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Transcriptional upregulation of fortilin in shrimp, *Penaeus* (*Metapenaeus*) *japonicus* fed diets containing recombinant VP28, an antigenic protein of white spot syndrome virus (WSSV)

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Abstract. White spot syndrome virus (WSSV) is considered as one of the serious viral pathogens of shrimp. There are several preventive measures that have been developed to curb the devastating effects of this virus in shrimp aquaculture. Juvenile shrimps, *Penaeus* (*Metapenaeus*) *japonicus* were fed with commercial feeds that were mixed with recombinant VP28, a structural protein antigen of WSSV for a period of 14 days. The immune response of the shrimp during oral administration of the medicated feed was determined by expression analysis of fortilin, a gene that is involved in the antiviral response. There was a significant increase in the level of expression of fortilin both in the gut and the gills in the fed group during the duration of feeding. The level of expression gradually decreased in the fed group, whereby at the 7th and 14th day after the last day of feeding with medicated feeds, no significant differences were observed between the fed and control groups in the expression at the gills and gut, respectively.
Key Words: shrimp, *Penaeus japonicus*, white spot syndrome virus, WSSV, VP28.

Introduction. White spot syndrome virus (WSSV) is the causative agent of a serious disease in penaeid shrimps worldwide. WSSV is a large, enveloped, rod- or elliptical-shaped dsDNA virus that has a unique tail-like extension at one end (Leu et al 2013) and is the type species of genus Whispoviridae in the Nimaviridae family (Van Hulten & Vlak 2004). This virus affects a broad range of crustaceans and causes severe mortalities resulting in huge economic loss (Lo et al 1996; Wang et al 1998). Among crustaceans, penaeid shrimps are highly susceptible to WSSV and could even lead to 100% mortality in the cultured stock within a few days following infection (Lightner 1996).

VP28 is one of the major envelope proteins of WSSV, and is also believed to have an important role in the initial steps during systemic infection of WSSV in shrimp (Van Hulten et al 2001). This has been used as a sub-unit vaccine against WSSV infection (Namikoshi et al 2004; Witteveldt et al 2004; Caipang et al 2008; Du et al 2013), and considered as one of the major immunogens of WSSV (Du et al 2013). Recombinant proteins of VP28 have been produced in various production systems and used to deliver to the shrimps orally with a high rate of survival following challenge with the virus (Namikoshi et al 2004; Witteveldt et al 2004; Caipang et al 2008; Fu et al 2010; Du et al 2013). It also stimulated the innate immune response of the shrimps particularly the production of iNOS (Fu et al 2010).

It is well known that shrimps lack a truly adaptive immune response system and they appear to rely on a variety of innate immune response systems, which act rapidly and efficiently recognize and destroy “non-self” materials (Lee & Söderhäll 2002). They can also recover from viral infection, and the hemolymph of survivors contain a humoral neutralizing factor that can counteract the virus (Wu & Muroga 2004). Recent studies have shown that a number of genes were differentially expressed in shrimps upon infection with WSSV (Rojtinnakorn et al 2002; Dhar et al 2003; He et al 2005; Pan et al

2005), and fortilin was one of those genes upregulated in the hemocytes of virus-resistant shrimp, *Penaeus japonicus* (He et al 2005).

Fortilin or the translationally controlled tumor protein (TCTP) is a highly conserved protein that is widely expressed in many species (Bangrak et al 2004; Hsu et al 2007). In shrimps, fortilin has been identified in *Penaeus monodon* (Bangrak et al 2004), *Fenneropenaeus chinensis* (Wang et al 2009) and *P. japonicus* (Chen et al 2009). Extensive studies on the functions of fortilin in *P. monodon* showed that it has a Ca^{++} binding domain (Tonganunt et al 2008), prevents cell death (Graidist et al 2006) and inhibits the expression of early and late genes of WSSV *in vitro* using an insect cell line (Nupan et al 2011). Injection of shrimps with recombinant fortilin resulted in high survival rates during challenge with WSSV and low levels of the virus present in the survivors (Tonganunt et al 2008). These results indicate that fortilin is an antiviral gene, which is one of the genes responsible in protecting the shrimps from WSSV infections.

The use of recombinant VP28 as an immunogen to stimulate the humoral factor and enhance the shrimp innate defense mechanisms is well studied. Its use as an immunostimulant has also resulted in higher survival rates of the shrimps upon viral challenge. However, there is no information as to what immune defenses are triggered in the gut, the tissues that are in immediate direct contact with the antigen during oral administration. Hence, in the present study we determined the expression of fortilin in the gut during oral administration of recombinant VP28 and also determined its expression at different time points after the antigen was administered. We also compared the expression of this gene in the gills to find out whether the expression of fortilin is systemic in the shrimps following oral administration of recombinant VP28.

Material and Method

Shrimp samples. Juvenile shrimps used in this study were obtained from a previous experiment (Caipang et al 2008). Five shrimps from the fed group were collected at 1, 3, 7 and 14 days during the feeding experiment. After this time, the shrimps were fed only with feeds without the recombinant VP28. The same number of shrimps was also collected from both groups at 1, 3, 7 and 14 days after the last feeding of the medicated feed. Five shrimps that were collected before the feeding experiment served as control of the study.

Gill tissues and the gut were aseptically excised from individual shrimp every sampling and placed in labeled microfuge tubes. These were immediately stored at -80^{o}C for subsequent extraction of total RNA.

Isolation of total RNA and cDNA synthesis. Total RNA was isolated from the gill and gut tissues of shrimp using TRIzol Reagent (Invitrogen). The tissues were homogenized and total RNA was subsequently isolated using chloroform and isopropanol following standard procedures according to the manufacturer's protocol. cDNA was generated from 10 μg of total RNA in a 25-μL reaction containing the following: 5 μL of 5x reaction buffer, 1 μL M-MMLV reverse transcriptase (Promega), 1 μL RNAsin (Toyobo), 2 μL of 0.1 M DTT, 1 μL oligo-dT primers (10 μg) (Proligo, Tokyo, Japan), 3 μL dNTP mix (10 mM) (Bioneer) and DEPC-treated water. The samples were heated to 70^{o}C for 5 min in order to dissociate secondary RNA structures, followed by incubation at 42^{o}C for 60 min. Reactions were terminated by heating for 5 min at 95^{o}C. The cDNA samples were normalized to a concentration of 100 μg mL^{-1} by the addition of 1x TE buffer and stored at -20^{o}C until use.

RT-PCR. A 292-bp fragment of *P. japonicus* fortilin gene was amplifed from the cDNA using the primers, PjfortFwd: GATGAGATGTTCACCGACACCTATA and PjfortRev: GCAAGATAATCCTTCTTGACTTGGA. The β-actin was used as control in all amplification reactions and was amplified using the primers developed by Rattanachai et al (2004). Two microliters of the normalized cDNA samples (100 μg mL^{-1} concentration) were used for PCR in a 25-μL reaction. Amplification conditions were as follows: initial denaturation at 95^{o}C for 3 min, followed by 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at

55°C for 30 sec, and extension at 72°C for 1 min, and a final elongation step at 72°C for 5 min.

PCR products (5 μL) were visualized on a 1.5% agarose gel stained with ethidium bromide using a densitometer (Atto, Tokyo, Japan). Semi-quantitative assessments of mRNA levels were determined by quantifying the intensity of each band of the PCR product using Image Gauge ver. 6 (Fuji, Tokyo, Japan). Relative expression of each mRNA transcript was derived by dividing it with the corresponding expression of β-actin.

Data analysis. The data are represented as mean±SD. Student's *t*-test for independent samples was used to compare the relative expression level of the gene at each particular time point with the value of the control. All significance levels were set at $p < 0.05$.

Results and Discussion. Juvenile shrimps that were fed with recombinant VP28, an antigenic protein of WSSV had increased expression of fortilin in the gills at different time points during oral administration of the medicated feeds (Figure 1). There was significant increase in the expression of the gene in the gills beginning the first day of feeding and this lasted until the 14th day of feeding. Monitoring of the level of expression after the last day of feeding (14th day), showed that the level of expression of the gene decreased but was still significantly higher at 1 and 3 days after the last day of feeding. Beginning the 7th day after the last day of feeding, no significant difference in the level of expression of fortilin in the gills was observed between the fed and the control groups.

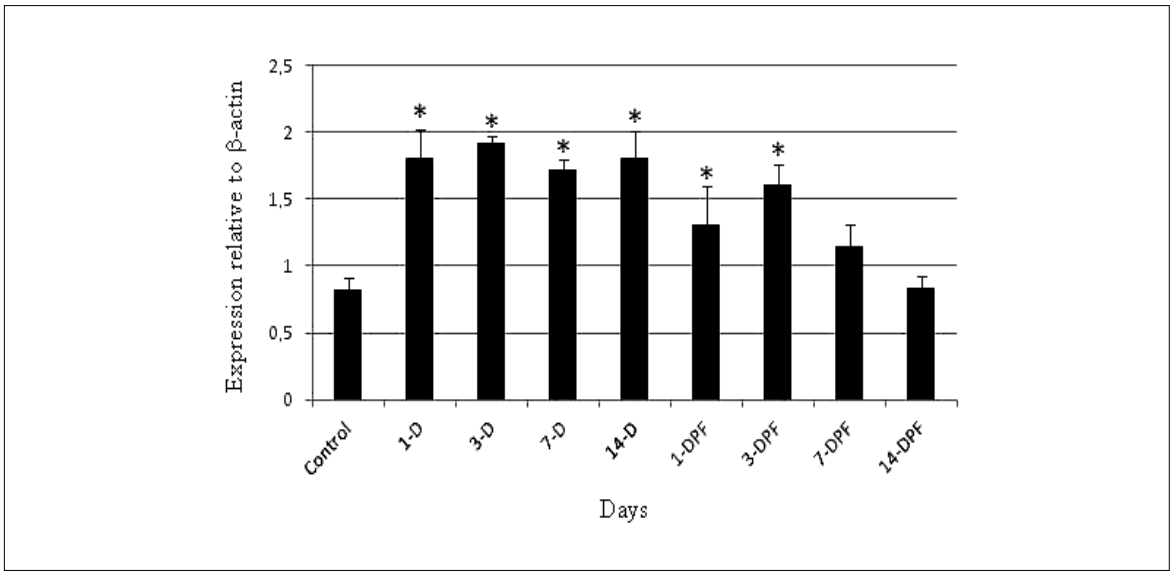

Figure 1. Relative expression levels of fortilin in the gills of *P. japonicus* during feeding (1-D, 3-D, 7-D and 14-D) and after the last day of feeding (1-DPF, 3-DPF, 7-DPF and 14-DPF) with medicated feeds. Column bars with asterisk indicate significant difference from the control at $p < 0.05$. N=5.

Figure 2 shows the expression profile of fortilin in the gut. During the entire duration of feeding the recombinant protein, there was a significant increase in the expression level in the gut of shrimps. At least a two-fold increase in the expression of this gene was observed. This was followed by a reduction in the expression levels in the succeeding days when administration of the medicated feed was stopped, although significant differences in the expression levels were still observed in the treated group versus the control. This lasted until the 7th day after the last day of feeding. On the 14th day, no significant differences were noted between the fed and the control group.

In the present study, the expression of fortilin in the gills and gut was used as an indicator of the immune response of juvenile shrimps upon oral administration of recombinant VP28. WSSV has five major structural proteins, and VP28, which is one of the major structural proteins showed potential as a vaccine candidate (Namikoshi et al 2004; Witteveldt et al 2004; Caipang et al 2008). This structural protein is used as a sub-unit vaccine because it can stimulate an innate immune response in the shrimp and

may have a role in providing protection against the disease (Fu et al 2010; Du et al 2013).

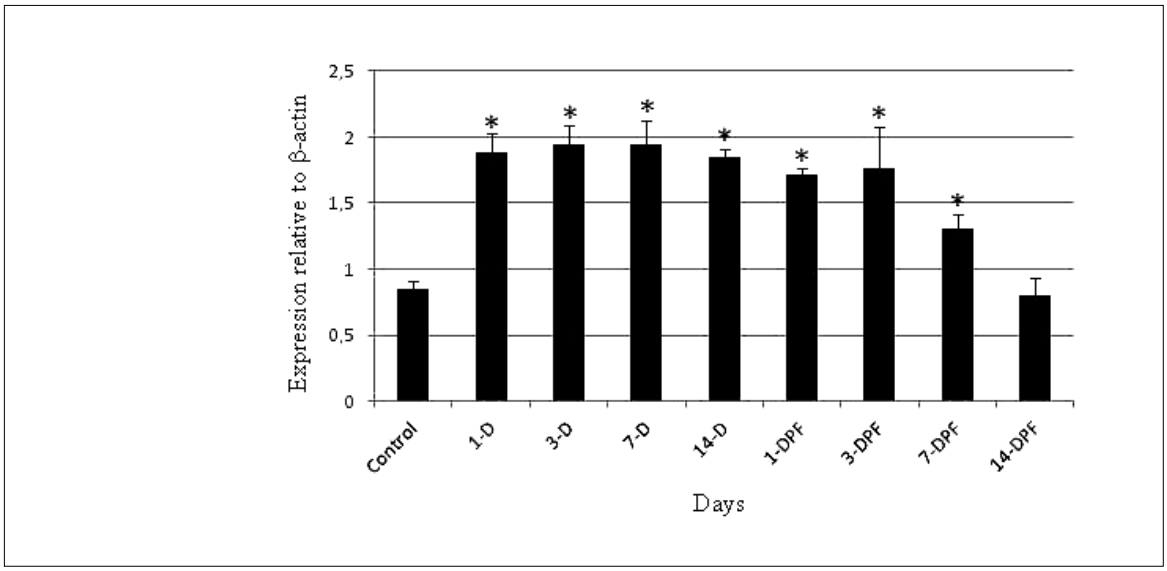

Figure 2. Relative expression levels of fortilin in the gut of *P. japonicus* during feeding (1-D, 3-D, 7-D and 14-D) and after the last day of feeding (1-DPF, 3-DPF, 7-DPF and 14-DPF) with medicated feeds. Column bars with asterisk indicate significant difference from the control at $p < 0.05$. N=5.

Fortilin has been found to be expressed in the hemocytes of WSSV-resistant *P. japonicus* (He et al 2005) and thus may have a role in the host during infection with the virus. As shown in another species of shrimp, *P. monodon*, this gene has calcium-binding activity (Bangrak et al 2004) and anti-apoptotic function (Graidist et al 2006). When *P. monodon* were infected with WSSV, there was a progressive increase in the expression of fortilin in the hemolymph during the course of infection, but decreased in moribund shrimp (Graidist et al 2006). Thus, it is not surprising that fortilin was expressed in the gills and gut during oral administration of a recombinant VP28.

The kinetics of the expression of fortilin in both the gills and the gut showed that there was a significant elevation in the expression levels in both tissues when the shrimps were fed with the recombinant protein. Regardless of the length of feeding with the recombinant protein, the expression was uniform in both tissues. This indicates that as long as cells come in contact with the antigen, they will synthesize immune-relevant molecules, such as fortilin as a response. This also implies that the length of exposure to the antigen does not correlate with a progressive increase in the production of an immune-related product. Rather, exposure to an antigen will lead to a significant increase in the response and the level is maintained as long as the antigen is present at the desired concentration. In this study, the shrimps were fed with the recombinant protein for two weeks. It will be interesting to determine in future studies whether the level can be maintained if the duration of exposure to the antigen is extended for more than two weeks. Another question that will be answered will be to find out whether the level of the response will decrease, which would likely indicate ”immune response fatigue or depression”.

The expression of fortilin in the gills and the gut were more or less similar during feeding with recombinant VP28. The high expression of this gene in the gut is expected because of the direct contact of the cells in the gut with the antigen, resulting in an immediate increase in expression as early as 1 day after feeding of the medicated feed. In an earlier study, Kulkarni et al (2013) demostrated that the receptors on the apical membrane of the shrimp enterocytes recognize recombinant VP28 efficiently. Once these receptors recognize the antigen, these will trigger a chain reaction leading to the upregulation of immune-relevant genes, as what was observed in the increased expression of fortilin in this study. Kulkarni et al (2013) also showed that the antigen can be translocated from the gut and comes in contact with the hemolymph, particularly at

the midgut. Once the antigen is in the hemolymph it can be circulated within the entire body. Because shrimps have an open circulatory system, the tissues and organs of the shrimp are directly exposed to the hemolymph and to the other substances that are dissolved in it including antigens. Thus, this likely explains that there was high expression of fortilin in the gills, which is also an immune-related organ. Another possibility is that the uneaten medicated feed could dissolve in the water, thereby releasing the antigen in the wáter column and could come in contact with the gills. This could also trigger the expression of fortilin.

After feeding the shrimps with the medicated feed for two weeks, the shrimps were then fed with the non-medicated commercial feed, and upon replacement of the medicated feed with the control diet, the expression of fortilin in both the gills and gut decreased until the levels of expression were no significantly different from the levels observed in the control. This indicates that there was gradual depletion of the antigen and consequently a decreased expression of fortilin. Interestingly, the level of expression in the gills returned to the pre-feeding level earlier than what was observed in the gut. This implies that the expression of fortilin as well as the rate of its decrease is likely affected by the amount of the antigen present. The gut likely contained more antigens than the gills considering that the former has a more direct contact with recombinant VP28. Thus, when the antigen is no longer added to the system, the gut will have more reserves of the antigen than the gills, and would therefore have a longer duration of significant expression of fortilin. This also indicates that fortilin is a component of the innate immune response in shrimps, and an enhanced expression of this gene in the immune-related organs of the shrimp even when there is no longer an exposure to the antigen does not mean an adaptive immunity but a quasi-immune response as proposed by Namikoshi et al (2004). This quasi-immune response has been implicated in the survival of shrimps after re-challenge with WSSV and fortilin could be involved in such response. Further studies are needed to establish the role of this gene in the prolonged innate response or quasi-immune response of the shrimp against WSSV infections.

Conclusions. In summary, we have shown that oral administration of recombinant VP28 for two weeks in juvenile shrimps resulted in the increased expression of fortilin in both the gills and the gut of the host, and regardless of the length of feeding with the recombinant protein, the expression was uniform in both tissues. When the feeding of the antigen was stopped and replaced by the control diet, the expression of fortilin in both tissues decreased and returned to the pre-feeding levels, with the expression in the gills returning earlier than in the gut. This indicates that fortilin has an involvement in the prolonged innate immune response or quasi-immune response in the shrimp and must be explored in future studies concerning resistance of the shrimp against WSSV infections.

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