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ORIGINAL RESEARCH

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Zebrafish U6 promoter driving short-hairpin RNA expression for PmRab7 knockdown to inhibit yellow head virus infection in shrimp hemocytes

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Abstract

RNA interference (RNAi) was investigated as potential antiviral strategy to mitigate losses in shrimp aquaculture. With this aim, an effective short-hairpin RNA (shRNA) expressed intracellularly from bacterial vectors incorporating eukaryotic promoters offers an alternative to an injected synthetic small-interfering RNA (siRNA) or long double-stranded RNA (dsRNA). The vector-based RNAi designed to contain a U6

In remembrance of Emeritus Professor Dr. Sakol Panyim (1943–2020).

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snRNA polymerase III promoter sequence from zebrafish (*Danio rerio*) for driving shRNA was previously introduced to shrimp cell extract and was able to express the shRNA. Here, four DNA plasmids containing putative zebrafish U6 promoter to drive shRNA against PmRab7-specific sequence were used to transfect primary hemocyte culture. The cells were subsequently infected by Yellow head virus (YHV). As results, when analyzed by RT-PCR at 24 hr post-transfection, *Penaeus monodon* Rab7 (PmRab7) mRNA transcription was inhibited most significantly by the pshPmRab7-2 construct. YHV replication in primary shrimp hemocyte cultures was shown to be inhibited substantially by this PmRab7 gene-specific hpRNA construct. Transfection of pshPmRab7-2 construct also reduced YHV replication most effectively when analyzed similarly between 24 hr until 72 hr post-infection. These results demonstrate a potential application of DNA-based shRNA construct as effective molecule for antiviral therapy in shrimp.

KEYWORDS
PmRab7, primary hemocyte culture, shRNA, yellow head virus (YHV), zebrafish U6 promoter

1 | INTRODUCTION

Diseases, especially caused by viruses, have been the most serious problem of shrimp production loss worldwide. Yellow head virus (YHV) is one of major causative agents that lead to high mortality rates in the black tiger shrimp, *Penaeus monodon* (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Flegel, 2006). The name of virus came from the gross signs of disease especially yellowish discoloration of cephalothorax caused by the underlying yellow hepatopancreas and overall light color of moribund shrimp. Then it leads to total mortality within a few days from the onset of symptoms (Chantanachookin et al., 1993; Limsuwan, 1991). YHV targets tissues of ectodermal and mesodermal origin including the lymphoid organ, gills, hematopoietic tissue, hemocytes, and spongy connective tissue (Chantanachookin et al., 1993; Lightner, 1996). Previous reports demonstrated that *Penaeus monodon* Rab7 is involved in YHV replication in shrimp by using dsRNA-mediated RNA interference (RNAi) technique (Ongvarrasopone, Chanasakulniyom, Sritunyalucksana, & Panyim, 2008). Besides YHV, silencing of PmRab7 expression could also inhibit several shrimp virus infections including WSSV (Attasart et al., 2009; Ongvarrasopone et al., 2008), Laem-Singh virus (LSNV) (Ongvarrasopone, Chomchay, & Panyim, 2010), and Taura syndrome virus (TSV) (Ongvarrasopone, Saejia, Chanasakulniyom, & Panyim, 2011).

Most of the studies based on RNAi technology to silence gene expression in shrimp utilized synthetic small interfering RNAs (siRNA) or in vitro synthesized double-stranded RNAs (dsRNA) directly injected into shrimp, resulting in short-term protection from virus infection. Additional doses of injection are required in order to increase its efficiency because siRNA or dsRNA is readily digested by nuclease and eliminated from the renal glomeruli before reaching the diseased tissues when they dispensed into the blood (Rojanarata, Opanasopit, Techaarpornkul,

Ngawhirunpat, & Ruktanonchai, 2008). To overcome that transient RNAi effects, DNA-based construct expressing long-hairpin dsRNA was developed, and it has shown to remain stable after injection into shrimp tissues for at least 30 days (Krishnan, Babu, Saravanan, Rajendran, & Chaudhari, 2009). Recently, primary hemocyte culture from *P. monodon* shrimp for RNA knockdown study was established as a platform to test DNA-based construct expressing long-hairpin RNA against YHV infection *in vitro* (Thedcharoen et al., 2020). In particular, long dsRNA molecules (>30 nt) widely used in shrimp serve as potent inducers of both sequence-independent and sequence-dependent silencing responses by interferon activation (Robalino et al., 2004, 2005; Yodmuang, Tirasophon, Roshorm, Chinnirunvong, & Panyim, 2006). Moreover, disadvantages of long dsRNA have been reported including non-specific mRNA degradation as well as induction of cytotoxicity (Aagaard & Rossi, 2007; Stark, 2007), and high risk of the off-target effects (Aleman, Doench, & Sharp, 2007; Ma, Creanga, Lum, & Beachy, 2006). To solve that effects, gene silencing approaches are now turned to short interfering RNAs (siRNAs) to avoid non-specific effects because of the interferon activation by long dsRNA (Conklin, 2003). In aquatic organism, siRNAs have been widely used for gene function analysis in zebrafish which is one of the most popular vertebrate model for genetic and developmental biology studies (Dodd, Chambers, & Love, 2004). However, there is a challenge in application for siRNA, as this approach usually conducts the transient loss-of-function phenotypes. To serve long-term gene silencing approach, DNA-based construct that stably expressed siRNA or short-hairpin RNA (shRNA) is previously introduced by using U6 snRNA genes as promoter in zebrafish (Boonanuntanasarn, Panyim, & Yoshizaki, 2008). Furthermore, zebrafish U6 promoter not only can express shRNA in zebrafish cell extract but also in aquaculture-related fish and shrimp cell extracts; Nile tilapia (*Oreochromis niloticus*) and shrimp species (*Penaeus monodon* and *Litopenaeus vannamei*) (Boonanuntanasarn, Panyim, & Yoshizaki, 2009). Thus, the putative zebrafish U6 promoter could provide the potential use for vector-based RNAi for long-term gene knockdown in these aquaculture-related species.

In this study, we applied short-hairpin RNA instead of long dsRNA to avoid non-specific effects. The DNA-based plasmid containing zebrafish U6 promoter to drive the expression of short hairpin RNA against PmRab7 gene has been constructed to study its effect on the knockdown of PmRab7 mRNA and YHV replication, when applied to the primary shrimp cell culture (Boonanuntanasarn et al., 2009).

2 | MATERIALS AND METHODS

2.1 | Zebrafish U6 promoter amplification

To obtain the U6 promoter sequence from zebrafish genome, genomic DNA extraction from dorsal fins of zebrafish (*D. rerio*) was carried out. Briefly, the dorsal fins were collected from individual zebrafish and then homogenized in DNA lysis buffer (50 mM Tris-HC1, pH 9.0, 100 mM EDTA, 50 mM NaCl), containing 2% SDS and 5 μg/ml of proteinase K). The total DNA was further extracted by phenol-chloroform-isoamyl alcohol and precipitated by absolute ethanol. The DNA pellet was collected by centrifugation and then re-suspended in TE buffer (10 mM tris–HCl, pH 8.0, 1 mM EDTA). The DNA concentration was determined by spectrophotometer (NanoDrop, Thermofisher Scientific, USA). For PCR amplification, two-step PCR amplification by using different primers was performed to obtain U6 promoter region. Firstly, one-step PCR amplification using specific primers U6-1F and U6-1R-1 (Table 1) was performed. About 100 ng of total zebrafish DNA were used. The PCR reagents and reaction setting were prepared using Invitrogen® *Taq* polymerase (Invitrogen, USA). A total 35 cycles of PCR amplification following initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C, 45 s, annealing at 70°C, 45 s, and extension at 72°C, 45 s with final extension at 72°C for 7 min. The second PCR reaction was performed by using U6-1F-Xbal and U6-1R-2 primers (Table 1) and the PCR product obtained from previous reaction was used as a template. The same PCR reaction setting and amplification cycles were performed similarly to the first PCR step. The PCR products were separated by using 1.2% agarose gel electrophoresis and further purified by using QIAquick Gel purification kit (Qiagen, USA). To verify DNA sequence, the purified PCR amplicon was ligated into pGEM®-T Easy vector (Promega, USA).

TABLE 1 Primers used in this study

Primer name	Sequence (5′-3′)	Tm (°C)	References
U6-1F	TCCATATTGCTGTTTTAGTGCGTGG	65.1	(Boonanuntanasarn et al., 2008)
U6-1R-1	TGCGCAGGGGCCATGCT	64.2	
U6-1F-XbaI	GTTCTAGATCCATATTGCTGTTTTAGTGCGTGG	68.0	In house design
U6-1R-2	CGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	66.3	
R1- shGFP	**TCTTGAA**CAGGACCATGTGATCGCGCTTCGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	79.9	In house design
R2-shGFP	AATCTAGATTGG**AAAAAAA***AGCGCGATCACATGGTCCTG***TCTCTTGAA**CAG	74.6	
R1-shPmRab7-1	**TCTTGAA**CAGGAGAATCTTCTTGCGAGACGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	78.6	In house design
R2-shPmRab7-1	AATCTAGATTGG**AAAAAAA**TCTCGCAAGAAGATTCTCCTG**TCTCTTGAA**CAG	72.8	
R1-shPmRab7-2	**TCTTGAA**ACCAATCTGTCATCAACCATACGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	78.0	In house design
R2-shPmRab7-2	AATCTAGATTGG**AAAAAAA**TATGGTTGATGACAGATTGGT**TCTCTTGAA**ACC	72.0	
R1-shPmRab7-3	**TCTTGAA**TAGCCTTGTTGTCATTGGTCACGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	78.6	In house design
R2-shPmRab7-3	AATCTAGATTGG**AAAAAAA**TGACCAATGACAACAAGGCTA**TCTCTTGAA**TAG	71.8	
R1-shPmRab7-4	**TCTTGAA**CAAGAGCATGCATCCTGTTTACGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	78.6	In house design
R2-shPmRab7-4	AATCTAGATTGG**AAAAAAA**TAAACAGGATGCATGCTCTTG**TCTCTTGAA**C	72.1	
R1-Scramble-1	**CTCTTGAA**AGCTACTACATCTTATGGCGCTCGAGAGATCAGCAGTCAGGCTCAGGGC	77.3	In house design
R2-Scramble-1	AATCTAGATTGG**AAAAAAA**GCCATAAGATGTAGTAGCT**TCTCTTGAA**AG	70.8	
PmRab7-F	TCTGGGATACAGCTGGTCAA	62.3	In house design
PmRab7-R	GTCGCACAACATGGTCAGTC	62.7	
GY2	CATCTGTCCAGAAGGCGTCTATGA	64.6	(Cowley et al., 2004)
GY3	ACGCTCTGTGACAAGCATGAAGTT	62.9	
Ef-1α-F	GGTGCTGGACAAGCTGAGGC	63.7	(Junkunlo et al., 2012)
Ef-1α-R	CGTTCCGGTGATCATGTTCTTGATG	62.4	

Note: The underline letters indicate specific restriction enzyme digestion site; 5′-TCTAGA-3′ (*Xba*I) and 5′-CTCGAG-3′ (*Xho*I), and underline or highlight letters present specific sequences: loop (TCTCTTGAA), underline as sense, underline as anti-sense, and terminator (AAAAAA).

The recombinant plasmids were transformed into *E. coli* JM109 and the positive clones were selected. The recombinant plasmids were extracted from bacterial cell culture and plasmid DNA sequencing was further analyzed by using T7 and SP6 primer (Macrogen Inc., Korea)

2.2 | Short-hairpin RNA-containing DNA plasmid construction

Prediction of the candidate siRNA sequences with highest efficiency of target gene suppression was determined by siRNA prediction software (GenScript siRNA Target Finder). The PmRab7 complete mRNA sequence from GenBank accession number DQ231062.1 was a reference nucleotide sequence used in this study. According to the algorithm based on GenScript siRNA Target Finder analysis tool, four regions of high efficient siRNA sequence were predicted (Figure S3). The specific primers containing four regions of predicted short hairpin nucleotide were designed. Two different short hairpin nucleotides including non-specific gene (EGFP) and scramble nucleotide sequence mimic to PmRab7 shRNA were also generated as a non-targeting siRNA.

For shRNA expression cassette, construction was synthesized by two-step PCR method (Figure S1). Briefly, the recombinant plasmid containing zebrafish U6 promoter which was obtained from previous step was used as a template for the PCR reaction. For the first step PCR, the forward primer (U6-1F-XbaI) and reverse primers (R1 sets; R1-shGFP, R1-shPmRab7-1, R1-shPmRab7-2, R1-shPmRab7-3, R1-shPmRab7-4, and R1-shScramble1, (shown in Table 1) which containing about 20 nucleotides of sense direction siRNA plus 9 nucleotides of loop region were applied in PCR reaction. Secondly, obtained PCR amplicons were subsequently applied for second round PCR amplification. The primers mixes containing forward primer (pU6-1F-XbaI) and various types of reverse primers including R2-shGFP, R2-shPmRab7-1, R2-shPmRab7-2, R2-shPmRab7-3, R2-shPmRab7-4, and R2-shScramble-1 (shown in Table 1) were applied in second PCR reaction. Each of reverse primers in set no.2 (R2) contained a specific 19 nucleotides of anti-sense direction siRNA plus 9 nucleotides of loop region and then followed by 6 nucleotides of termination site. The complete PCR amplicon for each shRNA expression cassette consisted of specific restriction enzymes digestion site for *Xba*I (TCTAGA) restriction site at 5′ end, a total ~ 500 bp of cloned U6-1 promoter, about 20 nucleotides of each siRNA in different direction which linked together by 9 nucleotides of the loop region (TTCAAGAGA). Six nucleotides of termination signal sequence (TTTTTT) and the specific restriction enzymes digestion site for *Xho*I were also incorporated into the shRNA expression cassette. Complete shRNA expression cassette amplicon was purified and then ligated into pGEM-T® easy vector (Promega, USA). The schematic diagram of recombinant plasmid construction was shown in Figure 1. The recombinant plasmids were multiplied by bacterial transformation and the positive clones were collected. The DNA plasmids were obtained by plasmid extraction kit and the correct shRNA-expression DNA cassette was confirmed by sequencing analysis (Macrogen Inc., Korea). Prediction of RNA folding structures was analyzed by MFOLD (http://unafold.rna.albany.edu/). The purified plasmid constructs were prepared for further in vitro transfection experiment.

2.3 | Preparation of YHV inoculum

The shrimp (*P. monodon*) with gross signs of disease especially yellowish discoloration of cephalothorax which are usually infected by YHV was selected. The hemolymph was collected from YHV-infected shrimp by using 21G needle equipped with 5 ml syringe containing equal volume of hemocyte culture medium (2X L-15, Gibco). The hemocytes were separated by centrifugation at 830*g* for 10 min at 4°C and the plasma was further sequentially filtrated through 0.45 and 0.22 μm filter membrane, respectively. The YHV copy number in plasmas was evaluated by real-time PCR using specific primers GY2 and GY3 (Table 1). The YHV stock composed of 3×10^7 copies per μl was obtained. The YHV stock was aliquot and kept at −80°C until use.

FIGURE 1 Schematic representation of a cloning strategy to generate shRNA expression constructs. pGEM-T easy vector was applied as a core template for shRNA expression cassette construction. The complete cassette for shRNA expression comprised upstream zebrafish U6 promoter, designed siRNA nucleotide sequences in both sense and anti-sense direction which linked by the loop region, and the termination site

2.4 | Preparation of primary hemocyte culture

Specific pathogen free (SPF) black tiger shrimp (*P. monodon*) was obtained from Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. The hemolymph was withdrawn using 21G needle equipped with 5 ml syringe containing equal volume of cell culture medium (2X L-15) and pooled hemocytes from all samples were collected by centrifugation at 250*g* for 10 min at 4°C. The plasma was discarded and hemocyte pellet was resuspended in fresh prepared complete culture medium containing double-strength Leibovitz's L-15 (2X L-15, Gibco) supplemented with 20% fetal bovine serum (FBS, Gibco), 2 g/L glucose, 100 UI/ml penicillin, 100 μg/ml streptomycin, and the osmolality was adjusted to 740 mOsm/L by adding 5 M NaCl. The total hemocyte count (THC) was determined by using hemocytometer and 2 ml of cell suspension were seeded into 6-well plate at 3×10^6 cells per well, and maintained at 27°C in moisturizing condition. After 2 hr of initial seeding, hemocytes were observed by inverted microscope (Olympus, Japan) before further experimental use.

2.5 | In vitro transfection of recombinant plasmids and YHV infection in hemocyte culture

To evaluate protection efficiency of individual shRNA-harboring DNA plasmid constructs against YHV infection in shrimp cells, the recombinant plasmids obtained from previous step were transfected into the primary hemocyte culture and then followed by YHV infection as previously described (Thedcharoen et al., 2020). In brief, after 2 hr of initial hemocyte seeding, cells were separately transfected with various DNA plasmid constructs including shGFP, shPmRab7-1, shPmRab7-2, shPmRab7-3, shPmRab7-4, and shScramble-1. Five μl of transfection reagent (Fugene® HD, Roche, USA) was mixed with 2 μg of DNA plasmids by the volume ratio of 2.5:1 (Transfection Reagent:DNA), and mixtures were added to hemocyte cells in a final volume of 1,000 μl followed by incubation at 27°C. At 24 hr post transfection, culture medium was replaced and the purified YHV stock was added at final multiplicity of infection (MOI) equal to 1.0. After 2 hr post YHV infection, cells were excessively washed and fresh complete medium was added. Hemocyte culture was further maintained and was collected at desired time-points post virus infection. Each group of treatments were performed at least triplicate.

2.6 | Hemocyte RNA extraction and RT-PCR analysis

To evaluate whether the short hairpin RNA could inhibit PmRab7 expression and YHV replication level in shrimp hemocyte post shRNA plasmid transfection, the total hemocyte RNA was collected and extracted by using RNeasy® plus minikit (Qiagen, Germany). Subsequently, semi-quantitative RT-PCR was performed to determine differential expression of target genes. RT-PCR reaction was prepared by using One-step RT-PCR kit (Roche, Germany) and 150 ng total RNA was used as a template for each reaction. The primers set specific for PmRab7 mRNA (PmRab7-F

and PmRab7-R) and YHV genome (GY2-GY3) are listed in Table 1. The RT-PCR amplification cycles for both PmRab7 and YHV genomes were performed at the same condition following an initial step at 50°C for 30 min, then started with denature at 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s with final extension at 72°C for 2 min. Expected PCR amplicons were analyzed by 1.5% agarose gel electrophoresis. Amplification of *P. monodon* elongation factor-1α (EF1α) was also performed as an internal control gene. The PCR products were visualized by ethidium bromide staining and analysis of PCR product intensities to determine relative gene expression comparing among experiments was performed by using ImageJ analysis software version 1.46r (https://imagej.nih.gov/ij/). The relative expression levels of PmRab7 and viral genome were normalized to shrimp Ef-1α expression level.

2.7 | Immunofluorescence detection of PmRab7 and YHV proteins in hemocyte culture

To evaluate whether the protein expression level of PmRab7 and YHV replication is corresponding to mRNA suppression by shRNA in hemocytes, the culture medium was removed and samples washed twice with PBS. The hemocytes were fixed with 4% paraformaldehyde for 20 min and then washed three times with 1X PBS, pH 7.4. Fixed hemocytes were permeabilized with 0.1% Triton X-100 for 5 min prior to incubated with blocking buffer (10% FBS in 1X PBS, pH 7.4) for 1 hr. Subsequently, hemocytes were incubated with polyclonal rabbit anti-PmRab7 primary antibody and mouse anti-gp64 monoclonal antibody targeting YHV (Soowannayan et al., 2003). Both primary antibodies were diluted in 1% FBS by ratio 1:250 and cells were incubated at 37°C for 1 hr. After incubation, cells were washed three times with 1X PBS and then further incubated with goat anti-rabbit Alexa-488® conjugated secondary antibody (Invitrogen, USA) at dilution 1:1000 and goat anti-mouse Alexa-633 conjugated secondary antibody (Invitrogen, USA) at dilution 1:500. Incubation of both antibodies was performed for 1 hr at 37°C and protect from light. Finally, cells were rinsed with PBS for three times and then anti-fade permount with DAPI for nucleus staining was added. Incubation of the secondary antibodies without primary antibody incubation step was also performed as negative control for staining. The fluorescence signals were observed under confocal microscope (Fluoview FV10i, Olympus, Japan).

2.8 | Statistical analysis

The relative mRNA expression of PmRab7 and YHV in each group was reported as mean ± standard error of the mean (SEM) and plotted in bar graph. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Significant difference was considered with p-value less than 0.05.

3 | RESULTS

3.1 | Construction of PmRab7-shRNA expression plasmids

Four plasmid constructs expressing shRNA (pshPmRab7-1, pshPmRab7-2, pshPmRab7-3, and pshPmRab7-4) specific for PmRab7 were obtained. In addition, two shRNA expression constructs harboring non-specific shRNA including GFP (pshGFP) and scramble PmRab7 shRNA (pshScramble-1) were also constructed as negative control. Schematic diagram showing a complete shRNA expression cassette containing upstream zebrafish U6 promoter sequence has been shown in method section (Figure 1). Approximately 500 bp of putative zebrafish U6 promoter sequence was successfully amplified by two-step PCR (Figure S2A,B) and multiple sequence alignment was revealed 97% identity

to the putative zebrafish U6-1 promoter (GenBank accession no. NW001514080.1, Figure S2C) in previous report (Boonanuntanasarn et al., 2008). Confirmation of recombinant plasmids containing zebrafish U6 promoter and predicted shRNA sequences was performed by DNA sequencing. Multiple sequence alignment of each shRNA plasmid constructs comparing with zebrafish U6 promoter region was revealed (Figure 2a). The specific Polymerase III recognition sites of U6 promoter including a consensus sequence for RNA transcription factor binding (CCAAT), Proximal sequence element (PSE), and TATA box consensus element are indicated. Each of the recombinant plasmids contained 500 bp upstream U6 promoter followed by four candidate siRNA sequences specific to PmRab7 (pshPmRab7-1 to pshPmRab7-4). The shRNA expression cassette composed of about 20 nucleotides in both sense (red labeled) and anti-sense (green labeled) direction which linked by 9 nucleotide of loop region (blue labeled). The termination signal (TTTTTT, black labeled) was also incorporated in adjacent to anti-sense shRNA nucleotides (Figure 2a). Based on multiple sequence alignment, only pshPmRAb7-3 showed one nucleotide mismatch at terminal signal. Two recombinant plasmids containing non-specific shRNA including pshGFP and scramble siRNA (pshScramble-1) were also constructed to use as negative control. All shRNA sequences were analyzed by MFOLD to predict RNA folding structures. A pentamer loop (CAAGA) formed correctly in all shRNA sequences into stem-loop structure (Figure 2b).

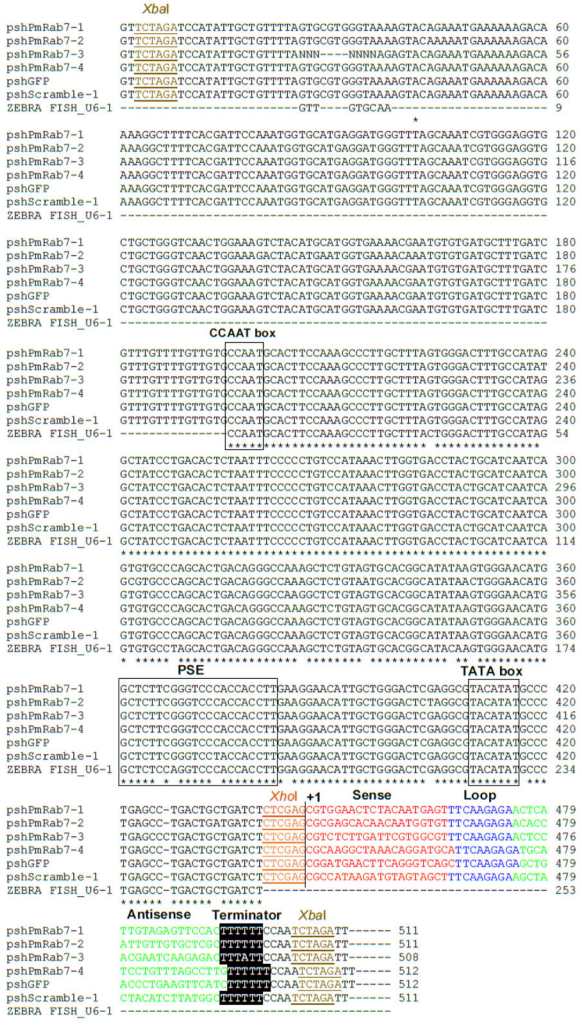
3.2 | Efficient PmRab7 suppression by shRNA plasmid constructs in shrimp hemocytes

The first important step has to be confirmed whether transfection could introduce our shRNA expression vector into cultured hemocytes. Two μg of purified recombinant plasmids were chemically transfected into hemocytes by Fugene® HD transfection reagent. The result showed that DNA plasmid constructs could be detected in hemocytes at 24 hr post transfection by PCR analysis using specific primer pairs (U6-1F-XbaI and U6-1R-2). Approximately, 500 bp PCR amplicon could be observed in hemocyte DNA derived from both pshGFP and pshPmRab7 transfections but not in the plasmid-transfected cell without transfection reagent (Figure 3a). This result suggested that the Fugene®-based transfection successfully introduced shRNA expression plasmid constructs into the primary cultured hemocytes. Subsequently, to evaluate suppression efficiency of PmRab7-shRNA expression plasmids, four recombinant plasmid constructs harboring different short hairpin (shRNA) sequences were tested by *in vitro* transfection in primary hemocytes culture. RT-PCR analysis was performed in order to determine the efficacy of siRNA suppression to PmRab7 gene. Agarose gel electrophoresis result indicated that various degrees of PmRab7 suppression were observed comparing among pshPmRab7 plasmid constructs. An efficient PmRab7 suppression was revealed by pshPmRab7-1 and pshPmRab7-2. Whereas moderate reduction level of PmRab7 was observed in hemocytes transfected by pshPmRab7-4 comparing to the hemocytes without plasmids transfection (Figure 3b). On the other hand, the pshPmRab7-3 transfected hemocytes showed no significant reduction of PmRab7 mRNA which might be because of the nucleotide mismatch at termination site (TTTATT) not consistent with those (TTTTTT) in the other constructs. Moreover, not only reduction in PmRab7 expression but also in Ef-1α expression in hemocytes transfected by non-specific shGFP were observed.

3.3 | Suppression of PmRab7 by shRNA plasmid constructs to inhibit YHV infection in shrimp hemocytes

Evaluation of shrimp PmRab7 involving YHV infection could be interrupted by reduction of host Rab7 expression by shRNA plasmid transfection following virus infection in hemocytes. In this experiment, hemocyte culture was transfected with shRNA plasmid constructs at 2 hr after initial seeding. After 24 hr post transfection, hemocytes were further infected for 2 hr by YHV at MOI of 1.0. The construct pshPmRab7-3 had nucleotide mismatch at terminator sequence, so this construct performed the poorest activity of the PmRab7 suppression. It was dropped from further

(a)



(b)

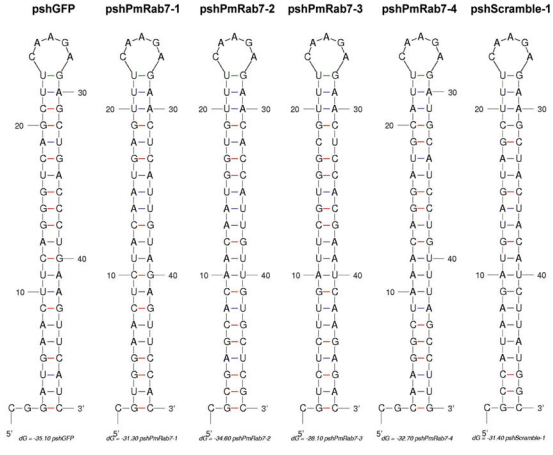


FIGURE 2 Multiple nucleotide alignments analysis of pshRNA containing short hairpin expression cassettes and RNA folding prediction. The alignments of nucleotide sequences in all types of recombinant plasmid constructs were demonstrated by MLS. The putative upstream U6 promoter sequence was indicated. Consensus regions of U6 promoter were indicated by box. shRNA expression cassettes for each specific shRNA were indicate by colors (a). The underline and highlight letters indicate specific restriction enzyme digestion site; TCTAGA (*Xba*I) and CTCGAG (*Xho*I). Prediction of RNA folding structures was demonstrated by MFOLD presenting a pentamer loop (CAAGA) in all shRNA sequences (b)

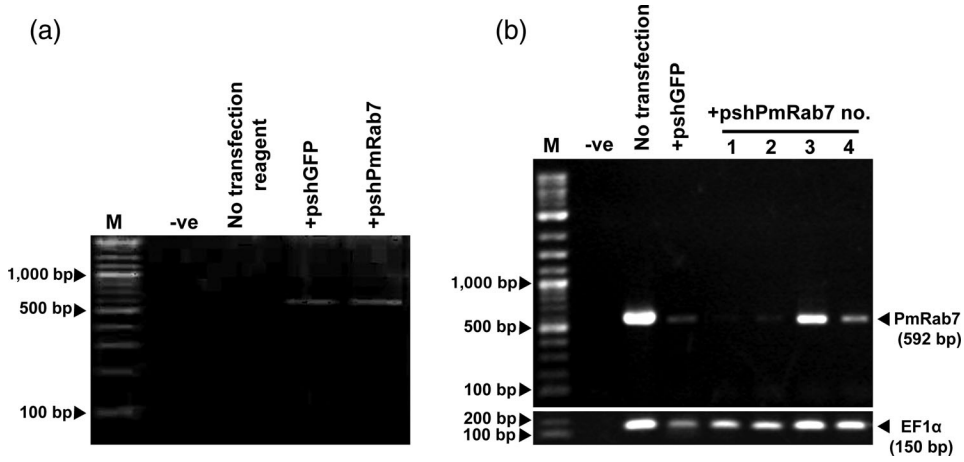


FIGURE 3 RT-PCR analysis for PmRab7 suppression efficiency of shRNA-expression constructs in shrimp primary hemocyte culture. PCR detection of shRNA-expression construct was confirmed that transfection was successful in shrimp primary hemocyte culture at 24 hr post transfection (a). Negative control (−ve) indicates no transfection of plasmid. RT-PCR detection of PmRab7 mRNA suppression efficiency by individual types of PmRab7 shRNA plasmid transfection in shrimp hemocytes (b). Negative control (−ve) indicates no cDNA templates

analysis. In addition, the GFP effect of pshGFP interferes the critical host transcripts, so the pshScramble-1 plasmid was designed as non-specific plasmid control instead of pshGFP. To follow the PmRab7 expression and YHV replication level, the hemocytes were then harvested at 24 and 48 hr post infection or the total duration post transfection for shRNA expression plasmids at 48 and 72 hr, respectively.

RT-PCR results showed that the trend of all specific shRNA against PmRab7 was able to suppress PmRab7 mRNA expression at 24 hr post virus infection (48 hr post plasmid transfection). Only the plasmid construct, pshPmRab7-2, had revealed significant inhibition of PmRab7 mRNA expression ($p < 0.05$) when compared to the YHV control group (Figure 4a). Meanwhile, significant reduction of YHV replication was also observed post pshPmRab7 transfection especially pshPmRab7-1 and pshPmRab7-2, respectively (Figure 4a). At 48 hr post virus infection, the significant reduction of both PmRab7 mRNA expression and virus replication level was still observed in only pshPmRab7-2 transfected cells, whereas mRNA expression of those in hemocytes transfected with pshPmRab7-1 or pshPmRab7-4 was recovered (Figure 4b). These results might indicate that prolongated siRNA suppression period may correspond to the region of inhibition and also the half-life of siRNA. Confirmation of PmRab7 protein expression correlated to YHV replication post plasmid transfection was demonstrated by immunofluorescence tracking of both PmRab7 and YHV protein (gp64) levels in hemocytes. Time-course analysis of PmRab7 and gp64 protein levels was determined at 24–72 hr post virus infection. There was no reduction of PmRab7 and YHV in the control treatment with pshScramble-1 transfected cells, but there was significant reduction of both proteins in pshPmRab7-2 transfected cells (Figures 5 and S4). After YHV infection, the pshPmRab7-2 transfected cells were examined at three time points, 24, 48, and 72 hr. The reduction in YHV protein was evident since 24 hr post virus infection (Figure 5), whereas clear reduction of PmRab7 was found at 72 hr post virus infection (Figure S4).

4 | DISCUSSION

RNAi-based technology has become an essential tool for preventive and therapeutic purposes for virus infection in shrimp. Most of the studies have relied on the use of synthesized siRNA or bacterially prepared dsRNA molecules by direct injection to trigger gene knockdown through the RNAi pathway. However, the effect is apparently transient

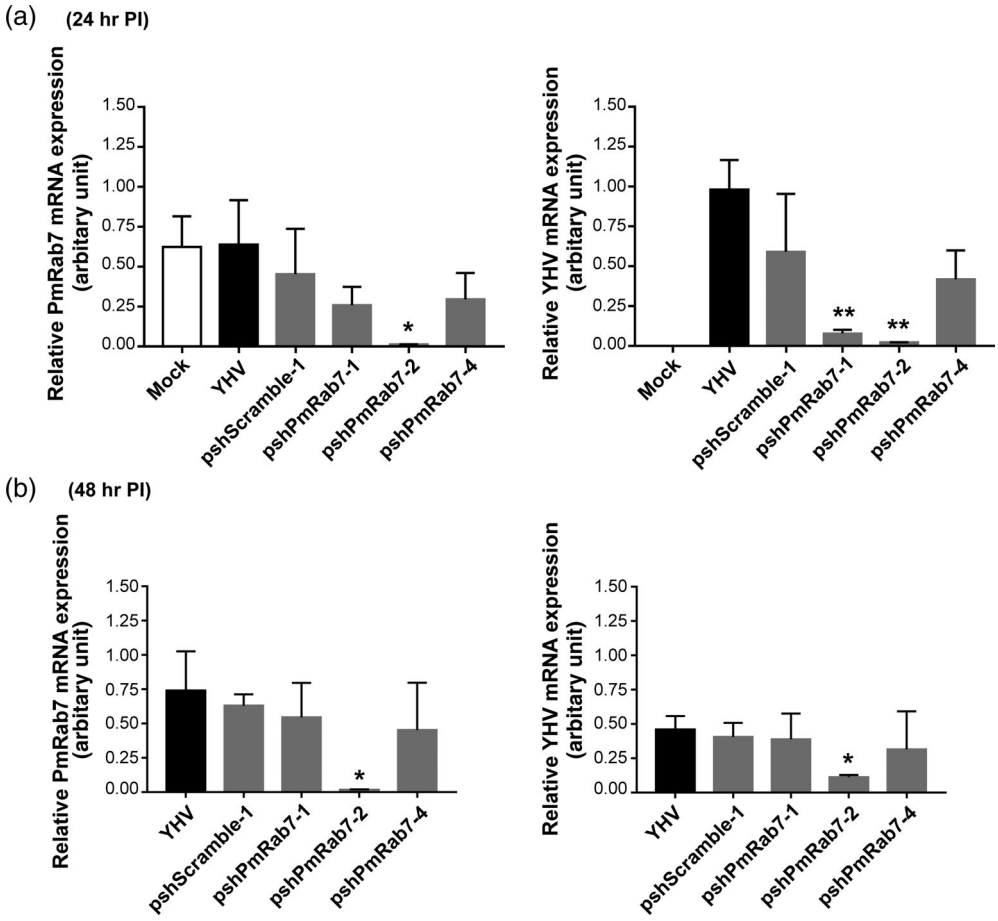


FIGURE 4 Effect of PmRab7 suppression by shRNAs against YHV replication in hemocytes. Time-course analysis of PmRab7 suppression efficiency and yellow head virus replication level in primary hemocyte culture was examined by different pshPmRab7 plasmid constructs. Each particular treatment group was transfected by different plasmid constructs for 24 hr prior to YHV infection. RT-PCR was performed to detect gene expression of PmRab7 and YHV. Relative mRNA expressions of PmRab7 and YHV in 24 hr (a) and 48 hr (b) post virus infection were expressed as mean ± SEM. Mock group was hemocytes without transfection of the plasmids and without viral infection, while the YHV group was no transfection of plasmid but infection with YHV. Elongation factor-1α (EF1α) transcript was used as reference for transcription levels. Statistically significant different between YHV control group and various treatment groups were performed, and *p*-value less than 0.05 and 0.01 presents as (*, **)

and thus might be inappropriate adaptation regarding to practical application in the field. In mammals, dsRNAs longer than 30 bp generally induce the interferon responses by binding to Toll-like receptors leading to the activation of double-stranded-RNA-activated protein kinase (PKR) and 2′, 5′-oligoadenylate synthetase-RNase L for a global protein synthesis inhibition as well as non-specific mRNA degradation (Aagaard & Rossi, 2007; Stark, 2007). Not only long dsRNA induces cytotoxicity, but it does risk the off-target effects from RNAi degradation of host, as many siRNA molecules were generated from a single dsRNA processed by an intracellular Dicer (Aleman et al., 2007; Ma et al., 2006). Recently, DNA plasmid constructs expressing long hairpin RNA have been shown to remain stable after injection into shrimp tissues for at least 30 days (Krishnan et al., 2009) and could be an alternative strategy to provide long-term inhibition of the expression of viral genes which in turn suppresses virus replication. For stable gene expression by DNA-based construct, a constitutive driving promoter is required. From previous report, zebrafish U6

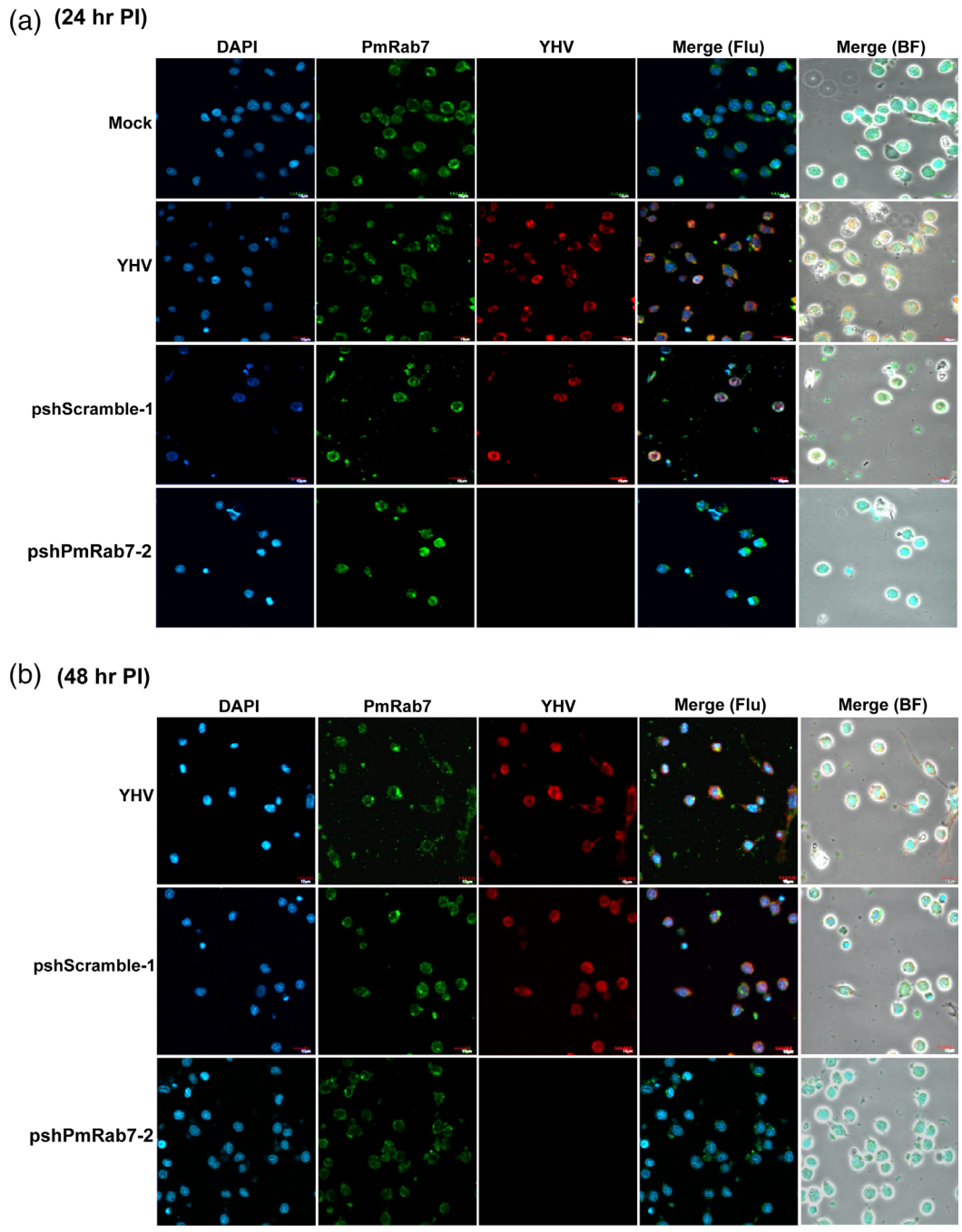


FIGURE 5 Immunofluorescence detection of PmRab7 and YHV protein post plasmids transfection in shrimp hemocytes. The protein expression levels of PmRab7 and YHV gp64 were determined by specific antibody at various time-points at 24 hr (a) and 48 hr (b) post virus infection. Mock group was hemocytes without transfection of the plasmids and without viral infection, while YHV group was no transfection of plasmid but infection with YHV. The treatment groups were transfected with pshScramble-1 or pshPmRab7-2 plasmid constructs prior to YHV infection. Nuclei were stained with DAPI, PmRab7 protein was stained with Alexa flour 488, and YHV capsid protein gp64 was stained with Alexa flour 633. Images merged with DAPI, Alexa 488 and Alexa 633 under fluorescent observation (Flu), and merged with bright field observation (BF). Scale bar = 10 μm

promoters were characterized and shown to drive the expression of shRNA *in vitro* using cell extracts prepared from two shrimp species, *Penaeus monodon* and *Penaeus vannamei* (Boonanuntanasarn et al., 2009). However, the transcription efficiency of zebrafish U6 promoters in shrimp cell extracts was around 70% of that in zebrafish cell extracts, so that would be because of the susceptible effect across species (Boonanuntanasarn et al., 2009). In this study, DNA-based plasmids were constructed to express shRNA under the control of zebrafish U6 promoter. The enhancer and basal elements including a CCAAT box, core region of proximal sequence element (PSE), and TATA box characteristic of type III RNA polymerase III (PolIII) promoter were all present in our cloned zebrafish U6-1 promoter (Figure S2) similarly to previous characterization (Boonanuntanasarn et al., 2008). The designed 19 nt stem of shRNA is advantageous for preventing triggering interferon system, as dsRNAs with longer than 30 bp can generally activate the interferon response, leading to a global shutoff in protein translation as well as non-specific mRNA degradation (Stark, 2007), and the shRNA could reduce the risk of off-target effects from RNAi mRNA degradation of host messengers (Aleman et al., 2007; Ma et al., 2006). This shRNA expression vector would be also no longer limited by the half-life of the applied siRNAs (Chen, Du, Zhang, Wahlestedt, & Liang, 2005; Grimm, 2009). Besides, duplexes of siRNA length 19–21 nucleotides could provide a successful RNAi purpose for gene functional analysis in cultured mammalian cell model (Elbashir et al., 2001) and in aquatic model (zebrafish) (Dodd et al., 2004). Thus, this new DNA-based plasmid would provide many advantages over the direct introduction of siRNA or dsRNA to knockdown a target gene. The small GTP binding protein Rab7 has a role in the late endocytic pathway and lysosome biogenesis (Vitelli et al., 1997). *Penaeus monodon* Rab7 (PmRab7) was isolated for the first time by virus-overlay protein binding assay, reported as a white spot syndrome virus (WSSV)-binding protein (Sritunyalucksana, Wannapapho, Lo, & Flegel, 2006). Later on, by silencing PmRab7 using dsRNA resulted in the inhibition of several viral infection in shrimp including YHV (Ongvarrasopone et al., 2008, 2010, 2011; Posiri, Ongvarrasopone, & Panyim, 2013) suggesting that most of these viruses possibly utilize this common endocytic mechanism for an entry into cell and infection in shrimp. Thus, PmRab7 becomes an attractive target for RNAi-based antiviral purposes in shrimp.

In this study, the DNA-based constructs of U6-pshPmRab7 were designed and transfected the primary shrimp hemocyte culture. The results demonstrated significant knockdown of PmRab7 mRNA and its cognate protein. Two of the plasmid constructs, pshPmRab7-1 and pshPmRab7-2, showed a great knockdown of PmRab7 mRNA after 24 hr post transfection; however, for YHV suppression, only the plasmid construct pshPmRab7-2 led to inhibit YHV replication from 24 to 48 hr post infection (Figure 4). The effect of PmRab7 knockdown by pshPmRab7-2 suggested the highest inhibitory efficiency on YHV replication on 24 hr post virus infection. At 48 hr post virus infection, the YHV replication started to be resumed, although the PmRab7 expression level was still as low as those observed at 24 hr post virus infection. It is hypothesized that there might be counteraction from the virus itself, which requires more experiment to clarify. Based on the primary structure of PmRab7 (GenBank accession no. DQ231062), the region (nt 471–489) designed for pshPmRab7-2 was not included in the putative domains of the protein (Figure S3). The success in silencing of PmRab7 expression indicated the PolIII driven-vector RNAi expression system could drive the expression of shRNA to induce RNAi for specific knockdown of target gene inside shrimp cells. Notably, YHV infection caused an upregulation of PmRab7 expression in hemocytes by 15% from 24 to 48 hr post virus infection compared to mock group (Figure 4). This upregulation effect of PmRab7 after YHV infection was previously reported (Ongvarrasopone et al., 2008), and the similar result from the different *Penaeus* species, *P. japonicus* Rab (PjRab) gene was upregulated after WSSV infection (Wu & Zhang, 2007). Nevertheless, shRNA with a scrambled sequence for a coding region of PmRab7 had a little effect on mRNA degradation of PmRab7 and YHV (Figure 4). In contrast to the other control plasmid, pshGFP plasmid showed a large reduction in both PmRab7 and Ef-1α mRNA expressions (Figure 3). This may suggest the sequence-independent effect of siRNA especially in GFP siRNA that can induce a non-specific innate immune response against WSSV in shrimp as previously reported (Westenberg, Heinhuis, Zuidema, & Vlak, 2005). It may lead to non-specific mRNA degradation as well as global inhibition in protein translation of critical host transcripts (Oates, Bruce, & Ho, 2000; Zhao, Cao, Li, & Meng, 2001). On the other hand, the sequence-independent effect of siRNA in scrambled shRNA was much lower than in GFP siRNA, and this result was consistent to previous report (Robalino et al., 2005) of which control siRNA (arbitrary sequence) was rarely able to

induce similar innate immune response against WSSV infection in shrimp. Thus, the further investigation of that effect between GFP siRNA and scrambled siRNA using as control is still needed.

Moreover, the pshPmRab7-2 showed a great inhibition in both mRNA and protein levels of YHV, but mRNA level of PmRab7 was not consistent with protein level from immunofluorescent result (Figure 5). When we considered the specific effect of pshPmRab7-2 at 24–48 hr post virus infection, the PmRab7 in mRNA level was almost completely inhibited (Figure 4). In contrast, PmRab7 in protein level was still observed in pshPmRab7-2 transfected hemocytes at 24–48 hr post virus infection. Surprisingly, when hemocytes transfected with pshPmRab7-2 were prolonged to 72 hr post virus infection (or 96 hr post transfection), the results showed that both PmRab7 and YHV's GP64 proteins had completely disappeared (Figure S4). Although we did not evaluate the mRNA expression of PmRab7 at 72 hr post virus infection, the PmRab7 knockdown effect of pshPmRab7-2 to YHV infection was still prolonged as shown in result from protein level (Figure S4). From the previous finding, the half-life of human Rab7 protein in HeLa cells is approximately 28 hr, and human Rab7 protein would completely degrade after about 56 hr (Ganley, Carroll, Bittova, & Pfeffer, 2004). This might be that PmRab7 protein expressing before gene knockdown still remained inside cell cytoplasm, but they were gradually degraded during 72–96 hr post transfection. Taken together with suppression for newly synthesized protein via specific interfering shRNA, the immunofluorescence signals of PmRab7 protein (green signal) clearly disappeared at 72 hr post virus infection (or 96 hr post transfection) (Figure S4). Similarly to the other PmRab, PmRab11 was almost completely knocked down at 96 hr post dsRNA injection (Kongprajug, Panyim, & Ongvarrasopone, 2017). In this accordance, it could be depicted that the newly synthesized PmRab7 protein was necessary for YHV transportation for infection and replication. YHV infection has relied on shrimp cell endocytic machinery especially PmRab7, so suppression of PmRab7 resulted in blocking of YHV in endosomal pathway being unable to further replicate process (Ongvarrasopone et al., 2008). Like in human, an overexpression of the dominant negative mutant of mammalian Rab7 led to accumulation of Semliki forest virus in early endosome (Vonderheit & Helenius, 2005). Therefore, YHV infection depended on several Rab proteins in shrimp; firstly PmRab5 supported clathrin-coated vesicle (CCV) to early endosome (EE) (Posiri, Panyim, & Ongvarrasopone, 2016), then PmRab7 required for EE to late endosome (LE) transportation (Ongvarrasopone et al., 2008), and PmRab11 involved in vesicle exocytosis from trans-Golgi network (TGN) to plasma membrane (PM) (Kongprajug et al., 2017).

Focusing on the Rab7 knockdown effects, Rab7 is a key regulator of the endocytosis pathway which controls multiple cellular and physiological functions. In shrimp, there was a concern that continuous knockdown of PmRab7 as a preventive antiviral purpose might also affect the well-being of the treated shrimp. To solve this problematic feature, several Pol III-based systems have been engineered to be inducible by tetracycline (Henriksen et al., 2007) or ecdysone (Gupta, Schoer, Egan, Hannon, & Mittal, 2004). Although inducible transcription systems applied in mammalian cells have been found to be somewhat leaky in the un-induced state (Aagaard & Rossi, 2007), it would be useful for future studies to improve DNA-based RNAi with controllable Pol III-based promoters which are suitable for gene functional analysis against viral-borne diseases in shrimp. To further apply in field study, an oral delivery of DNA construct expressing shRNA against genes involving viral-borne infection in shrimp via chitosan nanoparticle encapsulation as previously reported (Rajeshkumar et al., 2009) could be adopted to test the potential DNA-based RNAi vectors.

5 | CONCLUSIONS

This study demonstrated for the first time the successful utilization of DNA-based RNAi expressing shRNA driven by zebrafish U6 promoter for specific and efficient knockdown of the shrimp endogenous gene PmRab7 in primary hemocytes culture resulting in effective suppression of YHV replication. Moreover, this study proved that the newly synthesized PmRab7 protein was indispensable for YHV infection and replication in hemocytes. Overall, this DNA-based construct expressing short hairpin RNA vector could serve as tool for gene functional analysis or for testing as a novel antiviral vector against viral-borne diseases in shrimp.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

WT performed experimental works. YP analyzed and interpreted the data. HKK optimized hemocyte culture. LN trained the experimental techniques. WT and YP wrote the original draft of the manuscript. VS investigated thorough the study. ST prepared experimental shrimp and reviewed the manuscript. KS and SB conceived and designed the experiment, and edited the manuscript. SP and TWF conceptualized and supervised the research. All authors have approved the final version of manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and Supporting information file.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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