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Knocking down caspase-3 by RNAi reduces mortality in Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* challenged with a low dose of white-spot syndrome virus

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Abstract Apoptosis has long been observed in viral target organs of white-spot syndrome virus (WSSV)-infected shrimp and whether the phenomenon helps the shrimp to survive the infection or is a factor leading to mortality is still controversial. If the shrimp mortality is a result of triggered apoptosis, then inactivation of caspase-3, a key protein in the induction of apoptosis, should improve shrimp survival upon challenge with WSSV. To test this prediction, we identified and characterized a caspase-3 homologue (*cap-3*) from the Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* and used this information to silence *cap-3* expression by RNA interference prior to WSSV challenge. After confirming the efficacy of *cap-3* silencing, its effects on mortality at high and low doses of WSSV were evaluated. In a high-dose WSSV challenge, *cap-3* silencing had no significant effect on WSSV-induced mortality, except for a delay in mean time to death. However, at a low-dose WSSV challenge, *cap-3* silencing correlated with a lower level of cumulative mortality, relative to silencing of a control gene, suggesting that apoptosis may exacerbate rather than decrease mortality in WSSV-challenged shrimp.

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Introduction

White-spot syndrome virus (WSSV) is the causative agent of white-spot disease (WSD), which is a major cause of mass mortality in the shrimp farming industry today. The disease was first reported in China in 1993 [1,2] in the marine shrimp *Penaeus* (*Fenneropenaeus*) *chinensis* and has spread worldwide to all shrimp species including *Penaeus monodon* and *Penaeus* (*Litopenaeus*) *vannamei*, the two most cultured species. Histopathological features of WSSV-infected shrimp at the early stage of infection are nuclear hypertrophy, chromatin margination, and apoptosis of cells of ectodermal and mesodermal origin [3—5].

Apoptosis has been shown to play a critical role in vertebrate defense against viral pathogens [6,7]. Although apoptosis may suppress viral replication in some infected cells, other viruses can grow significantly in cells undergoing apoptosis [6—8]. In insects (which lack adaptive immunity), apoptosis is also reported to be extremely powerful in limiting viral replication, infectivity, and spread, through mechanisms that involve the premature lysis of infected cells [9,10]. In crustaceans, the occurrence of apoptosis upon viral infection has long been observed. Upon WSSV infection, apoptosis has been detected in several viral target tissues of shrimp, and the level of apoptosis seems to increase as WSD progresses towards shrimp death [3,5,11]. Several studies have investigated the changes in the level of apoptosis-related gene expression in WSSV-infected shrimp. So far, one down-regulated anti-apoptosis factor and several other up-regulated apoptosis factors have been detected [12—14]. It has been hypothesized in the viral accommodation theory that viral triggered apoptosis may be a major cause of mortality and that reduced rates of cell death may allow for attenuation of viral pathogenicity in shrimp [15,16]. Despite these findings, there is still no clear conclusion as to the relative contribution of apoptosis to viral pathogenicity and/or antiviral immune responses in shrimp.

Caspases are cysteine proteases that bring about most of the morphological changes that are collectively characterized as apoptosis. Elimination of caspase activities can slow down or even prevent those changes [17]. Phongdara *et al*. [12] discovered for the first time the caspase gene, *cap-3*, in the cultured banana shrimp, *Penaeus merguiensis*. This newly-found shrimp caspase gene is believed to function like human caspase-3, which is one of the key executioners of the apoptotic process. In mammals, the crucial task of caspase-3 in the programmed cell death process (both in the extrinsic and mitochondrial pathways) has been studied with the generation of caspase-3 deficient mice and caspase-3 mutated/deleted cell lines [18—21]. Collectively, these findings imply that, if apoptosis plays a role in the mortality of shrimp with WSD, then ablating *cap-3* should improve their survival.

Double-stranded RNA (dsRNA) injection induces in shrimp a classical RNAi-like effect, characterized by systemic down-regulation of endogenous gene expression in a sequence-specific manner [22]. Similarly, injection of dsRNA encoding gene sequences specifically encoding for essential viral proteins has been shown to block viral disease both *in vivo* and *in vitro* [23,24]. Double-stranded RNA is a common intermediate formed during the life cycle of many viruses, and in vertebrates it is a potent inducer of innate antiviral immunity. Similarly in shrimp, *in vivo* studies with *P. vannamei* showed that administration of dsRNA evoked limited innate antiviral immunity in a sequence-independent manner [25], a response that could be overwhelmed by a high-dose viral challenge. Here we take advantage of these distinct dsRNA-induced phenomena to determine the effect on shrimp survivability of *in vivo* knock-down of the *cap-3* gene of *P. vannamei* using its cognate dsRNA under both high- and low-dose WSSV challenge conditions.

Materials and methods

Shrimp and experimental viral infection

Shrimp were stocked and challenged as described previously [25,26]. Specific pathogen-free (SPF) *P. vannamei* (1 g BW) were stocked individually in 260-ml flasks and acclimatized for 2—3 days before experiments. After acclimatization, 37—43 shrimp each were treated with 5 μg of various dsRNA by intramuscular injection into the 2nd abdominal segment 2 days prior to high- or low-dose WSSV challenge by injection. Control shrimp were injected with SPF shrimp extract at an equivalent dilution. The injection volume of dsRNA and viral extract was 20 μl per shrimp. The dsRNAs used were *cap-3* dsRNA, complement subcomponents C1r-C1s/sea urchin protein Uegf/bone morphogenetic protein 1 (CUB) domain protein (CDP, GenBank accession no. AY907539) [24] dsRNA and VP19 dsRNA. High and low dose of WSSV was prepared by diluting the WSSV-containing extract 10^6 times and 24×10^6 times, respectively, with saline. The former caused 100% mortality while the latter caused approximately $80 \pm 5\%$ mortality. After WSSV injection, mortality was recorded daily over a period of 10—12 days. Hepatopancreas was collected into RNAlater (Qiagen) for total RNA isolation.

To ensure the knocking down of mRNA expression, *cap-3* dsRNA was injected into six shrimp in two separate occasions, with three shrimp in each occasion, and the *cap-3* expression was determined by RT-PCR.

Isolation of *P. vannamei cap-3* cDNA

Partial sequences of *P. vannamei cap-3* were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from the total RNA of normal and WSSV-infected shrimp, using primers that were designed from *P. merguiensis* (GenBank accession no. AY839873) [12] and *P. monodon cap-3* gene (GenBank accession no. DQ846887). The forward primers were CAP1F, 5′-AAG CTT GCT GCT CTC ATC TTC GCT CA-3′, and CAP2F, 5′-AAG CTT GTC TAG CCG GCA AAC CTA AG-3′, with a HindIII restriction site at the 5′end. The reverse primers were CAP1R, 5′-TCT AGA TCA GCC GTG AAG TTT AT C CA-3′, and CAP2R, 5′-TCT AGA CCA CTT CCC TGC TGA CTT T G-3′, with XbaI restriction site at the 5′end. To obtain the full length *cap-3* cDNA, 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE cDNA libraries (BD SMART™ RACE cDNA Amplification Kit; BD Biosciences Clontech) of hemocytes,