Activating Transcription Factor 4 and X Box Binding Protein 1 of *Litopenaeus vannamei* Transcriptional Regulated White Spot Syndrome Virus Genes *Wsv023* and *Wsv083*

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Abstract

In response to endoplasmic reticulum (ER) stress, the signaling pathway termed unfolded protein response (UPR) is activated. To investigate the role of UPR in *Litopenaeus vannamei* immunity, the activating transcription factor 4 (designated as *LvATF4*) which belonged to a branch of the UPR, the [protein kinase RNA (PKR)-like ER kinase, (PERK)]:eukaryotic initiation factor 2 subunit alpha (eIF2α)] pathway, was identified and characterized. The full-length cDNA of *LvATF4* was 1792 bp long, with an open reading frame of 1299 bp long that encoded a 432 amino acid protein. *LvATF4* was highly expressed in gills, intestines and stomach. For the white spot syndrome virus (WSSV) challenge, *LvATF4* was upregulated in the gills after 3 hpi and increased by 1.9-fold (96 hpi) compared to the mock-treated group. The *LvATF4* knock-down by RNA interference resulted in a lower cumulative mortality of *L. vannamei* under WSSV infection. Reporter gene assays show that *LvATF4* could upregulate the expression of the WSSV gene *wsv023* based on the activating transcription factor/cyclic adenosine 3’, 5’-monophosphate response element (ATF/CRE). Another transcription factor of *L. vannamei*, X box binding protein 1 (designated as *LvXBP1*), has a significant function in [inositol-requiring enzyme-1 (IRE1) – (XBP1)] pathway. This transcription factor upregulated the expression of the WSSV gene *wsv083* based on the UPR element (UPRE). These results suggest that in *L. vannamei* UPR signaling pathway transcription factors are important for WSSV and might facilitate WSSV infection.

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Introduction

The endoplasmic reticulum (ER) is the primary subcellular organelle of eukaryotic cells. This organelle is a site of lipid synthesis, protein folding and protein maturation. The proteins located in plasma membrane, Golgi apparatus and lysosomes, as well as the secreted proteins fold into their tertiary and quaternary structures in the ER [1]. Moreover, the ER is the major signal transducing organelle that recognizes and responds to homeostatic changes [2]. Conditions interferring with the function of the ER are collectively called ER-stress, which is induced by the accumulation of unfolded protein aggregates or by excessive protein traffic usually caused by viral infection [3].

The unfolded protein response (UPR) is the core pathway that eukaryotic cells use to cope with ER stress [4]. It is highly conserved and has been observed in eukaryotes. UPR contributes to cell survival by enhancing the protein folding capacity of the ER. Three pathways are involved in UPR, namely (1) [inositol-requiring enzyme-1 (IRE1)]-[X box binding protein 1 (XBP1)] pathway; (2) [double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK)]:[eukaryotic initiation factor 2α (eIF2α)]-[Activating transcription factor 4 (ATF4)] pathway, and (3) activating transcription factor 6 (ATF6) pathway [3, 4]. UPR decreases protein translation to restore normal cell functions, increases the production of molecular chaperones involved in protein folding, and activates the signaling pathways, resulting in the ubiquitination and subsequent degradation of misfolded proteins in the ER by proteasomes [5].

Under normal conditions, the immunoglobulin heavy chain binding protein (Bip) binds to the ER transmembrane protein sensor and inhibits their activation. Under ER-stress, Bip is occupied by the accumulated unfolded proteins, which frees the protein sensor [6]. In the PERK-eIF2α pathway, free PERK spontaneously dimerizes and activates eIF2α S(51) phosphorylation.
## Table 1. Summary of primers used in this study.

| Primers | Sequence (5’-3’) |
|---------|------------------|
| **For cDNA cloning** | |
| LvATF4-5RACE1 | TTGATTGTCAGGCTGAGCAAGCTGGGTC |
| LvATF4-5RACE2 | GAAGTTACAGCAGGGTTCATCAGGACATTA |
| LvATF4-3RACE1 | ACGTTCCCCATCAAAGAAGTGACAG |
| LvATF4-3RACE2 | GGTTAGCCGCCCTATCCGAAATAACGC |
| **For genes expression** | |
| pACB-LvATF4-Kpn1-F | AATGGTACCATGGAGTACAAACGGA |
| pACB-LvATF4-Apa1-R | ATAAGGGGCTTCTTTTTGAAAGATGCAG |
| pACB-LvXBP1u-F | TATGGTACCATGGCCAAGACGATCGTCATC |
| pACB-LvXBP1u-R | ATGGGGCCCAGCCTCAGGTTGATCCCG |
| **For reporter genes** | |
| pGL3B-wsv023-Kpn1-F | AATGGTACCCACTCTGGTCTTCTAACAAGCTAG |
| pGL3B-wsv023-Bgl2-R | TATAGATCCTGAAGGATGTTGATAG |
| pGL3B-wsv049-Kpn1-F | AATGGTACCCGAGCCAACGATCGTGTAG |
| pGL3B-wsv049-Hin3-R | TCGAAGGCTTTCAAGAGATGGGAGA |
| pGL3B-wsv064-Kpn1-F | AATGGTACCTTCAAAAGAATATGATAG |
| pGL3B-wsv064-Bgl2-R | TATAGATCCTTTCCAGCAGCTCGGAT |
| pGL3B-wsv069-Kpn1-F | AATGGTACCCTATAGATCCTTTCCGAT |
| pGL3B-wsv069-Bgl2-R | TATAGATCCTAAAGAGATGGGATG |
| pGL3B-wsv138-Kpn1-F | AATGGTACCACCTCTGAGTCTTCTAACAAGCTAG |
| pGL3B-wsv138-Bgl2-R | TATAGATCCTTAGATAGGATGTTGATAG |
| pGL3B-wsv242-Kpn1-F | AATGGTACCAAGCCTCGCACTTCTAGT |
| pGL3B-wsv242-Bgl2-R | TATAGATCCTCTCTAGCAACAGCTCAC |
| pGL3B-wsv256-Kpn1-F | AATGGTACCTTTCTCAACAATTTAACACAC |
| pGL3B-wsv256-Hin3-R | TCGAAGGCTTTCAAAAGAGATGGGATG |
| pGL3B-wsv282-Sac1-F | TATAGGCTCAAGTGTGGGTAGATAGAGAGT |
| pGL3B-wsv282-Bgl2-R | TATAGATCCTACCTGGTCTTCTCAGATG |
| pGL3B-wsv303-Kpn1-F | AATGGTACCAGGGGAAGGGGTGGTTGAA |
| pGL3B-wsv303-Bgl2-R | TATAGATCCTTGGAACAAAGACACCATGT |
| pGL3B-wsv306-Kpn1-F | AATGGTACCAAGGCTATGTTTATTTAGGQA |
| pGL3B-wsv306-Bgl2-R | TATAGATCCTGAGGCCATTTATGAAAT |
| pGL3B-wsv313-Kpn1-F | AATGGTACCAACAATCTGGAACGATGATTTCTTC |
| pGL3B-wsv313-Bgl2-R | TATAGATCCTAAACACTCCGACATCAGT |
| pGL3B-wsv321-Kpn1-F | AATGGTACCTTCGAGTCTTTGCTG |
| pGL3B-wsv321-Bgl2-R | TATAGATCCTGCAATAACAAATGGAGG |
| pGL3B-wsv343-Kpn1-F | AATGGTACCCGCTGTCTAGCCCTTTA |
| pGL3B-wsv343-Bgl2-R | TATAGATCCTGTTGAGCAGCATTGCTTAA |
| pGL3B-wsv406-Kpn1-F | AATGGTACCAAAACCCTTGATGACACAATTTT |
| pGL3B-wsv406-Bgl2-R | TATAGATCCTCTCTCCCAACAAATGTCGA |
| pGL3B-wsv453-Kpn1-F | AATGGTACCAACCCATCCGGGGCGGCG |
| pGL3B-wsv453-Hin3-R | TCGAAGGCTTTCAAAAGAGATGGGATG |
| pGL3B-wsv453-CRE-F | ACTCTCTAGGATCCACCATCG |
| pGL3B-wsv453-CRE-R | ctcgaaagcttccttagcttccttcaggg |
| pGL3B-wsv023ATF/CRE-F | GCAGATGTGGAATCTGTGATCTGAA |
| pGL3B-wsv023ATF/CRE-R | AATAGGCCCTTGAGTATCGGATG |
| pGL3B-wsv083-Kpn1-F | ATGGGCCCAGGAGGAGGAGGAGGGAAC |
| pGL3B-wsv083-CRE-F | aactacaCAGTGCAGTGTGAGG |
| pGL3B-wsv083-CRE-R | ATGGTAGATGATGGTATG |

*Relationship of *Litopenaeus vannamei* UPR and WSSV*
Phosphorylated eIF2a has a reduced frequency of AUG initiation codon recognition. However, selective mRNAs, such as the mRNA of the transcriptional activator in PERK-eIF2a pathway, ATF4, are effectively translated and activated [7,8]. In the IRE1-XBP1 pathway, the IRE1-Bip complex disintegrates, and IRE1 dimerizes. The IRE1 homodimers juxtapose the kinase domains for trans-autophosphorylation to stimulate the kinase and RNase activities that recognize the secondary structure of the XBP1 mRNA and remove the consensus motifs in the hairpin loops [9]. Thus, resulting in a transcription factor containing a basic region leucine zipper (BRIZ) domain that can recognize an activating transcription factor/cyclic adenosine 3', 5'-monophosphate response element (ATF/CRE) domain [10].

The Pacific white shrimp, Litopenaeus vannamei, is one of the most important penaeid shrimp in commercial fishing [11]. During culture, L. vannamei suffer from environmental stress, such as temperature shifts, sudden salinity changes, viral and bacterial infections, and heavy metal toxicity, which may induce ER-stress and harm the shrimp. Recently, the relationship between virus infection and the UPR in shrimp has become a constant concern, and researchers have found a complex relationship between them. For Penaeus monodon, heat shock protein70 (HSP70) responded to heat shock treatment, or WSSV challenge [14]. Previous studies have shown that WSSV successfully usurps the immune system of the host for its own gene regulation. For example, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway is part of the antiviral response of arthropods, however, in Penaeus monodon, PmSTAT is activated in response to WSSV infection, and WSSV uses the PmSTAT as a transcription factor to enhance the expression of viral gene iel [15]. Furthermore, two L. vannamei high-mobility group box proteins interact with transcription factors LvSTAT and LvDorsal to activate the promoter of WSSV iel [16]. The Toll-like receptor mediated NF-κB pathways are essential for inducing immune-related gene expressions against bacterial, fungal and viral infections. The Toll receptor in L. vannamei, namely, LvToll1, LvToll2, LvToll3, and three putative Spatze-like Toll ligands (LvSptz1-3) are upregulated after WSSV challenge [17]. The relationship between the WSSV genes and UPR transcription factors LAVTF4 and LVXBP1 was investigated in this research. We demonstrated that in L. vannamei UPR was also activated by WSSV infection and LvATF4 and LvXBP1 upregulated expression of WSSV genes wsv023 and wsv083, respectively.

## Materials and Methods

### 1. Cloning of LvATF4 from L. vannamei

Based on expression sequence tag (EST) sequences (available at www.marinegenomics.org, MGID428796), which are homologous to ATF4 of Danio rerio, Glossina morsitans morsitans, Camponotus floridanus, and some other species, specific primers were designed to obtain full length cDNAs. We obtained the 3'- and 5'-end cDNA sequences of L. vannamei ATF4 (LvATF4) by rapid amplification of cDNA ends (RACE). The cDNA template for RACE-polymerase chain reaction (PCR) was prepared using the BD SMART RACE cDNA Amplification Kit (Clontech, Japan). The LvATF4 5’ RACE1, and 5’ RACE2 primers were used for the first round 5’- and 3’-end RACE-PCR with a thermal cycler under the following conditions: denaturation at 94°C for 2 min, 7 cycles of 98°C for 10s, 68°C for 30s (decreasing by 1°C per cycle), and 68°C for 1.5 min, followed by 34 cycles of 98°C for 10s, 61°C for 30s, 68°C for 1.5 min, and final extension at 68°C for 5 min. The conditions for the second round 5’- and 3’-end PCR using LAVTF4 5’ RACE2

### Table 1. Cont.

| Primers            | Sequence (5’-3’)                                      |
|--------------------|------------------------------------------------------|
| For real-time RT-PCR |                                                      |
| QPCR-LvATF4-F      | GAAGTCCTGAGCTGAGACATCA                                 |
| QPCR-LvATF4-R      | CAGGGACTCAAAGGATGCTT                                    |
| QPCR-LvEF1a-F      | GCTGATTGCGGCGTACTCAT                                     |
| QPCR-LvEF1a-R      | TACCGGGTCTGCGGTCTT                                     |
| For RT-PCR         |                                                      |
| LvATF4-F           | GAGGGACTACAGCAATACCA                                     |
| LvATF4-R           | AGGGCAGCTTTGCTCAATGGTA                                  |
| For dsRNA templates amplification |                                                  |
| DsRNA-LvATF4-T7-F  | GGATCCTAAATAGCCTACACTATAGGATGCTTGTCCTCTGCTG             |
| DsRNA-LvATF4-R     | ACAGGGATGGGAAGGA                                        |
| DsRNA-LvATF4-F     | ATGTTGCTCCCTGCTG                                        |
| DsRNA-LvATF4-T7-R  | GGATCCTAATAGCCTACATAGGACAGGCGATGGAAGGGA                |
| DsRNA-eGFP-T7-F    | GGATCCTAATAGCCTACATAGGACAGGCGATGGAAGGGA                |
| DsRNA-eGFP-R       | ATGGGGGTGTTCTGCTGTAG                                    |
| DsRNA-eGFP-F       | CGACGTAAACGGGCCAAATG                                  |
| DsRNA-eGFP-T7-R    | GGATCCTAATAGCCTACATAGGACAGGCGATGGAAGGGA                |

*Nucleotides in bold indicate restriction sites introduced for cloning.

*Nucleotides in lower-case are the mutant sites.

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and LaATF4 3′ RACE2 primers were the same as the first round. The primer sequences are listed in Table 1. The PCR products were cloned into pMD-19 vector (TaKaRa, Japan) and subsequently sequenced (ABI PRISM, Applied Biosystems, USA).

2. Bioinformatics Analysis

The ATF4 protein sequences from other species in the database were searched and analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment

Figure 1. Nucleotide and deduced amino acid sequence of LvATF4. The ORF of the nucleotide sequences are shown in uppercase letters; the 5′- and 3′-UTRs are shown in lowercase letters. Nucleotides and amino acids are numbered on the left of the sequences. The conserved domains are shaded.

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was performed using the ClusterX v1.83 program. A neighbor-joining (NJ) phylogenetic tree was constructed based on the deduced amino acid sequences of LvATF4 and other known ATF4 proteins using MEGA 4.0 software. Bootstrap sampling was carried out 1,000 times. Protein domains were predicted using the SMART program (http://smart.embl-heidelberg.de/).

3. WSSV Challenge and RNA Extractions for Real-time RT-PCR Analysis

The shrimp (approximately 6.0 g mean body weight) were sacrificed and their eyestalks, gills, heart, hepatopancreas, stomach, intestines, nerves, muscles, pyloric caecum, hemocytes, and epidermis were collected for tissue expression analysis. A WSSV challenge experiment was performed to investigate the LvATF4 expression profile in the WSSV-infected shrimp. The WSSV challenge experiment method was described previously [18]. Moribund L. vannamei, which had white spots on the carapace and were confirmed to be WSSV-positive via one-step PCR, were used to prepare the inoculum for the challenge tests. The diseased shrimp muscle (2g) were homogenized in 20 mL of sterile 1x phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g K2HPO4, pH adjusted to 7.4, dH2O added to 1 L), and then centrifuged at 5000 × g for 15 min at 4°C. The supernatant was filtered through a 0.45-μm membrane and the sterilized supernatant was used for inoculation. Each healthy L. vannamei was injected intramuscularly at the second abdominal segment with 50 μL of WSSV inoculum (1 × 10^4 virions) that was prepared from WSSV-infected shrimp following a published protocol [19]. Total RNA from the gills and hemocytes were isolated immediately at 3, 6, 9, 12, 18, 24, 30, 36, 48, 72 and 96 h after injection. The total RNA samples were extracted and subsequently reverse transcribed into cDNA using PrimeScript RT Reagent Kit (TaKaRa). Real-time RT-PCR assays were performed as previously described. LvEF1α was used as the internal reference. The primer sequences are listed in Table 1.

4. Synthesis of Double-stranded RNA

The DNA templates of LvATF4 dsRNA (designated as dsLvATF4) were prepared via PCR using the primer pairs, DsRNA-LvATF4-T7-F/DsRNA-LvATF4-R and DsRNA-LvATF4-F/DsRNA-LvATF4-T7-R (Table 1). The products with a T7 promoter were confirmed via sequencing. Subsequently, the products were used as templates for the sense and antisense RNA strands, and subjected to in vitro transcription using RiboMAX™ Large Scale RNA production System-T7 (Promega) following the protocol of the manufacturer. After the reaction, the DNA templates were incubated at 37°C with RNase-Free DNase (1 U/1 g) for 15 min. The in vitro transcribed RNA products were then subjected to phenol/chloroform extraction, followed by isopropanol precipitation. An equal amount of sense and antisense RNAs were annealed to each other in 1x annealing buffer [20 mM potassium acetate, 6 mM Hepes-KOH (pH7.4), 6 mM MgOAc] at 90°C for 2 min to obtain dsRNA. The temperature was gradually decreased to 37°C, held for 1 h, and then placed at room temperature for another 1 h. The leftover single-stranded RNA template and the single-stranded overhang were degraded by incubating the annealing product with 0.1 μg of RNaseA at 37°C for 10 min followed by phenol/chloroform extraction and isopropanol precipitation. The LvATF4 dsRNA was 442 bp in length. The templates of eGFP dsRNA (designated as dsGFP) were prepared via PCR using the primer pairs DsRNA-eGFP-T7-F/DsRNA-eGFP-R and DsRNA-eGFP-F/DsRNA-eGFP-T7-R (Table 1). The dsGFP RNA was 504 bp in length.

5. Effect of Silencing LvATF4 upon a WSSV Challenge Test

The shrimp about 6.0 g body weight (50 shrimp/treatment obtained from the Zhuhai Shrimp Farm in Zhuhai, Guangdong Province, China) were intramuscularly injected with 5 g PBS. The shrimp were again injected after 48 h with either 50 μL of PBS (negative controls) or tissue homogenate at a 10^-3 dilution (1 × 10^4 virions, 50 μL volume). Shrimp were kept in culture tanks for approximately 7 d following infection. Cumulative mortality was recorded every 12 h. For investigating the efficiency of RNA interference (RNAi) in this study, shrimp treated with dsLvATF4 or dsGFP were sacrificed at
the third day after dsRNA injection [20], and the gills were collected for analyses. The gills of the untreated shrimp with dsRNA were also collected and used as a control. The total RNA extracted from the gills was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa) to serve as templates. Semi-RT-PCR assays were performed as described previously. LvEF1a was used as the internal reference. The primer sequences are listed in Table 1.

6. Dual-luciferase Reporter Assays

Expression vectors for the full length LvATF4, enhanced green fluorescent protein (eGFP), were constructed using pAcB5.1/V5-His B (Invitrogen, USA). The PCR products were amplified using the primers pairs pACB-LvATF4-F/pACB-LvATF4-R and pACB-eGFP-F/pACB-eGFP-R (Table 1), respectively. The PCR products were cleaved using the same restriction enzyme pairs and then ligated into the expression vector. The reporter genes, pGL3-wsv023, pGL3-wsv049, pGL3-wsv064, pGL3-wsv069, pGL3-wsv138, pGL3-wsv242, pGL3-wsv256, pGL3-wsv282, pGL3-wsv303, pGL3-wsv306, pGL3-wsv313, pGL3-wsv321, pGL3-wsv343, pGL3-wsv406 and pGL3-wsv453, were constructed by inserting the corresponding viral promoter regions into the pGL3-Basic, as described previously [16]. The pGL3-wsv083mUPRE (primers are shown in Table 1) was constructed based on pGL3-wsv083 using the TaKaRa MutanBEST Kit (TaKaRa).

Drosophila Schneider 2 (S2) cells maintenance, DNA transfection, and reporter gene assays were performed as described previously [16].

Figure 3. Phylogenetic analysis of the ATF4 proteins. Phylogenetic tree of the ATF4 proteins from invertebrates and vertebrates. The tree was constructed via a neighbor-joining algorithm using the Mega 4.0 program based on the multiple sequence alignment by ClusterX v1.83. The LvATF4 protein is marked with a box. CinATF4, Ciona intestinalis activating transcription factor 4 (GenBank accession no. BAE06319.1); AmaATF4, Amblyomma maculatum activating transcription factor 4 (GenBank accession no. AEO35749.1); CIATF4, Canponotus floridanus activating transcription factor 4 (GenBank accession no.EFN64595.1); GmATF4, Glossina morsitans morsitans activating transcription factor 4 (GenBank accession no.ADD20536.1); OnATF4, Oreochromis niloticus activating transcription factor 4 (GenBank accession no.XP_003443277.1); CidATF4, Ctenopharyngodon idella activating transcription factor 4 (GenBank accession no.AAS57931.1); DrATF4, Danio rerio activating transcription factor 4 (GenBank accession no.NP_001096662.1); XiATF4, Xenopus laevis activating transcription factor 4 (GenBank accession no.NP_001083212.1); GgATF4, Gallus gallus activating transcription factor 4 (GenBank accession no.NP_001291460.1); BrATF4, Bos taurus activating transcription factor 4 (GenBank accession no.ACJ15469.1); PpATF4, Pan paniscus activating transcription factor 4 (GenBank accession no.XP_003813296.1); CjATF4, Callithrix jacchus activating transcription factor 4 (GenBank accession no.XP_002763954.1); PtATF4, Pan troglodytes activating transcription factor 4 (GenBank accession no.NP_001239446.1); HaATF4, Homo sapiens activating transcription factor 4 (GenBank accession no.NP_087796.1); RnATF4, Rattus norvegicus activating transcription factor 4 (GenBank accession no.AA155859.1); MmATF4, Mus musculus activating transcription factor 4 (GenBank accession no.AAH85169.1).

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1. Cloning of Full Length LvATF4 cDNA and Phylogenetic Analysis

The full-length cDNA of *LvATF4* is 1972 bp long, includes a 213 bp 5' untranslated region (UTR) and a 460 bp 3'-UTR with a poly (A) tail. The open reading frame (ORF) of *LvATF4* is 1299 bp long, and encodes a putative 433 amino acid protein (Fig. 1). Conserved domain analysis using the SMART program shows that *LvATF4* contains a BRLZ domain (Fig. 2A), that is found in eukaryotic proteins with sequence-specific DNA binding activity [21].

To investigate the relationship between *LvATF4* and its homologues, multiple sequence alignment was performed (Fig. 2B) and phylogenetic trees were generated using the NJ method. These proteins were divided into three classes (Fig. 3).

Class 1 contained the Mammalia and Aves ATF4 proteins, including GgATF4, AmATF4, BtATF4, OaATF4, PpATF4, CjATF4, PaATF4, HsATF4, RnATF4, MmATF4. Class 2 contained the ATF4 protein of amphibian, XlATF4. Class 3 contained fish, arthropod and tunicates ATF4 proteins, including OnATF4, CidATF4, and DrATF4, CinATF4, AmaATF4, CfATF4, GmATF4, and LvATF4. *LvATF4* was most closely related to insect ATF4 proteins. The amino acid identity between AmaATF4 and LvATF4 was 47.4%. The ATF4 proteins showed a high homology with the amino acid sequence at the BRLZ domain (Fig. 2B).

2. LvATF4 Expression and Knockdown of LvATF4 in WSSV Challenged Shrimp

2.1. LvATF4 Expression in WSSV Challenged Shrimp.

*LvATF4* expressions were detected in all the tissues...
examined. The expressions of *LvATF4* was statistical significance different between each tissues. *LvATF4* was highly expressed in gills, intestines and stomach (Fig. 4). Considering that the *LvATF4* with higher expression in the gills and hemocytes are important for shrimp immunity, the *LvATF4* in shrimp gills and hemocytes were investigated after WSSV challenge. The expression level of the gene at 0 h was used as the baseline, and the corresponding expression in the PBS group was used as the control. In hemocytes, the *LvATF4* expression was not significantly different between the WSSV-infected groups and mock-infected groups except 9, 12, 24, 96 hpi (Fig. 5A). Expression of *LvATF4* was upregulated upon WSSV infection in gills after 3 hpi and increased by 1.9-fold (96 hpi) compared to the mock-treated group (Fig. 5B).

### 2.2. Knockdown of *LvATF4* reduced the cumulative mortality of WSSV infected shrimp
Experiments were carried out to explore the effect of the downregulation of the *LvATF4* expression upon WSSV infection. The injection of ds*LvATF4* reduced the expression of the *LvATF4* in the gills after 3 d, as shown in Fig. 6A. The injection of dseGFP did not induce the downregulation of the *LvATF4* expression in the gills. The shrimp treated with ds*LvATF4* had a lower cumulative mortality after WSSV infection. The cumulative mortality was 8.77%, 15.79%, 38.60%, and 54.39% in the ds*LvATF4* treatment group at 84, 108, 132, and 156 hpi, respectively. By contrast, the corresponding cumulative mortality in the control group was 54%, 80%, 90%, and 94%. The cumulative mortality was 100% after 168 hpi for the control group, whereas that of the ds*LvATF4* treatment group was just 57.89% (Fig. 6B). The dseGFP injection failed to protect the shrimp from viral infection, similar to the control group (Fig. 6B).

### 3. Upregulation of WSSV Genes wsv023 by *LvATF4* and wsv083 by *LvXBP1s* in S2 Cells
Based on the WSSV genome analysis, 15 genes (wsv023, wsv049, wsv064, wsv069, wsv138, wsv242, wsv256, wsv302, wsv306, wsv313, wsv321, wsv343, wsv406 and wsv453) containing at least one putative ATF/CRE [TGACGT(G/C)A] within their promoter regions were selected. A luciferase reporter assay was performed to examine the relationship between transcription factors and these viral genes (Fig. 7A). Expression of pACB-*LvATF4* significantly increased pGL3-wsv023 expression by approximately 10-fold (Fig. 7C). When the ATF/CRE element of wsv023 promoter was mutated or deleted (Fig. 7B), the pGL3-wsv023 expression was significantly reduced by approximately 93% and 87% respectively comparing to the wild type (Fig. 7C). The expression of pGL-wsv069 was also activated by *LvATF4*, increased by approximately 6-fold (Fig. 7A).
It had been proved that WSSV infection leaded to LvXBP1 mRNA splicing, and the spliced form LvXBP1 mRNA (LvXBP1s) encoding polypeptide with ATF/CRE BRLZ domain [14]. As a potent transcription activator, XBP1 binds to the UPRE and to the ER stress-response elements I and II (ERSE-I and ERSE-II) in the promoter regions of the target genes [22]. Another set of reporter genes, containing at least one putative UPRE \[TGACGTG(G/A)\] within their promoter regions were selected. pGL3-wsv083 expression significantly increased by approximately 20-fold by LvXBP1s, whereas the expression of the \textit{wsv083} promoter with UPRE element mutation was reduced 90% that compared with the wild type (Fig. 8).

**Discussion**

Previous studies have demonstrated that the \textit{L. vannamei} IRE1-XBP1 pathway is activated under WSSV challenge [14]. \textit{LvXBP1} knocked down by RNAi results in a lower cumulative mortality of \textit{L. vannamei} under WSSV infection. These results suggest that WSSV infection could cause ER stress and trigger UPR pathways in \textit{L. vannamei}. Moreover, IRE1-XBP1 may be utilized to respond to the virus infection. In this study, \textit{LvATF4}, a transcription factor of shrimp UPR, was identified and characterized. Under WSSV infection, the XBP1S spliced form of \textit{LvXBP1} mRNA is produced and may activate the UPR pathway, thereby increasing the expression of \textit{LvATF4} and \textit{LvCHOP}. This suggests that the coordinated activation of these genes may help \textit{L. vannamei} cope with WSSV infection.

**Figure 7. Activation of the \textit{wsv023} promoters by LvATF4.** (A) Screening of 15 WSSV gene promoters by LvATF4. (B) Schematic diagram of the \textit{wsv023} promoter regions in the luciferase reporter gene constructs. For pGL3-wsv023mATF/CRE, the ATF/CRE in the \textit{wsv023} promoters was replaced with (CTCGAAGG); and for pGL3-wsv023dATF/CRE, the ATF/CRE in the \textit{wsv023} promoters was deleted (showed as square frame). +1 denotes the transcription initiation site for \textit{wsv023}, and –1 indicates 1 bp before the translation initiation site. Luc denotes the firefly luciferase reporter gene. (C) Relative luciferase activity in S2 cells. The bars indicate mean values ± S.D. of the luciferase activity (n = 3). The statistical significance was calculated using Student’s t-test (* indicates \(p<0.05\) and ** indicates \(p<0.01\) compared with control).

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**Figure 8. Activation of the \textit{wsv083} promoter by LvXBP1s.** Schematic diagram of the \textit{wsv083} promoter regions in the luciferase reporter gene constructs. For pGL3-wsv083mUPRE, the UPRE in the \textit{wsv083} promoters was replaced with (TGACGTGG). The results were based on three independent experiments and expressed as mean values ± SD. The statistical significance was calculated using Student’s t-test (* indicates \(p<0.05\) and ** indicates \(p<0.01\) compared with control).

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**Figure 8.** Activation of the \textit{wsv083} promoter by LvXBP1s. Schematic diagram of the \textit{wsv083} promoter regions in the luciferase reporter gene constructs. For pGL3-wsv083mUPRE, the UPRE in the \textit{wsv083} promoters was replaced with (TGACGTGG). The results were based on three independent experiments and expressed as mean values ± SD. The statistical significance was calculated using Student’s t-test (* indicates \(p<0.05\) and ** indicates \(p<0.01\) compared with control).

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infection, the cumulative mortality of L. vannamei was lower in the dsLvATF4 treatment group than the control group. The reporter gene assays show that the transcription factor LvXBPls upregulated the expression of the WSSV gene wsv083 in the UPRE-dependent manner, and LvATF4 upregulated the expression of wsv023 in an ATF/CRE-dependent manner. These results suggest that WSSV triggers ER stress and uses the transcription factor of the UPR pathway for its gene transcription regulation.

Several viruses hijack and stimulate the host immunity system to facilitate their life cycle. Cytomegalovirus (CMV) alpha [immediately early (IE) genes] could use NF-kB as a target for CMV enhancer activation [23, 24]. Human immunodeficiency virus type I, human herpes virus 8, and Epstein Barr virus have incorporated aspects of the NF-kB signaling into their life cycle and pathogenicity, and can thus induce NF-kB activation [25]. As the primary viral pathogen of shrimp, WSSV infection could activate the NF-kB pathway of the host. The NF-kB pathway in L. vannamei is activated by the WSSV protein wsv494 to facilitate wsv393 and wsv371 expressions [26]. NF-kB binds to the wsv069 (iel) promoter of WSSV and upregulates its activity [27]. Thus, WSSV could utilize the shrimp immunity system for survival, similar to other viruses.

The UPR regulates ER protein folding upon ER stress, and plays an important role in innate immunity [28]. Environmental stresses, such as temperature shifts, heavy metal toxicity and viral infection increase the levels of unfolded proteins in the ER lumen [29, 30, 31]. Viruses cause ER stress and consequently UPR activation by inducing host cells to produce large amounts of viral proteins, some of which undergo glycosylation and other modifications in the ER. For example, several members of the flaviviruses, including the West Nile virus, Japanese encephalitis virus, and dengue virus, activate the UPR pathway in a variety of mammalian cells [32, 33, 34, 35]. Viruses both induce and manipulate UPR to protect the host cells from an ER stress-mediated death, thereby permitting the translation of viral proteins and efficient viral replication [36]. UPR has emerged as a key target of host cells and viruses for controlling infection. Murine CMV (MCMV) regulates UPR in a manner similar to that of human CMV. The UPR modulatory ability is triggered by virion entry and enhanced by viral immediate-early and early gene expression. While initially vulnerable, MCMV becomes resistant to exogenous ER stress at the later stage of the infection [37]. In the Chinese shrimp, F. chinensis, WSSV challenge significantly enhanced the 78 kDa glucose-regulated protein (GRP78) expression, which also known as Bip, an important chaperon of UPR [38]. In the current study, L. vannamei UPR was also triggered by WSSV infection and WSSV can manipulate UPR to its advantage.

LvATF4 and LvXBPl are the core transcription factors of L. vannamei UPR. LvATF4 upregulated WSSV genes in an ATF/CRE-dependent manner. Among the 15 genes (wsv023, wsv049, wsv064, wsv069, wsv130, wsv242, wsv256, wsv282, wsv306, wsv313, wsv321, wsv343, wsv406 and wsv453) with at least one putative ATF/CRE within their promoter regions wsv023 was upregulated significantly. Wsv023 is reportedly expressed at the early stage of WSSV infection, suggesting that it may be essential for WSSV [39], and its function still far from clear [40]. This study might give some cues to uncover the function of wsv023, while further research was needed. Wsv069 was also upregulated by LvATF4, but showed relatively smooth change comparing with that of wsv23. It had been reported that that WSSV protein IE1 function as transcriptional regulators and exhibit transactivation activity, DNA binding activity, and dimerization [41].

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Author Contributions

Conceived and designed the experiments: YHC XYL JGH. Performed the experiments: XYL LRP YGC. Analyzed the data: ZZZ SPW. Contributed reagents/materials/analysis tools: HTY. Wrote the paper: YHC XYL.

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