WSV399, a viral tegument protein, interacts with the shrimp protein PmVRP15 to facilitate viral trafficking and assembly

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Viral responsive protein 15 (PmVRP15) has been identified as a highly up-regulated gene in the hemocyte of white spot syndrome virus (WSSV)-infected shrimp Penaeus monodon. However, the function of PmVRP15 in host–viral interaction was still unclear. To elucidate PmVRP15 function, the interacting partner of PmVRP15 from WSSV was screened by yeast two-hybrid assay and then confirmed by co-immunoprecipitation (Co-IP). Only WSV399 protein was identified as a PmVRP15 binding protein; however, the function of WSV399 has not been characterized. Localization of WSV399 on the WSSV virion was revealed by immunoblotting analysis (in vitro) and immunoelectron microscopy (in vivo). The results showed that WSV399 is a structural protein of the WSSV virion and is particularly located on the tegument. Gene silencing of WSV399 in WSSV-infected shrimp reduced the percentage of cumulative mortality by 74%, although the expression level of a viral replication marker gene, vp28, was not changed suggesting that WSV399 might not involved in viral replication but viral assembly. Because it has already been known that tegument proteins function in capsid transport during viral trafficking and assembly, interaction between PmVRP15 on hemocyte nuclear membrane and the WSV399 viral tegument protein suggests that PmVRP15 might be required for trafficking and assembly of WSSV during infection.

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1. Introduction

White spot syndrome virus is one of the most severe shrimp pathogens. WSSV-infected shrimp population can reach a cumulative mortality of 100% within 3–10 days (Durand et al. 1997). The WSSV virion contains a rod-shaped nucleocapsid, typically measuring 65–70 nm in diameter and 210–350 nm in length. The nucleocapsids, which contain a DNA-protein core bound by a distinctive capsid layer giving it a cross-hatched appearance, are wrapped singly into an envelope to shape the virion (Durand et al. 1997; Nadala and Loh, 1998). About 40 WSSV proteins have been characterized (Escobedo-Bonilla et al. 2008). Most of these proteins are structural proteins in the envelope (VP12B, VP13B, VP14, VP19, VP28, VP31, VP32, VP33, VP38A, VP39B, VP41A, VP41B, VP51A, VP51B, VP53A, VP60A, VP90, VP110, VP124, VP180 and VP187), tegument (VP12A, VP26, VP36A, VP39A and VP95) and nucleocapsid (VP15, VP24, VP35, VP51C, VP60B, VP75, VP76, VP136A, VP190 and VP664). Some WSSV proteins that act as non-structural proteins are probably involved in transcriptional regulation (VP9), virus proliferation (WSV021) and regulation of DNA replication (WSV477). To date, many WSSV proteins are still uncharacterized.

Several WSSV-binding proteins have been identified in shrimp but their roles in signaling pathways related to immunity are still unclear. Viral-binding proteins are categorized into two types based on interaction with the virus: non-specific interaction and specific interaction. Non-specific interacting proteins like hemocyanin can bind to WSSV virion and act as antiviral factors of Penaeus monodon (Zhang et al. 2004). Meanwhile, many WSSV-binding proteins with specific interactions between host and viral protein have been reported. For example, the interaction between a major nucleocapsid
protein of WSSV like VP15 with PmFKB846 has been described and thought to be involved in genome packaging during virion assembly (Sangsuriya et al. 2011). Interaction between VP26, a major tegument protein of WSSV, with shrimp cytoskeletal protein β-actin and 3 kDa WSSV-binding protein (WB) was identified (Xie and Yang, 2005, Liu et al. 2011; Youtong et al. 2011) and its important role in WSSV infection was also revealed. The endosomal protein from the hemocytes of P. monodon, PmRab7, was identified as VP28-binding protein (Sritunyalucksana et al. 2006). PmRab7 gene silencing resulted in the decrease in WSSV replication suggesting that PmRab7 functions as an important regulator of intracellular trafficking (Ongvarrasopone et al. 2008). PlgC1qR, a receptor for globular head domain of complement component C1q, could bind to VP15, VP26, and VP28 of WSSV and possibly play a role in controlling antiviral mechanism (Watthanasurorot et al. 2010).

From our previous report (Vatanavicharn et al. 2014), a viral responsive gene, PmVRP15 that is highly abundant in WSSV-infected shrimp, was identified by suppression subtractive hybridization. PmVRP15 encodes for a deduced 137 amino acid protein containing a putative transmembrane helix and located around the nuclear membrane in shrimp hemocyte. The silencing of PmVRP15 gene in P. monodon significantly decreased gene expression and cumulative mortality suggesting its role in WSSV propagation pathway. Moreover, PmVRP15 also called PmERP15 was found to be involved in endoplasmic reticulum (ER) stress triggered by WSSV infection. From the results, PmERP15 was induced by ER stress and located in the ER. After silencing PmERP15 in WSSV-infected shrimp, the viral copy number did not change in the shrimp gill while the cumulative mortality was lower than control group. It was concluded that although PmERP15 was not involved in WSSV replication, it was essential for survival of WSSV-infected shrimp (Leu et al. 2015).

Despite the previously mentioned studies, the function of PmVRP15 protein was still unclear. To characterize the involvement of PmVRP15 in WSSV infection in shrimp, the interactions between PmVRP15 and viral proteins, were identified in this study. Also, the implication of the PmVRP15-binding protein in viral infection was revealed.

2. Materials and methods

2.1. Construction of bait plasmids containing the N-, C-terminus and open reading frame (ORF) of PmVRP15 gene

For yeast two-hybrid screening, three PmVRP15 bait vector containing ORF, N-terminus fragment, and C-terminus fragment, were constructed. Firstly, the open reading frame (ORF), N- and C-terminus fragment of PmVRP15 gene were amplified by PCR using gene specific primers (Table 1). The PCR conditions were 94 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min using RBC Taq polymerase (RBC Biosience). The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and purified using Nucleospin® Extract II kit (Macherery-Nagel). The purified PCR products were double-digested with restriction enzymes and cloned into-frame into pGBK7-7 vector, a bait vector; cut with the same restriction enzymes and transformed into an Escherichia coli XL1-Blue and then induced with 1 mM IPTG for 1 h to over-produce the recombinant PmVRP15 protein. The single colony of E. coli strain C43 (DE3) containing recombinant plasmid pET22b-PmVRP15 (a gift from Assist. Prof. Dr. Kuakarin Kruosong) was cultured in LB-amp medium with shaking at 250 rpm at 37 °C for overnight as a starter. The overnight culture was inoculated into fresh LB-amp medium until OD600 reached 0.5 then induced with 1 mM IPTG for 1 h to over-produce the recombinant PmVRP15. The cell pellet was collected, resuspended in 50 mM Tris–HCl, pH 7.0 and sonicated with a Bransonic 32 (Bandelin). The soluble fraction containing recombinant PmVRP15 was collected by centrifugation at 10,000 rpm for 20 min, and further centrifuged to isolate the membrane protein part of E. coli that contain PmVRP15 by ultracentrifugation at 100,000 × g for 1 h. The resulting pellet was homogenized in ice-cold solubilization buffer (50 mM Tris–HCl, pH 7.0, 20 mM Imidazole, 300 mM NaCl, 1% dodecyl-L-D-maltoside (DM) and 20% glycerol) at 4 °C for overnight. The crude supernatant of PmVRP15 protein was finally collected by ultracentrifugation at 100,000 × g for 30 min and subjected to purification through Ni-NTA column (GE healthcare). Protein was loaded onto the Ni-NTA column pre-equilibrated in the equilibration buffer (50 mM Tris–HCl, pH 7.0, 20 mM Imidazole, 0.1% DM and 10% glycerol) and incubated at 4 °C for 2 h. The column was washed with washing buffer (50 mM Tris–HCl, pH 7.0, 50 mM Imidazole, 0.1% DM and 5% glycerol) and PmVRP15 was eluted by elution buffer (50 mM Tris–HCl, pH 7.0, 300 mM Imidazole, 0.1% DM and 5% glycerol). The elution fraction was subjected to Amicon® Ultra-4 Centrifugal Filter Units cut-off 3 kDa (Millipore) to concentrate and exchange buffer to 10 mL Tris–HCl, pH 7.0, 0.07% DM and 2.5% glycerol. The purified protein was analyzed using 15% SDS-PAGE. The protein concentration was determined using the Bradford method.

For recombinant WSV399 protein production, WSV399 gene was amplified by PCR using gene specific primers with XhoI and EcoRI restriction sites, WSV399-XhoI-F and WSV399-EcoRI-R (Table 1). The PCR conditions were 94 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final
extension at 72 °C for 5 min using RBC Tag polymerase (RBC Bioscience). The purified PCR product was double digested with XhoI and EcoRI (New England Biolabs) and cloned into XhoI/EcoRI-digested pBAD/Myc-His-A (Invitrogen) and finally transformed into an E. coli TOP10. The recombinant plasmids were subjected to nucleotide sequencing (Macrogen Inc., Korea). The recombinant clone in the expression host was cultured and induced with 0.1% L-arabinose to over-produce the recombinant WSV399 (rWSV399). After induction for 4 h, cells were collected and resuspended in 1× PBS, pH 7.4 then sonicated. The crude soluble rWSV399 protein was collected and purified through Ni-NTA column (GE Healthcare) pre-equilibrated with 50 mM Sodium phosphate buffer, pH 7.4, 300 mM NaCl, and 10 mM Imidazole. Upon 2 h incubation at room temperature for 1 h. The bands of rWSV399 were collected and purified through Ni-NTA column (GE Healthcare) pre-equilibrated with 50 mM Sodium phosphate buffer, pH 7.4, 300 mM NaCl, and 20 mM Imidazole. The purified protein from elution fractions was selected and dialyzed against 1× TBS, pH 7.4. The protein was analyzed using 12.5% SDS-PAGE. The concentration of protein was determined using the Bradford method.

### 2.4. Co-immunoprecipitation (Co-IP)

The co-immunoprecipitation reaction was performed according to instructions of the Pierce c-Myc Tag IP/Co-IP Kit (Thermo scientific). The interaction between WSV399 and PmVRP15 was examined by incubating Myc-His-rWSV399 protein with His-rPmVRP15 at 30 °C for 2 h with gentle rocking. The anti-c-Myc antibody conjugated protein A beads (10 µl of 50% bed slurry) was incubated with protein mixture above at 4 °C for overnight with gentle rocking. The beads were washed 10 times with 500 µl of wash buffer (1× TBS) to remove non-specific binding protein. The supernatant obtained after adding SDS-loading sample buffer and heating at 100 °C for 15 min, was collected by centrifugation. Then, protein–protein complex was analyzed by SDS-PAGE and western blot analysis. For western blot analysis, the anti-c-Myc antibody (Clontech) and anti-His antibody (GE Healthcare) were used as primary antibodies for rWSV399 and PmVRP15 detection, respectively. The anti-c-Myc antibody diluted 1:7000 fold in 1× PBS-T (0.05% Tween-20 in 1× PBS buffer: 137 mM NaCl, 2.7 mM, 10 mM Na2HPO4, 2 mM K2HPO4, pH 7.4), was incubated with membrane at 37 °C for 3 h after blocking. The secondary antibody, an anti-mouse IgG conjugated alkaline phosphatase (diluted 1:5000) (Jackson ImmunoResearch Laboratories, Inc.) and the substrate NBT/BCIP solution, were used to detect rWSV399. The positive purple band size of about 26 kDa was detected. The membrane was then washed with 1× PBS-T for 3 times at 15 min each before incubating with the anti-His antibody diluted 1:3000 fold in 1× PBS-T at 37 °C for 3 h. This antibody can bind to both rPmVRP15 and rWSV399 proteins. Then, the secondary antibody, anti-mouse IgG conjugated alkaline phosphatase (diluted 1:7000 fold in 1× PBS-T) was incubated at room temperature for 1 h. The bands of rPmVRP15 (15 kDa) and rWSV399 (26 kDa) protein were identified after adding NBT/BCIP solution.

The control experiments were done simultaneously with the WSV399-PmVRP15 interaction experiment. They were performed as mentioned except that the anti-c-Myc antibody conjugated protein A bead was incubated with either rPmVRP15 or rWSV399.

### 2.5. Localization of WSV399 protein on WSSV intact virion

To localize viral protein on WSSV virion, the purified WSSV was prepared from WSSV infected-fresh water crayfish, Procambarus clarkii as described by (Xie et al. 2005). To determine where WSV399 is located on WSSV virion, envelope, tegument or nucleocapsid, the envelope and nucleocapsid proteins were separated by TritonX-100 treatment (Xie et al. 2006).

Ten micrograms of the purified WSSV virions and protein fraction of envelope and nucleocapsid were separated on 12.5% SDS-PAGE, and then transferred to a polyvinylidene difluoride (PVDF)
membrane. The membranes were incubated in blocking buffer containing 5% skim milk in 1X TBS-T (0.5% Tween 20 in Tris-buffered saline) at 4 °C overnight, followed by incubation with a purified polyclonal mouse anti-WSV399 antibody (1: 2,500 dilution in 2.5% skim milk, 1X TBS-T) for 3 h at room temperature. After the membrane was washed three times with TBS-T, it was incubated with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories, Inc.) diluted 5,000-fold in 2.5% skim milk in 1X TBS-T for 1 h at room temperature. The membrane was washed as described above, and protein band specific to WSV399 was detected by a chemiluminescence reagent, Western Lightning® Plus-ECL, (Perkin-Elmer, Inc.) followed by exposure the membrane to film at the appropriate time. The result can be visualized after film exposure and development.

According to the method described previously (Leu et al. 2005), a purified WSSV virion suspension was assayed to formvar-supported and carbon-coated nickel grids (150 mesh) and incubated for 5 min at room temperature. The primary antibody and pre-immune mouse serum were diluted 1:50 in an incubation buffer (0.1%Aurion Basic-c, 15 mM NaN₃, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4). The grids were blocked with the blocking buffer (5% bovine serum albumin, 5% normal serum, 0.1% cold water skin gelatin, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 15 min and then incubated with a diluted primary antibody or pre-immune mouse serum for 3 h at room temperature. After several washes with incubation buffer, the grids were incubated with a goat anti-mouse IgG secondary antibody conjugated with 18-nm-diameter gold particles (1:50 dilution in the incubation buffer) for 1 h at room temperature. The grids were then washed extensively with incubation buffer, washed twice more with distilled water to remove excess salt, and stained with 1% phosphotungstic acid (pH 7.2) for 2 min. Specimens were examined with a Transmission electron microscope (TEM, Hitachi/S-4800).

2.6. wsv399 gene expression profile in WSSV-infected shrimp hemocyte using RT-PCR technique

Semi-quantitative RT-PCR was used to examine the expression of wsv399 gene in WSSV-infected shrimp at various time points. P. monodon (approximately 15 g body weight) were injected with 100 μl of the diluted WSSV solution (a dose that causes 100% mortality of shrimp in 3 dpi). Hemocytes of 3 WSSV-infected shrimp were collected individually at 0, 3, 6, 12, 24, 36 and 48 h post infection (hpi). Total RNA was extracted and treated with RNase-free DNase to remove any residual DNA contamination. Total RNA concentration was determined using spectrophotometer. An equal amount of DNA-free total RNA from each individual was used for the first strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis kit (Fermentas). Wsv399 gene expression was analyzed by RT-PCR using WSV399-RT-F/R primers (Table 1). The EF-1α gene was determined by semi-quantitative RT-PCR using gene specific primers VP28-F/R (Table 1) to verify the viral replication in shrimp. The PCR conditions were 94 °C for 1 min, followed by 27 cycles of 95 °C for 30 s, 58 °C (for EF-1α) or 60 °C (for wsv399 and vp28) 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

Moreover, the percentage of cumulative mortality was also investigated after knocking down wsv399 gene in WSSV-infected shrimp. Ten P. monodon shrimps of approximately 3 g body weight per group were injected with wsv399 dsRNA or GFP dsRNA as above. The dosage of WSSV used in this experiment causes 100% mortality of shrimp in 3 dpi. The shrimp mortality was observed every 6 h after WSSV infection. This experiment was done in triplicate.

3. Results

3.1. Identification of PmVRP15-interacting protein by yeast two-hybrid screening

From our previous result (Vatanavicharn et al. 2014), PmVRP15 was shown to be involved in WSSV propagation. To better understand PmVRP15 function in WSSV infection in shrimp, yeast two-hybrid screening was used to identify its interacting-partner protein from WSSV. Firstly, the bait plasmids, pGBK7-PMvrp15, pGBK7-T-terPMvrp15 and pGBK7-C-terPMvrp15 were constructed and transformed into yeast cell, S. cerevisiae AH109. The toxicity and auto-activation were investigated. The results showed no toxicity and auto-activation after PmVRP15 protein expression (data not shown). Next, bait plasmid was used to screen for the interacting partner on the prey library containing WSSV ORF genes by yeast mating. The interaction between PmVRP15 and its protein partner was confirmed by co-transformation. Although, no interaction between PmVRP15 or C-terPmVRP15 with WSSV ORF were identified, interaction between a WSSV protein with N-terPmVRP15 was found (Fig. 1A). The prey plasmid was then extracted from the positive clone and identified by DNA sequencing. Searching against GenBank database using BLASTX showed a 100% sequence match to WSV399 (Accession No. NP_477921). Because no information about WSV399 function is available, further characterization is thus needed.
3.2. The production and purification of recombinant protein PmVRP15 and its interacting protein

To confirm the interaction between PmVRP15 and the identified interacting partner, the recombinant proteins of PmVRP15 and WSV399 were produced. E. coli strain C43 (DE3) containing pET22b-PmVRP15 was cultured and induced for rPmVRP15 expression by IPTG. The rPmVRP15 protein with the expected size of about 15 kDa could be detected by SDS-PAGE and western blot analysis using anti-His antibody (Fig. 1B). The membrane fraction containing rPmVRP15 was separated from soluble fraction, solubilized, and purified by Ni-NTA affinity column. After buffer-exchanged and concentrated, the purified rPmVRP15 was analyzed by 12.5% SDS-PAGE and used for further experiment.

The recombinant plasmid WSV399, pBAD-Myc/His containing WSV399, was constructed and transformed into E. coli strain TOP10. Production of the rWSV399 protein after L-arabinose induction at 4 h was confirmed by SDS-PAGE and western blot technique using antibody specific to the c-Myc tag. The expected size of rWSV399 is about 26 kDa as shown in Fig. 1B. The rWSV399 expressed in the soluble form was purified by Ni-NTA affinity column and determined by 12.5% Coomassie stained SDS-PAGE (Fig. 1B). The purified rWSV399 was further concentrated and buffer-exchanged before used.

3.3. Confirmation of PmVRP15 and WSSV protein interaction by co-immunoprecipitation (Co-IP)

To confirm the interaction between PmVRP15 and WSV399, co-immunoprecipitation assay was performed. Recombinant proteins of PmVRP15 (rPmVRP15) containing His6-tag and WSV399 (rWSV399) containing c-Myc tag and His6-tag were produced. In this experiment, the rPmVRP15 and rWSV399 were mixed and incubated with anti-Myc conjugated protein A bead. After unbound protein was removed by extensive wash, rPmVRP15-WSV399 complex was eluted and analyzed by western blot analysis (Fig. 1C). The rPmVRP15 and rWSV399 could be detected in the eluted complex indicating their true interaction. In the control experiments, the anti-Myc conjugated protein A bead incubated with either rPmVRP15 or rWSV399 only was analyzed by western blot simultaneously to confirm the specificity (Fig. 1C).

3.4. wsv399 gene expression profile in WSSV-infected shrimp hemocyte using RT-PCR

The expression profile of wsv399 gene in WSSV-infected shrimp hemocyte using RT-PCR...
P. monodon was investigated to further characterize wsv399 gene. In this study, the viral genes at different stages of viral replication, which are ie-1 (immediate early stage), wsv477 (early stage) and vp28 (late stage), were used as marker genes. The gene expression level of these viral genes in the hemocytes of WSSV-infected shrimp at 0, 3, 6, 12, 24, 36 and 48 hpi was analyzed by RT-PCR using EF-1α as an internal control. We found that wsv399 showed similar gene expression pattern as vp28 gene indicating that wsv399 transcript was a late gene (Fig. 2).

3.5. Localization of WSV399 protein on intact WSSV

To localize WSV399 on the WSSV virion, purified WSSV protein was separated by 12.5% SDS-PAGE and western blot analysis using anti-WSV399 antibody. The result showed that WSV399 is a structural protein (Fig. 3A), WSSV fractions, envelope and nucleocapsid were prepared to determine where WSV399 protein is located, whether on the envelope, nucleocapsid, or tegument part of WSSV virion. Immunoblot analysis revealed that WSV399 could be detected on both envelope and nucleocapsid fractions (Fig. 3B) indicating that WSV399 is possibly a WSSV tegument protein. The separation efficiency was determined by detecting VP28 protein, a known major envelope protein, using western blot analysis (data not shown). Moreover, the localization of WSV399 protein on the WSSV virion was confirmed by IEM. The WSV399 protein was detected using anti-WSV399 antibody and anti-mouse IgG conjugated with 18 nm-diameter gold particles. The electron micrograph showed that the position of gold particle labeled-WSV399 protein was located on the tegument part of WSSV (Fig. 3C). WSV399 is, therefore, a tegument protein of WSSV.

3.6. Effect of WSV399 gene knockdown in WSSV-infected shrimp

The involvement of wsv399 in WSSV replication was investigated by wsv399 gene silencing in WSSV-infected shrimp. The wsv399 gene knockdown did not affect the level of vp28 gene expression as compared to that of control shrimp injected with GFP dsRNA (Fig. 4A). We concluded that wsv399 might not be involved in WSSV replication. However, the wsv399 might have other functions. Therefore, the shrimp cumulative mortality upon wsv399 knockdown and WSSV infection was investigated in comparison with the control; WSSV-infected-shrimp with dsRNA GFP injection. At 72 hpi, the cumulative mortality of control group reached 100% whereas that of wsv399 knockdown shrimp was only about 26%, although it reached to 100% at 132 hpi (Fig. 4B). In this regard, wsv399 gene likely plays an important role in WSSV propagation in WSSV-infected shrimp. According to the results, the function of the PmVRP15-interacting WSSV tegument protein, WSV399, might be involved in viral assembly in WSSV-infected shrimp.

4. Discussion

Previously, PmVRP15 has been identified by suppression subtractive hybridization between normal and WSSV-infected shrimp hemocyte. PmVRP15 gene had highest up-regulation (9410 fold) in hemocyte at 48 h after WSSV infection (late stage of infection). PmVRP15 protein expression was localized on the nuclear membrane of both the uninfected and WSSV-infected shrimp hemocyte but highly induced in WSSV-infected shrimp hemocyte only. After WSSV infection, PmVRP15 protein was also over-expressed when compared with control and located around the nuclear membrane. After PmVRP15 gene knockdown, viral gene expression in various stages of WSSV infection, which are ie-1 for immediate early stage, wsv477 for early stage and vp28 for late stage, was reduced and also the cumulative mortality of WSSV-infected PmVRP15 knockdown shrimp was lower than control implying that PmVRP15 was involved in WSSV propagation in shrimp (Vatanavicharn et al. 2014). Recently, Leu et al. have characterized the function of PmVRP15 and found that it was induced by ER stress in WSSV-infected shrimp, finally conferring its name as ER stress-responsive protein or PmERP15. Silencing of this gene had no effect on WSSV multiplication but was deemed important for shrimp survival (Leu et al. 2015). The contrasting results of PmVRP15 and PmERP15 in the WSSV-infected-shrimps of the two studies may be attributed to different experimental parameters. In the experiments determining the effect of these genes on WSSV replication using RNA interference, parameters such as dose of WSSV infection, dose of PmVRP15 and PmERP15 dsRNA, shrimp tissue and time post infection, were different. The results of the two studies, although related, are not informative when compared. Agreeing results between PmVRP15 and PmERP15 were also find where the two genes were up-regulated and might be involved in WSSV infection in shrimp.

In the present study, PmVRP15-binding protein from WSSV was identified by yeast-two hybrid screening. When mature PmVRP15 protein was used to screen the WSSV ORF library, no interacting protein was found. This might be because the transmembrane structure of PmVRP15 protein may block the translocation of PmVRP15 into the nucleus and as a result, it cannot interact with other proteins and showed no transcription of the reporter gene. When the WSSV ORF library was screened with the N- and C-terminus fragments, which contain no transmembrane protein...
portion, WSV399 protein was found to have an interaction with \( Pm \) VRP15 N-terminus fragment only. Co-immunoprecipitation technique confirmed the interaction between the mature \( Pm \) VRP15 and WSV399 proteins.

The expression profile of \( wsv399 \) gene was characterized in hemocyte of WSSV-infected shrimp at various time points. The result showed that \( wsv399 \) gene was produced at 24 hpi, late stage of WSSV replication. Concordantly, \( Pm \) VRP15 gene expression profiling was also up-regulated at 24 hpi (Vatanavicharn et al. 2014). To date, limited information on WSV399 and its function has been found. Firstly, we performed WSV399 localization on WSSV virion by western blot analysis as well as IEM. WSV399 was found on both envelope and nucleocapsid parts of WSSV when virion was fractionated by Triton X-100 in low salt condition like other WSSV tegument proteins such as VP26 (the major tegument protein of WSSV), VP36A, VP39A and VP95. The known tegument proteins of WSSV, VP26 and WSV399 proteins, were detected on unenveloped WSSV virion by IEM (Tsai et al. 2006). We confirmed that WSV399 is, indeed, a tegument protein of WSSV.

Silencing \( wsv399 \) gene was performed in order to reveal its importance in viral replication. From the previous report, in Herpes Simplex Virus type 1, the functions of viral tegument proteins were characterized for: capsid transport during entry and egress; targeting of the capsid to the nucleus; nuclear egress of capsid; viral assembly and final egress (Kelly et al. 2009). In WSSV, the major tegument protein, VP26 can interact with \( \beta \)-actin, shrimp cytoskeleton protein (Xie and Yang, 2005). It was also evidenced that the host cytoskeleton protein was used in viral trafficking or viral assembly process (Lyman and Enquist, 2009). Because the \( wsv399 \) gene affected the cumulative mortality rate of WSSV-infected shrimp but did not alter viral gene expression, we hypothesized that WSV399 might be involved in viral trafficking or viral assembly.

In summary, \( Pm \) VRP15 is a highly WSSV-responsive transmembrane protein expressed in hemocyte. During WSSV infection, WSSV annexes a shrimp \( Pm \) VRP15 to facilitate infection via
Fig. 4. The effect of wsv399 gene silencing in WSSV-infected Penaeus monodon hemocytes. (A) Expression level of the WSSV gene, VP28, in wsv399 gene-silenced shrimp hemocytes were determined by RT-PCR. (B) The percentage of cumulative mortality rate of wsv399 gene knockdown in WSSV-infected shrimp (Grey line) was compared with that of the control, WSSV-infected GFP gene knockdown shrimp (Black line). Data are shown as the mean ± 1 S.D. and are derived from three independent repeats.

PmVRP15–WSV399 tegument protein interaction. The involvement of WSV399 in WSSV replication implied that PmVRP15 might play a role in viral entry and egress from cytoplasm to nucleus in P. monodon. This mechanism, however, needs to be further elucidated.

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