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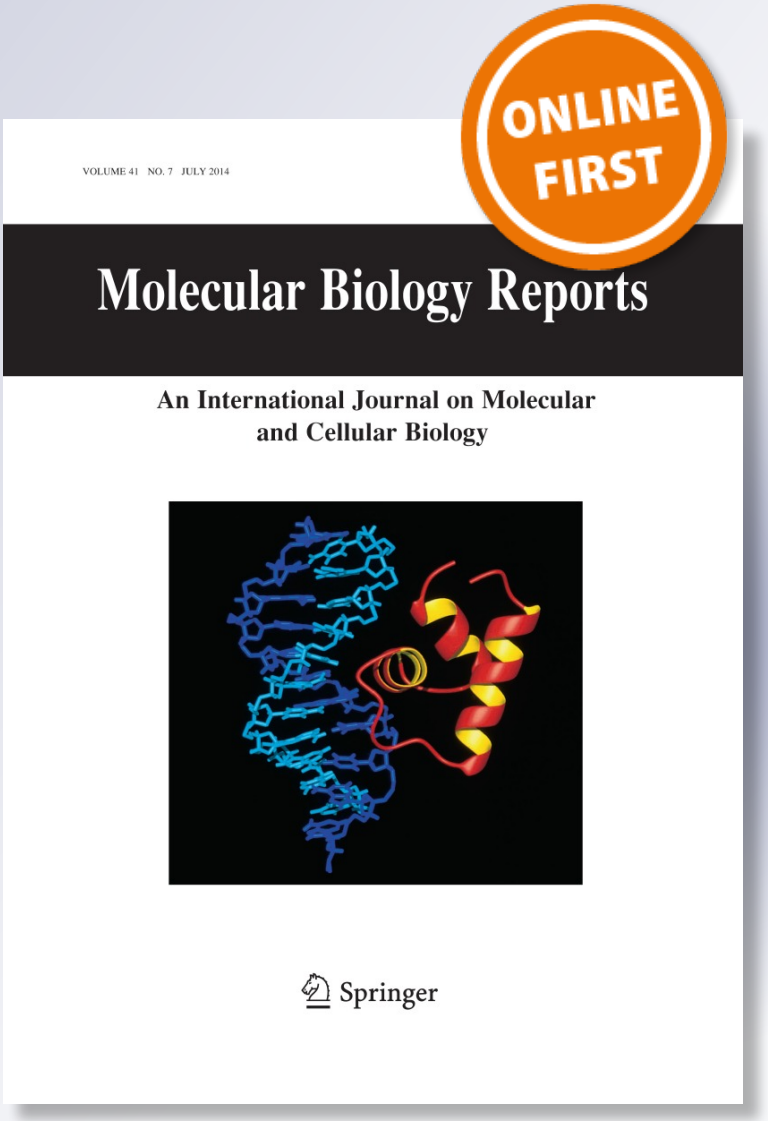

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High salinity induced expression profiling of differentially expressed genes in shrimp (*Penaeus monodon*)

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Abstract 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 high (55 ppt) salinity conditions. One each of the forward and reverse SSH cDNA libraries were developed from the gill and gut tissues of shrimp and clones having inserts larger than 300 bp were unidirectionally sequenced. Based on the sequence homology search, the identified genes were categorized for their putative functions related to a wide range of biological roles, such as nucleic acid regulation and replication, immune response, energy and metabolism, signal transduction, cellular process, structural and membrane proteins, stress and osmoregulation. Gene expression levels in response to high salinity conditions at 2 weeks post salinity stress for some of the differentially expressed genes (Na^+/K^+-ATPase α-subunit, glutathione peroxidase, intracellular fatty acid binding protein, elongation factor 2, 14-3-3 like protein, penaeidin, translationally controlled tumor protein, transglutaminase and serine proteinase inhibitor B3) identified from SSH cDNA libraries were analysed by real-time RT-PCR. The highest gene expression levels was observed for Na^+/K^+-ATPase α-subunit in gill tissues (15.23-folds) and antennal glands (12.01-folds) and intracellular fatty acid binding protein in gut tissues (14.05-folds) respectively. The differential and significant levels of gene expression indicate the functional role of these genes in shrimp salinity stress adaptive mechanisms.

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Keywords Gene expression profiling · *Penaeus monodon* · Salinity stress

Introduction

Many shrimp species are cultured under different farming conditions. Penaeid shrimps being euryhaline in nature have the ability to grow and survive in wide range of salinity. Shrimps are hence, reared and adapted to varying salinity conditions in many tropical and subtropical areas of the world. The optimal salinity is reported in range of 15–25 ppt for *Penaeus monodon* [1], 20–30 ppt for *Fenneropenaeus chinensis* [2], 22–34 ppt for *Penaeus latisulcatus* [3] and 30–35 ppt for *Penaeus semisulcatus* [4]. *Farfantepenaeus subtilis* showed higher growth in salinities above 25 ppt [5]. *Penaeus vannamei* which is native to the Pacific coast was observed to have best growth rates in salinity range of 33–40 ppt [6]. This species of shrimp has remarkable ability to tolerate very low salinities of 1–2 ppt [7] and is therefore preferred for culture in low salinity water also [8]. However, the changes in abiotic factors associated with climatic changes result in stress to the shrimps during culture period. Salinity and temperature are the two major factors of the seawater that influence culture of euryhaline penaeids. For example, survival and growth rate of *L. vannamei* post larvae were shown to depend on temperature, salinity and temperature-salinity interaction [6]. In general, the changes in the major physical factors such as temperature and salinity influence metabolism, growth, molting and survival of shrimps [5, 9, 10]. The salinity changes also results in altered hemato-immunological parameters and total hemocyte count (THC) was found to exhibit the greatest variation in shrimps in response to stress [11]. The changes in the

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salinity conditions results in extreme stress to shrimps undergoing ecdysis. The osmotic and chloride concentrations in *P. monodon* were shown to vary with both molt stage and salinity of the medium [1].

In euryhaline crustaceans, osmoregulation is an important regulatory function undertaken to regulate osmotic and ionic concentrations. Penaeids being very potent hyper and hypo-osmoregulators are able to maintain their internal osmotic concentration on exposure to various salinity conditions. Na^+/K^+-ATPase and carbonic anhydrase are the key enzymes involved in ion uptake in crustaceans. The sodium pump or Na^+/K^+-ATPase, is a major driving force involved in transepithelial movement of monovalent ions in gills by establishing an electrochemical gradient across epithelial cell membranes [12]. In crustaceans, carbonic anhydrase is involved in supplying H^+ and HCO^{3-}, through catalysis of respiratory CO_2, for counterions in cation and anion uptake, respectively [13]. The activation of the specific genes coding for these and other ion transport-related proteins may result in activation of osmoregulation. As, very less information is available on the changes associated at the molecular level in shrimps in response to salinity stress, the aim of this study was to identify and characterize the genes involved in the osmoregulatory process of *P. monodon* when exposed to abrupt salinity variations. Hence, suppression subtractive hybridization (SSH) cDNA libraries were constructed from the gill and gut tissues of the shrimp (*P. monodon*) exposed to high (55 ppt) salinity conditions to identify differentially expressed genes in response to salinity stress. The functional role of these genes in osmoregulatory mechanism is discussed.

Materials and methods

Collection of shrimps, salinity conditions and tissue samples

Penaeus monodon shrimps (10–15 g) at intermolt stage were divided into two groups of 30 numbers each and acclimatized to different salinity conditions. One group of shrimps was acclimatized to high salinity level (55 ppt) by increasing the salinity of sea-water by 2 ppt per day with brine. The second group of shrimps was kept in normal sea-water (28 ppt) as control. Both group of shrimps were maintained for a period of 2 weeks. At the end of 2 weeks, 53 % (16/30) shrimp mortality occurred in test group due to high salinity conditions. The gills, gut and antennal gland samples from six shrimps maintained at high (55 ppt) and 28 ppt salinity conditions were collected in RNA*later* (Qiagen, Germany) and stored in −80 °C till further use.

Construction of SSH cDNA libraries

Gill and gut tissues of the shrimp samples were used to construct four SSH cDNA libraries. Both forward and reverse cDNA libraries were constructed from each of these tissues using PCR-Select cDNA subtraction kit (Clontech, USA). Pooled gut or gill tissues (six numbers in each group) collected from the experimental (55 ppt) and control shrimp served as tester and driver respectively for the two forward SSH cDNA libraries. Whereas, control and experimental (55 ppt) shrimp served as tester and driver respectively for the two reverse SSH cDNA libraries. Total RNA was extracted using NucleoSpin RNA II kit (Macherey–Nagel, Germany) and the cDNA was synthesized using Super SMART-PCR cDNA Synthesis Kit (Clontech, USA).

Screening of SSH cDNA libraries

The PCR products obtained during construction of SSH cDNA libraries were cloned into pGEM-T Easy vector (Promega, USA). The recombinant clones were screened by colony PCR for the insert cDNAs with the vector primers (T7 forward and SP6) before sequencing (Scigenom technologies, India). The sequences were assembled using KEGG (Kyoto Encyclopedia of Genes and Genomes) software (www.genome.jp/kegg) and were grouped into contigs and singletons. Sequence analysis by BLASTX, BLASTN or TBLASTX (www.ncbi.nlm.nih.gov/blast) were used to identify differentially expressed genes in cDNA libraries.

Amplification and cloning of *P. monodon* differentially expressed genes

Amplification of nine *P.monodon* differentially expressed genes (Na^+/K^+-ATPase α-subunit, glutathione peroxidase, intracellular fatty acid binding protein, elongation factor 2, 14-3-3 like protein, penaeidin, translationally controlled tumor protein, transglutaminase and serine proteinase inhibitor B3 (serpinB3)) identified from SSH cDNA libraries were carried out using gene specific forward and reverse primers (Table 1). The PCR products were gel purified using QIAquick PCR purification kit (Qiagen, Germany), cloned into pGEM-T Easy vector (Promega, USA) and sequenced. Nucleotide sequence analysis of genes were performed by BLASTN (http://www.ncbi.nlm.nih.gov/blast).

Gene expression analysis by Real time PCR

Total RNA extracted using NucleoSpin RNA II kit (Macherey–Nagel, Germany) from gills, gut and antennal

Table 1 Gene specific primers used for amplification and real time PCR analysis of differentially expressed genes identified from SSH cDNA libraries

Gene	Primer sequence (5′-3′)	PCR	PCR product size (bp)	Reference
14-3-3	F: ATGTCGGACAAGGAAGAACA	Gene specific	741	AY903449
	R: TCAGTTTTGGTCGCCCTCGT			
	F: CGTGGGAGCTCGGAGAGGT	Real Time	80	KC731528
	R: TTTGTTGTTTTCGCTCTGAACCT			
Na^+/K^+ -ATPase α-subunit	F: ATGGCCGATTCTAAGAAAAAG	Gene specific	3,036	EF672699
	R: TTAATAGTAGGTCTCCAGTTC			
	F: AACCCATTCACCGACAAGCT	Real time	80	KF177338
	R: GCCAGGGCTTGAATCATACC			
Intracellular fatty acid binding protein	F: ATGGCCAAGATTGAAGGAA	Gene specific	411	DQ459988
	R: TTATTCTAAACGAGAGTAAAC			
	F: ATCACCAAGGACGGCGATAC	Real time	90	JN572542
	R: TTCAAATTCCTCCCCCAACTT			
Penaeidin	F: ATGCGTCTCGTGGTCTG	Gene specific	225	AF475082
	R: TCAACCATATGTCTGCTTTG			
	F: CAAGGGTACCAGGGTGGTTACA	Real time	91	JX961662
	R: TGTGGCATGAAGTACAAACA			
Translationally controlled tumor protein	F: ATGAAGGTCTTCAAGGATAT	Gene specific	507	EU492535
	R: TTATAGCTTCTCCTCTGTTA			
	F: GGAGGGAGCCAATCCATCAG	Real time	175	JX961663
	R: GCCTTCCAACTTTGCCTTTA			
Glutathione peroxidase	F: ATGGCTTCCTCCGCTATC	Gene specific	564	GQ996722
	R: TTACAGCAAATTTGCGATTTCA			
	F: CACCGAAGGAGAGTTGCTGAGT	Real time	90	JX912159
	R: ACGTCCACTTTGCCGAACAT			
Serine proteinase inhibitor B3	F: ATGCGTTCGTGCGTCGTT	Gene specific	1,232	GQ260130
	R: TCAGTCGAGCAGAGGCTC			
	F: GCCAGTTCGCCATGTTCTTC	Real time	90	GQ260130
	R: GTGTGTCGGCGTCGAGGTA			
Transglutaminase	F: ATGCCCACCGTGGACGCC	Gene specific	2,273	AY074924
	R: TCAAGCACTGGCCACGCT			
	F: GTCCCCATCGGCGATAATT	Real time	90	AY074924
	R: GGCGCAGGTCATCACTACGT			
Elongation factor 2	F: ATGGTGAACTTCACAGTGGA	Gene specific	2,540	EF426560
	R: TTACAGCTTGTCCAGGTAGT			
	F: ATCGAAGGCGGGTATTATTGC	Real time	85	EF426560
	R: AATGCATCGCTCCTGTTCATC			
β-actin	F: GAAGCTGTGCTACGTGGCTCTG	Real time	124	JN808449
	R: GAACCTCTCGTTGCCGATGGTG			

gland tissues of control and high salinity stressed shrimps were converted to cDNA using Protoscript M-MuLV first strand cDNA synthesis kit (New England Biolabs, USA). The cDNA was used to analyze the relative expression of the selected nine *P. monodon* differentially expressed genes (Na^+/K^+-ATPase α-subunit, glutathione peroxidase, intracellular fatty acid binding protein, elongation factor 2, 14-3-3 like protein, penaeidin, translationally controlled tumor protein, transglutaminase and serpinB3) identified from SSH cDNA libraries by real-time PCR using the Power SYBR green PCR master mix (Applied Biosystems, USA). Real time primers as shown in Table 1 were designed with the aid of Primer Express software (Applied Biosystems, USA). The shrimp β-actin gene amplified with primers (β-actin F and β-actin R) was used as an endogenous control. The relative quantification of the transcripts

were assessed by comparative $\Delta\Delta C_T$ method by measuring amplification of the target, endogenous control and reference sample. Amplification measurements were normalized using endogenous control. The relative quantification results were expressed as the fold change in levels of the gene expression by comparing normalized target quantity in each sample to normalized target quantity in the reference sample [14]. Statistical analysis of the data for comparison between groups was carried out by one-way ANOVA using Duncan's test and the values with $p < 0.05$ were considered significant.

Results

Identification and cloning of differentially expressed genes by SSH in salinity stressed shrimp *P. monodon*

The forward and reverse SSH cDNA libraries generated multiple colonies representing up-and down regulated genes respectively in the gill and gut tissues of the high salinity stressed *P.monodon* shrimps with the insert size ranging from 200 bp to 1.5 kb. Clustering of the ESTs using KEGG software generated 7 (contigs) and 35 (singletons) from the forward SSH cDNA library and 6 (contig) and 22 (singletons) from the reverse SSH cDNA library constructed from gill tissues of shrimp. Whereas, 22 (contigs) and 28 (singletons) from the forward SSH cDNA library and 18 (contigs) and 56 (singletons) were generated from the reverse SSH cDNA library constructed from gut tissues of shrimp. The SSH clones were grouped into putative functions based on the predicted functional category by BLAST analysis for the clones obtained from the forward SSH cDNA library (Table 2) and from the reverse SSH cDNA library (Table 3) constructed from gill tissues of shrimp. Putative functions were also assigned for the clones obtained from the forward SSH cDNA library (Table 4) and from the reverse SSH cDNA library (Table 5) constructed from gut tissues of shrimp. The percentage of differentially expressed genes identified from SSH cDNA library genes belonging to different putative functional categories is shown in Table 6.

Amplification of *P.monodon* differentially expressed genes

Amplification of *P.monodon* differentially expressed genes were confirmed by sequencing and the sequence information obtained for the six genes glutathione peroxidase (JX912159), elongation factor 2 (KF740505), penaeidin (JX961662), translationally controlled tumor protein (JX961663), transglutaminase (KF725626) and serpinB3 (KF525274) were submitted to the GenBank. The sequence information obtained for Na^+/K^+-ATPase α-subunit, intracellular fatty acid binding protein and 14-3-3 like protein were reported in our earlier study [18].

Gene expression analysis by Real time PCR

In gill tissues, the up-regulation with highest gene expression level was observed for Na^+/K^+-ATPase α-subunit (15.23-folds) followed by elongation factor 2 (9.31-folds), 14-3-3 like protein (3.94-folds), transglutaminase (3.32-folds), translationally controlled tumor protein (2.36-folds), glutathione peroxidase (2.10-folds), intracellular fatty acid binding protein (2.00-folds), serpinB3 (1.83-folds) and penaeidin was observed to be down-regulated with −0.18 folds (Fig. 1 a).

In gut tissues, the up-regulation with highest gene expression level was observed for intracellular fatty acid binding protein (14.05-folds) followed by Na^+/K^+-ATPase α-subunit (5.70-folds), 14-3-3 like protein (2.48-folds), elongation factor 2 (1.15-folds). The other genes penaeidin (−0.05-folds), serpinB3 (−0.18-folds), translationally controlled tumor protein (−0.24-folds), glutathione peroxidase (−0.66-folds) and transglutaminase (−0.75-folds) were observed to be down-regulated (Fig. 1b).

In antennal gland, the up-regulation with highest gene expression level was observed for Na^+/K^+-ATPase α-subunit (12.01-folds) followed by glutathione peroxidase (8.9-folds), transglutaminase (7.31-folds), translationally controlled tumor protein (5.00-folds), elongation factor 2 (4.76-folds), intracellular fatty acid binding protein (4.75-folds), 14-3-3 like protein (4.53-folds), serpinB3 (3.75-folds) and penaeidin (1.27-folds) (Fig. 1c).

Discussion

In the present study, forward and reverse SSH cDNA libraries constructed from gill and gut tissues of *P. monodon* resulted in identification of differentially expressed genes which could be classified for putative functions based on sequence homology search. We selected gills, gut and antennal gland of the shrimp for identification and characterization of differentially expressed genes in response to salinity stress as these organs are involved in crustacean osmoregulation [15]. Osmoregulatory capacity of shrimp reared in low salinity waters is well studied [8] and differentially expressed genes from shrimps exposed to low salinity stress have been characterized to some extent [16–18]. However, the culture of euryhaline decapod crustaceans at high salinities results in reduced growth and the causes for this reduced growth at high salinities are not clear as compared to those under lower salinities [19].

The optimal salinity for penaeids range from 15 to 40 ppt. For *P. monodon*, the optimum salinity for the

Table 2 Identification of differentially expressed genes from forward SSH cDNA library constructed from gill tissues of shrimp (*P. monodon*) exposed to high salinity (55 ppt) stress

Putative function (clones)	Length (bp)	Gene homology	GenBank Accession No.	Species homology	E-value	Similarity (%)	BLAST
Nucleic acid regulation and replication							
HSGUSE71	643	Helicase	EFX87750	*Daphnia pulex*	4.74 e−27	57	BlastX
HSGUSA13	690	Transposase	AM402994	*Listonella anguillarum*	2.54 e−8	92	BlastN
HSGUSB6	549	Transposase	AM402994	*Listonella anguillarum*	3.85 e−11	96	BlastN
Energy and metabolism							
HSGUSB7		Fructose-bisphosphate aldolase	ABW71268	*Phoronis muelleri*	6e−48	74	BlastX
HSGUC27	370	ATP synthase	NP038292	*Penaeus monodon*	3.3 e−20	83.85	BlastX
HSGUSE67	389	Ferritin light chain	ACR43472	*Rimicaris exoculata*	2.7 e−18	98	BlastX
Immune response							
HSGUSE24	624	Lysozyme	ABU75288	*Penaeus monodon*	2.87 e−22	61	BlastX
HSGUSF90	225	Glutathione peroxidase	ACB42236	*Metapenaeus ensis*	2.17e−6	72	BlastX
HSGUSE21	487	Basic salivary proline rich protein	XP003313554	*Pan troglodytes*	4.77 e−4	71	BlastX
HSGUSE180	617	Relish	JQ728539	*Penaeus monodon*	1.71 e−10	98	tBlastX
HSGUSE93	403	Relish	JQ728539	*Penaeus monodon*	1.22 e−52	100	tBlastX
Signal transduction							
HSGUSF3	565	Casein kinase 2 alpha1	CAQ14696	*Danio rerio*	4.98 e−34	44	BlastX
HSGUC21	261	Adenylate cyclase	ABK91814	*Artemia franciscana*	5.2 e−11	60.65	BlastX
HSGUSF61	372	14-3-3-like protein	AY903449	*Penaeus monodon*	1.43 e−40	100	tBlastX
Allergen protein							
HSGUC30	363	allergen Pen m 2	AAS98886	*Fenneropenaeus chinensis*	4.7 e−21	99.3	BlastX
Receptor protein							
HSGUSF39	260	Virus receptor protein	AAZ22828	*Penaeus monodon*	1.53 e−36	90	BlastX
Cytoskeletal protein							
HSGUSF58	511	Moesin/ezrin/redixin homolog	EFN67498	*Camponotus floridanus*	4.07e−9	84	BlastX
Reproduction							
HSGUC7	324	Vitellogenin like	HM217799	*Scylla paramamosain*	1.6 e−6	97	BlastN
Ion transport and osmoregulation							
HSGUSF110	516	Na/K ATPase	ABD59803	*Penaeus monodon*	5.60 e−44	98	BlastX
Ribosomal protein							
HSGUSB15	649	Ribosomal protein	ADN23582	*Hyalomma marginatum rufipes*	1.46 e−72	83	BlastX
HSGUSE120	310	Ribosomal protein	AEL22133	*Cherax quadricarinatus*	9.04 e−9	68	BlastX
HSGUSF8	396	Ribosomal protein	AEL23133	*Cherax quadricarinatus*	1.56 e−33	92	BlastX
HSGUSE52	495	Ribosomal protein	AEB54641	*Procambarus clarkii*	4.24 e−62	80	BlastX
Cellular process							
HSGUSE15	689	Catechol-O-methyltransferase	AEP84098	*Penaeus monodon*	5.5 e−13	66.7	BlastX
HSGUSE72	1,196	Elongation factor-2	ABR01223	*Penaeus monodon*	1.17 e−91	78	BlastX

Table 2 continued

Putative function (clones)	Length (bp)	Gene homology	GenBank Accession No.	Species homology	E-value	Similarity (%)	BLAST
HSGUSB12	549	Lysyl hydroxylase	NM001099604	*Takifugu rubripes*	8.48 e−7	88	BlastN
HSGUSC27	493	Lysyl hydroxylase	AB259759	*Takifugu rubripes*	6.63 e−14	81	BlastN
Hypothetical protein (15)							

The number of clones identified as hypothetical genes are represented in brackets

culture of shrimps is reported to be ~25 ppt and it was observed that salinity had a strong influence on various energy parameters. The salinity range outside 20–35 ppt led to significant reduction in shrimp growth mainly due to increased energy utilization for respiration, excretion and exuviae [20]. Therefore, we selected a very high salinity stress of 55 ppt which is outside the optimal range of salinity for penaeids, that would give insights to the molecular response of shrimp under high salinity stress conditions. The functional roles of nine differentially expressed genes which were subjected to gene expression analysis in gills, gut and antennal glands of shrimp is discussed.

Immune related genes

Innate immunity, also called as non-specific immune system is known to exist in shrimps in absence of an adaptive immune system to defend against various viral and microbial pathogens. Some of the shrimp immune-related proteins such as antimicrobial peptides, penaeidin, relish, lysozyme, glutathione peroxidase, anti-lipopolysacharride factor and mucin genes were differentially expressed in gill and gut tissues of *P. monodon* exposed to high salinity stress conditions.

Penaeidins, belong to family of antimicrobial peptides and show Gram-positive antibacterial activities. In shrimps, penaeidins were first characterized from *L. vannamei*, which were named as penaeidin (Pen)-1, -2 and -3 [21]. Penaeidins are reported to be constitutively synthesized and stored in granulocytes and released after microbial challenge [22]. In the present study, penaeidin gene which was differentially expressed in reverse SSH cDNA library constructed from gill and gut tissues, was found to be down- regulated in gill (−0.18-folds), gut (−0.05-folds) and up-regulated (1.27-folds) in antennal gland tissues of shrimp. The environmental stress factors influence the innate immune mechanism in shrimps. The down- regulation of penaeidin gene expression in gill and gut in shrimp exposed to high salinity stress, is in agreement with other studies in shrimps which have revealed lower expression of this gene against other stress factors such as heat shock [23]. The gene expression of penaeidins, which are constitutively produced and stored in the haemocytes of penaeid shrimp [24], may also be regulated through changes in haemocyte populations that occur in salinity stressed shrimp.

Glutathione peroxidase, is one of the wide range of enzymatic scavenger systems possessed by the cells to minimize the oxidative stress caused by reactive oxygen species (ROS). Glutathione peroxidase usually a selenoprotein [25], is a glutathione-dependent enzyme which occurs widely in eukaryotes. It plays an important role during phagocytosis or physiological metabolism in detoxifying lipids and hydrogen peroxide which are rapidly formed with the concomitant oxidation of glutathione [26, 27]. Recently, glutathione peroxidase was isolated from penaeid shrimp *Metapenaeus ensis*. It's specific expression in shrimp ovaries, pre-vitellogenic and mid-vitellogenic oocytes suggests its pivotal role in preventing oocytes from oxidative damage and balancing ROS production [28]. The role of glutathione peroxidases in shrimp host defense system has been reported [29, 30]. In the present study, glutathione peroxidase was found to be significantly down-regulated in gut (−0.66-folds) and significantly up-regulated in antennal gland tissues (8.9-folds) of shrimp indicating of oxidative stress response to salinity changes as observed in crustaceans [31].

An anti-apoptotic translationally controlled tumor protein (TCTP) gene named *Pm*–fortilin has been identified from *P. monodon* and its functional role has been established in immune defence against WSSV infection in shrimp [32–34]. TCTP which is a growth-related protein under transcriptional and translational control is reported to interact with the cytoplasmic domain of yeast and mammalian Na^+/K^+-ATPase isoforms and act as a cytoplasmic repressor of Na^+/K^+-ATPase. Overexpression of TCTP in HeLa cells inhibited the Na^+/K^+-ATPase activity but did not affect Na^+/K^+-ATPase at either mRNA or protein levels [35]. In the present study, TCTP was found to be significantly up-regulated in gill (2.36-folds) and antennal gland (5.00-folds) and down-regulated in gut (−0.24-folds)

Table 3 Identification of differentially expressed genes from reverse SSH cDNA library constructed from gill tissues of shrimp (*P. monodon*) exposed to high salinity (55 ppt) stress

Putative function (clones)	Length (bp)	Gene homology	GenBank Accession. No.	Species homology	E-value	Similarity (%)	BLAST
Immune response							
HSGDC3	185	Anti-lipopolysaccharide factor isoform 1	ABP73290	*Penaeus monodon*	6.11 e−9	65	BlastX
HSGDS55	632	Penaeidin 3a antimicrobial peptide	FJ686016	*Penaeus monodon*	0.0	99	BlastN
HSGDS238	536	peroxidase	DQ317315	*Musa acuminata*	2.71 e−8	85	BlastN
Energy and metabolism							
HSGDC4	254	Cytochrome c oxidase subunit III	YP002922052	*Farfantepenaeus californiensis*	1.34 e−19	87	BlastX
HSGDC10	661	Cytochrome c oxidase subunit II	NP038290	*Penaeus monodon*	3.16 e−43	95	BlastX
HSGDC11	480	NADH dehydrogenase subunit 1	NP038300	*Penaeus monodon*	6.75 e−76	89	BlastX
Reproduction							
HSGDS239	1,943	Nuclear progesterone receptor	GU906280	*Penaeus monodon*	1.06 e−46	100	BlastN
Signal transduction							
HSGDS70	551	Serine/threonine kinase	DQ459385	*Nicotiana tabacum*	8.14 e−4	86	BlastN
Nucleic acid regulation and replication							
HSGDS61	455	Transposase	AM402994	*Listonella anguillarum serovar*	1.07 e−5	90	BlastN
HSGDS48	455	Transposase	AM402994	*Listonella anguillarum serovar*	1.56 e−6	87	BlastN
Hypothetical proteins							
HSGDS45	788	Hypothetical protein	AM706411	*Eristalis tenax*	3.45 e−5	90	BlastN
HSGDS105	393	Hypothetical protein	XP001892466	*Brugia malayi*	2.11e−4	57	BlastX
Stress genes (16)		Cold-related Novel Gene	GQ844762	*Bombyx mori*			tBlastX

The number of clones identified as stress genes are represented in brackets

tissues of shrimp suggesting a possible new mechanism for the regulation and maintenance of ion homeostasis in shrimps by interacting with Na^+/K^+-ATPase. However, further work is necessary to elucidate the precise osmoregulatory role of TCTP in shrimps.

Transglutaminase in shrimps are known to be involved in haemolymph clotting which is one of the vital components of the innate shrimp immune response. Transglutaminase gene expression and protein levels changes in response to viral and bacterial infections. For example, the transglutaminase activity is reported to decrease and results in poor haemolymph coagulation in Taura Syndrome Virus infected *L. vannamei* infection [36] whereas, transglutaminase gene expression were observed to increase using *Vibrio harveyi* [37]. Transglutaminase is located in the haemocytes and haematopoietic tissue of shrimps [38–40]. Silencing of transglutaminase by RNAi mechanism led to a decrease in the expression of total haemocytes, suggesting its role in the proliferation of circulating haemocytes [41]. The differential expression with varying gene expression levels of transglutaminase observed in this study indicates its functional role in salinity stress. Enhanced activity of transglutaminase is reported to occur in presence of neutral salts such as NaCl or KCl in marine invertebrates [42]. We have previously shown that high salinity stress leads to decrease in total haemocyte counts in shrimps [43], therefore it would be interesting to estimate the transglutaminase activity and gene expression levels in haemocytes and transglutaminase mediated clotting reactions in shrimps exposed to salinity stress.

Down-regulation of penaeidin, glutathione peroxidase and TCTP in some of the shrimp tissues suggests that the expression of these immune genes is negatively affected by environmental stress such as salinity, which could lead to

Table 4 Identification of differentially expressed genes from forward SSH cDNA library constructed from gut tissues of shrimp (*P. monodon*) exposed to high salinity (55 ppt) stress

Putative functions (clones)	Length (bp)	Gene homology	GenBank Accession. No	Species homology	E-value	Similarity (%)	BLAST
Nucleic acid regulation and replication							
HSGTUC20	684	RNA polymerase	NP941973	*Uukuniemi virus*	0.021	38	Blast X
HSGTUC25	359	RNA polymerase	NP941973	*Uukuniemi virus*	0.031	48	Blast X
HSGTUS1	435	Transposase	CAL47051	*Listonella anguillarum*	0.027	70	Blast X
Energy and metabolism							
HSGTUS33	776	2-oxoglutarate ferredoxin oxidoreductase subunit beta	NP148404	*Aeropyrum pernix*	6.6	36	Blast X
HSGTUS57	319	Sulfite reductase alpha subunit (flavoprotein)	YP001938875	*Methylacidiphilum infernorum*	0.56	56	Blast X
HSGTUC1	583	Cytochrome b	NP038299	*Penaeus monodon*	3e−100	94	Blast X
HSGTUC5	600	Fructose 1,6-bisphosphate aldolase	NP001091766	*Bombyx mori*	2e−60	81	Blast X
HSGTUS12	143	Long-chain-fatty-acid-CoA ligase	EGU13426	*Rhodotorula glutinis*	5.3	50	Blast X
HSGTUS56	359	2-isopropylmalate synthase	ZP02963711	*Bifidobacterium animalis*	3.8	38	Blast X
HSGTUC27	431	senescence-associated protein	ABO20851	*Lilium longiflorum*	9e−37	85	Blast X
HSGTUS11	635	Acyl CoA Binding protein	ACU82846	*Fenneropenaeus chinensis*	4e−54	100	Blast X
HSGTUS21	650	Fatty Acid binding protein	ADK66280	*Litopenaeus vannamei*	1e−62	98	Blast X
Immune response							
HSGTUS45	1,049	Immunoglobulin heavy chain variable region	AAK67989	*Homo sapiens*	6.6	41	Blast X
Cellular process							
HSGTUC19	705	O-methyltransferase	AEP84098	*Penaeus monodon*	1e−35	92	Blast X
HSGTUS2	358	Glycoside hydrolase	ZP07918724	*Bacteroides sp*	0.82	43	Blast X
HSGTUS48	205	Glycosyl transferase	YP001518321	*Acaryochloris marina*	6.4	43	Blast X
HSGTUC14	448	Glucuronosyltransferase	NP001170967	*Danio rerio*	1e−04	45	Blast X
HSGTUC18	290	Cathepsin B	ABQ10737	*Penaeus monodon*	9e−68	100	Blast X
HSGTUS3	1,165	Elongation factor 2	ABR01223	*Penaeus monodon*	0.0	92	Blast X
HSGTUS47	1,102	Elongation factor 2	ABR01223	*Penaeus monodon*	3.6 e−16	91	Blast X
HSGTUS24	284	Signal peptidase I	ZP05082261	*Beta proteobacterium*	0.47	45	Blast X
HSGTUC8	262	alpha-amylase like	XP787209	*Strongylocentrotus purpuratus*	2e−15	65	Blast X
HSGTUC3	370	Ataxin-1-like protein	EHB10951	*Heterocephalus glaber*	7.7	37	Blast X
HSGTUS18	120	Proline-rich extensin-like family protein	AEE82632	*Arabidopsis thaliana*	5.0	47	Blast X
HSGTUC16	365	Amino acid transporter	XP001864053	*Culex quinquefasciatus*	3.8	40	Blast X
HSGTUS27	142	Small oligopeptide transporter, OPT family	XP002340616	*Talaromyces stipitatus*	2.8	38	Blast X
HSGTUS40	520	Transport protein	CBN81528	Dicentrarchus labrax	0.32	39	Blast X
Signal transduction							
HSGTUC17	511	Sensor histidine kinase	ZP07294336	*Streptomyces hygroscopicus*	7.8	32	Blast X

Table 4 continued

Putative functions (clones)	Length (bp)	Gene homology	GenBank Accession. No	Species homology	E-value	Similarity (%)	BLAST
HSGTUS14	298	ADP-ribosylation factor-related protein 1/GTP binding	EFN63993	Camponotus floridanus	1.3 e−46	89	Blast X
HSGTUS53	701	Protocadherin alpha-3-like	XP003480984	*Sus scrofa*	0.91	34	Blast X
Apoptosis							
HSGTUS43	221	Endonuclease G	YP008924	*Candidatus Protochlamydia amoebophila*	8.9	38	Blast X
Cellular and membrane proteins							
HSGTUC18	383	Collagen alpha-1(XI) chain	NP001077313	*Danio rerio*	3.6	50	Blast X
HSGTUC26	1,011	G2 virion envelope glycoprotein	AAY15205	*Mourilyan virus*	3e−28	52	Blast X
HSGTUC15	461	Membrane protein	YP002561656	*Streptococcus uberis*	4.0	48	Blast X
HSGTUS22	290	Inner-membrane translocator	YP533555	*Rhodopseudomonas palustris*	9.3	38	Blast X
Hypothetical proteins (15)							

The number of clones identified as hypothetical proteins are represented in brackets

reduced immune capacity of shrimp resulting in an increased susceptibility to disease outbreaks.

Osmoregulation related genes

Recently, Havrid et al., [44] meta-analysed the published reports that used quantitative polymerase chain reaction to examine expression of osmoregulatory genes in euryhaline animals. Na^+/K^+-ATPase, a critical component in osmotic/ionic regulation and for establishing electrochemical gradients across the cell membrane in the gills of euryhaline animals was identified as the most studied gene in osmoregulatory experiments. The changes in gene expression of Na^+/K^+-ATPase α-subunit in response to salinity stress have been observed in shrimps [45]. The expression of V–H ATPase α-subunit and Na^+/K^+-ATPase β-subunit in *L. vannamei* was found to be more sensitive to salinity stress when compared to other environmental stress (bacteria, pH, Cd and low temperature) responses [46]. In crustaceans, many reports have indicated that the change from a higher salinity to a lower salinity conditions also results in increased Na^+/K^+-ATPase gene expression and activity levels [12, 46–48]. The results in the present study suggest that *P. monodon* Na^+/K^+-ATPase gene which responded to salinity stress conditions with significant expression levels in gill (15.23-folds), gut (5.70-folds) and antennal gland (12.01-folds) tissues is involved in osmoregulatory process in shrimp.

Cellular process related genes

In eukaryotes, two soluble protein factors, designated elongation factor 1 (EF-1) and elongation factor 2 (EF-2) are associated with polypeptide chain elongation steps in eukaryotic protein synthesis [49]. Elongation factor 1-alpha (EF1A) and elongation factor 2 (EF2) from *L. vannamei* [50] and EF2 from *P. monodon* [51] have been isolated and characterized. Multiple sequence alignment of shrimp EF1A and EF-2 showed the high conservation with the other species (both vertebrate and invertebrate) indicating that the genes encoding EF1A and EF2 are highly conserved between species and might have similar functions in vertebrates and invertebrates [50, 51]. The deduced amino acid sequence of EF-2 of the present study, showed high similarity (99 %) when compared with EF-2 sequence reported from *P. monodon* (GenBank accession ABR01223). The protein sequence analysis by ExPASy (http://swissmodel.expasy.org/) revealed GTP- binding domains of GTP_EFTU (17–348),GTP_EFTU_D2 (395–472) and EFG_IV domain (606–723) confirming the presence of conserved domains in EF-2 of *P. monodon* (data not shown). To our knowledge, very little information of the functional roles of either EF1A or EF2 in shrimp is available. EF-2 isolated from *P. monodon* was observed to be constitutively expressed in ovary and was suggested to play an important role in the shrimp ovarian maturation stage [51]. In *L. vannamei*, up-regulation of EF1A and EF2 mRNA expression was observed following exposure to pH and cadmium stress, indicating that the EF1A and EF2 genes are inducible and involved in stress responses [50]. The expression of elongation factor 1Bγ (eEF1Bγ) a subunit of elongation factor 1 (EF1) was shown to be involved with molting cycle in freshwater crayfish, *Procambarus clarkii* [52]. Significant increase in the gene expression levels were observed for elongation factor 2 in gill (9.31-

Table 5 Identification of differentially expressed genes from reverse SSH cDNA library constructed from gut tissues of shrimp (*P. monodon*) exposed to high salinity (55 ppt) stress

Putative function (clones)	Length (bp)	Gene homology	GenBank Accession. No.	Species homology	E-value	Similarity (%)	BLAST
Nucleic acid regulation and replication							
HSGTDC11	1,845	S-adenosylmethionine synthetase	XM961585	*Tribolium castaneum*	7.23 e−6	84	BlastN
HSGTDS82	287	Polyadenylation factor subunit	XP002430712	*Pediculus humanus corporis*	1.42 e−9	55	BlastX
Immune response							
HSGTDS55	615	Antimicrobial peptide	AY859500	*Fenneropenaeus chinensis*	4.28 e−19	81	BlastN
HSGTDS102	275	Anti-lipopolysaccharide factor isoform 4	EF523563	*Penaeus monodon*	2.07e−13	100	BlastN
HSGTDS115	255	Relish	JQ728539	*Penaeus monodon*	1.36 e−12	100	BlastN
HSGTDS122	212	Penaeidin	FJ686017	*Penaeus monodon*	4.59 e−10	100	BlastN
HSGTDC8	271	Mucin-like protein	AAL85612	*Aedes aegypti*	5.96 e−4	43	BlastX
Energy and metabolism							
HSGTDC2	1,249	Ferritin	AY955373	*Litopenaeus vannamei*	2.91 e−16	100	BlastN
HSGTDS63	338	Fructose 1,6-bisphosphatase	BAJ23881	*Marsupenaeus japonicus*	1.50 e−30	9	BlastX
HSGTDS129	148	Ubiquitin-conjugating enzyme E2	ADK60919	*Fenneropenaeus chinensis*	1.30 e−48	97	BlastX
HSGTDS239	399	Ubiquitin/ribosomal S30 fusion protein	ADF45324	*Eriocheir sinensi*	2.97 e−17	64	BlastX
HSGTDS15	248	Triosephosphate isomerase	ABB81879	*Fenneropenaeus chinensis*	6.90 e−52	98	BlastX
HSGTDC3	394	NADH dehydrogenase subunit 2	NP038288	*Penaeus monodon*	2.87 e−46	98	BlastX
HSGTDC4	224	ATP synthase F0 subunit 6	NP038292	*Penaeus monodon*	2.0 e−27	94	BlastX
HSGTDS41	224	ATP synthase F0 subunit 6	NP038292	*Penaeus monodon*	5.05 e−30	95	BlastX
HSGTDS33	780	ATP synthase subunit 9	EU194608	*Litopenaeus vannamei*	2.76 e−16	95	BlastN
HSGTDS241	362	Triacylglycerol lipase	ACU57197	*Litopenaeus vannamei*	7.32 e−17	59	BlastX
HSGTDC19	312	NADH dehydrogenase subunit 1	NP038300	*Penaeus monodon*	1.91 e−85	91	BlastX
HSGTDS32	260	Cytochrome oxidase subunit I	ACD74583	*Penaeus monodon*	3.05 e−20	88	BlastX
HSGTDC17	308	Cytochrome oxidase subunit I	ACZ28891	*Macrobrachium lepidactylus*	3.28 e−98	92	BlastX
HSGTDS72	260	Cytochrome oxidase subunit I	ACD74583	*Penaeus monodon*	1.23 e−17	95	BlastX
HSGTDC13	229	Cytochrome c oxidase subunit II	NP038290	*Penaeus monodon*	2.96 e−39	93	BlastX
HSGTDS44	229	Cytochrome c oxidase subunit II	YP002922049	*Farfantepenaeus californiensis*	1.78 e−15	80	BlastX
HSGTDS189	263	cytochrome c oxidase subunit III	NP038293	*Penaeus monodon*	1.61 e−79	74	BlastX
HSGTDC10	263	cytochrome c oxidase subunit III	NP038293	*Penaeus monodon*	9.64 e−69	94	BlastX
HSGTDS11	263	cytochrome c oxidase subunit III	NP038293	*Penaeus monodon*	7.35 e−52	97	BlastX
HSGTDS37	263	cytochrome c oxidase subunit III	ABG65670	*Fenneropenaeus chinensis*	6.27 e−5	74	BlastX
HSGTDS163	263	cytochrome c oxidase subunit III	NP038293	*Penaeus monodon*	2.29 e−44	97	BlastX
Cellular process							
HSGTDC5	381	Chitin deacetylase 9 precursor	NP001103904	*Tribolium castaneum*	3.33 e−25	52	BlastX

Table 5 continued

Putative function (clones)	Length (bp)	Gene homology	GenBank Accession. No.	Species homology	E-value	Similarity (%)	BLAST
HSGTDS210	291	Clottable protein 2	EU082133	*Penaeus monodon*	5.19 e−13	98	BlastN
HSGTDS187	168	Translationally controlled tumor protein	ACD13588	*Penaeus monodon*	6.41 e−35	100	BlastX
HSGTDS10	659	Fibrinogen and fibronectin	XP001849757	*Culex quinquefasciatus*	1.97 e−7	60	BlastX
HSGTDS34	735	Transglutaminase	AY074924	*Penaeus monodon*	0.0	88	BlastN
HSGTDS206	130	Calponin	XP002834486	*Pongo abelii*	1.20 e−7	78	BlastX
HSGTDS209	601	Ribophorin I	ACA83751	*Penaeus monodo*	1.17 e−51	98	BlastX
HSGTDS208	389	Ribophorin I	EU369695	*Penaeus monodon*	0.0	97	BlastN
HSGTDC16	193	Serine proteinase inhibitor B3	GQ260130	*Penaeus monodon*	2.33e−90	98	BlastN
Signal transduction							
HSGTDS81	189	Calreticulin	HQ259085	*Penaeus monodon*	2.76 e−89	99	BlastN
HSGTDS109	57	ADP ribosylation factor 4	GQ279375	*Marsupenaeus japonicus*	8.43 e−14	94	BlastN
Structural proteins							
HSGTDS42	160	Arthrodial cuticle protein	ABC26005	*Callinectes sapidus*	9.33 e−18	67	BlastX
HSGTDS49	281	Alpha-I tubulin	ACY66451	*Scylla paramamosain*	3.26e−10	96	BlastX
Mitochondrial genes (9)							
Ribosomal proteins (23)							
Hypothetical proteins							
HSGTDS98	56	Hypothetical protein	ACJ50595	*Lutzomyia shannoni*	1.01 e−8	85	BlastX

The number of clones identified as mitochondrial and ribosomal genes are represented in brackets

folds), gut (1.15-folds) and antennal gland (4.76-folds) tissues of shrimp on exposure to salinity stress in this study.

Serpins, which are a superfamily of proteins play an important role in inhibition of serine proteinases. Serpins are ubiquitous in multicellular higher eukaryotes and several serpin-like genes have been identified from humans, animals, poxviruses, plants and insects except fungi. Very few serpins have been identified in shrimp in which they are generally reported to be immune responsive. Based on immunocytochemistry using anti-*Pm*SERPIN6 polyclonal antiserum, SERPIN6 identified from *P. monodon* revealed pathogen response in the late phase of infection with WSSV and *V. harveyi* [53]. A serpin (*Fc*-serpin) isolated from *F. chinensis* was reported to have potential roles in the innate immunity of shrimp challenged with bacterial and WSSV pathogens [54]. The serpin identified in this study was similar to serpin B3 previously isolated from *P. monodon* (GenBank accession ADC42877). The functional role of this serpin has been associated in bacterial response in shrimp *P. monodon* with increased expression level of the gene in shrimp hemocytes after microbial challenge with *V. harveyi* [55]. The serpin isolated in this study indicates its potential role in salinity stress response in shrimp with significant increase in gill (1.83-folds) and antennal gland (3.75-folds).

Signal transduction related genes

The different isoforms of 14-3-3 protein which are expressed in a wide range of organisms and tissues play an important role in phosphorylation and modulating protein interactions. The diverse functional roles of 14-3-3 proteins include cell signaling, regulation of cell cycle progression, intracellular trafficking/targeting, cytoskeletal structure and transcription [56] and in regulating plant plasma membrane H^+-ATPase and K^+ channels [57]. In crustaceans such as crab *Pachygrapsus marmoratus*, the N terminus of the D form of Na^+/K^+-ATPase α-subunit, was shown to contain a 14-3-3 protein binding site suggesting that the Na^+/K^+-ATPase may be responsive to this regulatory protein [58]. In shrimps, the expression analysis of two splice variants of the 14-3-3 epsilon from *L.vannamei* indicate their cellular functional role during WSSV infections [59]. 14-3-3 gene was observed in the present study to express at the highest level (4.53-folds) in antennal gland when compared to gill tissues (3.94-folds) and gut

Table 6 Percentage of differentially expressed genes from SSH cDNA library belonging to different putative functional categories

Putative function	Gill tissues		Gut tissues	
	forward SSH cDNA library	reverse SSH cDNA library	forward SSH cDNA library	reverse SSH cDNA library
Nucleic acid synthesis and replication	7 %	7 %	6 %	3 %
Energy and metabolism	7 %	11 %	18 %	28 %
Immune response	12 %	11 %	2 %	7 %
Signal transduction	7 %	4 %	6 %	1 %
Allergen proteins	3 %			
Receptor proteins	2 %			
Ion transport and osmoregulation	2 %			
Cytoskeletal proteins	2 %			3 %
Cellular process	10 %		28 %	14 %
Cellular and membrane proteins			8 %	
Reproduction	2 %	3 %		
Ribosomal proteins	10 %			31 %
Mitochondrial genes				12 %
Apoptosis			2 %	
Stress genes		57 %		
Hypothetical proteins	36 %	7 %	30 %	1 %

(2.48-folds) tissues of shrimp. These results suggest that *P. monodon* 14-3-3 gene which responded with significant expression levels, may play an important role in shrimp adaptive mechanism to salinity stress. This is in agreement with report of Kaeodee et al., [60] who observed significant changes in the expression of 14-3-3B transcripts in the osmoregulatory tissues such as gills and epipodites, suggesting that 14-3-3B is likely to be involved in the hyper-osmotic regulation in *P. monodon* .

Energy and metabolism related genes

Some of the differentially regulated genes belonging to energy and metabolism were identified as NADH dehydrogenase, fructose bisphosphate aldolase, ferritin, ATP synthase and Pen m 2 allergen (an arginine kinase 4).

Intracellular fatty acid-binding proteins (FABPs) belonging to superfamily of lipid-binding proteins, are involved in wide range of biological roles which includes transport, cellular uptake and cytoplasmic trafficking of fatty acids [61]. In shrimp, the role of FABPs are mainly attributed to immunity against WSSV [62, 63] and in general antibacterial defenses [64]. The expression of FABP isolated from freshwater crayfish *P. leniusculus* was shown to get induced by all-trans retinoic acid (ATRA) treatment, indicating retinoic acid-dependent signaling may be present in crustaceans [65]. A variety of fatty acids are known to regulate the activity of specific Na^+, K^+, Ca^{2+} and Cl^- ion channels. The action of fatty acids can be through either indirect effects such as metabolic conversion of arachidonic acid to active oxygenated metabolites, which in turn affect the ion channels or through interaction with the ion channels directly [66]. The FABPs may therefore be indirectly involved in osmoregulatory responses mediated by fatty acids. This may be a reason that amongst all the genes, the expression level of FABP gene was found to be maximum in gut (14.05-folds) tissues of salinity stressed shrimp.

In conclusion, current study was successful in identifying differentially expressed genes by SSH cDNA library in response to high salinity stress (55 ppt) in shrimp. Together with our previous reports indicating the role of catechol-*O*-methyltransferase [67], acyl-CoA binding protein [68] and other differentially expressed genes [18] it has been ascertained that several genes are significantly up-regulated in shrimp in response to salinity stress.

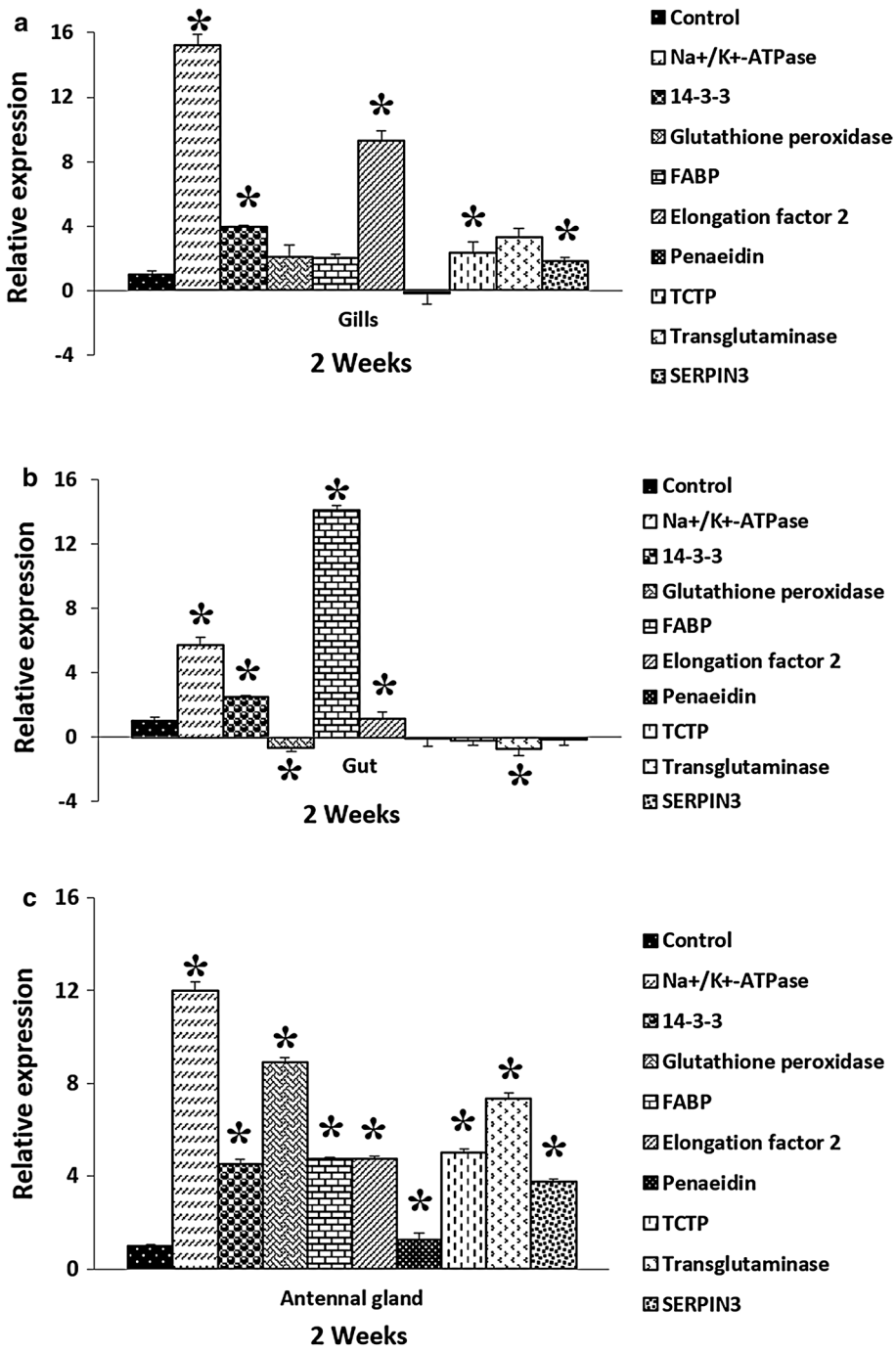

Fig. 1 Real time PCR analysis of differentially expressed genes of shrimp *P. monodon* from (**a**) gills (**b**) gut (**c**) antennal gland. The nine genes which were analysed for gene expression were Na^+/K^+-ATPase α-subunit, 14-3-3 like protein, glutathione peroxidise, intracellular fatty acid binding protein (FABP), elongation factor 2, penaeidin, translationally controlled tumor protein (TCTP), transglutaminase and serine proteinase inhibitor B3 (serpinB3). The significant difference ($p < 0.05$) in gene expression levels is indicated with *asterisks*

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