Fish and Shellfish Immunology 93 (2019) 997–1006

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Genome organization and definition of the *Penaeus monodon* viral responsive protein 15 (*Pm*VRP15) promoter

Phattarunda Jaree[a,1], Taro Kawai[b], Chu-Fang Lo[c], Anchalee Tassanakajon[a], Kunlaya Somboonwiwat[a,*]

[a] Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd., Bangkok, Thailand
[b] Laboratory of Molecular Immunobiology, Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Nara, Japan
[c] Institute of Bioinformatics and Biosignal Transduction, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan, ROC

A B S T R A C T

The viral responsive protein 15 from the black tiger shrimp *Penaeus monodon* (*Pm*VRP15) is a highly responsive gene upon white spot syndrome virus (WSSV) challenge. It is identified from hemocyte and important for WSSV trafficking and assembly. However, the knowledge of *PmVRP15* gene regulation is limited. In the present study, the genome organization and 5′upstream promoter sequences of *PmVRP15* gene were investigated. The *PmVRP15* gene was found to contain 4 exons interrupted by 3 introns and the start codon was located in the exon 2. The transcription start site and TATA box were also determined from the 5′ upstream sequence. By using the narrow down experiment, the 5′ upstream promoter active region was determined to be at the nucleotide positions -525 to +612. Mutagenesis of the putative transcription factor (TF) binding sites revealed that the binding site of interferon regulatory factor (IRF) (-495/-479) was a repressor-binding site whereas those of the octamer transcription factor 1 (Oct-1) (-275/-268) and the nuclear factor of activated T-cells transcription factor (NFAT) (-228/-223) were activator-binding sites. This is the first report on the transcription factors that might play essential roles in modulating the *PmVRP15* gene expression. Nevertheless, the underlying regulation mechanism of *PmVRP15* gene expression needs further investigation.

## 1. Introduction

Due to the serious impact of WSSV infection on shrimp aquaculture, the understanding of mechanisms involved in WSSV pathogenesis in shrimp is needed. So far, little information on the underlying mechanisms of host responses during the WSSV infection in naturally infected shrimp is available. Although several immune-related proteins have been characterized, there are still many more uncharacterized WSSV-responsive proteins that play crucial roles in antiviral immunity.

Upon WSSV infection, it is known that signal transduction pathways in shrimp; for example, the Toll pathway and IMD pathway, are triggered [1]. The WSSV also hijacks the immune-related JAK-STAT and NF-κB pathways in order to promote the expression of the immediately early gene 1 [2,3]. Among the WSSV highly responsive gene/protein identified, the hemocyte homeostasis-associated protein (*Pm*HHAP) from *P. monodon* [4] is vital to survival of shrimp and plays an essential role in hemocyte homeostasis by controlling apoptosis during WSSV infection [5]. The *Pm*HHAP and its interacting-protein WSSV134 act as inhibitors of caspase-induced activation of *Pm*Casp [6].

The viral responsive protein 15 (*Pm*VRP15) from *P. monodon* or alternatively named as *Pm*ERP15, is highly up-regulated at both transcription and translation levels during WSSV infection [7]. The *PmVRP15* gene knockdown reduces viral gene expression and the cumulative mortality of WSSV-infected shrimp. As an endoplasmic reticulum (ER) stress-induced protein, its importance in the survival of WSSV-infected shrimp has been shown [8]. Moreover, the *Pm*VRP15 protein can interact with the viral tegument protein, WSV399, and is probably involved in WSSV trafficking and assembly during infection [9]. Recently, it has been reported that the *Pm*VRP15 plays a role in viral exit from the nucleus because the newly synthesized viral genomes accumulate in the nucleus in the absence of *Pm*VRP15 [10].

According to the fact that the transcriptional level of *PmVRP15* gene is low in the unchallenged shrimp but is extremely high after WSSV infection [7], it is believed that the expression of *PmVRP15* is tightly regulated at the transcriptional level. In this present work, the genome organization and genome walking were performed to identify the promoter sequence. The locations of possible transcription factor binding sites were verified. The putative regulatory elements responsible for the regulation of *PmVRP15* gene expression were identified. The Interferon regulatory factor (IRF) might act as the repressor whereas the Octamer transcription factor binding site 1 (Oct-1) and the Nuclear factor of activated T-cell transcription factor (NFAT) act as the activator. The

* Corresponding author.
*E-mail address:* kunlaya.s@chula.ac.th (K. Somboonwiwat).
[1] Current address: Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand.

https://doi.org/10.1016/j.fsi.2019.08.026
Received 28 May 2019; Received in revised form 6 August 2019; Accepted 10 August 2019
Available online 11 August 2019

modes of modulating the *PmVRP15* gene expression implicated.

## 2. Materials and methods

### 2.1. Genomic organization of PmVRP15 gene

The genomic DNA of *P. monodon* was extracted from shrimp pleopods (swimming leg). Two to three frozen shrimp swimming legs were mixed with extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose and 50 mM $Na_2EDTA$) and homogenized on ice. After that, 10% SDS was added and the mixture was incubated at 65 °C for 1 h proteinase K (50 ng/μL) was subsequently added and incubated at 65 °C for another 3 h. Protein was precipitated by 5 M potassium acetate solution. After centrifugation at 12,500 × *g* for 15 min, the supernatant was transferred into a new microcentrifuge tube. The extracted DNA was treated with 1 μg of RNase A at 37 °C for 30 min. The contaminated protein was removed by phenol:chloroform extraction. Genomic DNA was ethanol precipitated and the pellet was washed with 75% ethanol. The pellet was air-dried and resuspended with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Genomic DNA was stored at 4 °C until used. The quality and quantity of genomic DNA were analyzed by 1% (w/v) agarose gel electrophoresis and UV spectrophotometer.

To determine the organization of *PmVRP15* gene in genome, a primer pair specific to *PmVRP15* gene, ORF-PmVRP15-F and ORF-PmVRP15-R (Table 1), were designed and used for amplification of genomic DNA by PCR. The PCR conditions were 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and then a final extension at 72 °C for 5 min using Advantage® 2 polymerase (Clontech). The PCR product was analyzed by 1% (w/v) agarose gel electrophoresis and the positive band was purified and cloned into a T&A cloning vector (RBC Bioscience). The genomic DNA sequences were compared with the *PmVRP15* cDNA sequences using multiple sequence alignment software, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The exon–intron boundaries with the consensus GT at the 5′ end and AG at the 3′ end of the intron were used to identify the exon and intron regions of the *PmVRP15* gene.

### 2.2. Identification of the promoter active region of PmVRP15 gene

The 5′ upstream promoter sequence of *PmVRP15* gene were determined using genome walking technique. The genomic libraries were constructed using the GenomeWalker Universal Kit (Clontech). The *P. monodon* genomic DNA was completely digested by *Eco* RV, *Stu* I, *Pvu* II, and *Dra* I, respectively. The digested DNA was purified and ligated to GenomeWalker adaptor to construct four genomic libraries of *P. monodon*. The genomic libraries were used as the templates for PCR amplification.

In this experiment, four gene-specific primers were designed according to the *PmVRP15* genomic sequence available (Table 1). The first-round of genome walking used GSP1-1_PmVRP15 and GSP2-1_PmVRP15. The GSP1-2_PmVRP15 and GSP2-2_PmVRP15 were used for the second-round genome walking. Using each of four genomic DNA libraries as a template, the primary PCR was carried out with GSP1 and AP1 (adaptor primer, Clontech) as primers. The PCR reaction contained 100 ng DNA template, 1 × Advantage®2 reaction buffer, 0.2 mM dNTP, 400 nM each primer and Advantage®2 polymerase (Clontech). The primary PCR condition was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and then a final extension at 72 °C for 5 min.

The secondary PCR was performed using GSP2 and AP2 primer pair (adaptor nested primer, Clontech), using each primary PCR product as a template. The PCR reaction was the same as the primary PCR. The nested-PCR products were analyzed by 1% (w/v) agarose gel electrophoresis. The largest DNA fragment was purified by Nucleospin® Extract II Kit (Macherey-Nagel). The purified DNA fragment was cloned into a pGEM-T® easy vector (Promega) and subjected to DNA sequencing (Macrogen, Korea). The promoter sequence motifs were then analyzed using Neural Network Promoter Prediction and Promoter 2.0 Prediction Server.

### 2.3. Construction of the luciferase reporter plasmid containing the PmVRP15 promoter

The promoter sequence of *PmVRP15* gene from -2047 to +612 position was cloned into a reporter plasmid, pGL3-basic (Promega), containing the firefly luciferase gene for measuring the promoter activity. This recombinant plasmid, p(-2047/+612), was a parental plasmid for the experiment that narrowed down the DNA sequence to identify the promoter active region of *PmVRP15* gene. The narrowed-down *PmVRP15* promoter constructs containing the nucleotide sequence regions of -1621/+612, -1147/+612, -907/+612, -727/+612, -525/+612, -427/+612, -387/+612, -287/+612, -208/+612, -92/+612 and -34/+612, were constructed by PCR using the parental construct as a template and specific primers containing restriction sites, *Sac* I and *Xho* I, at the 5′ end of forward and reverse primers, respectively (Table 1). The PCR condition was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and then a final extension at 72 °C for 5 min using KOD *Taq* polymerase (TOYOBO). The PCR products were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid), then cut with *Sac* I and *Xho* I, and cloned in-frame into a pGL3-basic vector. The constructed plasmids were subjected to nucleotide sequencing to verify the sequences of inserts (MB Mission Biotech, Taiwan).

The pGL3 plasmids containing *PmVRP15* promoter regions, p(-2047/+612), p(-1621/+612), p(-1147/+612), p(-907/+612), p(-727/+612), p(-525/+612), p(-427/+612), p(-387/+612), p(-287/+612), p(-208/+612), p(-92/+612) and p(-34/+612) were purified using Qiagen Plasmid Mini Kit (Qiagen). The plasmids were analyzed by 1% (w/v) agarose gel electrophoresis, measured the plasmid concentration ($A_{260}$) and quality ($A_{260/280}$ and $A_{260/230}$) by UV spectrophotometer.

### 2.4. Promoter activity assay

Since no stable shrimp cell line is available, in this experiment, the *Drosophila Schneider* 2 (S2) hemocyte cell line, was used instead. For the transfection, S2 insect cells were seeded onto a 24-well plate ($8 \times 10^5$ cells/well) and grown in the complete Schneider's Drosophila Medium containing 10% heat-inactivated FBS and antibiotics (50 units penicillin G and 50 μg streptomycin sulfate per mL of medium, Invitrogen) for an overnight at 27 °C. Then, 200 ng of each pGL3 plasmid containing *PmVRP15* promoter region or the parental plasmid, pGL3-basic vector (Promega), as a negative control were co-transfected with 5 ng of the Renilla luciferase plasmid phRL/AcMNPV ie1 into the S2 cells using the Effectene Transfection Reagent (Qiagen) [3].

The promoter activities were determined using the Dual-Luciferase® Reporter Assay (Promega). Briefly, after 60 h post transfection, S2 cells were collected by centrifugation at 1000 × *g* for 10 min at 4 °C and washed with 1 × PBS buffer. The cells were lyzed with 1 × PLB (passive lysis buffer) and transfer to 96 well plate. The substrate of Firefly luciferase, LARII solution, was added into the wells and mixed. The Firefly luciferase activity was determined by measuring the luminescence signal in Relative Luminescence Units (RLU) using SpectraMax M5 Multi-Mode Microplate Reader (Molecular Device). After that, a Stop & Glo solution was added to stop the Firefly luciferase activity and the luminescence signal of the Renilla luciferase activity was measured.

The RLU value of Firefly luciferase activity was normalized to that of the Renilla luciferase to correct for transfection efficiency. The data is expressed as relative luciferase activity. Independent triplicate experiments were performed for each promoter construct, and the mean and standard deviation (SD) were calculated. The statistical significance of differences among means was calculated by the paired-samples *t*-test

**Table 1**
List of primers.

| Genome walking | |
|---|---|
| Primer name | Sequence (5′-3′) |
| ORF-PmVRP15-F | ATGTTAACAGAGGACTTA |
| ORFPmVRP15-R | ATGCTCTACTGACATGTTGTG |
| GSP1-1 PmVRP15 | AAGACGCCCAAGGGGCCCATATACAG |
| GSP2-1 PmVRP15 | TGAGACGAATGGTATGGAAGCGCACTGA |
| GSP1-2 PmVRP15 | TGTTCACTACGGGTGAGCCCACCCTGA |
| GSP2-2 PmVRP15 | TGGATTTTCTCGCATTTTCGAGGACTGCA |
| AP1 | GTAATACGACTCACTATAGGGC |
| AP2 | ACTATAGGGCACGCGTGG |
| **Amplification of *Pm*VRP15 promoter regions** | |
| **Primer name** | **Sequence (5′-3′)** |
| promo-2047SacIF | CGCGGAGCTC-CCTAGATGGAAAGAAGAAAAAAAATACTTTTGA |
| promo-1621SacIF | ACAGAGCTC-CCACTCAGGCCAGATGTGGTATACAGTT |
| promo-1147SacIF | AGCGAGCTC-TGGGAGCTGCTCTTATCCAATGTGATGC |
| promo-907SacIF | AGCAGGAGCTC-TGAGTCCGATGCTCTAACACTCG |
| promo-727SacIF | AGCAGGAGCTC-GCTGAACCTGTGTTTTCTGCTTGT |
| promo-525SacIF | TATGAGCTC-CCGTAGTGAACAGCTCCGGGTCTT |
| promo-427SacIF | AGCAGGAGCTC-TCACTCCTGCTCGGTTTTCC |
| promo-387SacIF | AGCAGGAGCTC-GTCGAGAGATTTTTTGTTGGGCATCA |
| promo-287SacIF | AGCAGGAGCTC-TGAAGTATTTATATTTGTATTTTGTAACATTAATGA |
| promo-208SacIF | AGCAGGAGCTC-ACCTATGCGCTGATAATGGCTT |
| promo-92SacIF | GGCGAGCTC-CTTCAGGTTCAAGTGCAAAACGTCATCC |
| promo-34SacIF | AGCAGGAGCTC-CTCAGTAATATAAAGCACAGTTCCCACG |
| promo + 612XhoIR | GCAGTCTCGAG-TGCGTAGCTAATGGGATGAGGA |
| **Promoter deletion assay** | |
| **Primer name** | **Sequence (5′-3′)** |
| p(-727/ + 612) del (-525/-428)F | TCACTCCTGCTCGGTTTTCCCGCC |
| p(-727/ + 612) del (-525/-428)R | GTGAGCCCACCCTGACCCCAGGT |
| p(-387/ + 612) del (-287/-209)F | ACCTATGCGCTGATAATGGCTTT |
| p(-387/ + 612) del (-287/-209)R | AAAAGTATGTAAAATTAGATACTAACGCTTCTTATC |
| **Site-directed mutagenesis of transcription factor binding sites** | |
| **Primer name** | **Sequence (5′-3′)** |
| p(-525/ + 612)muC/EBP_F | CTTAAACCGTACGGGTCTGA |
| p(-525/ + 612)muC/EBP_R | CCATTGATAGAGCGCGATCT |
| p(-525/ + 612)muPU.1_F | ACCATGAAACCGATAGATCGC |
| p(-525/ + 612)muPU.1_R | GAATTGTAGTGAAAGACCCGGA |
| p(-525/ + 612)muMyb_F | ATTTCCGGGTCTTTCACTACAAAAGAGGATG |
| p(-525/ + 612)muMyb_R | GCGCACTACGGGAGCTCGGTA |
| p(-525/ + 612)muRel_F | TGGCACTACAAAAGAGGATGAAACC |
| p(-525/ + 612)muRel_R | GATTCGGAGCTGTTCACTACGGG |
| p(-525/ + 612)muIRF_F | CAATAGATCGCGCTCTATCTTTGGC |
| p(-525/ + 612)muIRF_R | GCGTCATCCTCTTTTGTAGTGAAAGA |
| p(-525/ + 612)muNFYB_F | GCAAGATCGCGCTCTATCTTTGG |
| p(-525/ + 612)muNFYB_R | GATTTCATCCTCTTTTGTAGTGAAAGACCC |
| p(-287/ + 612)muOct-1_F | ATCGTTTGTAACATTAATGATAATGTCA |
| p(-287/ + 612)muOct-1_R | TACGATAAATACTTCAGAGCTCGG |
| p(-287/ + 612)muC/EBP_F | ATGGCATTAATGATAATGTCATATAATCTT |
| p(-287/ + 612)muC/EBP_R | AAGGTACAAATATAAATACTTCAGAGCTCG |
| p(-287/ + 612)muNFAT_F | GGAAAGAAATGAGAATAAACCTATGC |
| p(-287/ + 612)muNFAT_R | TAATTCTAAAGATTATATGACATTATCATTAATG |
| p(-287/ + 612)muUbx_F | CAATAATGTCATATAATCTTTAGAAGGAAAAAAG |
| p(-287/ + 612)muUbx_R | TCGATGTTACAAAATACAAATATAAATACTTCA |

where the significance was accepted at the *P*-value < 0.05.

### 2.5. Confirmation of the regulatory element by deletion assay

From the narrow down promoter assay experiment, the *PmVRP15* promoter active region that is required for the regulation of *PmVRP15* gene expression was identified. Two deletion constructs of -525/-428 and -287/-209 regions were cloned. The p(-727/ + 612) was used as a template and a primer pair; p(-727/ + 612) del (-525/-428) F and R (Table 1) was used for p(-727/ + 612) del (-525/-428) construction. The p(-387/ + 612) del (-287/-209) was constructed using p(-387/ + 612) as a template and primers such as p(-387/ + 612) del (-287/-209) F and R.

The rolling PCR condition was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 7 min, and then a final extension at 72 °C for 30 min using KOD *Taq* polymerase (TOYOBO). The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis and purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid). After ligation, the p(-727/ + 612) del (-525/-428) or p(-387/ + 612) del (-287/-209) constructs were transformed into an *E. coli* DH5α (RBC Bioscience) using calcium chloride heat-shock transformation. The plasmids were extracted and subjected to nucleotide sequencing to verify the sequences (MB Mission Biotech, Taiwan). The promoter activities of p(-727/ + 612) del (-525/-428) and p(-387/ + 612) del (-287/-209) were compared with those of p(-727/ + 612) or p(-387/ + 612), respectively, using Dual-Luciferase® Reporter Assay (Promega). Independent triplicate experiments were performed for each plasmid, and the mean and standard deviation (SD) were calculated. The statistical significance of differences among means was calculated by the paired-samples *t*-test where the significance was accepted at the *P*-value < 0.05.


**Fig. 1.** Genomic and deduced amino acid sequences of *Pm*VRP15. The position of transcription factor start site (+1) was predicted using Neural Network Promoter Prediction and TATA box located on -25 position is highlighted in grey with bold letters. Sequences of the four exons are highlighted in grey and the deduced amino acids are shown underneath in single letter code. The start codon (ATG) and stop codon (TAA) are boxed. The polyadenylation signal (AATAAA) is dotted underlined.

### *2.6. Prediction of transcription factor binding sites on the PmVRP15 promoter region by bioinformatics approach*

Upon identification of *PmVRP15* promoter regulatory sequence, the transcription factor binding sites were predicted by TF search Ver.1.3, Match 1.0 (TRANSFAC® Public database) and Alibaba (TRANSFAC® Public database) programs. The results were compared with the JASPAR database to confirm the DNA sequence of the putative transcription factor binding sites.

### *2.7. Identification of transcription factors regulating PmVRP15 gene expression by site-directed mutagenesis*

The predicted transcription factor binding sites in the *PmVRP15* promoter region were mutated and assayed for the promoter activity to confirm the importance of each transcription factor binding site in regulating the *PmVRP15* gene expression. A pair of specific primer was designed for site-directed mutagenesis for each transcription factor binding site on the *PmVRP15* promoter region.

The mutated constructs of these putative transcription factor binding sites were prepared by rolling PCR using their specific primers (Table 1). The PCR condition was same as in section 2.5. The mutated plasmid was subjected to nucleotide sequencing to verify the sequences (MB Mission Biotech, Taiwan). The promoter activity of each mutated-transcription factor binding site construct was measured and compared with that of wild type *PmVRP15* promoter plasmid using Dual-Luciferase® Reporter Assay (Promega). Independent triplicate experiments were performed for each mutated plasmid, and the mean and standard deviation (SD) were calculated. The statistical significance of differences among means was calculated by the paired-samples *t*-test where the significance was accepted at the *P*-value $< 0.05$.

## 3. Results

### *3.1. Genome organization of PmVRP15 gene and its promoter region*

Regulation of gene expression depends on different regulatory elements located in the noncoding and coding region of the genome. The *PmVRP15* gene is highly induced in the WSSV-challenged shrimp with unknown mechanism [7]. The clues may possibly lie in the *PmVRP15* gene region. The genome organization and promoter region of *PmVRP15* gene were therefore characterized. The corresponding genomic region was amplified by gene-specific primers using *P. monodon* genomic DNA as a template. The amplicon size of about 1 kb

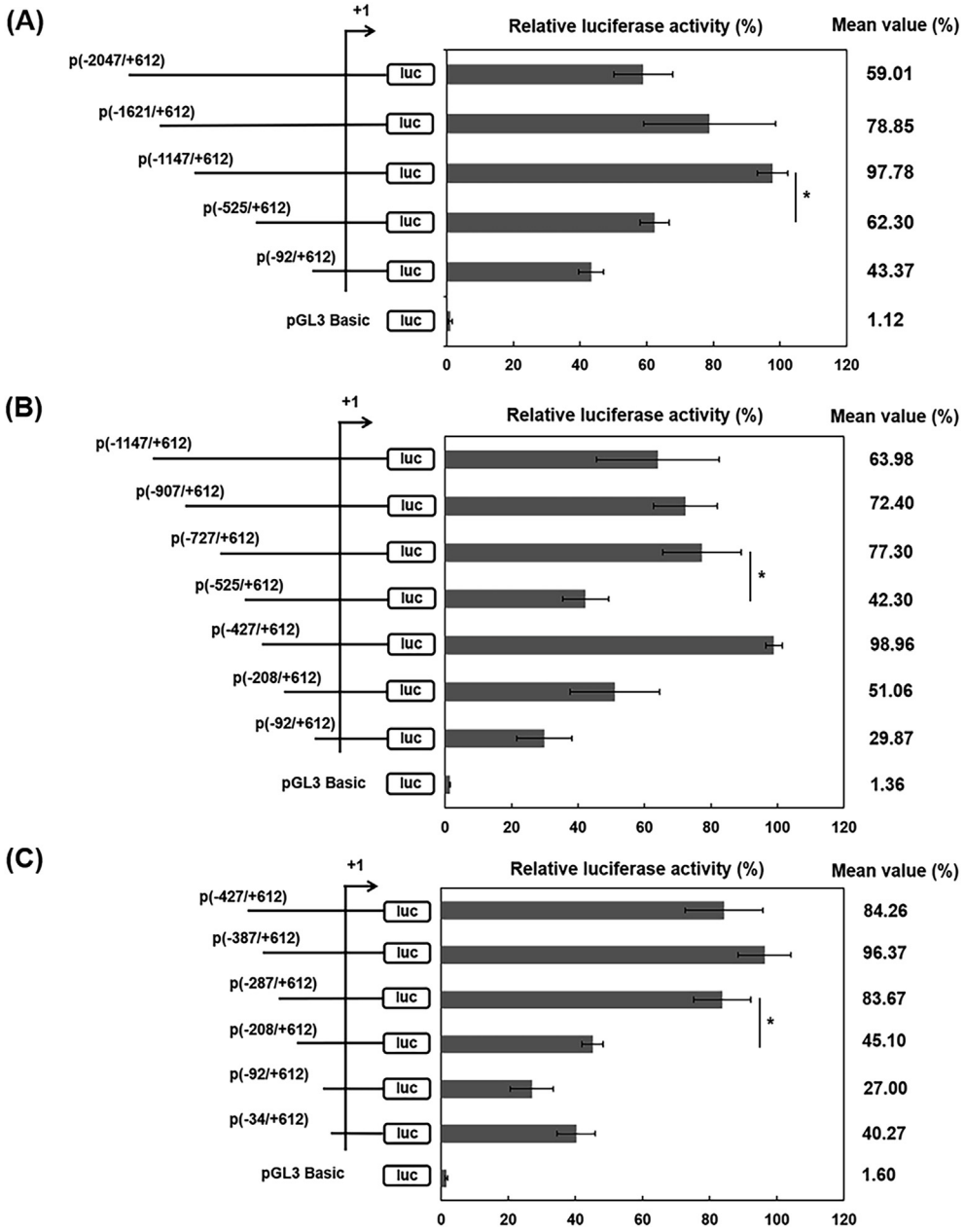


**Fig. 2.** Functional mapping of the deletion *Pm*VRP15 promoter from positions -2047 to -92 (A), -1147 to -92 (B) and -427 to -34 (C). Relative luciferase activity has been normalized to the activity of the p(-1147/+612) plasmid which is arbitrarily set to 100%. Data represents the means from triplicate experiments. Error bars show ± SD. * indicates significant differences with $P < 0.05$. The plasmid numbers in parentheses specify the beginning and end positions of the promoter fragments, and the arrow labeled +1 marks the transcription start site.

which was larger than the *PmVRP15* cDNA fragment, was obtained and sequenced. The DNA sequence analysis showed that the *PmVRP15* gene contained 3 exons interrupted by 2 introns with the exon and intron boundaries (AG/GT).

Furthermore, to study the regulation of *PmVRP15* gene expression, the 5′ upstream promoter sequence of *PmVRP15* gene was also determined by genome walking technique. The 5′ upstream sequence of *PmVRP15* gene of about 2 kb was obtained. Intriguing, another exon and intron were identified within the 5′ upstream region. Collectively, the *PmVRP15* gene contains 4 exons interrupted by 3 introns and the start codon was located in the exon 2 (Fig. 1). Based on the computational analysis, the transcription start site and TATA box were predicted as shown in Fig. 1.

### 3.2. Identification of PmVRP15 promoter active region

The *PmVRP15* promoter active region that is involved in the regulation of *PmVRP15* gene expression was identified by promoter narrow-down assay. The 5′ upstream sequence of *PmVRP15* gene to the translation start site at position +612 considered as the parental promoter region was amplified and cloned into a pGL3-basic vector containing the Firefly luciferase gene and was named as p(-2047/+612). In our first attempt, the *PmVRP15* parental promoter was randomly 5′ narrowed down. The 5′ narrowed-down plasmids including p(-2047/+612), p(-1621/+612), p(-1147/+612), p(-525/+612) and p(-92/+612) were constructed. The plasmids were used for the promoter activity assay by co-transfection with the phRL-null containing AcMNPv *ie*-1 promoter as an internal control into the *Drosophila* S2 cells. Of those tested, the p(-1147/+612) construct showed a significantly high level

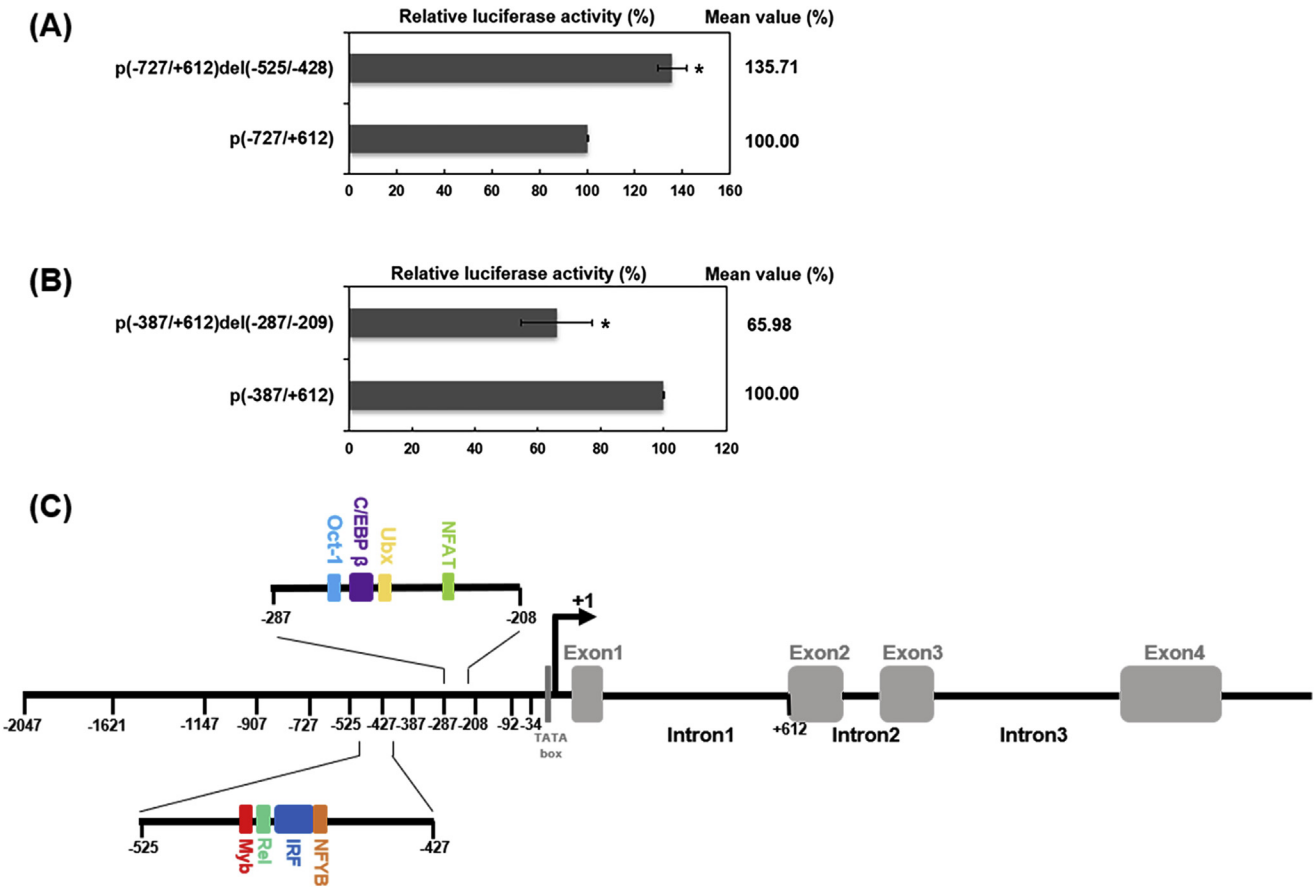


**Fig. 3.** Effect of deleting the nucleotide fragment from the *Pm*VRP15 promoter region. (A)The deletion plasmid p(-728/+612) del (-525/-428) was assayed for promoter activity in comparison with the parental plasmid p(-728/+612). (B) The deletion plasmid p(-387/+612) del (-287/-209) was assayed for promoter activity in comparison with the parental plasmid p(-387/+612). The experiment was performed in triplicate. The relative luciferase activity is expressed as mean ± SD. * indicates significant differences with $P < 0.05$. (C) Schematic diagram of the *Pm*VPR15 genome organization and the delineated TF-binding sites is shown.

of promoter activity suggesting that the sequence region of -1147/+612 might take part in controlling the *PmVRP15* gene expression (Fig. 2A). However, this promoter region was too long to be used for the identification of specific regulatory sites. Thus, the promoter sequence at positions -1147 to -93 was narrowed-down further.

The second set of narrowed-down promoter sequences including p(-907/+612), p(-727/+612), p(-427/+612) and p(-208/+612), were constructed and tested for promoter activity together with p(-1147/+612), p(-525/+612) and p(-92/+612). The sharp increase in promoter activity by 56.66% was observed when the promoter region -525 to -428 was deleted suggesting that the position between -525 and -428 might contain the repressor-binding site. Moreover, the promoter activity of p(-92/+612) was significant decreased for 69.09% as compared with that of p(-427/+612) suggesting the importance of DNA sequence between positions -427 to -93, i.e. the presence of activator binding site (Fig. 2B).

To define the promoter boundary closer, the promoter was further narrowed down from positions -427 to -34. A series of deletion plasmids including p(-427/+612), p(-387/+612), p(-287/+612), p(-208/+612), p(-92/+612), and p(-34/+612) were constructed and assayed for the promoter activity. The fact that the p(-208/+612) has much lower activity by 38.66% as compared to that of p(-287/+612) indicating that the sequence from positions -287 to -209 was important for the expression of *PmVRP15* gene for it might possess the activator-binding site (Fig. 2C).

### 3.3. Confirmation of the regulatory elements by deletion assay

From the above experimental results, two promoter regions, (-525 to -428) and (-287 to -209) were found to be essential for efficient *PmVRP15* gene regulation. If either one of them or both were absent, they would have profound effect on gene expression. These promoter regions were deleted and the promoter activity was assayed. For (-525/-428) promoter region, the promoter activity of the deletion construct p(-727/+612) del (-525/-428) was higher by 35.71% than that of the parental construct p(-727/+612) indicating that the promoter sequence between -525 and -428 region had the repressor-binding site as predicted above (Fig. 3A). On the other hand, the promoter activity of the (-287/-209) deletion construct, p(-387/+612) del (-287/-209), was decreased by 34.02% compared with the control p(-387/+612) suggesting that the promoter region between positions -287 to -209 contained the activator-binding site (Fig. 3B).

### 3.4. Prediction of transcription factor binding sites in the PmVRP15 promoter fragments

The transcription factor binding sites of the interested *PmVRP15* promoter regions were predicted by searching against the JASPAR transcription factor binding site database. The sequence at positions -525 to -428, predicted to be the repressor-binding site, was found to contain a few putative transcription factor binding sites such as Myb proto-oncogene protein (Myb) at the nucleotide positions -517 to -511, proto-oncogene c-Rel transcription factor (Rel) at positions -508 to -499, interferon regulatory factor (IRF) at positions -495 to -479 and

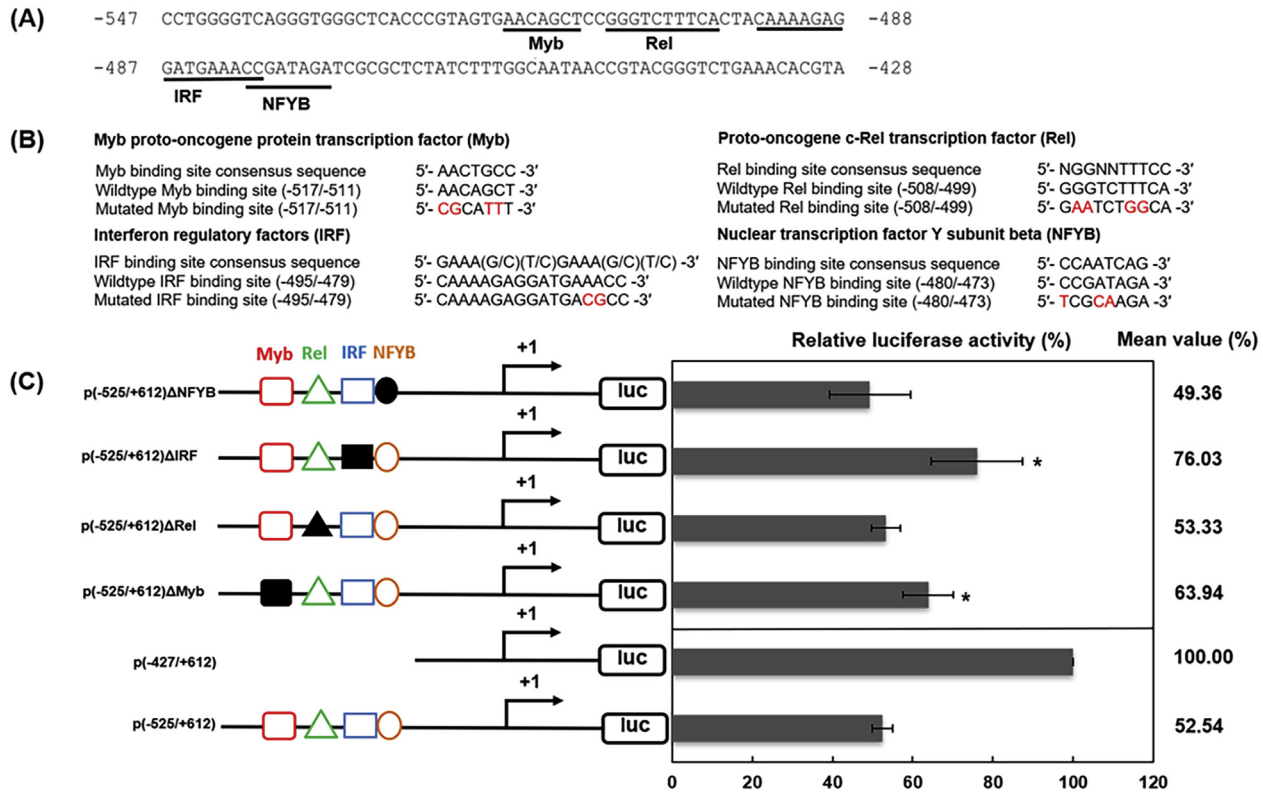


**Fig. 4.** Site-directed mutagenesis of transcription factor binding sites in the sequence positions -525 to-428) of *PmVRP15* promoter region. (A) The putative *cis*-acting elements in the 5′ flanking promoter region are underlined and designated according to bioinformatic analysis. (B) The nucleotide sequence of each transcription factor binding region on *Pm*VRP15 is shown in comparison to the consensus sequence and its mutated nucleotides indicated by red letters. (C) The promoter activity assay of the mutated sequences. The sites that are mutated are indicated by closed black symbols in the *PmVRP15* promoter active region in p(-525/+612). Relative luciferase activities of *PmVRP15* promoter constructs with wild-type or mutated transcription factor binding sites are shown. Data represent the means ± SD from three independent experiments. Means with an asterisk (*) shows significantly higher promoter activity than the control, p(-525/+612), with $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nuclear transcription factor Y subunit beta (NFYB) at positions -480 to -473 (Figs. 3C and 4A). For the (-287/-209) promoter region, predicted to be the activator-binding site, a few transcription factor binding sites were found such as octamer transcription factor binding site 1 (Oct-1) at positions -275 to -268, CCAAT/enhancer binding protein beta (C/EBP β) at positions -268 to -260, Ultrabithorax (Ubx) at positions -257 to -252 and nuclear factor of activated T-cells transcription factor (NFAT) at positions -228 to -223 (Figs. 3C and 5A).

### 3.5. Identification of the transcription factor that could regulate the *PmVRP15* promoter by site-directed mutagenesis

To further identify which predicted transcription factor binding sites play a role in regulating the *PmVRP15* gene expression, the putative transcription factor binding sites, Myb (-517/-511), Rel (-508/-499), IRF (-495/-479) and NFYB (-480/-473), in the p(-525/-428) were mutated by site-directed mutagenesis (Fig. 4B). The promoter activity was assayed in comparison with the wild type plasmid, p(-525/-428). It was found that the mutation only at the IRF binding site resulted in the increase in the promoter activity by 23.49% as compared with the control (Fig. 4C) indicating that the IRF binding site was a repressor binding site of *PmVRP15* promoter.

For the other *PmVRP15* promoter regions at positions -287 to -208, the Oct-1 (-275/-268), C/EBP β (-268/-260), Ubx (-257/-252) and NFAT (-228/-223) were mutated and assayed for promoter activity (Fig. 5B). It was found that the mutation at Oct-1 and NFAT binding sites decreased the promoter activity by 60.48% and 48.82%, respectively, when compared with the control (Fig. 5C). These indicated that the binding sites for Oct-1 and NFAT were the functional sites for the regulation of the *PmVRP15* gene expression.

## 4. Discussion

In *P. monodon*, the WSSV-responsive protein, *Pm*VRP15 has been shown previously to have a crucial role in WSSV pathogenesis. It is transcriptionally activated in response to WSSV infection in shrimp hemocytes, involved in trafficking and assembly of WSSV during infection through the interaction with WSV399 tegument protein of the virus, and functions in viral exit from the nucleus [4,7,9,10]. Moreover, a homolog from *Marsupenaeus japonicus* was identified. Although the amino acid sequence similarity was only 34%, it showed similar functional characteristics when compared to the *Pm*VRP15 [11].

Herein, the gene regulation of the viral responsive *PmVRP15* gene expression was characterized in order to gain more insight into its response upon WSSV infection. From our study, the gene structure of *PmVRP15* was determined. The gene contained 4 exons interrupted by 3 introns and the start codon was located in the exon 2. The 5′ upstream promoter active region was delineated and the transcription factor binding sites essential for efficient *PmVRP15* gene expression were identified within. Deletion at specific promoter region and site-directed mutagenesis at specific nucleotides of the transcription factor binding sites demonstrated that the *PmVRP15* promoter contained the putative repressor binding site for IRF located at the positions -495 to -479 and the putative activator binding sites for Oct-1 at -275 to -268 and NFAT at -228 to -223.

The human interferon regulatory factor (IRF) genes were up-regulated after viral infection or interferon (IFN) stimulation. The IRF proteins are not only the activators that induce the expression of IFNs

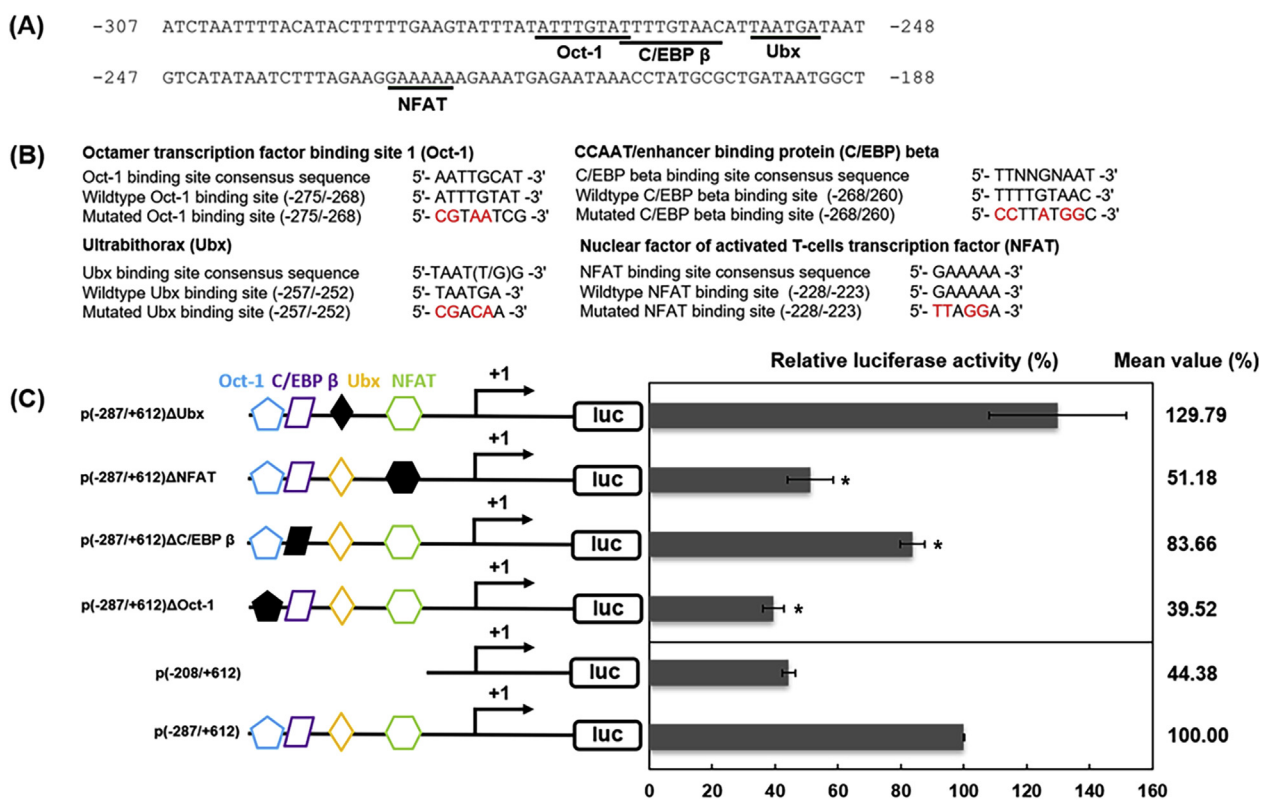


**Fig. 5.** Site-directed mutagenesis of transcription factor binding sites in the sequence positions -287 to-209) of *PmVRP15* promoter region. (A) The putative *cis*-acting elements in the 5′ flanking promoter region are underlined and designated according to bioinformatic analysis. (B) The nucleotide sequence of each transcription factor binding region on *PmVRP15* is shown in comparison to the consensus sequences and its mutated nucleotides indicated by red letters. (C) The promoter activity assay of the mutated sequences. The sites that are mutated are indicated by closed black symbols in the *PmVRP15* promoter active region in p(-287/+612). Relative luciferase activities of *PmVRP15* promoter constructs with wild-type or mutated transcription factor binding sites were shown. Data represent the means ± SD from three independent experiments. Means with an asterisk (*) shows significantly lower promoter activity than the control, p(-287/+612), with $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

but some of them also acted as the repressors [12]. The IRFs play roles in the regulation of host defense mechanisms including adaptive and innate immune responses and oncogenesis [13]. Moreover, the involvement of IRFs in innate and adaptive immune responses that are regulated by Toll-like receptors and cytosolic pattern-recognition receptors has been reported [14].

A couple of IRF-like genes have been identified and characterized in crustaceans. The pearl oyster IRF-2 is involved in the immune response to LPS and poly (I:C) stimulation *via* stimulating interferon and NF-κB signal pathway [15]. In 2015, the first shrimp, *Litopenaeus vannamei*, IFN regulatory factor (IRF)-like gene was discovered. It was reported that it could be activated during virus infection. Moreover, the *L. vannamei* IRF can bind to the 5′ UTR of *L. vannamei Vago4* gene, the IFN-like gene in invertebrate, and activates the *Vago4* gene expression. It is proposed that the IRF-Vago-JAK/STAT regulatory axis may possess an IFN system-like antiviral mechanism [16]. In our hands, our result implied that the shrimp IRF might act as a repressor that negatively regulated the *PmVRP15* gene expression by binding to the *PmVRP15* promoter at positions -525 to -428. Therefore, we hypothesized that the expression of *PmVRP15* was regulated through the action of IRF repressor. Without infection stress, the *Pm*VRP15 might not have a necessary role in shrimp and hence the IRF repressed the expression of *PmVRP15* gene.

The activator binding sites located at position -287 to -209 of *PmVRP15* promoter were putative Oct-1 and NFAT binding sites that might cause high *PmVRP15* production after WSSV infection. The Octamer consensus sequence located at position -275 to -268 of *PmVRP15* promoter region was possibly recognized by the POU/Oct transcription factor. This transcription factor has a conserved regulatory mechanism among mammals and *Drosophila* [17].

In *Drosophila*, the nubbin gene (*nub*), the class II POU/Oct 1 subfamily, encodes two transcription factor isoforms which are Nub-PB and Nub-PD. They regulate the immune homeostasis where the Nub-PB acts as an activator and the Nub-PD acts as a repressor to maintain the expression level of immune genes after pathogen invasion. Moreover, the stimulating immune system *via* Nub-PB activated JNK and JAK/STAT pathways to promote a proinflammatory and increased apoptosis and stem cell proliferation [18]. Like the WSSV-infected shrimp, several shrimp immune pathways are activated including prophenoloxidase activating system, apoptosis, JAK/STAT pathway and antimicrobial peptide production that protect the pathogen spreading in shrimp [1,19]. The few pieces of information about Oct-1 in shrimp are available. Although, the Oct-1 transcription factor controlling shrimp gene expression remains uncharacterized, the Oct-1 motif was found on the 5′ upstream sequence of the antimicrobial peptide, ALFs, which play an important role in shrimp antiviral immunity [20,21]. Oct-1 binding sites located on the promoter of ALF*Pm*2 and ALF*Pm*3, from the black tiger shrimp, *Penaeus monodon* were predicted [22]. Later, the regulatory elements of ALF*Pm*3 were characterized. However, the Oct-1 binding site of ALF*Pm*3 promoter was not located on the strong regulatory elements [23]. In the Chinese shrimp, *Fenneropenaeus chinensis*, the Oct-1 motif was probably an activator-binding site on the *ALFFc* promoter [24]. The regulation of Oct-1 transcription factor on the *PmVRP15* promoter needs to be further investigated in shrimp under WSSV infection condition.

The nuclear factor activated T-cell (NFAT) is a putative transcription factor that potentially stimulates the expression level of *PmVRP15* gene. The NFAT protein family has a highly conserved DNA-binding

domain that belongs to the Rel-family transcription factors. The conserved core region of NFAT proteins consists of two tandem domains; a regulatory domain, which is also known as the NFAT-homology region (NHR), and a DNA binding domain called Rel homology region, RHR. The NFAT proteins also have crucial roles in the development and function of the immune system. The NFAT targets many genes that control alternative functions in activated T cells, such as cell-cycle progression and activation-induced cell death. The highly flexible structure of the NFAT DNA-binding domain allows several surfaces to be available for interaction with different transcriptional partners on DNA, thereby, allowing the NFAT to integrate into many signaling pathways [25]. Moreover, the NFAT plays a role in Simian Virus 40 (SV40) infection. After inhibition of NFAT activity, the infectivity of SV40 is reduced suggesting the importance of NFAT in the transcription of viral gene [26]. Unlike the situation in vertebrates, especially mammals, little is known about NFATs in invertebrates. The *Drosophila* NFAT homolog, *Dm*NFAT5, was identified that activated in response to osmotic stress [27]. Moreover, the NFAT from *Branchiostoma belcheri* is firstly reported that involved in the innate immunity as its expression was induced by LPS stimulation [28]. The expression of PfNFAT, the NFAT homolog from the pearl oyster, *Pinctada fucata*, was found to be significantly up-regulated after stimulation with LPS and poly I:C [29]. The NFAT protein function is also involved in calcium signaling pathway. After the $Ca^{2+}$ is up-taken into the cytoplasm, the NFAT protein in cytoplasm is dephospholyrated and translocated into the nucleus to regulate the gene expression [30]. According to the conserved regions of NFAT, NHR and RHR are similar to that of the NF-kB transcription factor [31]. Moreover, the activation of the NF-κB pathway in response to pathogen infection is also the hallmark of shrimp innate immunity [32]. In shrimp humoral immunity, the NF-κB is related to signaling pathways such as Toll and Imd pathways. In response to WSSV infection, the activation of Toll and IMD signaling pathways results in the up-regulated activities of the NF-κB transcription factors Dorsal, Relish, and AP-1 that activate the antimicrobial peptides to decrease viral loads and shrimp mortality after WSSV infected shrimp [33]. *L. vannamei* NF-κB might play important roles in WSSV replication. WSSV might hijack the shrimp NF-κB system to overproduce the viral immediate-early genes [34]. According to our research, the presence of NFAT binding sequence on *PmVRP15* promoter led us to speculate that the responses of shrimp NFAT after WSSV infection might activate the *PmVRP15* gene expression *via* binding to *PmVRP15* promoter upon WSSV infection causing a sharply up-regulated *PmVRP15* expression for it enhanced the WSSV infection in shrimp. However, this hypothesis needs further experimental confirmation.

The information above suggested that the *PmVRP15* gene, one of the key players in enhancing WSSV infectivity, might be regulated by several transcription factors and through a certain signaling pathway that was activated upon WSSV infection. Among the putative transcription factor binding sites identified, the binding site of IRF, Oct-1, and NFAT were confirmed for their involvement in *PmVRP15* gene regulation *in vitro*. As a point of concern, most of the candidate transcription factors that might regulate *PmVRP15* gene were uncharacterized in crustaceans and the specific function of *Pm*VRP15 was still unclear. Therefore, the candidates of the transcription factors will be further sought and characterized.

## Acknowledgements

The authors would like to thank Prof. Dr. Vichien Rimphanitchayakit for the valuable comment and suggestion on the manuscript. This research was supported by the Thailand Research Fund (TRF Research Career Development Grant No. RSA5580024). The authors would also like to thank the support from Chulalongkorn University under the Outstanding Research Performance Program: Chulalongkorn Academic Advancement into Its 2nd Century Project (CUAASC). The fellowship granted to PJ by the Royal Golden Jubilee Ph.D. Program, Thailand Research Fund is greatly appreciated. The short-term research fellowships to K.S. from NAIST Global Collaboration Program and Chulalongkorn University Office of International Affairs Scholarship for Short-term Research are also acknowledged.

## References

[1] F. Li, J. Xiang, Signaling pathways regulating innate immune responses in shrimp, Fish Shellfish Immunol. 34 (4) (2013) 973–980.
[2] I.T. Chen, T. Aoki, Y.T. Huang, I. Hirono, T.C. Chen, J.Y. Huang, G.D. Chang, C.F. Lo, H.C. Wang, White spot syndrome virus induces metabolic changes resembling the warburg effect in shrimp hemocytes in the early stage of infection, J. Virol. 85 (24) (2011) 12919–12928.
[3] W.J. Liu, Y.S. Chang, A.H. Wang, G.H. Kou, C.F. Lo, White spot syndrome virus annexes a shrimp STAT to enhance expression of the immediate-early gene ie1, J. Virol. 81 (3) (2007) 1461–1471.
[4] A. Prapavorarat, T. Vatanavicharn, K. Soderhall, A. Tassanakajon, A novel viral responsive protein is involved in hemocyte homeostasis in the black tiger shrimp, *Penaeus monodon*, J. Biol. Chem. 285 (28) (2010) 21467–21477.
[5] K. Apitanyasai, P. Amparyup, W. Charoensapsri, S. Senapin, A. Tassanakajon, Role of *Penaeus monodon* hemocyte homeostasis associated protein (*Pm*HHAP) in regulation of caspase-mediated apoptosis, Dev. Comp. Immunol. 53 (1) (2015) 234–243.
[6] K. Apitanyasai, P. Amparyup, W. Charoensapsri, P. Sangsuriya, A. Tassanakajon, Shrimp hemocyte homeostasis-associated protein (*Pm*HHAP) interacts with WSSV134 to control apoptosis in white spot syndrome virus infection, Fish Shellfish Immunol. 76 (2018) 174–182.
[7] T. Vatanavicharn, A. Prapavorarat, P. Jaree, K. Somboonwiwat, A. Tassanakajon, *Pm*VRP15, a novel viral responsive protein from the black tiger shrimp, *Penaeus monodon*, promoted white spot syndrome virus replication, PLoS One 9 (3) (2014) e91930.
[8] J.H. Leu, K.F. Liu, K.Y. Chen, S.H. Chen, Y.B. Wang, C.Y. Lin, C.F. Lo, The novel white spot syndrome virus-induced gene, PmERP15, encodes an ER stress-responsive protein in black tiger shrimp, *Penaeus monodon*, Dev. Comp. Immunol. 49 (2) (2015) 239–248.
[9] P. Jaree, S. Senapin, I. Hirono, C.F. Lo, A. Tassanakajon, K. Somboonwiwat, WSV399, a viral tegument protein, interacts with the shrimp protein *Pm*VRP15 to facilitate viral trafficking and assembly, Dev. Comp. Immunol. 59 (2016) 177–185.
[10] K. Jaturontakul, T. Jatuyosporn, P. Laohawutthichai, S.Y. Kim, T. Mori, P. Supungul, T. Hakoshima, A. Tassanakajon, K. Krusong, Molecular characterization of viral responsive protein 15 and its possible role in nuclear export of virus in black tiger shrimp *Penaeus monodon*, Sci. Rep. 7 (1) (2017) 6523.
[11] S. Elbahnaswy, K. Koiwai, V.H. Zaki, A.A. Shaheen, H. Kondo, I. Hirono, A novel viral responsive protein (MjVRP) from *Marsupenaeus japonicus* haemocytes is involved in white spot syndrome virus infection, Fish Shellfish Immunol. 70 (2017) 638–647.
[12] H. Harada, K. Willison, J. Sakakibara, M. Miyamoto, T. Fujita, T. Taniguchi, Absence of the type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated, Cell 63 (2) (1990) 303–312.
[13] T. Taniguchi, K. Ogasawara, A. Takaoka, N. Tanaka, IRF family of transcription factors as regulators of host defense, Annu. Rev. Immunol. 19 (2001) 623–655.
[14] K. Honda, T. Taniguchi, IRFs: master regulators of signaling by Toll-like receptors and cytosolic pattern-recognition receptors, Nat. Rev. Immunol. 6 (9) (2006) 644–658.
[15] X.D. Huang, W.G. Liu, Q. Wang, M. Zhao, S.Z. Wu, Y.Y. Guan, Y. Shi, M.X. He, Molecular characterization of interferon regulatory factor 2 (IRF-2) homolog in pearl oyster *Pinctada fucata*, Fish Shellfish Immunol. 34 (5) (2013) 1279–1286.
[16] C. Li, H. Li, Y. Chen, Y. Chen, S. Wang, S.P. Weng, X. Xu, J. He, Activation of Vago by interferon regulatory factor (IRF) suggests an interferon system-like antiviral mechanism in shrimp, Sci. Rep. 5 (2015) 15078.
[17] K.R. Nitta, A. Jolma, Y. Yin, E. Morgunova, T. Kivioja, J. Akhtar, K. Hens, J. Toivonen, B. Deplancke, E.E. Furlong, J. Taipale, Conservation of transcription factor binding specificities across 600 million years of bilateria evolution, Elife 4 (2015).
[18] B.G. Lindberg, X. Tang, W. Dantoft, P. Gohel, S. Seyedoleslami Esfahani, J.M. Lindvall, Y. Engstrom, Nubbin isoform antagonism governs Drosophila intestinal immune homeostasis, PLoS Pathog. 14 (3) (2018) e1006936.
[19] A. Tassanakajon, V. Rimphanitchayakit, S. Visetnan, P. Amparyup, K. Somboonwiwat, W. Charoensapsri, S. Tang, Shrimp humoral responses against pathogens: antimicrobial peptides and melanization, Dev. Comp. Immunol. 80 (2018) 81–93.
[20] S.H. Li, S.Y. Gu, F.H. Li, J.H. Xiang, Characterization and function analysis of an anti-lipopolysaccharide factor (ALF) from the Chinese shrimp *Fenneropenaeus chinensis*, Dev. Comp. Immunol. 46 (2) (2014) 349–355.
[21] T. Methatham, P. Boonchuen, P. Jaree, A. Tassanakajon, K. Somboonwiwat, Antiviral action of the antimicrobial peptide ALF*Pm*3 from *Penaeus monodon* against white spot syndrome virus, Dev. Comp. Immunol. 69 (2017) 23–32.
[22] S. Tharntada, K. Somboonwiwat, V. Rimphanitchayakit, A. Tassanakajon, Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci, Fish Shellfish Immunol. 24 (1) (2008) 46–54.
[23] P. Kamsaeng, A. Tassanakajon, K. Somboonwiwat, Regulation of

antilipopolysaccharide factors, ALF*Pm*3 and ALF*Pm*6, in *Penaeus monodon*, Sci Rep-Uk 7 (2017).
[24] T. Tang, L.X. Li, L.L. Sun, J.C. Bu, S. Xie, F.S. Liu, Functional analysis of *Fenneropenaeus chinensis* anti-lipopolysaccharide factor promoter regulated by lipopolysaccharide and (1,3)-beta-D-glucan, Fish Shellfish Immunol. 38 (2) (2014) 348–353.
[25] F. Macian, NFAT proteins: key regulators of T-cell development and function, Nat. Rev. Immunol. 5 (6) (2005) 472–484.
[26] K. Manley, B.A. O'Hara, W.J. Atwood, Nuclear factor of activated T-cells (NFAT) plays a role in SV40 infection, Virology 372 (1) (2008) 48–55.
[27] P. Keyser, K. Borge-Renberg, D. Hultmark, The Drosophila NFAT homolog is involved in salt stress tolerance, Insect Biochem Molec 37 (4) (2007) 356–362.
[28] X. Song, J. Hu, P. Jin, L. Chen, F. Ma, Identification and evolution of an NFAT gene involving *Branchiostoma belcheri* innate immunity, Genomics 102 (4) (2013) 355–362.
[29] X.D. Huang, G.J. Wei, H. Zhang, M.X. He, Nuclear factor of activated T cells (NFAT) in pearl oyster *Pinctada fucata*: molecular cloning and functional characterization, Fish Shellfish Immunol. 42 (1) (2015) 108–113.
[30] P.G. Hogan, L. Chen, J. Nardone, A. Rao, Transcriptional regulation by calcium, calcineurin, and NFAT, Genes Dev. 17 (18) (2003) 2205–2232.
[31] I.A. Graef, J.M. Gastier, U. Francke, G.R. Crabtree, Evolutionary relationships among Rel domains indicate functional diversification by recombination, P Natl Acad Sci USA 98 (10) (2001) 5740–5745.
[32] P.H. Wang, Z.H. Gu, D.H. Wan, M.Y. Zhang, S.P. Weng, X.Q. Yu, J.G. He, The shrimp NF-kappa B pathway is activated by white spot syndrome virus (WSSV) 449 to facilitate the expression of WSSV069 (ie1), WSSV303 and WSSV371, PLoS One 6 (9) (2011).
[33] C. Li, S. Weng, J. He, WSSV-host interaction: host response and immune evasion, Fish Shellfish Immunol. 84 (2019) 558–571.
[34] X.D. Huang, L. Zhao, H.Q. Zhang, X.P. Xu, X.T. Jia, Y.H. Chen, P.H. Wang, S.P. Weng, X.Q. Yu, Z.X. Yin, J.G. He, Shrimp NF-kappa B binds to the immediate-early gene ie1 promoter of white spot syndrome virus and upregulates its activity, Virology 406 (2) (2010) 176–180.