Functional and global expression analyses of crustin-like peptide by RNA interference and microarray and identification of variants from EST information in kuruma shrimp Marsupenaeus japonicus.
FUNCTIONAL AND GLOBAL EXPRESSION ANALYSES OF CRUSTIN-LIKE PEPTIDE BY RNA INTERFERENCE AND MICROARRAY AND IDENTIFICATION OF VARIANTS FROM EST INFORMATION IN KURUMA SHRIMP Marsupenaeus japonicus

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Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
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## Table of contents

Acknowledgements .......... v

Abstract .......... vii

**Chapter I**

General Introduction .......... 1

**Chapter II**

Role of *Marsupenaeus japonicus* crustin-like peptide (MjCRS) against *Vibrio penaeicida* and white spot syndrome virus infection .......... 21

**Chapter III**

Identification of MjCRS variants from EST information in kuruma shrimp *Marsupenaeus japonicus* .......... 54

**Chapter IV**

Changes in global gene expression profile of kuruma shrimp *Marsupenaeus japonicus* associated with specific long dsRNA .......... 74

**Chapter V**

General Conclusion .......... 106
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ABSTRACT

World shrimp productions have increased in the past decades due to the rapid expansion of the cultured shrimp industry. However, development was seriously affected by outbreak of diseases. Therefore, understanding the shrimp’s immune system is important for the continuity of the industry. With the advent of RNA interference (RNAi) technology, utilization of EST information and application of microarray technology, more immune genes were identified. Using RNAi, this study aimed to determine the role of *Marsupenaeus japonicus* crustin-like peptide in shrimp immunity. It also aimed to identify crustin-like peptide variants from expressed sequence tag (EST) information. Lastly, with the application of microarray technology, the effects of crustin-like peptide silencing on global gene expression were determined.

Antimicrobial peptides (AMPs) are essential component of the shrimp’s innate immune system. One important AMP that has been identified in crustacean is crustin. In *M. japonicus*, crustin-like peptide (*MjCRS*) has been identified, however, its antimicrobial activity has not been elucidated. The role of *MjCRS* was examined *in vivo* by RNAi using double-stranded RNA (dsRNA). Tissue expression analysis revealed that *MjCRS* transcripts are highly expressed in hemocytes. Treatment with double-stranded RNA specific to *MjCRS* led to a significant reduction of *MjCRS* transcripts within the hemocytes. When *MjCRS* was silenced and subsequently infected with *Vibrio penaeicida* final mortality was significantly higher compared with control groups. On the other hand, final mortalities of *MjCRS* silenced and control group (PBS) were similar and are both significantly high. *V. penaeicida* infection significantly decreased *MjCRS* expression at 3, 6, 12 and 24 hours followed by significant increase at 48 hours.
and sustained until day-5 post-infection. On the contrary, white spot virus infection significantly increased MjCRS expression at 6 and 12 hours and decreased at 48 hours and diminished at day-5 post-infection. dsRNA treatment alone decreased total hemocyte counts (THCs) and subsequent V. penaeicida or white spot virus infection further decreased THCs. VP28 gene expression was both similarly and significantly increased in PBS treated and MjCRS silenced group at 24 and 48 hours post-infection. Results provided evidences on possible involvement of MjCRS in antibacterial defense. In addition, it also showed that MjCRS might not have critical function against viral infection.

EST databases have been developed in several crustacean species including shrimps. With EST information in the data bank, a variety of immune related genes have been identified. Of the immune related genes, multiple isoforms and types or variants have been reported to coexist. Using EST information from kuruma shrimp, we identified 44 sequences with match in the existing crustins. Sequences were subjected to phylogenetic analysis and the results showed 13 possible crustin variants. Comparison with existing crustins exhibits variable degrees of identity ranging from 37-100% and showed similarities with shrimps, crabs and lobster. Tissue expression analysis revealed different patterns of expression. Locus 1748 and 3 have similar pattern of expression with the previously identified MjCRS where they are ubiquitously expressed in different tissues. Locus 3886 (1), 16076, 11885, 5882, 5809 and 2365 are all highly expressed in gills. Locus 7363 was expressed in several tissues like hepatopancreas, intestines and stomach and Locus 3886 (2) was specifically expressed in hepatopancreas. Locus 11885 and 16076 were further studied and analyzed and renamed as MjCRS6 and MjCRS7. MjCRS6 and MjCRS7 sequence
analysis revealed predicted ORFs of 543 and 504 bp encoding a putative protein of 180 and 167 amino acid residues respectively. The MjCRS6 putative signal peptide cleavage is in between the 20th and 21st amino acid while MjCRS7 is in between 18th and 19th amino acid. Both MjCRS6 and MjCRS7 contained the conserved 12 cysteine-rich region and a WAP domain of 49 and 51 amino acids at the C-terminal end respectively. In this study, two new MjCRS variants were identified containing conserved crustin domains. Further studies are needed to determine their possible immune function.

dsRNA is a potent regulator of gene expression. The injection of dsRNA with specific target leads to the degradation of targeted mRNA. However, there are evidences that dsRNAs could affect global gene expression. Here, we employed RNAi and microarray technology to gain further insights on the effect of specific silencing on global gene expression in kuruma shrimp. The components that were affected at day-2 post treatment were dissected and the possible causes in global gene expression were addressed. Specific long dsRNA was used to suppress the expression of MjCRS (dsCRS). At day-2 post treatment, 401 genes were differentially expressed genes by a factor of 4. 188 are upregulated and 213 are downregulated. Shrimps were also treated with non-specific dsRNA for green fluorescent protein (dsGFP). The results showed that dsGFP treated shrimps have 248 differentially expressed genes by a factor of 4. 174 are upregulated and 74 are downregulated. Several upregulated and downregulated genes were selected for RT-qPCR analysis to validate results of microarray. Results of RT-qPCR were in agreement with the microarray results. Sequences of dsRNA used were compared to the downregulated non-target gene to check for possible cross-hybridization to non-target mRNAs. dsCRS and dsGFP sequences
were 18-59% and 21-31% similar with the downregulated non-target mRNAs respectively. The results of dsCRS treated shrimps were compared with dsGFP treated shrimps. dsCRS treatment have higher number of differentially expressed genes compared to dsGFP. Genes that are specifically and mostly upregulated by dsCRS treatment are those involved in cell cycle, DNA synthesis, repair and replication and gene expression, regulation and protein synthesis. On the other hand, genes that were specifically and mostly downregulated by dsCRS treatment are those with defense and homeostasis function. It is possible that the changes in global gene expression are due to sequence similarities or the interconnection of biological pathways and that perturbation in the system could result to the impairment of the pathways affecting expression of other genes. Further studies are needed to check whether these changes are detrimental or beneficial to shrimps overall status.

Taken together, the results came out with three major findings, first through RNAi studies, it was determined that \( Mj\)CRS have antibacterial function. Second, two new \( Mj\)CRS variants, \( Mj\)CRS6 and \( Mj\)CRS7, were characterized from EST information. And lastly, \( Mj\)CRS silencing affected the global gene expression in kuruma shrimp. RNAi, EST information and microarray technology were also shown to be an effective method in determining gene function, variant identification and global gene expression analysis.
Chapter I

General Introduction

Shrimps are one of the most widely traded and cultured species of crustacean in the world. The contribution of the shrimp industry in the world aquaculture commodity is valued at about $11 billion USD or almost 20% of the total value of all aquatic products sold in the international market (Fig. 1). This high contribution is attributed to the rapid expansion of shrimp farming industry. However, diseases plague the development of the shrimp farming industry. This poses a big problem to both shrimp consuming and producing countries. The two most devastating pathogen of shrimps are viruses and bacteria. It is estimated that approximately 60% of disease losses in shrimp aquaculture have been caused by viral pathogens and 20% by bacterial pathogens (Flegel, 2012). For viral pathogens white spot virus and yellow head virus are the most lethal while for bacterial pathogens, *Vibrio* species are the most important. These diseases if not controlled will hinder continuous supply in the market and will put sustainability at risk. New approaches are urgently required to enhance yield by improving broodstock and larval sourcing, promoting best management practices by farmer and supporting cutting-edge research that aims to harness the natural abilities of invertebrates to mitigate assault from pathogens (Stentiford et al., 2012). In Japan, one of the most cultured species of shrimp is the kuruma shrimp, *Marsupenaeus japonicus*. The annual aquaculture production has been steadily increasing since 1953 until the industry was struck by a devastating viral infection caused by the white spot virus in the early years of 1990 (Fig. 2). Current researches are now focused on understanding more of the shrimp immune
system and how pathogens affect shrimp to come up with valuable information that could lead to development of disease prevention methods and solution to disease problems.

Fig. 1. Overview of fish production, utilization, consumption and trade based on 2012 data (Food and Agriculture Organization).

Fig. 2. Annual aquaculture production of *M. japonicus* in Japan from 1953-2011 (Food and Agriculture Organization).
Shrimp innate immune system

In an environment teeming with opportunistic microorganisms, shrimps have developed an effective innate immune system to fight infections. Lacking an adaptive immunity, combating pathogens are mediated by innate immune system that are divided into cellular and humoral. Cellular responses include cell-mediated reactions like phagocytosis, nodule formation and encapsulation. Humoral responses are characterized by the release of antimicrobial peptides present in the hemocytes along with reactions such as activation of proPO and clotting cascade and release of antioxidant enzymes. Infection with pathogens activates cellular and humoral responses via signal transduction pathways (Borregaard et al., 2000). A schematic model of the shrimp immune system based on current information is presented in Fig. 3 (Tassanakajon et al., 2013). It is initiated when components of the pathogenic organisms, the pathogen-associated molecular patterns (PAMPs) like viral protein antigen, peptidoglycan, lipopolysaccharide (LPS) and β-1-3 glucan (BG) activate pattern recognition receptors (PRRs) like peptidoglycan recognition protein (PGRP), Toll-receptor or lipopolysaccharide-and β-1, 3-glucan binding protein (LGBP). Recognition of LPS or BG by LGBP triggers the activation of serine proteinases (SPs), leading to a final clip-domain serine proteinase designated as proPO-activating enzyme (PPAE). Subsequently, the inactive proPO zymogen is converted to active phenoloxidase (PO) by PPAE to produce quinones, which can cross-link neighboring molecules to form melanin around invading microorganisms (Amparyup et al., 2013). In addition, it also activates the immune deficiency (IMD) pathway where the central component relish (Rel) is translocated into the nucleus to promote the release of antimicrobial peptides (Li et al., 2009; Wang et al., 2012; Wang et al., 2009). On the other hand, when Toll pathway is activated by PAMPs, the transcription factor dorsal and dorsal-related immunity factor
(DIF) also result to translocation into the nucleus to regulate the transcription of antimicrobial peptides (Huang et al., 2010; Li and Xiang, 2013). Clotting system combats tissue damage or intrusion by microbial pathogen. Hemolymph clots form a physical barrier to prevent loss of hemolymph and further entry of microbial pathogen in the body. Clotting is achieved by the polymerization of the clottable protein that catalyzed by the Ca$^+$ dependent covalent linkage of the large dimeric clottable protein by transglutaminase into long chains (Tassanakajon et al., 2013). Among the diverse array of innate immune responses, the release of AMP is considered as one of the most important component of the shrimp immune system.

Fig. 3. A schematic model of the shrimp immune system modified from Tassanakajon et al., 2013. For abbreviations and explanation see the text.
Antimicrobial peptides – AMPs

The release of antimicrobial peptides (AMPs) is considered as the hallmark of humoral immune response and widely distributed in all living organisms. They act as first line of defense against pathogen invasion (Brown and Hancock, 2006; Hancock et al., 2006; Hancock and Diamond, 2000). Antimicrobial peptides encompass a wide variety of structural motifs and occur in different types or isoforms. In shrimps, they are mainly but not strictly produced by and stored in hemocytes, which are key cells in shrimp immune system. The majority of AMPs are cationic and amphipathic but there are also hydrophobic \( \alpha \)-helical peptides, which possess antimicrobial activity (Epand and Vogel, 1999). AMPs are normally small in size, usually less than 150-200 amino acid and display a myriad of antimicrobial activities (Tassanakajon et al., 2013). AMPs exhibit diverse spectrum of activities against viruses, bacteria, fungi, yeast and parasites (Tassanakajon et al., 2011). Generally, their modes of microbial killing are pore formation on microbial membranes or disrupting membrane integrity which are accomplished by the presence of amphipathic structures allowing them to bind to membrane interface (Epand and Vogel, 1999). In addition to antimicrobial actions they also agglutinate bacterial cells, have protease inhibitory activities, perform cytokine-like functions and have LPS and lipotheichoic acid (LTA) binding and neutralizing activities (Amparyup et al., 2008a; Amparyup et al., 2008b; Destoumieux et al., 1999; Jia et al., 2008; Krusong et al., 2012; Li et al., 2010; Nagoshi et al., 2006; Somboonwiwat et al., 2008; Somboonwiwat et al., 2005; Supungul et al., 2008; Tharntada et al., 2009). In shrimps, several AMPs have been identified and characterized including penaeidins, antilipopolysaccharide factor (ALF), lysozyme, crustins and the recently identified stylincins (Hancock and Diamond, 2000; Rolland et al., 2010; Tassanakajon et al., 2013).
At present, with the use of high throughput genomic and proteomic approaches, a lot of immune genes have been identified contributing greatly in better understanding of the shrimp’s enigmatic immune system. The discovery of RNA interference, utilization of data obtained from expressed sequence tags stored in the data bank and microarray technology paved the way of investigating gene function on a whole-genome scale.

**RNA interference – RNAi**

RNA interference (RNAi) is mechanism of gene regulation demonstrated in *Caenorhabditis elegans* that results to post-transcriptional gene silencing (Fire et al., 1998). It occurs in a wide variety of organisms including protozoans, plants, insects and shrimps (Kennerdell and Carthew, 1998; Maningas et al., 2008; Robalino et al., 2004; Tijsterman et al., 2002; Ullu et al., 2004; Waterhouse et al., 1998). Silencing phenomenon is triggered by double-stranded RNA (dsRNA), which suppresses gene expression by a post-transcriptional mechanism resulting to specific suppression of gene expression (Montgomery and Fire, 1998; Montgomery et al., 1998). Silencing of specific genes gives a window of opportunity to allow characterization of gene functions. Additionally, RNAi has also been widely exploited as a method that aids in the identification of genes that have immune functions or involved in disease processes. In shrimps, the *in vivo* efficiency of RNAi technology in determining gene function in the shrimp immune system have been evidenced in several knockdown experiments. Penaeidin depleted shrimps fail to produce more penaeidin-positive granulocytes at the site of wound tissue but recovered after administration of recombinant penaeidin or its proline-rich domain suggesting that penaeidin acts as a pro-inflammatory cytokine and attracts penaeidin-positive granulocytes toward the inflammatory site by autocrine activity through integrin-dependent cell migration. (Li and Song, 2010). The *in vivo*
functions of crustin in response to microbial infection was also examined by RNAi revealing that absence of crustin will make shrimps susceptible to bacterial infection emphasizing its important role in combatting the Gram-negative bacteria, *Vibrio penaeicida* (Shockey et al., 2009). Gene knockdown experiments have also uncover essential functions of ALFs in shrimps against the bacteria, fungus and white spot virus (de la Vega et al., 2008; Ponprateep et al., 2012). In *Marsupenaeus japonicus*, silencing of c-type lysozyme and prophenoloxidase made the shrimp incapable of controlling bacterial population resulting to significant increase in mortality revealing their essential functions in shrimp survival (Fagutao et al., 2009a; Kaizu et al., 2011). The crucial function of shrimp transglutaminase and clotting protein in shrimp blood coagulation as well as their antimicrobial functions against bacterial and viral infection have also been demonstrated *in vivo* by RNAi (Maningas et al., 2008).

In addition, several knockdown experiment in shrimps have evidenced that silencing of specific genes by dsRNA also affected the amount of circulating hemocytes. Silencing of proPO, transglutaminase, c-type lysozyme and hemocyte homeostasis-associated protein in shrimps led to significant fall of circulating hemocytes (Fagutao et al., 2009a; Fagutao et al., 2012; Kaizu et al., 2011; Prapavorarat et al., 2010). Injection of dsRNA with specific targets silences the expression of the specifically targeted gene. However, previous studies showed that specific silencing also affected expression of non-target genes (off-target effects). Silencing of genes like proPO, transglutaminase and c-type lysozyme caused an increase or decrease in expression level of non-target genes including AMPs and other immune related genes as a result of sequence similarity or possible connection of the targeted gene to the affected genes (Fagutao et al., 2009a; Fagutao et al., 2012; Kaizu et al., 2011)
Expressed sequence tag – EST

Shrimp genome size is approximately $2 \times 10^9$ bp containing high percentage of repetitive sequences (Alcivar-Warren et al., 2006; Chow et al., 1990). At present, the information on shrimp genome has largely been obtained from the analysis of expressed sequence tags (ESTs) stored in the EST data bank. ESTs are short sequences ranging from 100-800 nucleotide bases in length and randomly selected single-pass sequence reads derived from cDNA libraries. cDNA is cloned to make libraries representing a set of transcribed genes of the original cell, tissue or organism. ESTs are sequenced from both directions resulting to either partial or full-length sequences. ESTs have been utilized in many ways including the discovery of novel genes, aid in gene structure identification, alternative splicing, single-nucleotide polymorphism (SNP) characterization and proteome analysis (Nagaraj et al., 2007). Analysis of EST libraries generated from various shrimp cells and tissues have provided information on tissue-specific profiles of gene expression (Lehnert et al., 1999). Comparison of EST libraries of infected and uninfected shrimps at various times of infection also provided information on genes with immune functions (Tassanakajon et al., 2013). In addition, a number of genes involved in immune defense, growth and sex differentiation have also been identified from 15 shrimp cDNA libraries (Tassanakajon et al., 2006). Among the many uses of EST information include the construction of microarray chips. For example, shrimp DNA microarray chips used in investigating gene expression after bacterial or viral infection, antibiotic treatment or peptidoglycan or exposure to environmental stress were constructed based on EST sequences (Aoki et al., 2011; de la Vega et al., 2007a; Dhar et al., 2003; Fagutao et al., 2008; Fagutao et al., 2009b; Leu et al., 2007; Pongsomboon et al., 2011; Robalino et al., 2007; Veloso et al., 2011; Wang et al., 2006; Wang et al., 2008; Wongpanya et al., 2007).
Shrimp EST sequences (Table 1) available are relatively few compared to EST sequences obtained from other model organisms like mouse, Mus musculus + domesticus (4,853,570 ESTs as of January, 2013), zebrafish, Danio rerio (1,488,275 ESTs as of January 2013) and fruit fly, Drosophila melanogaster (821,005 ESTs as of January, 2013) and comprises about 0.3% of 74,186,692 EST sequences deposited in the GenBank. Whole genome sequencing of L. vannamei is presently being undertaken and a significant amount of information will be generated in the future. However, at the moment, ESTs have been effectively used as an alternative to extract useful biological information.

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of EST entries</th>
</tr>
</thead>
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<tr>
<td>Penaeus/Litopenaeus vannamei</td>
<td>161,248</td>
</tr>
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<td>Penaeus monodon</td>
<td>39,397</td>
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<tr>
<td>Fenneropenaeus chinensis</td>
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</tr>
<tr>
<td>Macrobrachium rosenbergii</td>
<td>4,427</td>
</tr>
<tr>
<td>Marsupenaeus japonicus</td>
<td>3,156</td>
</tr>
<tr>
<td>Penaeus/Litopenaeus stylirostris</td>
<td>416</td>
</tr>
</tbody>
</table>

**cDNA microarray technology and its application**

The principle of a microarray experiment is that mRNA from a given cells or tissue is used to generate a labeled sample, which is hybridized in parallel to a large number of DNA sequences and immobilized on a solid surface in an ordered array (Schulze and Downward, 2001). The cDNA microarray is one of the most powerful technologies developed for studying global gene expression. Using microarray technology, parallel quantification of large numbers of mRNA transcript could be done providing detailed insight to the cellular processes involved in the regulation of gene expression. It has become an invaluable tool that is widely applied in plants, animals,
yeast and human (DeRisi et al., 1997; Schena, 1996; Shalon et al., 1996). Microarray has led the way from studies of the individual biological functions of few related genes, proteins or, at best pathways towards more global investigations of cellular activity (Hoheisel, 2006). It can be used in profiling disease related genes providing an opportunity for discovery of novel immune related of gene and their functions or how the global gene expressions are affected by an infection, a certain kind of treatment or stimulus (Xiang and Chen, 2000). In shrimps, cDNA microarray-based gene expression profiling has been used to identify genes whose transcriptional expression is changed after pathogen infection by comparing gene expression of infected to uninfected cells or tissues (Dhar et al., 2003; Pongsomboon et al., 2011; Wang et al., 2006; Wongpanya et al., 2007). As a result, altered expressions have been found revealing their possible involvement or function in the shrimp immune system. It was also used in the identification of reproduction or stress related genes (de la Vega et al., 2007b; Karoonuthaisiri et al., 2009). By microarray various biochemical pathways can also be predicted (DeRisi et al., 1997) or relationships in gene regulation (Fagutao et al., 2009a; Fagutao et al., 2012). Microarray have also been used to monitor changes in response to drug treatment leading to drug discovery and development. cDNA microarray is a powerful approach for simultaneous analysis of relative expression of a large number of genes and its application and suitability to a specific objective has been convincingly demonstrated. Microarray studies provided better understanding of the shrimp’s innate immune responses in a global scale.
Objectives and outline of the thesis

With the occurrence of pathogenic microorganisms and their damaging effect, understanding the innate immune responses of shrimp against invading microbes is of prime importance. Discovery of genes and their function in shrimp immunity with the use of different methods are now in focus. The simple yet very effective RNAi technologies have been widely used in determining gene functions. By the application of high throughput screening approach utilizing EST information and using microarray analysis, more immune related genes or proteins related to bacteria or viral infection or stresses are identified. The objective of this dissertation is to provide an additional understanding and information on shrimp immune response against microbial infections and treatments by incorporating methods like RNAi technology, utilization of EST information and microarray technology.

Chapter I discussed some important information regarding the shrimp industry and the diseases that affected it. Detailed information of shrimp innate immunity was also presented with special emphasis on shrimp antimicrobial peptides. The procedures used in the study like RNAi technology, ESTs and cDNA microarray technology were also briefly discussed. Chapter II described the investigation of the role of *Mj*CRS against microbial infection using RNAi technology. Chapter III presented the newly identified *Mj*CRS variants from *M. japonicus* EST information. Chapter IV tackled the global changes in gene expression in *M. japonicus* after treatment with dsRNA. And lastly, in Chapter V, based on the results general conclusions were stated.
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Hancock, R.E., Diamond, G., The role of cationic antimicrobial peptides in innate host

Hoheisel, J.D., Microarray technology: beyond transcript profiling and genotype


18


Chapter II

Role of *Marsupenaeus japonicus* crustin-like peptide (*MjCRS*) against *Vibrio penaeicida* and white spot syndrome virus infection

Abstract

Crustins are important AMP that has been identified in crustaceans. In this study, the role of *Marsupenaeus japonicus* crustin-like peptide (*MjCRS*) was examined *in vivo* by RNA interference (RNAi) using double-stranded RNA (dsRNA). Tissue expression analysis revealed that *MjCRS* transcripts are expressed in different tissues tested with the highest expression observed in hemocytes. Treatment with double-stranded RNA specific to *MjCRS* led to a significant reduction of *MjCRS* transcript within the hemocytes. When *MjCRS* was silenced and subsequently infected with *Vibrio penaeicida* final mortality was significantly higher compared with PBS and dsGFP treated groups. On the other hand, final mortalities of *MjCRS* silenced and PBS injected groups were both similarly and significantly high but not significantly different with each other after infection with white spot virus. *V. penaeicida* infection significantly decreased *MjCRS* expression at 3, 6, 12 and 24 hours followed by significant increase at 48 hours post-infection. On the contrary, white spot infection significantly increased *MjCRS* expression at 6 and 12 hours and decreased at 48 hours post-infection. dsRNA treatment alone decreased total hemocyte counts (THCs) and subsequent *V. penaeicida* or white spot virus infection further decreased THCs. VP28 gene
expression was both similarly increased in PBS injected group and *Mj*CRS silenced group at 24 and 48 hours post-infection. Results suggest that *Mj*CRS may be involved in antibacterial defense and might not have critical function against viral infection.

Keywords: Crustin, antimicrobial peptide, RNA interference, shrimp, Vibrio penaeicida, WSSV

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**Introduction**

World shrimp productions have increased in the past decades due to the rapid expansion of the cultured shrimp industry. However, development was seriously affected by outbreak of viral and bacterial diseases (Lightner and Redman, 1998). In addition to prevention and control of diseases, understanding the shrimp’s immune system must also be prioritized for the continuity of this industry. Vibriosis and white spot syndrome virus are the two of the most devastating diseases of shrimps. Lacking an adaptive immune system, shrimps rely solely on their innate immunity, an ancient immune response comprised of cellular and humoral responses to combat infections from the moment of first contact to pathogenic organisms (Bachère et al., 2004; Krimbrell and Beutler, 2001; Lee and Söderhäll, 2002). Cellular responses involve encapsulation, nodule formation and phagocytosis while humoral responses include the synthesis and release of antimicrobial peptides (AMPs), proteinase inhibitors and cytokine-like factors, activation of the prophenoloxidase system and the release of stress responsive proteins. Antimicrobial peptides are major component of the innate
immune defense system in marine invertebrates (Tincu and Taylor, 2004). They act as first line of defense by providing immediate and rapid response against invading microorganisms (Hancock and Diamond, 2000). A variety of AMPs have been identified in several shrimp species including penaeidins, crustins, anti-lipopolysaccharide factor, lysozyme and stylicins and exhibit a broad spectrum of activities against bacteria, viruses and fungi (Tassanakajon et al., 2013).

Crustins are multi-domain cationic AMPs expressed in circulating hemocytes of crustaceans. They are characterized by a cysteine-rich region and a single WAP domain that is predicted to form a four-disulfide core (4DSC) at the C-terminal end (Smith et al., 2008). Proteins containing the WAP domain exist in many species and proven to have antimicrobial and protease inhibitory functions (Amparyup et al., 2008a; Hagiwara et al., 2003; Hiemstra et al., 1996; Nile et al., 2006; Sallenave, 2002; Wiedow et al., 1990). They are classified into three types: Type I, II and III, and are distinguished based on the organization of regions in between the N-terminal signal sequence and the C-terminal WAP domain. Type I crustins contain cysteine-rich region between signal sequence and WAP domain and are mainly found in crabs, crayfish and lobster. Type II crustins contain not only cysteine-rich region but also glycine-rich region adjacent to the signal sequence and are common in shrimps. Type III crustins lack both the glycine-rich and cysteine-rich region instead it contains a proline-arginine region and are also present in shrimps (Smith et al., 2008). The first crustacean crustin identified was carcinin, an 11.5 kDa cationic and hydrophobic protein isolated from the granular hemocytes of shore crab, *Carcinus maenas* with antimicrobial activity against Gram-positive bacteria (Relf et al., 1999; Schnapp et al., 1996; Smith and Chisholm, 2001).
In shrimps, more than 41 crustins have been isolated and identified (Tassanakajon et al., 2011) including *Litopenaeus vannamei* and *L. satiferus* (Gross et al., 2001), *Penaeus monodon* (Chen et al., 2004; Supungul et al., 2002, 2004), *M. japonicus* (Rattanachai et al., 2004), *Fenneropenaeus chinensis* (Zhang et al., 2007) and *Macrobrachium rosenbergii* (Arockiaraj et al., 2013). Crustins are widely regarded as antimicrobial effectors and exhibit a variety of responses depending on species or even different isoforms of the same species. Several studies have evidenced the importance of crustin against bacterial infection (Amparyup et al., 2008b; Krusong et al., 2012; Pongsomboon et al., 2011; Shockey et al., 2009; Sun et al., 2010; Supungul et al., 2008). However, the antiviral mechanism of action and function of crustins in shrimps are still largely unknown. Several researches addressed its antiviral function in shrimp immune system and showed that crustin may also play important roles in antiviral defense (Amparyup et al., 2008a; Antony et al., 2011; Pongsomboon et al., 2011; Prapavorarat et al., 2010a).

Although *MjCRS* has been identified in *M. japonicus*, its antimicrobial activity against bacterial and viral infection has not been studied. In this study, expression profiles in different tissues were examined. Effectivity of the in vitro synthesized *MjCRS* dsRNA was checked at transcript level. Temporal changes in gene expression after *V. penaeicida* and white spot virus infection were also analyzed. In vivo silencing by RNAi and challenge tests was conducted to determine its antimicrobial role. The effect of dsRNA and microbial infections on total hemocyte counts (THCs) and VP28 gene expression were also investigated to gain further insights in the involvement of *MjCRS* in shrimp immunity.
Materials and methods

Experimental shrimp

Kuruma shrimps (*M. japonicus*) with an average body weight of 10 g were purchased from a local commercial shrimp farm in Miyazaki, Japan. Shrimps were kept in a recirculating water tank system with continuous aeration. Water temperature and salinity were maintained at 22-25 °C and 30 ppt respectively. Shrimps were fed daily with a commercial diet and conditioned for a week prior to all experimental procedures.

Tissue distribution analysis

Tissues (gills, lymphoid organ, hepatopancreas, muscle, intestine, heart, nerve, stomach and eyes) from three healthy shrimps were dissected out and suspended immediately in RNAiso Plus (TaKaRa, Japan). For hemocyte collection, hemolymph was drawn out from the ventral sinus using a sterile 2.5 ml syringe and 23Gx1 1/4” needle with 1 ml of pre-cooled anticoagulant (19.3 mM NaCl, 239.8 mM sodium citrate, 182.5 mM glucose, 0.5 M EDTA, pH 7.0). Hemocytes were immediately separated from the plasma by centrifugation at 3000 x g for 3 min at 4 °C. The plasma was discarded and the resulting hemocyte pellets were washed with anticoagulant and resuspended in 500 µl of RNAiso Plus (TaKaRa, Japan).

Total RNA isolation and cDNA synthesis

Tissue samples and hemocytes were homogenized in RNAiso Plus (TaKaRa, Japan) and total RNAs were extracted following the manufacturer’s instructions. Samples were then treated with RQ1 RNase-Free DNase (Promega,
U.S.A.) to remove contaminating genomic DNA. Total RNA concentrations were determined by spectrophotometry at A260 nm and the purity was assessed by $A_{260}/A_{280}$ absorbance ratio using NanoDrop Lite spectrophotometer (Thermo Scientific, U.S.A.).

First strand cDNA was generated using M-MLV Reverse Transcriptase (Invitrogen, U.S.A.) in a 20 µl reaction volume containing 1 µg of total RNA, 10 mM oligo (dT) 12-18, 10 mM dNTP, 5X First-Strand Buffer, 0.1 M DTT, 40 U of RNaseOUT™ recombinant ribonuclease inhibitor and 200 U M-MLV reverse transcriptase. The reaction was incubated at 37 for 1 h followed by an inactivation step at 70 °C for 15 min.

**Production of double-stranded RNA and in vivo silencing**

Double stranded RNAs (dsRNAs) were synthesized by *in vitro* transcription using T7 RiboMAX™ Express Large Scale RNA Production System (Promega, U.S.A.) following the manufacturer's instructions. Set of primers for specific ($Mj$CRS) and non-specific (green fluorescent protein - GFP) targets were designed with T7 promoter sequence attached to the 5′ end (Table 1) to generate sense and anti-sense strands separately. Only one primer set was used for silencing of $Mj$CRS and was designed from the conserved region of $Mj$CRS type 1-5 (GenBank accession numbers AB121740.1, AB121741.1, AB121742.1, AB121743.1 and AB121744.1). PCR products used as templates for the generation of single-stranded RNAs (ssRNAs) were purified using Amicon® Ultra-0.5 (Millipore, Ireland). Single-stranded RNAs were created by incubation at 37 °C for 30 min. Then, equal amounts of ssRNAs were mixed and annealed by incubations at 70 °C for 10 min. Double stranded RNAs were treated with
RQ1 RNAse-Free DNase (Promega, U.S.A.), purified using phenol:chloroform:isoamyl alcohol (25:24:1) extraction and quantified in NanoDrop Lite spectrophotometer (Thermo Scientific, U.S.A.). Formations of dsRNAs were visualized in 1% agarose gel electrophoresis. For in vivo experiments, twelve shrimps were injected intramuscularly with (1 µg/g shrimp) of MjCRS specific dsRNA (dsCRS), a non-specific control GFP dsRNA (dsGFP) or phosphate buffer saline (PBS) as negative control. Hemocytes were sampled at different time points, day-0, -1, -3 and -5 after dsRNA and the silencing efficiency of was determined by RT-qPCR as described below.

**Gene expression analysis**

Tissue specific expressions of MjCRS from unchallenged shrimps were checked by real-time quantitative PCR (RT-qPCR). Total RNA was extracted and first strand cDNA was generated as described above. Primers were designed using Primer Express 3 (Applied Biosystems, U.S.A.) and RT-qPCR was carried was ran in Applied Biosystems 7300 real-time PCR system (Applied Biosystems, U.S.A.). Amplifications were performed in a 96-well plate in a 20 µl reaction volume containing 1× THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan), 0.3 µM each of the gene-specific forward and reverse primers (Table 1), 1× ROX reference dye and 2 µl of cDNA. Relative expressions were determined by $2^{-\Delta\Delta C_t}$ method using elongation factor-1 alpha (EF-1α) as reference gene and presented as relative expression ratio.

Temporal expression after *V. penaeicida* and white spot virus infection were also examined. Shrimps were injected with either PBS, *V. penaeicida* or white spot virus and hemocytes were sampled at 0, 3, 6, 12, 24 and 48 h post-infection from
3 shrimps in each sampling points. Challenge tests were conducted twice using 18 shrimps from each group and MjCRS expression was determined by RT-qPCR as described previously. After dsRNA treatment and prior to microbial infections, MjCRS expressions were first checked from 3 individual shrimps by semi-quantitative PCR using gene-specific primers (Table 1) to confirm that MjCRS was effectively silenced. EF-1α was used as standard control. Total RNA was extracted and cDNA was generated as described above. One microliter of first-strand cDNA was subjected to PCR in a 20 µl reaction volume containing 2 µl of 10X Ex Taq buffer, 1.6 µl of 2.5 mM dNTP mix, 0.4 µl of 10 µM of each of the primers, 0.1 µl of Ex Taq DNA polymerase (TaKaRa, Japan). The reaction was amplified with the following thermal profile: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min. Five microliters of PCR products were electrophoresed and visualized on a 1% agarose gel electrophoresis.

**Challenge experiments**

To further investigate the role of MjCRS in response to microbial infection, shrimps were first injected with dsCRS, dsGFP or PBS and challenge tests were conducted three days after dsRNA treatment. Shrimps were infected by intramuscular injection between the 5th and 6th abdominal segment of 100 µl solution containing 10⁴ CFU ml⁻¹ and 10⁻⁵ dilution of *V. penaeicida* and white spot virus respectively. Bacterial concentration and viral dilution correspond to LD50 conditions (Maningas et al., 2008) and were used in all the experimental infections. Mortality experiments were conducted twice (n = 15 in Trial 1 and
\( n = 18 \) in Trial 2 for *V. penaeicida* and \( n = 18 \) in Trial 1 and \( n = 16 \) in Trial 2 for white spot virus infection) and mortalities were recorded daily for 8 days.

**Total hemocyte counts and gene expression analysis of VP28 after dsRNA treatment and microbial infection**

Total hemocyte count (THC) was performed to determine the effects of dsRNA treatment and microbial infection in the total number of circulating hemocytes per milliliter of shrimp hemolymph. Five hundred microliter of hemolymph was drawn out from the ventral sinus of 3 individual shrimps using a sterile 2.5 ml syringe and 23Gx1 1/4” needle containing 500 µl of pre-cooled anticoagulant with 10% formalin. Hemolymph and anticoagulant mixture was mixed thoroughly after collection. Ten microliter of the sample was loaded in C-Chip DHC-N01 (Digital Bio, U.S.A.) disposable hemocytomer and counted under a light microscope. THCs were checked at different time points, day-0 and -3 to check the effect of dsRNA treatment in THCs. These dsRNA treated shrimps were then infected with either *V. penaeicida* or white spot virus and THCs were again determined at day-0, -1, -3 and -5 post-infection.

Expression of one essential viral gene, the VP28 was also checked from dsRNA treated and white spot virus infected samples. Shrimps were first treated with either PBS, dsCRS or dsGFP followed by white spot virus infection after 3 days. Gills were sampled at 0, 6, 24 and 48 h post-infection. Total RNA was extracted, cDNA was synthesized and VP28 gene expression was determined by RT-qPCR following the same procedure described above.
Statistical analysis

Statistical analysis for gene expression, mortality and THCs were performed in SPSS Statistics Version 21 (SPSS Inc., U.S.A.). The data are presented as mean ± S.E. (SEM). Differences were considered significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$.

Results

MjCRS in healthy M. japonicus was highly expressed in hemocytes

Gene expression analysis of MjCRS transcript in hemocytes, gills, lymphoid organ, hepatopancreas, muscle, intestine, heart, nerve, stomach and eyes were assessed by RT-qPCR. Transcripts were detected in all tissues tested where the highest level of expression was observed in hemocytes. Lower expression levels were observed in gills, lymphoid organ, intestines and stomach and least expressed in heart, nerve, hepatopancreas, eyes and muscle (Fig. 1).

![Fig. 1. Expression of MjCRS in various tissues.](image)

Fig. 1. Expression of MjCRS in various tissues. Relative expressions were determined by $2^{-\Delta\Delta C_t}$ method using EF-1α as reference gene and presented as relative expression ratio. Hm, hemocytes; Gil, gills; L.O., lymphoid organ; Hep, hepatopancreas; Msc, muscle; Int, intestines; Hrt, heart; Ner, nerve; Sto, stomach; Eye, eyes. The data were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 3 individual shrimps. Asterisks indicate significant differences between tissues ($* P < 0.001$).
Table 1. Primers used for dsRNA synthesis, RT-PCR and RT-qPCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MjCRS-F1</td>
<td>ATGAAGGGCTTCAAAGCGGT</td>
</tr>
<tr>
<td>MjCRS-R1</td>
<td>TTATCCGAAAGAGGTTTCC</td>
</tr>
<tr>
<td>T7MjCRS-F2</td>
<td>TAATACGACTCACTATAGGGTGTTATCCGAAAGGTTTCC</td>
</tr>
<tr>
<td>T7MjCRS-R2</td>
<td>TAATACGACTCACTATAGGGTGTTATCCGAAAGGTTTCC</td>
</tr>
<tr>
<td>GFP-F2</td>
<td>ATGGTGAGCAAGGCGAGGA</td>
</tr>
<tr>
<td>GFP-R2</td>
<td>TTACTTGTACAGCTCGTCCA</td>
</tr>
<tr>
<td>T7GFP-F2</td>
<td>TAATACGACTCACTATAGGGTGAGCAAGGCGAGGA</td>
</tr>
<tr>
<td>T7GFP-R2</td>
<td>TAATACGACTCACTATAGGGTGAGCAAGGCGAGGA</td>
</tr>
<tr>
<td>MjCRSqPCR-F3</td>
<td>AACTACTGCTGCAAGAGGTCTCA</td>
</tr>
<tr>
<td>MjCRSqPCR-R3</td>
<td>GGCAGTCCAGTGCTGCTGCTGTA</td>
</tr>
<tr>
<td>EFqPCR-F3</td>
<td>ATTGCCACACCGCTCACA</td>
</tr>
<tr>
<td>EFqPCR-R3</td>
<td>TCGATCTTGTACAGCTCGTCCA</td>
</tr>
<tr>
<td>VP28qPCR-F3</td>
<td>ATGAAAACCTCCGCATTCCTG</td>
</tr>
<tr>
<td>VP28qPCR-R3</td>
<td>CCAAGGTGCTGCTGCTGAAAG</td>
</tr>
</tbody>
</table>

1 dsRNA synthesis and RT-PCR; 2 dsRNA synthesis; 3 RT-qPCR; MjCRS (crustin-like peptide); GFP (green fluorescent protein); EF (elongation factor-1 alpha); VP28 (viral protein 28 of white spot virus).

*MjCRS expression was effectively silenced at transcript level*

Treatment with dsRNA specific to *MjCRS* resulted to a significant fall in transcript abundance within the hemocytes as revealed by RT-qPCR (Fig. 2). Significant reduction in expression level was observed at day-1 post-treatment and persisted until day-5. Silencing was observed as early as 3 h post-treatment and reduced levels persisted until day-9 (data not shown). Silencing was specific to the group injected with dsCRS, such reduction was not observed in PBS or dsGFP injected groups. In a separate experiment using microarray analysis, the designed MjCRS primer set has been found to effectively silence all the 5 types of MjCRS (data not shown). In addition, the effect of dsCRS treatment alone to shrimp mortality was also checked. Results showed that dsCRS treatment would not
cause a significant increase in mortality over a two-week period provided that there is no infection (Fig. 3).

Fig. 2. Transcript expression of MjCRS after dsRNA treatment. MjCRS transcript expression in hemocytes at day-0, -1, -3, and -5 post-treatment with 1 µg/g shrimp of dsRNA. Relative expressions were determined by $2^{-\Delta\Delta Ct}$ method using EF-1α as reference gene and presented as relative expression ratio. dsCRS, double-stranded RNA specific to crustin-like peptide; dsGFP, double-stranded RNA for green fluorescent protein as non-specific control and PBS, phosphate-buffered saline as negative control. The data were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 3 individual shrimps. Asterisks indicate significant differences between groups (*$P < 0.001$).

Fig. 3. Effect of MjCRS treatment in shrimp mortality. Mortality was recorded daily over a 15-day period. dsCRS, double-stranded RNA specific to crustin-like peptide; dscLYS, double-stranded RNA specific to c-type lectin as positive control; dsGFP, double-stranded RNA for green fluorescent protein as non-specific control and PBS, phosphate-buffered saline as negative control. The data were analyzed by one-way ANOVA and differences in final mortalities between groups were considered significant at $P < 0.05$. 
Silencing of MjCRS rendered shrimps higher susceptibility to bacterial infection but not viral infection

The antimicrobial role of MjCRS in shrimp immunity was assessed by dsRNA-mediated silencing. Experimental group was intramuscularly injected with dsCRS while control groups were injected with dsGFP or PBS. Three days after dsRNA treatment shrimps were subsequently infected with either V. penaeicida or white spot virus. At day-3 post-treatment with dsRNA prior to microbial infections, MjCRS expression was checked, a significant decrease in MjCRS transcript were observed in dsCRS treated groups while no significant changes were observed in dsGFP and PBS treated groups (Fig. 4B and D). Subsequent infection with V. penaeicida resulted to a significantly higher final mortality compared to control groups. Mortality started to increase significantly as early as day-1 post infection. Mean final mortalities over the 8-day period were 97%, 38% and 48% for dsCRS, dsGFP and PBS treated groups respectively (Fig. 4A). On the other hand, a similar mortality pattern between dsCRS and PBS treated group were observed after white spot virus infection. No significant differences were observed between the two groups, however, both are significantly higher compared to dsGFP treated group. Mean final mortalities were 91%, 41% and 97% for dsCRS, dsGFP and PBS treated groups respectively (Fig. 4C). Groups treated with dsGFP did not exhibit a significantly high final mortality rate after infection with either V. penaeicida or white spot virus.

Expression of MjCRS was affected by bacterial and viral infection

Temporal changes of MjCRS expression in V. penaeicida and white spot virus infected shrimps were examined by RT-qPCR. Microbial infections
displayed different pattern of expressions. Expression level of MjCRS was significantly downregulated at 3 h and more dramatically at 6, 12 and 24 h followed by significant increase at 48 h post-infection with V. penaeicida (Fig. 5A). On the contrary, a significant increase in MjCRS transcript abundance was observed at 6 and 12 h followed by decreased level at 48 h post-infection with white spot virus (Fig. 5C). In later stages of V. penaeicida infection, MjCRS expression level was sustained (Fig. 5B) while in white spot virus infection, MjCRS expression level continued to decrease and became almost undetectable until day-5 post-infection as revealed by RT-PCR (Fig. 5D). These significant changes in gene expressions were not observed in the control group, which was mock injected with PBS.

**dsRNA treatment and microbial infection decreased the total hemocyte counts and increased VP28 gene expression**

The effects of dsRNA treatment and subsequent microbial infection on THCs were investigated. THCs were checked at day-0 and -3 after dsRNA treatment and after subsequent infection with V. penaeicida or white spot virus at day-0, -1, -3 and -5 post-infection. dsRNA treatment alone significantly decreased THC at day-3 in dsCRS treated group. Although decrease in THC was also observed at day-3 in PBS and dsGFP treated groups these changes are not significantly different compared to day-0. The decreased THC in dsCRS treated group was further decreased significantly at day-1, -3 and -5 post-infection with V. penaeicida (Fig. 6A). THC was significantly decreased at day-1 and recovered at day-3 and -5 in PBS treated group while no significant changes in THCs were observed in dsGFP treated group after infection (Fig. 6A). Similarly, dsCRS and
dsGFP treatment significantly decreased THCs at day-3 prior to white spot virus infection while no significant changes were observed in PBS treated group. Subsequent infection with white spot virus did not significantly affect the amount of circulating hemocytes at day-1 but significantly declined both at day-3 and day-5 in PBS, dsGFP and dsCRS treated groups (Fig. 6B).

To further investigate the role of \( Mj \)CRS against white spot virus VP28 gene expression was also checked. PBS and dsCRS treated groups exhibited similar significant increase in VP28 transcript abundance at 24 and 48 h post-infection with white spot virus. No significant increase in expression was observed in the dsGFP treated group (Fig. 6).

**Discussion**

Crustins are proteins that display a diversity of antimicrobial activities. They are identified in different crustaceans and structure and function vary according to species. Previously, \( M. japonicus \), crustin-like peptide has been identified and characterized. Analysis of its amino acid sequences revealed the presence of glycine-rich region adjacent to signal sequence, the highly conserved 12 cysteine-rich region and a single WAP domain that is predicted to form a four-disulfide core (4DSC) at the C-terminal end and thus classifying it to Type II crustin. In this study the role of \( Mj \)CRS was examined *in vivo* using RNA interference.
Fig. 4. Effect of *Mj*CRS silencing and microbial infection on shrimp mortality. Mortality results of dsRNA treated and *V. penaeicida* (A) or white spot virus (C) infected samples. Confirmation of crustin-like peptide suppression three days after dsRNA treatment prior to microbial infections (B and D). PBS + Vp, PBS treated and *V. penaeicida* infected; dsCRS + Vp, dsCRS treated and *V. penaeicida* infected and dsGFP + Vp, dsGFP treated and *V. penaeicida* infected. PBS + WSV, PBS treated and WSV infected; dsCRS + WSV, dsCRS treated and WSV infected and dsGFP + WSV, dsGFP treated and WSV infected. Challenge tests were conducted twice and mortality was recorded daily over an 8-day period. The data were analyzed using one-way ANOVA and presented as mean ± S.E. Differences in final mortalities between groups were considered significant at $P < 0.01$. 
Fig. 5. MjCRS transcript expression in hemocytes after microbial infection. MjCRS transcript expression in hemocytes at 0, 3, 6, 12, 24 and 48 h post-infection with *V. penaeicida* (A, RT-qPCR and B, RT-PCR) and white spot virus (C, RT-qPCR and D, RT-PCR). Results of RT-qPCR were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 3 individual shrimps. Asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01 and ***P < 0.001 for *V. penaeicida* infection and *P < 0.01 and **P < 0.001 for WSSV infection).
Fig. 5. Effect of MjCRS and microbial infection in total hemocyte counts. Total hemocyte counts (THC) at day-0 and -3 post-treatment with dsRNA and day-0, -1 and -3 and -5 post-infection with *V. penaeicida* (A) and white spot virus (B). d3 dsRNA; d0 Vp represents day-3 of dsRNA or PBS treatment and day-0 of *V. penaeicida* infection while d3 dsRNA; d0 WSSV represents day-3 of dsRNA or PBS treatment and day-0 of WSSV infection. dsCRS (double-stranded RNA specific to crustin-like peptide), dsGFP (double-stranded RNA for green fluorescent protein) as non-specific control and PBS (phosphate-buffered saline) as negative control. THCs were first compared to day-0 after day-3 of dsRNA or PBS injection using Students $t$-test. After infections, THCs were compared to day-3 of PBS or dsRNA treatment that represents day-0 of microbial infections by one-way ANOVA. Asterisks indicate significant differences between groups ($*P < 0.05$ and $**P < 0.01$).
Fig. 7. Transcript expression of VP28 after dsRNA treatment and white spot virus infection. VP28 transcript expression in hemocytes at 0, 6, 24 and 48 h post-treatment with 1 µg/g shrimp of dsRNA and white spot virus infection. Relative expressions were determined by $2^{-\Delta\Delta Ct}$ method using EF-1α as reference gene and presented as relative expression ratio. PBS + WSV, PBS treated and WSV infected; dsCRS + WSV, dsCRS treated and WSV infected and dsGFP + WSV, dsGFP treated and WSV infected. The data were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 3 individual shrimps. Asterisks indicate significant differences between groups (*P < 0.05 and **P < 0.01). ND, not detected.

Crustacean hemocytes are considered the major site of AMP synthesis and storage and function as a defender through direct sequestration, killing of infectious agents or synthesis and exocytosis of a series of bioactive molecules (Iwagana and Kawabata, 1998; Muñoz et al., 2002; Smith and Chisholm, 1992). Broad spectrums of antimicrobial activities and transcripts of AMPs have been observed and identified in crustacean hemocytes (Chisholm and Smith, 1992, 1995; Haug et al., 2002; Muñoz et al., 2002; Supungul et al., 2004). The result of tissue distribution analysis showed expression at varying levels in all tissues examined. The highest expression was detected in hemocytes, low levels in gills, lymphoid organ, intestines, stomach, heart and almost undetected in nerve, hepatopancreas, eyes and muscle. This is in contrast with the previous report where it was detected only in hemocytes (Rattanachai et al., 2004). The
differences in expression may be due to the use of RT-qPCR, which is a more sensitive method in determining gene expression, compared to RT-PCR that was used in the earlier study. The present results are in agreement to previous researches where in healthy, unchallenged shrimps, most crustins are constitutively expressed on high levels in hemocytes and in lower amounts or almost undetectable in other tissues (Amparyup et al., 2008a,b; Gross et al., 2001; Smith et al., 2008; Sun et al., 2010; Supungul et al., 2004; Wang et al., 2007; Zhang et al., 2007). Crustin expression however, is not strictly highly abundant in hemocytes, they also vary according to different isoforms (Vatanavicharn et al., 2009). Presence of expression in other tissues may be due to the infiltration of hemocytes in various tissues since shrimps posses an open circulatory system allowing the hemocytes to infiltrate and adhere to different tissues (Antony et al., 2011). Hemocytes play a major role in shrimp immune response. The presence of antimicrobial peptides in the hemolymph, both in the plasma and hemocytes are involved in both systemic as well as site-specific protection from pathogen invasion in invertebrates (Hancock et al., 2006). High expression of MjCRS in hemocytes may indicate its major area of synthesis and expression in different tissues might suggest various roles in the shrimp immune system.

RNA interference has been widely exploited in crustaceans as a method to study the function of genes in vivo and in vitro. Treatment with dsRNA specific to MjCRS successfully silenced its expression at transcript level as revealed by RT-qPCR. Such changes were not observed in control groups indicating the effectivity and specificity of silencing of MjCRS dsRNA in dsCRS treated group. When the MjCRS was silenced and infected with V. penaeicida it exhibited significant increase in mortality as early as day-1. This result was similar to the
previous report in *L. vannamei* where the crustin silenced shrimps had significant increase in mortality 48 h after *V. penaeicida* infection (Shockey et al., 2009). Crustins show a wide range of antibacterial activities. Recombinant peptides of crustin demonstrated antibacterial activities of varying responses when tested against several Gram-positive and Gram-negative bacteria (Amparyup et al., 2008b; Krusong et al., 2012; Sun et al., 2010; Supungul et al., 2008). Differences in responses may be attributed to the methods used. It is also notable that structural differences or shrimp species caused the variable responses. In the present study, high mortality rate in the absence of *Mj*CRS suggests that it is an important AMP against the Gram-negative bacteria *V. penaeicida*.

AMPs are known to participate in fending off infection against viruses as well by controlling viral replication or decreasing shrimp’s susceptibility to viral infection (Liu et al., 2006; Woramongkolchai et al., 2011). Enhanced production of crustin-like AMP in *P. monodon* fed with marine yeast and probiotic bacteria conferred significant protection against white spot virus infection (Antony et al., 2011). Our results showed that absence of *Mj*CRS followed by white spot virus would result to a mortality pattern similar to PBS treated group. This observation indicates that *Mj*CRS may not be involved in antiviral defense.

Vibriosis caused by a variety of *Vibrio* spp. is one of the major causes of mortalities in crustacean. Microbial challenge using *Vibrio* infection exhibits two distinct phases of immune response. The first phase results in a dramatic decrease in the total number of circulating hemocytes in early hours approximately 12 h post-infection. It is then followed by recovery phase corresponding to 24 to 48 h post-infection where the amounts of circulating hemocytes return to basal levels (Bachère et al., 2004). In addition, AMP transcript expression exhibits similar
kinetics corresponding to the phases of immune response. A fall in *Litvan*-Pen3-1 mRNA concentration in early phase was followed by an increase at 72 h post-infection (Destoumieux et al., 2000). *L. stylirostris* crustin exhibited a fall in transcript abundance at 12 h followed by a tendency to return to levels observed in uninfected shrimp at 24 h post-infection with *V. penaeicida* (de Lorgeril et al., 2008). In our study *Mj*CRS transcript expression significantly decreased during the early phases of infection followed by recovery in later phase. This result is similar to the responses in phases of immune response described above. The increase in transcript expression in the later phase might imply that the shrimp’s immune system is responding to successfully eliminate *V. penaeicida* and indicate the important role of *Mj*CRS against *V. penaeicida* infection.

Viral infection showed a different response, crustin expression levels tend to be upregulated in early stages followed by significant downregulation in later stages of viral infection (Amparyup et al., 2008a; Prapavorarat et al., 2010a). Our results on *Mj*CRS transcript expression results followed a similar trend after white spot virus infection. Although the upregulation of *Mj*CRS expression is observed in early stages of white spot virus infection the response is transient and may not be a critical factor in protection against viral infection as reflected in the mortality experiment.

The number of circulating hemocytes has been used as an indicator to evaluate the health status of shrimps. Several researches have evidenced that the number of circulating hemocytes are affected by dsRNA treatment. Silencing of hemocyte homeostatis associated protein (HHAP) in *P. monodon* was correlated with significant decrease in THC as a result of hemocyte deformation and lysis (Prapavorarat et al., 2010b). In *M. japonicus*, it has been reported that RNAi
mediated silencing by dsRNA of transglutaminase, c-type lysozyme and prophenoloxidase without any artificial bacterial infection led to a decreased amount of hemocytes in the hemolymph accompanied by increased bacterial number leading to increased mortality (Fagutao et al., 2009, 2012; Kaizu et al., 2011). Their results pointed the importance of these peptides in controlling bacterial population in shrimp hemocytes to maintain homeostasis. Our results show a similar effect where dsRNA treatment alone significantly lowered the THCs regardless of specificity. In contrast though, silencing of *Mj*CRS did not result in significant mortality over a 15-day period provided that there is no artificial infection. This observation show that *Mj*CRS may not be involved in controlling bacterial population in shrimp hemolymph which is critical in shrimp survival. This, however, needs further clarification to check whether *Mj*CRS silencing has effect on bacterial population in shrimps. When the shrimps are subsequently infected with *V. penaeicida* THCs declined further at day-1, -3 and -5 in *Mj*CRS silenced group and at day-1 in PBS injected group. Similar observation was seen in vitellogenic females of *P. indicus* where THC was significantly reduced 24 h post-infection with *V. penaeicida* (Avarre et al., 2003). Upon entry of pathogens, hemocytes leave the circulation and migrate to the site of infection to exert their antimicrobial effects (Hancock et al., 2006; van de Braak et al., 2002). White spot virus infection in shrimps showed signs of apoptosis including chromatin margination and nuclear condensation and fragmentation as early as 6 h and increases the percentage of apoptotic hemocytes (Sahul Hameed et al., 2006; Wongprasert et al., 2003). The decline in THCs of *V. penaeicida* and white spot virus infected *M. japonicus* might be caused by the hemocytes leaving the circulation and accumulation at the infected tissues and viral-induced apoptosis.
VP28 is an envelope protein and involved in systemic infection of white spot virus in shrimps (van Hulten et al., 2001). Our results show that absence or presence of *Mj*CRS will both result to significant increase in VP28 gene expression. It is notable that in dsCRS treated group VP28 gene expression is higher at 24 h post-infection compared to PBS group. It is possible that the absence of *Mj*CRS resulted to faster viral multiplication at this stage however as we speculated earlier, *Mj*CRS function against white spot virus may not be as critical compared to other AMPs.

The significant increase in mortality results of the bacterial and viral infection raises an important question whether these are combined effects of the dsRNA treatment that decreased the THCs prior to infection and the infection itself. Hemocyte number is unlikely to affect the shrimp survival because depletion of up to 95% circulating hemocyte is replaced by rapid hemocyte production and release in the circulation (Prapavorarat et al., 2010b). However, in other cases mentioned above, the decrease in THC and increase in mortality may be affected depending on the function of the gene being silenced. The exact mechanism why dsRNA treatment regardless of specificity lowers THC needs further clarification. In this study, we speculate that the significant increase in mortality in *Mj*CRS silenced and *V. penaeicida* infected shrimps was due to the decreased levels of *Mj*CRS. In agreement with previous research, THC levels were found to be related to *Mj*CRS transcript abundance. In the case of *V. penaeicida* infection, *Mj*CRS expressions fall together with the decrease in THC in early stages and recovered in later stages in the control group. These observations are not seen in the *Mj*CRS silenced group where the THC continued to decrease and lost the ability to recover after *V. penaeicida* infection. These observations are
also exhibited in white spot virus infection in later stages of infection when THC levels decreased and was accompanied by decrease in MjCRS transcript expression.

In summary, RNAi silencing by dsRNA allowed the investigation of MjCRS function \textit{in vivo}. Consistent with previous results, MjCRS transcripts were highest in hemocytes indicating its primary site of synthesis. Absence of MjCRS makes shrimp susceptible to \textit{V. penaeicida} but not white spot virus infection. \textit{V. penaeicida} infection decreased MjCRS transcript during the early phase of infection and recovered in the late phase which is in agreement with previous studies on phases of immune response during bacterial infection. Transient change in MjCRS expression transcript during early stages of viral infection and VP28 expression similar to control group suggests that it may not have critical immune function against viral infection. This study also showed the importance of circulating hemocytes in shrimp immunity. Taken together, our results provided additional understanding on the important involvement of MjCRS in fending off infections like \textit{V. penaeicida}. However, the exact mechanism how MjCRS eliminate bacterial infection is still unknown and requires more clarification. Further studies are needed to address this question.

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Chapter III

Identification of MjCRS variants from EST information in kuruma shrimp Marsupenaeus japonicus

Abstract

EST databases have been developed in several crustacean species including shrimps. With EST information in the data bank, a variety of immune related genes have been identified. Of the immune genes, multiple isoforms and types or variants have been reported to coexist. Using EST information, this study was conducted to identify MjCRS variants. 44 EST sequences with match in the existing crustins were identified. Sequences were subjected to phylogenetic analysis and the results showed 13 possible MjCRS variants. Comparison with existing crustins exhibits variable degrees of identity ranging from 37-100%. Sequences showed similarities with shrimps, crabs and lobster. Primers were designed from the EST sequences and expression in different tissues was checked. Locus 1748 and 3 have similar pattern of expression with the previously identified MjCRS where they are ubiquitously expressed in different tissues. Locus 3886 (1), 16076, 11885, 5882, 5809 and 2365 were highly expressed in gills. Locus 7363 was expressed in several tissues like hepatopancreas, intestines and stomach and Locus 3886 (2) was specifically expressed in hepatopancreas. Locus 11885 and 16076 were further studied and analyzed and renamed as MjCRS6 and MjCRS7 respectively. Sequence analysis of MjCRS6 revealed a predicted ORF of 543 bp.
encoding a putative protein of 180 amino acid residues and signal peptide cleavage between the 20th and 21st amino acid. *MjCRS6* contained the conserved 12 cysteine-rich region and a WAP domain of 49 amino acids at the C-terminal end. On the other hand, *MjCRS7* revealed a predicted ORF of 504 bp encoding a putative protein of 167 amino acid residues. The predicted signal peptide cleavage between the 18th and 19th amino acid. Similarly, *MjCRS7* also contained the conserved 12 cysteine-rich region and a WAP domain of 51 amino acid residues. In this study, two new *MjCRS* variants were identified and further studies are needed to determine their possible immune function.

**Introduction**

Analyses of expressed sequence tags (ESTs) generated from cDNA libraries are important approaches in gene identification (Adams et al., 1991; Leu et al., 2011). ESTs stored in the database are rich resources of gene expression analysis and can provide an informative overview of major transcripts in specific tissues (Liu et al., 2011). Tissue-specific expression profiles based on ESTs have been successfully characterized, which are highly useful in understanding the gene functions, tissue physiology and transcriptomics (Lehnert et al., 1999; Leu et al., 2011; Liu et al., 2011; O'Leary et al., 2006). In crustaceans, EST databases have been developed and genes associated with immunity have been identified (Gross et al., 2001; Leu et al., 2011; Supungul et al., 2004). From the generated EST data, several isoforms or types or variants of immune related genes were isolated and characterized. In *P. monodon*, 5 different isoforms of antilipopolysaccharide factors have been isolated based on EST information (Supungul et al., 2004). In 2006, a large-scale expressed EST sequencing project
was undertaken for the purpose of gene discovery in *P. monodon* (Tassanakajon et al., 2006). From *P. monodon* EST database, different isoforms of crustin have been found (Supungul et al., 2008; Tassanakajon et al., 2006). In addition, 12 and 6 kazal-type proteinase inhibitors are identified in crayfish, *Pasifastacus leniusculus* and shrimp *P. monodon* by utilization of EST data (Cerenius et al., 2010). Several isoforms of crustins were also identified from two species of Penaeid shrimps, *Litopenaeus vannamei* and *L. satiferus* (Bartlett et al., 2002).

Generally, shrimp crustins contain 12 cysteine residues at the carboxyl terminal end together with the whey-acidic protein domain or the WAP domain containing the 4DSC signature. There are three types of crustins (see Chapter II for detailed discussion). Most of shrimp crustins belong to Type II and several Type III crustins have also been identified.

Previously, in *Marsupenaeus japonicus*, 5 variants of crustin-like peptide have been identified (Rattanachai et al., 2004). At present, although smaller in number compared to other shrimp ESTs available in the database, considerable number of EST data from *M. japonicus* have been generated. Utilization of this information could lead to identification of new genes, variants and isoforms of previously identified genes. This study aimed to identify possible crustin-like peptide in *M. japonicus* (*MjCRS*) variants. EST sequences with similarities from the previously identified crustins were selected for further analysis. Sequences were analyzed as well as tissue expressions.
Materials and methods

Experimental shrimp

Kuruma shrimps (*M. japonicus*) with an average body weight of 10 g were purchased from a local commercial shrimp farm in Miyazaki, Japan. Shrimps were kept in a recirculating water tank system with continuous aeration. Water temperature and salinity were maintained at 22-25 °C and 30 ppt respectively. Shrimps were fed daily with a commercial diet and conditioned for a week prior to tissue collection.

Tissue collection for EST generation and EST sequence retrieval

For EST sequence generation, tissues including gills, muscle, hepatopancreas and lymphoid organ were dissected from 3 individual shrimps. Tissue samples were suspended immediately in RNAiso Plus (TaKaRa, Japan). For hemocyte collection, hemolymph was drawn out from the ventral sinus using a sterile 2.5 ml syringe and 23Gx1 1/4” needle with 1 ml of pre-cooled anticoagulant (19.3 mM NaCl, 239.8 mM sodium citrate, 182.5 mM glucose, 0.5 M EDTA, pH 7.0). Hemocytes were immediately separated from the plasma by centrifugation at 3000 x g for 3 min at 4 °C. The plasma was discarded and the resulting hemocyte pellets were washed with anticoagulant and resuspended in 500 µl of RNAiso Plus (TaKaRa, Japan).

From the Genome Science Laboratory EST database of *M. japonicus*, 44 EST sequences with similarities to existing crustins were selected. Primers were designed (Table 1) and tissue expression analysis by RT-PCR was conducted.
**Tissue collection for gene expression analysis**

For tissue expression analysis gills, lymphoid organ, hepatopancreas, muscle, intestine, heart, nerve, stomach and eyes were collected from three individual shrimps as well as hemocytes as described above.

**Total RNA isolation and cDNA synthesis**

Tissue samples and hemocytes were homogenized in RNAiso Plus (TaKaRa, Japan) and total RNAs were extracted following the manufacturer’s instructions. Samples were then treated with RQ1 RNAse-Free DNase (Promega, U.S.A.) to remove contaminating genomic DNA. Total RNA concentrations were determined by spectrophotometry at A260 nm and the purity was assessed by A$_{260}$/A$_{280}$ absorbance ratio using NanoDrop Lite spectrophotometer (Thermo Scientific, U.S.A.).

First strand cDNA was generated using M-MLV Reverse Transcriptase (Invitrogen, U.S.A.) in a 20 µl reaction volume containing 1 µg of total RNA, 10 mM oligo (dT) 12-18, 10 mM dNTP, 5X First-Strand Buffer, 0.1 M DTT, 40 U of RNaseOUT™ recombinant ribonuclease inhibitor and 200 U M-MLV reverse transcriptase. The reaction was incubated at 37 for 1 h followed by an inactivation step at 70 °C for 15 min.

**Tissue expression analysis**

Tissue specific expressions of MjCRS variants from unchallenged shrimps were checked by semi-quantitative RT-PCR. One microliter of first-strand cDNA was subjected to PCR in a 20 µl reaction volume containing 2 µl of 10X Ex Taq buffer, 1.6 µl of 2.5 mM dNTP mix, 0.4 µl of 10 µM of each of the primers, 0.1 µl
of Ex Taq DNA polymerase (TaKaRa, Japan). The reaction was amplified with the following thermal profile: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min. Five microliters of PCR products were electrophoresed and visualized on a 1% agarose gel electrophoresis.

Cloning of locus 11885 (MjCRS6) and locus 16076 (MjCRS7)

Based on the results of tissue expression analysis, locus 11885 and 16076 were selected for cloning and sequencing and designated at MjCRS6 and MjCRS7 respectively. Putative open reading frames (ORF) were checked using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orf1.cgi). New sets of primers were designed (Table 1) covering the putative ORF and amplified by PCR following the protocols described above. The reaction was amplified with the following thermal profile: pre-denaturation at 95 °C for 5 min, followed by 28 (MjCRS7) and 30 (MjCRS6) cycles of denaturation at 95 °C for 30 s, annealing at 55.5 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min. Five microliters of PCR products were electrophoresed and visualized on a 1% agarose gel electrophoresis.

PCR products corresponding to the expected sizes were cloned into pGEM-T easy vector system (Promega, U.S.A.). Positive recombinant clones were identified and isolated. Plasmid DNA was extracted and sequencing was performed using 3130xl Genetic Analyzer (Applied Biosystems, U.S.A.).
Table 1. Primers used for tissue expression and sequencing analysis.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MjCRS-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ATGAAGGGCTTCAAAGCGGT</td>
</tr>
<tr>
<td>MjCRS-R&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TTATCCGAAAAGAGGTTC</td>
</tr>
<tr>
<td>Locus 1748-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>CAAAGACTGATGGAGCCTT</td>
</tr>
<tr>
<td>Locus 1748-R&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TACGACATCATCGTCGACCT</td>
</tr>
<tr>
<td>Locus 3-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GCGACAGATCTGAGGTT</td>
</tr>
<tr>
<td>Locus 3-R&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 7363-F&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 7363-R&lt;sup&gt;1&lt;/sup&gt;</td>
<td>CAAAGACTGATGGAGCCTT</td>
</tr>
<tr>
<td>Locus 3886(1)-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 3886(1)-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 16076-F&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Locus 16076-R&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 11885-F&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Locus 11885-R&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 5882-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 5809-F&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 5882-R&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 5809-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 2365-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 2365-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 2760-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 2760-R&lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td>Locus 2701-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 2701-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locus 9331-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Locus 9331-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>EF-1α-F&lt;sup&gt;1&lt;/sup&gt;</td>
<td>EF-1α-R&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>RT-PCR; <sup>2</sup>cloning and sequencing.
**Sequence analysis**

The cloned sequences were analyzed for identity and similarity by BLASTX. The signal peptide and putative cleavage site was predicted by the SignalIP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). WAP domain was identified using SMART™ (http://smart.embl-heidelberg.de). Multiple sequence alignments with previously identified MjCRS type 1-5 (GenBank accession nos. (GenBank accession numbers AB121740.1, AB121741.1, AB121742.1, AB121743.1 and AB121744.1) were performed using CLUSTALW (http://www.genome.jp/tools/clustalw/).

**Results**

**EST sequences**

EST Sequences were subjected to BlastX and BlastN program against the nr and nt databases. 44 sequences with similarities to existing crustins were identified and retrieved. Sequence similarities ranged from 37-100% with crustins identified from *M. japonicus, Farfantepenaeus subtilis, L. satiferus, L. vannamei, Farfantepenaeus paulensis, P. monodon, Macrobrachium rosenbergii, Panulirus japonicus, Portunus trituberculatus* and *Scylla paramamosain* (Table 2). Phylogenetic analysis revealed 13 possible crustin variants (Figure 1).

**Tissue expression analysis**

Primer sequences were designed from the EST sequences and gene expression analysis was conducted using RT-PCR. The results showed variable expression in different tissues. Locus 1748 with 100% similarity to previously identified MjCRS showed similar expression pattern. Locus 3 with 37-44%
similarity to *M. rosenbergii* crustin also showed a similar pattern except for higher expression in eyes. Others were only expressed in several or one tissue. Locus 7363 was detected highly in hepatopancreas, intestines and stomach. Locus 3886(2) on the other hand was only specifically detected in hepatopancreas while 3886(1) was specifically expressed in gills. Locus 16076, 11885, 5882, 5809 and 2365 were highly expressed in gills (Fig 2). Locus 2760, 2701 and 9331 to were not successfully amplified.

**Table 2. Sequence identities of 44 EST nucleotide sequences to existing crustins.**

<table>
<thead>
<tr>
<th>Locus</th>
<th>% Identity</th>
<th>Organism</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1748</td>
<td>100</td>
<td><em>M. japonicus</em></td>
<td>Kuruma shrimp</td>
</tr>
<tr>
<td>2760</td>
<td>85 – 88</td>
<td><em>F. subtilis</em></td>
<td>Southern brown shrimp</td>
</tr>
<tr>
<td>3886 (1)</td>
<td>72 – 78</td>
<td><em>L. satiferus</em></td>
<td>White prawn</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>F. subtilis</em></td>
<td>Southern brown shrimp</td>
</tr>
<tr>
<td>16076</td>
<td>73</td>
<td><em>L. vannamei</em></td>
<td>Pacific white shrimp</td>
</tr>
<tr>
<td>23635</td>
<td>72</td>
<td><em>L. satiferus</em></td>
<td>White prawn</td>
</tr>
<tr>
<td>5809</td>
<td>71</td>
<td><em>P. japonicus</em></td>
<td>Japanese spiny lobster</td>
</tr>
<tr>
<td>2701</td>
<td>67</td>
<td><em>P. japonicus</em></td>
<td>Japanese spiny lobster</td>
</tr>
<tr>
<td>9331</td>
<td>52</td>
<td><em>P. trituberculatus</em></td>
<td>Swimming crab</td>
</tr>
<tr>
<td>5882</td>
<td>47 – 49</td>
<td><em>P. japonicus</em></td>
<td>Japanese spiny lobster</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>F. paulensis</em></td>
<td>Pink shrimp</td>
</tr>
<tr>
<td>3886 (2)</td>
<td>45</td>
<td><em>S. paramamosain</em></td>
<td>Green mud crab</td>
</tr>
<tr>
<td>7363</td>
<td>41</td>
<td><em>P. monodon</em></td>
<td>Black tiger shrimp</td>
</tr>
<tr>
<td>3</td>
<td>37 – 44</td>
<td><em>M. rosenbergii</em></td>
<td>Giant freshwater prawn</td>
</tr>
<tr>
<td>11885</td>
<td>37</td>
<td><em>F. paulensis</em></td>
<td>Pink shrimp</td>
</tr>
</tbody>
</table>

**Sequence analysis of MjCRS6**

From the results of tissue expression analysis, locus 11885 whose expression was high in gills was selected for further analysis and renamed as *MjCRS6*. The predicted ORF of *MjCRS6* is 543 bp and encoding a putative protein of 180 amino acid residues. The signal peptide is composed of 20 amino acids and the putative cleavage site is in between 20th (Ala) and 21st (Leu) amino acids. Following the signal sequence is glycine-rich repeat region. However, in contrast to previously identified *MjCRS*, the glycine residues are only 13
compared to 50-67 glycine present in \textit{Mj}CRS type 1-5. The carboxyl terminal end of \textit{Mj}CRS6 possesses the 12 conserved cysteine-rich rich residues that are found in all crustin-encoding ESTs from several crustaceans. With the carboxyl terminus, a WAP domain of 49 amino acids was identified based on consensus identification by SMART\textsuperscript{TM} database (Fig 3).

The highest homology of the predicted ORF of \textit{Mj}CRS6 was with crustin of \textit{M. rosenbergii} (AFO68120, 46\% identity), crustin isoform 2 of \textit{L. schmitti} (ABQ96198, 46\% identity), crustin mature isoform 2 of \textit{F. paulensis} (ADF80918, 42\%). The sequence homology of \textit{Mj}CRS6 to the previously identified \textit{Mj}CRS type 1-5 was only 41\%.

Fig. 1. Phylogenetic analysis from amino acid sequences of 44 EST sequences with similarities to previously identified crustins.
Fig. 2. Expression of possible *MjCRS* variants in various tissues. Hm, hemocytes; Gil, gills; L.O., lymphoid organ; Hep, hepatopancreas; Msc, muscle; Int, intestines; Hrt, heart; Ner, nerve; Sto, stomach; Eye, eyes.

![Signal peptide and glycine-rich region for MjCRS variants](image)

**Fig. 3.** Comparison of amino acid sequences of *Mj*CRS type 1-5 to *MjCRS*6. (*) Indicates identical amino acids, (:) indicates very similar amino acids while (.) indicates amino acids that are more or less similar. The signal peptides were underlined and glycine and cysteine-rich regions were indicated by gray...
Sequence analysis of MjCRS7

Locus 16076 whose expression was also high in gills was renamed and designated as MjCRS7. The predicted ORF of MjCRS7 is 504 bp and encoding a putative protein of 167 amino acid residues. The signal peptide is composed of 18 amino acids and the putative cleavage site is in between 18th (Ala) and 19th (Asp) amino acids. Following the signal sequence is glycine-rich repeat region and contains 27 glycine repeats, higher compared to MjCRS6. Similar to MjCRS type 1-6, the carboxyl terminal end of MjCRS7 possesses the 12 conserved cysteine-rich residues and a WAP domain of 51 amino acids (Fig 4).

Fig. 4. Comparison of amino acid sequences of MjCRS type 1-5 to MjCRS7. (*) Indicates identical amino acids, (:) indicates very similar amino acids while (.) indicates amino acids that are more or less similar. The signal peptides were
underlined and glycine and cysteine-rich regions where were indicated by gray boxes above the amino acid residues. The 12 conserved cysteine residues were indicated by bold letters. WAP domains were shaded gray and the 4DSC signature were indicated by italicized letters.

The highest homology of the predicted ORF of MjCRS7 was with crustin-like AMP of P. monodon (ACL51682, 77% identity), crustin I of L. vannamei (AAS59735, 75%) and crustin of L. schmitti (ABM63362, 74%). The sequence homology of MjCRS7 to the previously identified MjCRS type 1-5 was between 66-67%.

Discussion

Crustins are antimicrobial proteins discovered in crustaceans. There are three main types in which all possess a signal sequence at the amino terminus and a WAP domain at the carboxyl terminal end (Smith et al., 2008). They are classified based on the structure in between signal sequence and WAP domain. In M. japonicus, five variants of crustin-like AMP (MjCRS type 1-5) were first reported in 2004 (Rattanachai et al., 2004). Based on their sequence structure, MjCRS type 1-5 belong to Type II crustins as they contain both the glycine and cysteine-rich region in between the signal sequence and WAP domain.

In this study new variants of MjCRS, MjCRS6 and MjCRS7 have been identified from kuruma shrimp EST information. Molecular studies in shrimps have evidenced the existence of more than one isoform, type or variants of crustin (Antony et al., 2010; Amparyup et al., 2008a; Rosa et al., 2007; Zhang et al., 2007; Chen et al., 2004; Rattanachai et al., 2004; Supungul et al., 2004; Bartlett et al., 2002).

Most reported crustins are highly expressed in hemocytes. Crustin expression however, is not strictly highly abundant in hemocytes, they also vary
according to different isoforms. In *P. monodon*, one isoform, crustin*Pm*5 is uniquely and mainly expressed in epipodite and eyestalks and no expression was detected in hemocytes (Vatanavicharn et al., 2009). In contrast, transcripts of Chinese shrimp crustin, *CruFc*, were found to be both abundantly expressed in hemocytes and gills (Zhang et al., 2007). Tissue expression analysis revealed a unique expression of *Mj*CRS6 and *Mj*CRS7. While *Mj*CRS type 1-5 and other shrimp crustins are highly expressed in hemocytes (Smith et al., 2008), the newly identified variants were both highly expressed in gills and undetected in hemocytes and in low expression in other tissues examined. Variation in tissue distribution may indicate diversity of functions of crustins in shrimp immunity that needs clarification through further studies.

Crustin putative signal sequence normally comprises of 16-24 amino acids and not markedly conserved (Smith et al, 2008). Signal peptide length varies between species or between isoforms. *P. monodon* crustins have signal peptide containing 17 to 19 amino acids (Krusong et al., 2012). Signal peptide of spider crab and red king crab crustins are composed of 23 and 18 amino acids respectively (Sperstad et al., 2009). The signal peptides of *Mj*CRS type 1-5 are all composed of 18 amino acids with identical sequences (Rattanachai et al., 2004). In contrast, *Mj*CRS6 and *Mj*CRS7 contain signal sequence of 20 and 18 amino acids respectively and are composed of totally different amino acid sequences compared with *Mj*CRS types 1-5. These differences show diversity in amino acid sequences and length between crustin variants or isoforms that exist is different shrimp species.

Putative cleavage sites are usually between alanine and glycine, although in some crustins it lies between glycine and glutamine, alanine or threonine
(Smith et al., 2008). In contrast to the usual cleavage sites, the putative cleavage sites of \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) are between alanine and leucine and alanine and aspartic acid respectively. Again in this regard, the \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) are both in contrast to the previously identified \( Mj\text{CRS} \) type 1-5 where the signal cleavage site is between alanine and glycine. These differences in cleavage sites are in agreement with other shrimp crustins wherein diversity between different species or different isoforms in the same species have also been observed and evidenced (Krusong et al., 2012; Zhang et al., 2007; Rosa et al., 2007).

\( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) also contain the characteristic WAP domain and the conserved 12 cysteine residues. The WAP domain, in contrast to the signal sequence, is highly conserved and has been described in proteins containing a variety of functions (Moreau et al., 2008; Smith et al., 2008). Proteins containing WAP domain have antimicrobial and antiproteinase activities (Krusong et al., 2012; Amparyup et al., 2008b; Hagiwara et al., 2003; Sallevane, 2000). The WAP domains of \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) are composed of 49 and 51 amino acids respectively, which contain the 4DSC signature. 4DSC signatures especially in shrimps are arranged where the aspartic acid and lysine residues are positioned as follows: -C-XX-D-XX-C-XXXD-K-CC-X-D (Smith et al., 2008). 4DSC signature of \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) conform to this arrangement. The conserved 12 cysteine residues are also observed in \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \). The cysteine rich region participates in the formation of disulphide bonds (Antony et al., 2011). Cysteine residues found in the WAP domain are important in maintaining the crustin’s tertiary structures (Gross et al., 2001).

The glycine residues of \( Mj\text{CRS6} \) and \( Mj\text{CRS7} \) however are few, 13 and 27 respectively compared to \( Mj\text{CRS} \) type 1-5 that contain 44-67 glycine residues in
the glycine-rich region. Similar low glycine residues were also observed in several isoforms of *P. monodon* crustins. *P. monodon* crustin*Pm*1, crustin*Pm*5, crustin*Pm*6 and crustin*Pm*7 only contained 16, 24, 28 and 21 glycine residues respectively in contrast to crustin*Pm*1 that has 106 (Krusong et al., 2012). The low sequence similarity of *Mj*CRS6 to *Mj*CRS 1-5, only 41%, is probably due to the difference in the length of glycine-rich repeats at the amino terminus. Crustins are one of the most diverged representatives of AMP families (Tassanakajon et al., 2011). Clearly, the present results provided additional evidence that high sequence diversity within shrimp crustins in terms of lengths and primary sequences occur.

In summary, based on EST information there are 13 more possible *Mj*CRS variants and their expressions vary in different tissues. Based on tissue expression analysis, two variants that were highly expressed in gills were selected for further sequence analysis and designated as *Mj*CRS6 and *Mj*CRS7. Although differences were observed between the new variants and previous crustins, both the new variants contain the conserved characteristics of crustins. To understand more of their function in shrimp immunity, further studies are recommended.

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Chapter IV

Changes in global gene expression profile of kuruma shrimp Marsupenaeus japonicus associated with specific long dsRNA

Abstract

dsRNA is a potent regulator of gene expression. The injection of dsRNA with specific target leads to the degradation of targeted mRNA. However, there are evidences that specific dsRNAs could also lead to degradation of non-targeted mRNAs that happen to contain a cross hybridizing region (off-target effects) with the non-targeted mRNAs. The connections between immune responses and immune genes in shrimps have not been fully explored. This study was conducted to gain insights on the effect of dsCRS treatment in gene expression profile of kuruma shrimp. The components that were affected at day-2 post treatment were dissected and the possible causes in global gene expression were addressed. Specific long dsRNA was used to suppress the expression of MjCRS (dsCRS). At day-2 post treatment, 401 genes were differentially expressed by a factor of 4. 188 are upregulated and 213 are downregulated. Shrimps were also treated with non-specific dsRNA for green fluorescent protein (dsGFP). The results showed that dsGFP treated shrimps have 248 differentially expressed genes by a factor of 4. 174 are upregulated and 74 are downregulated. Several upregulated and downregulated genes were selected for RT-qPCR analysis to validate the results of microarray. Results of RT-qPCR were in agreement with the microarray
results. Sequences of dsRNA used were compared to the downregulated non-target gene to check for possible cross-hybridization to non-target mRNAs. dsCRS and dsGFP sequences were 18-59% and 21-31% similar with the downregulated non-target mRNAs respectively. The results of dsCRS treated shrimps were compared with dsGFP treated shrimps. dsCRS treatment have higher number of differentially expressed genes compared to dsGFP. Genes that are specifically and mostly upregulated by dsCRS treatment are those involved in cell cycle, DNA synthesis, repair and replication and gene expression, regulation and protein synthesis. On the other hand, genes that were specifically and mostly downregulated by dsCRS treatment are those with defense and homeostasis function. It is possible that the changes in global gene expression are due to sequence similarities or the interconnection of biological pathways and that perturbation in the system could result to the impairment of the pathways affecting expression of other genes.

A broader picture of the effect of dsRNA injection in global gene expression was elucidated. The results suggest that dsCRS treatment altered the expression of wide array of genes and provides a framework for further studies. With special focus on immune related genes, the data generated from this study may provide insight to the mechanism of shrimp immunity. On the basis of the results it would be of great importance if a detailed connections of the responses after dsRNA treatment could be generated to provide a more comprehensive understanding on the regulation of shrimp biological, physiological and immunological processes.
Introduction

RNA interference (RNAi) is an evolutionary conserved biological response to double stranded RNA (dsRNA) resulting to gene silencing (Hannon, 2002). Naturally, dsRNAs are endogenously produced by RNA-templated RNA polymerization or by hybridization of overlapping transcripts (Meister and Tuschl, 2004). dsRNA serves as a potent signaling molecule that triggers this phenomenon and was first demonstrated in plants and nematode worm, *Caenorhabditis elegans* (Fire et al., 1998; Matzke et al., 1989; Napoli et al., 1990; van der Krol et al., 1990). This homology-dependent silencing was first recognized as antiviral mechanism that protect organism from RNA viruses and transposable elements and has also been known as key player in generating antiviral immunity in arthropods (Baulcombe, 2004; Qu and Morris, 2005; Robalino et al., 2005; Robalino et al., 2004; Wang et al., 2006; Waterhouse et al., 2001; Zambon et al., 2006).

The key steps involved in the silencing pathway are the processing of dsRNA by the RNAse III enzyme dicer (Aliyari and Ding, 2009; Baulcombe, 2005; Knight and Bass, 2001) into short RNA duplexes of 21-23 nucleotides in length with symmetric 2-3 nucleotide 3’ overhangs and 5’-phosphate and 3’-hydroxyl groups known as short-interfering RNA (siRNA) (Dykxhoorn et al., 2003). siRNAs are then incorporated and bound into RNA-inducing silencing complex (RISC) (La Fauce and Owens, 2012). Through the action of RNA helicase present in RISC (Nykanen et al., 2001), siRNA will unwind producing the single-stranded RNA known as the guide strand. This activates the RISC and then searches the transcriptome for homologous or complementary mRNA sequences to bind to. The sequence-specific binding of the active siRNA cleaves
the mRNA resulting to rapid degradation of the target mRNA and subsequently, the protein for which it encodes is not produced (La Fauce and Owens, 2012; Meister and Tuschl, 2004).

Robalino and his group first reported RNAi in shrimp in *Litopenaeus vannamei* in 2004. In their study, shrimp mounted increased resistance to infection by two unrelated viruses, white spot syndrome virus and Taura syndrome virus after dsRNA treatment. Their results demonstrated that an invertebrate immune system could recognize dsRNA as a virus-associated molecular pattern, resulting in the activation of innate antiviral response. At present, RNAi technology has been increasingly used in shrimp research as a powerful tool in determining gene function, understanding biological and physiological processes and also to decipher host-pathogen interactions (Labreuche and Warr, 2013). Gene knockdown experiments revealed important function of immune related genes in shrimps. Silencing of penaeidin in *Penaeus monodon* revealed its important function as a pro-inflammatory cytokine (Li and Song, 2010). Involvement of *L. vannamei* antiliposaccharide factor (ALF1) against bacterial and fungal infection was discovered using gene knockdown experiments (de la Vega et al., 2008). The role of crustins in *L. vannamei* against *Vibrio penaeicida* infection was also elucidated by an in vivo approach using RNAi (Shockey et al., 2009). Moreover, with the application of RNAi technology, the important roles Toll and immune deficiency gene in regulating the transcription of antimicrobial peptides (AMPs) has been determined (Hou et al., 2014).

Injection of dsRNA with specific gene silences the expression of target genes. This provides a window of opportunity of studying the function of the gene of interest. The usefulness of this approach depends on the assumption that the
silencing is highly specific and that, knockdown of the targeted gene does not interfere the expression of other genes (Kulkarni et al., 2006). In mammalian and insect cells, studies showed that RNAi could lead to silencing or degradation of non-targeted mRNAs. This unintended degradation affecting expression of other genes is known as the “off-target effects”. The presence of cross hybridizing region to the siRNA trigger could result to such effect (Jackson et al., 2003; Scacheri et al., 2004). dsRNAs containing ≥19-nucleotide perfect matches could result to off-target effects (Kulkarni et al., 2006). Seven-nucleotide complementation was also found to downregulate non-targeted genes (Lin et al., 2005).

In shrimps, microarray technology has revealed important information on changes in global gene expression after gene-specific silencing. With the combination of RNAi and microarray technology, several researches have evidenced that targeted gene silencing could also affect expression of non-target genes by either decreasing or increasing their expressions. In *Marsupenaeus japonicus*, transglutaminase (TGase) silencing affected the expression of genes in shrimp and caused significant downregulation of the expressions of AMPs like crustin and lysozyme (Fagutao et al., 2012). Sequence comparison analysis, showed that the TGase dsRNA sequence used to specifically target TGase was 43% and 47% identical to lysozyme and crustin sequences respectively. Suppression of prophenoloxidase (proPO) was also shown to downregulate expression of several AMPs including penaeidin, crustin and lysozyme (Fagutao et al., 2009). Knockdown experiment of *M. japonicus* c-type lysozyme increased the expression of some putative immune related genes such as histone, double
stranded binding protein, proteases, protease inhibitor and hemocyanin (Kaizu et al., 2011).

In this study RNAi and microarray technology were used to gain further insights on the effect of specific silencing on global gene expression in shrimp. Specific long dsRNA was used to suppress the expression of *M. japonicus* crustin-like peptide (*MjCRS*). After silencing, differential gene expression were analyzed using microarray. dsRNA sequence that was used to suppress the expression of *MjCRS* was compared to the affected non-targeted genes to determine the cause of off-target effects. The results were also compared to shrimps treated with non-specific gene (green fluorescent protein – GFP) to elucidate whether the changes in gene expression after specific silencing are due to dsRNA treatment or due to the relationships in shrimp biological components.

**Materials and methods**

*Experimental shrimp*

Kuruma shrimp (*Marsupenaeus japonicus*) used in this study were purchased from a local commercial shrimp farm in Miyazaki, Japan. Shrimps with an average body weight of 14 g were used in the experiment. Prior to all experimental procedures shrimps were kept for 5 days in artificial seawater with salinity of 30 ppt, temperature ranging from 22-25 °C and fed daily with a commercial diet.

*Production of double-stranded RNA and in vivo silencing*

Double-stranded RNAs (dsRNAs) were generated *in vitro* using T7 RiboMAX™ Express Large Scale RNA Production System (Promega, U.S.A.)
following the manufacturer’s instructions. Primers used to generate dsRNA specific for *M. japonicus* crustin-like peptide (*Mj*CRS) and non-specific green-fluorescent protein (GFP) were listed in Table 1. Templates for the generation of single-stranded RNAs (ssRNAs) for specific and non-specific genes were amplified by PCR in a 20 µl reaction volume containing 2 µl of 10X Ex Taq buffer, 1.6 µl of 2.5 mM dNTP mix, 0.4 µl of 10 µM of each of the primers, 0.1 µl of Ex Taq DNA polymerase (TaKaRa, Japan). The reaction was amplified with the following thermal profile: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min. Five microliters of PCR products were electrophoresed and visualized on a 1% agarose gel electrophoresis. The resulting PCR products were purified using Amicon® Ultra-0.5 (Millipore, Ireland). Single-stranded RNAs were created by incubation at 37 °C for 30 min. Then, equal amounts of ssRNAs were mixed and annealed by incubations at 70 °C for 10 min. Double stranded RNAs were treated with RQ1 RNase-Free DNase (Promega, U.S.A.), purified using phenol:chloroform:isoamyl alcohol (25:24:1) extraction and quantified in NanoDrop Lite spectrophotometer (Thermo Scientific, U.S.A.). Formations of dsRNAs were visualized in 1% agarose gel electrophoresis.

A total of 16 shrimps were used in the *in vivo* silencing study. For specific silencing, 8 shrimps were intramuscularly injected with 100 µl solution containing either *Mj*CRS specific dsRNA (dsCRS, n=4) at a concentration of 1 µg/g shrimp or phosphate buffer saline (PBS, n=4) that served as negative control. For non-specific dsRNA treatment, 8 shrimps were also used and were intramuscularly injected with 100 µl solution containing either non-specific dsRNA for GFP
(dsGFP, n=4) at a concentration of 3 µg/g shrimp or PBS (n=4) as negative control.

**Hemocyte collection**

Two days after dsCRS, dsGFP or PBS treatment, hemolymph was drawn out from the ventral sinus using a sterile 2.5 ml syringe and 23Gx1 1/4" needle with 1 ml of pre-cooled anticoagulant (0.82% NaCl, 0.55% citric acid, 1.98% glucose and 0.88% sodium citrate, pH 5.6). After centrifugation at 3000 x g for 3 min at 4 °C plasma was discarded. The hemocytes were washed with 1X PBS and resuspended in 500 µl of RNAiso Plus (TaKaRa, Japan).

**Total RNA isolation**

Immediately after collection, hemocytes were homogenized in RNAiso Plus (TaKaRa, Japan). Total RNAs were extracted and purified using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer’s instructions. The integrity of the purified total RNAs were checked using Agilent 2200 TapeStation (Agilent, U.S.A.) system in Agilent R6K ScreenTape (Agilent, U.S.A.). Ribosomal RNA (rRNA) ratio (28S/18S) and RNA integrity number (RIN²) was used to assess intactness and the integrity of total RNA. Acceptable values were defined as: rRNA ratio (28S/18S) > 0.9 and RIN² value > 8.0.

**cRNA labeling and hybridization**

Two hundred nanograms of total RNA was reversed transcribed to cDNA during which a T7 sequence was introduced into cDNA. The cDNA was converted into labeled complementary RNA (cRNA) with nucleotides coupled to
a fluorescent dye, cyanine 3 (Cy3) using the Low Input Quick Amp Labeling Kit (version 6.6 protocol) (Agilent Technologies, U.S.A.). It was then purified using Agilent RNeasy Mini Kit (Qiagen, Germany) and the quality and quantity of the resulting labeled cRNA was assessed using a NanoDrop Lite spectrophotometer (Thermo Scientific, U.S.A.). A total of 16 individually labeled cRNA were used for hybridization. Equal amounts of Cy3 labeled (1.5 µg) cRNA were hybridized to an 8 × 15 microarray format for *M. japonicus* high-density oligonucleotide microarray chip containing 13,875 probe sets (120424_Mj_v3) for 17 hours at 65 °C. The hybridized microarrays where then washed following the manufacture’s recommended conditions and scanned using an Agilent G2565CA DNA Microarray Scanner (Agilent Technologies, U.S.A.).

**Microarray data collection and analysis**

Data were extracted from the scanned image using Agilent Technologies’ Feature Extraction Software version 8.5 (FE8.5). Statistical analysis and visualization were all performed using GeneSpring GX software (Agilent Technologies, U.S.A.). Mean PBS values were used as control for dsCRS and dsGFP treated groups. Differences were calculated using ANOVA and considered significant at *P* < 0.05. To determine changes in gene expression after dsCRS or dsGFP treatments, only genes that are either upregulated or downregulated by 4-fold were selected and considered differentially expressed. Genes that are differentially expressed after dsCRS or dsGFP treatments were compared to determine genes that are specifically affected by dsCRS, both dsCRS and dsGFP or dsGFP. Classifications of gene functions were based in *P. monodon* studies of Tassanakajon et al., 2013 and Pongsomboon et al, 2011.
Sequence comparison of dsRNA sequences to differentially expressed genes

The sequences of dsRNAs used in the study were compared to the differentially expressed genes to check sequence similarities or determine nucleotide matches that could possibly result to cross hybridization. Multiple sequence comparison was done using Geneious R6 (Biomatters Limited, New Zealand).

cDNA synthesis

First strand cDNA was generated using M-MLV Reverse Transcriptase (Invitrogen, U.S.A.) in a 20 µl reaction volume containing 1 µg of total RNA, 10 mM oligo (dT) 12-18, 10 mM dNTP, 5X First-Strand Buffer, 0.1 M DTT, 40 U of RNAseOUT™ recombinant ribonuclease inhibitor and 200 U M-MLV reverse transcriptase. The reaction was incubated at 37 °C for 1 hour followed by an inactivation step at 70 °C for 15 min.

Quantitative real-time PCR

Real-time quantitative PCR (RT-qPCR) was used to validate selected data from microarray. Several upregulated (HSP90 - heat-shock protein 90; c-type lectin; HHAP - hemocyte homeostasis associated protein and ALF - antilipopolysaccharide factor) and downregulated (serpin6 - serine protease inhibitor 6 and SP - serine protease) genes from dsCRS treated group were selected from the microarray data to validate results. In addition, MjCRS silencing were also checked. Primers were designed using Primer Express 3 (Applied Biosystems, U.S.A.) (Table 1) and RT-qPCR was carried out in Applied Biosystems 7300 real-time PCR system (Applied Biosystems, U.S.A.). Amplifications were performed
in a 96-well plate in a 20 µl reaction volume containing 1× THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan), 0.3 µM each of the gene-specific forward and reverse primers (Table 1), 1× ROX reference dye and 2 µl of cDNA. The relative expressions were determined by $2^{ΔΔCt}$ method and were used to validate results of the microarray data and presented as relative expression ratio. Elongation factor-1 alpha (EF-1α) was used as reference gene for all data.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MjCRS-F1</td>
<td>ATGAAGGGCTTCAAAGCGGT</td>
</tr>
<tr>
<td>MjCRS-R1</td>
<td>TTATCCGAAAAGAGGTTC</td>
</tr>
<tr>
<td>T7MjCRS-F1</td>
<td>TAATACGACTCACTATAGGGATGAAGGCTTCAAAGCGGT</td>
</tr>
<tr>
<td>T7MjCRS-R1</td>
<td>TAATACGACTCACTATAGGGTTATCCGAAAGAGGTTC</td>
</tr>
<tr>
<td>GFP-F1</td>
<td>ATGGTGAGCAAGGGCGAGGA</td>
</tr>
<tr>
<td>GFP-R1</td>
<td>TTACTTGACAGCTCGTCA</td>
</tr>
<tr>
<td>T7GFP-F1</td>
<td>TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA</td>
</tr>
<tr>
<td>T7GFP-R1</td>
<td>TAATACGACTCACTATAGGGTTACTTGTCAGCTCGTC</td>
</tr>
<tr>
<td>MjCRS qPCR-F2</td>
<td>AACTACTGCTGCAGGAAGGTCTCA</td>
</tr>
<tr>
<td>MjCRS qPCR-R2</td>
<td>GGCAGTCCAGTGCTGGA</td>
</tr>
<tr>
<td>Serpin6 qPCR-F2</td>
<td>GGGCGGAGGTGACATG</td>
</tr>
<tr>
<td>Serpin6 qPCR-R2</td>
<td>CCTGCGAAGACCTATCTTTAAAGA</td>
</tr>
<tr>
<td>SP qPCR-F2</td>
<td>TGTCGCGCGCGTGATC</td>
</tr>
<tr>
<td>SP qPCR-R2</td>
<td>GCCAAGCAAGCAGCACCATCCA</td>
</tr>
<tr>
<td>HSP90 qPCR-F2</td>
<td>TGCATCGTCACTCCAGTCCAGTA</td>
</tr>
<tr>
<td>HSP90 qPCR-R2</td>
<td>GGGCCTGAGCCCTTCATGA</td>
</tr>
<tr>
<td>HHAP qPCR-F2</td>
<td>TCATGCAAACAGCGACGTGA</td>
</tr>
<tr>
<td>HHAP qPCR-R2</td>
<td>GGCGGCTCATCGCTCTT</td>
</tr>
</tbody>
</table>
Results

Identification of differentially expressed genes after dsCRS or dsGFP treatment

Global expression analysis of mRNA expression profiles from hemocytes of dsCRS or dsGFP treated *M. japonicus* was conducted. After dsCRS treatment, among the 13,875 target genes in the array, globally, 401 (2.9%) genes were differentially expressed. 188 were upregulated in which known genes (with BLAST hits) identified were 184 while unknown genes were 4. 213 genes were downregulated in which 207 and 7 genes were known and unknown respectively (Table 2). On the other hand, out of 13,875 target genes, dsGFP treatment resulted to a total of 248 (1.9%) differentially expressed genes. 174 genes were upregulated in which 170 were known and 4 were unknown. Only 74 genes were downregulated in which 72 were known while only 2 were unknown (Table 2).

The number of differentially expressed genes was higher after dsCRS treatment compared to dsGFP treatment. dsCRS treatment also resulted to higher number of downregulated (213) than upregulated genes (188). This is in contrast to dsGFP treatment where upregulated genes (174) were higher compared to downregulated genes (74).
Table 2. Overview of differentially expressed genes in *M. japonicus* hemocytes after dsCRS or dsGFP treatment.

<table>
<thead>
<tr>
<th>Sampling period/treatments</th>
<th>d2 dsCRS</th>
<th>d2 dsGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of spotted genes</td>
<td>13,875</td>
<td>13,875</td>
</tr>
<tr>
<td>Differentially expressed genes</td>
<td>401</td>
<td>248</td>
</tr>
<tr>
<td>Upregulated genes (&gt;4-fold change)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known genes</td>
<td>184</td>
<td>170</td>
</tr>
<tr>
<td>Unknown genes</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Downregulated genes (&gt;4-fold change)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known genes</td>
<td>206</td>
<td>72</td>
</tr>
<tr>
<td>Unknown genes</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

Validation of microarray results by RT-qPCR

RT-qPCR was used to validate selected values from microarray after dsCRS treatment. Expression levels that were validated included HSP90, c-type lectin, HHAP, ALF, serpin6, SP and *Mj*CRS. The samples that were used in the RT-qPCR were the same as in the microarray study. Gene expression analysis by RT-qPCR showed that HSP90, c-type lectin, HHAP and ALF were significantly upregulated (Fig. 1). On the other hand serpin6 and SP were significantly downregulated (Fig. 2). The results of the RT-qPCR analysis were consistent with the results obtained from the microarray.

![Fig. 1. Gene expression analysis of upregulated genes based on microarray results. Relative expressions were determined by 2^{ΔΔCt} method using EF-1α as reference gene and presented as relative expression ratio. The data were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 4 individual shrimps. Asterisks indicate significant differences between tissues (*P < 0.05).](image-url)
HSP90 - heat-shock protein 90; HHAP - hemocyte homeostasis associated protein and ALF - antilipopolysaccharide factor.

Fig. 2. Gene expression analysis of downregulated genes based on microarray results. Relative expressions were determined by $2^{-\Delta\Delta C_t}$ method using EF-1α as reference gene and presented as relative expression ratio. The data were analyzed by one-way ANOVA and presented as mean ± S.E. calculated from 4 individual shrimps. Asterisks indicate significant differences between tissues (*$P < 0.05$).

**Sequence comparison of dsRNA sequences to differentially expressed genes**

Sequences of the dsRNAs used were compared with the downregulated non-target gene sequences to check sequence similarity and determine possible cross-hybridization resulting to downregulation. dsCRS sequence that was used to silence *Mj*CRS were 18 to 59 % similar with the downregulated non-target genes. On the other hand dsGFP sequence with no specific target were 21 to 31% similar with the downregulated non-target genes.

**Genes affected by dsCRS, both dsCRS and dsGFP or dsGFP**

In order to find relevant information from the effect of specific and non-specific dsRNA treatment in global gene expression, genes that are differentially expressed after dsCRS and dsGFP treatments were compared. Genes that are specifically affected by dsCRS, both dsCRS and dsGFP and dsGFP were grouped
and analyzed. Comparison and analysis of the differentially expressed genes after dsCRS and dsGFP treatment revealed that 294 (106 upregulated and 188 downregulated) and 141 (92 upregulated and 49 downregulated) were specifically affected by dsCRS and dsGFP respectively. 107 (82 upregulated and 25 downregulated) genes were affected both by dsCRS and dsGFP (Table 3).

**Table 3. Number of genes specifically affected by dsCRS, both dsCRS and dsGFP or dsGFP**

<table>
<thead>
<tr>
<th></th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dsCRS</td>
</tr>
<tr>
<td><strong>Number of genes</strong></td>
<td>294</td>
</tr>
<tr>
<td><strong>Upregulated (&gt;4-fold change)</strong></td>
<td></td>
</tr>
<tr>
<td>Known</td>
<td>106</td>
</tr>
<tr>
<td>Unknown genes</td>
<td>104</td>
</tr>
<tr>
<td><strong>Downregulated (&gt;4-fold change)</strong></td>
<td></td>
</tr>
<tr>
<td>Known</td>
<td>188</td>
</tr>
<tr>
<td>Unknown genes</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

**Genes specifically affected by dsCRS treatment**

 Majority of the genes upregulated specifically by dsCRS treatment were those involved in cell cycle, DNA synthesis, repair and replication followed by genes with functions on gene expression, regulation and protein synthesis, signaling and communication. Several genes involved in defense and homeostasis including antilipopolysaccharide factor, heat shock cognate 70 protein, mannose-binding protein, low density lipoprotein receptor-related protein isoform cra_a, protease inhibitor epi11 and transferrin were also upregulated. Other genes that were upregulated are those involved in development and differentiation, structural and cytoskeleton related proteins, reproduction, transport and energy and metabolism.

In contrast, a significant number of genes that are specifically downregulated by dsCRS treatment are those involved in defense and homeostasis like c-type lysozyme, crustin I, serine protease, serine protease
easter, c-type lectin, ficolin 1-like, protein NLRC5, annulin (homologous to transglutaminase), chitinase 1 and its precursor, alkylglycerol monooxygenase, cklf-like marvel transmembrane domain-containing protein 4-like, glutathione s-transferase m, laccase 1, fibrinogen and fibronectin and glutaredoxin duf547 domain-containing protein. These are followed by genes that have functions on gene expression, regulation and protein synthesis, transport, signaling and communication, structural and cytoskeleton related proteins. Genes that have roles on reproduction, neurosensory and endocrine system, DNA repair and energy and metabolism were also downregulated.

**Genes specifically affected by dsGFP treatment**

While the majority of genes upregulated by dsCRS were involved in cell cycle, DNA synthesis, repair and replication, a considerable amount of genes that were specifically upregulated by dsGFP are those that have functions on gene expression, regulation and protein synthesis followed by cell cycle, DNA synthesis, repair and replication and transport. Important RNAi related genes were also upregulated like argonaute 2, sid-1 like protein, helicase mov-10 and dicer 2. Expressions of several immune related genes were also upregulated like caspase-2 and e3 ubiquitin ligase. Genes involved in growth and reproduction and signaling and communication are also increased. Genes with roles on cell growth and survival, neurosensory and endocrine system, energy and metabolism and structural and cytoskeleton related proteins were least affected.

dsGFP treatment have been known to trigger antiviral immune response by stimulating antiviral immune genes. However, it also downregulated some important immune related genes like ALF2, ALF6, chitinase 5, c-type lectin like
protein and cathepsin C. Only a few number of genes were downregulated including those involve in gene expression, regulation and protein synthesis, energy and metabolism and transport.

**Genes both affected by dsCRS and dsGFP treatment**

Several immune related genes were upregulated by both dsCRS and dsGFP treatments. These include caspase apoptosis-related cysteine peptidase, caspase nc-like, calreticulin, HSP90 and hemocyte homeostasis-associated protein. These are followed by genes involved in gene expression, regulation and protein synthesis, cell cycle, DNA synthesis, repair and replication, signaling and communication and neurosensory and endocrine system.

dsCRS designed to target *Mj*CRS effectively silenced *Mj*CRS type 1-5 by 498-563 fold. Interestingly, dsGFP treatment also downregulated *Mj*CRS type 1-5 by 5-6 fold, much lower compared to dsCRS treated shrimps. Both the dsCRS and dsGFP treatment also affected a gene that is involved in defense, the serpin6. Genes with function on energy and metabolism, gene expression, regulation and protein synthesis, signaling and communication and structural and cytoskeleton related protein were also downregulated.

**Discussion**

Microarray technology was used to determine the changes in global gene expression by checking differentially expressed genes in shrimp after treatment with long dsRNA with (dsCRS) and without (dsGFP) specific targets. In general, organism's biological system is composed of pathways or networks. In biological network, there are molecules that work independently and there are molecules
that work in connection with other molecules in the pathway or share the same pathway but results to different endpoints. In mammalian cell line experiments like perturbations or treatment with small bioactive molecules (i.e., drugs, compounds sharing same molecular targets, molecules that act on gene expression, molecules without targets, disease, and inhibitory RNAs), it has been evidenced through the use of connectivity map that there are connections among small molecules sharing a mechanism of action, chemical and physiological processes (Lamb et al., 2006). 175 dsRNA-stimulated genes and 95 dsRNA-repressed genes were observed in dsRNA treated GRE cells demonstrating interconnections among disparate signaling pathway (Geiss et al., 2001).

The observations mentioned above have also been observed in crustaceans. In this study, perturbation through the introduction of dsRNA with or without specific targets resulted in a considerable number of differentially expressed genes. At day-2 post-treatment with dsCRS (with specific target, MjCRS), 401 genes were differentially expressed with higher number of downregulated compared to upregulated genes. In shrimps, genetic perturbation by introduction of dsRNA with specific targets has been shown to affect global gene expression. Transglutaminase silencing in M. japonicus resulted to 624, 280 and 507 differentially expressed genes at day-1, -3 and -5 post-treatment respectively (Fagutao et al., 2012). Similarly, silencing of prophenoloxidase in M. japonicus resulted to 1042 and 965 differentially expressed genes at day-1 and -3 respectively (Fagutao et al., 2009). Injections of dsRNA with specific targets have been shown to affect global gene expression.

The effect of non-specific silencing on global gene expression by injection of dsGFP was also examined. Similar to specific silencing, global gene expression
was also affected. 294 genes were differentially expressed and are much lower compared to genes affected by specific silencing. Interestingly, in contrast to specific silencing, the number of upregulated genes is much higher compared to downregulated genes. In agreement to previous studies, the present results showed that specific and non-specific silencing could affect gene expression and provided additional proof that perturbations in biological system of shrimps by RNAi could result to changes in global gene expression. These results also show possible connection in shrimp biological, molecular, physiological or immunological pathways and that disturbing one important molecule could affect expression or regulation of other molecules.

Off-target effects have been evidenced in several studies. Several reports have suggested that non-specific effects can be induced by long dsRNA or siRNA. In *Drosophila melanogaster*, long dsRNAs containing ≥19-nucleotide perfect matches to unintended targets may contribute to off-target effects (Kulkarni et al., 2006). In addition, siRNA-mediated silencing could also result to off-target effects by a 7-nucleotide complementation (Lin et al., 2005). Downregulation of unintended targets have also been observed in *M. japonicus* (Fagutao et al., 2012). In their study, silencing of transglutaminase downregulated several AMPs including lysozyme and crustin. Alignment analysis of transglutaminase dsRNA sequence showed that it is 43% identical to lysozyme and 47% identical to crustin sequences. Although the sequences do not contain several nucleotide perfect complementations with the affected unintended targets, repression has been observed. In addition to sequence similarity as a possible reason why these AMPs are downregulated, they also speculated that transglutaminase may be involved in the regulation of AMP and that its absence affected lysozyme and crustin.
In the present results, sequences of dsRNA used were also compared to the downregulated non-target genes. Results showed 18 to 59% (dsCRS) and 21 to 31% (dsGFP) sequence identities between dsRNAs and the unintended targets. In addition, correlations with identity and downregulation were not observed. In this regard, it may questionable to speculate that the observed off-target effects after dsRNA treatment are due to sequence similarities, however, the possibility cannot be disregarded. It is also possible as mentioned above that the downregulation/off-target effects are due to interconnections in the pathway regulating the affected genes.

dsRNA injection has been shown to affect global gene expression, however, this observation poses some questions whether the effects are due to the dsRNA itself or the perturbation in the biological system caused by the dsRNA. Are these changes detrimental or beneficial to shrimp’s overall biological status? The genes that are upregulated and downregulated by either dsCRS or dsGFP were grouped to determine which genes are specifically affected by dsCRS, both dsCRS and dsGFP and dsGFP only. The majority of genes upregulated specifically by dsCRS are involved in cell cycle, DNA synthesis, repair and replication followed by genes with functions on gene expression, regulation and protein synthesis, signaling and communication. In Chapter II results, \textit{Mj}CRS silenced shrimps survived provided that there is no infection. These observations regarding the upregulated genes showed that silencing of \textit{Mj}CRS possibly stimulated important biological functions to maintain molecules important for survival and normal functioning.

In addition to the downregulated expression of \textit{Mj}CRS, dsCRS treatment also downregulated a number of important immune related genes including c-
type lysozyme, crustin I, serine protease, serine protease easter, c-type lectin, ficolin 1-like, protein NLRC5, annulin (homologous to transglutaminase), chitinase 1 and its precursor, alkylglycerol monooxygenase, cklf-like marvel transmembrane domain-containing protein 4-like, glutathione s-transferase m, laccase 1, fibrinogen and fibronectin and glutaredoxin duf547 domain-containing protein. Can these downregulation compromise the shrimp’s health stability? The genes downregulated by dsCRS have been identified in shrimps and other organisms. Their important roles in immune response have been previously studied.

C-type lysozyme is an important shrimp AMP that catalyzes glycosidic linkages in bacterial cell wall and has a broad antibacterial activity (Tassanakajon et al., 2013). In *M. japonicus*, its absence resulted in increased proliferation of Gram-negative bacteria in the hemolymph (Kaizu et al., 2011). Lysozyme was also found to be upregulated in white spot virus-resistant *L. vannamei* indicating its possible role against viral infection (Zhao et al., 2007). Serine proteases are part of shrimp proteolytic cascades that are one of the key components of the shrimp immune system performing multifunctional antimicrobial role and are also involved in apoptosis and melanization (Tassanakajon et al., 2013). Clip domain serine proteinases (clip-SPs) and their homologs (clip SPHs) are involved in the shrimp innate immunity (Jiang and Kanost, 2000). Serine proteases are upregulated upon pathogen infection and have been shown to have activity against Gram-positive bacteria and have binding activity to *V. harveyi* and LPS (Jitvaropas et al., 2009; Ren et al., 2009; Ren et al., 2011). Recombinant clip-SPHs have been shown to display cell adhesion activity (Lin et al., 2006). SPH516 specifically interacts with the metal ion-binding (MIB) domain of YHV,
suggesting that it might play an important role in viral infection (Sriphaijit et al., 2007). Ficolin 1-like is a pattern recognition protein and found to be upregulated by WSSV and YHV infections in *P. monodon* indicating its possible involvement against viral infections (Pongsomboon et al., 2011). NLRC5 is another pattern recognition receptor implicated in innate immunity to viruses potentially by regulating interferon activity (Cui et al., 2010; Kuenzel et al., 2010; Neerincx et al., 2010). Annulin is homologous to the transglutaminase (Singer et al., 1992). Transglutaminase in shrimp is involved in blood coagulation and its absence can render shrimp susceptible to both bacterial and viral infections (Fagutao et al., 2012; Maningas et al., 2008). Chitinases are essential enzymes in crustaceans for molting and digestion of foods containing chitin. Although involved in molting and in digestion, chitinases and its precursor are found to be upregulated in WSSV resistant *P. japonicus* (Pan et al., 2005), WSSV and YHV infected *P. monodon* (Pongsomboon et al., 2011) indicating their important roles in viral infections. Glutathione s-transferase mu is an oxidative enzyme involved in fighting oxidative stress. It was found to be upregulated by a variety of infections including WSV, YHV and *Vibrios* (Pongsomboon et al., 2011). Laccases are copper-containing oxidase enzymes that are found in many plants, fungi, and microorganisms. Gene profiling showed that laccase was upregulated in white spot virus-resistant *L. vannamei* (Zhao et al., 2007). Fibrinogens are family of protein acting as pattern recognition receptors. It has a wide antimicrobial activity and can bind to PGs, LPS, bacteria and VP28 of WSSV (Chai et al., 2012). Clearly, the downregulated immune related genes after dsCRS treatment are important in shrimp immunity and may indicate that silencing of *MjCRS* might compromise the shrimp’s immune status against microbial infection.
In shrimps, treatment with non-specific dsRNA trigger or activates antiviral immunity (Robalino et al., 2005; Robalino et al., 2004). In contrast to the upregulated genes in dsCRS treated shrimps that are mainly involved in cell cycle, DNA synthesis, repair and replication, dsGFP upregulated genes that have important roles in gene expression, regulation and protein synthesis. This is of significant importance as it has been evidenced that non-specific injection of dsRNA in shrimps could result to resistance against pathogens. In *L. vannamei*, non-specific dsRNA treatment showed increased resistance to infection by two unrelated viruses, white spot syndrome virus and Taura syndrome virus (Robalino et al., 2004).

Interestingly, dsGFP downregulated several important immune related genes like ALF2, ALF6, chitinase 5, c-type lectin-like protein and cathepsin C. In previous researches, only a certain degree of protection or resistance can be elicited by non-specific dsRNA injection. The suppression of the above mentioned immune related genes might be one of the reasons why protection acquired after non-specific dsGFP treatment was only partial. Although the present results provided very basic information, the link that connects the non-specific dsRNA treatment and disease resistance needs to be explored further. These observations provided some information on what are the possible molecules activated/induced in the shrimp system that results to resistance.

Genes that are both effected by dsCRS and dsGFP were analyzed. Both treatments upregulated the following genes with putative immune function: caspase apoptosis-related cysteine peptidase, caspase nc-like, calreticulin, HSP90, and hemocyte homeostasis-associated protein. Caspases play an important role in the different stages of programmed cell death or apoptosis. *M. japonicus* caspase
(PjCaspase) was found to be significantly upregulated in survivors of WSSV challenged shrimp and in its absence, the WSSV induced apoptosis was significantly inhibited coupled with increased viral copy number indicating its important role in antiviral immunity (Wang et al., 2008). The immune response of HrCas8 and HrCas3, two caspases in red abalone, Haliotis rufescens, was evaluated in response to V. anguillarum and were both increased after infection (Chavez-Mardones and Gallardo-Escarate, 2014). Calreticulin plays important roles in Ca$^{2+}$ homeostasis and molecular chaperoning. WSSV infection upregulated calreticulin protein in L. vannamei and F. chinensis inferring its potential as an antiviral gene (Luana et al., 2007; Wang et al., 2007). Heat shock proteins function as molecular chaperones and are expressed when shrimps are exposed to physiologically stressful conditions. It has been evidenced that M. japonicus HSP90 (MjHSP90) respond to temperature stress and WSSV challenge suggesting its various role against stress and viral infection (Danwattananusorn et al., 2011).

Regarding the important immune related genes that are both specifically downregulated by dsCRS and dsGFP, only one gene with important immune function was downregulated, the serpin6. Serpin is a family of high structural conserved inhibitors that act as suicide like-substrates (Huntington, 2011). They are localized in the hemocytes and expressed after pathogen challenges (Homvises et al., 2010; Liu et al., 2009; Somnuk et al., 2012).

Taken together, a broader picture of the effect of dsRNA injection in global gene expression was elucidated. The results suggest that dsCRS treatment altered the expression of wide array of genes and provides a framework for further studies. With special focus on immune related genes, the data generated
from this study may provide insight to the mechanism of shrimp immunity. On the basis of the results it would be of great importance if detailed connections of the responses after dsRNA treatment could be generated to provide more comprehensive understanding on the regulation of shrimp biological, physiological and immunological processes.

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Chapter V

General conclusion

Shrimps provide considerable contribution as source of food for human consumption. Majority of its production are sourced from cultured species. At present, pathogenic microorganism endangering its sustainability is challenging the shrimp aquaculture industry. It is therefore important to understand the shrimp immune system, its responses and important molecules involved in combatting the most deadly diseases. In this research, the shrimp immune response against microbial infections, the molecules involved and responses to treatments were studied. By incorporating high-throughput molecular approaches like RNAi technology, utilization of EST information and microarray technology, additional information regarding shrimp immune responses was elucidated.

The results have provided evidences on the role of \textit{Mj}CRS in shrimp immunity against microbial infection. Here we showed that introduction of dsRNA specific to \textit{Mj}CRS could silence its expression. In addition to silencing, dsRNA treatment also decreased the THCs and that subsequent infection further decreased THCs. It was also determined that the absence of \textit{Mj}CRS would make the shrimps highly susceptible to the pathogenic Gram-negative bacteria, the \textit{V. penaeicida}. We have also provided evidence that \textit{Mj}CRS may not be an important AMP against viral infection specifically to WSSV. \textit{Mj}CRS levels were significantly decreased in early stages, however, increased and the level was
maintained in later stages of *V. penaeicida* infection suggesting that it is effectively fighting the infection. On the contrary, *Mj*CRS was only transiently increased in early stages of WSSV infection and diminished in later stages of infection indicating that *Mj*CRS may not have important function against viral infection. We also showed that VP28 significantly increased in similar level with the absence or presence of *Mj*CRS in WSSV infected shrimps. With these results, the antimicrobial role of *Mj*CRS was clarified and showed that RNAi technology was an effective approach in determining gene function. These important findings have provided additional knowledge and better understanding of the shrimp immune system.

Thirteen possible *Mj*CRS variants were identified from EST information. Two new *Mj*CRS variants, *Mj*CRS6 and *Mj*CRS7 were further studied and characterized. Both of the new variants were interestingly highly expressed in gills which is in contrast to previously identified crustins whose expressions were highly observed in hemocytes. *Mj*CRS6 and *Mj*CRS7 contained signal sequences and WAP domain with 4DSC signature. In between the signal sequence and WAP domain, glycine and cysteine-rich regions were also detected. The results showed the presence of more than five variants of *Mj*CRS and may indicate the diversity of its sequences in a single organism. The discovery of additional *Mj*CRS variants showed that myriads of immune molecules are present in shrimps and several variants or isoforms exist.

We have also provided insights on shrimp’s response to perturbations. By introduction of dsRNA and application of microarray technology, the changes in global gene expression were determined. Here we showed that dsRNA treatment with or without specific target would result to changes in global gene expression.
At present, the exact reasons are unclear why such changes occurred. It is speculated that the changes are either due to sequence similarities or connections in the biological and signaling pathways and that perturbation of one important molecule could subsequently affect other genes. This experiment provided additional insights that perturbation in the shrimp’s system could affect the global gene expression.

Taken together, the studies conducted provided additional understanding on the essential role of AMPs in the shrimp immune response. This work also provided evidences on the existence of more than 5 variants of MjCRS offering an opportunity for further immune function studies. Furthermore, our results also showed the effects of dsRNA treatment in global gene expression and discovered potential connection of MjCRS with other molecules, thus, providing further information on the complex interconnection in the shrimp system.