# A review of the strategies evolved by WSSV to thwart host responses to infection and ensure successful virus replication in cells - apoptosis and anti-apoptosis strategies

JIANN-HORNG LEU[1] and CHU-FANG LO[2]

[1] *Center for Marine Bioscience and Biotechnology, National Taiwan Ocean University No. 2, Pei-Ning Road, Keelung 20224, Taiwan, ROC*
[2] *Institute of Zoology, National Taiwan University, No.1, Sec. 4, Roosevelt Road Taipei 10617, Taiwan, ROC*

## ABSTRACT

During virus infection, premature cell death (*apoptosis*) blocks production of progeny virus. Some viruses have evolved ways to block apoptosis to ensure their replication, and some viruses can even induce apoptosis to assist their own dissemination. White spot syndrome virus (WSSV) infection induces apoptosis in shrimp, and the infected tissues display the characteristic signs of apoptosis, *i.e.* nuclear disassembly, fragmentation of DNA into a ladder, and increased caspase-3 activity. WSSV-induced apoptosis occurs in bystander, non-infected cells, whereas the infected cells are non-apoptotic. Although the factors that induce apoptosis are currently unknown, it has become clear that shrimps use apoptosis as a protective response to prevent the spread of WSSV. To counter this, WSSV is now known to produce at least two anti-apoptosis proteins that block apoptosis and thus facilitate viral multiplication.

**Key words:** apoptosis**,** anti-apoptosis**,** WSSV**,** shrimp

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Corresponding author: Grace Lo, gracelow@ntu.edu.tw

## WSSV-INDUCED APOPTOSIS: THE BEGINNING

Henderson and Stuck (1999) were first to report that apoptosis levels were increased in moribund *Penaeus* (*Litopenaeus*) *vannamei* shrimp infected with WSSV. In a subsequent report (Sahtout *et al*., 2001), *Penaeus monodon* shrimp from commercial cultivation ponds were diagnosed first through histological examinations and then PCR, and some of them were found to be WSSV-positive. All of the shrimp were then examined with TUNEL and agarose gel electrophoresis of DNA to detect the occurrence of apoptosis. Sahtout *et al*. (2001) found that in grossly normal shrimp negative for WSSV by PCR, no TUNEL-positive cells were detected. Conversely, shrimp showing gross signs of WSSV infection had TUNEL-positive cells, and the number of TUNEL-positive cells was proportionally related to the severity of the WSSV infection. Lightly infected shrimp (2-step PCR positive) gave mean counts of 16 ± 8% TUNEL-positive cells, whereas heavily infected shrimp (1-step PCR positive) had mean counts of 40 ± 7% TUNEL-positive cells. Among the examined tissues, abdominal epithelium had the highest level of TUNEL-positive cells, followed by the stomach epithelium, hepatopancreatic interstitial cells, gills and muscle. Another indication of apoptosis was that agarose gel analysis of DNA extracts from the WSSV-infected shrimp showed DNA smears or ladders. The authors suggested that apoptosis induced by WSSV in numerous tissues/organs might contribute to shrimp death.

Although the above study showed that shrimp naturally infected with WSSV contained numerous apoptotic cells, there remained the possibility that the observed apoptosis may have been due to other viral pathogens. Viral co-infection in shrimp is quite common, and it is hard to tell whether apoptosis in naturally infected shrimp is in fact induced by WSSV or by another viral pathogen. The possibility of this kind of confounding factor is especially problematic when there is no information about how long the shrimp have been infected or how soon after infection the apoptosis occurred. Two years after the Sahtout *et al*. (2001) study, Wongprasert *et al*. (2003) experimentally infected *P. monodon* shrimp and performed a time course study to evaluate apoptosis levels in various tissues. DAPI staining showed that hemocytes with condensed and fragmented nuclei, *i.e.* apoptotic cells, were first detected at 24 hpi, and their numbers increased significantly thereafter. At 60 hpi, 20% of the hemocytes were apoptotic. For other tissues, the subcuticular epithelium displayed the highest level of TUNEL-positive cells (10% at 36 hpi), and these were detected as early as 6 hpi. In gills and hematopoietic tissues, TUNEL-positive cells were first detected at 24 hpi and their numbers significantly increased by 36 hpi. The occurrence of apoptosis in various tissues was further confirmed by TEM. More importantly, TEM showed that apoptotic features such as chromatin margination and nuclear condensation and fragmentation only occurred in cells that contained no virions; in cells where WSSV virions were present, no signs of apoptosis were observed. This study also reported that caspase-3 activity of the subcuticular epithelium was 6-fold higher in WSSV-infected shrimp.

Both Sahtout *et al*. (2001) and Wongprasert *et al*. (2003) reported that some cells with hypertrophied nuclei (*i.e.* WSSV-infected cells), were TUNEL-positive. Under TEM, Wongpraset *et al* . 2003) confirmed that the chromatin in such cells was not condensed and

did not break up into apoptotic bodies. They proposed that either these cells followed a non-typical apoptotic pathway or that the fragmentation of the DNA was caused by an activity of WSSV that was unrelated to apoptosis. It now appears quite possible that WSSV intentionally degrades host DNA for its own benefit, as two WSSV proteins have recently been implicated in DNA degradation: a nuclease protein (Li *et al*., 2005) and a novel multifunctional protein called ICP11 are both able to promote DNA degradation (Wang *et al*., 2008).

The apoptosis response in WSSV-infected *P. vannamei* shrimp has also been investigated. The observations reported by (Granja *et al.,* 2003) were in general agreement with those for infected *P. monodon*. In both species, apoptosis occurs in WSSV-infected tissues, and the incidence of apoptotic cells increases with time. One discrepancy noted by Granja *et al.* (2003) was that their TUNEL-positive cells did not have hypertrophied nuclei, although this is a characteristic that would be expected in WSSV-infected cells. Granja *et al.* (2003) considered this unexpected result to be accurate because they used the less sensitive colorimetric TUNEL method, and this allowed the morphology of the cells to be observed under the light microscope, thus reducing the risk of misinterpretation and false positives.

Wu and Muroga (2004) used TUNEL to investigate apoptosis responses in the lymphoid organ (LO) and stomach epithelium of WSSV-infected kuruma shrimp. The shrimp were experimentally infected with high or low doses of WSSV. In LO, high infection doses produced higher numbers of apoptotic cells while the incidence of apoptotic cells decreased with the progress of WSSV infection. The highest number of apoptotic cells was observed at 12hpi (4.5%±3.8) and then decreased thereafter. Curiously, there were only very few TUNEL-positive cells in the stomach epithelium, regardless of whether the infection dose was high or low. Compared to *P. monodon* and *P. vannamei*, Kuruma shrimp (*Penaeus japonicus*) therefore seem to have different apoptosis responses toward WSSV infection. In this study, tissue sections adjacent to those used for TUNEL staining were also subjected to *in situ* hybridization (ISH) with a WSSV probe, and comparison of these neighboring sections showed that TUNEL-positive cells were ISH-negative, confirming that cells infected with WSSV were non-apoptotic, and that apoptotic cells had no WSSV.

A comparative study in hemocytes for WSSV-infected *P. monodon* and *Penaeus indicus* using flow cytometry showed that the number of apoptotic hemocytes was higher in *P. indicus* than in *P. monodon* (60% vs. 20%), and that in both species, the number of apoptotic hemocytes increased with time (Sahul Hameed *et al*., 2006). This suggested that *P. indicus* hemocytes are more sensitive to WSSV infection than *P. monodon* hemocytes.

There are relatively few studies of WSSV-induced apoptosis in crustaceans other than penaeid shrimp. In crayfish, WSSV infection induces hemocytes to undergo apoptosis, and the percentage of apoptotic hemocytes is quite low: 1.5% in crayfish (Jiravanichpaisal *et al*., 2006) vs. 20% in shrimp (Wongprasert *et al*., 2003).

Overall, based on these studies, we can conclude that WSSV infection induces apoptosis in crustaceans, and that the incidence of apoptosis is both species-specific and tissue-

specific, and is related to the severity of WSSV infection. Further, the apoptosis occurs only in uninfected by-stander cells, whereas WSSV-infected cells are non-apoptotic.

## WSSV ANTI-APOPTOSIS GENES

There is evidence that at least two WSSV proteins are involved in anti-apoptosis: ORF390 (we tentatively called this protein WSSV anti-apoptosis protein-1, AAP-1, and we use this name throughout this paper; Wang *et al*., 2004) and WSSV222 (He *et al*., 2006). The gene encoding AAP-1 was identified through a genetic complementation assay for rescuing the multiplication of a mutant insect virus in host cells. Figure 1 shows a schematic summary of this assay.

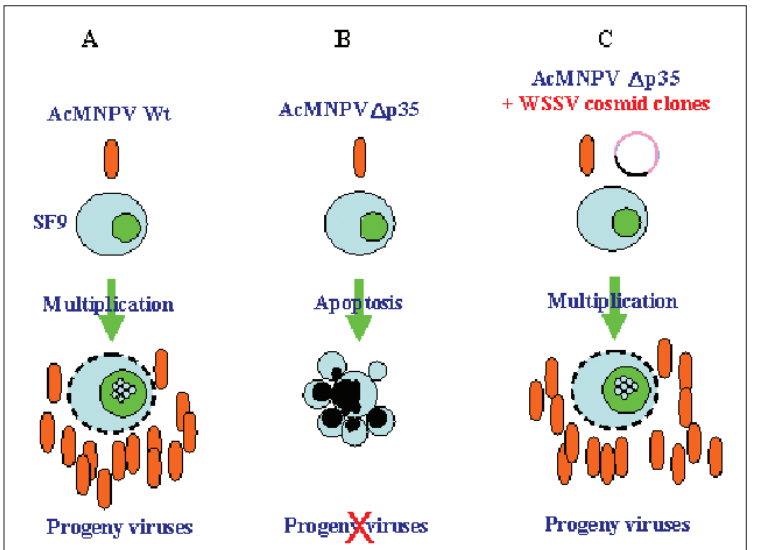


**Figure 1.** The genetic complementation assay that was used to identify the non-structural WSSV protein ORF390 as an anti-apoptotic Protein (AAP-1). A. The wild type AcMNPV has an anti-apoptosis gene that prevents apoptosis in the Sf9 host cells and thus allows the virus to replicate; B. When this anti-apoptosis gene –p35- is deleted, then apoptosis occurs in the Sf9 cells and the virus fails to replicate; C. If you co-infect with a WSSV cosmid and you find that apoptosis is prevented, then you know that the cosmid contains an anti-apoptotic gene.

Briefly, the insect virus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), contains an anti-apoptosis gene, *p35*, that is essential for the multiplication of AcMNPV in SF cells. Mutation/deletion of *p35* induces apoptosis during AcMNPV infection, and this severely impairs the production of progeny virus. However, if multiplication of the *p35*-mutated AcMNPV in SF cells is restored by overexpresssion of an unknown anti-apoptosis protein, then it follows that the candidate must be capable of blocking apoptosis. Based on this strategy, a WSSV cosmid library was constructed and the cosmid vectors were

co-transfected with *p35*-mutated AcMNPV DNA into SF cells to isolate WSSV genes that could restore the multiplication of *p35*-mutated AcMNPV. By repeating the experiments many times, WSSV anti-apoptosis protein AAP-1 was finally identified, and it appeared to function like AcMNPV P35 protein (Wang *et al*., 2004). In insect cells this protein blocked apoptosis induced by Actinomcyin D, *Drosophila* pro-apoptosis protein Rpr, and *P. monodon* caspase3 (Pm caspase3) (Wang *et al*., 2004; Leu *et al*., 2008a,b). The fact that AAP-1 could block apoptosis induced by different stimuli, suggests that it is acting at a conserved, central point in the apoptosis pathway. We recently (Leu *et al*., 2008b) showed that AAP-1 was both a substrate and inhibitor for Pm caspase-3, and that only cleaved APP1 directly bound to Pm caspase-3. Accordingly, we hypothesized that after AAP-1 is recognized and cleaved by caspase, it functions by directly binding to the active site of caspase, preventing the caspase from further accessing other substrates. AAP-1 contains a caspase-3 cleavage site, which seems to be responsible for both the cleavage by and also the inhibition of caspase. Our unpublished data shows that point mutation at this caspase-3 cleavage site abolishes the anti-apoptosis activity of AAP-1.

Another anti-apoptosis protein is WSSV222. This is one of the four WSSV proteins (the other three are WSV199, WSV249 and WSV403) that are predicted to have a RING-H2 finger motif. This motif is involved in ubiquitin-conjugating enzyme (E2)-dependent ubiquitination, and many proteins containing a RING finger play a key role in the ubiquitination pathway. He *et al*. (2006) first showed that WSSV222 had RING-H2-dependent E3 ligase activity *in vitro* and then used a yeast two-hybrid assay to identify its interaction partner, the tumor suppressor-like protein (TSL). They further showed that WSSV222 could ubiquitinate the TSL, and that the ubiquitinated TSL then underwent proteasome-dependent degradation in a mammalian cell line and in a primary shrimp tissue culture of shrimp cells infected with WSSV. Since transient expression of TSL in BHK mammalian cells caused apoptosis, and this could be blocked by coexpressing WSSV222 in the same cells, they hypothesized that in WSSV-infected shrimp, WSSV222 functions as an anti-apoptosis protein through ubiquitin-mediated degradation of TSL. By preventing apoptosis, WSSV222 thus facilitates the propagation of WSSV.

Although both AAP-1 and WSSV222 exhibit anti-apoptosis activity, they apparently act through different mechanisms. AAP-1 is a direct caspase inhibitor and it can evidently block apoptosis triggered by different stimuli, whereas WSSV222 only blocks apoptosis triggered by a specific but currently unknown protein(s) through ubiquitin-mediated degradation. We also note that WSSV222 is an early protein while AAP-1 is a late protein. Thus WSSV evidently deploys different anti-apoptotic strategies at different stages of infection.

## INHIBITION OF APOPTOSIS: PROS AND CONS FOR THE HOST AND VIRUS

As WSSV uses at least two different anti-apoptosis proteins and at least two different mechanisms to suppress the occurrence of apoptosis in infected cells, it can be inferred that the induced apoptosis must be an anti-viral response that is deleterious to the multiplication

of WSSV. But to what extent is apoptosis beneficial or detrimental to the shrimp host itself? At low levels, apoptosis can remove the infected cells without harm to the shrimp host, but when extensive apoptosis occurs, dysfunction or failure of the affected tissues/organs might lead to the demise of the shrimp. Therefore, apoptosis is a double-edged sword. Two studies have explored this question by manipulating the expression of caspase. Caspases are a group of structurally related cysteine proteases that play important role in apoptosis. Based on their functions and structural features, caspases are classified into two different groups, initiator and effector caspases. At least three different caspases have been cloned from penaeid shrimp: two effectors and one initiator (Phongdara *et al*., 2006; Leu *et al*., 2008b; Wang *et al*., 2008). An initiator caspase gene, *Pjcaspase*, was identified in *Masupenaeus japonicus* (Wang *et al*., 2008). This gene was upregulated in survivors of WSSV-challenged shrimp. To investigate the importance of this initiator caspase gene against WSSV infection, the authors silenced this gene through siRNA. In WSSV-infected shrimp, when *Pjcaspase* was down regulated, caspase-3 activity and the number of apoptotic hemocytes were both decreased and, conversely, the number of copies of WSSV in the hemolymph was increased. This paper demonstrated that apoptosis could indeed be regarded as a host anti-viral defense system, and that the down-regulation of an initiator caspase gene favored the multiplication of WSSV. However, this study did not investigate the extent to which the WSSV-induced apoptosis may have contributed to the death of the shrimp.

In contrast to the above report for an initiator caspase, when an effector caspase gene was silenced in WSSV-infected shrimp, the opposite result was produced. Rijiravanich *et al*. (2008) used dsRNA to suppress the expression of *P. vannamei* caspase-3 genes, and when the shrimp were challenged with a high dose of WSSV, there was no observed effect on shrimp mortality. On the other hand, when challenged with a low dose of WSSV, the caspase-3 dsRNA injection group exhibited a lower mortality (27%) compared to the nonspecific dsRNA injection group (52%). The authors therefore concluded that silencing this caspase-3 gene partially protected the shrimp against death induced by WSSV infection. However, the authors did not report whether the number of apoptotic cells, the caspase-3 activity or the number of WSSV copies were changed in the caspase-3 silenced shrimp. Currently, although the dsRNA-based gene silencing technique is widely used in shrimp, the underlying mechanism remains largely unknown. Even so, it is known that dsRNA produces non-specific anti-viral activity (Robalino *et al*., 2005; 2006), so it is quite possible that the protection effect of caspase-3 dsRNA was due to such non-specific activity. We also note that, as would be expected, the control dsRNA group showed increased protection against WSSV infection when compared to the WSSV-infected shrimp with no dsRNA injection, which had a mortality of 79% (Rijiravanich *et al.,* 2008).

The administration of an apoptosis inhibitor to shrimp is a more direct method to evaluate the importance of apoptosis to WSSV infection and shrimp death. Wang and Zhang (2008) experimentally injected shrimp with the apoptosis inhibitor z-VAD-FMK, and then infected the shrimp with WSSV. They found that the WSSV-infected shrimp with z-VAD-FMK had a lower number of apoptotic hemocytes, increased WSSV copies, and increased mortality.

Since the inhibition of apoptosis resulted in a reduction of the number of surviving shrimp, the authors concluded that the net effect of apoptosis is to protect the infected shrimp from demise, rather than to cause the shrimp to die.

The notion that apoptosis protects the WSSV-infected shrimp from mortality coincides with a study by Granja *et al*. (2003), which investigated the mechanism involved in the beneficial effect of hyperthermia for WSSV-infected *P. vannamei* shrimp. Hyperthermia (32°C) increased the survival of WSSV-infected shrimp. Compared to shrimp kept at normal temperature (25°C), *in situ* hybridization showed a decrease in the number of WSSV-positive cells. Conversely, the incidence of apoptotic cells increased under hyperthermic conditions. Granja *et al*. (2003) suggested that at 32°C, the increased incidence of apoptosis might be the mechanism by which the shrimp were able to avoid death.

## CONCLUSION

Some progress has been made toward the understanding of WSSV's anti-apoptosis mechanisms However, it remains unclear how WSSV induces apoptosis in by-stander cells. As for the host, compared to other model organisms, the study of the mechanisms and molecules involved in shrimp apoptosis is still in its infancy. Studies of vertebrate/virus interactions reveal that host cells have many cellular sensors that can detect the activities of an infecting virus and then transmit the pro-apoptosis signals to initiate cell death. These cellular sensors include death receptors, protein kinase R, mitochondrial membrane potential, p53 and the endoplasmic reticulum (Everett and McFadden, 1999). We hypothesize that detecting sensors should exist in shrimp cells as well. During the course of WSSV infection, these sensors could transmit signals, such as the binding of WSSV to its receptors, the transcription of WSSV genes or the replication of the WSSV genome, to trigger apoptosis. Before or during the execution of the apoptosis program, WSSV anti-apoptosis proteins such as WSSV222 and AAP-1 would begin to function and block further progress of apoptosis, so that WSSV could complete its replication cycle. In the case of apoptotic by-stander, cells, we speculate that these cells might have actually been infected with WSSV, but that for some reason either the two anti-apoptosis proteins do not function well or the pro-apoptotic signals are too strong to be blocked; consequently, apoptosis begins and the multiplication of WSSV is prevented.

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