Fish & Shellfish Immunology 47 (2015) 572–581

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

YHV-responsive gene expression under the influence of *Pm*Relish regulation

Suwattana Visetnan [a], Premruethai Supungul [a, b], Sureerat Tang [a, b], Ikuo Hirono [c], Anchalee Tassanakajon [a], Vichien Rimphanitchayakit [a, *]

[a] *Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand*

[b] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 10120, Thailand*

[c] *Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo 108-8477, Japan*

ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form
28 September 2015
Accepted 29 September 2015
Available online 3 October 2015

Keywords:
Relish
Suppression subtractive hybridization
Yellow head virus
Penaeus monodon
Antimicrobial proteins

ABSTRACT

In animals, infection by Gram-negative bacteria and certain viruses activates the Imd signaling pathway wherein the a NF-κB transcription factor, Relish, is a key regulatory protein for the synthesis of antimicrobial proteins. Infection by yellow head virus (YHV) activates the Imd pathway. To investigate the expression of genes involved in YHV infection and under the influence of *Pm*Relish regulation, RNA interference and suppression subtractive hybridization (SSH) are employed. The genes in forward library expressed in shrimp after YHV infection and under the activity of *Pm*Relish were obtained by subtracting the cDNAs from YHV-infected and *PmRelish*-knockdown shrimp with cDNAs from YHV-infected shrimp. Opposite subtraction gave a reverse library whereby an alternative set of genes under YHV infection and no *PmRelish* expression were obtained. Nucleotide sequences of 252 and 99 cDNA clones from the forward and reverse libraries, respectively, were obtained and annotated through blast search against the GenBank sequences. Genes involved in defense and homeostasis were abundant in both libraries, 31% and 23% in the forward and reverse libraries, respectively. They were predominantly antimicrobial proteins, proteinases and proteinase inhibitors. The expression of antimicrobial protein genes, *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5* were tested under *PmRelish* silencing and Gram-negative bacterium *Vibrio harveyi* infection. Together with the results using YHV infection previously reported, the expression of *penaeidin5* and also *penaeidin3* but not *ALFPm3* and *crustinPm1* were under the regulation of *Pm*Relish in the Imd pathway.

1. Introduction

Humans and animals alike are prone to be infected by disease-producing entities, the pathogens, which lead to sickness and sometimes death. However, nature has provided them means of immunity to defense against these pathogens. Besides the adaptive immunity found in vertebrate animals, innate immunity is the basic defense against pathogens in all animals. Once infected by the pathogens, the innate immunity recognizes certain characteristics of the pathogens though pattern recognition proteins. The interaction between pattern recognition proteins and pathogens leads to triggers in probably several signaling pathways to prepare the animals for the pathogenic invasion and for several cellular responses, such as defense responses, stress responses, inflammation, cell proliferation, etc. [1–3].

In terms of defense responses, there are two main defense signaling pathways, the Toll and Imd pathways, that respond to different types of pathogens. The Toll pathway is activated by Gram-positive bacteria and fungi whereas the Imd pathway is activated by Gram-negative bacteria. The invasion triggers these two pathways through the binding of activated pattern recognition proteins or other downstream activated carrier proteins to the cell membrane receptors. The receptors are activated and relayed the signal into the cytoplasm through a series of protein components. The cytoplasmic inactive NF-κB transcription factors are activated and translocate into the nucleus to regulate the expression of

* Corresponding author.
E-mail address: kpvr2@yahoo.com (V. Rimphanitchayakit).

http://dx.doi.org/10.1016/j.fsi.2015.09.053
1050-4648/

antimicrobial protein genes. The antimicrobial proteins are synthesized and secreted into the circulation to counter attack and directly kill the pathogens. It is presumed that different antimicrobial proteins are expressed in the two signaling pathway responses [2–4].

In shrimp and other crustaceans, it is certain that Toll and Imd signaling pathways exist for the related protein components of the pathways, such as the Toll receptors from *Penaeus monodon* and *Litopenaeus vannamei* [5,6], MyD88 from *Fenneropenaeus chinensis* [7], Cactus from *F. chinensis* [8], and Dorsal from *L. vannamei* [9] in Toll pathway, the IMDs from *L. vannamei* and *F. chinensis* [10,11] and Relish from *L. vannamei* and *F. chinensis* [12,13] in Imd pathways, have been identified. The two pathways are responsive to bacterial infection for the pathway protein members are up-regulated in response to bacterial infection. The response to white spot syndrome virus (WSSV) infection has been reported. The LvTolls, LvDorsal and LvRelish from Pacific white shrimp, the FcIMD from Chinese white shrimp and the PcIMD from crayfish are up-regulated after WSSV infection [6,11,14]. Moreover, the WSSV replication seems to partly rely on the activation of LvDorsal and LvRelish or, in other words, the WSSV gains certain benefit in their multiplication from the Toll and Imd pathways [14].

We have shown previously that the expression of *Pm*Relish, a NF-κB transcription factor in the Imd pathway from the black tiger shrimp, was up-regulated upon *Vibrio harveyi*, YHV and WSSV challenges [15]. The up-regulation is relatively stronger with bacterial infection than viral infection. Furthermore, the shrimp are more susceptible to YHV if the defense response from the Imd pathway is absence. Therefore, the Imd pathway is responsive to the YHV infection. In this study, we further explore the consequence of YHV infection in shrimp to fish out genes that are under the influence of the Imd pathway using suppression subtractive hybridization (SSH) technique. The expression of some genes identified from the SSH libraries is tested to confirm that it is under the effect of *Pm*Relish activation by YHV. The expression of some antimicrobial proteins is also investigated under infection by Gram-negative bacterium, a bacterium that activate the Imd pathway, to determine whether the expression is under the *Pm*Relish regulation.

2. Materials and methods

2.1. Shrimp and pathogen challenge

Healthy shrimp, *Penaeus monodon*, with body weight of 7–10 g for gene silencing experiment and 10–15 g for other experiments were purchased from a local farm and maintained in aerated water with a salinity of 15 ppt for at least 7 days before use.

The Yellow Head Virus (YHV) and *V. harveyi* 639 solutions were prepared as previously described [16]. Appropriate dilution of YHV in the volume of 20 μl was injected into each shrimp in the abdominal muscle. This amount of YHV caused a cumulative mortality of 50% at about 3 days after injection. The *V. harveyi* 639 suspension of 20 μl in 0.85% NaCl (normal saline) having 2×10^4 CFU was used for injection into each shrimp.

2.2. Silencing of PmRelish using dsRNA interference

To investigate the response of shrimp under *PmRelish* silencing on YHV infection, the double-stranded RNA (dsRNA)-mediated gene knock-down of *PmRelish* gene was employed by using the T7 RiboMAX™ Express RNAi System (Promega). The *PmRelish* dsRNA and *GFP* dsRNA as a control dsRNA were prepared as described in our previous study [15]. The specific primers KD_PmRelish_T7F/KD_PmRelish_R and KD_PmRelish_F/KD_PmRelish_T7R (Table 1) were used for the synthesis of upper and lower RNA strands, respectively. The *GFP* dsRNA was produced using primers KD_GFP_T7F/KD_GFP_R and KD_GFP_F/KD_GFP_T7R (Table 1).

Briefly, a plasmid clone containing either *PmRelish* gene or *GFP* gene was used as a template in the PCR amplification reactions. The 25-μl reaction contained 1 μl plasmid DNA template, 1 × PCR buffer, 0.2 mM each dNTP, 0.4 μM each primer and 1.5 unit RBC *Tag* DNA polymerase (RBC Bioscience). The reaction was pre-denatured at 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, 58 °C for *GFP* or 60 °C for *PmRelish* for 30 s and 72 °C for 30 s, and the final extension step at 72 °C for 10 min. The PCR products were analyzed with 2% agarose gel electrophoresis.

Then, 1 μg of each PCR products was used as templates for in vitro transcription using T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) according to the manufacturer's recommendation. Equal amount of the two complementary RNA strands were annealed. The quality and amount of RNAs were verified by 1.5% agarose gel electrophoresis and measured spectrophotometrically, respectively.

The dsRNAs were tested for their efficacy on gene silencing. Four groups of six shrimp were given injection of 20 μl of normal saline (NaCl control group), normal saline (YHV group), *GFP* dsRNA (5 μg/g shrimp) (*GFP* dsRNA-YHV group) and *PmRelish* dsRNA (5 μg/g shrimp) (*PmRelish* dsRNA-YHV group). After 24 h, shrimp in NaCl control group were injected again with the same solutions and shrimp in the other three groups were injected again with the same solutions plus YHV. The hemocytes were collected at 3 and 6 h after the second injection. Total RNA was extracted by a FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen). One microgram of total RNA was used for the synthesis of first strand cDNA using SMARTer™ PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions.

The RT-PCR was performed to evaluate the silencing of target *PmRelish* gene transcript in NaCl control group, *GFP* dsRNA-YHV group and *PmRelish* dsRNA-YHV group using the specific primers PmRelish_RT_F and PmRelish_RT_R (Table 1) and the PCR condition as described above. The PCR products were analyzed by 2.5% agarose gel electrophoresis. The expression level of silenced gene was reported relative to that of *EF-1α*.

2.3. Construction of cDNA libraries using suppression subtractive hybridization

To do the suppression subtractive hybridization (SSH), the combined 3-h and 6-h cDNAs from *GFP* dsRNA-YHV group and *PmRelish* dsRNA-YHV group were used. The SSH was performed in two direction, forward and reverse using the PCR-select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. For the forward subtraction, cDNAs of *PmRelish* dsRNA-YHV group were used as driver to subtract from those of tester, *GFP* dsRNA-YHV group. For the reverse subtraction, cDNAs of *GFP* dsRNA-YHV group were used as driver to subtract from those of tester *PmRelish* dsRNA-YHV group. The efficiency of SSH was analyzed using elongation factor-1α (*EF-1α*) gene.

The subtracted mixture was nested PCR-amplified and cloned into a pGEM-T easy vector (Promega), transformed into *Escherichia coli* StrataClone™ SoloPack® competent cells (Algilent). After plating, the positive clones were randomly selected and purified by FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen). The SSH positive clones were sequenced using M13 forward primer by Macrogen Inc., South Korea.

2.4. Bioinformatic analysis of genes derived from the SSH libraries

The gene sequences obtained from SSH experiment were