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PmEEA1, the early endosomal protein is employed by YHV for successful infection in *Penaeus monodon*

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ABSTRACT

Yellow head disease (YHD) is an infectious disease of *Penaeus monodon* which is caused by the yellow head virus (YHV). YHV infection invariably leads to 100% shrimp mortality within 3–5 days. Currently, an effective method to prevent or cure shrimp from YHV infection has not been elucidated. Therefore, the molecular mechanism underlying YHV infection should be examined. In this study, early endosome antigen 1 (EEA1) protein that was involved in the tethering step of the vesicle and early endosome fusion was investigated during YHV infection. The open reading frame of *P. monodon* EEA1 (PmEEA1) was cloned and sequenced (3000 bp). It encoded a putative protein of 999 amino acids and contained the zinc finger C_2H_2 domain signature at the N-terminus and the FYVE domain at the C-terminus. Suppression of PmEEA1 by specific dsRNA in shrimp showed inhibition of YHV replication after 48 h post YHV injection (hpi). On the other hand, shrimp received only NaCl without any dsRNA showed high YHV levels at approximately one hundred thousand times at 24 hpi and 48 hpi. Moreover, silencing of PmEEA1 by specific dsRNA followed by YHV challenge demonstrated a delay in shrimp mortality from 60 hpi to 168 hpi when compared to the control. These results indicated that YHV required PmEEA1 for trafficking within the infected cells, strongly suggesting that PmEEA1 may be a potential target to control and prevent YHV infection in *P. monodon*.

1. Introduction

YHD outbreaks have been reported since 1990 as epizootic events in the eastern, central and southern parts of Thailand, and resulted in shrimp production loss worldwide. A causative agent of this disease is the Yellow head virus (YHV) [1]. YHV is a positive-sense single stranded RNA virus that is classified into genus *Okavirus*, family *Roniviridae*, in the order *Nidovirales* [2,3] and is closely related to gill-associated virus (GAV) from Australia particularly in terms of the nucleotide and amino acid sequences, histological, and morphological observations [4,5]. YHV is approximately 50–60 × 190–200 nm in size, with enveloped bacilliform surrounded by prominent peplomers or spikes [6]. The genome of the virus is approximately 27 kb in length and consists of ORF1a, ORF1b, ORF2 and ORF3. The enveloped glycoprotein, gp116, which is involved in the entry process of YHV into the cells is encoded from ORF3 [7].

Currently, it is believed that the major route of YHV entry into the cells of *Penaeus monodon* is by clathrin-mediated endocytosis via clathrin heavy chain and AP17 protein [8,9]. The clathrin-coated vesicle containing YHV requires a small GTPase Rab5 protein to traffic it from the plasma membrane towards the first sorting station, the early endosome. *P. monodon* Rab5 was identified and showed to colocalize with YHV particles [10,11]. At the early endosomal compartment, Rab5 protein binds to the Rab5 effector protein, the early endosome antigen 1 (EEA1), to promote the fusion between the vesicular and early endosomal membranes, causing the cargo proteins including the virus to be transported inside of the early endosome [12].

Rab5 effector EEA1 works with SNAREs (Soluble NSF (N-Ethylmaleimide-Sensitive Factor) Attachment Protein Receptors) complex which consists of synaptobrevin, syntaxin and two SNAP-25 proteins. This interaction helps to promote the transported vesicle-endosome membrane tethering and fusion [12]. EEA1 location is associated with the early endosome and showed colocalization with Rab5 protein [13]. The EEA1 structure is a long parallel coiled coil homodimer that

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contains C_2H_2 zinc finger (ZF) domain at the N-terminus and FYVE domain at the C-terminus [14]. Interactions between the N-terminal ZF domain of EEA1 with Rab5 protein and the SNAREs complexes facilitate the docking and fusion processes. Furthermore, the fusion step of the vesicle and the early endosome requires phosphatidylinositol-4-5-bisphosphate 3-kinase (PI(3)K) activity whereby the C-terminal FYVE domain of EEA1 can bind directly to the product of PI(3)K, phosphatidylinositol-3-phosphate (PtdIns3P). Taken together, the specific binding of Rab5-GTP bound form, together with PI(3)K activity are needed in the vesicle-early endosome membrane fusion [15–18]. Furthermore, EEA1 has been shown to colocalize with Semliki forest virus (SFV) [19] and hepatitis C virus (HCV) [20]. Our previous study found that YHV utilized clathrin heavy chain, Rab5 and Rab7 proteins to get into the shrimp cells [9,11,21]. It is thus possible that YHV also requires Rab5 effector early endosome antigen 1 (EEA1) protein during infection. Therefore, in this study, the roles of EEA1 during YHV infection and shrimp mortality in *P. monodon* was investigated.

2. Materials and methods

2.1. The black tiger shrimp

Penaeus monodon or the black tiger shrimps were obtained from Choochai farm in Chonburi province and also from the Shrimp Genetic Improvement Center (SGIC, BIOTEC) in Surat Thani province, Thailand. Shrimps were acclimatized for at least 2 days and maintained in large containers with oxygenated sea water at 10–30 ppt salinity before used. They were fed with commercial feed every day. The water was changed every 2 days.

2.2. Cloning of the full-length PmEEA1

Coding region of EEA1 from *Marsupenaeus japonicus* kindly provided by Dr. Hidehiro Kondo, Tokyo University of Marine Science and Technology, Japan was used to design specific primers to amplify a region of PmEEA1. First, total RNA from ovary was extracted using Tri Reagent (Molecular Research Center) and cDNA was synthesized using Improm-II reverse transcriptase (Promega) according to the manufacturer's protocol. The full-length open reading frame of EEA1 was amplified using Q5 DNA polymerase (New England Biolabs) wtih cdEEA1-F and cdEEA1-R primers (Table 1), with an expected size of approximately 3000 bp. The PCR condition was: denaturation at 98 °C for 30 s, then 35 cycles of 98 °C for 10 s, 55 °C for 20 s, and 72 °C for 3 min. The PCR product was purified and cloned into pGEMT-easy vector and sequenced using T7, SP6, EEA1-Fseq, EEA1-Rseq and asEEA1-R2 primers (Table 1) by First Base Co, Ltd. (Malaysia).

2.3. PmEEA1 nucleotide and protein sequence analysis

The nucleotide sequence of PmEEA1 was confirmed and analyzed by BLASTN program using search under nucleotide database. Predictions of molecular weight and isoelectric point (pI) of the protein were performed by Expert Protein Analysis System (www.expasy.org). Conserved motifs of the deduced amino acids were scanned using ScanProsite tool (http://prosite.expasy.org/scanprosite). EEA1 protein sequences from several organisms were obtained from GenBank database. Phylogenetic analysis was performed using Phylogeny.fr with "A la Carte" mode (http://www.phylogeny.fr) based on neighbor-joining method and 1000 replicates of bootstrap with distance methods [22,23].

2.4. Construction of two dsRNAs targeting PmEEA1 mRNA

Recombinant plasmid containing stem-loop of dsRNA of PmEEA1 was constructed in pET-17b (Novagen) vectors. Two plasmids containing dsRNA-Cter and dsRNA-Nter whose targets were close to the stop and start codons of *PmEEA1* gene, respectively, were constructed. Sense-loop regions of the dsRNAs located near stop and start codons were amplified from the first-strand cDNA using specific primers, sEEA1-F1 and lEEA1-R1 for dsRNA-Cter, and nSLEEA1-F1 and nSLEEA1-R1 for dsRNA-Nter (Table 1). In addition, antisense regions were amplified using asEEA1-F2 and asEEA1-R2 for dsRNA-Cter, and nASEEA1-F2 and nASEEA1-R2 for dsRNA-Nter (Table 1). All PCR fragments were gel-purified and verified by restriction enzyme digestion. The purified fragments of the sense-loop and antisense of the Cter region were digested with *Eco*RI and ligated together using T4 DNA ligase (NEB). Then, the sense fragment was digested by *Xba*I whereas the antisense fragment was cut by *Xho*I. The ligated fragment of the sense and antisense of PmEEA1 was cloned into pET-17b to obtain pET-17b-dsRNA-Cter. In addition, the PCR fragment of sense-loop of the Nter region was cloned into pGEM-3Zf+ at *Xba*I and *Kpn*I sites and then the antisense fragment of the Nter region was subsequently cloned into *Kpn*I and *Eco*RI site of this recombinant plasmid. Then, the sense-loop and antisense fragments of the Nter region in pGEM-3Zf+ was cut and subcloned into pET-17b vector to construct recombinant plasmid named pET-17b-dsRNA-Nter. Both of the recombinant plasmids were used for dsRNA production by *in vivo* bacterial expression.

Table 1
Primer sequences used in this study.

Name	Sequence (5'→ 3')	Purposes
cdEEA1-F	ATGTCAGAGAGAGGAATG	Amplification of full-length cDNA coding region of PmEEA1
cdEEA1-R	TCACATTTTTGAAGTGAG	
T7	TAATACGACTCACTATAGGG	Sequencing of PmEEA1 nucleotides
SP6	ATTTAGGTGACACTATAG	
EEA1-Fseq	GCAGGGTTGAAGGAAGAGATG	
EEA1-Rseq	CCCTTAGCAGCTTCTCTCTCC	
PRT	CCGGAATTCAAGCTTCTAGAGGATCCTT TTTTTTTTTTTTT	The first strand cDNA synthesis
sEEA1-F1	*Xba*I GCTCTAGAACAAAATGAAGCCAAGCAGC	Construction of the recombinant plasmid expressing dsRNA-Cter targeting PmEEA1 mRNA
lEEA1-R1	*Eco*RI GGAATTCTAGCAACCTCAGCCTCCAG	
asEEA1-F2	*Xho*I CCGCTCGAGACAAAATGAAGCCAAGCAGC	
asEEA1-R2	*Eco*RI GGAATTCGGCATCAATTTCAAGCTGG	
nSLEEA1-F1	*Xba*I GCTCTAGAGGGCTTCTTGTGTCCAAC	Construction of the recombinant plasmid expressing dsRNA-Nter targeting PmEEA1 mRNA
nSLEEA1-R1	*Kpn*I GGGGTACCACCTTTTCAGCTTGTAGGG	
nASEEA1-F2	*Eco*RI GGAATTCGGGCTTCTTGTGTCCAAC	
nASEEA1-R2	*Kpn*I GGGGTACCCGCAAGGAACTGTTCAAC	
PmEEA1-F	AGCTTGAAATTGATGCCCAGAAG	Detection of PmEEA1 mRNA level
PmEEA1-R	TTGTTGCAGCTGTGGCAATTTAG	
PmActin-F	GACTCGTACGTGGGCGACGAGG	Detection of PmActin mRNA level
PmActin-R1	AGCAGCGGTGGTCATCTCCTGCTC	
qYHV-F	ATCATCAGCTCACAGGCAAGTTCC	Detection of YHV mRNA level by realtime PCR
qYHV-R	GGGTCTAAATGGAGCTGGAAGACC	
YHV(hel)-F	CAAGGACCACCTGGTACCGGTAAGAC	Detection of YHV mRNA level by RT-PCR
YHV(hel)-R	GCGGAAACGACTGACGGCTACATTCAC	
EF-1α-F	GAACTGCTGACCAAGATCGACAGG	Detection of EF-1α mRNA level by realtime PCR
EF-1α-R	GAGCATACTGTTGGAAGGTCTCCA	

2.5. Production of dsRNAs by in vivo bacterial expression

The recombinant plasmids, pET-17b-dsRNA-Cter and –Nter, were transformed into a RNase III mutant HT115 *E. coli* strain. DsRNAs

expression were induced by 0.1 mM IPTG. Then, they were extracted by using ethanol method [24,25]. The quality of the dsRNAs were characterized by ribonuclease digestion assay using RNase A and RNase III digestions. Concentration of dsRNA was estimated visually using agarose gel electrophoresis by comparing the target band intensity to that of the 100 bp DNA marker.

2.6. Yellow head virus (YHV) preparation

YHV stock was prepared using the hemolymph of YHV-infected moribund black tiger shrimp. The hemolymph was drawn and mixed with anticoagulant (AC-1) solution (27 mM Sodium citrate, 34.33 mM NaCl, 104.5 mM Glucose, 198.17 mM EDTA, pH 7.0), at ratio 1:1, and centrifuged at 20,000 ×g for 20 min at 4 °C to remove hemocyte debris. YHV was separated from the hemolymph by ultracentrifugation at 100,000 ×g for 1 h. YHV contained pellet was then dissolved with 150 mM NaCl and stored at −80 °C until used. The viral titer that caused 100% mortality within 3–4 days was used in this study. For confirmation of YHV infection, total RNA was extracted from the YHV-infected hemolymph that used to prepare the YHV stock and RT-PCR was performed to detect the level of YHV. In addition, the pellet obtained after ultracentrifugation of the hemolymph was dissolved with 150 mM NaCl and injected into shrimp. After 3–4 days of YHV injection, YHV levels can be detected in all dead shrimp, suggesting that the pellet contained YHV.

2.7. Injection of P. monodon with dsRNAs

The efficiency of dsRNA-Cter and dsRNA-Nter were examined by injection of dsRNAs into shrimp hemocoel. Shrimps were injected with 2.5 μg. g^{-1} shrimp of dsRNA-Cter, -Nter, C + Nter or unrelated dsRNA-GFP dissolved in 150 mM NaCl. Injection of 150 mM NaCl was used as control. After 24 h post dsRNA injection, gills of the individual shrimp were collected for total RNA extraction. Suppression effect of dsRNAs was analyzed by reverse-transcription PCR (RT-PCR) to determine PmEEA1 mRNA level.

2.8. Study of the knock down effect by dsRNAs upon YHV infection and shrimp mortality assay

In order to investigate the silencing effect of PmEEA1 upon YHV infection, shrimp were injected with 2.5 μg. g^{-1} shrimp of the combination of dsRNA-C and dsRNA-Nter (1.25 μg. g^{-1} shrimp each) or unrelated dsRNA-GFP, followed by YHV challenged after 24 h post dsRNA injection. For each group, 5 shrimp were used. Twenty-four and 48 h post YHV injection (hpi), gills of individual shrimp were collected and analyzed for PmEEA1 and YHV levels. To determine whether YHV levels could be detected at 60, 72, 84, and 96 hpi, gills from the living shrimp were collected. Moreover, shrimp mortality was also recorded every 12 h post YHV injection (hpi) for 144 hpi. Three replicates of the experiment were performed. Gills from the dead shrimp were also collected to determine YHV and PmEEA1 levels.

2.9. Total RNA extraction and RT-PCR analysis

Total RNA from gill tissue was isolated using Tri Reagent® (Molecular Research Center) following the manufacturer's protocol. Two microgram of the total RNA was used to produce first-strand cDNA using Improm-II™ reverse transcriptase (Promega) with PRT primer (Table 1). PCR products were amplified by using Taq DNA polymerase (New England Biolabs). Multiplex PCR of PmEEA1 (PmEEA1-F and PmEEA1-R1 primers, and PmActin (PmActin-F and PmActin-R1) (Table 1) were amplified under this condition: 95 °C for 5 min, then 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 68 °C for 45 s, followed by 68 °C for 7 min. YHV mRNA level was amplified using primers, YHV (hel)-F and YHV(hel)-R (Table 1). The multiplex PCR condition for YHV and PmActin was performed according to this condition: 95 °C for 5 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; followed by 72 °C for 7 min. The PCR products were analyzed on 1.5% agarose gel. The intensity of each band was quantitated using Scion Image program. Relative mRNA transcript levels of PmEEA1 was normalized with PmActin intensity and recorded as arbitrary unit.

2.10. Detection of YHV mRNA levels by quantitative real time PCR (qPCR)

For qPCR analysis, cDNA template was diluted at 1:4 and mixed with qPCR reaction using KAPA™ SYBR® Fast qPCR master mix (2X) ABI Prism™ (KAPA Biosystems) following manufacturer's protocol. The qPCR was analyzed using Mastercycler RealPlex4 (Eppendorf). qYHV-F and qYHV-R primers (Table 1) were used to amplify YHV mRNA. For internal control, EF1-α, was used (EF1α-F and EF1α-R primers) (Table 1). The qPCR condition was as followed: 95 °C for 3 min; 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The cycle threshold (C_t) values of YHV and EF1-α were compared and calculated using $2^{-\Delta\Delta CT}$ method [26].

2.11. Statistical analysis

The relative transcription levels of PmEEA1 and YHV that were normalized with PmActin or EF1α were presented as mean ± SD. In addition, cumulative percent shrimp mortality was plotted as mean ± SD. A significant difference of the experiment groups was examined by analysis of variance (ANOVA). A probability *(P)* value less than 0.05 was accepted as significant difference.

3. Results

3.1. Cloning and sequence analysis of PmEEA1 coding region

The full-length sequence of PmEEA1 is 3000 bp, including the stop codon (TGA), thus encoded a protein of 999 amino acids (GenBank accession number MK775551; Supplementary Fig. 1). The predicted molecular weight and pI of PmEEA1 protein are 112.6 kDa and 5.04, respectively. Sequence analysis revealed that PmEEA1 protein contains conserved domain of EEA1 which are Zinc finger C_2H_2 type domain at the amino acids 43–64 and 73–94, and the FYVE domain at the amino acids 938–996. These domains are conserved in both invertebrates and vertebrates including CeEEA1, DrEEA1, HsEEA1, LaEEA1, LpEEA1, and RnEEA1 (Fig. 1 and Table 2). Phylogenetic tree analysis revealed that PmEEA1 was closely related to the invertebrate group (Fig. 2).

3.2. Silencing of PmEEA1 mRNA levels by two dsRNAs

Two dsRNAs, dsRNA-Cter and dsRNA-Nter, targeting the PmEEA1 mRNA and dsRNA-GFP that used as unrelated dsRNA control could be cleaved by RNase III but not by RNase A, suggesting that they were of good quality. The expected sizes of these dsRNAs were approximately 400 bp (Supplementary Fig. 2). The effectiveness of dsRNA-Cter and dsRNA-Nter on silencing of PmEEA1 mRNA were investigated. Shrimp were injected with dsRNAs targeting PmEEA1 or unrelated dsRNA-GFP for 24 h. NaCl injection was employed as an experimental control. The result found that shrimp received either dsRNA-Cter or dsRNA-Nter showed a significant reduction of PmEEEA1 mRNA levels at 81% and 76%, respectively. Moreover, in shrimp injected with dsRNA-Cter and dsRNA-Nter (dsRNA-C + Nter), the level of PmEEA1 mRNA could hardly be detected (91% suppression). Interestingly, PmEEA1 mRNA level could be silenced at about 33% in dsRNA-GFP injected shrimp (Fig. 3).

3.3. Knockdown effect of PmEEA1 reduced YHV replication levels

The role of PmEEA1 on YHV level was next investigated during YHV replication. The combination dsRNA-C + Nter was used to silence

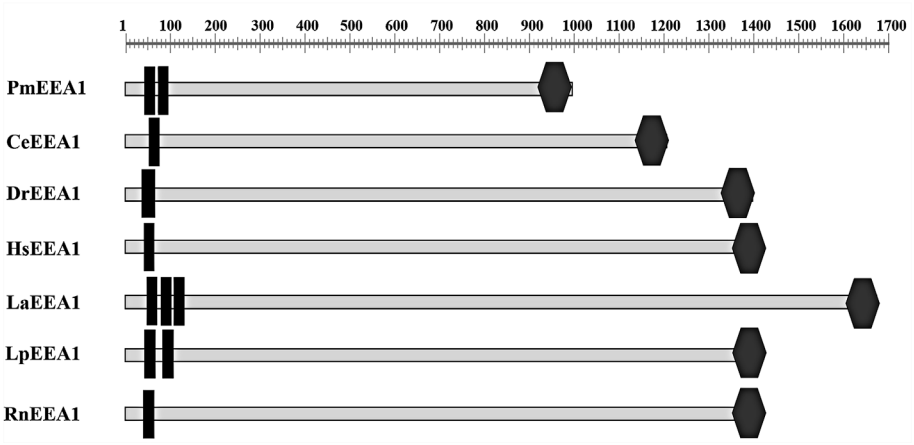

Fig. 1. Schematic of the predicted protein domain of the early endosome antigen 1 (EEA1) from *P. monodon* and other species. The size of EEA1 protein varied among organisms but all contain the signature motifs of Zinc finger C_2H_2 type domain (▮) at the N-terminus and the FYVE domain (⬮) at the C-terminus. *Penaeus monodon* EEA1 (PmEEA1) possess 999 amino acids which compose of two domains of Zinc finger C_2H_2 signature motifs at the position aa 43–64 and aa 73–94 and one FYVE domain at aa 938–996. EEA1 protein from several species including *Penaeus monodon* (PmEEA1; accession number MK_775551), *Caenorhabditis elegans* (CeEEA1; accession number NP_001024127.1), *Danio rerio* (DrEEA1; accession number XP_003200485.1), *Homo sapiens* (HsEEA1; accession number NP_003557.2), *Lingula anatine* (LaEEA1; accession number XP_013419201.1), *Limulus Polyphemus* (LpEEA1; accession number XP_013777228.1), *and Rattus norvegicus* (RnEEA1; accession number NP_001101556.1) were used for domain analysis.

Table 2
Early endosome antigen 1 (EEA1) proteins used for multiple sequence alignment and phylogenetic analysis.

Abbreviations	Species	Accession number
AcEEA1	*Aplysia californica*	XP_005095892.2
BaEEA1	*Balaenoptera acutorostrata scammoni*	XP_007166165.1
BgEEA1	*Biomphalaria glabrata*	XP_013093515.1
CeEEA1	*Caenorhabditis elegans*	NP_001024127.1
DrEEA1	*Danio rerio*	XP_003200485.1
FhEEA1	*Fundulus heteroclitus*	XP_012731526.1
HsEEA1	*Homo sapiens*	NP_003557.2
LaEEA1	*Lingula anatina*	XP_013419201.1
LpEEA1	*Limulus polyphemus*	XP_013777228.1
NgEEA1	*Nannospalax galili*	XP_008822059.1
ObEEA1	*Octopus bimaculoides*	XP_014782058.1
PmEEA1	*Penaeus monodon*	MK_775551
TcEEA1	*Tupaia chinensis*	ELW_61492.1
TrEEA1	*Takifugu rubripes*	XP_003967354.1
RnEEA1	*Rattus norvegicus*	NP_001101556.1
XtEEA1	*Xenopus tropicalis*	XP_002935361.1

PmEEA1 mRNA. Shrimp received the dsRNA after 24 h were challenged with YHV. After 24 and 48 h post YHV injection (hpi), the level of PmEEA1 and YHV mRNAs were determined from the gills. Injected shrimp with dsRNA-C + Nter following YHV challenge showed significant reduction of PmEEA1 at 24 and 48 hpi about 82% and 78% when compared to NaCl→YHV control group, respectively. Moreover, shrimp injected with unrelated dsRNA-GFP → YHV showed no difference in PmEEA1 mRNA levels at both 24 and 48 hpi when compared to the control (Fig. 4A).

The level of YHV mRNAs were determined by real time PCR and expressed as mean ± SD of the fold change of YHV mRNA levels compared to the NaCl injected group at 24 hpi. At 24 hpi, the fold change of YHV mRNA level in the PmEEA1 knock down group injected with dsRNA-C + Nter showed no significant difference when compared to the control groups injected with NaCl or dsRNA-GFP. At 48 hpi, the fold change of the YHV level increased more than 100,000 and 25,000 times for NaCl- and dsRNA-GFP- injected groups, respectively. However, the fold change of the YHV level at 48 hpi (1.28 ± 1.31 fold) remained at very low level and showed no significant difference when compared at 24 hpi (1.16 ± 0.53 fold) of the PmEEA1 knock down group (Fig. 4B). In addition, the expression of YHV mRNA levels were detected in the gills collected from the living shrimp between 48 and 96 hpi. The results showed that knockdown of PmEEA1 resulted in complete inhibition of YHV mRNA levels at 48 hpi. The expression of YHV mRNA levels can be detected at very low levels at 60 hpi and increased from 72 to 96 hpi in living shrimp. High levels of YHV mRNA could be detected in both control groups between 48 and 72 hpi. All shrimp in the control groups died at 72 hpi. These results suggested that knockdown of PmEEA1 restricted the YHV in the early compartment of the endosome. YHV levels could be detected but not high enough to cause shrimp dead between 60 and 96 hpi. Therefore, shrimp mortality was delayed in the dsRNA-C + Nter injected group (Supplementary Fig. 4).

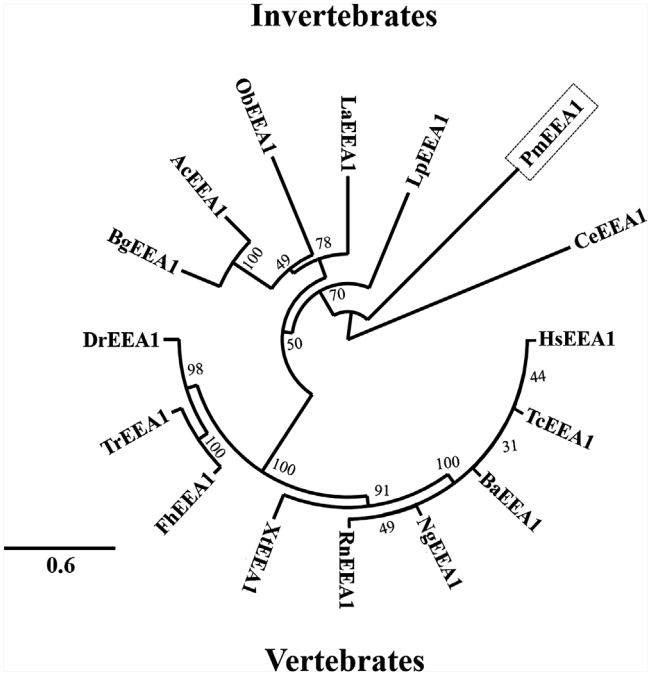

Fig. 2. Phylogenetic analysis of EEA1 protein. The phylogenetic tree was reconstructed using the Neighbor-joining method based on the full amino acid sequences of EEA1 with bootstrap value of 1000. Organisms used for the tree reconstruction were *Aplysia californica* (AcEEA1), *Balaenoptera acutorostrata scammoni* (BaEEA1), *Biomphalaria glabrata* (BgEEA1), *Caenorhabditis elegans* (CeEEA1), *Danio rerio* (DrEEA1), *Fundulus heteroclitus* (FhEEA1), *Homo sapiens* (HsEEA1), *Lingula anatine* (LaEEA1), *Limulus Polyphemus* (LpEEA1), *Nannospalax galili* (NgEEA1), *Octopus bimaculoides* (ObEEA1), *Penaeus monodon* (PmEEA1), *Tupaia chinensis* (TcEEA1), *Takifugu rubripes* (TrEEA1), *Rattus norvegicus* (RnEEA1), *and Xenopus tropicalis* (XtEEA1).

3.4. Silencing of PmEEA1in YHV-infected shrimp decreased shrimp mortality

The shrimp mortality was further observed to investigate the involvement of PmEEA1 on YHV infection. There were 10–15 shrimp per group and the experiments were performed three times. Shrimp

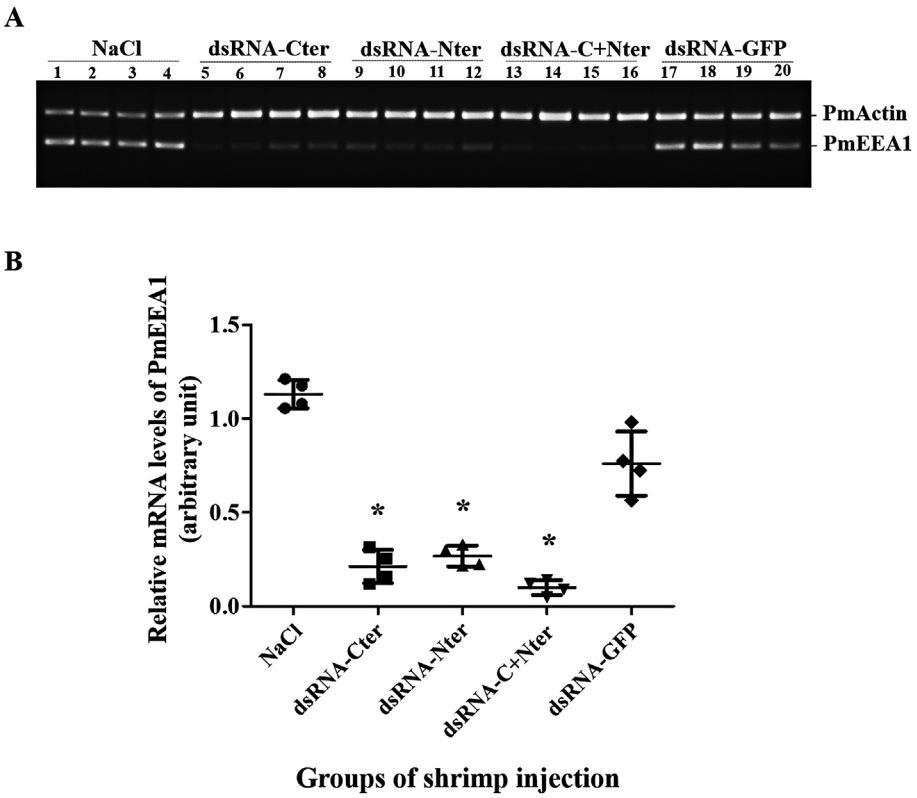

Fig. 3. Effectiveness of two types of dsRNAs targeting PmEEA1 mRNA. A representative gel of RT-PCR products was presented as expression levels of PmEEA1 and PmActin of shrimp injected with NaCl control (lanes 1–4), each dsRNA targeting PmEEA1 which are dsRNA-Cter (lanes 5–8) and dsRNA-Nter (lanes 9–12), the combination between dsRNA-Cter and -Nter (dsRNA-C + Nter) (lanes 13–16) and unrelated dsRNA-GFP (lanes 17–20) at 2.5 μg g^{-1} shrimp at 24 h post dsRNA injection (n = 4) (A). The relative mRNA expression levels of PmEEA1 normalized with PmActin are shown as mean ± SD (n = 4) (B). (*) Statistically significant difference between dsRNA injected shrimp as compared to NaCl injected group ($P < 0.05$).

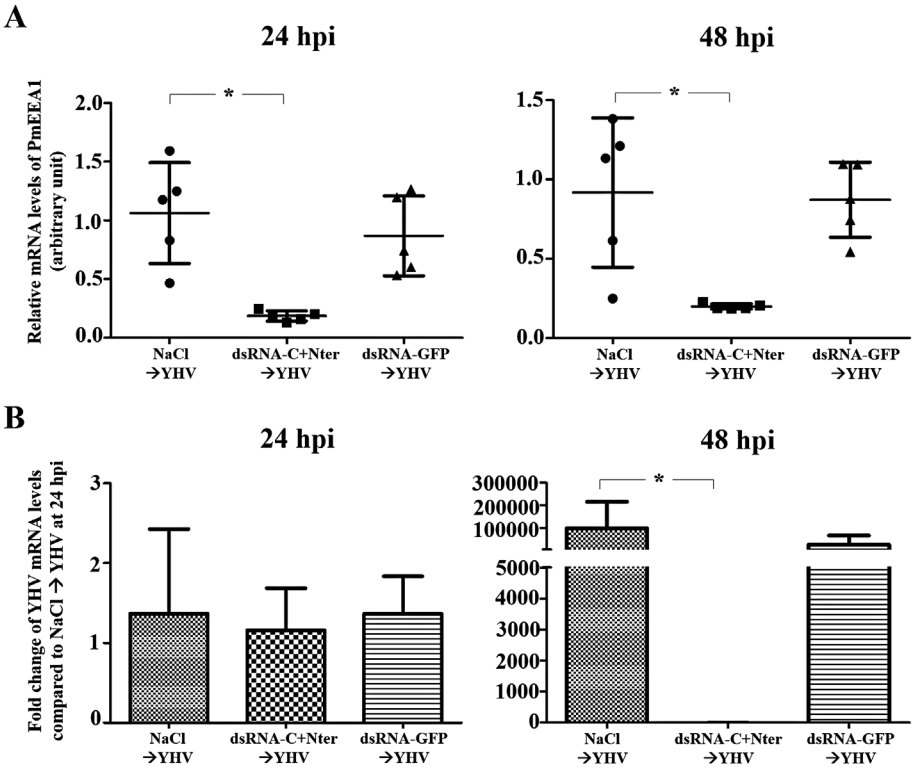

Fig. 4. Effect of PmEEA1 depletion during YHV infection. Shrimp were injected with NaCl alone, dsRNA-C + Nter, and dsRNA-GFP at 2.5 μg g^{-1} shrimp for 24 h followed by YHV challenge and detection at 24 and 48 hpi from gill tissues (n = 5). The relative mRNA levels of PmEEA1 normalized with PmActin are presented as dot graphs of arbitrary unit of mean ± SD (A). Bar graphs represent the quantitative RT-PCR of fold change of YHV mRNA levels compared to NaCl → YHV at 24 hpi. The result was demonstrated as mean ± SD (B). Asterisks indicate significant differences between experimental group and the control group ($P < 0.05$).

received dsRNA-C + Nter followed by YHV challenge showed a significant delay in shrimp mortality as observed in 60 hpi to 168 hpi when compared to NaCl → YHV and unrelated dsRNA-GFP → YHV groups (P value = 0.05). At 60 hpi, no shrimp in dsRNA-C + Nter → YHV group died whereas shrimp in the control groups of NaCl → YHV and unrelated dsRNA-GFP → YHV had 80% and 50% shrimp mortality, respectively. In addition, at 84 hpi, shrimp in both control groups showed 100% mortality while the dsRNA-C + Nter → YHV group demonstrated only 40% mortality (Fig. 5A). In addition, expression of YHV can be detected in all dead shrimp (Fig. 5B).

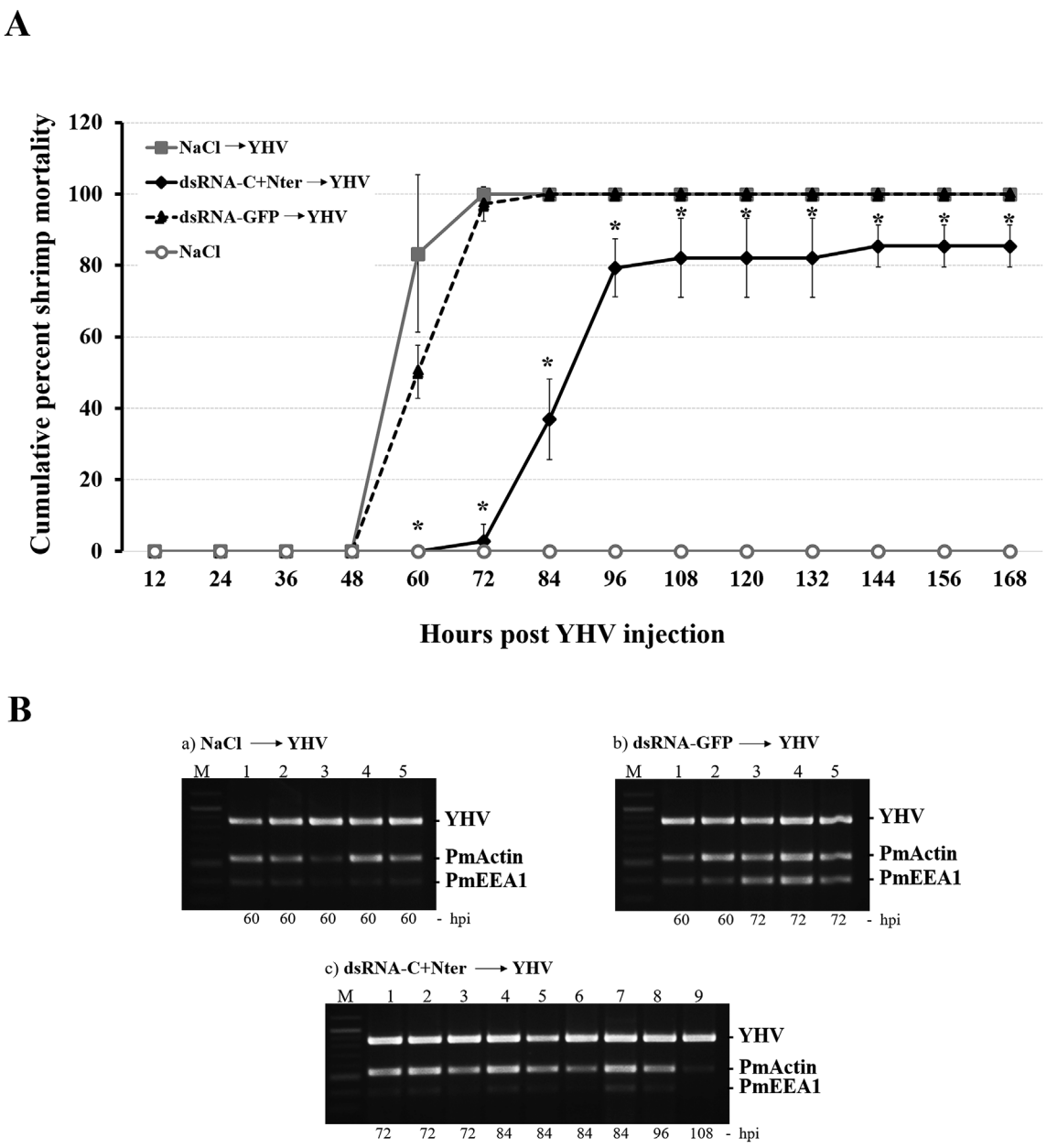

Fig. 5. Silencing of PmEEA1 by the combination dsRNA-C + Nter on YHV infection delayed shrimp mortality. (A) The cumulative percent mortality of shrimp injected with NaCl, dsRNA targeting PmEEA1 (dsRNA-C + Nter), or unrelated dsRNA-GFP at 2.5 μg g^{-1} shrimp followed by YHV injection were observed. Dead shrimp were recorded every 12 hpi. The graph was plotted as mean ± SD from three replicates per group (n = 12–15 shrimp per group). Asterisks represents statistically significant difference between NaCl → YHV and dsRNA-C + Nter → YHV group ($P < 0.05$). (B) A representative gel of RT-PCR products of YHV and PmEEA1 mRNA levels from gill of the dead shrimp in the 3 groups. PmActin was used as an internal control. PmAcin expression level is low in the dead shrimp samples that showed high levels of YHV expression. This result was also. demonstrated in the previous studies [9,15]. The number on the bottom of each lane represents the time (hours of post YHV challenge, hpi) that the shrimp die.

4. Discussion

Clathrin-mediated endocytosis is the major route of YHV penetration inside the shrimp host cell [8,9]. After internalization, YHV requires a small GTPase Rab5 protein to regulate the transportation from plasma membrane to early endosome. Colocalization between YHV and *Penaeus monodon* Rab5 (PmRab5) was observed in the hemocytes from 10 min to 3 h post YHV infection [11]. Rab5 proteins on vesicle membrane are tethered with Rab5 effector early endosome antigen 1 (EEA1) that appeared on the surface of the early endosome to promote the fusion of the two membrane [12,17,18]. Previously under transmission electron microscopy, it was found that YHV particles are inside the early endosomes of shrimp cells [27]. PmEEA1 protein contains 999 amino acids, while other species including CeEEA1, DrEEA1, HsEEA1, LaEEA1, LpEEA1, and RnEEA1 have more than 1200 amino acids (Fig. 1). Although the size of the protein from different species present a wide range of sizes, but they all showed signature characteristics of EEA1, two Zinc finger C_2H_2 domains and FYVE domain of N- and C-terminal sites [14]. Moreover, the function of EEA1 during YHV infection in *P. monodon* was examined. Two dsRNAs targeting N- and C-terminus of PmEEA1 were produced and used to knockdown PmEEA1 transcripts. A combination of two dsRNAs were used to improve the inhibition of YHV infection in shrimp [28]. Since, dsRNA targeting C-terminus of PmEEA1 region (the first construction) was located near 3′ end of the open reading frame (dsRNA-Cter). This dsRNA was first injection into shrimp resulting in inhibition of PmEEA1 mRNA levels only

50–80% compared to the control (data not shown). Local protein factors involving in termination of protein synthesis may cause the positional effect that interrupts the accessibility of RISC-siRNA complex to the local target [29]. After obtaining the full-length coding region of PmEEA1, another dsRNA targeting PmEEA1 mRNA was constructed. This dsRNA was located on near the 5′ end of the open reading frame (dsRNA-Nter). The result demonstrated that injection of the combined two types of dsRNA targeting PmEEA1 could silence the mRNA levels more than using only one type of the dsRNA (Fig. 3). This is probably due to a variety of siRNA population generated from the combined two dsRNAs. It could increase the efficiency of siRNA to bind to the mRNA target. The siRNA could bind with the secondary structure of mRNA as stem and loop structure better than the hairpin structure [30,31].

After testing the effectiveness of the dsRNA, the role of PmEEA1 during YHV infection was investigated. Shrimp that were injected with NaCl → YHV at 48 hpi showed increased YHV replication at about 100,000 times when compared to 24 hpi. On the other hand, injected shrimp with dsRNA targeting PmEEA1 (using the combination between dsRNA-Cter and –Nter (dsRNA-C + Nter)) followed by YHV challenge demonstrated no significant difference of YHV levels at 24 and 48 hpi (Fig. 4B). In addition, dsRNA-C + Nter → YHV group showed a delay in shrimp mortality when compared to the control NaCl → YHV group (Fig. 5). Injection of dsRNA targeting PmEEA1 alone has no effect on shrimp mortality (Supplementary Fig. 3). Shrimp started dying after receiving dsRNA-C + Nter followed by YHV challenge at 84 to 96 hpi (about 108–120 h post dsRNA injection) is probably caused by the loss of the effectiveness of its dsRNA and the increasing number of virus progenies. An increasing levels of YHV expression could be detected between 60 and 96 hpi in gills collected from the living shrimp. However, it did not cause shrimp die (Supplementary Fig. 4). High levels of YHV expression could be detected in all dead shrimp samples (Fig. 5B). Longevity of dsRNA inside the shrimp cells is about five days (120 h) after dsRNA injection and YHV challenge [32]. Therefore, to improve the effectiveness of dsRNA-C + Nter targeting PmEEA1 during YHV infection, multiple injections of dsRNA every 72 h may be performed in order to reduce shrimp mortality. Based on this study, silencing of PmEEA1 which is not lethal is possibly used as an alternative approach to prevent YHV replication. Taken together, this study demonstrated the crucial role of PmEEA1 during YHV transportation inside the cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.054.

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