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Characterization of candidate genes involved in growth of black tiger shrimp *Penaeus monodon*

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ABSTRACT

The optic lobe of the female-shrimp eyestalk was selected as the target tissue for suppressive subtractive hybridization (SSH) because it is a place for synthesis of many hormones and peptides/enzymes involved in molting and growth. SSH was performed to screen genes differentially expressed in the optic lobe between large female (LF; body weight>90 percentile of weight distribution curve) and small female (SF; body weight<10 percentile). A total of 426 recombinant clones were obtained from the two directions. After sequencing and analysis less than 30% of the resulting expressed sequence tag (EST) exhibited high homology to known records at GenBank (BlastX with *E*-value$<10^{-4}$) indicating little available molecular information on the optic lobe. Among the EST with relatively high homology to GenBank records, 5 candidate ESTs had homology to records for genes involved in cell differentiation/proliferation, cell cycle and hormone processing. These included genes resembling cyclophilin, cyclophilin A, fibrillarin, SPARC and PC2. These candidate ESTs were selected to confirm the reproducibility of the SSH data using semi-quantitative RT-PCR normalized with EF-1α. Pearson's correlation analysis confirmed that the index of relative cyclophilin, SPARC and fibrillarin-like expression was negatively correlated with body weight ($p<0.05$) and this supported the SSH data indicating their low relative expression in LF shrimp when compared to SF shrimp. The index of relative cyclophilin-like expression showed the highest correlation coefficient with body weight ($r=-0.678$). The relationship could be expressed by inverse fitted equation: body weight (g) = 38.243/(index of relative cyclophilin-like expression). This fitted inverse model could predict the body weight of female shrimp with 91.6 % coefficient of determination (R^2) based on the index of relative cyclophilin-like expression in the optic lobe of female *P. monodon*.

1. Introduction

Thailand has been the world's leading shrimp-exporting nation with most of the quantity going to the United States of America and Japan. Thai shrimp industry exported mostly farmed black tiger shrimp *Penaeus monodon*. Since 2001, Thai shrimp farmers have faced a new disease problem called monodon slow growth syndrome (MSGS) that is characterized by slow growth that leads to small harvest sizes and thus low prices and loss of profit (Chayaburakul et al., 2004). The cause of MSGS is still unknown, although evidence suggests that a new virus called Laem Singh virus (LSNV) plays an important role in its clinical manifestation (Pratoomthai et al., 2008).

Apart from viral infections, it is clearly known from the Thai domestication and breeding program for *P. monodon* that genotypes or genetic differences among families play a very important role in differential growth rate. Within the same family, i.e., with closely related genetics, heterogeneous individual growth (HIG) among siblings has been noticed, even when they are grown in the same pond or the same environment. As all the siblings are exposed to the same rearing conditions, HIG is considered to arise from certain internal factors such as levels of neuropeptides that play an important role in regulating a wide variety of physiological processes in crustaceans. Examples include such things as blood glucose level, stress responses, coordination of reproduction and yolk biosynthesis (Fingerman, 1987; Quackenbush, 2001; Lorenzon et al., 2004), ecdysteroid biosynthesis and periodic molting cycles (Spaziani et al., 1989) that are strongly associated with growth. Crustacean growth and development is not continuous because of the rigid exoskeleton. Thus, periodic shedding (ecdysis) of the exoskeleton and its replacement are associated with growth (Keller, 1992). A longstanding model proposes that molt control in crustaceans is achieved by the interplay of hormones including molt-inhibiting hormone (MIH) from the X-organ-sinus gland (XO-SG) complex that suppresses the

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synthesis or secretion of molting hormones (ecdysteroids) from Y-organs in the cephalothorax during much of the molting cycle (principally intermolt). It is proposed that the molting sequence is initiated when MIH secretion diminishes (Skinner, 1985). The XO-SG complex located in each eyestalk represents a major neuroendocrine center in decapod crustaceans. It produces and stores MIH members of the crustacean hyperglycemic hormone (CHH) family (Keller, 1992). These are examples of several physiological processes that are controlled by neurohormones from eyestalks (Fingerman and Nagabhushanam, 1992) that may harbor other peptides/enzymes or receptors directly or indirectly involved in growth regulation.

Suppression subtractive hybridization (SSH) is widely used to isolate differentially expressed genes in two closely related samples/specimens/species. SSH should facilitate the identification of genes involved in growth which could assist the domestication and selective breeding programs of *P. monodon*. In this paper, we used SSH technique to examine differential expression of optic lobe genes of large and small female siblings of *P. monodon* reared in the same pond. We found candidate expressed sequence tag (EST) that showed negative correlation with body weight of female shrimp.

2. Materials and methods

2.1. Experimental animal model and tissue collection

Black tiger shrimp *P. monodon* aged 7 months were obtained from a commercial pond of Charoen Pokphand Feeds (public) Company, Thailand. The shrimp were siblings generated from a single pair of broodstock. Average body weight of the shrimp pond population sample ($n=400$) used for SSH was 36.25 ± 9.29 g and the coefficient of size variation was 25.46%. Only female shrimp were studied because female of *P. monodon* exhibit approximately 10–20% greater growth than males (Browdy, 1998). The large and small sizes of female shrimp were selected, respectively, from the >90 and <10 percentile regions of the growth distribution curve. Large females (LF) and small females (SF) sub-population had average body weights of 52.02 ± 4.13 g and 19.40 ± 5.11 g, respectively. By semi-quantitative RT-PCR, female shrimp aged 5 months with average weight (34.10 ± 9.91 g) and 29.07% coefficient of size variation were subjected to analysis of correlation between index of relative gene expression and body weight.

Pleopod samples ($n=10$) were tested for the presence of yellow head virus/gill-associated virus (YHV/GAV), white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV; also called *Penaeus stylirostris densovirus* or PstDNV) by IQ2000 test kits (Farming Intelligene, Taipei) whereas the hepatopancreatic parvovirus (HPV) and Monodon Baculovirus (MBV) were tested by Ezee gene kit (SBBU, Thailand). The presence of Laem-Singh virus (LSNV) in eyestalks was tested by nested RT-PCR (Sritunyalucksana et al., 2006).

Eyestalks of shrimp were cleaned with 70% ethanol, dissected from live animals and washed with RNase free water. The optic lobes were isolated from individual eyestalk using a stereo microscope (Olympus SZ 6045 TRPT) and kept in RNA*later* solution (Ambion) at 4 °C overnight before being stored at −20 °C until used.

2.2. RNA isolation

Total RNA was extracted from each pair of optic lobes using Trizol reagent (Invitrogen, USA) according the manufacturer's manual. Purity and quantity of RNA was determined from 260:280-nm ratios. Total RNA extracted from approximately 50 pairs of optic lobe in each group were combined and poly $(A)^+$ mRNA was purified by using an mRNA isolation kit (Amersham Bioscience).

2.3. Suppression subtractive hybridization

SSH was performed to generate subtracted cDNA libraries between LF and SF shrimp using a PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Subtraction was performed simultaneously in two directions: a forward SSH library was prepared using mRNA from the SF shrimp as the tester and LF shrimp as driver and a reverse library was prepared vice versa. To evaluate the efficiency of the subtraction, the relative amounts of EF-1α cDNA present in the subtracted and unsubtracted cDNA populations after SSH were examined by PCR amplification according to the manufacturer's manual. The subtracted PCR products representing tester specific sequences, were cloned into the pGEM-T Easy vector (Promega) and transformed into competent *Escherichia coli* JM109 cells. Recombinant clones obtained from each SSH library (>200 cloned) were isolated and grown overnight in LB–ampicillin broth at 37 °C. Plasmid DNA was purified (GeneJet™ plasmid Miniprep kit, Fermentas) and inserts were sequenced (Macrogen, Korea).

2.4. Nucleotide sequence and bioinformatics analyses

Nucleotide sequences obtained from SSH were compared to the sequences deposited in public databases using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov). Matches were considered significant when the probability (*E*-value) was less than 10^{-4} and clones were tentatively classified according the functional categories of their homologues. Nucleotide sequences that significantly matched hypothetical proteins and/or functionally unidentified proteins were regarded as unidentified cDNA whereas those that did not match any sequence in GenBank (E-value $\geq 10^{-4}$) were regarded as unknown genes.

2.5. Semi-quantitative by RT-PCR

Total RNA isolated from the optic lobe of individual shrimp was treated with DNase I (RNase-free DNase; Promega) at 37 °C for 1 h to remove genomic DNA contamination and was purified by phenol–chloroform–isoamyl alcohol (25:24:1) extraction. A total of 2.0 μg of total RNA was reverse-transcribed into cDNA using Superscript™ III reverse transcriptase with Oligo (dT_{20}) as primers and the resulting product was used as the template in the succeeding PCR reactions. The expression of 5 candidate ESTs including those with homology to cyclophilin, cyclophilin A, fibrillarin, prohormone convertase 2 (PC2) and secreted protein, acidic and rich in cysteine (SPARC) were determined by semi-quantitative RT-PCR with specific primers designed using the Primer 3 program (http://frodo.wi.mit.edu/). Primer sequences, annealing temperatures and optimized number of PCR cycles shown in Table 1. For semi-quantitative RT-PCR, the optimal number of PCR cycles was adjusted with the assumption for equal 100 ng total RNA templates that amplicons visible at low cycle numbers. The PCR protocol was as follows: 3 min at 94 °C followed by the optimized number of PCR cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 30 s followed by an additional extension at 72 °C for 5 min. The EF-1α gene was used as an internal control to normalize cDNA quantity. Amplicons of candidate genes and an internal control were electrophoretically analyzed in the same gel. The band intensity of the candidate genes and internal control in each gel was digitalized by GeneTools software (SYNGENE) and the index of relative gene expression was analyzed by ratio of band intensity of candidate gene to EF-1α. This formula is valid only for EF1-α intensity in a range of 0.49–1.05 E06 unit.

2.6. Statistical analysis

Data was subjected to statistical correlation analysis using SPSS for Windows (version 17). The Kolmogorov–Smirnov (KS) test was