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Profiling of Up-Regulated Genes Response to Acute Hypo-Osmotic Stress in Hepatopancreas and Gill of the Pacific White Shrimps (*Litopenaeus vannamei*)

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Abstract

Suppression subtractive hybridization (SSH) was applied to screen responsively up-regulation genes in hepatopancreas and gill of Litopenaeus vannamei induced by acute hypo-osmotic stress. In the hepatopancreas, 196 clones were randomly selected and sequenced. 131 non-redundant transcripts, corresponding to 41 genes, were found with elevated expressions. They were functionally clustered into eight biological processes which were protein synthesis and processing, carbohydrate metabolism and energy production, transport, cell growth, apoptosis, cytoskeletal component, cell defense and homeostasis, signal transduction, accounting for 26.2%, 14.3%, 16.7%, 4.7%, 4.7%, 2.3%, 11.9% and 7.1%, respectively. When confirmed by real time qRT-PCR, the gene expression levels of MnSOD, glutathione and glutamine synthetase showed significant increases (2.64-folds, 3.44-folds and 2.16-folds, respectively) in hepatopancreas (P<0.05). Totally 81 expressed sequence tags (ESTs) were obtained by random sequencing from the gill SSH cDNA library, and 52 unigenes, including 15 contigs and 37 singlets, after CP3 online matching were gained by clustering and assembling. The sequence alignment results revealed that 30 of them had significant homology to previously identified Genbank database sequences, while 22 unigenes did not match any sequence in the datebase and presumably represented unidentified cDNAs. Functionally, the unigenes were classified into six clusters, including ion channels and transport proteins (1); protein synthesis, translation and transcription factor (2); stress resistance and antioxidant factors (3); energy metabolism (4); signal receptor and transduction (5); cell fibrin and cytoskeletal proteins (6) with proportion of 23.3%, 20.0%, 20.0%, 16.7%, 10.0% and 10.0% respectively. Real time qRT-PCR confirmation revealed significant elevation of Arginine kinase, Carbonic anhydrase and NaK-ATPase-α-subunit expressions (3.73-folds, 2.55-folds and 5.83-folds, respectively) in the gill after acute low-osmotic stress ($P < 0.05$). These results provided insight into critical physiology processes and pathways constituting the hypo-osmotic stress adaption program in hepatopancreas and gill of *L. vannamei*.

Keywords: *Litopenaeus vannamei*, hepatopancreas, gill, suppression subtractive hybridization (SSH), hypo-osmotic stress

1. Introduction

Litopenaeus vannamei, has been cultured widely in many parts of the world, such as the US (Cuzon, Lawrence, Gaxiola, Rosas, & Guillaume, 2004), Thailand (Thongsawad et al., 2007), China (Bi, Huang, Gu, Wang, & Wang, 2008), Brazil (dos Santos Braz, Reis, Martins, de Sales, & Meissner, 2009) and Indonesia. Recently, *L. vannamei* has been cultured in brackish water as well as freshwater because of its strong hypo-osmotic regulation. The tolerance of shrimps to acute salinity change dependent on their capability of osmotic physiological regulation, such as improvements of ion-transport enzymes activities (Jasmani, Jayasankar, Shinji, & Wilder, 2010; L.-Q. Pan, Zhang, & Liu, 2007), changes of free amino acids (FAAs) (Marangos, Brogren, Alliot, & Ceccaldi, 1989; Silvia, Antonio, Francisco, & Georgina, 2004) , enhances of digestive enzymes activities (Li et al., 2008), increases of ammonia afflux rate (Pillai & Diwan, 2002), elevations of oxygen consumption rate (Zhang, Zhang, Li, & Gao, 2009) and optimization of genes transcript levels (Dong, Wang, Tian, Guo, & Liu, 2011; Jayasundara, Spanings-

Pierrot, & Towle, 2004; Luquet, Weihrauch, Senek, & Towle, 2005; Tiu, He, & Chan, 2007). Most published studies on osmotic physiological adaption were focused on physiological apparent changes, while few of them dig into the molecular mechanism of the procedure.

In crustaceans, gill is the most important organ for respiration and osmoregulation (Pequeux, 1995). The osmo-regulatory mechanism of *L. vannamei* in low salinity stress is accomplished through the coordination of a number of functional genes, usually accompanied by the modulation of mRNA expression levels of a series of osmotic-related genes (Zhao, Pan, Ren, & Hu, 2015). However, the classes and pathways of osmotic related genes are not yet fully revealed. The gill epithelial cells are the primary sites of shrimps for ion exchange with the environment. The ion channels and transmembrane proteins at the apical and basolateral membrane play important roles in the osmoregulation (Lovett et al., 2006). The hepatopancreas is regarded as one of the most important tissues involved in the innate immune defense of shrimp, and a digestive gland, which involved in digestion, absorption of nutrients, storage of reserves, synthesis and excretion of digestive enzymes (Franceschini-Vicentini et al., 2009). Shrimps produced more B cells in hepatopancreas tubules at salinity 3 ppt than at salinity 17.0 ppt, which indicated that the high rate of synthesis and release of digestive and antioxidant enzymes accelerated the mobilization of nutrients in hepatopancreas tubules, by which more energy is supplied for omosregulation of shrimps at low salinity, and to adapt to environment stress (E. Li et al., 2008).

Suppression subtractive hybridization (SSH), an effective method to identify differently expressed genes (Diatchenko et al., 1996), was adopted to find involved genes and genes which have never been discovered. Employing SSH, Foil et al., (Fiol, Chan, & Kültz, 2006) discovered 20 genes cluster together in six molecular processes that are rapidly activated in *Tilapia* gills upon salinity transfer; James et al., (James, Thampuran, Lalitha, Rajan, & Joseph, 2010) identified 17 novel proteins which invovled in immunity in hepatopancreas of *Fenneropenaeus*; Pan et al., (D. Pan, He, Yang, Liu, & Xu, 2005) found 8 novel genes related to virus-resistant in hepatopancreas of panaeid shrimps. Regarding osmoregulation of shrimps, Shekhar et al.,identified differentially expressed genes in response to salinity stress with suppression subtractive hybridization (SSH) through cDNA library constructed from gill and gut tissues of the giant tiger shrimps (*Penaeus monodon*) exposed to low (Shekhar, Kiruthika, & Ponniah, 2013) and high (Shekhar, Kiruthika, Rajesh, & Ponniah, 2014) salinity conditions. The resulted expressed sequence tags (EST) provided a partial description of the transcribed regions or activities of a genome in a tissue or an organism under specific experimental conditions. As a commercially important aquaculture spice, *L. vannamei* has been extensively studied. However, the up-regulation of its genes, particularly those involved in the physiological adaptation of the hepatopancreas to low saline condition, is rarely reported. This study aimed to identify the up-regulated genes induced by acute hypo-osmotic stress using SSH in the hepatopancreas and gill of *L. vannamei* and to provide a valuable insight into the immunity, osmoregulation and metabolic adaption mechanisms of *L. vannamei* under hypo-osmotic conditions.

2. Method

2.1 Animals

L. vannamei (10.7 ± 0.4 g) were obtained from a farm in Zhanjiang, Guangdong, China, and then acclimatized for 2 weeks in a pool (at 30 °C) filled with aerated seawater (30 ± 2 ppt). The shrimps were fed on a commercial shrimp diet four times a day with 25% seawater exchanged every day.

2.2 Hypo-Osmotic Stress and Samples Collection

Shrimps with similar length and weight (12.4 ± 0.2 g) were randomly distributed (n=20/tank) to 300L experimental containers with either seawater (Control group) or low salinity dilute seawater (Treatment group). The salinity was gradually decreased from 30 to 4 ppt over a period of 12 h in the treatment group, while kept at 30 ppt in the control group. Shrimps were sacrificed by removing their carapace from backside with a jerk 2 days after the salinity transfer. Hepatopancreata were quickly dissected out, flash frozen in liquid nitrogen and stored at -80 °C until used for total RNA extraction. The experimental conditions were as follows: water temperature, 28.5±2 °C; salinity, 30 or 4 ppt; dissolved oxygen, 10.30–10.50 mg L^{-1}; pH, 7.8±0.5.

2.3 RNA Extraction and SMART cDNA Synthesis

Total RNA was extracted from gills and hepatopancreata of *L.vannamei* using Unizol reagent (GENEray biotechnology, China) according to the manufacturer's protocol. Purity of RNA was confirmed by reading absorbance at 260 and 280 nm. The ratio of background–free absorbance at 260/280 nm was always between 1.8 and 2.0. Reverse transcription of mRNAs from Control and Treatment were performed using SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions.

2.4 Suppression Subtractive Hybridization

The hepatopancreata and postgills of *L.vannamei* in hypo-osmotic stress (4 ppt) were used as the Tester with those from normal seawater (30 ppt) as the Driver respectively. SSH was performed to generate a SSH library using the PCR-Select cDNA Subtractive Kit (Clontech) according to the manufacturer's protocol. Briefly, SMART cDNAs from both group were digested with Rsa I at 37 °C overnight to generate shorter blunt-ended double-strand cDNA and purified with Axyprep Clean Kit (AXYGEN). Aliquots of Rsa I-digested Tester cDNA were ligated with adaptor1 and adaptor2R (Table 1) respectively at 5'-end and hybridized at 68 °C for 8 h with the excess of driver cDNA after denaturation at 98 °C for 90 s. After the first hybridization, the two samples were mixed together without denaturation and hybridized with the excess of fresh denatured driver cDNA for overnight incubation at 68 °C. The resulted mixture was diluted in 200 μL dilution buffer and submitted to two rounds PCRs to enlarge the target cDNAs. Primer 1 (Table 1) against adaptor1 and adaptor2R was used to selectively amplify differentially expressed cDNA in the primary PCR, followed the secondary PCR through a set of nested primers with the product of the first PCR as template. Finally, the SSH efficiency was evaluated by PCR with β-actin forward and reverse primers performed on tester (unsubtracted) and subtracted cDNA s for 18, 23, 28or 33 cycles.

2.5 Cloning, Sequencing and Homology Analysis

cDNAs were inserted directly into PED-T vector (Promega) and transformed into DH-5α competent *E. coli*, followed by spreading onto LB agar plates containing AMP, X-Gal and IPTG. Blue clones were selectively sequenced. The sequences were clustered by CAP3 (http://pbil.univ-lyon1.fr/cap3.php), and then searched in the NCBI GenBank database for homology with Blastn and Blatstx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The significant matches presented E-values lower than $1e^{-6}$.

2.6 Real Time qRT-PCR

Real time qRT-PCR was used to confirm the differential expression of up-regulated genes in salinity-stress shrimps. Total RNA was extracted from hepatopancreas and gill. The first-strand cDNA was synthesized using the iScript™ cDNA synthesis kit (BIO-RAD). The reaction was conducted in a 20 μL reaction volume consisting of RNA (100 ng), 4 μL iScript reaction mix, 1 μL iScript reverse transcriptase, and Nuclease-free dH_2O at 25 °C for 5 mins, then at 42 °C for 30 mins, and followed by 5 mins at 85 °C.. The real time qPCR amplification was performed in a 10 μL reaction volume consisting of 5 μL SsoFast™ EvaGreen® supermix(BIO-RAD), 0.3 μL forward primer (Table 1), 0.3 μL reverse primer (Table 1), RNase/Dnase-free water and 1 μL cDNA. The specific primers of ten up-regulated genes picked from the SSH libraries of hepatopancreas and gill (MnSOD, Ferritin, glutathione, destabilase I, glutamine synthetase, arginine kinase, Carbonic anhydrase, Serine protease, NaK-ATPaseαsubunit, NaK-ATPaseβ-2 subunit) used for real time analysis are shown in Table 1. The relative expression was normalized against β-actin using the comparative Ct method with the formula $2^{-\Delta\Delta Ct}$ (whereΔ $\Delta Ct = \Delta Ct$ sample – ΔCt reference).

Table 1. Primers used for SSH library construction and gene specific real time PCR analysis of identified up-regulated genes

Genes/ Names	Forward (5' to 3')	Reverse (5' to 3')
Adaptor 1	CATTACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT	GGCCCGTCCA
Adaptor 2R	CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT	GCCGGCTCCT
Primer 1	CTAATACGACTCACTATAGGGC	
Nested PCR 1	TCGAGCGGCCGCCCGCCCGGCAGG	
Nested PCR 2R	AGCGTGGTCGCGGCCGAGGT	
β-actin	CCTGTTCCAGCCTCAT	GTCCACGTCGCACTTCA
MnSOD	GCCAGCGTTGGAGTGAAAGG	TTGGTCGCCACGAGAAGTCA
Ferritin	CGTTTACCTTTCTATGGCTTAC	CACTTGTTCCTCCAGATACTCA
glutathione	GGCACTGAGTTCGAGGAGA	TTCGTAGGTGACGGTAAAGA
destabilase I	CGCATCACCATTCTTCCTG	CGTAGTCCTCATTCATCCC
Glutamine synthetase	GGCATGGAGCAGGAGTA	CGCCGCAGTAGTAGGGT
arginine kinase	GCTGACGCTGCTGTTATTGAGA	GGTGGCGGCTTCAAGCTT
Carbonic anhydrase	CCCCACCTTGCTATGAATGC	TCCTGGGACAGCTGAATGG
Serine protease	TTCGTTGTTGCGCGTTTCT	CAGTGCGCAGCGTTTGG
NaK-ATPase-α-subunit	ACGTTTCAGGATGGGAGTGTT	CCTCTGCAACCGTGCTGAAT
NaK-ATPase-β-subunit	CGCTCACAATTCCACACAACA	GCACCCCAGGCTTTACACTT

3. Results

The subtraction efficiency of our study was evaluated by the constitutively expressed gene β-actin. The amount of β-actin transcript was hardly detectable in the subtracted library comparing with that in the unsubtracted sample, suggesting that SSH procedure successfully suppressed cDNA constantly expressed in the normal and acute low salinity stressed shrimps.

3.1 Up-Regulated Genes in Hepatopancreas SSH cDNA Library of Litopenaeus Vannamei

196 clones were sequenced in hepatopancreas SSH cDNA library. 131 potential transcripts included 21 contigs and 110 singlets were identified by assembling and clustering. 41 with significant homologies to known genes were obtained and categorized according to their putative functions (Table 2), out of which ribosomal protein S15, ribosomal protein S2, ATP/ADP translocase, vesicle-associated membrane protein 2, cytochrome P450 and glutamine synthetase were firstly uncovered in *L.vannamei*. The genes were functionally clustered into eight biological processes(Figure 1) including protein synthesis and processing, carbohydrate metabolism and energy production, transport, cell growth, apoptosis, cytoskeletal component, cell defense and homeostasis and signal transduction. They accounted for 26.2%、14.3%、16.7%、4.7%、4.7%、2.3%、11.9% and 7.1% of the identified genes respectively (Figure 2). The most abundant genes in the subtractive library were related to protein synthesis, indicating protein metabolism in hepatopancreas was rnhanced by acute hypo-osmotic stress. The identification of glucose dehydrogenase and phosphoenolpyruvate carboxykinase also implied the activiation of gluconeogenesis pathway in hepatopancreas. The up-regulation of transport and signal transduction genes suggested the involvement of metabolic pathways and mediated pathways in physiological adaption of *L.vannamei* to hypo-osmotic water.

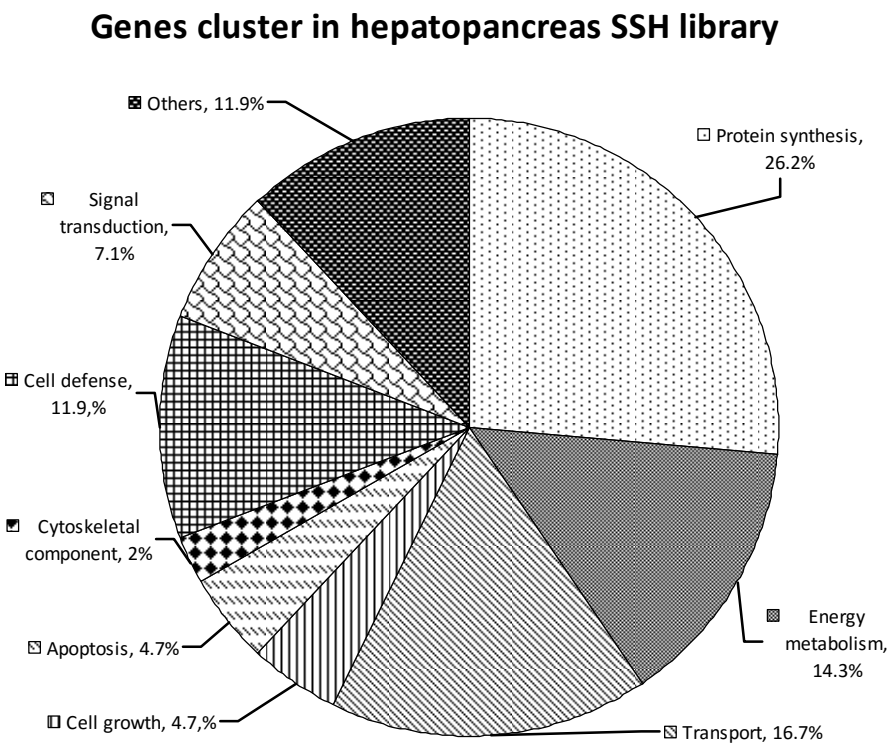

Figure 1. The genes were in hepatopancreas SSH library clustered together according to their predictable functions of protein

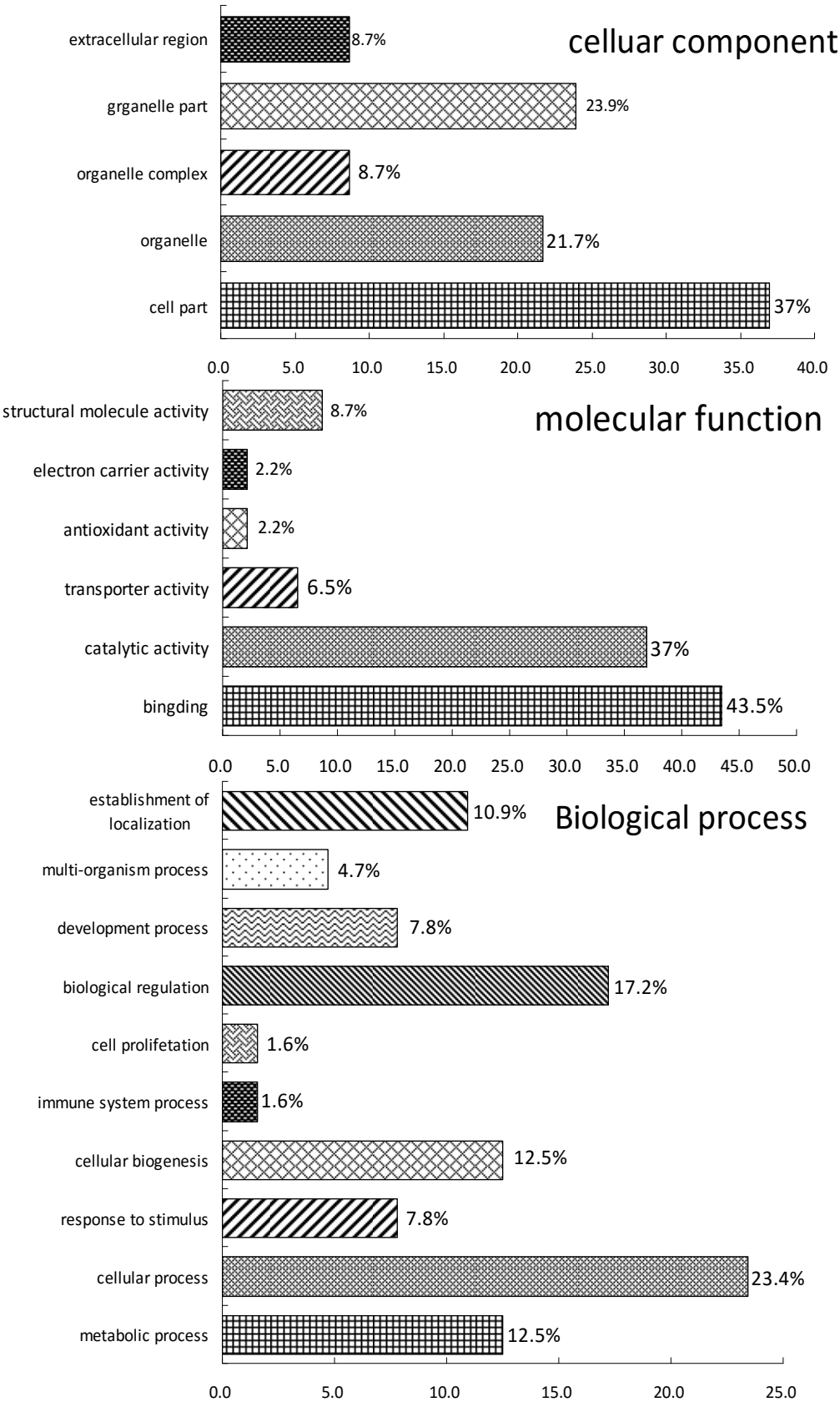

Figure 2. Up-regulation genes were classified according to biological process, molecular function and celluar component

Table 2. Up-regulated genes indentified in the hepatopancreas of *Litopenaeus vannamei* after sudden salinity stress by suppression subtractive hybridization (SSH)

Clones	Genes	the closest species	GeneBank accession	clones in library	E-value(%identity)
Protein metabolism and processing					
contig1	eukaryotic translation initiation factor 5A	*Penaeus monodon*	DQ851145.1	1	5e-67/96
contig19	elongation factor 1-alpha	*Litopenaeus vannamei*	GU136229.1	1	2e-97/98
contig21	ribosomal protein S15	*Spodoptera frugiperda*	AF400212.1	1	9e-77/80
A82-M13F-A08	18S ribosomal RNA	*Farfantepenaeus duorarum*	FJ943445.1	4	1e-30/100%
A57-M13F-H04	ribosomal protein S2	*Bombyx mori*	NM_001044099.1	1	1e-13/76%
A29-M13F-F09	glutamine synthetase	*Fenneropenaeus chinensis*	EU428821.1	1	1e-98/92
F7-M13F_C06	ribosomal protein L8	*Litopenaeus vannamei*	DQ316258.1	1	2e-32/97
A41-M13F-F05	Proteasome subunit alpha type-3	*Harpegnathos saltator*	EFN81786.1	1	4e-36/69
Carbohydrate metabolism and energy production					
contig7	glucose dehydrogenase	*Aedes aegypti*	XP_001648298.1	1	4e-15/57
contig20	phosphoenolpyruvate carboxykinase (pepck gene)	*Penaeus vannamei*	AJ250829.1	1	2e-32/98
A46-M13F-E03	ATP/ADP translocase	*Marsupenaeus japonicus*	EF077712.1	1	1e-40/97
B3-M13F_C01	putative mitochondrial ATP synthase	*Graphocephala atropunctata*	ABD98767.1	1	7e-10/68
C3-M13F_G02	ATP-binding cassette sub-family A member 3	*Bos taurus*	NP_001107218.1	1	1e-34/42
A75-M13F-B07	24-dehydrocholesterol reductase	*Capsaspora owczarzaki*	EFW46219.1	1	1e-17/41
Transport					
contig5	vesicle-associated membrane protein2	*Xenopus laevis*	NP_001080944.1	1	6e-19/70
contig11	hemocyanin	*Penaeus monodon*	AF431737.1	3	2e-90/92
contig17	ferritin	*Penaeus monodon*	ABP68819.1	2	9e-13/90
A54-M13F-E04	fatty acid binding protein 10	*Litopenaeus vannamei*	DQ398572.1	1	7e-69/99
Cell growth					
contig6	sterol carrier protein x-like	*Litopenaeus vannamei*	DQ858901.1	1	0/98
F1-M13F_A05	Cytochrome P450	*Harpegnathos saltator*	EFN78521.1	1	7e-8/47
Apoptosis					
B02_1011006300	mitochondrion	*Litopenaeus vannamei*	DQ534543.1	1	7e-145/99
A89-M13F-H08	senescence-associated protein	*Penaeus vannamei*	AF124597.1	1	3.3e-28/89

Cytoskeletal component					
contig9	Glutamic acid-rich protein	*Plasmodium falciparum FC27*	P13816.1	1	2e-5/30
Cell defense and homeostasis					
contig13	kazal-type proteinase inhibitor VANPI-2	*Litopenaeus vannamei*	DQ230989.1	1	2e-35/79
A25-M13F-B09	chitinase	*Litopenaeus vannamei*	AY576684.1	1	0/88
A38-M13F-G10	glutathione S-transferase	*Litopenaeus vannamei*	AY573381.1	1	4e-30/97
A94-M13F-E09	MnSOD	*Litopenaeus vannamei*	DQ005531.1	1	2e-53/100
G12-M13F_D08	lectin A isoform 1	*Marsupenaeus japonicus*	ADG85666.1	1	7e-20/62
Signal transduction					
A72-M13F-G06	Adapter molecule Crk, putative	*Pediculus humanus corporis*	XP_002427598.1	1	2e-42/82
A77-M13F-D07	chitinase precursor	*Litopenaeus vannamei*	AF315689.1	1	8.8e-21/99
D5-M13F_C04	nuclear progesterone receptor	*Penaeus monodon*	GU906280.1	1	0/92
Others					
A78-M13F-E07	estrogen sulfotransferase	*Penaeus vannamei*	AF124597.1	1	3.3e-28/89
A93-M13F-D09	hypothetical protein	*Daphnia pulex*	EFX80592.1	1	5e-16/55
C4-M13F_H02	TAR RNA-binding protein 2	*Fenneropenaeus chinensis*	ACG60900.1	1	1e-61/100
G11-M13F_C08	tryptophanyl-tRNA synthetase	*Taeniopygia guttata*	XP_002200606.1	1	1e-50/67
A_2-M13F_B04	destabilase I	*Litopenaeus vannamei*	DQ398564.1	1	3e-129/98

Table 3. Different expression genes indentified in gills of *L vannamei* induced by low-osmotic stress by suppression subtractive hybridization (SSH)

Oringnal	Genes	Reference Species	E-Value (%identity)	Clones in library
Ion channels and transport proteins				
Contig3	Vesicle transport protein	*Mus musculus*	6e-10/54	1
M_T1_E7_G03	Carbonic anhydrase	*Litopenaeus vannamei*	2e-32/98	1
M_T1_F8_A05	Vesicle-associated membrane protein	*Xenopus laevis*	1e-38/53	1
M_T1_H4_G06	Sodium/potassium -ATPase alpha subunit	*Litopenaeus vannamei*	3e-55/93	2
M_T1_H4_G03	Sodium/potassium- ATPase beta-2 subunit	*Litopenaeus vannamei*	1e-53/64	1
M_T1_C9_G01	Calcium-activated chloride channel regulator 1	*Homo sapiens*	2e-11/78	1
Protein synthesis, translation and transcription factor				
M_T1_C9_G02	Mitochondrion 12S	*Drosophila melanogaster*	2e-135/95	1
Contig1	Single VWC domain protein	*Litopenaeus vannamei*	6e-11/45	1
Contig2	Probable elongation factor 1-delta	*Drosophila melanogaster*	8.2e-06 /69	1
M_T1_E5_F03	Ribosomal protein L7a	*Gallus gallus*	7e-46/72	1
M_T1_E7_G06	40S ribosomal protein	*Drosophila melanogaster*	2.e-67/99	1
Contig7	Eukaryotic translation initiation factor	*Gallus gallus*	3e-26/87	1
Stress resistance and antioxidant factors				

Contig9	Major allergen Cr-PII	*Periplaneta Americana*	1e-19/79	1
M_T1_C4_ B08	Beta-lactamase	*Escherichia coli*	4.6e-34/74	1
M_T1_C9_G04	Ferritin heavy chain	*Drosophila melanogaster*	1e-46/54	2
Contig12	Thioredoxin 1 (antioxidant protein)	*Equus caballus*	2e-53/79	1
M_T1_E12_D07	Serine protease	*Drosophila melanogaster*	4e-12	1
Energy metabolism				
M_T1_F8_A08	NADH dehydrogenase subunit 3	*Drosophila melanogaster*	1e-46/90	1
M_T1_H4_A06	NADH ubiquinone oxidoreductase	*Drosophila melanogaster*	1e-21/71	2
M_T1_H2_D09	Mitochondrial ATP synthaseαsubunit precursor	*Gallus gallus*	3e-85/98	1
Contig11	Arginine kinase	*Penaeus monodon*	5e-42/90	1
Signal receptor and transduction				
M_T1_H4_F03	Ornithine decarboxylase antizyme	*Mus musculus*	2e-20/67	1
Contig8	Ectonucleotide pyrophosphatase	*Sus scrofa*	1.1e-06/47	1
M_T1_E7_F06	Signal sequence receptor beta-like protein	*Danio rerio*	1e-38/74	1
Cell fibrin and cytoskeletal proteins				
M_T1_H4_F09	Actin depolymerizing factor	*Drosophila melanogaster*	5e-46/84	1
Contig5	Fibrinogen and fibronectin	*Mus musculus*	5e-9/74	1
M_T1_E5_E06	Beta actin	*Litopenaeus vannamei*	2e-78/100	1

3.2 Up-Regulated Genes in Gills SSH cDNA Library of Litopenaeus vannamei

Totally 81 expressed sequence tags (ESTs) were obtained by randomly sequencing from the gill SSH cDNA library, and 52 unigenes, including 15 contigs and 37 singlets, after CP3 online matching were gained by clustering and assembling. The sequence alignment results revealed that 30 of them had significant homology to previously identified Genbank database sequences, while 22 unigenes did not match any sequence in the database and presumably represented unidentified cDNAs. Functionally, the unigenes were classified into 6 clusters (Figure 3), including ion channels and transport proteins (1); protein synthesis, translation and transcription factor (2); stress resistance and antioxidant factors (3); energy metabolism (4); signal receptor and transduction (5); cell fibrin and cytoskeletal proteins (6) with proportion of 23.3%, 20.0%, 20.0%, 16.7%, 10.0% and 10.0% respectively (Figure 4).

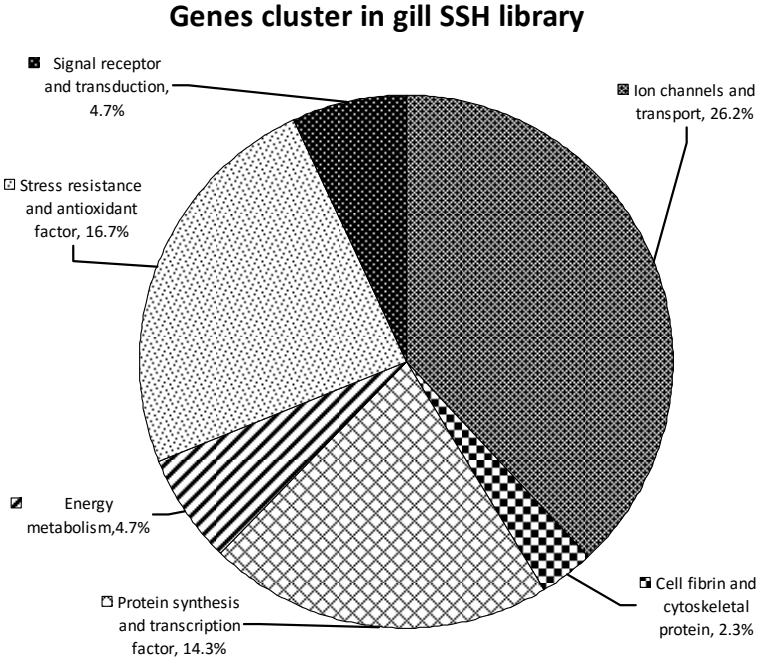

Figure 3. The genes were in hepatopancreas SSH library clustered together according to their predictable functions of protein

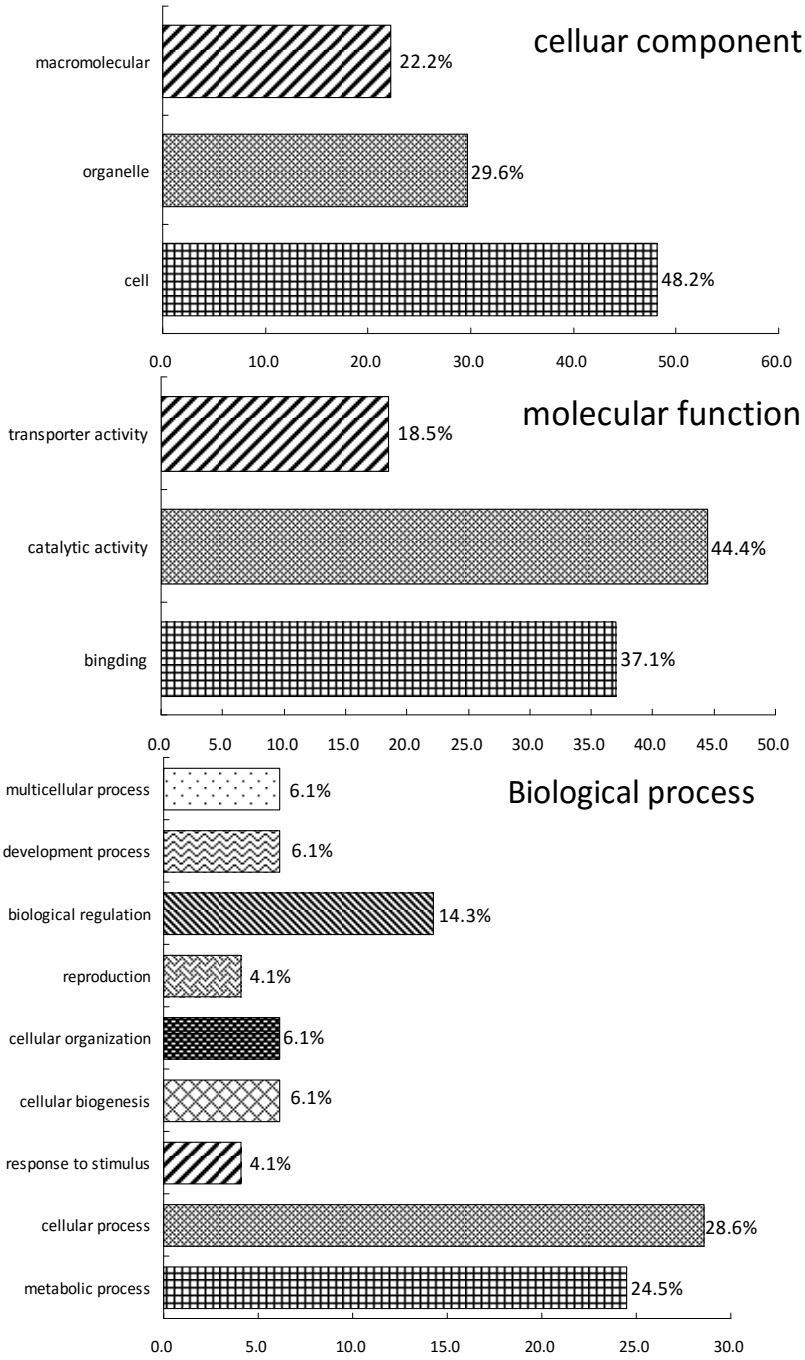

Figure 4. Up-regulated genes were classified according to biological process, molecular function and celluar component

3.3 qRT-PCRs Check of Up-Regulation of 10 Identified Genes

Real time qRT-PCRs were conducted to confirm the up-regulation of 10 identified genes including MnSOD, Ferritin, Glutathione, Destabilase I, Glutamine synthetase, Arginine kinase, Carbonic anhydrase, Serine protease, NaK-ATPase-α-subunit and NaK-ATPase-β- subunit in both hepatopancreas and gill(Figure 5). The expression levels of MnSOD, glutathione and glutamine synthetase showed significant increases (2.64-folds, 3.44-folds and 2.16-folds, respectively) in the hypo-osmotic inducecd hepatopancreas (P<0.05). Significantly elevated expressions of Arginine kinase, Carbonic anhydrase and NaK-ATPase-α-subunit were also revealed (3.73-folds, 2.55-folds and 5.83-folds, respectively) in the acute low-osmotic stressed gill (P<0.05). However, the up-regulations of Ferritin, Destabilase I, Serine protease and NaK-ATPase-β- subunit were not significant (Figure 5).

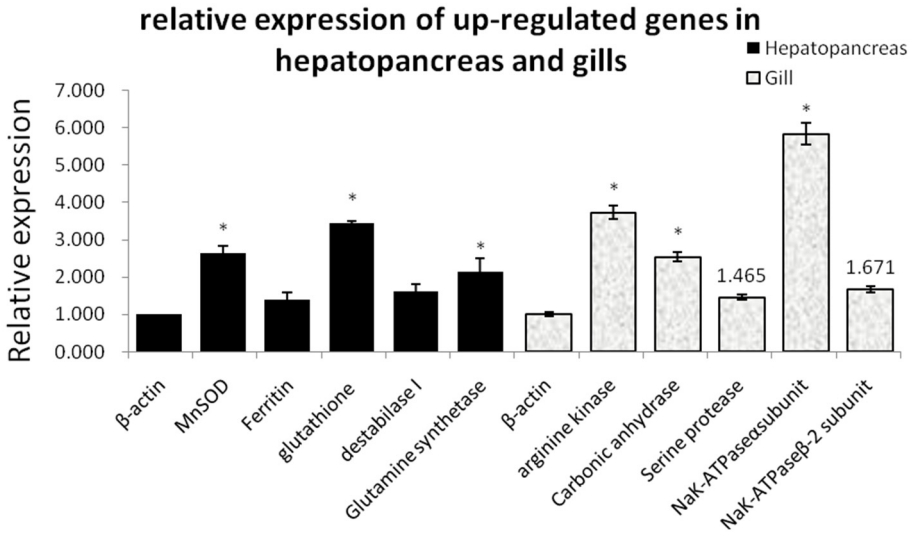

Figure 5. Real time qRT-PCR of the up-regulated genes of hepatopancreas and gills in response to acute low salinity stress

4. Discussion

Suppression subtractive hybridization (SSH) is a powerful method for screening abundant transcripts. Preechapho et al., isolated 109 known sequences and identified several reproduction-related transcripts in ovaries of the giant tiger shrimps (*P. monodon*) using SSH (Preechaphol, Klinbunga, Khamnamtongand, & Menasveta, 2010). Adisak et al., obtained 185 transcripts and uncovered 8 novel YHV-responsive genes from haemocyte libraries of *P. monodon* induced by yellow head virus after 24h and 48/72h (Prapavorarat, Pongsomboon, & Tassanakajon, 2010). Four suppression subtractive hybridization (SSH) cDNA libraries were constructed to identify differentially expressed salinity stress responsive genes of black tiger shrimp, *Penaeus monodon* exposed to low (3 ppt) salinity and high (50 ppt) salinity conditions (Shekhar et al., 2013; Shekhar et al., 2014). We also performed uppression subtractive hybridization (SSH) in juvenile L. vannamei exposed to long-term low salinity (2 ppt) for 56 days (Gao et al., 2012). In this study 41 and 30 unigenes were profilled with elevated espression from the hepatopancreas and gill library using SSH respectively. They were clustered into five functional categories in accordance with the blast sequences and functional analysis. We will discuss the functions of these genes clustered in functional groups as follows.

4.1 Cell Defense, Homeostasis and Immune

The differential expression of stress-induced and antioxidative-related genes in the hepatopancreas suggested that there might be a novel stress-reponse and remediation patterns. The stress-induced and antioxidative-related genes revealed in the subtractive library included original Cr-pII, the β-lactamase, ferritin heavy chain, thioredoxin and serine proteases and others. Thioredoxin is a redox regulation protein, which widely presents in prokaryotes and eukaryotes *in vivo*. In addition, thioredoxin maintains oxidation-reduction balance by reducing the target protein. Thioredoxin, thioredoxin reductase (TrxR), and NADPH compose the thioredoxin system, which catalyzes the reduction of hydrogen peroxide and organic peroxides involved in numerous physiological processes (Aispuro-Hernandez et al., 2008). The reactive oxygen species (ROS) is a major oxidative stress factor in cells. The thioredoxin can reduce the oxidized disulfide bonds to repair oxidative damage *in vivo* (Garcia-Orozco et al., 2012).The serine protease and homologue may contribute in the innate immunity of the Chinese shrimp (*Fenneropenaeus chinensis*). Ren et al., reported that the serine protease was activated in normal tissues of the Chinese shrimp (*F. chinensis*) (Ren et al., 2010). However, the homologue of serine protease was unactivated until stimulated by *Vibrio*. Serine protease inhibitors (serpins) are widely known to its inhibitory role on proteases involved in the immune responses. *Lvserpin7* transcripts were significantly up-regulated in the early stage upon pathogens infection. *Lv*serpin7 was implicated in the shrimp immunity via the inhibition of bacterial proteases and proteases involved in prophenoloxidase system (Y. Liu, Hou, Wang, & Liu, 2015). Despite of previous reports that the innate immunity of *L.vannamei* was depressed at low salinity (Joseph & Philip, 2007; C.-C. Li, Yeh, & Chen, 2010).Five up-regulated immune-related genes were discovered in the hepatopancreas SSH library, including kazal-type proteinase inhibitor vanpin-2, chitinase, glutathione S-transferase, MnSOD and lectin A isoform 1. The up-regulation of these genes indicated that shrimps may keep homeostasis of internal environment

in hepatopancreas at low salinity. The induction of immune and antioxidant genes under low salinity contributed to the consolidation of the immune defense of *L. vannamei.*

4.2 Protein Metabolism and Processing

The transcriptional regulation mechanism of osmoregulation-related genes is modulated by the direction of salinity change and the developmental background of the gill (Zhao et al., 2015). The proportion of protein synthesis and transcription factors was just less than that of ion channels and transport-related genes in the gill forward SSH cDNA library of *L. vannamei* induced by low salinity stress. These genes included mitochondria 12s, domain protein, elongation factor, ribosomal protein L7a, 40S ribosomal protein and initial eukaryotic transcription factor. The up-regulation of those genes suggested that the low salinity stress effectively activated the transcription and translation of osmoregulation-related genes and confirmed that the osmoregulation process was a post-translational mechanism (Towle et al., 2001). Researches in *Penaeus japonicus* showed that the free amino acid (FAA) pool in muscle was directly related to osmoregulationwhile the FAA pool in the hepatopancreas was related to energy expenditure and protein synthesis (Marangos et al., 1989). However, shrimps at 3.0 ppt salinity had significantly higher soluble protein concentration than those at 17.0 and 32.0 ppt in the hepatopancreas (ErChao et al., 2009). We also cloned and identified the Glutamine synthetase of *L. vannamei* from the hepatopancreas forward SSH library. The LvGS can participate in physiological osmotic adaptation to hypo-osmotic water and aid *L. vannamei* in resisting acute salinity changes via the manipulation of nitrogen metabolism (H.-y. Liu et al., 2012). The number of genes related to protein synthesis and processing was the highest (26.2%) in the hepatopancreas SSH library, including eukaryotic translation initiation factor 5A, elongation factor 1-alpha, ribosomal protein S15, 18S ribosomal RNA, ribosomal protein S2, ribosomal protein L8, and proteasome subunit alpha type-3. These genes whose expression levels were up-regulated possibly accelerated protein synthesis in the hepatopancreas and contributed to the tolerance of low salinity of *L. vannamei.*

4.3 Carbohydrate Metabolism and Energy Production

Glucose, as an important source of energy, increased rapidly in the hemolymph of *L. vannamei* in hypoxia and up to 4.7 and 5.0-fold in response to 1.5 mg/L Do for 1 h (Soñanez-Organis et al., 2009). Glucose dehydrogenase and phosphoenolpyruvate carboxykinase are key enzymes involved in gluconeogenesis (Enes, Panserat, Kaushik, & Oliva-Teles, 2009). These enzymes catalyze macromolecular substances into glucose, thereby maintaining the glucose concentration in hemolymph sufficient for energy expenditure. Numerous genes involving in gluconeogenesis and energy production were uncovered in the SSH library, including glucose dehydrogenase, phosphoenolpyruvate carboxykinase, ATP/ADP translocase, mitochondrial ATP synthase, 24-dehydrocholesterol reductase, and ATP-binding cassette sub-family A member 3. These results indicated the activation of the gluconeogenesis pathway by hypo-osmotic stress and suggested that more energy was consumed for osmoregulation under hypo-osmotic environment. The nicotinamide dehydrogenase (NADH dehydrogenase) and oxidoreductase (NADH ubiquinone oxidoreductase) are involved in the glycolysis and citric acid cycle of cellular respiration. Arginase is a key enzyme involved in ornithine cycle of nitrogen metabolism and catalyzes the hydrolysis of L-arginine to form ornithine and urea. As a part of the urea cycle, arginase is widely expressed and plays a major role gill, hepatopancreas and muscle of crustaceans animals (Chen & Chen, 1997). The transfer of shrimp and other crustaceans from high to low salinity water results in a diffusive loss of salts from the haemolymph to the medium and, consequently, uptake of water from the medium. As water intake increases in response to low salinity, tissues and cells take up water and increase their volume (Pequeux, 1995). Ornithine decarboxylase is the first ***rate-limiting enzyme*** in the dopamine synthesis pathway. It can be activated by the volume increase of tissues and cells induced by low salinity stress (Watts, Yeh, & Henry, 1996). Biogenic amine synthetase, such as ornithine decarboxylase, is activated to produce biogenic amines, such as dopamine (Sommer & Mantel, 1988). Dopamine plays an important role in neurotransmission and causes energy metabolism, osmoregulation, and respiratory response modulation. The dopamine can also stimulate Na^+/K^+-ATPase activity and NaCl uptake to maintain the osmolality balance via a cAMP-mediated phosphorylation (Trausch, Forget, & Devos, 1989).

4.4 Transport

In the gill forward SSH cDNA library constructed in this study, transport-related genes made up the largest proportion (23.3%), including vesicle transport protein, calcium chloride channel protein stimulated vesicle membrane protein-coupled, Na^+/K^+-ATPase (Na^+/K^+-dependent adenosine triphosphatase enzyme) and carbonic anhydrase enzyme important genes. Moreover, the identification of stimulated calcium chloride channel protein was novel in *L. vannamei*. The Na^+/K^+-dependent adenosine triphosphatase (Na^+/K^+-ATPase), composed of three subunits (α, β, γ), is the most important transmembrane protein and predominances in basolateral membrane

regions of the epithelium (Pequeux, 1995). The α-subunit provides the catalytic function of the Na^+/K^+- ATPase, binding and hydrolyzing ATP and itself becoming phosphorylated during the transport cycle. The gill SSH library showed that expression of both α and β subunits of Na^+/K^+-ATPase were simultaneously induced by low salinity stress. During acclimation to dilute seawater, the euryhaline crab *Chasmagnathus granulatus* Na^+/K^+-ATPase α-subunit mRNA increased 35–55 folds in posterior gill within the first 24 h. However, the Na^+/K^+-ATPase β subunit was thought to participate in anchoring the complex in the basolateral membrane (Therien & Blostein, 2000). Our results were similar to study by Wang et al., who revealed those two genes are sensitive and involved in all different stress responses and are more sensitive to salinity than other stresses may have relationship with the anti-stress mechanism induced by environment stress in shrimp (Wang et al., 2012). The carbonic anhydrase (CA) is a zinc metalloenzymes which involves in many biological processes, including pH adjustment, ion exchange, CO_2 transport, respiration, biosynthesis and so on (Henry, Gehnrich, Weihrauch, & Towle, 2003). In gill epithelial cells, the HCO_3^- is provided by carbonic anhydrase which facilitates CO_2 excretion, while NH_4^+ substitutes for K^+ in basal ATPase and for H^+ in the apical exchange (Morris, 2001). The vesicle transport protein can transport synthesized proteins from endoplasmic reticulun to the apical and basolateral membrane. The up-regulation of vesicle transport protein in the gill forward SSH library indicated that the increasing of protein synthesis activated the vesicle transport protein. The detections of carbonic anhydrase and vesicle transport protein in *L. vannamei* are also novel. Transport genes showed up-regulation, suggesting metabolic pathways were involved in physiological adaption to hypo-osmotic environment. The organics synthesized and catabolized in the hepatopancreas are transported to other tissues through these proteins, including vesicle-associated membrane protein 2 which directs protein movement out of a cell, within a cell, or between cells. hemocyanin is synthesized in the hepatopancreas binds oxygen and carries oxygen to other tissues (Lehnert & Johnson, 2002); fatty acid binding protein 10 which is intracellular lipid-binding invovles in absorption, transport and metabolism of lipid (Arena et al., 2011; Venkatachalam, Thisse, Thisse, & Wright, 2009). The up-regulation of these genes may improve the transport efficiency of organics required bythe maintenance of osmotic balance.

4.5 Cell Growth, Signal Transduction and Cytoskeletal Component

The expression of several genes related to cell growth, apoptosis and cytoskeletal component were higher after hypo-osmotic stress, including sterol carrier protein x, cytochrome P450, mitochondrion, senescence-associated protein, glutamic acid-rich protein and glucuronosyl transferases, indicating the acceleration of corporeal cells renewing and its participation in the adaption to low salinity. These results are similar to the report by Shekhar (Shekhar et al., 2013) Numerous genes detected in the SSH library, such as Adapter molecule Crk, putative, chitinase precursor, nuclear progesterone receptor, estrogen sulfotransferase, and destabilase I, are involved in signal transduction and other biological progress. The increased expression of these genes in shrimps with hypo-osmotic stress indicated that signal transductions and other biological progresses are involved in osmotic physiological adaption. The phosphodiesterases (PDEs) can hydrolyze and degrade the intracellular second messengers (cAMP, cyclic adenosine monophosphate, or cGMP, cyclic guanosine monophosphate) to end the biochemical effects conducted by those second messengers (Soderling & Beavo, 2000). In addition, the signal sequence receptors have important functions in numerous physiological processes involved in the recognition of signal sequence, modification of nascent peptide chain and formation of the transport channels (Pequeux, 1995). The cytoskeletal protein and fiber-related genes were found in the gill forward SSH cDNA library. The presence of these genes suggested that transcriptional expression of membrane proteins and cytoskeletal proteins were activated by low salinity stress. This activation resulted in specializations of the gill epithelial membrane structure and ion transport channels to adapt to the changes in the external environment (Towle et al., 2001). The up-regulation of signal transduction genes probably suggested the activation of their mediated pathways by hypo-osmotic stress. However, the function and progress involved in the adaption to low salinity environment requires further study.

In summary, up-regulated genes in the hepatopancreas and gill of *L. vannamei* involved in physiological adaption to hypo-osmotic environment were screened and identified by SSH. The results indicate that cell defense, homeostasis and immune, protein metabolism and processing, carbohydrate metabolism and energy production, transport, cell growth, apoptosis and cytoskeletal component were activated by hypo-osmotic stress. These data are helpful in providing insight into osmotic physiological adaption to hypo-osmotic environment in the hepatopancreas and gill of *L. vannamei*. The functions and molecular mechenism of these genes revealed from the hepatopancreas and gills SSH library require further investigation.

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