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Review

Antiviral immunity in crustaceans

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ABSTRACT

Viral diseases of shrimp have caused negative effects on the economy in several countries in Asia, South America and America, where they have numerous shrimp culture industries. The studies on the immunity of shrimp and other crustaceans have mainly focused on general aspects of immunity and as a consequence little is known about the antiviral responses in crustaceans. The aim of this review is to update recent knowledge of innate immunity against viral infections in crustaceans. Several antiviral molecules have been isolated and characterized recently from decapods. Characterization and identification of these molecules might provide a promising strategy for protection and treatment of these viral diseases. In addition dsRNA-induced antiviral immunity is also included.

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1. Crustacean viruses

Viruses are the most common pathogens in the sea and they are present up to ten billion per litre and several of them can infect many organisms [1]. Hence, viral infections are common diseases in crustaceans such as penaeid shrimp, which can be infected by more than twenty different viruses [2]. In crustaceans, research on viruses has increased considerably since the first report on the occurrence of a virus in the crab *Macropipus depurator* [3]. Crustacean viruses belong to or are related to various viral families like the *Baculoviridae*, *Bunyaviridae*, *Herpesviridae*, *Picornaviridae*, *Parvoviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae*, *Iridoviridae* or a new virus family, the *Nimaviridae* [4]. During the past decades, both extensive and intensive shrimp cultures have been established and as a result more detailed knowledge of viral-host interactions has been gained. Among these viruses, the most intensively studied viruses have been characterized from cultured penaeids such as the white spot syndrome virus (WSSV), yellow head virus (YHV), and Taura syndrome virus (TSV). Much less details are available for other viruses present in wild crustaceans [5]. Infection with these viruses has caused detrimental diseases in shrimp culturing, resulting in serious economic losses. WSSV, YHV, and TSV have been regarded as the most serious shrimp viruses (Fig. 1) [6].

WSSV is an enveloped large circular double stranded DNA virus containing about 300 Kbp [7,8]. The enveloped virions (120–150 nm × 270–290 nm) are symmetrical and ellipsoid to bacilliform in shape similar to baculovirus [9]. Phylogenetic analysis suggests that WSSV is a new virus family named as the *Nimaviridae*, genus *Whispovirus*. WSSV has been widely studied and here we mainly focus on the interaction between this virus and its decapod hosts. The WSSV has a wide host range including all species of shrimps, some other crustaceans (e.g. crayfish, crab, and spiny lobster), and has even been detected in insects (Fig. 2) [10] and can be transmitted from broodstock to the offspring [11]. After infection, the shrimp exhibits very high mortality rates and the cumulative mortality usually reaches 100% within 3–10 days after the onset of the first visible gross signs. Histopathological studies have shown that WSSV targets many different tissues in the freshwater crayfish, *Pacifastacus leniusculus* (Fig. 3) [12].

YHV is an enveloped viral particle (40–60 nm × 150–200 nm) with rounded ends containing a sense single-stranded RNA (approximately 26 Kbp). This virus contains three major structural proteins (gp116, gp64 and p20) and belongs to the genus Okavirus, *Roniviridae*, *Nidovirales* [13–16]. The shrimps usually die within a few days after infection with a widespread necrosis in the

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Fig. 1. Here is an intensive shrimp culture farm in Thailand. The main viral diseases in shrimp culture are caused by white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome virus (TSV). Of these white spot disease is one of the most serious disease. It reduces shrimp production and caused serious economic losses worldwide. Mortality is very high and can reach 100% within 3–10 days from the onset of diseases.

lymphoid organ (LO), gills, connective tissues, hemocytes, and hematopoietic organs [17]. The LO is probably the primary target of YHV based on the histopathological studies and a YHV receptor study [18,19].

TSV was originally reported in 1992 [20] and the virus was first isolated and characterized by Bonami et al. [21]. The viral particle is non-enveloped, icosahedral in shape with a diameter of 31–32 nm, and replicates within the cytoplasm of host cells. It contains a linear positive-sense ssRNA genome of about 10, 205 nucleotides, and the capsid possesses three major (55, 40, and 24 kDa) and one minor (58 kDa) polypeptides. This virus has been classified as a member of *Picornaviridae* [22,23]. TSV is the first characterized picorna-like virus infecting an invertebrate other than insects. The cuticular epithelium of the foregut, gills, appendages, hindgut, and general body cuticle has been described as the predominant targeted cell type by TSV [6].

Unlike vertebrate immunity which is composed of both innate and adaptive responses, a general opinion is still that invertebrates rely on multiple innate defence reactions to combat infections. These reactions include the protection by physical barriers together with local (e.g. epithelial immunity) and systemic immune responses. Two main components, the humoral and cellular

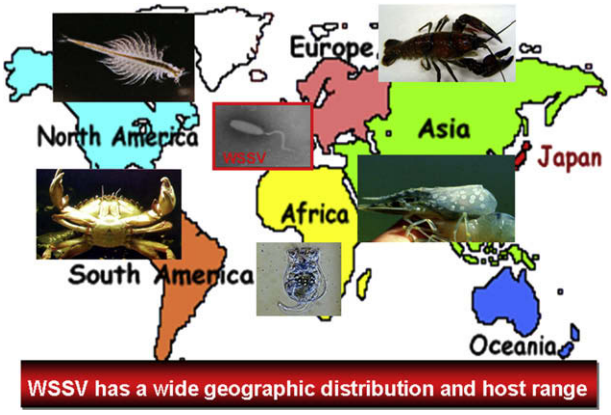

Fig. 2. WSSV spreads to all countries which have shrimp farming from Asia to North and South America. This virus not only infects shrimp but also other crustaceans such as crayfish, crab, artemia and recently it was reported to infect rotifer in a shrimp pond.

systems, are involved in the systemic innate immune response in invertebrates, both of which are activated upon immune challenge. Several reviews have described the histopathology, diagnostic techniques, epidemiology, and genome organization of these important viruses [6,24–26]. This review is mainly focused on recent antiviral immunity described on these three major viruses in crustaceans.

2. Virus entry

Knowledge of virus entry into permissive cells is mainly from vertebrates and both cellular and viral factors are recruited when virus enters into cells. Most cells are capable of endocytosis through several distinct mechanisms including clathrin-coated vesicles, phagocytosis, macropinocytosis and caveolae [27]. Several animal viruses take advantage of the cell endocytic machinery for a successful infection. In this process, viruses are taken up into the cytoplasm, where they produce dsRNA and initiate replication. The incoming virus particles are entering endosomal structures, lysozymes, the endoplasmic reticulum (ER), and occasionally the Golgi complex. The mildly acidic pH in the endosome will trigger penetration and uncoating. During this process, cytoskeleton and cellular proteins are often recruited by the invading viruses.

How invertebrate viruses enter host cells remains largely unknown. Coronaviruses have been suggested to use envelope proteins as recognition molecules for viral-host binding and cell entry [28]. This is also supported by an in vitro study showing that a polyclonal antibody against gp116 (YHV glycoprotein 116) inhibited YHV entry into LO cells, but not an antibody to gp64 (YHV glycoprotein 116) [29]. YRP65 has been identified as a YHV receptor in *Penaeus monodon*. Both antibodies against YRP65 and silencing of the *YRP65* are able to specifically inhibit the entry of the YHV into the Oka or lymphoid organ cells [18]. *Pm*Rab7, a shrimp small GTPase protein binding directly to VP28 of WSSV, was suggested to be involved in WSSV infection [30]. Silencing of *Pm*Rab7 dramatically inhibited WSSV-VP28 RNA and protein expression. Future work is still necessary to elucidate if this *PmRab7* localizes on the surface of the host cells and how this protein is involved in virus transport into the cells. Further, the silencing of *PmRab7* also inhibited YHV replication in the YHV-infected shrimp. These data indicate that *Pm*Rab7 is a common cellular factor required for WSSV or YHV replication in shrimp [31]. However, in a latter study, some

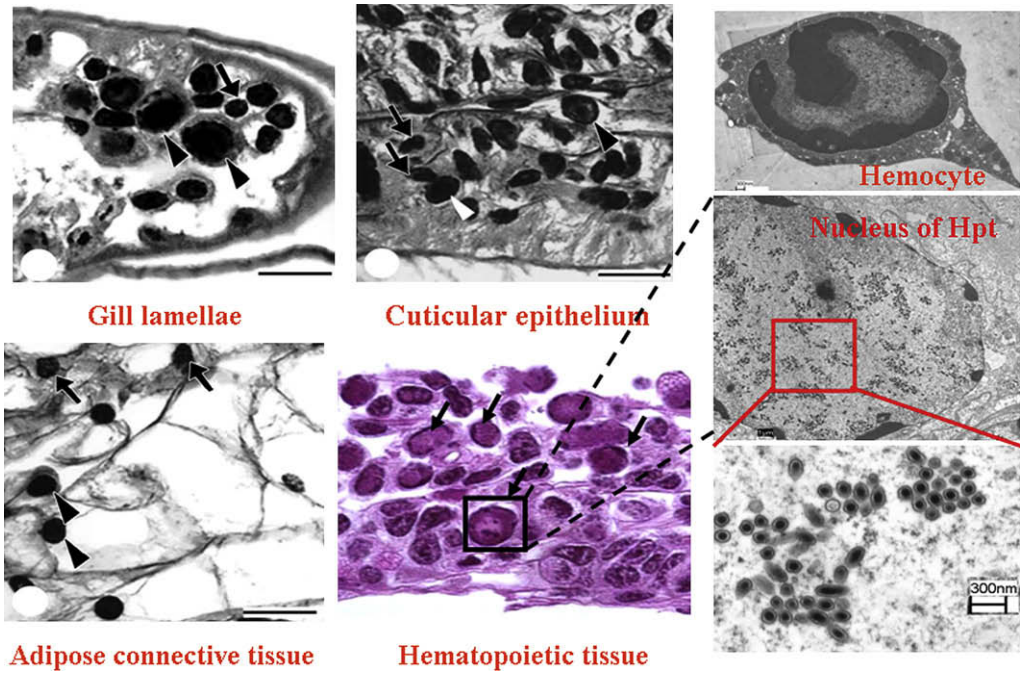

Fig. 3. Target tissues of WSSV infection. Histopathological changes were found in various tissues of infected crayfish. For example gill, cuticular epidermis under shell and around the stomach, adipose connective tissue, hematopoietic tissue even hemocytes. The nuclei of infected cells are hypertrophied and the chromatin is marginated along the nuclear border (arrows). The arrow heads in the gill lamellae show infected hemocytes. By TEM observation inside these enlarged nuclei contains a massive of virus particles.

shrimp injected with dsRNA did not show any antiviral protection and some of the RT-PCR results of host gene/viral gene are not in agreement with each other in the quantitation of their transcripts. Thus, the data presented in this study may need more detailed investigations for a fully understanding of the mechanism by which *Pm*Rab7 interacts with WSSV. Besides, another Rab GTPase from *Penaeus japonicus* was reported to regulate shrimp hemocytic phagocytosis through a proposed protein complex consisting of the *Pj*Rab, beta-actin, tropomyosin, and an envelope protein VP466 of WSSV, indicating a possible role of phagocytosis involved in virus evasion in shrimp [32]. A VP28 of WSSV was suggested to bind to shrimp cells as an attachment protein and in this way could help the virus to enter into the cytoplasm [33], but this speculation needs to be documented by more experimental data.

WSSV exhibits higher tropism to semigranular cells (SGCs) than to granular cells (GCs) in both crayfish and shrimp [12,34]. Both the percentage of degranulated cells and cell spreading in WSSV-infected crayfish were significantly lower than that of sham infected crayfish when hemocyte lysate supernatant (HLS) or phorbol 12-myristate 13-acetate (PMA) was used [35]. Peroxinectin, a cell adhesive and opsonic peroxidase, has been shown to mediate attachment and spreading of the hemocytes in crayfish. This molecule can also trigger degranulation by a regulated exocytosis [36,37]. Binding of peroxinectin to the cell surface via an extracellular superoxide dismutase (SOD) may produce a signal into the cell through an integrin for further cellular responses [38]. The cellular responses have been suggested to be triggered via a pathway that includes protein kinase C (PKC) activation and elevated protein tyrosine phosphorylation of a cellular protein of ~80 kDa [39]. Hemocyte lysate supernatant (HLS) containing peroxinectin treated GCs and SGCs from WSSV-infected crayfish resulted in three different cell-reactions: non-spread, spread, and degranulated cells. The non-spread cell groups from both GCs and SGCs exhibited more WSSV positive cells than the degranulated cells. Taken these data together, it is likely that the PKC pathway might be somehow affected by WSSV during its replication within the cells [35].

3. Viral infection and hematopoiesis in crustaceans

In crustaceans, the number of freely circulating hemocytes varies in response to environmental stress, endocrine activity during the moulting cycle, and infection. Exposure to non-self molecules can cause a dramatic drop in total hemocyte count and the animals may die of an infection that they otherwise usually resist [37,40]. WSSV affects the hemocytes in crayfish *P. leniusculus*. For instance, infection of WSSV has a significant effect on the proportion of different hemocyte types in the animals. The number of GCs was significantly higher in WSSV-injected crayfish compared to control animals [12]. Studies from crayfish and shrimp suggest that SGCs are more susceptible to WSSV and the virus replicates more rapidly in SGCs than GCs resulting in the gradually decreased SGCs from blood circulation [34,35]. Other studies showed that the total numbers of circulating hemocytes in shrimp were dramatically decreased after infection by WSSV [41–43]. On the other hand, immune-relevant molecules from crustacean hemocytes are essential in defence against invasive pathogens [37,44]. The data from recent studies also demonstrate that silencing of immune-relevant genes like anti-lipopolysaccharide factor (ALF) from crayfish hemocytes led to rapid infection of WSSV [45]. Therefore, immediate regeneration of hemocytes is critical for the survival of the animals against pathogenic intruders and hence the hematopoiesis, a process in which mature hemocytes are produced and subsequently released from the hematopoietic tissue, is therefore an important process during an infection with a microorganism.

So far, little is known about hematopoiesis under a viral infection in crustaceans. A previous study showed that the hematopoietic tissue (Hpt) of crayfish is the organ in which proliferation of hemocytes occurs. Injection of a β-1,3-glucan caused a severe loss of hemocytes followed by a rapid recovery due to release from the Hpt organ [46]. This study also indicated that the hemocytes were synthesized and partly differentiated in the Hpt, but the final differentiation into functional hemocytes was not completed until they were released into the circulation. Hematopoiesis is tightly

regulated by the lineage restricted activity of various factors that promote cell diversification [47–49]. The hematopoietic tissue has been studied in several crustaceans, but the mechanisms for the release of blood cells into circulation in crustaceans are still unclear. The identification of three different proteins/transcripts can be used as markers/indicators for hematopoietic cell proliferation and specific differentiation into SGCs or GCs. Thus, the study of hemocyte lineage marker proteins would be useful for future investigations of hematopoiesis under normal or microbial challenge conditions in crustaceans [50].

4. Antiviral immune responses

Viral components like genomic DNA and RNA or dsRNA generated in virally infected cells can be sensed by host pattern recognition receptors (PRRs). After recognition, PRRs trigger effective and appropriate antiviral responses, including production of various cytokines and induction of inflammatory and adaptive immune reactions [51]. The molecular mechanisms that underlie the majority of crustacean antiviral immune responses are still unknown and are only starting to be addressed. So far, few host cellular genes/proteins involved in TSV and YHV pathogenesis have been studied. An increasing number of immune responsive genes/proteins involved in WSSV pathogenesis have been described in shrimp and crayfish. Here we summarize the major progresses made in anti-WSSV response in shrimp, crayfish, and other crustaceans.

4.1. Antiviral-related proteins/genes in crustaceans

To find antiviral substances, the substances have been isolated from tissue extracts of shrimp, blue crab, and crayfish. These extracts can bind to various DNA and RNA viruses such as Sindbis virus, vaccinia virus, vesicular stomatitis virus, mengovirus, Banzi virus, and poliomyelitis virus. The mechanism of this inhibitory activity remains unclear [52]. Recently, many studies using different techniques have been carried out on host-WSSV interactions in crustaceans [45,53–56]. It has been reported that virus-inhibiting proteins could be produced and some genes were up-regulated upon viral infection in crustaceans [52,57–61]. Genes induced by viral infections and genes whose expression are associated with the ability of shrimp to survive from viral infections have been reported but their importance to produce antiviral substances is little known (Table 1).

A well-known cationic protein, the anti-lipopolysaccharide factor (ALF) originally isolated from the horseshoe crab *Limulus polyphemus* [62,63] has been well studied in crustaceans for its antibacterial activity [64,65]. Interestingly, the crayfish ALF was up-regulated by a WSSV challenge and was shown to be involved in antiviral response against WSSV. Silencing of ALF specially resulted in higher rates of WSSV propagation both in the animals and in a cell culture of Hpt from crayfish. In contrast, enhanced expression of ALF in the animals by the administration of UV-treated WSSV led to lower viral replication and a partial protection against a subsequent challenge with the active virus [45]. ALF from shrimp was also up-regulated in *Litopenaeus vannamei* upon WSSV challenge [66]. Silencing of *Lv*ALF1 resulted in a significant increase of mortality in *L. vannamei* challenged by *Vibrio penaeicida* and *Fusarium oxysporum* indicating that *Lv*ALF1 has a role in protecting shrimp from both bacterial and fungal infections, but not to a WSSV infection even if it was up-regulated by WSSV infection [65]. This is different from crayfish ALF which showed antiviral property both in vivo and in vitro [45]. The mechanism for the antiviral activity of crayfish ALF is still unknown. Further studies have shown that recombinant ALF*Pm*3, from shrimp *P. monodon* affected the viral infection by inhibiting the attachment of virus to the crayfish Hpt cells suggesting that ALF may act during the initial stages of virus entry to host cells (unpublished data). The mechanism of this inhibition still needs further investigations. A gene named as *Pm*AV was found to be up-regulated in virus resistant shrimp and the *Pm*AV protein has a C-type lectin-like domain (CTLD). Recombinant *Pm*AV protein displayed a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cells in vitro. Further experiments showed that *Pm*AV did not bind to the WSSV implying that the antiviral mechanism of this protein was not due to inhibition of the attachment of virus to target host cell [67]. A novel C-type lectin (*Lv*CTL1) showed strong affinity to WSSV and interacted with several envelope proteins of WSSV. Binding of recombinant *Lv*CTL1 to WSSV could protect the shrimp from viral infection and could delay the survival of shrimps against WSSV infection [68]. Besides, a beta-integrin was found to interact with a WSSV envelope protein VP187 containing the RGD motif. Soluble integrin, integrin-specific antibody and an RGD containing peptide could block the WSSV infection in vivo and in vitro. Silencing of beta-integrin efficiently inhibited the virus infection. These data suggest that this beta-integrin may function as a cellular receptor for WSSV infection [69]. Injection of recombinant shrimp fortilin followed by WSSV challenge resulted in decreased viral infection by an unknown mechanism in *P. monodon* [70]. A syntenin and its protein partner alpha-2-macroglobulin co-precipitated with each other and both of them were up-regulated in the acute-phase of a WSSV infection [71]. Hemocyanin, the respiratory protein of arthropods and molluscs, was found to exhibit non-specific antiviral properties [72] and to delay the infection of WSSV in vivo in *P. japonicus* [73]. Other host proteins like a chitin-binding protein [74] and actin microfilaments have been shown to interact with WSSV component proteins [75]. Some other genes have been found to be up-regulated as a response to WSSV infection in the animals, but no mechanistic studies have been performed. These genes include a lipopolysaccharide and beta-1,3-glucan binding protein gene [58], a C-type lectin [76], a catalase gene [77], a Ras-related nuclear protein (Ran protein) gene [78], a caspase-3 like gene [79], calreticulin [80], a Rab GTPase gene [81], manganese superoxide dismutase [82], *Fc*lectin [83], and a syntenin-like protein gene (Table 1) [84]. The

Table 1
Proteins/genes involved in anti-WSSV responses or interacting with WSSV in crustaceans.

Proteins/genes involved in anti-WSSV or interacting with WSSV	Species
Actin	*Litopenaeus vannamei* [75]
ALF	*Pacifastacus leniusculus* [45]
Beta-integrin	*Marsupenaeus japonicus* [69]
Calreticulin	*Fenneropenaeus chinensis* [80]
Caspase-3 like gene	*Penaeus monodon* [79]
C-type lectin	*Litopenaeus vannamei* [76]
C-type lectin (LvCTL1)	*Litopenaeus vannamei* [68]
*Fc*Lectin	*Fenneropenaeus chinensis* [83]
Fortilin	*Penaeus monodon* [70]
Hemocyanin	*Marsupenaeus japonicus* [73]
	Penaeus monodon [72]
LGBP	*Penaeus stylirostris* [58]
Manganese superoxide dismutase	*Fenneropenaeus chinensis* [82]
*Pm*AV	*Penaeus monodon* [67]
*Pm*CBP	*Penaeus monodon* [74]
*Pm*Rab7	*Penaeus monodon* [30]
Rab GTPase	*Marsupenaeus japonicus* [81]
Ran protein	*Marsupenaeus japonicus* [78]
Syntenin	*Penaeus monodon* [71]
Syntenin-like protein gene	*Penaeus monodon* [84]

functional characterization of these genes/proteins will bring interesting insights into the antiviral defence in crustaceans.

4.2. Antiviral activity induced by antibody or immune stimulants

WSSV proteins have been studied as possible immune therapeutic agents to prevent WSSV infections. For instance, a number of WSSV envelope proteins, such as VP28, have been proposed to be involved in viral infectivity based on the ability of specific antibodies to attenuate WSSV-induced mortality in vivo. When injected intramuscularly or administered orally with VP28, the shrimps obtained a higher and prolonged survival rates after WSSV challenge [85,86]. Different protein/DNA vaccinations against WSSV infection were reported for the protection of shrimp/crayfish [87–94]. Other antibodies against WSSV envelope proteins, such as VP68, VP281 and VP466 were also shown to reduce and delay the mortality of shrimp challenged with WSSV [95]. However, strong inactivation of WSSV by some normal rabbit sera was observed in a manner independent of anti-VP28 antibodies questioning the potential of anti-VP28 preimmune antibodies to specifically neutralize WSSV [96]. Thus, further investigations are necessary for the availability of these antibodies against different viral proteins. Preincubation of WSSV with mature synthetic mytilin (a synthetic antibacterial peptide from *Mytilus galloprovincialis*) significantly reduced shrimp mortality. This production was suggested to be caused by the interaction between the peptide and virus, but no mechanism was present [97].

Some other immune stimulants have also been described such as: glucans derived from yeast [98,99], LPS from bacteria [100], inactivated viruses [101,102], and dsRNA [103–106]. Among these immune stimulants, viral proteins and dsRNA might be of particular interest for further investigations, because as virus-associated molecules they are likely to be the targets of immune recognition in the context of natural viral infections [107]. Recently, grossly normal shrimp has been found sometimes to be infected with one or more viruses [108,109]. A viral accommodation concept has been introduced to the shrimp–virus interaction which provides more clues for a better understanding of the crustacean–virus interaction [110].

4.3. Cytokine activation mediated antiviral response

In vertebrates, most cell types respond to invading viral infection by rapidly releasing antiviral cytokines such as interferon alpha/beta (IFN-α/β). IFN-α/β is able to trigger activation of multiple noncytolytic intracellular antiviral pathways that can interfere with many steps in the life cycles of virus, thereby limiting the amplification and speed of the virus and attenuating the infection [111]. After the cells are activated by IFNs, signal transduction through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) system will induce the expression of hundreds of genes [112,113]. The widely studied type I IFN-induced genes are the dsRNA-activated serine/threonine protein kinase (PKR), the myxovirus-resistance (Mx) proteins, oligoadenylate synthetase, RNaseL, RNA-specific adenosine deaminase ADAR and IFNs themselves [114]. These self-amplifying systems can be triggered not only by IFN, but also directly by viral components. Cytokine activation through JAK/STAT pathway of a number of genes has been suggested in countering viral infection in *Drosophila* [115]. Flies deficient in the JAK kinase Hopscotch show increased susceptibility to *Drosophila* C virus and contain a higher viral load. These data indicate that flies produce antiviral molecules in a JAK-STAT-dependent way [116]. The JAK-STAT signaling pathways are also considered as a part of the antiviral response in mosquitoes [117].

The WSSV immediate early gene (*ie1*) was shown to employ a shrimp STAT as a transcription factor to enhance its expression and this resulted in high promoter activity in the host cells (Fig. 4) [118]. A further study showed that shrimp STAT was activated in response to WSSV infection and the WSSV does not disrupt JAK-STAT pathway but benefits from STAT activation in the shrimp [119]. WSSV ie1 protein also exhibits transactivation, dimerization, and DNA binding activity. This might provide more insights into the mechanism of how the replication of WSSV is manipulated in the host cells [120]. In addition, some components of the Toll pathway (Toll and Dif) have also been shown to be of importance for the resistance against *Drosophila* X virus [121]. But although a few Toll receptors have been found in shrimp [122,123], their importance in any innate immune reactions in crustacean is totally unknown.

4.4. Apoptosis

Apoptosis is a critical cellular process for removing unnecessary or potentially harmful cells and for development [124]. Apoptosis is also regulated as an innate cellular response to limit virus production and also decrease or eliminate spread of progeny virus in the host [125]. Many viruses have evolved genes encoding proteins to effectively suppress or delay apoptosis for producing sufficient quantities of progeny. For instance, *Baculovirus* contains p35 and inhibitor of apoptosis proteins [126] which can inhibit multiple caspases [127–129]. Besides, an increasing number of viruses are shown to induce apoptosis actively at late stages of infection. This process may function as a final and key step in the spread of progeny virus to neighbouring cells and also to evade from host immune inflammatory responses and protect progeny virus from enzymes and antibodies [130]. Increasing numbers of cells showed DNA fragmentation as the animals approach death following WSSV or YHV infections, suggesting that apoptosis occurs after infections by WSSV [43,131,132] or YHV [133] in shrimp. Caspases are central effectors in apoptosis [134] and if in a shrimp, *Marsupenaeus japonicus*, the *Pjcaspase* gene was silenced, the WSSV-induced apoptosis was significantly inhibited and this resulted in an increase of viral copies, indicating that apoptosis may

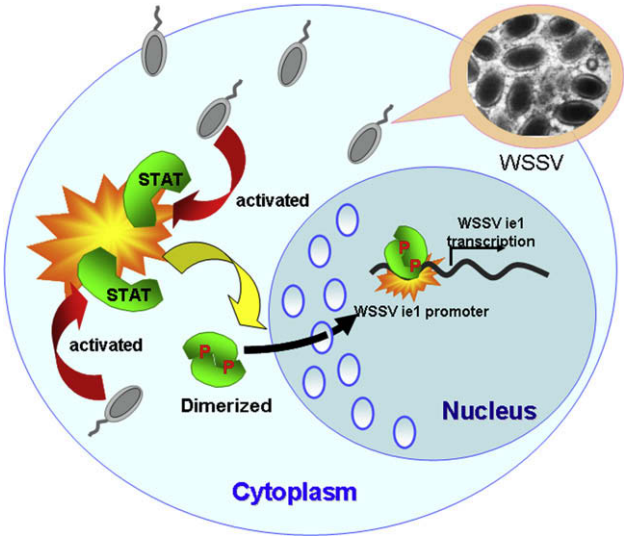

Fig. 4. STAT–WSSV interaction based on Lo's work [118]. This model is modified from Arbouzova et al. [182]. This model suggests that the host STAT can be activated after WSSV invasion. Activated STAT will dimerize and be phosphorylated, which then enters the nucleus and activates the promoter of WSSV ie1 gene for viral transcription.

play a role in antiviral processes of shrimp [135]. This proposal however needs to be ascertained by more experiments.

The percentage of apoptotic hemocytes in WSSV-infected crayfish was very low, but it was significantly higher than that in the sham-injected crayfish on day 3 or 5 post-infection [35]. Apoptosis has been considered to constitute a defence responsible for eliminating virus in naturally TSV infected shrimp [136]. However, other studies [132,137] suggest that apoptosis cannot be taken as an effective protection against WSSV in shrimp. For example by knocking down caspase-3 by RNAi this reduces mortality in Pacific white shrimp challenged with a low dose of WSSV but not if a high-dose of WSSV is used. This suggests that apoptosis may increase rather than decrease mortality in WSSV-challenged shrimp [138]. Another study indicates that shrimp with gross signs of WSSV infection from shrimp farms exhibited up to 40% apoptotic cells, and it was suggested that apoptosis might be implicated in shrimp death [132]. Similarly, the widespread and progressive occurrence of apoptosis in *P. monodon* infected with YHV is a major cause of dysfunction and death of the host. YHV-infected cells show the signs of viral-triggered apoptosis like nuclear pyknosis and karyorrhexis [133]. The expression of ribophorin I was up-regulated and remained high until the moribund stage in YHV-infected shrimp [139]. Ribophorin I belongs to the oligosaccharyltransferase (OST) complex involved in apoptosis [140]. Whereas, the transcriptional level of defender against apoptotic death 1 (DAD1), a negative regulator of apoptosis [141], decreased dramatically after YHV challenge in *P. monodon*, suggesting that DAD1 plays a role in mortality caused by YHV [142]. A shrimp translationally controlled tumor protein (TCTP) is an anti-apoptotic protein which has been implied to play a key role in shrimp defence responses or in control over viral-triggered apoptosis [143]. Whereas a hepatopancreatic nuclease is shown unlikely to be involved in viral-triggered apoptosis in shrimp [144]. The DNA fragmentation for WSSV-infected shrimp is the possible result of WSSV nuclease activity rather than shrimp nuclease activity. WSSV nuclease was identified and clearly shown to be expressed in the hepatopancreas of WSSV-infected shrimp [145]. However, signs of endogenous apoptosis have been shown in the shrimp lymphoid organ without known viral infections [133,136]. Thus, still quite a lot of controversy remains for the role of apoptosis in antiviral responses in crustaceans and this may be due to different species used for the assays, the big variations of animals, different experimental manipulations, etc. Besides, apoptosis is a complex process involving the interaction of many proteins. Few of these proteins have been characterized from crustaceans. Therefore, studies are still wanted of the process and the role of apoptosis in the antiviral response in crustaceans.

4.5. Antiviral activity induced by RNA interference or injection of dsRNA

RNA interference (RNAi) is triggered by dsRNA processed into shorter 21–25 bp small interfering RNAs (siRNA) by the type III endonuclease Dicer [146]. The siRNA are then incorporated into the RNA-induced silencing complex (RISC) [147,148], which facilitates the binding of the siRNA to the homologous mRNA upon which the targeted mRNA will be then degraded. RNAi has been proven to be a natural antiviral mechanism in plants [149], fruit flies [150–153], mosquitoes [154], nematodes [155,156], and mammalian cells [157–159]. The RNAi technique is now explored as an alternative and more specific approach to counteract virus infections in shrimps. Injection of dsRNA/siRNA specific to viral genes can block viral disease progression. This effect has been confirmed with different unrelated viruses. For instance, viral replication was efficiently suppressed with injection of WSSV-specific dsRNA/siRNA targeting VP19, VP28, VP281, or WSSV protein kinase in penaeid shrimp [103,160,161]. Lower YHV replication was observed in shrimp primary cell cultures by transfecting the cells with dsRNA targeted to the viral nonstructural genes [162]. Inhibition of YHV replication by cognate dsRNA significantly resulted in lower mortality in the black tiger shrimp [105,163]. All these data strongly imply the important role of RNAi in antiviral protection, which might also offer applied practice in shrimp culture in the near future.

On the other hand, dsRNA is a potent inducer of the IFN response in vertebrates. It is a molecule that often forms during viral infection as a result of viral genomic replication and viral RNAs with extensive secondary structure [164]. In mammals, dsRNA is recognized by TLR3, which activates myeloid differentiation factor 88 (Myd88)-dependent and an independent signal transduction cascade, resulting in expression of IFN-β [165,166]. DsRNA can also induce antiviral responses intracellularly by directly activating PKR, which results in inhibition of cellular and viral protein synthesis via phosphorylation of eukaryotic translation initiation factor 2 α (eIF2α) [167]. Further, some of the components of the pro-apoptotic pathways (e.g. PKR, RNaseL, IFN regulatory factor 3, and c-Jun N-terminal kinase) can be activated by dsRNA [168]. Hence, dsRNA is involved in both signaling viral infection and inducing apoptosis. It has been generally agreed that these dsRNA-induced IFNs are lacking in invertebrates [169–171]. Recent studies reveal the existence of both innate (non-sequence specific) and RNAi related (sequence specific) antiviral phenomena in a crustacean model [103,104,107,172]. But the protection induced by dsRNA could be overwhelmed by a higher dose (8-fold) of infectious virus, which means that sequence independent dsRNA induces quite a low induction of resistance to virus infection. The mechanism of this difference between higher and lower amount of infectious virus remains unknown. The protective efficiency for WSSV infection by specific dsRNAs is also varied between different viral genes targeted and no explanations have been addressed for these differences in this study. Other studies have shown that viral RNAs exhibit different sensitivities to RNAi attack [173]. This may results from protective proteins that bind to the viral RNA, or protective RNA structure because the viral RNA presents in a virus particle or a subcellular compartment that is not accessible to the RNAi machinery [173]. It remains unclear whether the antiviral protection by virus-specific long dsRNA is the result of RNAi mechanism alone or the combination of innate immune activation and RNAi in shrimp. Further, the readout from these studies was mainly based on the cumulative mortalities and no mechanism so far is available for these antiviral phenomena. Importantly, these studies suggest a possible evolutionary link (recognition of dsRNA) between innate antiviral immunity in invertebrates and vertebrates (Fig. 5). DsRNAs have been implied as important regulators for gene expression [174,175]. The distinction between self and non-self dsRNA is suggested by different structural features including 5′ triphosphate, 3′ protruding end or other specific motifs. Following the identification of RNA helicases, retinoic acid inducible gene-I, melanoma differentiation associated gene-5, and TLRs 7/8, more details will be elucidated on how different dsRNAs are selectively recognized by different sensors of the innate immune responses [168]. Based on the antiviral activity induced by dsRNA in shrimp, it would be also interesting to elucidate the possible role, potency and application of dsRNA in antiviral immunity in crustaceans.

5. Concluding remarks

Crustaceans (e.g. crayfish, crabs, lobster and shrimp) mount strong innate immune responses against microbes such as bacteria,

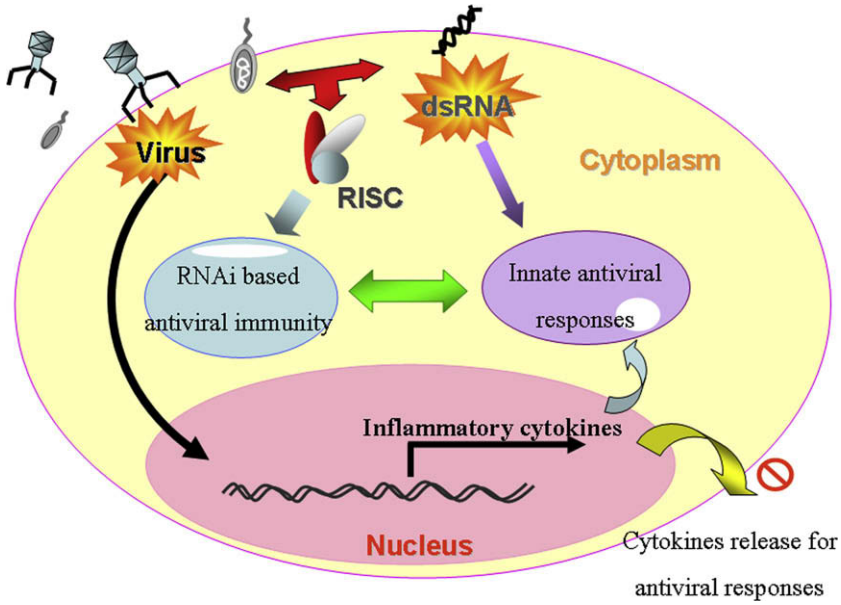

Fig. 5. A hypothetical scheme for dsRNA-induced antiviral immunity in crustacean (modified from Robalino et al. 2007) [107]. Viral dsRNA or extracellular dsRNA enters the RNAi pathway, and/or activates innate antiviral responses directly or indirectly. Signal transduction triggered by dsRNA recognition may regulate transcriptomic expression leading to antiviral reactions. These pathways may function independently as well as interact together to facilitate the antiviral responses.

fungi, and viruses. Studies on the mechanism of these responses have been hampered by absence of genome, tools for genetic manipulation and mutants, and stable long-term cell lines for in vitro studies. We have succeeded in developing an Hpt cell culture from crayfish, which can be a useful tool for gene functional studies in crustaceans [46]. This Hpt cultures can also be used to replicate WSSV and to study host–virus interactions (Fig. 4) [176]. A novel dsRNA mediated RNAi technology has been developed for crustacean cells [177], which is useful for antiviral studies in crustaceans.

RNAi is a powerful tool for antiviral responses [173]. Compared to other pharmacological interventions, RNAi is an attractive antiviral therapeutic because it allows interference with the target gene in a highly sequence-specific manner, and therefore essential viral genes can be targeted by design with little or no risk of unexpected off-target effects. Recent studies have demonstrated that the viral disease progression can be blocked by injecting shrimp with dsRNA/siRNA specific to viral genes. This strategy is effective against three unrelated viruses: WSSV, TSV and YHV [104,105]. The mechanism for this phenomenon is still not clear. In these studies, positive strand RNA virus (e.g. TSV and YHV) and DNA virus (e.g. WSSV) often induce the formation of dsRNAs during their infectious cycles such as genomic replicative intermediates, intramolecular interactions within viral transcripts, and bi-directional transcription [178]. And these dsRNAs might engage the shrimp RNAi pathway, resulting in effective antiviral responses. Thus it will be of interest to investigate whether viral dsRNA accumulates in crustacean cells infected with WSSV, TSV, or YHV. Two components of RNA silencing, Argonaute and Dicer-1, have been characterized from *P. monodon* [179–181]. Based on the studies in crustaceans above, plants [149], fruit flies [150–152], mosquitoes [154], and nematodes [155,156], it is likely that RNAi indeed exists as a natural antiviral mechanism in several organisms including crustaceans. These studies and further investigations of genes involved in RNAi in crustaceans should provide the possibility by reverse-genetic approaches to test this hypothesis directly.

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