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Recent Advances in Researches on Shrimp Immune Pathway Involved in White Spot Syndrome Virus Genes Regulation

Yihong Chen[1], Xiaoyun Li[2] and Jianguo He[1,2*]

[1]MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Marine Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, P. R. China
[2]School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, P. R. China

Abstract

Shrimp is an important aquaculture species. *Litopenaeus vannamei, Fenneropenaeus chinensis, Marsupenaeus japonicus*, and *Penaeus monodon* are the main cultivated species in the world. The yield of shrimp culture was about 3,130,000 tons in the year 2013. Though shrimp industry has been made great progress in the last decade, shrimp diseases still endangering the healthy development of this industry. On the one hand, occurring of shrimp disease was the result of kinds of pathogenic microorganism infection, such as WSSV. On the other hand, environmental stress also played an important role in disease outbreak. Researchers have paid more attention to the shrimp immune system, and mechanism of environmental stress responding nowadays. Shrimp immune system, including physical barriers, cellular immunity and humoral immunity, was important for shrimp to combat diseases. Among these, humoral immunity was the most well studied. Currently, the main pathways of the shrimp immune system, including TLRs pathway, IMD pathway, JAK-STAT pathway, RNAi pathway, P38 MAPK pathway, and JNK pathway have been proved to play important roles in shrimp immunity. Meanwhile, shrimp unfolded protein response (UPR) was considered to be the core of its environmental stress resistance system. In addition to ATF6 pathway, the other two branches of UPR, the IRE1-XBP1 pathway and PERK-eIF2α pathway have been shown to exist, and took effect in shrimp environmental stress responding. These studies also revealed a secret of WSSV: it activated and used a number of immune pathways as well as the UPR of shrimp, to increase its genes replication. This review intends to present the latest development in shrimp innate immunity, UPR, and their regulation on WSSV. It will also help us to understand the relationship between shrimp humoral immune response/UPR and WSSV infection.

Keywords: Shrimp immunity; Humoral immunity; Environmental stress; Unfolded protein response; WSSV

Introduction

There are two significant systems for internal homeostasis protection of invertebrate, the innate immunity system and the environmental stress responding system, which worked together to protect cells from biotic and abiotic stresses. In response to pathogen infection, the host innate immune system activated microbial clearing pathways and cellular stress relief pathways that needed to be balanced for insufficient or excessive immune responses leading to deleterious consequences. Shrimp is an invertebrate with open circulatory system, especially prone to alter in the environmental change. In addition, as an aquatic livestock, shrimp exposed to temperature shifts, viral infections, sudden salinity changes, and heavy metal toxicity, as well as various pathogens, which may interfered with shrimp internal homeostasis. In most cases, the innate immunity system and UPR helped to against these pathogens, while sometimes they worked on the contrary [1,2].

Immune responses are typically distinguished in two main systems, the adaptive and the innate immune response. Shrimp seems to just rely on the innate immune system, which refers to the evolutionary ancient and presumably conserved first-line host defense against the early phases of microbial infection. It is believed that it recognized broadly conserved microbial moieties, and defense against pathogens. Humoral immune response is a vital part of shrimp innate immunity system. It is mainly containing blood cells (hemocytes), which floating in the hemolymph participates in phagocytosis and encapsulation of foreign invaders, for example, some components of shrimp blood cells asked for melanization. The larval fat body, a functional analog of the mammalian liver and the main larval energy reservoir, is the chief site of the shrimp larva's humoral (or systemic) immune response, while in adults other tissues can take on major roles for immune surveillance [3]. Beside, shrimp has physical barriers (exoskeleton, chitinous epithelial lining of gut and trachea) to prevent microbial from entering into its body.

Recent studies demonstrate that living organisms have developed strategies to cope with many kinds of environmental changes. However, when confront acute changes, they suffer stresses. The main damage of stresses for living organisms is protein denaturation. In other words, these proteins lose their native, functional configuration. The metabolism and function of hemocytes, which are significant part of defense in marine invertebrates, must be adaptable to the changing ambient conditions for survives. Therefore, stress responses may play a more direct role in the immune function of hemocytes besides insuring survival. Unfolded protein response (UPR), initiated in endoplasmic reticulum stress (ER-stress), is a mechanism that eukaryotic cells using to cope with stresses [4]. It is a cascade of responses against the accumulation of unfolded or misfolded proteins in the lumen of the ER. This mechanism is highly conserved and has been observed in all mammalian species, as well as in worms and yeasts [5,6]. UPR contributes to cell survival during ER-stress by enhancing the protein folding capacity in the ER [7]. In most species, three well-characterized

***Corresponding author:** Jianguo He, School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, P.R. China, Tel: +86-20-39332988; Fax: +86-20-39332849; E-mail: lsshjg@mail.sysu.edu.cn

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pathways are involved in UPR [8-10]. Each pathway contains a class of transmembrane ER-resident signaling components, named inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)–like ER kinase (PERK), and activating transcription factor 6 (ATF6) [11]. The response to ER-stress is mediated at the transcriptional and translational levels [12,13]. In mammals, the transcriptional activation of UPR-responsive genes is regulated by two distinct pathways, the ATF6 pathway and the IRE1-XBP1 pathway. The PERK-eIF2α pathway mainly works at translational level [14]. UPR inhibits protein translation to restore normal cell functions, activates the signaling pathways to increase the production of molecular chaperones involved in protein folding, also activates the signaling pathways that result in the targeting of misfolded proteins in the ER for ubiquitination and subsequent degradation by proteasomes [15]. When ER-stress is not relieved, UPR results in apoptosis [16-19].

Shrimp Humoral Immune Response

Toll like receptors (TLRs) pathway

In Drosophila, Toll pathway mainly contains Spätzle, Toll-like receptors (TLRs), Pelle, SARM, Tollip, Tube, MyD88, TRAF6, Cactus, Dorsal, and Dorsal-related immunity factor (DIF) [20]. It is involved in Gram-positive bacteria and fungi responding by leading signaling cascades, and regulating some immune-relative genes, such as antimicrobial peptide (AMPs), lysozymes as well as components of the melanization and clotting cascades. Both shrimp and Drosophila are arthropod, and they have similar TLRs pathway. Nowadays, most components of the TLRs pathway have been discovered in shrimp, and studying about them proved essentials to our understanding of this pathway in shrimp (Table 1).

TLRs: So far, all investigated species have more than one TLR. TLRs, which are receptors that could recognize pathogen-associated molecular pattern (PAMP) derived from microbes, play a key role in the innate immune system. Once the microbes breached physical barriers, such as the shell or intestinal tract mucosa, TLRs could recognize them and activate the antimicrobial response [21]. In penaeid shrimp, several types of TLRs were isolated. The first TLR gene (designated as LvToll) encoding 926 amino acid residues was reported in *L. vannamei* [22]. It contains an extracellular domain containing 16 leucine-rich repeats (LRRs) flanked by cysteine-rich motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain. The TIR domain showed high similarity to Apis mellifera Toll and Drosophila melanogaster Toll, with 59.9% and 54.3% identity respectively [22]. Another study showed that knock-down LvToll does not increase mortality after challenge with white spot syndrome virus (WSSV) [23,24]. Recently, two TLRs (LvToll2 and LvToll3) were isolated from L. vannamei. LvToll2 has 1009 amino acid residues with an extracellular domain containing 18 LRRs and a cytoplasmic TIR domain of 139 residues. 1244 residues with an extracellular domain containing 23 LRRs and a cytoplasmic TIR domain of 138 residues constitute of LvToll3, which was localized to the membrane and cytoplasm like LvToll, while LvToll2 was confined to the cytoplasm. LvToll2 but not LvToll or LvToll3 could significantly activate the promoters of AMPs, which were controlled by NF-κB pathway. In the gill, they were all up-regulated with WSSV challenge [25].

A TLR in Penaeus monodon (PmToll, accession NO. ADK55066) shows 59% similarity to a Toll-related protein of Apis mellifera. LRRs of PmToll contained no obvious PAMP-binding insertions. Phylogenetic analysis with the insect TLRs family show it clustering with Toll1 and Toll5, and it is less related to Toll3 and Toll4. Furthermore, RT-qPCR shows that PmToll is constitutively expressed in gut, gill and hepatopancreas, and challenge with WSSV shows equal levels of expression in these organs [26]. Counterpart of LvToll in Fenneropenaeus chinensis (FcToll, accession NO. EF407561) also be cloned, and it showed similar characteristic and function with LvToll [27]. There were also two TLRs in *Marsupenaeus japonicus* (MjToll, accession NO. BAF99007; MjToll2, accession NO. BAG68890) as reported [28].

Spätzle: In Drosophila, Spätzle (Spz) was upstream of Toll, and roled in both the embryonic development and innate immune response [29]. In the dorsal–ventral pattern development of embryonic or as a pro-Spz processing enzyme in immune response, Spz is cleaved by a serine protease Easter, and then activated. Gram-positive bacteria or fungi could induce a protease cascade to activate the pro-Spz, and resulted in production of AMPs in adult flies [30]. It has been reported that three Spz-like proteins (LvSpz1~3) were cloned from *L. vannamei* [25]. All these Spz-like proteins have a putative C-terminal cysteine-knot domain. LvSpz1 and LvSpz3 in gill were up-regulated after WSSV challenge, but LvSpz2 showed no obvious change [25]. From hemocytes of *Fenneropenaeus chinensis*, a Spz-like protein (FcSpz) was isolated too. It consisted of 997 bp with an ORF of 768 bp, which encoded a protein of 255 amino acids with a signal peptide of 19 residues. FcSpz was up-regulated in shrimp after challenging with WSSV. The recombinant protein of FcSpz C-terminal active domain could upregulate the expression of crustin in crayfish [31]. These results suggested that shrimp Spz might play a role in the innate immune defense.

Pelle: Downstream of the activated TLRs, MyD88-Tube-Pelle form a heterotrimeric complex via their death domains (DD) [32]. Activation of Pelle leads to cascade reaction, which results in degradation of Cactus (the homolog of mammalian IkB). After that, NF-κB family protein Dorsal is released and gets into the nucleus for the transcriptional induction of immune-related genes [33]. The cDNA of *L. vannamei* Pelle (designated as LvPelle) consists of 1706 bp nucleotide with an ORF of 1611 bp encoding 537 amino acid residues. The LvPelle genome is 8031 bp containing eight exons and nine introns. It contains an N-terminal death domains and a C-terminal protein kinase domain, showing 24~40% identity with IRAK family proteins from insect to human. LvPelle death domain consists of six α-helices, which is similar to Drosophila Pelle and mouse IRAK4. It was constitutively expressed in different tissues of shrimp and showed different expression profiles in tissues when shrimp was infected by WSSV or bacteria [1]. LvPelle localized in the cytoplasm of S2 cells, which was consistent with the putative function of LvPelle in forming a receptor complex with MyD88 and Tube.

TRAF6: Tumor necrosis factor receptor (TNFR)–associated factor 6 (TRAF6), a key signaling adaptor protein for the TNFR superfamily and the Interleukin-1 receptor/Toll-like receptor (IL-1/TLR) superfamily, is the down-stream target of Pelle [34,35]. Full-length cDNA of *L. vannamei* TRAF6 (named LvTRAF6) was 2823 bp long, with an ORF encoding a putative protein of 594 amino acid residues. It constitutively expressed in various tissues, and could activate the promoters of AMPs. With *V. alginolyticus* or WSSV challenge, the expression of LvTRAF6 was changed. This indicated that LvTRAF6 might play a crucial role in antibacterial and antiviral responses through regulated AMPs [36].

Dorsal: Dorsal is a Rel/NF-κB transcription factor, which is activated in the successive signaling cascade of TLRs pathway for antifungal and antibacterial responses [37]. Dorsal homolog was cloned

Component	Species	Gene name	Accession number	Tissues: variation at transcription level		References
				Response to bacteria	Response to WSSV	
Toll	*L. vannamei*	LvToll	DQ923424.1	G: up	G: up	[22]
	L. vannamei	LvToll2	JN180637.1	G: none	G: up	[25]
	L. vannamei	LvToll3	JN180638.1	G: none	G: up	[25]
	F. chinensis	FcToll	EF407561.1	L: modulated	L: down	[125]
	M. japonicus	MjToll1	BAF99007	/	/	[28]
	M. japonicus	MjToll2	BAG68890	/	/	Unpublished
Spätzle	*L. vannamei*	LvSpz1	JN180646.1	G: up	G: up	[25]
	L. vannamei	LvSpz2	JN180647.1	G: down	G: none	[25]
	L. vannamei	LvSpz3	JN180648.1	G: up	G: up	[25]
	F. chinensis	Fc-Spz	/	G, Hc, Hp, I,	G, Hc, Hp, I,	[31]
				Ht:up	Ht: up	
Pelle	*L. vannamei*	LvPelle	JN180645.1	G:none;	G: up;	[1]
				I:down;	Hp, I: down	
				Hp:modulate;		
TRAF6	*L. vannamei*	LvTRAF6	HM581680.1	G, Hp: none;	G, Hp: up;	[36]
				I: down	I: down	
Dorsal	*L. vannamei*	LvDorsal	FJ998202.1	/	/	[39]
	F. chinensis	FcDorsal	EU815056.1	L, Hc: up	L, Hc:	[38]
					modulated	
SARM	*L. vannamei*	LvSARM	JN185615.	I, Hp: none;	I, Hp: none;	[46]
				Hc:down;		
				G: up;	G, Hc: up;	
Tillip	*L. vannamei*	LvTillip	JN185616	I, Hc: down;	Hp: none;	[47]
				Hp:none;	G, Hc, I: up;	
					M:modulated	
IMD	*L. vannamei*	LvIMD	FJ592176.1	Hc, Hp: up;	Hp, G: none;	[50]
				G: none	Hc: up	
Relish	*L. vannamei*	LvRelish	FJ416145.1	/	/	[54]
	L. vannamei	sLvRelish	FJ416146.1	/	/	
	F. chinensis	FcRelish	EU815055.1	Hc: down;	Hc: modulate;	[53]
				L:modulated	L: up	
IKK	*L. vannamei*	LvIKKβ	AEK86518	I, G, Hp: up	I, G: up	[55]
	L. vannamei	LvIKKε	AEK86519	G, Hp: up;	G, Hp, Hc: up	
				Hc: dowm;		
				I: modulated		
STAT	*L. vannamei*	LvSTAT	HQ228176	/	/	[57]
	P. monodon	PmSTAT (L)	EU367985	/	/	Unpublished
	P. monodon	PmSTAT	AY327491	/	Ce: down	[2]
	M. japonicus	MjSTAT	AB501344	/	/	Unpublished
P38	*L. vannamei*	Lvp38	JX990130	G: up	/	[78]
Dicer1	*L. vannamei*	LvDcr1	EU676241	/	Hc, G: up	[69]
	P. monodon	PmDcr1	EF446324	/	Hl: none	[68]
	M. japonicus	MjDcr1	GU265733	/	/	Unpublished
Dicer2	*L. vannamei*	LvDcr2	HQ541163	/	G: up	[70]
	P. monodon	PmDcr2	JX624789	Hp: up	Hp: up	[71]
	M. japonicus	MjDicer2	AFB82635	/	/	Unpublished
Ago	*L. vannamei*	Lv-Ago1	ADK25180.1	/	/	[126]
	P. monodon	PmAgo1	DQ663629	/	/	[65]
	M. japonicus	MjAgo1A	FJ593185	/	L, Hp: up	[64]
	M. japonicus	MjAgo1B	FJ593186	/	L, Hp: up	[64]
	M. japonicus	MjAgo1C	JX170715	/	/	[64]
	L. vannamei	Lv-Ago2	HM234690.1	/	/	[126]
	P. monodon	PmAgo3	JX845575	/	/	[65]
TRBP	*F. chinensis*	Fc-TRBP1	EU679001	Hc, S, G, I,	Ht, G, I: up;	[72]
				Ht, Hp: up;	Hc, Hp:down	
	F. chinensis	Fc-TRBP2	EU679001.1	Hc, S, G, I,	Ht, G, I: up;	[72]
				Ht, Hp: up;	Hc, Hp: down	
	F. chinensis	Fc-TRBP3	FJ573168.1	Hc, S, G, I,	Ht, G, I: up;	[72]
				Ht, Hp: up;	Hc, Hp: down	

	L. vannamei	LvTRBP1	HQ541157	/	/	[70]
	L. vannamei	LvTRBP2	/	/	/	Unpublished
	L. vannamei	LvTRBP3	/	/	/	Unpublished
	P. monodon	PmTRBP1	JX465430	Hp: up	Hp: up	[74]
Drosha	*M. japonicus*	MjDrosha	JQ918355	/	L: up	[127]
Ars2	*L. vannamei*	LvArs2	HQ692888	/	/	[76]
Pasha	*L. vannamei*	LvPasha	HQ692889	/	/	[76]
SID1	*L. vannamei*	Lv-SID1	HM234688.1	/	/	[126]

Note: Ce, Cephalothoraces; G, gill; Hp, hepatopancreas; Hc, hemocytes; Ht, heart; Hl, Hemolymph; I, intestine; L, lymphoid organ; M, muscle; S, stomach; up, differentially up-regulated; down, differentially down-regulated; modulated, different regulation trends at different post-infection time; none, no obvious difference; /, not available

Table 1: P38 mitogen-activated protein kinases (MAPKs).

from *F. chinensis* (designated as FcDorsal) and *L. vannamei* (named LvDorsal). ORFs of FcDorsal and LvDorsal encoded 357 amino acid residues and 400 amino acid residues, respectively. And both of them contained a Rel homolog domain (RHD) and an IPT/TIG (Ig-like, plex-ins and transcription factors) domain. FcDorsal had the highest expression level in the hemocytes and lymphoid organ (Oka), and its expression profiles in hemocytes and lymphoid organ were apparently modulated when shrimp were stimulated by bacteria or WSSV. Knock-down of FcDorsal by dsRNA led to the decrease of the transcription of pen5 [38]. And for LvDorsal, dual-luciferase reporter assays indicated that LvDorsal could trans-activate the pen4 and Drosophila Atta [39].

Cactus: NF-κB pathways play important roles in innate immune response, and IκB is the main cytoplasmic inhibitor of this pathway [40]. Drosophila Cactus inhibited dorsal from binding DNA [40]. *L. vannamei* Cactus (LvCactus) contained six predicted ankyrin repeats, which was the same as those from insects. LvCactus localized in cytoplasm and interacted with *L. vannamei* Dorsal. Over-expression of LvCactus appeared downregulated the activities of shrimp AMP promoters. Promoter of LvCactus was predicted to contain five putative NF-κB binding motifs, and four of them were proved to be bound by LvDorsal. LvCactus was promoted by LvDorsal and inhibited by LvCactus itself, which indicating a feedback regulatory pathway between LvCactus and LvDorsal. LvCactus was up-regulated after Lipopolysaccharides, poly (I:C), *V. parahaemolyticus,* and *Staphylococcus aureus* injections, suggesting an activation response of LvCactus to bacterial and immune stimulant challenges. On the contrary, expression of LvCactus was inhibited by WSSV. It is possible that WSSV modified the feedback regulatory pathway of LvCactus/LvDorsal during infection [41].

Cactus also was cloned in *F. chinensis*. Genomic DNA sequence of FcCactus was more than 17698 bp, included seven exons and six introns. Spatial expression profiles showed that FcCactus mRNA had the highest expression level in muscle, hemocytes, heart and lymphoid organ. Gram-positive bacteria (Micrococcus lysodeikticus) and Gram-negative bacteria (*V. anguillarium*) injection to shrimp caused the modulation of FcCactus at the transcription level. Knock-down FcCactus by dsRNA showed that FcCactus could regulate the expression of different AMPs and antiviral factor (AV) [42]. These data showed that Cactus might play a critical role in regulating the immune response of shrimp.

Myeloid differentiation factor 88: Myeloid differentiation factor 88 (MyD88) is a universal and pivotal adapter protein in IL-1/TLR mediated NF-κB pathway activation [43]. Two MyD88 protein variants (LvMyD88 and LvMyD88-1) were identified in *L. vannamei*. LvMyD88 cDNA was 1848 bp in length and contained an ORF of 1428 bp; LvMyD88-1 cDNA was 1719 bp in length and had an ORF of 1299 bp. Both variants encoded proteins with DD and TLR/interleukin-1 receptor (IL-1R)-related (TIR) domain and shared 91% sequence identity. In healthy *L. vannamei*, the LvMyD88 was highly expressed in hemocytes, and with a low level in the hepatopancreas. LvMyD88s were induced in hemocytes after challenge with lipopolysaccharide, CpG-ODN2006, *V. parahaemolyticus*, *S. aureus*, and WSSV, but not by poly I:C. Overexpression of LvMyD88s in Drosophila S2 cells led to activation of AMPs and wsv069 (ie1), wsv303, and wsv371[44].

MyD88 homolog from *F. chinensis* (FcMyD88) was 477 amino acids with a DD and TIR domain. The expression level of FcMyD88 mRNA was significantly up-regulated at 1 hour (h), 12 h and 24 h with both V. anguillarum and *M. lysodeikticu*. The expression level of FcMyD88 was 2-fold up-regulated at 12 h post injection (hpi) of inactivated *V. anguillarum*, while it did not change after *M. lysodeikticu* injection during this period. After WSSV injection, the expression level of FcMyD88 mRNA remained relatively constant, and the FcMyD88 protein was significantly up-regulated at 12 and 24 hpi [45]. These results suggested that MyD88 might take part in antibacterial and antiviral response in shrimp.

Sterile-alpha and armadillo motif-containing protein (SARM) and Toll-interacting protein (Tollip): Over-activation of TLRs pathway may leads to severe damage to the host, such as in chronic inflammatory diseases and cancer, therefore this pathway need tightly controlled. And in mammals, SARM negatively regulated TLRs pathway. *L. vannamei* SARM (LvSARM) shared signature domains with and exhibited significant similarities to mammalian SARM. Expression of LvSARM was responsive to *V. alginolyticus* and WSSV infections in the hemocytes, gill, hepatopancreas and intestine. In Drosophila S2 cells, LvSARM significantly inhibited the promoters of the NF-kB pathway-controlled AMPs, and silencing LvSARM increased the expression levels of PENs and anti-LPS factors, while it was strange that mortality rate was increased after *V. alginolyticus* infection. These results revealed that LvSARM might be a novel component of the shrimp Toll pathway, which negatively regulated shrimp AMPs [46].

Tollip is another important factor for negative regulation TLRs pathway in mammalian. *L. vannamei* Tollip (LvTollip) shared significant similarities to mammalian Tollips, and contained a centrally localized protein kinase C conserved region 2 (C2) domain and a C-terminal CUE domain. Expression levels of LvTollip were altered in the gill, hemocytes, hepatopancreas, intestine, and muscle tissues after challenges with WSSV or *V. alginolyticus*. LvTollip significantly inhibited the promoter activities of the NF-κB pathway-controlled pen4 in Drosophila S2 cells. Knock-down LvTollip increased the expression level of pen4. While the mortality rate of LvTollip-knockdown shrimp in response to WSSV or *V. alginolyticus* infections was not significantly different from those of the control group [47].

Immune deficiency (IMD) pathway

IMD: IMD pathway is the vital signaling cascades which regulate

the expression of AMPs in Drosophila [48]. IMD and Relish are the two key components of this pathway (Table 1). IMD preferentially recognizes Gram-negative bacteria, and its mutation showed severe defects in resistance to Gram-negative bacteria but has normal response to fungi and Gram-positive bacteria [49].

Full-length cDNA of *L. vannamei* IMD (LvIMD) was 758 bp with an ORF of 483 bp encoding a putative protein of 160 amino acids including a DD at the C-terminus. LvIMD showed similarity to that of Drosophila IMD and human receptor interacting protein 1 (RIP1) of the TNFR pathway, respectively. LvIMD mRNA expressed in most tissues and was induced in hepatopancreas and hemocytes after immune challenge. Luciferase reporter assays confirmed that LvIMD was able to induce the expression of AMPs [50]. The above data indicated that LvIMD participated in innate signaling to activate the expression of AMPs in *L. vannamei*.

Relish: Relish is triggered by the IMD pathway in response to infection by Gram-negative bacteria [51]. Stimulation results in the Relish N-terminal RHD and C-terminal ANKs cleaved. Then RHD translocates into the nucleus and induces the expression of AMPs [51,52]. The full length cDNA of *F. chinensis* Relish (FcRelish) consisted of 2157 bp, including a 1512 bp ORF, encoding 504 amino acids. FcRelish was highest expression in the hemocytes and lymphoid organ. Its expression was affected by *V. anguillarium* and *M. lysodeikticus* stimulation. Silencing FcRelish through RNAi greatly changed the transcription profile of AMPs [53]. And in L. vannamei, two full length cDNAs of Relish homolog (designated LvRelish and sLvRelish) were obtained. Long form LvRelish with an ORF encoding 1207 amino acids, contained a conserved RHD, a nucleus localization signal, an IκB-like domain (six ankyrin repeats), and a DD. The short form LvRelish (sLvRelish) cDNA was 1051 bp encoding 317 amino acids. SLvRelish had the same RHD region as LvRelish. Electrophoretic mobility shift assay showed that recombinant RHD of LvRelish in S2 cells bound specifically to *D. melanogaster* NF-κB motifs in vitro. Furthermore, LvRelish could regulate the transcription of pen4 [54].

IKKβ and IKKε: Two IκB kinases homologs, LvIKKβ and LvIIKK ε are cloned from *Litopenaeus vannamei*. Both of them are widely expressed in different tissues. LvIKKβ but not LvIKKε activates the promoters of NF-κB pathway-controlled AMPs, such as PENs in Drosophila S2 cells. While in HEK 293T cells, both of them activate an NF-κB reporter. Knock-down the expression of LvIKKβ or LvIKKε using sequence-specificity dsRNA decreased the expression of PENs, lysozyme and crustins in *L. vannamei* [55].

JAK-STAT pathway

JAK-STAT signaling pathway transmits chemical signals outside into cell nucleus, which causes DNA transcription and activity in the cells [56]. It consists of three main components: (1) a receptor (2) Janus kinase (JAK) and (3) Signal Transducer and Activator of Transcription (STAT). Except for STAT, members of this pathway have not been cloned in shrimp (Table 1).

P. monodon STAT (PmSTAT) had two isoforms, the short one with 774 amino acids, and the long one with 790 amino acids. They shared identities of 99%. Alignment and comparison with the deduced amino acid sequences of other STATs identified several important conserved residues and functional domains, including the DNA binding domain, SH2 domain and C-terminal transactivation domain. Phylogenetic analysis suggested that shrimp STAT belonged to the ancient STAT family, while the presence of the functional domains suggested that shrimp STAT might share similar functions and regulating mechanisms with the well-known STATs isolated from model organisms [2]. The *L. vannamei* STAT were cloned, and its function in ie1 transcriptional regulation was investigated [57].

RNA interference (RNAi)

RNAi is a critical regulator of many diverse biological functions, such as transcriptional gene regulation, post-transcriptional gene silencing, heterochromatin remodeling, suppression of transposon activity, and antiviral defenses [58,59]. Nowadays, RNAi has been reported to be relatively well conserved in species of different phyla. It has been proved that there is functional RNAi system in penaeid shrimp (Table 1). Yet its immune function remains still unclear [60].

Argonaute (Ago): Argonaute (Ago) proteins are the central component of the RNAi pathway, and plays important roles in host innate antiviral immunity. Each Organism always has a large number of different Ago proteins and isoforms. Several Ago genes have been cloned in shrimp, two in *L. vannamei* (Lv-Ago1 and Lv-Ago2), three in *P. monodon* (PmAgo1, PmAgo2, and PmAgo3) and three in *M. japonicus* (MjAgo1A, MjAgo1B, and MjAgo1C) [61-65]. Shrimp Ago proteins contain a PAZ domain and a PIWI domain, which both exhibit highest homology to their counterparts in D. melanogaster [61]. Ago interacts with other proteins, such as Dicer2 or Tudor staphylococcal nuclease, and then form the RNA-induced silencing complex (RISC) [65]. It also involves in anti-viral immunity as reported [64].

Dicer: Dicer, a member of the RNase III family, catalyzes the cleavage of double-stranded RNA to small interfering RNAs (siRNAs) and microRNAs (miRNAs), and finally directs sequence-specific gene silencing [66]. Invertebrate has two dicer proteins, Dicer1 involved in miRNAs pathway, and Dicer2 involved in siRNAs pathway [67]. Both *P. monodon* Dicer1 (PmDcr1) and *L. vannamei* (LvDcr1) had an ORF 7422 bp encoding a polypeptide of 2473 amino acids [68,69]. During the developmental stages from fertilized egg to postlarva VII, LvDcr1 was constitutively expressed at all examined development stages, but the expression levels varied significantly. The highest expression level was observed in fertilized eggs then decrease from fertilized egg to nauplius I stage, and the higher levels of expression were detected at nauplius V and postlarva stages. LvDcr1 expression regularly increased at the upper phase of nauplius, zoea and mysis stages than their prophase. The different expression of LvDcr1 in the larval stages could provide clues for understanding the early innate immunity in the process of shrimp larval development. It was suggested that both PmDcr1 and LvDcr1 took part in antiviral defense in adult shrimp [69].

Dicer2 (Dcr2) is the key protein of the siRNA pathway. The full-length cDNA of *L. vannamei* Dcr2 (LvDcr2) was 5513 bp long, with an ORF encoding a putative protein of 1502 amino acids. It was demonstrated that LvDcr2, LvAgo-2, and *L. vannamei* transactivating response RNA-binding protein isoform 1 (LvTRBP1) interacted with each other, and formed the RISC complex. A luciferase reporter assay indicated that LvDcr2 engaged in LvSVC1, LvSVC4, LvSVC5, and DmSVC Vago (DmVago) activation, which were potential antiviral genes. LvDcr2 also be up-regulated in immune responses against Poly(C-G) or WSSV challenge. These results suggested that LvDcr2 might engage in non-specific activation of anti-viral immunity [70].

Full length cDNA of Penaeus monodon Dicer2 (designated as PmDcr2) contains a 5′ untranslated region (UTR) of 109 bp, an open reading frame (ORF) of 4509 bp and a 3′ UTR of 842 bp. PmDcr2 shared the highest similarity of 91.8% and 90.7% with LvDcr2 and MyDcr2,

respectively. PmDcr2 was widely expressed in almost all examined tissues except muscle, with high expression in gill, hemocytes and lymph. The expression of PmDcr2 in hepatopancreas was up-regulated by *V. vulnificus*, WSSV, dsRNA poly (I:C) and ssRNA R484, but not by *S. aureus*. These data reflected that PmDcr2 was response to the gram negative bacterial infection and viral infection in black tiger shrimp [71].

TRBP1: TRBP plays an important role in many biological processes, and is a critical component of the RISC, which is a key complex in the RNAi. It is showed that TRBP1 interacts with eIF6. There were three TRBP family genes cloned from *F. chinensis*. Expression of Fc-TRBP was detected in many tissues, with elevated expression in the heart, gill, and intestine in the early stages of infection by the WSSV, and enhanced expression in most tissues following challenge with *V. anguillarum*. The expression pattern of eIF6 was also analyzed and its expression was up-regulated in intestine of WSSV-challenged shrimp. The replication of WSSV was reduced after injection of FcTRBP. These results indicated that FcTRBP and FceIF6 might be components of the RISC, and thereby played a crucial role in the antiviral defense response of shrimp [72]. Similar function was detected in *M. japonicus* TRBP [73].

There also cloned three *L. vannamei* TRBP genes (LvTRBP1, LvTRBP2, and LvTRBP3) and a *P. monodon* TRBP gene (PmTRBP1). LvTRBP1 was found to interact with LvDicer2, and to form RISC [70]. PmTRBP1 was evolutionarily closest to LvTRBP1. Both of them owned three double-stranded RNA-binding motifs, a character of TRBP family. Tissue expression profile analysis showed that PmTRBP1 was with a predominant expression in the lymphatic organs and with the weakest expression in the ovaries. PmTRBP1 expression was upregulated when elicited by systemic injections of *S. aureus*, *V. vulnificus*, and WSSV, thereby revealing its pathogen inducibility. Furthermore, exogenous viral nucleoside analogs (dsRNA poly(I:C) as well as ssRNA R484) were remarkably induced PmTRBP1 transcription at 9 h and 48 h post-injection, respectively, which suggested that PmTRBP1 might function in tiger prawn antibacterial and antiviral response [74].

ARS2, Pasha and Drosha: It has been reported that Ars2 regulates both miRNA and siRNA dependent silencing and suppresses RNA virus infection in Drosophila [75]. The full-length cDNA of *L. vannamei* arsenite resistance gene 2 (LvArs2) was 3470 bp, including a 5′ untranslated region (UTR) of 167 bp, a 3′ UTR of 639 bp, and an ORF of 2664 bp encoding 887 amino acid residues with an estimated molecular mass of 102.5 kDa. Co-Immunoprecipitation demonstrated that LvArs2 interacted with LvDcr2 and *L. vannamei* Pasha (LvPasha) in S2 cells, suggesting that LvArs2 might be involved in regulation of the miRNA/siRNA pathways in L. vannamei. Subcellular localization assays demonstrated that both LvArs2 and LvPasha mainly presented in the nucleus. After Poly(C–G) stimulation, the expression of LvArs2 was suppressed and expression of LvPasha was enhanced in shrimp gills. These results suggested that LvArs2 and LvPasha might participate in the defense against RNA viruses in *L. vannamei* [76].

The RNase III Drosha is a critical component for miRNA maturation. Shrimp Drosha was characterized in *M. japonicus* (MjDrosha). The sequence analysis revealed that it encoded a 1081-amino-acid peptide, which comprised two tandem ribonuclease III C terminal domains and a double-stranded RNA binding motif. The MjDrosha was homologous with those of other animal species. It was highly expressed in lymphoid organ and was significantly up-regulated in response to WSSV challenge, which suggesting that MjDrosha was involved in the antiviral immunity of *M. japonicus*. Knock-down MjDrosha led to the defect of miRNA maturation, and subsequent higher virus loads in *M. japonicus*. Therefore, MjDrosha played important roles in the antiviral defense of *M. japonicus.*

P38

P38 mitogen-activated protein kinases (MAPKs) are broadly expressed in eukaryotes, from yeasts to mammals, and are involved in the regulation of responding to various extracellular stimulus [77]. It was found that p38 MAPK gene (designated as Lvp38) in *L. vannamei* contained the conserved Thr-Gly-Tyr (TGY) motif as well as the substrate-binding site, Ala-Thr-Arg-Trp (ATRW) (Table 1) [78]. Lvp38 was highest expressed in hemocytes, nerves, and intestines. It was upregulated in gills and hemocytes after infection with the *V. alginolyticus* and the *S. aureus*. It was showed that Lvp38 activated the expression of AMPs in Drosophila and shrimp. Knock-down Lvp38 resulted in a higher mortality of *L. vannamei* with *V. alginolyticus* and *S. aureus* infection, and a down regulation of shrimp AMPs pen4, crustin, and ALF2. It seems that Lvp38 roled in defending against bacterial infections.

Antimicrobial peptide (AMP)

One important products of immune response are AMPs whose primary function is killing or cleaning the infected pathogens. Their production was regulated by Toll and IMD pathway in Drosophila [79]. The penaeidins, initially characterized from the shrimp *L. vannamei,* are a family of antimicrobial peptides that appear to be expressed in all penaeid shrimps [80]. In shrimp the penaeidins exhibited activities against both Gram-positive and negative bacteria and fungi [81]. Antilipopolysaccharide factor (ALF), a small basic protein, was initially isolated and characterized in amebocytes of the horseshoe crab *Limulus polyphenus* [82]. An ALF gene cloned form hemocytes of *F. chinensis* (ALFFc) was constitutively expressed in hemocytes. In responding to Vibrio infection, ALF transcription level appeared significant enhancement [83].

Crustins also is an important AMP in shrimp. The first crustin was isolated from the granular hemocytes of the shore crab C. maenas [84]. In *F. chinensis*, different types of crustins were reported, and a characteristic of the crustins is containing a whey-acidic protein (WAP) domain at the C-terminal. In *M. japonicus*, a gene with two WAP domains (Mj-DWD) was isolated. During the early phase of WSSV infection, the Mj-DWD expression was significantly upregulated. The recombinant Mj-DWD, expressed by *P. pastoris*, showed specific protease inhibitory activity on *Bacillus subtilis* [85]. The above data showed that diversified crustins existed in shrimp and they seemed to have function divergence, anti-bacteria or anti-virus activity.

In summary, the members of humoral immune response make up of a fine defense in shrimp, which can respond quickly to microbial invasion. Most of them took part in antiviral defense. WSSV simulate the humoral immune response, however, WSSV could utilize immune factors to regulate viral genes expression that make the cleaning of WSSV become difficult.

Shrimp UPR

As an aquatic livestock, *L. vannamei* is exposed to temperature shifts, viral infections, sudden salinity changes, heavy metal toxicity and so on, which may induce ER-stress and injure it. Some genes involved in UPR, including (immunoglobulin heavy chain-binding protein) Bip, heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), protein disulfide isomerase (PDI), hypoxia-inducible factor

(HIF), and calreticulin (CRT), have been cloned in shrimp. Their roles in the ER-stress have been studied [86-93] (Table 2). The relationship between the virus and UPR in shrimp has also been investigated. There are three branches for UPR, except ATF6 pathway, two branches have been investigated in *L. vannamei*.

IRE1-XBP1 pathway

The inositol-requiring enzyme-1 (IRE1)-X-box binding protein 1 (IRE1-XBP1) pathway is the main branch of the UPR [4]. Full-length cDNA of *L. vannamei* IRE1 (LvIRE1) was 4908 bp long, with an ORF encoding a putative 1174 amino acid protein. Full-length cDNA of *L. vannamei* XBP1 (LvXBP1) is 1746 bp long [94]. It contained two ORFs encoding putative 278 amino acid and 157 amino acid proteins, respectively. A predicted IRE1 splicing motifs CNG'CNGN were located within the loop regions of two short hairpins on LvXBP1 mRNA. It was interesting that the splicing fragment induced by ER-stress showed a 3 bp or 4 bp frame shift from the predicted sites. The spliced form LvXBP1 (LvXBP1s) fused the two ORFs together, and encoded a putative 463 amino acid protein. LvXBP1s activated the promoter of important UPR effector LvBip. RT-PCR showed that LvXBP1 was spliced upon heat shock treatment or WSSV challenge. These results suggested that the IRE1-XBP1 pathway was important for *L. vannamei* environmental stress resistance, and *L. vannamei* IRE1-XBP1 may be activated by WSSV and be annexed to serve the virus.

PERK-eIF2α pathway

PERK-eIF2α pathway is another important branch of UPR [4]. In *L. vannamei* immunity, PERK and eIF2αstill not cloned. Yet activating transcription factor 4 (designated as LvATF4), which was the key transcription factor of PERK-eIF2α pathway, has been identified and

Component	Species	Gene name	Accession number	Tissues: response to stress		References
				stresses	Response	
XBP1	*L. vannamei*	LvXBP1	JQ265944	heat	Hc: up	[94]
IRE1	*L. vannamei*	LvIRE1	JQ265943	/	/	[94]
ATF4	*L. vannamei*	LvATF4	JX908828	WSSV	Hc : none; G: up	[94]
HSP	*L. vannamei*	LvHSP60	FJ710169.	bacterial	G, Hp, Hc: up	[98]
	L. vannamei	LvHSP70	EF495128	bacterial	Hc, He: up	[98,100]
	F. chinensis	FcHSP70	FJ167398.1	heat,	Ce, Ht: up (heat);	[101]
				heavy	Ce: up (Copper);	
				metal	Ce: dwom (Cadmium)	
	P. monodon	PmHSP70	AF474375.1	heat	Hc: up	[128]
	F. chinensis	FcHSP90	EF032650.1	heat, hypoxia	Hc, G: modulated	[102]
					(hypoxia);	
					W: up (heat)	
	P. monodon	PmHSP21	/	heat,	P: up (heat);	[103]
				WSSV	P: down (WSSV)	
	Artemia	p26	DQ310577	/	/	[105]
	franciscana					
	M. japonicus	MjHSP40	AB520825	WSSV	Hc, L: up	[106]
	M. japonicus	MjHSP70	AB520826	WSSV	Hc, L: up	[106]
	M. japonicus	MjHSP90	AB520827	WSSV,	Hc, L: up (WSSV);	[106]
				heat	G: up (heat)	
GRP78	*F. chinensis*	FcGRP78	EF032651	heat,	Ce: up (heat);	[108]
				WSSV	Hc: down; Hp, L: up	
	L. vannamei	LvGRP78	JQ265942	/	/	[94]
CRT	*P. monodon*	PmCRT	JX961661.1	heat	Hc: up; Hp,G: none	[112]
	F. chinensis	FcCRT	DQ323054.1	heat,	Ce: up (heat);	[111]
				WSSV	Hc, Hp: modulated	
					(WSSV)	
HIF	Palaemonetes	gsHIF-1α	AY655698	hypoxic	Hp: none	[92]
	pugio					
	L. vannamei	LvHIF-1α	FJ807918	hypoxic	G, M, Hp: down	[91]
	L. vannamei	LvHIF-1β	FJ807919	hypoxic	G:down; M: up;	[91]
					Hp: none	
PDI	*L. vannamei*	LvPDI	FJ179395	bacterial	Hc: up	[93]
	P. monodon	PmPDIA6	JF290430	/	/	[129]
	F. chinensis	FcPDI1	HQ630061	bacteria,	Hp, G: up (bacterial);	[130]
				WSSV	O: down (bacterial)	
	F. chinensis	FcPDI2	HQ630062	bacteria,	Hc, Hp, G: up	[130]
				WSSV	(WSSV);	
					O: down (WSSV)	

Note: Ce, Cephalothoraces; G, gill; Hp, hepatopancreas; Hc, hemocytes; Ht, heart; L, lymphoid organ; M, muscle; O, ovary; Pleopod, P; S, stomach; W, whole shrimp; up, differentially up-regulated; down, differentially down-regulated; modulated, different regulation trends at different post-infection time; none, no obvious difference; /, not available.

Table 2: Heat shock proteins (HSPs).

characterized. The full-length cDNA of LvATF4 was 1972 bp long with an ORF 1299 bp long, encoded protein with 432 amino acids. Reporter gene assays showed that LvATF4 could upregulate the expression of WSSV gene wsv023 in an ATF/CRE-dependent manner. Knock-down LvATF4 also resulted in a lower cumulative mortality of *L. vannamei* under WSSV infection. Under the WSSV challenge, LvATF4 was upregulated in gills. It looks like that in *L. vannamei* UPR signaling pathway transcription factors were important for WSSV infection [95].

Heat shock proteins (HSPs): HSP are highly conserved proteins that are found in diverse organisms from bacteria to mammals. HSPs participate in a variety of cellular processes, such as folding or unfolding of proteins, translocation of proteins into organelles and so on [96]. HSPs have been considered as an appropriate biomarker for the measurement of stress resistance in shrimp. So far, *L. vannamei* hsp 60, hsp70 [97-100], *F. chinensis* hsp 70, hsp 90 [101,102], *P. monodon* hsp21, hsp70 and hsp90 [103,104], Artemia franciscana small hsp belonging to the α-crystalline family of proteins, referred to as p26 [105]. *M. japonicus* hsp40, hsp 70 and hsp 90 genes [106] have been cloned, and their functions in shrimp immunity and stresses resistance have been investigated (Table 2).

GRP78: The 78 kDa glucose-regulated protein (GRP78), also known as BiP, is an essential regulator of ER homeostasis because of its multiple functions in protein folding, ER calcium binding, and controlling of the activation of transmembrane ER stress sensors [107]. The first reported GRP78 gene in crustacean is *F. chinensis* GRP78 (FcGRP78) with an ORF 1,968 bp encoding 655 amino acids. The deduced amino acid sequence of FcGRP78 shared 86%, 87% and 85% identity with GRP78s of D. melanogaster, *A. aegypti* and *B. mori,* respectively [108]. Heat shock at 35°C significantly enhanced the expression of FcGRP78 at the first hour, reached the peak value at 4 h post heat shock treatment, then fell back and resumed to the normal level until 48 h of post recovery at 25°C. Besides, when shrimp were challenged by WSSV, differential expression levels of FcGRP78 were detected in hemocytes, hepatopancreas and lymphoid organ [88]. It was suggested that FcGRP78 might play important roles in chaperoning, protein folding and immune function of shrimp.

Calreticulin (CRT): Calcium signaling has its roles in growth, reproduction and molting in crustaceans [108]. And Calreticulin (CRT) is a main protein involved in calcium homeostasis of eukaryotes [109]. The full-length cDNA of CRT in *P. monodon* was 1682 bp, containing an ORF of 1221 bp corresponding to a deduced protein of 406 amino acids. Genomic sequence of PmCRT was 3006 bp in length, composing of 4 exons and 3 introns. In hemocytes of juvenile *P. monodon*, PmCRT was upregulated at early stage (0 and 1 h) post treatment at 35°C for 3 h. But the expression levels in gills and hepatopancreas after the temperature stress (0-48 hpt) showed no significantly difference with the control groups. Expression profile of PmCRT in hemocytes was up-regulated at 0 and 3 hpt for about 25 fold, then reduced to about 5 fold between 3 and 12 hpt and returned to the baseline level at 24 and 48 hpt. Furthermore, PmCRT exhibited an ability to form a complex with recombinant Endoplasmic Reticulum protein 57 of *P. monodon* (rPmERp57). *F. chinensis* calreticulin (FcCRT) had the same length and similar function with PmCRT. Above results suggested that shrimp CRT might work at Ca^{2+} homeostasis, chaperoning and immune function in shrimp [88], and be a suitable biomarker for temperature stress responses [110].

In summary, there are two pathway of UPR, IRE1-XBP1 and PERK-eIF2α pathway, which have been proved to exist in shrimp. Besides helping shrimp to cope with environment stress, they involved in WSSV genes expression. The molecular chaperones (HSP70, Bip, and CRT) which are essential regulators of ER homeostasis, also relate to WSSV infection.

Shrimp Humoral Immune Response and WSSV Genes Regulation

The host responding to the pathogens helps to clean pathogenic microorganisms. For successful infection, pathogens developed strategies to evade these responding, even to use them. As the most dangerous pathogens for shrimp, WSSV could benefit from the host responding in several ways.

Shrimp TLR-Dorsal pathway upregulated expression of WSSV ie1, wsv303 and wsv371

The immediate-early gene ie1 of WSSV expresses highly throughout the infection cycle, and exhibits very strong promoter activity [111]. A NF-κB binding motif was found in the ie1 promoter region. EMSA indicated that the recombinant RHD bound to the putative NF-κB site within the ie1 promoter region. A transactivity assay demonstrated that LvRelish could increase ie1 promoter activity in S2 cells. These results showed that LvRelish transactivated WSSV ie1 gene expression, and contributed to its high promoter activity. Further transactivation assays showed that WSSV IE1 protein expression upregulated the promoter activities of WSSV ie1 gene, as well as AMP genes (Figure 1). These results suggested that WSSV might annex the shrimp NF-κB pathway to enhance the expression of viral immediate-early genes [112].

Through whole genome sequence assay, wsv449 was found with 15.7~19.4% identity to Tube, which was an important component of the insect Toll pathway. Further research showed that wsv449 activated promoters of Toll pathway-controlled AMPs. Therefore, wsv449 has a similar function with host Tube in activating the NF-kB pathway. A promoter screening suggested that the promoter activities of ie1, wssv303 and wssv371 could be highly induced by the shrimp NF-kB family protein LvDorsal, as well as wsv449 [1].

Shrimp IMD-Relish pathway upregulated expression of ten WSSV genes

In the RNAi experiment, it was found that *L. vannamei* were resistant to WSSV infection when knock-down LvIKKβ or LvIKKε. The authors hypothesized that IKK-NF-κB signaling pathway modulating viral genes expression was required for successful infection with WSSV. A reporter gene assays with 147 WSSV genes screening revealed that the wsv051, wsv059, wsv069, wsv083, wsv090, wsv107, wsv244, wsv303, wsv371 and wsv445 promoters could be activated by LvIKKβ or LvIKKε in Drosophila S2 cells [55].

Shrimp STAT activated the promoter of WSSV ie1

Although the JAK/STAT signaling pathway is usually involved in antiviral defense, recent study suggested that it might be annexed by WSSV to enhance the expression of a viral immediate early gene in infected shrimps. Shrimp STAT belonged to the ancient STAT family, and the presence of the functional domains suggested that shrimp STAT shares similar functions and regulating mechanisms with the well-known STATs isolated from model organisms. Shrimp STAT showed a decreased transcription level after WSSV infection, but increased level of phosphorylated (activated) STAT in the lymphoid organ. It was also showed that STAT was translocated from the cytoplasm to the nucleus and activated, in a primary culture of lymphoid organ cells derived

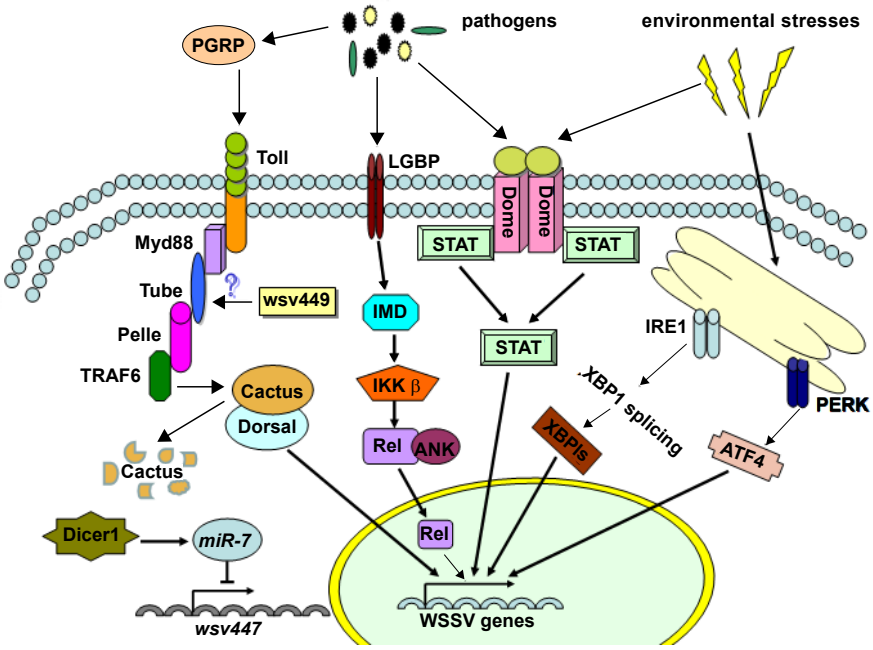

Figure 1: WSSV IE1 protein expression upregulated the promoter activities of WSSV ie1 gene, as well as AMP genes.

from WSSV-infected shrimp [2]. This result suggested that shrimp STAT was activated by WSSV.

Via a series of deletion and mutation assays, an element containing STAT binding motif was found to be important for the full level of WSSV ie1 promoter activity. PmSTAT bound to this element was detected by EMSA. It also found that in WSSV-infected *P. monodon*, levels of activated PmSTAT were higher than that in WSSV-free *P. monodon*. And increasing the level of rPmSTAT resulted in dose-dependent increases the ie1 promoter activity. It was concluded that WSSV successfully annexed a putative shrimp defense mechanism to enhance the expression of viral immediate-early genes [113].

RNAi pathway and WSSV genes regulation

RNAi plays an important role in fine-tuning virus-host interactions via gene expression, which guided by miRNAs. Increasing evidence suggests that viral and cellular miRNAs are involved in virus infection. In *M. japonicus* miR-7 was found to be upregulated in response to WSSV infection. Shrimp miR-7 could target the 3′UTR of wsv477, suggesting that miR-7 might involve in viral DNA replication (Figure 1). Synthesized miR-7 could significantly decreased the expression level of the fluorescent construct bearing the 3′UTR of wsv477 in insect High Five cells, and that could be rescued by miR-7 blocking. In vivo, synthesized miR-7 injection would reduce the numbers of WSSV genome copies/mg gills about 1,000-fold at 72 and 96 h compared to the control. Besides, blocking of endogenous miR-7 by AMO-miR-7 led to about 10-fold increases of WSSV genome copies/mg gills in WSSV-infected *M. japonicus* comparing with the control WSSV only [114]. Thus these results indicated that *M. japonicus* miRNAs roled in host-virus interactions by targeting the viral early gene. 35 miRNAs were identified, and a correlation of WSSV infection with the expression levels of 22 miRNAs was discovered in *M. japonicus* [115].

On the other hand, based on WSSV miRNA microarray and northern blot analyses, WSSV was proved to have the capacity encoding 40 viral miRNAs, which was about 360 times greater than that of human genome in content. The egregious high content of viral miRNAs of WSSV, suggested that WSSV miRNAs might greatly contribute to viral variability for dealing with host environment and responding. As reported, 80% of WSSV miRNAs were expressed during early stages of viral infection, which highlight their importance in initial infective processes. Besides host Drosha, Dicer1 and Ago1, biogenesis of viral miRNAs was demonstrated to be dependent on viral miRNAs too, such as WSSV-miR211 and WSSV-miR212. These viral miRNAs were proved to be required for successful WSSV infection. Therefore, during WSSV infection, numerous viral genes were targeted by WSSV miRNAs [116].

The results as mentioned above indicate that the shrimp humoral immune responses which designed to clean pathogenic microorganisms were annexed by WSSV to enhance the expression of viral genes. It means that activated immune responses are essential to WSSV infection.

Shrimp UPR and WSSV Genes Regulation

L. vannamei XBP1 transcriptional regulated wsv083

After WSSV challenging, LvXBP1 was upregulated during the experiment and the percentage of the spliced form continuously declined after 18 h of infection. Besides, LvXBP1 upregulated wsv 083 in a UPRE-dependent manner [95]. Wsv083, an immediate early gene of WSSV, worked as a protein Ser/Thr kinase and inhibited the activity of focal adhesion kinase (FAK), involving in the processes of cell adhesion and spreading, which were crucial for invertebrate immune system [117]. It was suggested that wsv083 was important for WSSV to evade the host immune response. Knock-down LvXBP1 resulted in a lower cumulative mortality of *L. vannamei* under WSSV infection. Taken together, these results confirmed that the *L. vannamei* IRE1-XBP1 may activated by WSSV and be annexed to serve the virus.

L. vannamei ATF4 upregulation expression of wsv023

ATF4 is the main transcription factor of PERK-eIF2α pathway,

and was reported to take part in viral genes regulation. For example, human ATF4 interacted with Tax and engaged in T-cell leukemia virus type 1 (HTLV-1) Tax protein activating viral transcription [118]. Based on the WSSV genome analysis, 15 genes (wsv023, wsv049, wsv064, wsv069, wsv138, wsv242, wsv256, wsv282, wsv303, 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. Expression of pACB-LvATF4 significantly increased pGL3-wsv023 expression by approximately 10-fold. When the ATF/CRE element of wsv023 promoter was mutated or deleted, the pGL3-wsv023 expression was significantly reduced by approximately 93% and 87% comparing to the wild type, respectively. The expression of pGL-wsv069 was also activated by LvATF4, increased by approximately 6-fold. Taken together, these results suggested that in *L. vannamei* UPR signaling pathway transcription factors were important for WSSV infection [95].

HSP70 and WSSV genes regulation

High temperature (32-33°C) has been shown to reduce mortality in WSSV infected shrimps, but the mechanism still remains unclear. It was found that in WSSV infected shrimps cultured at 32°C, transcriptional levels of representative immediate early, early, and late genes of WSSV were initially higher than that at 25°C. However, neither IE1 nor VP28 proteins were detected at 32°C, suggesting that high temperature might inhibit WSSV protein synthesis. Two-dimensional gel electrophoresis analysis revealed two proteins, NAD-dependent aldehyde dehydrogenase (ALDH) and proteasome alpha 4 subunit (proteasome α4), were markedly up-regulated in WSSV-infected shrimps at 32°C. RT-PCR analysis also showed hsp70 was up-regulated at 32°C. When knocked-down ALDH or hsp70, shrimps challenging with WSSV became severely infected at 32°C. While the proteasome α4 knock-down shrimps remained uninfected [87].

The above researches shown that UPR transcription factor XBP1s and LvATF4 could directly bind to WSSV genes and upregulate their expression, and the molecular chaperones ALDH and Hsp70 both play an important role in the inhibition of WSSV replication at high temperature. The relationship between UPR and WSSV infection is complicated. WSSV infection could trigger ER stress and in turn utilize UPR to expression IE gene, and then UPR inhibit WSSV protein synthesis.

Conclusion and Perspective

Similar to other eukaryotes, shrimp innate immunity and UPR are important for its survival. So far, TLRs pathway, IMD pathway, JAK-STAT pathway, and RNAi pathway have been identified in shrimp. Meanwhile, shrimp IRE1-XBP1 pathway and PERK- eIF2α pathway also have been investigated. More innate immunity relative pathways and UPR relative pathways will be found with the dramatic increase in sequence data. Considering their essentiality and necessity, shrimp innate immunity and UPR occupy important positions in the shrimp homoeostasis. They play important roles in innate immune responses to against various pathogens, such as bacterium, fungus and viruses, and they are also involved in environmental stress resisting. Generally speaking, viruses can be seen as either a pathogen or a kind of environmental stress. Among the shrimp viral pathogens, WSSV is well studied. An increasing evidences showed that WSSV successfully activated, and employed the shrimp innate immunity and UPR for its genes transcription.

Particularly noteworthy is the shrimp UPR. It has been reported that WSSV are widespread among the culture shrimp. Considering that environmental catastrophe, such as rainstorm and cold snap always following outbreak of WSS, it is possible that WSS outbreaks caused by environmental stress. Furthermore, UPR is not only critical for environmental stress resisting, but also cross-talk with several innate immunity signal pathways, such as TLRs pathway, autophagy pathway and apoptosis pathway [118-121]. Therefore, it seems that shrimp UPR play central role in the shrimp homoeostasis. Based on the previous research, we found the clues that WSSV could hijack and stimulate the shrimp innate immunity and UPR to facilitate its life cycle. To take advantage of the innate immunity responding or UPR, WSSV has to activate them first. However, we still have limited knowledge about that, even the functions of wsv023 and wsv083 in WSSV infection. There are already research teams that focus on the study of WSSV gene function. Further work on interaction between shrimp homoeostasis signal pathways and WSSV, especially the UPR and WSSV infection, would be helpful for developing new strategies to control shrimp disease, and their application in aquaculture would be of great significance [89,122-126].

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