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

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4 Phattarunda Jaree^a, Saengchan Senapin^{b,c}, Ikuo Hirono^d, Chu-Fang Lo^e, Anchalee
5 Tassanakajon^a, and Kunlaya Somboonwiwat^{a*}

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7 ^a*Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry,*
8 *Faculty of Science, Chulalongkorn University, Phayathai Rd., Bangkok 10300, Thailand*

9 ^b*Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, Rama VI*
10 *Rd., Bangkok 10400, Thailand*

11 ^c*National Center for Genetic Engineering and Biotechnology, National Science and Technology*
12 *Development Agency, Pathumthani 12120, Thailand*

13 ^d*Laboratory of Genome Science, Graduate School of Marine Science and Technology, Tokyo*
14 *University of Marine Science and Technology, Tokyo, Japan*

15 ^e*Institute of Bioinformatics and Biosignal Transduction, College of Bioscience and Biotechnology,*
16 *National Cheng Kung University, Tainan, Taiwan, Republic of China*

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18

19 *Corresponding author. Tel.: +66 2218 5438.

20 E-mail address: kunlaya.s@chula.ac.th (K. Somboonwiwat).

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23 **1. Introduction**

24 White spot syndrome virus is one of the most severe shrimp pathogens.
25 WSSV-infected shrimp population can reach a cumulative mortality of 100% within
26 3–10 days (Durand *et al.* 1997). The WSSV virion contains a rod-shaped
27 nucleocapsid, typically measuring 65-70 nm in diameter and 210-350 nm in length.
28 The nucleocapsids, which contain a DNA-protein core bound by a distinctive capsid
29 layer giving it a cross-hatched appearance, are wrapped singly into an envelope to
30 shape the virion (Durand *et al.* 1997, Nadala and Loh 1998). About 40 WSSV
31 proteins have been characterized (Escobedo-Bonilla *et al.* 2008). Most of these
32 proteins are structural proteins in the envelope (VP12B, VP13B, VP14, VP19, VP28,
33 VP31, VP32, VP33, VP38A, VP39B, VP41A, VP41B, VP51A, VP51B, VP53A,
34 VP60A, VP90, VP110, VP124, VP180 and VP187), tegument (VP12A, VP26,
35 VP36A, VP39A and VP95) and nucleocapsid (VP15, VP24, VP35, VP51C, VP60B,
36 VP75, VP76, VP136A, VP190 and VP664). Some WSSV proteins that act as non-
37 structural proteins are probably involved in transcriptional regulation (VP9), virus
38 proliferation (WSV021) and regulation of DNA replication (WSV477). To date, many
39 WSSV proteins are still uncharacterized.

40 Several WSSV-binding proteins have been identified in shrimp but their roles
41 in signaling pathways related to immunity are still unclear. Viral-binding proteins are
42 categorized into two types base on interaction with the virus: non-specific interaction
43 and specific interaction. Non-specific interacting proteins like hemocyanin can bind to
44 WSSV virion and act as antiviral factors of *Penaeus monodon* (Zhang *et al.* 2004).
45 Meanwhile, many WSSV-binding proteins with specific interactions between host
46 and viral protein have been reported. For example, the interaction between a major
47 nucleocapsid protein of WSSV like VP15 with PmFKBP46 has been described and

48 thought to be involved in genome packaging during virion assembly (Sangsuriya *et al.*
49 2011). Interaction between VP26, a major tegument protein of WSSV, with shrimp
50 cytoskeletal protein β -actin and 3 kDa WSSV-binding protein (WBP) was identified
51 (Xie and Yang 2005b, Liu *et al.* 2011, Youtong *et al.* 2011) and its important role in
52 WSSV infection was also revealed. The endosomal protein from the hemocytes of *P.*
53 *monodon*, PmRab7, was identified as VP28-binding protein (Sritunyalucksana *et al.*
54 2006). PmRab7 gene silencing resulted in the decrease in WSSV replication
55 suggesting that PmRab7 functions as an important regulator of intracellular
56 trafficking (Ongvarrasopone *et al.* 2008). PlgC1qR, a receptor for globular head
57 domain of complement component C1q, could bind to VP15, VP26, and VP28 of
58 WSSV and possibly play a role in controlling antiviral mechanism (Watthanasurorot
59 *et al.* 2010).

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

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

78

79 **2. Materials and Methods**

80 *2.1 Construction of bait plasmids containing the N-, C- terminus and open reading* 81 *frame (ORF) of PmVRP15 gene*

82 For yeast two-hybrid screening, three *PmVRP15* bait vector containing ORF,
83 N-terminus fragment, and C-terminus fragment, were constructed. Firstly, the open
84 reading frame (ORF), N- and C-terminus fragment of *PmVRP15* gene were amplified
85 by PCR using gene specific primers (Table 1). The PCR conditions were 94 °C for 1
86 min, followed by 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30
87 sec, and then a final extension at 72 °C for 5 min using RBC Taq polymerase (RBC
88 Bioscience). The PCR products were analyzed by 1.5 % (w/v) agarose gel
89 electrophoresis and purified using Nucleospin® Extract II kit (Macherey-Nagel). The
90 purified PCR products were double-digested with restriction enzymes and cloned in-
91 frame into pGBKT7 vector, a bait vector, cut with the same restriction enzymes and
92 transformed into an *Escherichia coli* XL1-blue and then selected on LB agar plate
93 containing 30 µg/ml kanamycin. The recombinant plasmids were subjected to
94 nucleotide sequencing to verify the sequences of inserts (Macrogen Inc., Korea).

95

96 *2.2 Yeast two-hybrid screening*

97 Yeast two-hybrid library construction and screening were performed.
98 *Saccharomyces cerevisiae* AH109 was co-transformed with pGBKT7-*PmVRP15*,
99 pGBKT7-N-ter*PmVRP15*, or pGBKT7-C-ter*PmVRP15* (bait vectors) and the prey
100 pGADT7-WSSV ORF library (Sangsuriya *et al.* 2014). The transformants were
101 selected on a synthetic defined (SD) medium lacking leucine and tryptophan (SD/-
102 Leu/-Trp). Interaction between the bait and prey fusion proteins was indicated by the
103 growth and blue color change on the double dropout SD/-Leu/-Trp media containing
104 X-alpha-Gal (DDO/X). In order to ascertain that the positive clones contained the
105 interacting proteins, the positive blue colonies were subsequently grown on a higher
106 stringency quadruple dropout medium lacking leucine, tryptophan, adenine and
107 histidine (SD/-Leu/-Trp/-Ade/-His) but containing X-alpha-Gal (QDO/X). Prey
108 plasmids were then isolated from those positive clones that had all four yeast reporter
109 genes [ADE2, HIS3, MEL1 (encodes for α -galactosidase) and lac Z (encodes for β -
110 galactosidase)] activated, transformed into *Escherichia coli* XL1-Blue to recover the
111 plasmid, and subjected to DNA sequencing. The obtained sequences were searched
112 against the NCBI GenBank database using BLASTX to identify the clones. To
113 confirm the screening results, recovered prey plasmids were co-transformed with the
114 bait plasmid into *S. cerevisiae* AH109 and plated onto QDO/X medium according to
115 the manufacturer's instructions (BD Biosciences).

116

117 2.3 Production and purification of recombinant *PmVRP15* protein and *WSSV* protein

118 The single colony of *E. coli* stain C43 (DE3) containing recombinant plasmid
119 pET22b+-*PmVRP15* (a gift from Assist. Prof. Dr. kuakarun Krusong) was cultured in
120 LB-amp medium with shaking at 250 rpm at 37 °C for overnight as a starter. The
121 overnight culture was inoculated into fresh LB-amp medium until OD₆₀₀ reached 0.5

122 then induced with 1 mM IPTG for 1h to over- produce the *rPmVRP15*. The cell pellet
123 was collected, resuspended in 50 mM Tris-HCl, pH 7.0 and sonicated with a
124 Bransonic 32 (Bandelin). The soluble fraction containing *rPmVRP15* was collected
125 by centrifugation at 10,000 rpm for 20 min, was further centrifuged to isolate the
126 membrane protein part of *E. coli* that contain *rPmVRP15* by ultracentrifugation at
127 $100,000 \times g$ for 1 h. The resulting pellet was homogenized in ice-cold solubilization
128 buffer (50 mM Tris-HCl, pH7.0, 20 mM Imidazole, 300 mM NaCl, 1% dodecyl-L-
129 D- maltoside (DM) and 20% glycerol) at 4°C for overnight. The crude supernatant of
130 *rPmVRP15* protein was finally collected by ultracentrifugation at $100,000 \times g$ for 30
131 min and subjected to purification through Ni-NTA column (GE healthcare). Crude
132 protein was loaded on to the Ni-bead column pre-equilibrated in the equilibration
133 buffer (50 mM Tris-HCl, pH 7.0, 20 mM Imidazole, 0.1% DM and 10% glycerol) and
134 incubated at 4 °C for 2 h. The column was washed with washing buffer (50 mM Tris-
135 HCl, pH 7.0, 50 mM Imidazole, 0.1% DM and 5% glycerol) and *rPmVRP15* was
136 eluted by elution buffer (50 mM Tris-HCl, pH 7.0, 300 mM Imidazole, 0.1% DM
137 and 5% glycerol). The elution fraction was subjected to Amicon® Ultra-4 Centrifugal
138 Filter Units cut-off 3 kDa (Millipore) to concentrate and exchange buffer to 10 mM
139 Tris-HCl, pH7.0, 0.07% DM and 2.5% glycerol. The purified protein was analyzed
140 using 15% SDS-PAGE. The protein concentration was determined using the Bradford
141 method.

142 For recombinant WSV399 protein production, WSV399 gene was amplified
143 by PCR using gene specific primers with *XhoI* and *EcoRI* restriction sites, WSV399-
144 *XhoI*-F and WSV399-*EcoRI*-R (Table1). The PCR conditions were 94 °C for 1 min,
145 followed by 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec, and
146 then a final extension at 72 °C for 5 min using RBC *Taq* polymerase (RBC

147 Bioscience). The purified PCR product was double digested with *Xho*I and *Eco*RI
148 (New England Biolabs) and cloned into *Xho*I/*Eco*RI-digested pBAD/Myc-His/A
149 (Invitrogen) and finally transformed into an *E. coli* TOP10. The recombinant plasmids
150 were subjected to nucleotide sequencing (Macrogen Inc., Korea). The recombinant
151 clone in the expression host was cultured and induced with 0.1% L-arabinose to over-
152 produce the recombinant WSV399 (rWSV399). After induction for 4 h, cells were
153 collected and resuspended in 1× PBS, pH 7.4 then sonicated. The crude soluble
154 rWSV399 protein was collected and purified through Ni-NTA column (GE
155 Healthcare) pre-equilibrated with 50 mM Sodium phosphate buffer, pH 7.4, 300 mM
156 NaCl, and 10 mM Imidazole. Upon 2 h incubation at room temperature, flow-through
157 was discarded and the bead was washed with washing buffer (50 mM Sodium
158 phosphate buffer, pH 7.4, 300 mM NaCl, and 20 mM Imidazole). The purified-
159 rPmVRP15 was eluted using 50 mM Sodium phosphate buffer, pH 7.4, 300 mM NaCl
160 containing 40, 80, 100, 250 and 500 mM Imidazole. The purified protein from elution
161 fractions was selected and dialyzed against 1× TBS, pH 7.4. The protein was analyzed
162 using 12.5% SDS-PAGE. The concentration of protein was determined using the
163 Bradford method.

164

165 2.4 Co-immunoprecipitation (Co-IP)

166 The co-immunoprecipitation reaction was performed according to instructions
167 of the Pierce c-Myc Tag IP/Co-IP Kit (Thermo scientific). The interaction between
168 WSV399 and PmVRP15 was examined by incubating Myc-His-rWSV399 protein
169 with His-rPmVRP15 at 30 °C for 2 h with gentle rocking. Next, the anti-c-Myc
170 antibody conjugated protein A bead (10 µl of 50% bed slurry) was incubated with
171 protein mixture above at 4 °C for overnight with gentle rocking. The beads were

172 washed 10 times with 500 µl of Wash buffer (1× TBS) to remove non-specific
173 binding protein. The supernatant obtained after adding SDS-loading sample buffer
174 and heating at 100 °C for 15 min, was collected by centrifugation. Then, protein-
175 protein complex was analyzed by SDS-PAGE and western blot analysis. For western
176 blot analysis, the anti-c-Myc antibody (Clontech) and anti-His antibody (GE
177 Healthcare) were used as primary antibodies for rWSV399 and *Pm*VRP15 detection,
178 respectively. The anti-c-Myc antibody diluted 1:7,000 fold in 1× PBS-T (0.05%
179 Tween-20 in 1× PBS buffer: 137 mM NaCl, 2.7 mM, 10 mM Na₂HPO₄, 2 mM
180 KH₂PO₄, pH 7.4), was incubated with membrane at 37 °C for 3 h after blocking. The
181 secondary antibody, an anti-mouse IgG conjugated alkaline phosphatase (diluted
182 1:5,000) (Jackson ImmunoResearch Laboratories, Inc.) and the substrate NBT/BCIP
183 solution, were used to detect rWSV399. The positive purple band size of about 26
184 kDa was detected. The membrane was then washed with 1× PBS-T for 3 times at 15
185 min each before incubating with the anti-His antibody diluted 1:3,000 fold in 1× PBS-
186 T at 37 °C for 3 h. This antibody can bind to both *rPm*VRP15 and rWSV399 proteins.
187 Then, the secondary antibody, anti-mouse IgG conjugated alkaline phosphatase
188 (diluted 1:7,000 fold in 1× PBS-T) was incubated at room temperature for 1 h. The
189 bands of *rPm*VRP15 (15 kDa) and rWSV399 (26 kDa) protein were identified after
190 adding NBT/BCIP solution.

191 The control experiments were done simultaneously with the WSV399-
192 *Pm*VRP15 interaction experiment. They were performed as mentioned except that the
193 anti-c-Myc antibody conjugated protein A bead was incubated with either *rPm*VRP15
194 or rWSV399.

195

196 *2.5 Localization of WSV399 protein on WSSV intact virion*

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

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

216 According to the method described previously (Leu *et al.* 2005), a purified
217 WSSV virion suspension was adsorbed to Formvar-supported and carbon-coated
218 nickel grids (150 mesh) and incubated for 5 min at room temperature. The primary
219 antibody and pre-immune mouse serum were diluted 1:50 in an incubation buffer
220 (0.1% Aurion Basic-c, 15 mM NaN₃, 10 mM phosphate buffer, 150 mM NaCl, pH
221 7.4). The grids were blocked with the blocking buffer (5% bovine serum albumin, 5%

222 normal serum, 0.1% cold water skin gelatin, 10 mM phosphate buffer, 150 mM NaCl,
223 pH 7.4) for 15 min and then incubated with a diluted primary antibody or pre-immune
224 mouse serum for 3 h at room temperature. After several washes with incubation
225 buffer, the grids were incubated with a goat anti-mouse IgG secondary antibody
226 conjugated with 18-nm-diameter gold particles (1:50 dilution in the incubation buffer)
227 for 1 h at room temperature. The grids were then washed extensively with incubation
228 buffer, washed twice more with distilled water to remove excess salt, and stained with
229 1% phosphotungstic acid (pH 7.2) for 2 min. Specimens were examined with a
230 Transmission electron microscope (TEM, Hitachi/S-4800).

231

232 *2.6 wsv399 gene expression profile in WSSV infected-shrimp hemocyte using RT-PCR* 233 *technique*

234 Semi-quantitative RT-PCR was used to examine the expression of *wsv399*
235 gene in WSSV-infected shrimp at various time points. *P. monodon* (approximately 15
236 g body weight) were injected with 100 µl of the diluted WSSV solution (a dose that
237 causes 100% mortality of shrimp in 3 dpi). Hemocytes of 3 WSSV-infected shrimp
238 were collected individually at 0, 3, 6, 12, 24, 36 and 48 hours post infection (hpi).
239 Total RNA was extracted and treated with RNase-free DNase to remove any residual
240 DNA contamination. Total RNA concentration was determined using
241 spectrophotometer. An equal amount of DNA-free total RNA from each individual
242 was pooled. One µg of pooled DNase-free total RNA was used for the first strand
243 cDNA synthesis using the RevertAid First Strand cDNA Synthesis kit (Fermentas).
244 To investigate the expression level of viral genes, which are *ie-1* (immediate early
245 gene), *wsv477* (early gene), *vp28* (late gene) and *wsv399*, RT-PCR was performed
246 using gene specific primers, IE-1-F/R, WSV477-F/R, VP28-F/R and WSV399-RT-

247 F/R, respectively (Table 1). EF-1 α gene as a reference gene was amplified using EF-
248 1-F/R primers. The reaction was pre-denatured at 94 °C for 1 min and followed by 35
249 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension
250 at 72 °C for 30 sec, and final extension at 72 °C for 7-10 min. The PCR products were
251 analyzed by 1.5% (w/v) agarose gel electrophoresis.

252

253 2.7 The effect of *wsv399* gene knockdown in WSSV- infected shrimp

254 The *wsv399* dsRNA was prepared using WSV399-recombinant plasmid as a
255 template for producing sense and anti-sense DNA templates of *in vitro* transcription.
256 DNA templates containing the T7 promoter sequence at the 5'-end were generated by
257 PCR using WSV399-T7-F and WSV399-R for the sense strand template, the other
258 with WSV399-F and WSV399-T7-R (Table 1). The dsRNA was prepared using T7
259 RiboMAX™ Express RNAi System (Promega).

260 *P. monodon* (approximately 3 g body weight) were divided into two groups of
261 three individuals each. The first (control) group was injected with 10 μ g/g shrimp of
262 GFP-dsRNA, while the second groups were injected with 10 μ g/g shrimp WSV399-
263 dsRNA, respectively. Diluted WSSV solution at dosage that causes 100% mortality of
264 shrimp in 3 days post infection (dpi), was mixed with dsRNA and injected into the
265 respective group of shrimp. Hemocytes of individual shrimp were collected at 24 hpi
266 and total RNA was extracted, treated with RNase-free DNase and determined for
267 concentration. An equal amount of DNA-free total RNA from three shrimp was
268 pooled and 1 μ g of pooled total RNA was used for the first strand cDNA synthesis
269 using the RevertAid First Strand cDNA Synthesis kit (Fermentas). *wsv399* gene
270 expression was analyzed by RT-PCR using WSV399-RT-F/R primers (Table 1). The
271 *EF-1 α* gene as an internal control using the EF-1 α -F/R primer pair (Table 1) was

272 amplified. The expression level of *vp28* gene was determined by semi-quantitative
273 RT-PCR using gene specific primers VP28-F/R (Table 1) to verify the viral
274 replication in shrimp. The PCR conditions were 94 °C for 1 min, followed by 27
275 cycles of 95 °C for 30 sec, 58 °C (for *EF-1α*) or 60 °C (for *wsv399* and *vp28*) 30 sec,
276 and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The PCR products
277 were analyzed by 1.5% (w/v) agarose gel electrophoresis.

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

284

285 **3. Results**

286 *3.1 Identification of PmVRP15-interacting protein by yeast two-hybrid screening*

287 From our previous result (Vatanavicharn *et al.* 2014), *PmVRP15* was shown
288 to be involved in WSSV propagation. To better understand *PmVRP15* function in
289 WSSV infection in shrimp, yeast two-hybrid screening was used to identify its
290 interacting-partner protein from WSSV. Firstly, the bait plasmids, pGBKT7-
291 *PmVRP15*, pGBKT7-N-ter*PmVRP15* and pGBKT7-C-ter-*PmVRP15* were
292 constructed and transformed into yeast cell, *Saccharomyces cerevisiae* AH109. The
293 toxicity and auto-activation were investigated. The results showed no toxicity and
294 auto-activation after *PmVRP15* protein expression (data not shown). Next, bait
295 plasmid was used to screen for the interacting partner on the prey library containing
296 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-ter-*PmVRP15* with WSSV ORF were identified, interaction between a WSSV protein with N-ter-*PmVRP15* 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. *Escherichia coli* strain C43 (DE3) containing pET22b⁺-*PmVRP15* was cultured and induced for r*PmVRP15* expression by IPTG. The r*PmVRP15* 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 r*PmVRP15* was separated from soluble fraction, solubilized, and purified by Ni-NTA affinity column. After buffer-exchanged and concentrated, the purified r*PmVRP15* 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* stain 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%

322 Coomassie stained SDS-PAGE (Fig. 1B). The purified rWSV399 was further
323 concentrated and buffer-exchanged before used.

324

325 3.3 Confirmation of *PmVRP15* and *WSSV* protein interaction by co- 326 immunoprecipitation (co-IP)

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

338

339 3.4 *wsv399* gene expression profile in *WSSV*-infected shrimp hemocyte using RT-PCR

340 The expression profile of *wsv399* gene in *WSSV*-infected shrimp *P. monodon*
341 was investigated to further characterize *wsv399* gene. In this study, the viral genes at
342 different stages of viral replication, which are *ie-1* (immediate early stage), *wsv477*
343 (early stage) and *vp28* (late stage), were used as marker genes. The gene expression
344 level of these viral genes in the hemocytes of *WSSV*-infected shrimp at 0, 3, 6, 12,
345 24, 36 and 48 hpi was analyzed by RT-PCR using *EF-1 α* as an internal control. We

346 found that *wsv399* showed similar gene expression pattern as *vp28* gene indicating
347 that *wsv399* transcript was a late gene (Fig. 2).

348

349 *3.5 Localization of WSV399 protein on intact WSSV*

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

365

366 *3.6 Effect of WSV399 gene knockdown in WSSV- infected shrimp*

367 The involvement of *wsv399* in WSSV replication was investigated by *wsv399*
368 gene silencing in WSSV-infected shrimp. The *wsv399* gene knockdown did not affect,
369 the level of *vp28* gene expression as compared to that of control shrimp injected with
370 GFP dsRNA (Fig. 4A). We concluded that *wsv399* might not be involved in WSSV

371 replication. However, the *wsv399* might have other functions. Therefore, the shrimp
372 cumulative mortality upon *wsv399* knockdown and WSSV infection was investigated
373 in comparison with the control; WSSV infected-shrimp with dsRNA GFP injection.
374 At 72 hpi, the cumulative mortality of control group reached 100% whereas that of
375 *wsv399* knockdown shrimp was only about 26%, although it reached to 100% at 132
376 hpi (Fig. 4B). In this regard, *wsv399* gene likely plays an important role in WSSV
377 propagation in WSSV-infected shrimp. According to the results, the function of the
378 *PmVRP15*-interacting WSSV tegument protein, WSV399, might be involved in viral
379 assembly in WSSV-infected shrimp.

380

381 **4. Discussion**

382 Previously, *PmVRP15* has been identified by suppression subtractive
383 hybridization between normal and WSSV-infected shrimp hemocyte. *PmVRP15* gene
384 had highest up-regulation (9,410 fold) in hemocyte at 48 h after WSSV infection (late
385 stage of infection). *PmVRP15* protein expression was localized on the nuclear
386 membrane of both the uninfected and WSSV-infected shrimp hemocyte but highly
387 induced in WSSV-infected shrimp hemocyte only. After WSSV infection, *PmVRP15*
388 protein was also over-expressed when compared with control and located around the
389 nuclear membrane. After *PmVRP15* gene knockdown, viral gene expression in
390 various stages of WSSV infection, which are *ie-1* for immediate early stage, *wsv477*
391 for early stage and *vp28* for late stage, was reduced and also the cumulative mortality
392 of WSSV-infected *PmVRP15* knockdown shrimp was lower than control implying
393 that *PmVRP15* was involved in WSSV propagation in shrimp (Vatanavicharn *et al.*
394 2014). Recently, Leu *et al.* have characterized the function of *PmVRP15* and found
395 that it was induced by ER stress in WSSV-infected shrimp, finally conferring its name

396 as ER stress-responsive protein or *PmERP15*. Silencing of this gene had no effect on
397 WSSV multiplication but was deemed important for shrimp survival (Leu *et al.*
398 2015). The contrasting results of *PmVRP15* and *PmERP15* in the WSSV-infected
399 shrimps of the two studies may be attributed to different experimental parameters. In
400 the experiments determining the effect of these genes on WSSV replication using
401 RNA interference, parameters such as dose of WSSV infection, dose of *PmVRP15*
402 and *PmERP15* dsRNA, shrimp tissue and time post infection, were different. The
403 results of the two studies, although related, are not informative when compared.
404 Agreeing results between *PmVRP15* and *PmERP15* were also find where the two
405 genes were up-regulated and might be involved in WSSV infection in shrimp.

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

416 The expression profile of *wsv399* gene was characterized in hemocyte of
417 WSSV-infected shrimp at various time points. The result showed that *wsv399* gene
418 was produced at 24 hpi, late stage of WSSV replication. Concordantly, *PmVRP15*
419 gene expression profiling was also up-regulated at 24 hpi (Vatanavicharn *et al.* 2014).
420 To date, limited information on WSV399 and its function has been found. Firstly, we

421 performed WSV399 localization on WSSV virion by western blot analysis as well as
422 IEM. WSV399 was found on both envelope and nucleocapsid parts of WSSV when
423 virion was fractionated by Triton X-100 in low salt condition like other WSSV
424 tegument proteins such as VP26 (the major tegument protein of WSSV), VP36A,
425 VP39A and VP95. The known tegument proteins of WSSV, VP26 and WSV399
426 proteins, were detected on unenveloped WSSV virion by IEM (Tsai *et al.* 2006). We
427 confirmed that WSV399 is, indeed, a tegument protein of WSSV.

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

439 In summary, *PmVRP15* is a highly WSSV-responsive transmembrane protein
440 expressed in hemocyte. During WSSV infection, WSSV annexes a shrimp *PmVRP15*
441 to facilitate infection via *PmVRP15*-WSV399 tegument protein interaction. The
442 involvement of WSV399 in WSSV replication implied that *PmVRP15* might play a
443 role in viral entry and egress from cytoplasm to nucleus in *P. monodon*. This
444 mechanism, however, needs to be further elucidated.

445

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458

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523

524

525 **Figure legends**

526

527 **Figure 1** Interaction between N-terminal fragment of *Pm*VRP15 and WSV399
528 protein. (A) The *Pm*VRP15-interacting protein, WSV399, was identified by yeast
529 two-hybrid screening and the binding was confirmed by co-transformation. The pBD-
530 N-ter *Pm*VRP15 and pAD-WSV399 plasmids were co-transformed into the yeast
531 *Saccharomyces cerevisiae* strain AH109, grown on selective media, DDO/X and
532 QDO/X media. Negative and positive controls are the yeast, *S. cerevisiae* strain
533 AH109, containing the murine p53 and either lamin or the SV40 large T antigen,
534 respectively. (B) The recombinant proteins, r*Pm*VRP15 and rWSV399 were produced
535 in *Escherichia coli* and analyzed by Coomassie-stained SDS-PAGE (left panel) and
536 western blot analysis (middle panel) using the anti-His antibody (lane 1: 0 h after
537 induction, lane 2: 1 h for r*Pm*VRP15 or 4 h for rWSV399 after induction). The
538 r*Pm*VRP15 and rWSV399 were purified by Ni-NTA column (right panel). (C) The
539 interaction between *Pm*VRP15 and WSV399 was confirmed by co-
540 immunoprecipitation. The r*Pm*VRP15- rWSV399 complex was analyzed by western
541 blot using specific antibodies, which are anti-His antibody for *Pm*VRP15 and anti-c-
542 Myc antibody for WSV399.

543

544 **Figure 2** The expression profile of *wsv399* gene in hemocyte of WSSV- infected
545 shrimp *P. monodon* after 0, 3, 6, 12, 24, 36 and 48 hpi. The expression level of viral
546 gene in various stages which are *ie-1* (immediate early stage), *wsv477* (early stage)
547 and *vp28* (late stage), was used as marker. *EF-1α* gene was used as an internal
548 control. Four individuals were analyzed for each group. The representative data of
549 two individuals per group is shown.

550

551 **Figure 3** Localization of WSV399 on WSSV virion. (A) WSV399 protein was
552 identified on purified-WSSV structural protein by western blot analysis using anti-
553 WSV399 antiserum. The pre-immunized antibody was used as a control showing the
554 specificity of antibody. (B) Localization of WSV399 on WSSV protein fractions. The
555 presence of WSV399 protein on the intact WSSV virion protein, envelope protein and
556 nucleocapsid protein was determined by western blot analysis using the purified anti-
557 WSV399 polyclonal antibody. (C) Localization of WSV399 on WSSV virion by
558 immunoelectron microscopy. WSV399 on WSSV virion was detected using the
559 purified anti-WSV399 antibody as a primary antibody and a 18 nm-diameter gold-
560 labeled secondary antibody. As compared to the control (pre-immune serum) where
561 the gold particle was undetectable, the positive signals were observed on the tegument
562 of the virion as indicated by black arrows.

563 **Figure 4** The effect of *wsv399* gene silencing in WSSV-infected *Penaeus monodon*
564 hemocytes. (A) Expression level of the WSSV gene, *vp28*, in *wsv399* gene-silenced
565 shrimp hemocytes were determined by RT-PCR. (B) The percentage of cumulative
566 mortality rate of *wsv399* gene knockdown in WSSV-infected shrimp (Grey line) was
567 compared with that of the control, WSSV-infected GFP gene knockdown shrimp
568 (Black line). Data are shown as the mean ± 1 S.D. and are derived from three
569 independent repeats.

Table 1 List of primers used in this study

Primers used to amplify <i>PmVRP15</i> gene for bait plasmids construction	
ORF- <i>PmVRP15</i> - <i>Nco</i> I-F	GGCCATGGAG-TTAACAGAGGACTTA
ORF- <i>PmVRP15</i> - <i>Bam</i> HI-R	ACGGATCCTTA-ATGCTCTACTGA
N- <i>PmVRP15</i> - <i>Nde</i> I-F	GCGCATATG-TTAACAGAGGACTTAGTAAACCTG
N- <i>PmVRP15</i> - <i>Eco</i> RI-R	GTGCTGAATTCTTA-TGTTGAGACGAATGGTATGG
C- <i>PmVRP15</i> - <i>Nde</i> I-F	GCGCATATG-TATGCTAGGGGGAAGTTCAAA
C- <i>PmVRP15</i> - <i>Eco</i> RI-R	GCTCGGAATTCTTA-ATGCTCTACTGACATGTTGTG
WSV399- <i>Xho</i> I-F	CGCCTCGAGG-TTCCAGAAATGGTTTGAATCGTT
WSV399- <i>Eco</i> RI-R	GCCGAATTCGC-TTTGTTTGATAATACAATTTTCACCTTGT
Primers used to study the various stage of WSSV gene expression	
IE-1-F	GACTCTACAAATCTCTTTGCCA
IE-1-R	CTACCTTTGCACCAATTGCTAG
WSV477-F	CGCGGATCCATGTATATCTTCGTCGA
WSV477-R	CCGGAATTCTTATAAGAAATGTACAA
VP28-F	TCACTCTTTCGGTCGTGTCG
VP28-R	CCACACACAAAGGTGCCAAC
WSV399-F	CGCCTCGAGGTTCCAGAAATGGTTTGAATCGTT
WSV399-R	GCCGAATTCGCTTTGTTTGATAATACAATTTTCACCTTGT
EF-1 α -F	GGTGCTGGACAAGCTGAAGGC
EF-1 α -R	CGTTCCGGTGATCATGTTCTTGATG
Primers used to construct dsRNA-WSV399 and dsRNA-GFP	
WSV399-T7-F	GGATCCTAATACGACTCACTATAGGTTCCACATCGCATTCGCA
WSV399-T7-R	GGATCCTAATACGACTCACTATAGGCACGGGGATCAATATCTTGGA
WSV399-RT-F	TTCCACATCGCATTCGCA
WSV399-RT-R	CACGGGGATCAATATCTTGGA
GFP-T7-F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGGGAGGA
GFP-T7-R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA
GFP-F	ATGGTGAGCAAGGGGGAGGA
GFP-R	TTACTTGTACAGCTCGTCCA

Figure 1

A

B

C

Figure 2

Figure 3

A **B**