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**1. Introduction**

Thailand is the world’s important exporter of shrimp products, especially the black tiger shrimp, *Penaeus monodon*. At present, losses of shrimp production are caused by various viral diseases. One of the major causative agents is the yellow head virus (YHV) which causes extensive and rapid mortality in black tiger shrimp [1,2]. YHV is a positive sense, single-stranded RNA virus that is classified as a member of Gill-associated virus, belonging to the genus Okavirus, family Roniviridae in the order Nidovirales [3]. YHV genome is approximately 26 kb, containing four long open reading frames (ORF1a, ORF1b, ORF2, and ORF3) [4–6]. The enveloped rod-shaped YHV particles are 150–200 nm in length and 40–50 nm in diameter [1,7]. The virion has prominent surface spikes and contains internal helical nucleocapsid [8,9]. It contains three structural proteins, the transmembrane glycoproteins gp116 and gp64 are found in the viral envelope, and the nucleoprotein p20 [6,10].

Recently, the mechanism of YHV transportation has been proposed. After YHV enters into the cell, it is transported via endosomal compartments [11,12] and released its genome into the cytoplasm for replication. Then, the nucleocapsid is synthesized and transported to the ER-Golgi compartment where the envelope is formed [2,13]. Finally, the enveloped viral particle is exocytosed at the plasma membrane. However, this exocytosis process of YHV out of the cells is not well understood. Normally, enveloped viruses are budded at the membrane in order to generate the envelope that surrounds the nucleocapsid [14]. In this process, the nucleocapsid is wrapped in a cellular membrane containing virus-specific envelope proteins. The viral envelope protein serves to target the host cell receptor [15]. Some viruses bud at the plasma membrane (PM), whereas others are assembled and budded at intracellular membranes along the secretory pathway such as the nuclear envelope, rough and smooth endoplasmic reticulum (ER), endosomes, intermediate or pre-Golgi compartment, Golgi cisternae and the trans-Golgi-network (TGN) [16]. However, the cytoplasmic transportation of viral genome or viral particle to plasma membrane

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requires the host proteins.

One of the cellular proteins that is involved in intracellular trafficking process is Rab11. Rab11 is a small GTPase protein belongs to the Ras superfamily, whose function is in transportation of the vesicles through the TGN [17], apical recycling of endosomes [18] and the perinuclear recycling of endosomal compartments before redirecting the vesicular cargo back to the apical plasma membrane. The Rab GTPase protein acts as molecular switches that shuffle between two conformational states, the GTP bound ‘active’ form and the GDP-bound ‘inactive’ form. In the active form, Rab protein is associated with membranes by hydrophobic geranylgeranyl groups at the C-terminal and recruited the specific effector molecules such as sorting adaptors, tethering factors, kinases, phosphatases and motor proteins in vesicle transport process [18]. Our previous studies found that several Rab proteins are hijacked by YHV [11,12]. Specifically, the transportation of YHV particles required PmRab5 which is a key protein in vesicles transport to early endosome; whereas, PmRab7 is involved in YHV transportation from early endosome to late endosome and lysosome. The silencing effects of PmRab5 or PmRab7 in YHV-infected P. monodon inhibited YHV expression, suggesting that PmRab5 and PmRab7 are involved in intracellular trafficking of YHV [11,12]. Recently, several evidence revealed that some RNA viruses such as vesicular stomatitis virus, sendai virus, influenza A, measles virus, mumps virus, and hantavirus use Rab11-dependent pathway to assemble and release out of the cells [19–25]. Whether YHV which is also a e RNA virus requires Rab11 for its transportation out of the cell remains to be elucidated. Furthermore, subtractive hybridization study found that Rab11 is one of the responsive genes that is upregulated after YHV infection [26]. Therefore, here, molecular cloning of the full-length cDNA in P. monodon Rab11 (PmRab11) and its probable function during YHV infection were performed.

### 2. Materials and methods

#### 2.1. Black tiger shrimp culture

Juvenile viral-free pathogen black tiger shrimps (P. monodon) of about 10–30 g were obtained from commercial shrimp farms in Thailand. Before use, shrimps were tested for YHV and white spot syndrome virus infection by using diagnostic strip test (Pacific Biotech Co., Ltd., Thailand). Shrimps were grown in a plastic box containing oxygenated seawater at 10 ppt salinity and were acclimated for 2 days before the experiment was carried out. They were fed with commercial shrimp feed every day. The salt water was changed every 2 days.

#### 2.2. Yellow head virus (YHV) stock

The infectious YHV was propagated by injection of YHV to viral-free shrimp. Then, hemolymph was collected from YHV-infected moribund shrimp and mixed with AC-1 solution (27 mM Sodium citrate, 34.33 mM NaCl, 104.5 mM Glucose, 198.17 mM EDTA, pH 7.0). Next, the hemolymph was centrifuged at 20,000×g for 20 min at 4 °C to remove hemocyte debris. Free YHV particles were collected by ultracentrifugation (100,000×g) for 1 h. Virus pellets were suspended with 150 mM NaCl and stored at −80 °C until used. The viral nucleic acid was purified from the YHV stock using high pure viral nucleic acid kit (Roche Diagnostics, Germany) and subjected to RT-PCR to determine viral titer using primers YHV_F: 5′-CAAGGACCACTCTTGACCGTTAAGAC-3′ and YHV_R: 5′-GCCGAAACGACTAGCGCTACATTCCAC-3′ [11].

#### 2.3. Molecular cloning of the full length PmRab11cDNA

The partial sequence of PmRab11 served as template to design primers for 5′ and 3′ RACE. 5′ and 3′ RACE were performed as previously described [11]. Briefly, 5′ RACE method was performed by using 5Rab11_R1 specific primer (Table 1) to generate the first-strand cDNAs by Superscript III reverse transcriptase (Invitrogen). Then, 5Rab11_R1 and PRT primers (Table 1) were used to synthesize the first PCR product which was diluted to 1:100 for used as template in the nested PCR with 5Rab11_R2 and PM1 primers (Table 1). To obtain the 3′ end of PmRab11 cDNA, 3′ RACE PCR was performed by PRT primer to generate the first-strand cDNAs. Then, the PCR reaction containing 3Rab11_F1 and PM-1 primers was performed. Next, two nested PCRs were performed using 3Rab11_F2 and PM-1 primers and 3Rab11_F3 and PM-1 primers (Table 1). Then, all expected bands of the 5′ and 3′ RACE PCR products were purified by Gel/PCR fragments extraction kit (Geneaid), cloned into pGEM-T-easy vector (Promega) and sequenced (First Base Co. Ltd, Malaysia).

The sequences of the 5′ and 3′ ends obtained from RACE-RCR were assembled together with the partial sequence of PmRab11 in order to obtain the full-length cDNA by using ContigExpress tool from Vector NTI Advance 11.3.1 program. Finally, a PCR was performed to amplify the full-length PmRab11 cDNA using Taq DNA polymerase (New England Biolabs) with primers that were designed based on the 5′ and 3′ end sequences, fullRab11_F and fullRab11_R primers (Table 1). PCR condition was: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1.30 min and 72 °C for 7 min in the final step. Finally, the PCR product was purified, cloned and subjected to sequence following the protocol that was described earlier.

#### 2.4. Sequence analysis of PmRab11

The full-length cDNA sequence of PmRab11 was analyzed against the NCBI’s blastn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The coding sequences were translated to amino acid sequence by using Expasy’s tool (http://web.expasy.org/translate/). Amino acid sequence identity between PmRab11 and Rab11 proteins of other species was performed by using alignX of Vector NTI. In addition, the initiation codon and poly-A signal were identified by using ATGpr (http://atgpr.dbcls.jp/) and Poly (A) signal miner (http://dnafmminer.bic.nus.edu.sg/PolyA.html). Multiple sequence alignment of PmRab11 was performed to identify the conserved domains of Rab11 protein by using the conserved domain database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Prenylation site was identified by using Prenylation Prediction Site (http://mendel.imp.acat/PrePS/). Phylogenetic tree analysis of PmRab11 was performed based on the neighbor-joining methods (http://www.phylogeny.fr/) [27]. The phylogeny was constructed by phylogeny.fr under “One Click” mode. The pipeline is already set up to run and connect well recognized programs: MUSCLE for multiple alignment, Gblocks for automatic alignment curation, PhyML for tree building and TreeDyn for tree drawing.

#### 2.5. Construction of recombinant plasmid expressing dsRNA-PmRab11

A recombinant plasmid containing sense-loop and antisense fragment of PmRab11 was constructed using pGEM®-3zf (+) vector (Promega) as a plasmid backbone. A sense-loop fragment of size 494 bp was amplified from PmRab11 cDNA using sRab11_F and sRab11_R primers containing XhoI and KpnI sites, respectively (Table 1). The amplification of the antisense fragment of size 404 bp was performed using asRab11_F and asRab11_R primers containing
Table 1
List of synthetic oligonucleotide primers.

| Name          | Sequences (5’→3’)                              | Experiments       |
|---------------|-------------------------------------------------|-------------------|
| PRT           | GGCGGTAATCCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTTTT | Reverse transcription |
| 5Rab11_R1     | GGCCTGCTTACCTAGTATGGGAC                        | 5’RACE            |
| 5Rab11_R2     | ATTTTGATCTCTAGTATGGGAC                        | 3’RACE            |
| 3Rab11_F      | CAGCTTCTCTCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTT |                   |
| 3Rab11_F2     | GGCCTGCTTACCTAGTATGGGAC                        |                   |
| 3Rab11_F3     | AAGCCATTTCCGAAAAGGAGAGGAC                      |                   |
| PM1           | CCGGATATATACGCTCTAGTATGGGAC                    |                   |
| FullRab11_F   | GAAAGGCATTTGCAGAAAAGGAGGGAC                    |                   |
| FullRab11_R   | GGCTCTCTCTCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTT |                   |
| slRab11_F     | GGCCTGCTTACCTAGTATGGGAC                        | Sense-loop of dsRNA |
| slRab11_R     | GGCCTGCTTACCTAGTATGGGAC                        |                   |
| asRab11_F     | GGAATCCCCCTCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTT | Antisense of dsRNA |
| asRab11_R     | GGCCTGCTTACCTAGGATCCTTTTGGTTTTTTTTTTTTTTTTTTTTT |                   |
| PmRab11_F     | ATGCGGAAAGCGGACAGACAGGTATATG                  | Detection of Pmrab5 |
| PmRab11_R     | GGCCTTCTCTCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTT |                   |
| PmRab5_F      | GACGCTCTACCTTTGTTACACACAGAC                   | Detection of Pmrab5 |
| PmRab5_R      | GCTCTGCTTACCTTTGTTACACACAGAC                  |                   |
| PmRab7_F      | ATGCGGAAAGCGGACAGACAGGTATATG                  | Detection of Pmrab7 |
| PmRab7_R      | GGCCTTCTCTCAGGTTCCTAGGATCCTTTTGGTTTTTTTTTTTTT |                   |
| PmActin_F     | GACGCTCTACCTTTGTTACACACAGAC                   | Detection of PmActin |
| PmActin_R1    | GCAAGCAGGACAGCAGACAGGTATATG                   |                   |
| PmActin_R2    | GTCAGTCTGCTGCTAGGACAGACAGAC                   |                   |
| YHV_F         | CAAAGACACACCTTTGTTACACACAGAC                  | Detection of YHV  |
| YHV_R         | GCGGAAAGCCAGGACAGACAGGTATATG                  |                   |

EcoRI/Xhol and KpnI, respectively (Table 1). Then, the sense and antisense fragments were cloned into pGEM™-3zf(+) vector to obtain a recombinant clone containing the stem-loop fragment of PmRab11. Then, the stem-loop fragment, size 989 bp, was digested by HindIII and Xhol and subcloned to an expression vector, pET-17b to produce dsRNA-PmRab11 by in vivo bacterial expression. The region of dsRNA-PmRab11 is located at the nucleotides 124–510 from the start codon. In addition, a recombinant plasmid containing a stem-loop fragment of GFP (kindly provided by Asst. Prof. Witoon Tiraphsophon) was used to express dsRNA-GFP which served as an unrelated dsRNA [28].

2.6. Expression and extraction of dsRNA-PmRab11

A recombinant plasmid pET-17b containing a stem-loop of PmRab11 was transformed into E. coli HT115 which is a ribonuclease III mutant strain to produce dsRNA-PmRab11 [28]. dsRNA-PmRab11 expression was induced by 0.1 mM IPTG. dsRNA-PmRab11 was extracted and purified by ethanol method [29]. The quality of dsRNA was characterized by ribonuclease digestion assay using RNase A and RNase III (New England Biolab, USA). Yield of dsRNA-PmRab11 was determined by agarose gel electrophoresis and compared to the intensity of 2-log DNA marker. The intensity was measured by an ImageJ program.

2.7. Suppression of PmRab11 expression by dsRNA-PmRab11

In order to determine specific inhibition of PmRab11 expression, injection of dsRNA-PmRab11 was performed. Shrimps were injected in the muscle with 1.25 or 2.5 μg/g shrimp of dsRNA-PmRab11. Injection of unrelated dsRNA-GFP and 150 mM NaCl were used as controls. Then, hemolymph from individual shrimp was collected at 24, 48 and 72 h post-dsRNA or NaCl injection and mixed with TRI REAGENT™ (Molecular Research Center). Hemocytes and supernatant were extracted by TRI REAGENT™ (Molecular Research Center) following the manufacturer’s procedure. The RNA concentration was measured by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total RNAs (2 μg) was used as template to generate the first-strand cDNA by Improm-II™ reverse transcriptase (Promega) using primer (Table 1). PmRab11 expression was monitored by multiplex PCR using PmRab11-F and PmRab11-R primers and PmActin-F and PmActin-R1 primers for PmActin detection which served as an internal control. The PCR was carried out according to this condition: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 45 s and 72 °C for 7 min in the final step. Expressions of PmRab5, PmRab7 and YHV were determined according to the

2.8. Suppression effect of PmRab11 during YHV infection

To investigate the function of PmRab11 on YHV infection, shrimps were intramuscularly injected with dsRNA-PmRab11 at 1.25 μg/g shrimp. Injection of 150 mM NaCl and unrelated dsRNA-GFP were used as control groups. After 24 h, shrimps were challenged with 10^2 dilution of YHV (dose of YHV that leads to 100% mortality within 3 days). The hemolymph was collected at 24, 48, 72 and 96 h post-YHV challenge. Then, the hemocytes and supernatant were fractionated by centrifugation at 650×g for 15 min. Total RNAs in hemocytes and supernatant were extracted by TRI REAGENT® and TRI REAGENT® LS (Molecular Research Center), respectively. The levels of PmRab11 and YHV expression were monitored by semi-quantitative RT-PCR using PmRab11 and YHV specific primers (Table 1), respectively. PmActin was used as an internal control. Finally, the relative expressions of PmRab11 and YHV in hemocytes were normalized by PmActin. However, PmActin cannot be amplified from the supernatant fraction, thus the YHV levels was analyzed from equal amount of the total RNAs.

2.9. RNA isolation and RT-PCR

Total RNA from hemocytes or supernatant was isolated by Trizol® reagent (Molecular Research Center) following the manufacturer’s procedure. The RNA concentration was measured by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total RNAs (2 μg) was used as template to generate the first-strand cDNA by Improm-II™ reverse transcriptase (Promega) using primer (Table 1). PmRab11 expression was monitored by multiplex PCR using PmRab11-F and PmRab11-R primers and PmActin-F and PmActin-R1 primers for PmActin detection which served as an internal control. The PCR was carried out according to this condition: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 45 s and 72 °C for 7 min in the final step. Expressions of PmRab5, PmRab7 and YHV were determined according to the
previous methods [11,12]. PCR products were analyzed on 1.5% agarose gel electrophoresis. The intensity of each band after subtracting the background was quantified by using ImageJ program (Version 1.50b). The relative expression level of the gene of interest was normalized against PmActin level and expressed as an arbitrary unit.

2.10. Investigation of protein levels of YHV in hemocytes and supernatant

In order to investigate YHV protein levels in hemocytes and supernatant of the complete PmRab11 knockdown shrimp, shrimps were injected with dsRNA-PmRab11 at 1.8 μg/g shrimp by intramuscular injection. Injection of NaCl and unrelated dsRNA-GFP were used as control groups. After 24 h, shrimps were challenged with 10^{-2} dilution of YHV by intramuscular injection. The hemolymph was collected at 24, 48, 72 and 96 h post-YHV injection. The hemolymph from 3 shrimps in the same group at the same time was pooled. Then, total RNA from 200 μl of the pooled hemolymph was extracted by TRI REAGENT® L5 method and RT-PCR was performed in order to detect PmRab11 and YHV expression at mRNA level. To extract total protein for Western blot analysis, 2 ml of the pooled hemolymph was fractionated to separate hemocytes and supernatant by centrifugation at 650 g. Total proteins from hemocytes were extracted by using 100 μl buffer T (8M urea, 2 M thiourea, 0.4% Triton X-100, 60 mM DTT, 1 mM PMSF, 1 × Protease inhibitor cocktail (Sigma)). Whereas, total proteins from 100 μl of supernatant were precipitated by 10% TCA at final concentration and dissolved in 100 μl of buffer T. Then, Western blot was performed to detect YHV level in both fractions.

2.11. Western blot analysis

Hemocyte and supernatant proteins were extracted by using buffer T. The protein lysate (50 μg) was electrophoresed in 12% SDS-polyacrylamide gel (SDS-PAGE). Then, proteins were gel transferred onto a nitrocellulose membrane (Bio-Rad) by electrophoresis with 1X transfer buffer [0.025 M Tris–HCl pH 8.3, 0.192 M glycine, and 20% (v/v) methanol]. Then the membrane was blocked with blocking solution [5% skimmed milk in 0.2% PBST (0.2% (v/v) Tween-20 in 1X PBS)] for 2 h. Next, the membrane was soaked with 0.05% PBST (0.2% (v/v) Tween-20 in 1X PBS) containing primary antibody for 2 h. To detect YHV levels, the mouse anti-gp64 antibody was used as primary antibody at the dilution 1: 1000 in 0.2% PBST. In addition, dilution 1: 1000 in 0.2% PBST of the rabbit anti-Rab11 antibody was used as primary antibody at the dilution 1: 1000 in 0.2% PBST. In addition, dilution 1: 1000 in 0.2% PBST of the rabbit anti-β-tubulin primary antibody was used to detect β-tubulin which is an internal control. Then, the primary antibody was removed. The membrane was washed with 0.2% PBST for 10 min, 3 times and incubated with blocking solution containing horseradish peroxidase-conjugated secondary antibody (Sigma) in dilution 1:5000. Then, the membrane was washed 1 time with 0.2% PBST and 3 times with 1X PBST. Finally, the signal was detected by adding the Lumina® Forte Western HRP Substrate (Millipore Corpora- tion) for 5 min and exposed to X-ray film. PmRab11 and β-tubulin have sizes of about 23 and 60 kDa, respectively.

2.12. Shrimp mortality assay

Suppression effects of PmRab11 with or without YHV challenge on shrimp mortality were investigated. Shrimps size about 1 g (n = 15 shrimps per group) were injected with 1.25 or 2.5 μg/g shrimp of dsRNA-PmRab11 24 h with or without YHV challenge. Injection of 150 mM NaCl and unrelated dsRNA-GFP were used as controls. The experiment was performed in triplicates and the number of dead shrimps were recorded every day for 10 days.

2.13. Immunofluorescence assay of YHV and PmRab11 in YHV-infected hemocytes

Shrimp was injected with 1.8 μg/g shrimp of dsRNA-PmRab11 24 h prior to YHV challenge. The injection of 150 mM NaCl and unrelated dsRNA-GFP were used as control groups. Then, 250 μl of hemolymph was collected at 24, 48 and 72 h after YHV challenge. The hemocytes were separated by centrifugation at 550 × g for 10 min at 4 °C. Cell pellet was resuspended in 500 μl of L-15 medium. Then, hemocytes mixture was seeded in 24 wells plate containing a coverslip and incubated at room temperature. After for 2 h, culture media was discarded and 500 μl of ice cold 4% (w/v) paraformaldehyde was added into each wells. After incubation at room temperature for 20 min, the supernatant was removed and fixed hemocytes were washed with 1X PBS for 5 min, 3 times. Then, they were permeabilized by adding 350 μl of 0.1% (v/v) Triton X-100 in 1X PBS for 5 min. After the supernatant was discarded and the permeabilized hemocytes were proceeded to immunofluorescence staining according to the previous method [10]. Except that the rabbit polyclonal anti-Rab11 antibody (ab3612, Abcam, USA) and mouse monoclonal anti-gp64 of YHV antibody (kindly provided by Professor Paisarn Sithigongul, Department of Biology, Faculty of Science, Srinakharinwirot University) were used. After washing, the secondary antibodies which are goat anti-rabbit IgG, Alexa Fluor® 596 and goat anti-mouse IgG, Alexa Fluor® 488 (Invitrogen) were used for detection of PmRab11 and YHV. The nuclei were stained by TO-PRO®-3 iodide (Invitrogen). Finally, a cover slip was mounted with 8 μl of ProLong antifade (Invitrogen). The slide was kept at 4 °C until visualized under confocal microscope (Fluoview FV10i – DOC, Olympus).

2.14. Statistical analysis

The relative mRNA levels of PmRab11 normalized with PmActin were presented as mean ± standard error of mean (SEM). The statistical analysis of the relative mRNA expression levels was tested by using analysis of variance (ANOVA). A probability (P) value at less than 0.05 was used to define significant difference. Cumulative percent mortality was plotted as mean ± SEM.

3. Results

3.1. Full-length cDNA cloning and sequences analysis of PmRab11

The full-length cDNA was obtained from 5’ and 3’ RACE protocol. It has 2 forms of sizes 1217 and 1065 bp with the same coding sequences of 645 bp. The 5’ and 3’ untranslated region (UTR) have sizes 247 bp and 324 bp, respectively (Fig. 1). The sequence of PmRab11 cDNA of 2 variants were submitted into the GenBank database under the accession number KY241479 and KY241480, respectively. PmRab11 protein has size of 214 aa. The theoretical pi and molecular weight are 5.22 and 23.85 kDa, respectively. In addition, this protein shared the characteristics of Rab11 protein family when compared to others species such as five GTP binding domains (G1-G5), five Rab family domains (RabF1-5), four Rab subfamily domains (RabSF1-4) and a prenylation site that usually terminated in CC or CXC motif at the C-terminus (Fig. 2). The amino acid sequence identity showed that PmRab11 protein shared 100% amino acid sequence identity with Rab11 of Pacific white shrimp (Lito-peneus vannamei) and shared approximately 80% with Rab11 of vertebrates such as zebrafish (Danio rerio). In addition, the phylogenetic tree revealed that PmRab11 protein was closely related to the Rab11 of vertebrate and arthropods (Fig. 3). It was not clustered with other Rab proteins including Rab5, Rab6 and Rab7. These results indicate that PmRab11 is a highly conserved protein.
3.2. Suppression of PmRab11 expression by dsRNA-PmRab11

To investigate whether the PmRab11 expression can be suppressed by using RNAi approach. Double-strands RNA targeting PmRab11 was produced and injected into shrimp muscle. The expression levels of PmRab11 in dsRNA-PmRab11 injected shrimp were significantly reduced at more than 90%, 24 h post-dsRNA-PmRab11 injection at both dosages (1.25 and 2.5 μg/g shrimp) when compared to 0 h. The suppression effect of PmRab11 was maintained for at least 72 h post-dsRNA injection (P < 0.001, n = 4/time point) (Fig. 4A). As the nucleotide sequences of dsRNA-PmRab11 contained many conserved domains such as Rab family domains, Rab subfamily domains and GTP binding domains, it is possible that some of the regions of dsRNA-PmRab11 are similar to other Rab genes. Therefore, a specific inhibition to PmRab11 expression was investigated. The results showed that suppression of PmRab11 by injection of dsRNA-PmRab11 has no effect on the levels of PmRab5 and PmRab7 expression from 24 to 72 h post-dsRNA injection (Fig. 4B). Large standard deviation of PmRab7 after 24 h dsRNA injection may be due to varying expression of PmRab7 among shrimp samples.

3.3. Suppression effect of PmRab11 during YHV infection

To investigate whether PmRab11 protein is essential for YHV transport out of the cell, suppression of PmRab11 was performed 24 h prior to YHV challenge. PmRab11 expression level in hemocytes was significantly decreased approximately 50% in shrimp injected with dsRNA-PmRab11 within 48 h post-dsRNA-PmRab11 injection. The knockdown effect of PmRab11 was increased to 75% at 72 and 96 h post-dsRNA injection (P < 0.001, n = 4/time point) (Fig. 5A). In addition, YHV levels in hemocytes and supernatant of PmRab11 knockdown group were significantly decreased from 48 to 72 h post-YHV infection when compared to NaCl injected group (P < 0.01 for hemocytes and P < 0.001 for supernatant, n = 4/time point) (Fig. 5B and C). Notably, shrimps in 2 control groups died at 96 h while shrimp in PmRab11 knockdown group still alive at this time point.

Protein analysis revealed that an envelope glycoprotein gp64 of YHV cannot be detected in the hemocytes and supernatant of the PmRab11 knockdown group from 24 to 72 h post-YHV challenge. At 96 h post-YHV challenge, low levels of YHV can be detected in both hemocytes and supernatant while shrimp in both control groups died at this time point. On the other hand, high YHV level can be detected in hemocytes and supernatant of NaCl-injected groups at 48 and 72 h. However, low level of YHV expression can still be observed in dsRNA-GFP injected group at 48 h and even increased at 72 h post-YHV infection (Fig. 6A and B, Supplementary Fig. 1).

3.4. Shrimp mortality assay

To investigate the suppression effect of PmRab11 on shrimp mortality, shrimp was injected with 1.25 μg/g shrimp of dsRNA-PmRab11. Then, shrimp mortality was recorded for 10 days. The results showed that injection of dsRNA-PmRab11 caused 100% shrimp death at day 7. In contrast, injection of 1.25 μg/g shrimp dsRNA-GFP and NaCl resulted in less than 10% shrimp mortality within 10 days (Fig. 7A). In addition, the cumulative mortalities of
shrimp that injected with dsRNA-PmRab11 at 1.25 μg/g shrimp followed by YHV challenge was delayed at least 2 days when compared to the control groups (Fig. 7B).

3.5. Co-localization of YHV and PmRab11 in YHV infected hemocytes

The PmRab11 protein level in individual hemocytes was observed using immunofluorescence assay. Shrimp was injected with dsRNA-PmRab11 at 1.8 μg/g shrimp, then PmRab11 protein levels was detected at various time points. The result demonstrated that the PmRab11 protein was gradually decreased from 24 to 96 h post-dsRNA-PmRab11 injection (Fig. 8A). Second, the localization of PmRab11 and YHV was investigated by injection with dsRNA-PmRab11 at 1.8 μg/g shrimp and followed by YHV. The low signals of PmRab11 protein and YHV glycoprotein 64 (gp64) can be observed inside the hemocytes of PmRab11 knockdown shrimp at 24 h post-infection (Fig. 8B). In contrast, high signals of PmRab11 and gp64 can be detected in shrimp that was injected with dsRNA-GFP and NaCl followed by YHV challenge at this time point. In addition, co-localization of PmRab11 and YHV can be clearly observed at 24—72 h post-infection in hemocytes of both control groups. In contrast, the PmRab11 knockdown group showed low level of YHV and PmRab11 at these time points (Fig. 8C and D).
Fig. 3. Phylogenetic analysis of Rab11 of P. monodon compared with Rab11, Rab5, Rab6 and Rab7 of other species. The amino acid sequences of the coding region of Rab proteins of P. monodon and of other species were used to perform phylogenetic tree analysis based on the neighbor-joining methods by using the program from (http://www.phylogeny.fr/). The abbreviation of species is as follows: Homo sapiens (Hs), Mus musculus (Mm), Litopenaeus vannamei (Lv), Panaeus monodon (Pm), Aspergillus parasiticus (Ap), Marsupeneus japonicus (Mj), Rattus norvegicus (Rn), Drosophila melanogaster (Dm), Entamoeba histolytica (Eh), Candida albicans (Ca), Neurospora crassa (NC), and Danio rerio (Dr). The accession number is in the parenthesis. PmRab11 cDNA of 2 variants were submitted into the GenBank database under the accession number KY241479 and KY241480, respectively.

Fig. 4. Suppression of PmRab11 gene by dsRNA-PmRab11. (A) Relative mRNA expression levels of PmRab11 in shrimps injected with dsRNA-PmRab11 at 1.25 and 2.5 μg/g shrimp compared with 1.25 μg/g shrimp of dsRNA-GFP injection are presented as mean ± SEM, n = 4/time point. (B) Relative mRNA expression levels of PmRab11, PmRab5 and PmRab7 are presented as mean ± SEM, n = 3–4/time point. The relative mRNA expression levels of these genes were normalized with PmActin. (*) statistically significant difference between dsRNA-PmRab11 and dsRNA-GFP injected groups at each time point.

4. Discussion

At present, the mechanisms of YHV internalization and transportation inside the cell have been proposed. The viral glycoprotein gp116 on YHV envelope binds to the YHV binding protein, PmYRP65 on the cell membrane [30,31] then internalizes into the cell via clathrin-dependent endocytosis [32,33]. After that, the vesicle containing YHV particle is transported to the early and late endosome by Rab5 and Rab7, respectively [11,12] before the viral genome is released to the cytoplasm for replication and translation. Electron microscopy revealed that enveloped YHV virion is formed at TGN [2,13]. However, the mechanism of the transport of YHV genome is released to the cytoplasm for replication and translation.

The earlier studies of other ssRNA viruses such as avian leukosis virus [1] and flaviviruses and coronaviruses found that the viral budding from TGN to PM is not well understood. The earlier studies of other species have been reported and shown to localize differently at specific organelles. Rab11A and B localize at the Golgi, RE and early endosomes and may be involved in membrane trafficking pathway by transportation of the target protein from TGN/RE to plasma membrane [39]. While Rab11C is expressed only in certain epithelial cells and may be involved in vesicular trafficking from recycling endosome to plasma membrane [39,40]. The deduced PmRab11 protein shared a characteristic signature of Rab11 with other species and closely related to Rab11 in invertebrate, especially in shrimp species (100% amino acid sequence identity with Rab11 of Pacific white shrimp, Litopenaeus vannamei). The PmRab11 function was characterized by using RNAi approach. Knockdown of PmRab11 by dsRNA-PmRab11 specifically inhibited PmRab11 expression within 24 h but not PmRab5 and PmRab7 expression. Suppression of PmRab11 led to 100% shrimp death at day 7. Similar results were demonstrated in the knockdown effect of endogenous genes that are involved in the trafficking process in shrimp including PmRab5, PmRab7 and clathrin heavy chain [11,12,33]. In addition, Rab11 is an essential gene in the regulation of transportation of the endocytosed proteins [18,41,42].

The role of Rab11 during YHV infection was investigated by suppression of PmRab11. The PmRab11 expression was suppressed approximately 50% at 48 h post-dsRNA injection (or 24 h post-YHV infection) (Fig. 5A). This is in contrast to the result of PmRab11 knockdown alone (without YHV infection) that PmRab11 expression was inhibited more than 90% at this time point (Fig. 4A).
Interestingly, subtractive hybridization found that Rab11 was upregulated at 24 h post-YHV infection [26]. Therefore, it is possible that an incomplete PmRab11 knockdown is caused by upregulation of PmRab11 expression in response to YHV infection. In addition, viral titre inside and outside the cell was determined by semi-quantitative RT-PCR. The results showed that YHV levels in hemocytes and supernatant of PmRab11 knockdown group were significantly decreased from 48 to 72 h post-YHV infection when compared to the NaCl-injected group. Notably, not only PmRab11 expression was recovered at 120 h post-dsRNA injection, but also the YHV released was increase at this time point. This relationship demonstrated that PmRab11 is required for YHV infection. Similar results were observed in the plaque assay of virus infected cells that were treated with siRNA targeting Rab11 such as hantavirus [25], Influenza A [43], and hepatitis C virus [44].

To estimate the YHV’s protein level in hemocytes and supernatant, Western blot was performed using antibody against YHV-gp64 which is one of the viral glycoproteins on YHV envelope. Unfortunately, our Rab11 antibody cannot be used to detect Rab11 protein by Western blot under the denaturing condition, suggesting that it required the native structure of epitope. The results revealed that YHV protein level cannot be observed in both fractions of all experimental groups at 24 h post-dsRNA injection (Fig. 6). Previous study in PmRab5 knockdown during YHV infection found that YHV is internalized and transported via PmRab5 from PM to early endosome around 10 min–6 h post-infection [10]. After that, viral replication occurred and nucleocapsid was found in cytoplasm at 24 h post-YHV infection [13]. Thus, the, low level of YHV-gp64 protein at the early phase of infection and the failure to detect the protein in Western blot at this time point. At 48 h post-dsRNA injection, high YHV protein level in the NaCl-injected group can be observed. Whereas, the dsRNA-GFP injected group showed low YHV protein level at this time point. Many studies found that the innate antiviral immunity in invertebrates including shrimp was induced by any dsRNA in a sequence-independent manner [45,46]. High levels YHV protein in hemocytes and supernatant of the NaCl injected group and dsRNA-GFP injected group could be observed at 72 h. In contrast, YHV levels could not be detected in both fractions of PmRab11 knockdown group at 24–72 h post-dsRNA injection. For the (+) ssRNA virus including YHV, the replication process occurs in the cytoplasm [47] and the viral envelop glycoproteins are co-translationally inserted into ER membranes. Then, modification of the glycoproteins occurs during their passage through the secretory pathway. For example, all structural proteins of members of the Flaviviridae family such as hepatitis C and dengue virus are co-translated as a single polyprotein and are inserted into the ER membrane [48]. An individual glycoprotein is generated by proteolytic cleavage and the RNA-binding core protein that is known as the nucleocapsid is assembled within the ER, and transported to Golgi apparatus. It is possible that these events may also occur for YHV which is one of the (+) ssRNA. The single polyprotein containing gp116 and gp64 is co-translationally inserted into the ER membrane. The proteolytic cleavage resulted in individual gp116 and gp64 that exposed the N-terminal domain to the cytosolic side [7]. Then, the viral nucleocapsid and glycoproteins are transported to the Golgi apparatus for maturation. At TGN, the budding of YHV envelope virion can be observed under electron microscope [2,13]. Normally, Rab11 is involved in the vesicle transport from TGN to the PM [49]. After PmRab11 knockdown, YHV cannot bud and has no target organelle to transport to. Therefore, the YHV protein in Golgi network might be transported to lysosome for degradation. However, the mechanism in this process remains to be elucidated. Similar results can be observed in the dengue virus [50]. In addition, the mortality assay revealed that injection of dsRNA-PmRab11 can delay shrimp mortality when compared to both control groups. Similar results of the delay in shrimp mortalities can be demonstrated for the knockdown effects of other Rab proteins including PmRab7 and PmRab5 during YHV infection [11,12].

Co-localization between YHV and PmRab11 was observed by immunofluorescence assay. The results showed co-localization of YHV-gp64 and PmRab11 was seen from 24 to 72 h post-YHV injection in both control groups (Fig. 8). Accumulation of YHV can be found at the PM of hemocytes. Similar results were also seen from other viruses that used Rab11-dependent pathway in the
transportation. Such as sendai virus [20], influenza A virus [21,22,43], measles virus [23], mumps virus [24], hantavirus [25], hepatitis C virus [44] and dengue virus [50]. In contrast, low level of co-localization signals between PmRab11 and YHV can be observed in the perinuclear region at 24–72 h in the PmRab11 knockdown group. This is supported by an overexpression of Rab11 dominant negative mutant in hepatitis C, hantavirus or dengue infected cell. The virus was accumulated at the perinuclear region that was identified as TGN [25,44,50]. In addition, overexpression of Rab11 constitutively active mutant in influenza A virus infection showed that Rab11 is aggregated with vRNP at the perinuclear region and cannot be transported out of the cell [21–23]. These evidence suggested a possible role of Rab11 in transportation of YHV.

Other Rab such as Rab6 has been shown to play important roles in virus infection of shrimp. Rab6 is involved in the regulation of phagocytosis against white spot syndrome virus (WSSV) infection through the interaction with the WSSV envelope protein VP466, β-actin, and tropomyosin which could activate the GTPase activity of Rab6 and subsequently induce the rearrangement of actin fibers in invertebrates [51,52]. An increase in Rab6 activity was demonstrated in WSSV resistant shrimp. Silencing of Rab6 by a specific siRNA significantly increased WSSV replication in shrimp [53,54]. In addition, Rab6 plays an important role in the regulation of phagocytosis against bacterial infection in Marsupenaeus japonicus [55].

In summary, the virus budding process and its cellular trafficking inside the cell are core study in virology, Therefore, the identification of the routes for viral infection and release including the discovery of the cellular and viral components involved in various processes are essential. These are an important knowledge that will provide insights into host-pathogen interaction in order to improve a potential approach for therapeutic intervention against viral diseases.
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Supplementary data

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