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# A laminin-receptor-like protein regulates white spot syndrome virus infection by binding to the viral envelope protein VP28 in red claw crayfish *Cherax quadricarinatus*

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ABSTRACT

White spot syndrome virus (WSSV) is a lethal pathogen of shrimp and many other crustaceans, which has been causing huge economic losses in global aquaculture. Laminin receptor (LR) is a cell surface receptor which participates in the interactions between cells as well as cells and extracellular matrix. Previously, we found that a *CqLR-like* gene was responsive to WSSV infection in the hematopoietic tissue (Hpt) cells from red claw crayfish *Cherax quadricarinatus*. To further reveal the role of *CqLR-like* gene involved in WSSV infection, the full-length cDNA of *CqLR-like* gene was cloned with 1000 bp, and the open reading frame encoded 308 amino acids with a conserved laminin-binding domain. Importantly, both the WSSV entry and viral replication were strongly reduced in Hpt cells after loss-of-function of *CqLR-like* gene by gene silencing. Protein interaction assay demonstrated that the recombinant *CqLR-like* protein could bind to WSSV virion in vitro by enzyme-linked immunosorbent assay and the binding affinity was in a dose-dependent manner. Furthermore, recombinant *CqLR-like* protein was found to bind to WSSV envelop protein VP28, but not other envelop proteins tested including VP19, VP24, and VP26, by pull down assay in HEK293T cells. In regarding to that LR is mainly localized on many types of cells' membrane, these data together suggested that *CqLR-like* protein was likely to function as a putative recognition molecule towards WSSV and act in the viral entry into a crustacean host cell, which may benefit the elucidation of the WSSV pathogenesis and further the pharmaceutical target for the possibly effective control of WSSV disease.



## 1. Introduction

White spot syndrome virus (WSSV) is a rod-shaped, circular double-stranded DNA virus, which belongs to a member of the family *Nimaviridae* consisting of a single genus *Whispovirus* (Mayo, 2002). WSSV virion protein mainly includes envelope, nucleocapsid and tegument between these two components. As one of the biggest genome sequenced animal virus, the WSSV's genome is around 300 kb (Escobedo-Bonilla et al., 2008; van Hulten et al., 2001; Yang et al., 2001). With a wide range of hosts in crustaceans, WSSV has been a major causative agent that leads to high mortality in shrimp and crayfish, which has ever been reported to cause total mortality within 7–10 days of its onset and result in huge economic losses in shrimp farming (Lightner, 1996). However, there is no efficient therapeutic treatment till now due to the lack of understanding on WSSV pathogenesis. Thus, understanding of the infection mechanism of WSSV will definitely benefit the efficient control of WSSV disease.

Laminin receptor (LR), a cell surface receptor, is an important protein which instructs interaction between cells, and between cells and extracellular matrix. In previous studies, the search for cell-surface proteins mediating their effects identified receptors of two main classes: the integrin and the non-integrin laminin receptors. The first to be revealed was the non-integrin 67-kDa laminin receptor, identified via binding to immobilized laminin-1

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(α1β1γ 1) from rat *Rattus norvegicus* (Lesot et al., 1983; Malinoff and Wicha, 1983) as a binding protein of laminin, an extracellular matrix glycoprotein component of basement membranes. Further study demonstrated this protein might arise from a 32–33 kDa precursor that migrates at approximately 37 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (Wewer et al., 1986). Later, a protein called p40 in a different line of investigation was found to be equivalent (Mccaffery et al., 1990) and later confirmed to be a component of the 40S ribosome now called ribosomal protein SA (RPSA) in rat *R. norvegicus* (Tohgo et al., 1994). Hence, LR has been given many names including 37/67-kDa laminin receptor, 32 kDa laminin binding protein (LBP), 32 kDa laminin binding protein precursor and p40 and ribosomal protein SA (RPSA) (DiGiacomo and Meruelo, 2016). Thus, LR has been recognized as a multifunctional protein involved in a broad range of biological processes such as cell adhesion, mobility and differentiation (Castronovo, 1993).

Notably is that LR has been recently identified as a receptor for many virus on the cell membrane. When virus infects cells, it is a precondition for the virion to be absorbed on the surface of host cell. For examples, LR has been shown to be a kind of receptor for Sindbis virus, Venezuelan equine encephalitis, dengue virus and tick-bone encephalitis virus spread by ticks (Thepparit and Smith, 2004). Lately, LR has also been identified as a cellular attachment receptor for classical swine fever virus (CSFV), and helps the CSFV attach to the cell surface but not involved in the viral entry (Chen et al., 2015). In shrimp, LR was first identified as a receptor protein for Taura syndrome virus (TSV) via binding to TSV capsid proteins VP1 (Senapin and Phongdara, 2006). Additionally, LR was shown to act as a binding protein for a shrimp RNA virus yellow head virus (YHV) via interaction with its envelope protein gp116 (Busayarat et al., 2011). Meanwhile, LR was recently shown to play as an important cellular attachment receptor which bound to the WSSV envelope protein VP31 and mediated WSSV infection (Liu et al., 2016). Previously, we found that the *CqLR-like* gene transcription was responsive to WSSV infection in the hematopoietic tissue (Hpt) cells from red claw crayfish *Cherax quadricarinatus* (Liu et al., 2011). Whereas, whether LR affected the WSSV entry and further the viral replication in crustacean is not clear. To explore the mechanism of LR in the WSSV-host interaction of a crustacean, we carried out the *CqLR-like* gene -WSSV interaction studies in red claw crayfish Hpt cells, and the results indicated that *CqLR-like* protein was likely to act as an interacting partner which bound to the WSSV envelope protein VP28 and thus mediated WSSV infection.

## 2. Materials and methods

### 2.1. Animals, Hpt cell cultures and virus

The healthy red claw crayfish *C. quadricarinatus* free of WSSV were purchased from Tenglong Xianyou, Fujian Province, China. The animals were acclimatized in freshwater tanks and the ambient temperature was 25 °C.

Hpt cells were prepared from hematopoietic tissue of *C. quadricarinatus* and cultured as described by Söderhäll et al. and Liu et al. (Liu et al., 2011; Söderhäll et al., 2003). Hpt cells were cultured in 24-well plates ($5 \times 10^5$ cells/500 μL) and 96-well plates ($10^5$ cells/100 μL) at 20 °C, and red claw crayfish plasma containing crude astakine were added after cell attachment for about 30 min (Söderhäll et al., 2005). WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China). The virus was prepared as described by Xie et al. and quantified via absolute quantification by PCR (Xie et al., 2005).

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from different tissues of crayfish using the TRIzol reagent (Roche, USA), according to the manufacturer's instructions. RNase-Free DNase I (Ambion, USA) was used to eliminate genome DNA contamination in the extracted RNA. First strand cDNA synthesis was performed using PrimeScript™ RT Reagent Kit (TaKaRa) following the manufacturer's instructions.

### 2.3. The full-length cDNA cloning and sequence analysis of CqLR-like gene

A partial *CqLR-like* gene cDNA sequence of *C. quadricarinatus* was isolated from a transcriptome library of Hpt cells post WSSV infection in our lab (Liu et al., 2011). In order to clone the full-length cDNA sequence of *CqLR-like* gene, 5′ and 3′ Rapid Amplification of cDNA Ends (RACE)-PCR was carried out using SMART RACE cDNA Amplification kit (Clontech, USA). The gene-specific primers for RACE, *CqLR-like*-5′RACE-R and *CqLR-like*-3′RACE-F, were shown in Table 1. The PCR conditions were as follows: 5 min at 98 °C; 30 cycles of 98 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s; and 72 °C for 10 min. All amplified PCR products were gel-purified using a Gel Extraction Kit (Sangon Biotech, Co., Ltd., Shanghai, China). The recovered DNA fragment was ligated into pMD18-T vector (TaKaRa), and then the vector was transformed into *E. coli* DH5α cells, and positive clones containing the expected size of the insert were picked and sequenced at Xiamen Borui Biotechnology Company, China.

The similarity analysis of *CqLR-like* gene sequence was conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). A phylogenetic analysis of *CqLR-like* gene was conducted using the MEGA 7.0 program (Kumar et al., 2016).

### 2.4. The distribution of CqLR-like gene transcript in various tissues of red claw crayfish

As mentioned above, total RNA was extracted from 12 tissues of 3 healthy crayfish which were selected randomly, including Hpt, heart, nerves, gonads, muscle, haemocytes, intestines, gills, hepatopancreas, stomach, eyestalk and epithelial tissue for cDNA synthesis. With these templates, a pair of specific primers of *CqLR-like* gene (*CqLR-like*-qRT-F and *CqLR-like*-qRT-R; Table 1) was designed in Primer Premier 5.0, and the transcriptional level of *CqLR-like* gene mRNA in different tissues was tested by real-time quantitative PCR (qRT-PCR) with 16S ribosomal gene (GenBank: AF135975.1) of crayfish as the internal standard (Table 1). Each reaction system included 10 μL of SYBR Green Master (2 × ) (Roche, USA), 0.5 μL of sense primer and antisense primer respectively, 5.0 μL of 50 times diluted cDNA and 4 μL of sterile water. The procedure of qRT-PCR was as following: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample had three replicates. The data of qRT-PCR were processed by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and presented as the mean ± SD from three independent experiments and analyzed statistically using Student *t*-test. Significant differences were accepted at $p < 0.05$.

### 2.5. Effect on WSSV infection by gene silencing of CqLR-like gene in red claw crayfish Hpt cells

To knockdown the gene expression of *CqLR-like gene*, an RNAi assay was performed by double-strand RNA (ds*CqLR-like*) transfection. A pair of primers for dsRNA synthesis were designed (ds*CqLR-like*-F and ds*CqLR-like*-R; Table 1) with incorporation of a T7 promoter. The dsRNA was synthesized by using the MegaScript kit (Ambion, Austin, TX, USA) according to the manufacturer's

**Table 1**
Primers used in this study.

| Primers | Sequence (5′-3′) |
|---|---|
| **cDNA amplification** | |
| *CqLR-like*-5′RACE-R | GTGAACACCATCCTGACGCCTCTTGAA |
| *CqLR-like*-3′RACE-F | TTGGTGGATGTTGGCTCGTGAAGTTCT |
| *CqLR-like*-ORF-F | CCGGAATTCATGTCGGGAGGACTTGCTGTTATGA |
| *CqLR-like*-ORF-R | ACGCGTCGACTTACCAGTTGGTACCATCACCTGCA |
| **qRT-PCR** | |
| *CqLR-like*-qRT-F | TCCTGCTGATGTGTATGTGAT |
| *CqLR-like*-qRT-R | TAAGATGCTTCAGTTATGGGC |
| 16S-F | AATGGTTGGACGAGAAGGAA |
| 16S-R | CCAACTAAACACCCTGCTGATA |
| VP28-qRT-F | AAACCTCCGCATTCCTGT |
| VP28-qRT-R | GTGCCAACTTCATCCTCATC |
| IE1-qRT-F | CTGGCACAACAACAGACCCTACC |
| IE1-qRT-R | GGCTAGCGAAGTAAAATATCCCCC |
| **RNAi** | |
| ds*CqLR-like*-F | TAATACGACTCACTATAGGGCCTGCTTCGGACCATCAGCCCAT |
| ds*CqLR-like*-R | TAATACGACTCACTATAGGGACCACCCCAATCATCGCCAGTTC |
| dsGFP-F | TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT |
| dsGFP-R | TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC |
| **Pull down** | |
| VP19-EcoR I-F | CCGGAATTCATGGCCACCACGACTAACACT |
| VP19-BamH I-R | CGCGGATCCCTGCCTCCTCTTGGGGTAAG |
| VP24-EcoR I-F | CCGGAATTCATGCACATGTGGGGGGTTTAC |
| VP24-BamH I-R | CGCGGATCCTTTTTCCCCAACCTTAAACAGATCA |
| VP26-EcoR I-F | CCGGAATTCATGGAATTTGGCAACCTAACAA |
| VP26-BamH I-R | CGCGGATCCCTTCTTCTTGATTTCGTCCTTGATA |
| VP28-EcoR I-F | CCGGAATTCATGGATCTTTCTTTCACTCTTTCGG |
| VP28-BamH I-R | CGCGGATCCCTCGGTCTCAGTGCCAGAGTAGG |

instructions. Green fluorescent protein (GFP) served as the control and dsGFP RNA was synthesized in the same way, which was used as a control treatment.

For dsRNA transfection, 400 ng of dsRNA/well (24-well plates) and 100 ng of dsRNA/well (96-well plates) in RNase-free water was mixed with Cellfectin II Reagent (Life Technologies), incubated for 10 min at room temperature, then supplied with medium and added into the cell cultures. The dsRNA transfection was repeated at 24 h after the first dsRNA transfection. WSSV infection was performed in 24-well plates (MOI = 1) for detecting the transcription and replication of WSSV and 96-well plates (MOI = 10) for detecting the WSSV entry at 36 h after the second transfection. The cells in 24-well plates were collected with lysis buffer at the time of 3 h, 6 h after WSSV infection. The total RNA was extracted using the GenElute™ Mammalian Total RNA miniprep kit (Sigma) according to the protocol and cDNA synthesis was described in section 2.2 using the PrimeScript™ RT Reagent Kit (TaKaRa). Then, qRT-PCR was carried out to determine whether gene silencing of *CqLR-like* had any effect on viral replication. The gene expression of *CqLR-like*, an immediate early gene (IE1), and a late gene (VP28) of WSSV were analyzed during WSSV infection. The primers of IE1 (IE1-qRT-F, IE1-qRT-R) and VP28 (VP28-qRT-F, VP28-qRT-R) were shown in Table 1. The 16S RNA was used as an internal standard. For the cells in 96-well plates, the samples were collected for detection of virus entry into cells by Western blotting after 1 h of WSSV infection, and β-Actin was used as the internal reference as previously described (Chen et al., 2016). The experiments were repeated three times.

### 2.6. Recombinant expression and purification of CqLR-like protein

The coding region of *CqLR-like gene* was amplified from cDNA of Hpt in *C. quadricarinatus* through PCR with a pair of primers (*CqLR-like*-ORF-F and *CqLR-like*-ORF-R; Table 1). The condition of PCR amplification was 98 °C for 5 min; 30 cycles of 98 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and 72 °C for 10 min. Then the recombinant vector pET28a-LR-like and pGEX-4T-1-LR-like was digested with EcoR I and Sal I restriction enzyme (TaKaRa), and cloned into expression vector pET-28a and pGEX-4T-1. The recombinant vector pET28a-LR-like and pGEX-4T-1-LR-like was transformed into *E. coli* (BL21:DE3), respectively, to express CqLR-like-His and CqLR-like-GST protein induced by 0.1 mM isopropylthiogalactoside at 16 °C for 20 h. Following centrifugation (10,000 × g for 30 min), *E. coli* cells precipitation of CqLR-like-His was resuspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 20 mM imidazole) and then put them together on the ice for ultrasonic processing. Cellular debris was removed by centrifugation (10,000 × g for 30 min). The recombinant protein CqLR-like-His was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose column, and washed by washing buffer1 (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 40 mM imidazole), washing buffer2 (50 mM Tris-HCl [pH7.4], 300 mM NaCl, 80 mM imidazole) and washing buffer3 (50 mM Tris-HCl [pH7.4], 300 mM NaCl, 120 mM imidazole). Finally, CqLR-like-His was processed by elution buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 250 mM imidazole), collected and dialyzed by 1 × PBS. For the CqLR-like-GST, *E. coli* cells precipitation was resuspended in 1 × PBS and then with ultrasonic processing. Cellular debris was removed by centrifugation at 10,000 × g for 30 min. The recombinant protein CqLR-like-GST was purified by glutathione sepharose 4B resins (GE Healthcare), and washed by 1 × PBS. Then CqLR-like-GST was eluted by elution buffer (50 mM Tris-HCl [pH 7.4], 500 mM NaCl, 20 mM L-Glutathione reduced), collected and dialyzed by 1 × PBS.

### 2.7. Proteins interaction between CqLR-like-His with WSSV by ELISA

For ELISA, 100 μL of the coating solution was added to the 96-well ELISA plate (Corning Costar) with 1 × $10^7$ copies virus particles per well and incubated overnight at 37 °C followed by wash for three times with wash buffer (1 × PBS containing 0.05% Tween 20). Then 200 μL of 0.3% BSA was added to each well and blocked at 37 °C for 1 h. After washing with the wash buffer, various

concentrations of the CqLR-like-His and GFP-His dilution were added to the plate and then incubated at room temperature for 2 h. Plates were washed and a blocking buffer diluted detection antibody (mouse anti-His antibody, 1:8000) was added (100 μL per well), and then incubated at room temperature for 1 h. After washing five times, HRP-labeled goat anti-mouse antibody (1:10000) was added to the plate and the reaction mixture was incubated at room temperature for an extra hour. The reaction was visualized using the HRP substrate TMB (3, 3′, 5, 5′-tetramethylbenzidine; Solarbio), stopped by adding 100 μL of 2 mol/L $H_2SO_4$. The absorbance was immediately read at 450 nm using an ELISA reader.

### 2.8. Protein interactions between CqLR-like-GST and the main envelope proteins of WSSV

Pull-down assays were performed to explore whether *CqLR-like* protein could interact with the main envelope proteins of WSSV (VP19, VP24, VP26 and VP28), which occupies a greater proportion in the envelope proteins (Xie et al., 2006). The empty vector PB513B-Flag was used to construct the plasmids for WSSV VPs expression in human embryonic kidney 293T (HEK293T). The open reading frame (ORF) of VPs (Genbank accession number AF332093.3) was amplified by gene-specific primers (Table 1) and cloned into the PB513B-Flag vector. For DNA transfection, HEK293T were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher) at 37 °C. The PB513B-VPs-Flag plasmid was transfected into the HEK293T with Sofast®Transfection Reagent (Sofast). The whole cell was lysed at 72 h after transfection with Western and IP cell lysis buffer (Beyotime) on ice for 30 min. After centrifugation at 10,000 × g for 5 min, 5 μg of CqLR-like-GST protein or GST protein and 20 μL of glutathione sepharose 4B resins (GE Healthcare) were added to the supernatant and rotated at 4 °C for 2 h. After incubation, the beads were washed with PBS for 5 times and added in 2 × SDS-PAGE sample buffer (Solarbio) and denatured by boiling for 10 min. The protein samples were electrophoresed in 15% SDS-PAGE gels and transferred to PVDF membranes (GE Healthcare). The membranes were blocked for 1 h at room temperature in 5% skim milk dissolved in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6), and subsequently incubated with anti-flag monoclonal antibodies (Sigma, 1:10000) at 4 °C overnight. The membranes were then washed for five times with TBST buffer followed by incubation for 1 h at room temperature with HRP-linked secondary antibodies (1:5000). The GST protein was used as a control.

## 3. Result and discussion

### 3.1. Characterization of the full-length cDNA sequence of CqLR-like gene

The full-length cDNA sequence of *CqLR-like* gene and its deduced amino acid sequence were shown in Fig. 1. The *CqLR-like* gene cDNA was 1000 bp with an open reading frame of 927 bp encoding 308 amino acids, a 51 bp of 5′-Untranslated Regions (5′-UTR) and a 22 bp of 3′-UTR (Genbank accession no. MG148351). The calculated molecular mass of the deduced mature *CqLR-like* protein was about 35 kDa with a theoretical isoelectric point of 4.6. The phylogenetic tree was also constructed with MEGA 7.0 according to the amino acid sequences with other species, including *Litopenaeus vannamei* (ABH10628.1); *Meretrix meretrix* (AFV15300.1); *Mus musculus* (J02870.1); *Chelonia mydas* (EMP25493.1); *Penaeus monodon* (DT044263); *Homo sapiens* (NP_002286.2); *Xenopus laevis* (NP_001089106.1); *Lineus viridis* (ABZ04275.1); *Callorhinchus milii* (AFM85544.1); *Bombyx mori* (NP_001106143.1); *Drosophila melanogaster* (AAA28667.1) and *Danio rerio* (AAQ91246.1) (Fig. 2A), which suggested that *CqLR-like* gene was similar to shrimp LRs, including *L. vannamei* and *P. monodon*, but was with lower homology to the *H. sapiens* and *M. musculus*. Sequence alignments of *CqLR-like* gene with other homologs using ClustalW program

```
1     ACGCGGGCTTCTTCTTGATGTGATCCTGCCGAGGAGCGGATAGCCGCCATCATGTCGGGAGGACTTGCTGTTATGAGCCTTGAGGAGAAT
1                                                        M  S  G  G  L  A  V  M  S  L  E  E  N
91    GATGTGACAAGGTTTCTTGCAGCATCAACTCATTTGGGTGCCAACAATGCAAACTTTCAGATGGAACAATACGTCTTCAAGAGGCGTCAG
14     D  V  T  R  F  L  A  A  S  T  H  L  G  A  N  N  A  N  F  Q  M  E  Q  Y  V  F  K  R  R  Q
181   GATGGTGTTCACATCATCCATTTGCGCAAGACCTATGAGAAGATCCTGCTAGCAGCACGTGCAATTGCTGCCATTGAAAATCCTGCTGAT
44     D  G  V  H  I  I  H  L  R  K  T  Y  E  K  I  L  L  A  A  R  A  I  A  A  I  E  N  P  A  D
271   GTGTATGTGATATCATCACGCCCCATGGGACAGAGAGCTGTACTCAAATTTGCAAGATACACTGGTGCCACTCCAATTGCTGGGCGCTTC
74     V  Y  V  I  S  S  R  P  M  G  Q  R  A  V  L  K  F  A  R  Y  T  G  A  T  P  I  A  G  R  F
361   ACTCCTGGAGCATTCACCAACCAAATCCAGGCTGCTTTCCGTGAACCTCGACTGTTAGTTGTGACTGACCCTGCTTCGGACCATCAGCCC
104    T  P  G  A  F  T  N  Q  I  Q  A  A  F  R  E  P  R  L  L  V  V  T  D  P  A  S  D  H  Q  P
451   ATAACTGAAGCATCTTATGTTAATATCCCTGTTATTGGATTTTGCAACACTGATTCTCCCCTTCGTTTTGTGGACGTTGCTATCCCATGT
134    I  T  E  A  S  Y  V  N  I  P  V  I  G  F  C  N  T  D  S  P  L  R  F  V  D  V  A  I  P  C
541   AATAACAAGAGTCCTCACTCAGTTGGTCTGATTTGGTGGATGTTGGCTCGTGAAGTTCTACGTCTGCGTGGCACCATTTCCCGCAACCTT
164    N  N  K  S  P  H  S  V  G  L  I  W  W  M  L  A  R  E  V  L  R  L  R  G  T  I  S  R  N  L
631   CCTTGGGAGACTGACGTTATGCCTGATTTGTTCTTCTACCGTGACCCTGAGGAACAGGAGAAGGAAGAAGCTGCCAAAGCTGAGGCTGCC
194    P  W  E  T  D  V  M  P  D  L  F  F  Y  R  D  P  E  E  Q  E  K  E  E  A  A  K  A  E  A  A
721   AAGGCTGAAGCAGAGGCAGCTAAGGCAGAAGTTCCAGCCCCAGAGACTTGGGTCAATGATGTAGCTGATGCAGAGGCTCCTGTTGCTGCA
224    K  A  E  A  E  A  A  K  A  E  V  P  A  P  E  T  W  V  N  D  V  A  D  A  E  A  P  V  A  A
811   CCTGCCACACCTGTTGCTGCTAGTACAGCTCCAGTGGCTGGTGTGGGCATACCACCTGCTGCTCCTGCAACTGTTGATGACTGGGGTCAA
254    P  A  T  P  V  A  A  S  T  A  P  V  A  G  V  G  I  P  P  A  A  P  A  T  V  D  D  W  G  Q
901   GCTGGTGATGACTGGGCTGCTCCTGCTGCTGGAACTGGCGATGATTGGGGTGGTGCAGGTGATGGTACCAACTGGTAAACCTCAATAAAT
284    A  G  D  D  W  A  A  P  A  A  G  T  G  D  D  W  G  G  A  G  D  G  T  N  W  *
991   AATAGTCGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

**Fig. 1.** The full-length cDNA sequence and the deduced amino acid sequence of *CqLR-like* gene from the *C. quadricarinatus*. The conserved putative laminin-binding site of the laminin receptor family was shaded; the stop codon was indicated by the *.

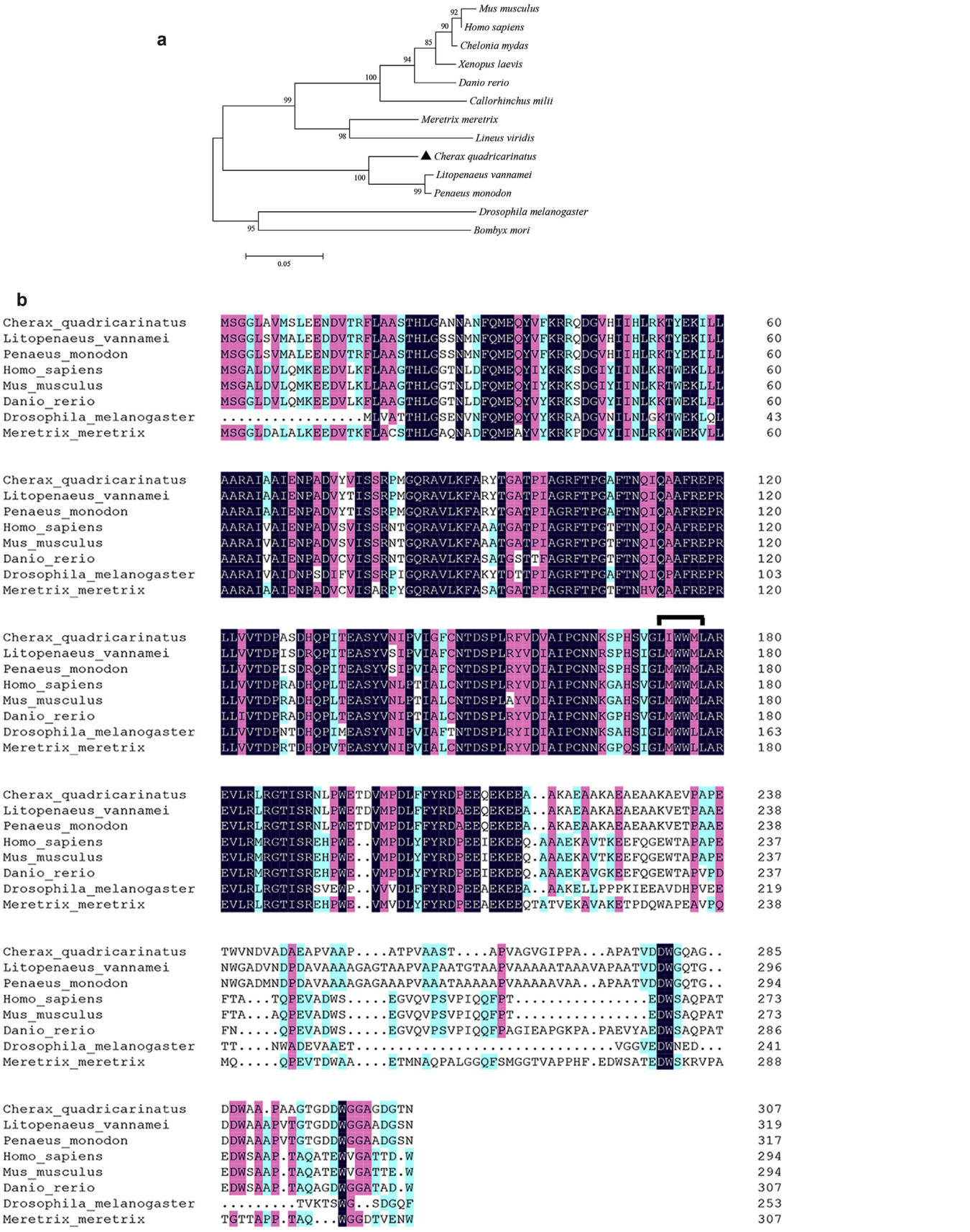


**Fig. 2.** The bioinformatics analysis of *CqLR*-like gene. (A) The phylogenetic tree of *CqLR*-like gene with LR from other species. The values on each branch represent the bootstrap values (%) for 1000 replications, and the bar indicates the distance of evolution. The Genbank ID of sequences were shown as follows: *Litopenaeus vannamei* (ABH10628.1); *Meretrix meretrix* (AFV15300.1); *Mus musculus* (J02870.1); *Chelonia mydas* (EMP25493.1); *Penaeus monodon* (DT044263); *Homo sapiens* (NP_002286.2); *Xenopus laevis* (NP_001089106.1);

revealed that *CqLR-like* gene had 81% identity of amino acid sequence with *LvLR* and *PmLR* (Fig. 2B), suggesting that *CqLR-like* gene might have similar function with shrimp LRs such as recognition and interaction with virus (Liu et al., 2016; Senapin and Phongdara, 2006). Besides, *CqLR-like* gene gave 51–58% of similarity to LR of *H. sapiens*, *M. musculus*, *D. rerio*, *D. melanogaster* and *M. meretrix*. Furthermore, an LIWWML sequence was speculated as the laminin-binding domain in *C. quadricarinatus*, regarding to that the laminin-binding site of LR has been reported to form the six amino acid palindrome LMWWML (Castronovo et al., 1991; Jaseja et al., 2005; Magnifico et al., 1996) of which two consecutive tryptophan (W) residues are considered to be suitable for protein–protein interaction (Jaseja et al., 2005).

### 3.2. The distribution of CqLR-like gene transcript in various tissues of red claw crayfish

To analyze the distribution of *CqLR-like* gene mRNA in different tissues of red claw crayfish, qRT PCR was used to analyze the presence of the transcript in different tissues, including haemocytes, stomach, gonads, muscle, nerves, intestines, heart, Hpt, hepatopancreas, gills, epithelial tissue and eyestalk. As shown in Fig. 3, *CqLR-like* gene mRNA was expressed in all examined tissues with the highest expression in muscle and gonads, which was followed by the relatively lower expression in heart, hepatopancreas, Hpt, but lowest expression in eyestalk, gill and epithelial tissue, suggesting that *CqLR-like* gene was a constitutively and widely expressed gene in red claw crayfish. The wide tissue distribution indicated that *CqLR-like* gene was likely to play important roles in red claw crayfish. In consideration to that LR was a multifunctional protein involved in a broad range of biological processes such as cell adhesion, mobility and differentiation (Castronovo, 1993), the high expression of *CqLR-like* gene in muscle and gonads might suggest its important function in the development and differentiation of red claw crayfish. Besides, it is well known that Hpt and haemocytes play key roles in immune response of crustacean, and LR serves as a cell surface receptor and can be dedicated to cell adhesion and migration. Thus, LR may connect between haemocytes and haemocytes, as well as between haemocytes and other tissues during blood circulation, then participating in the innate immunity.

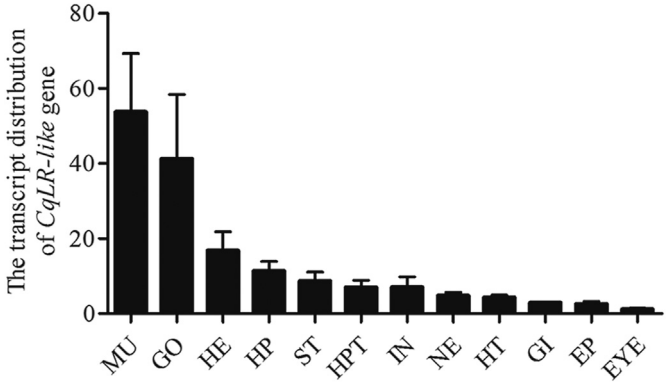


**Fig. 3.** The mRNA expression of *CqLR-like* gene in different tissues from *C. quadricarinatus*. HE: haemocytes; Hpt: hematopoietic tissue; IN: intestine; GI: gill; ST: stomach; GO: gonad; NE: nerve; EP: epithelial tissue; MU: muscle; HP: hepatopancreas; HT: heart; EYE: eyestalk. The relative transcript levels of *CqLR-like* gene in eye were employed as the calibrator (value set as 1). The experiment was repeated three times. Bars represent ± standard error of the mean.

### 3.3. Inhibition on WSSV infection by loss-of-function of CqLR-like gene in crayfish Hpt cell cultures

Previously, we found that *CqLR-like* gene expression was significantly up-regulated after WSSV infection in crayfish Hpt cells (Liu et al., 2011), indicating that *CqLR-like* gene might be involved in WSSV infection. To further reveal how *CqLR-like* gene affected on WSSV infection efficiency, the *CqLR-like* gene was silenced in Hpt cells followed by WSSV infection. By using RNAi assay, the gene knockdown efficiency of *CqLR-like* gene was more than 95% in comparison to control cells (Fig. 4A). Importantly, the relative amount of a viral envelope protein VP28, an indication of the entered WSSV at the early viral evasion stage within 1 h post infection (Chen et al., 2016), was obviously decreased, suggesting a strong suppression on the WSSV entry into host cells by loss-of-function of *CqLR-like* gene (Fig. 4B). Meanwhile, the transcript of both the immediate early gene IE1 and the late viral gene VP28 of WSSV exhibited significant decrease than that of control groups at both 3 h and 6 h, respectively, after the viral infection in *CqLR-like* gene silenced Hpt cells (Fig. 4C). These results together implied that the presence of *CqLR-like* gene had clear positive effect on WSSV entry into Hpt cells of red claw crayfish. It has been reported that LR participated in tumor metastasis and prion diseases in cultured cells (Chen et al., 2009; Leucht et al., 2003; Shammas et al., 2006). Loss-of-function of LR resulted in lower viral infection efficiency of grass carp reovirus in ctenopharyngodon idella kidney cells from grass carp (Wang et al., 2016). Besides, the classical swine fever virus (CSFV) titer was significantly reduced after gene silencing of LR in porcine kidney cell lines (PK-15 and SK6 cells) (Chen et al., 2015). Taken together, our findings suggested that *CqLR-like* gene might serve as a receptor or key co-factor in the WSSV entry into host cells.

### 3.4. Prokaryotic expression and purification of recombinant CqLR-like protein

To explore the biological activity of *CqLR-like* protein, two recombinant *CqLR-like* (rCqLR-like) proteins with His-fusion tag and GST-fusion tag, respectively, were produced in *E. coli* (BL21:DE3). Two rCqLR-like proteins were named CqLR-like-His and CqLR-like-GST. The CqLR-like-His protein was purified via single-step purification using a Ni-NTA affinity column and the CqLR-like-GST protein was purified using glutathione sepharose 4B resins. As shown in Fig. 5, the CqLR-like-His was approximately 44 kDa in molecular weight and the CqLR-like-GST was about 70 kDa, which were both similar to that of a shrimp LR recombinant protein expressed in an *E. coli* expression system (Busayarat et al., 2011; Liu et al., 2016). Besides, the purity of two rCqLR-like proteins were more than 90% (Fig. 5) and the deduced recombinant protein sequence of rCqLR-like proteins was further confirmed with several peptide fragments corresponding to *CqLR-like* protein by MALDI-TOF/TOF mass spectrometry analysis (data not shown).

### 3.5. rCqLR-like protein bound to WSSV in a dose dependent manner via interaction with VP28

As mentioned above, *CqLR-like* gene strongly interfered with

*Lineus viridis* (ABZ04275.1); *Callorhinchus milii* (AFM85544.1); *Bombyx mori* (NP_001106143.1); *Drosophila melanogaster* (AAA28667.1); *Danio rerio* (AAQ91246.1). (B) Amino acid sequence alignment of CqLR-like gene with other known LRs: *L. vannamei*, *P. monodon*, *H. sapiens*, *M. musculus*, *D. rerio*, *D. melanogaster* and *M. meretrix*. The conserved laminin-binding site is shown by a black line.

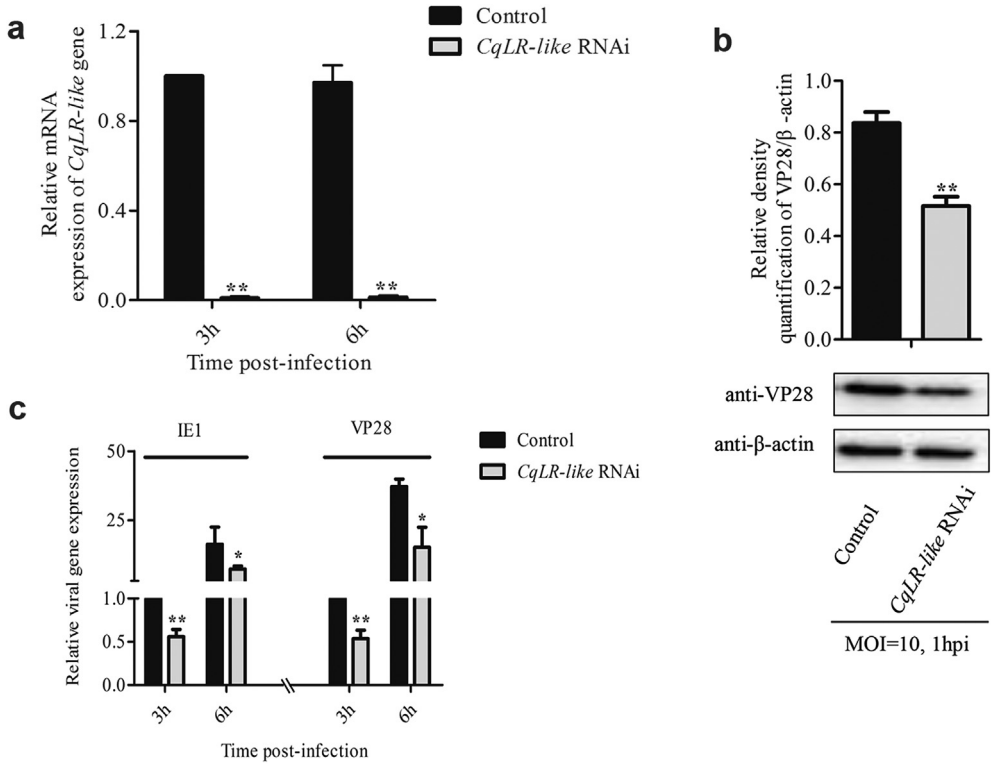


**Fig. 4.** Reduced WSSV entry and viral replication by gene silencing of *Cq*-LR-like gene in crayfish Hpt cells. (A) The mRNA expression of *CqLR*-like gene during WSSV infection was determined by qRT-PCR. The expression of *CqLR*-like gene was reduced significantly compared to control groups during WSSV infection. GFP dsRNA treatment was used as the control groups. (B) Inhibition on WSSV entry by gene knocking down of *CqLR*-like gene: the major viral envelope protein VP28 was immunoblotted with a monoclonal antibody against VP28 at 1 hpi. (C) Relative gene expression of a viral immediate early gene IE1 and a late gene VP28 were examined, respectively, in *CqLR*-like gene silenced Hpt cells post WSSV challenge at 3 h and 6 h. The relative transcript levels in GFP control of 1hpi were employed as the calibrator (value set as 1). This experiment was repeated for three times. The asterisk indicated significant difference compared with those of controls ($^{*}p < 0.05$, $^{**}p < 0.01$).

WSSV entry at an early stage of viral infection. According to the literature that LR was mainly located on the cell membrane and serves as a cell surface receptor in HeLa cells (Gauczynski et al., 2001), kidney cell of grass carp (Wang et al., 2016) and porcine kidney cell (Chen et al., 2015), we speculated that *CqLR-like* protein might be recruited as a receptor or key co-factor to recognize WSSV and then mediate the viral infection. To determine the possible interaction between rCqLR-like protein and WSSV in vitro, the CqLR-like-His and WSSV binding assay was performed by using ELISA. As shown in Fig. 6, the binding affinity of CqLR-like-His to WSSV was clearly enhanced as accompanied with the increased concentrations of CqLR-like-His protein in a dose dependent manner (from 0.1 to 1.6 μg) if compared to that of GFP-His control protein, indicating that *CqLR-like* protein was able to bind directly to WSSV virion.

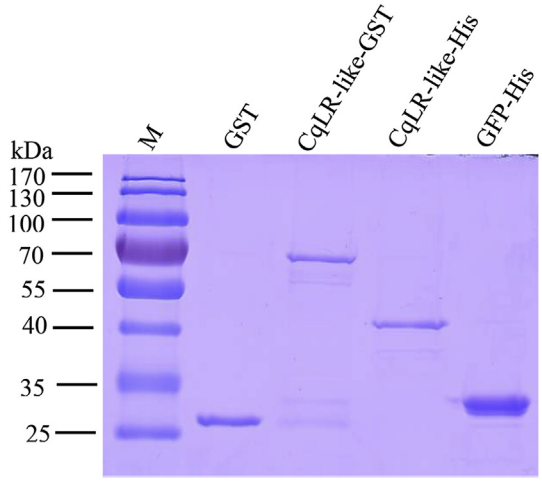


**Fig. 5.** SDS–PAGE analysis of purified recombinant GST, CqLR-like-GST, CqLR-like-His and GFP-His protein. Lane M: molecular weight marker (kDa).

As one of the four major WSSV envelope proteins, VP28 is located on the viral envelope surface, which helps to adhere to the surface of the host cell, and helps WSSV enter into the cell (Yi et al., 2004); VP24 has been recognized as a core protein to associate with other structural protein partners and form an envelope protein complex, serving as a hub protein to function in cell recognition, cell attaching and guidance of virus entry (Li et al., 2011; Sun et al., 2016); VP26 is located on the surface of WSSV and act as an adapter protein between the membrane protein and the nucleocapsid protein (Tang et al., 2007); VP19 is also an envelope protein, which contains two putative transmembrane domains, and may anchor this protein in the WSSV envelope (van Hulten et al., 2002). To further explore which key envelope protein(s) of WSSV could be recognized by CqLR-like protein, the pull down assay was carried out between CqLR-like-GST and four major WSSV envelope proteins, including VP28, VP26, VP24 and VP19, respectively. As indicated in Fig. 7, VP28, but not VP19, VP24 or VP26, was pulled down by CqLR-like-GST, clearly suggesting that *CqLR-like* protein could bind to the envelope protein VP28 of WSSV. This result differed from the shrimp LR binding to WSSV, in which a *PmLR* was reported to bind to the WSSV structural protein VP31, and *PmLR* was found to be co-localized with VP31 in *Drosophila* S2 cells by immunofluorescence assay (Liu et al., 2016). In consideration to that VP28 may

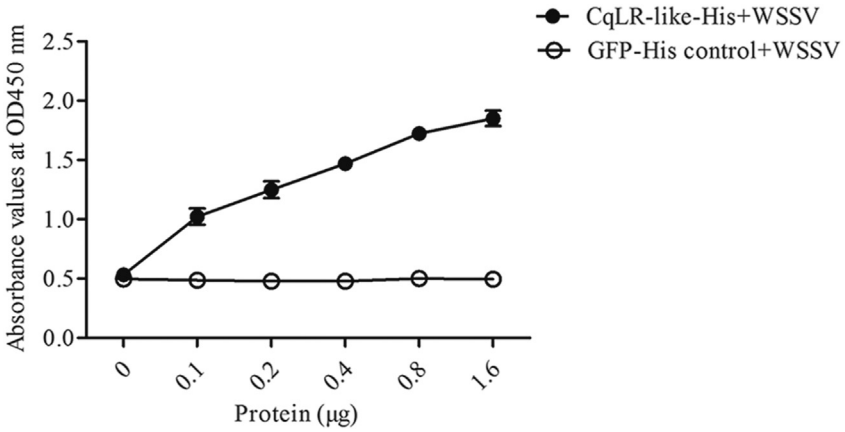


**Fig. 6.** Determination of rCqLR-like protein bound to WSSV virion by ELISA assay. Binding of CqLR-like-His to WSSV was in a protein dose-dependent manner. WSSV ($10^7$ copies/well) was coated to a 96-well plate and incubated with various amounts of purified CqLR-like-His. GFP-His was added as negative control. Mean (±SEM) is from three independent experiments.

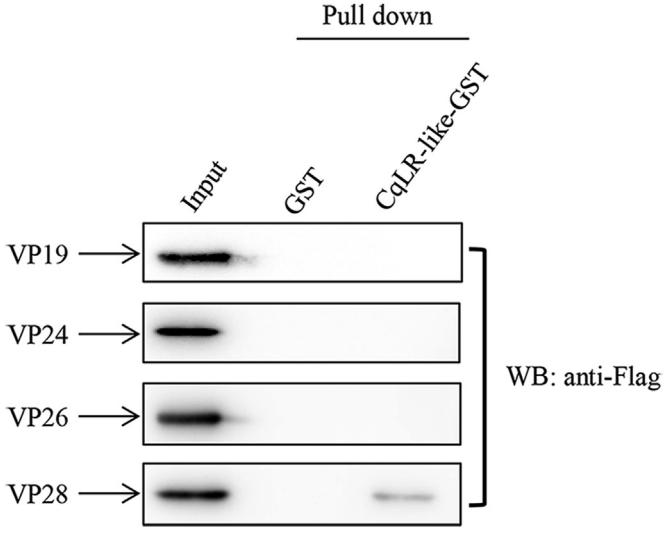


**Fig. 7.** Protein interaction between rCqLR-like protein and WSSV envelope proteins (VP19, VP24, VP26 and VP28). HEK 293T cells were transiently co-transfected with the FLAG-tag VPs. At 72 h after transfection, cells were lysed and subjected to pull down assay with glutathione MagBeads and CqLR-like-GST followed by Western blot (WB) determination with anti-FLAG antibody. The whole cell lysates (Input) were used to confirm the successful expression of VPs in HEK 293T cells.

rivet on the surface of the host cell membrane to assist WSSV infection (Tang et al., 2007), we thus speculated that *CqLR-like* protein, possibly as a cellular receptor or co-factor, may interact with VP28 and mediate the process of WSSV entry into Hpt cells, which needs further studies such as localization of *CqLR-like* protein on Hpt cell membranes as well as its co-localization with WSSV particle on the Hpt cell surface or not.

## 4. Conclusion

In summary, we identified a laminin-receptor-like gene *CqLR-like* from red claw crayfish *C. quadricarinatus*. Functional study revealed that *CqLR-like* protein could recognize the WSSV by binding to viral envelope protein VP28 and loss-of-function of *CqLR-like* gene clearly reduced both the WSSV entry and the viral replication in the crayfish Hpt cells. Taken together, these data strongly suggested that *CqLR-like* protein was likely to act as a key viral recognition molecule involved in the WSSV infection, which can be employed as a putative blocking target against WSSV entry and further benefit the control of WSSV disease in aquaculture.

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