.5 kDa and isoelectric point of 10.07. The SMART indicated that the amino acid sequence of LvQM contained a series of functional motifs, including a ribosomal protein L10 signature motif (ADRLQTGMRGAFGKPQGT- VARV), an N-acylation site (GMGR), two acylamidation sites (MGRR; LGRK), two putative antibiotic binding sites (GRI; NK) and two putative protein kinase C phosphorylation sites (SVR; SRK). Moreover, the polyadenylation signal sequence (ATTAAA) is in bold and underlined. The predicted SH3-binding motif (RPARCY) is underlined, an N-acylation site (GMRGAF) is double-boxed in shaded, two putative SH3-binding motifs (RPARCYR)[32]. Actually, neither signal peptide nor nuclear localization signal (NLS) was detected in LvQM.

3.2. Homology and phylogenetic analysis of LvQM

Homology analysis of the deduced amino acid sequence of the LvQM in L. vannamei (GenBank Accession No. X880087) showed an overall similarity to the QM protein sequence. Furthermore, the polyadenylation signal sequence (ATTAAA) is in bold and underlined. The predicted SH3-binding motif (RPARCY) is underlined, an N-acylation site (GMGR) is boxed in shaded, two putative antibiotic binding sites (GRI; NK) and two putative protein kinase C phosphorylation sites (SVR; SRK). Similar with abalone QM, LvQM also contained one putative SH3-binding motif (RPARCVR) [32]. Actually, neither signal peptide nor nuclear localization signal (NLS) was detected in LvQM. Moreover, the tertiary structure prediction of LvQM together with P. japonicus QM is shown in Fig. 2. The tertiary structures, containing two α-helices and six β-sheets, are highly conserved.
japonicus (96%), P. clarkia (91%), Callinectes sapidus (90%), Caligus
clemensi (81%), Haliotis discus discus (81%), Drosophila melanogaster
(81%), Mus musculus (81%), Xenopus tropicalis (79%) and Danio rerio
(79%), respectively. Among the QM molecules, the N-terminal and
internal region of the peptides are well conserved, while the C-
terminal differs significantly from the corresponding region in all
known QM sequences. Moreover, it was revealed that the SH3-
binding motif, protein kinase C phosphorylation site, antibiotic
binding sites and ribosomal protein L10 signature were highly
conserved during the evolution of QM proteins (Fig. 3).

To study the molecular evolutionary and the relatedness of the
LvQM with known QM sequences, a neighbor-joining (NJ) distance-
based phylogenetic tree based on the amino acid sequences of 20
different species was constructed. It was clearly to find that the QM
group was mainly divided into two clusters, invertebrates and
vertebrates. The invertebrates group could also be subdivided into
different clusters, crustaceans, insect, molluscs, sponges, cnidarians
and flatworms. Moreover, the tree indicated that LvQM had closest
genetic relationship to PmQM, and with MjQM were clustered in the
crustacean group (Fig. 4).

3.3. Tissue expression of LvQM

The relative levels of LvQM transcripts from gill, hepatopancreas,
muscle, hemocytes, intestine, ovary and eyestalk were examined by
qRT-PCR. The β-actin gene was used as an internal control. As
shown in Fig. 5, the expression of LvQM was detected in all tested
tissues and most highly expressed in gill, moderately expressed in
ovary and muscle, and less expressed in hemocytes, eyestalk,
hepatopancreas and intestine.

3.4. Time-dependent expression of LvQM in hepatopancreas and
hemocytes after bacterial infection

In order to determine transcriptional response of LvQM after V.
anguillarum challenge, three animals from each group were
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putative tumor suppressor gene from the Pacific white shrimp, Litopenaeus vannamei, Fish & Shellfish Immunology (2014), http://dx.doi.org/
10.1016/j.fsi.2014.01.005
Fig. 4. Phylogenetic tree of the deduced amino acid sequence of LvQM with other proteins of the QM family constructed using the NJ-method with 1000 trials by MEGA 5.0 software. Sequences used in the phylogenetic tree are: L. vannamei (AGA165781.1); P. monodon (AVC70626.1); M. japonica (ABS45569.1); Procambarus clarkii (ABE54638.1); Callinectes sapidus (AW71145.1); Bombyx mori (AAK73358.1); Drosophila melanogaster (AAC16108.1); Hadolotis discus discus (AB0267001.1); Croatiaestia arionikensis (A070021.1); Pinctada fascata (AAN855781.1); Pinctada martensi (AB023588.1); S. domuncula (CAC80049.1); Hydra vulgaris (AAQ13347.1); Schistosoma japonicum (CAX78008.1); Ctenopharyngodon idella (A0715991.1); Danio rerio (ABQ23588.1); Callinectes sapidus (AAC16108.1); Xenopus tropicalis (AAB27665.1) and Caenorhabditis elegans (CA083081.1).

selected at each time point (0, 2, 6, 12, 24, 48 hpi) and qRT-PCR were employed to determine the expression levels of LvQM in hepatopancreas and hemocytes. In hepatopancreas, the expression of the LvQM increased gradually and reached the peak at 6 hpi (compared to the blank group, P < 0.05) and then gradually dropped back to the original level (Fig. 6A). Interestingly, the expression of the LvQM in hemocytes also increased to the highest at 2 hpi (P < 0.05) and then recovered to the original level at 24 hpi (Fig. 6B). While in the control group, no significant difference was observed in the expression of LvQM during the experiment period.

3.5. Impact of rLvQM on bacterial clearance

The rLvQM was expressed as soluble protein (Fig. 7A), and purified with a commercial kit. The purified protein was analyzed on 12% SDS-PAGE, and an apparent 45 kDa target protein band was visualized, which is closed to the size of calculated molecular mass of the fusion protein (Fig. 7B). To make certain that the rLvQM protein have an important role in shrimp immune system, a bacterial clearance assay was carried out. Live V. anguillarum (4 × 10^8 CFU/ml) mixed with 5 μM rLvQM were injected into shrimp. The control group was injected with equal volume of 0.85% NaCl mixed with V. anguillarum (4 × 10^8 CFU/ml). Then the hemolymph were collected at 5 min, 30 min and 2 h post-injection and plated onto the 2216E-agar to count the bacterial colonies. It was found that the bacterial counts were not significantly different at 5 min after injection, but were significantly decreased comparing with the control group at 30 min (7.14 folds, P < 0.05) and 2 h (13.53 folds, P < 0.05) post-injection (Fig. 8).

3.6. Suppression of LvQM decreased the transcription of proPO

To further explore the role of QM in shrimp immunity, LvQM was knocked down in vivo by dsRNA-mediated RNA interference. Quantitative results showed that a dramatic decrease in the mRNA level of LvQM in hemocytes was observed at 24 hpi of LvQM dsRNA (P < 0.05), and the LvQM transcript could be barely detected at 48 hpi (P < 0.05). However, the LvQM gene was normally transcribed when treated with LvEGFP dsRNA and PBS, and there was no observably change in the mRNA level of LvQM during the whole experiment period (Fig. 9A). In this case, the expression of LvproPO in shrimp hemocytes was analyzed. It was found that the expression of LvproPO was dramatically decreased comparing with the control shrimps at 24 h post-injection (P < 0.05). Notably, no significant difference was detected at 36 and 48 h post-injection (P > 0.05) (Fig. 9B).

3.7. Suppression of LvQM led to increased mortality

It was amazed to find that the mortality of shrimp injected with LvQM dsRNA was remarkably increased, especially at 18–24 h post-injection. As shown in Fig. 10, the final mortality rates reached 63.6%, 10.0% and 16.7% for LvQM dsRNA, LvEGFP dsRNA and PBS groups, respectively. It might be concluded that the reduction of LvQM expression could affect the basic survival ability of shrimp.

3.8. Regulation of PO activity by RNAi assay

When LvQM expression in vivo was knocked down by dsRNA-mediated RNAi, the PO activity of these treated shrimps was assessed. Results showed that the PO activity in shrimp hemolymph began to decrease at 24 h, and was significantly decreased at 36 and 48 hpi of LvQM dsRNA (P > 0.05), whereas no marked difference of PO activity was observed in the control group (Fig. 11). The fact might reveal the activity regulation role of LvQM on phenoloxidase, a key enzyme involved in the proPO-AS of shrimp immunity.

3.9. Regulation of PO activity by rLvQM injection

Live V. anguillarum (4 × 10^8 CFU/ml) mixed with 5 μM rLvQM were injected into shrimp, in which the hemolymph collected at 5 min, 30 min and 2 h post-injection were subjected to phenol oxidase activity assay. The PO activity in selected shrimp hemolymph began to increase at 5 min post-injection, and was significantly increased at 30 min and 2 h post-injection (P < 0.05) compared with the control group, which was injected with equal volume of 0.85% NaCl mixed with V. anguillarum (4 × 10^8 CFU/ml) (Fig. 12). The fact further revealed the regulation of LvQM on phenoloxidase activity.
4. Discussion

Before our study, QM genes have been identified in varieties of animals. In the present study, we first reported the identification and molecular characterization of LvQM cDNA sequence from the Pacific white shrimp, *L. vannamei*. The deduced amino acid sequence of LvQM was subsequently found to contain a series of characteristic features of QMs. The ribosomal protein L10 signature, which was conformity to the pattern of ribosomal protein L10 signature, indicated that LvQM was an orthologue of QM family. Moreover, the SH3-binding motif, two protein kinase C phosphorylation sites and two antibiotic binding sites found in LvQM indicated the high conservation of QM. The highly conserved domains might suggest the important role of QM in the basic functions. Besides, no signal peptide and NLS identified from the deduced amino acid sequence of LvQM, pointed out that LvQM may not be a secretory protein and remain localized in the cytoplasm. A study which was carried out on mammalian cells and yeast confirmed the result that QM localized to the cytoplasmic face of the rough ER [25,36]. In addition, there were seven cysteine residues in LvQM, and six of them (C8, C12, C23, C49, C71, C105) were highly conserved with all selected QM proteins, suggested that three intrachain disulfide bonds may be potentially involved in secondary structure formation of LvQM.

Since it was first identified as a putative tumor suppressor [24], the QM gene has attracted wide interests and been...
Fig. 9. Temporal expression analysis of LvQM (A) and LvproPO (B) mRNA in shrimp hemocytes after knocked-down the LvQM by dsRNA-mediated RNAi. Injection of LvEGFP dsRNA and Phosphatase Buffer Saline (PBS) were used as control. The change in fold-expression was calculated by the \(2^{-\Delta\Delta CT}\) method using \(\beta\)-actin as a reference gene. Data are presented as the mean \(\pm\) SD (N = 3). Significant difference compared to the control group is marked with an asterisk at \(P < 0.05\).

Fig. 10. Cumulative mortalities of LvQM silenced shrimp. Mortality was measured in each treatment group (\(n = 30\)) and was recorded every 6 h post-injection. The Pearson’s ch-square test was used to compare different cumulative mortality. Significant differences in shrimp mortality are marked with asterisks.

LvQM in gill demonstrated that LvQM might cooperate to resist the invasion of pathogens. Besides, the wide distribution of LvQM might further suggest the multiplex biological functions of LvQM in shrimps.

In shrimp, many stress and pathogen infection related proteins were expressed in hepatopancreas [40,41]. The up-regulation of LvQM in hepatopancreas at 6 h post-\(V.\) anguillarum injection might reveal its involvement in the early immune defense against bacteria in shrimp. Previous researches have shown that the proPO system is activated early upon the pathogen infection [42,43], and the observably up-regulation of LvQM in hepatopancreas in early phase might also suggest the important role of LvQM in shrimp immunity, and probably participated in the proPO-AS. As we know, when pathogens passed the first defense line of shrimps, their effective cellular and humoral innate immune responses would be induced to resist pathogen invasion [44]. Thus the proPO-AS, one of the crucial immune responses employed by arthropods, would be activated by microbial elicitors like \(1,3\)-glucan, lipopolysaccharide (LPS), and peptidoglycan (PG), and functioned as a cleaner of pathogens passed the invading bacteria and viruses [45,46]. Along with the activation of proPO system, a mass of hemocyanin was generated in hepatopancreas [40,41]. The up-regulation of LvQM in gill demonstrated that LvQM had a remarkable increase after VHSV challenged in gill of abalone [32]. Hence, the highest expression of LvQM in gill demonstrated that LvQM might cooperate to resist the invasion of pathogens. Besides, the wide distribution of LvQM might further suggest the multiplex biological functions of LvQM in shrimps.

Fig. 11. Gene silencing of shrimp LvQM decreased the phenol oxidase activity of shrimp hemolymph. Shrimp hemolymph was drawn at 24, 36, 48 h post-injection. Vertical bars are presented as the mean \(\pm\) SD (\(N = 3\)). Significant difference compared to the control group is marked with an asterisk at \(P < 0.05\).

Fig. 12. Phenol oxidase activity of shrimp hemolymph after injection with 5 \(\mu\)M \(r\)LvQM mixed with \(V.\) anguillarum. The control group was injected with \(V.\) anguillarum only. Shrimp hemolymph was drawn at 5 min, 30 min and 2 h post-injection. Vertical bars are presented as the mean \(\pm\) SD (\(N = 3\)). Significant difference compared to the control group is marked with an asterisk at \(P < 0.05\).
LvQM displayed 96% amino acid identity to PjQM, moreover, the predicted tertiary structures of LvQM and PjQM are highly conserved which suggested the similar role in the proPO-AS. Hence, it might be inferred that the high expression of LvQM in hepatopancreas would boost the interaction of QM protein with hemocyanin.

To further reveal the participation of LvQM in shrimp immunity, a dsRNA-mediated RNA interference was processed. Knocking down LvQM expression in vivo led to a significant decrease of the transcript of LvproPO in hemocytes. As the precursor of PO, proPO play important role in the proPO-AS. The decrease of LvproPO might suggest the inactivity of proPO. Coincidentally, suppression of LvQM significantly dropped the PO activity. The result was in accord with P. japonicas, in which the PO activity of shrimp hemolymph was significantly decreased at 36, 48 and 72 hpi of the PjQM-siRNA [23].

Furthermore, the PO activity was markedly increased after the injection of LvQM protein. In general, suppression of LvQM expression led to the decrease of PO activity while over-expression of LvQM induced the increase of PO activity. All the facts here further confirmed that LvQM had vital function in shrimp immunity, and might function as positive regulator to the phenoloxidase activity.

It is generally known that phagocytosis, endocytosis and encapsulation were mediated by hemocytes and depended on phenoloxidase activity to involve in the immune and defense function [13,49]. In crustaceans, it was also reported that hemocytes could defend the invaders through direct sequestration, killing of infectious agents or synthesis and exoythysis of a series of bioactive molecules [50]. The up-regulation of LvQM in hemocytes after challenged with V. anguillarum might suggest the important immune regulation effect of LvQM in L. vannamei. In order to confirm the important role of LvQM in shrimp immunity, bacterial clearance of shrimp was analyzed. The rLvQM together with V. anguillarum was injected into the shrimp to determine the extent of bacterial clearance. The results clearly showed that the bacterial density of shrimp was significantly decreased with the addition of rLvQM protein. Here, we guessed that the rLvQM injection could cause the enhancement of the bacterial clearance in shrimp. Since it is well known that the proPO system is activated early upon the pathogen infection [42,43], the likely explanation for such decrease in bacterial density was that the rLvQM presumably involved in the proPO-AS. Moreover, the high cumulative mortality in LvQM knocked down shrimps probably revealed that the suppression of LvQM expression could affect the basic survival ability of shrimp. Taking all the facts above into account, we infer that LvQM may play an important role in bacterial defense, probably participate in the activity regulatory of phenoloxidase. Surely, the precise mechanism of QM protein function as a defender in the proPO-AS still badly needs exploration.

In summary, we have successfully cloned the cDNA sequence of LvQM from the Pacific white shrimp, L. vannamei. Tissue expression analysis indicated that LvQM was detected in all of the tissues examined, and higher expressed in gills, ovary and muscle. The LvQM was significantly increased both in hepatopancreas and hemocytes after challenged with V. anguillarum. Silencing of LvQM in vivo significantly decreased the transcript of proPO, the PO activity in shrimp hemolymph and also led to the high cumulative mortality. In addition, we successfully obtained the purified rLvQM, and injection of rLVQM protein significantly increased the clearance rate of bacteria and the PO activity in the shrimp hemolymph.

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