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ORIGINAL ARTICLE

Gene Co-Expression Network Analysis Reveals the Correlation Patterns Among Genes in Euryhaline Adaptation of *Crassostrea gigas*

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Abstract The Pacific oyster *Crassostrea gigas* is a dominant aquaculture species in many intertidal zones throughout the Pacific and Atlantic Oceans and can tolerate a wide range of salinity. Studying the gene expression profiles of oyster gills had found differentially expressed genes (DEGs) involved in salinity tolerance. A systematic study of cellular response to salinity stress may provide insights into the mechanism of acquired salinity tolerance. Here, weighted gene co-expression network analysis (WGCNA) was carried out using RNA-seq data from gill transcriptome in response to different salinity. A total of 25,463 genes were parsed into 22 gene modules, of which 5 gene modules were identified as salinity-related modules. Brown module was the only one significantly correlated with salinity and free amino acids (FAAs) contents, which was associated with cellular metabolism, biosynthesis of amino acids, oxidation reduction, electron transport, nitrogen compound metabolism, and others. The enriched pathways in brown module were mainly about FAAs metabolism. The other four modules were significantly correlated with certain FAAs, and were over-represented in certain salinity. These results indicated that *C. gigas* triggered different FAAs in different salinity stress. This study represents the first RNA-seq gene network analysis in oysters responding to different salinity stresses. These results provide a systems-level framework to help understand the complexity of cellular process in response to osmotic stress and show the function and regulated genes of different FAAs at the molecular level.

Keywords Weighted gene co-expression network analysis · Osmotic stress · *Crassostrea gigas*

Introduction

Oysters are a major group of marine bivalves, which usually inhabit intertidal zones with fluctuating environmental factors, and they can tolerant a wide range of natural and anthropogenic stressors such as the changes of salinity and temperature, anoxia, acidification, and a variety of toxicants. The Pacific oyster *Crassostrea gigas* is a dominant species in many intertidal zones throughout the Pacific and Atlantic Oceans (Guo 2009). Because of their sessile lifestyle, they have evolved to acquire powerful defense mechanisms to withstand the highly dynamic and stressful environments. Meanwhile, with the genome of *C. gigas* released (Zhang et al. 2012a), they become an excellent model for studying stress adaption on the molecular levels.

Osmotic stress is one of common stresses that oysters usually face when there is rain or drying between tidal inundations (Barnes 1999). *C. gigas* can experience rapid and dramatic salinity fluctuations, from below salt 10 to in excess of salt 35 (Pauley et al. 1988). In previous studies, it has been shown that hypo-osmotic tolerance is rather a complex

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physiological trait incurring the coordination of cellular responses from transcriptome (Zhao et al. 2012) and proteome (Zhang et al. 2014). Salt stress effectors and free amino acids metabolism pathways were suggested to be a powerful ability to adapt to fluctuating salinities (Hosoi et al. 2007; Meng et al. 2013). A systematic study of cellular response to salinity would provide new insights into the mechanism of osmotic tolerance. Meng et al. (2013) have performed the transcriptome data of the Pacific oyster in seven different salinity gradients using RNA-seq technology. Although they uncovered several hundred marker genes for monitoring physiology status of oysters and the osmotic conditions, their analysis just focused on individual genes with high statistical significance and ignored gene interactions. Such analysis might lack scientifically sound hypotheses.

Gene co-expression network analysis is a system biology method for describing the correlation patterns among genes across a large-scale gene expression data, which is the merging of network theory with gene expression data analysis techniques (Fuller et al. 2011). Genes with similar expression patterns may participate in pathways and in regulatory and signaling circuits (Eisen et al. 1998). Weighted gene correlation network analysis (WGCNA) is an approach to network modeling that relies on easily understood statistical methods and improves on simple correlation networks (Zhang and Horvath 2005). This approach can identify modules of co-expressed genes, and relate these modules to phenotypic traits. Although, it has been widely applied in gene expression studies of humans and model organisms (Iancu et al. 2015; Lee et al. 2004; Liu and Ye 2014; Malki et al. 2013), the related studies on non-model organisms are still in its infancy (Fu et al. 2014; Zhang et al. 2012b).

Here, we constructed the co-expressed gene network of *C. gigas* transcriptome by reanalyzing RNA-seq datasets (Zhang et al. 2012a) to identify salinity stress-related modules and candidate key genes.

Materials and Methods

Transcriptome Data Acquisition

The transcriptome data from Zhang et al. (2012a) were downloaded from the Gene Expression Omnibus website, under the GEO accession number GSE31012 (GSM748453 ~ GSM768459).

Data Preprocessing

All the seven different gene expressed libraries were mapped to the genome of *C. gigas* (Zhang et al. 2012a) using tophat2 (Kim et al. 2013). Of the 28,027 coding sequences in the genome, 25,463 sequences could be mapped in at least two libraries. The transcript expression levels (FPKM) were estimated by RSEM software (Li and Dewey 2011), which were retained in subsequent analysis. Considering salt 30 as optimal salinity, differential expression analysis was performed using the R package DESeq (Anders 2010). Here, transcripts with nominal P value <0.05 and |log2(foldchange)| >1 were considered as differentially expressed genes (DEGs) to avoid omitting the positives.

Gene Co-Expression Network Construction

Gene network was constructed using R package WGCNA following the procedure described in Langfelder and Horvath (2008). Firstly, we clustered the samples to see if there were any obvious outliers. As a result, the group of salt 5 was removed as an outlier. Secondly, a power of nine was chosen so that the resulting networks exhibited approximate scale-free topology (model fitting index $R^2 = 0.8$). Thirdly, all the 25,463 genes in the other six libraries were hierarchically clustered using the topological overlap-based dissimilarity measure (Zhang and Horvath 2005). Finally, the resulting gene dendrogram was used for module detection using the dynamic tree cut method (minModuleSize = 100 and mergeCutHeight = 0.25). In a weighted gene co-expression network, any two genes were connected, and the edge weight was determined by the topology overlap measure provided in WGCNA. The weights ranged from 0 to 1, and reflected the strength of the communication between the two genes. Connectivity was defined as the sum of the weights across all the edges of a node, and the top 1 % (or 5 %) of the genes with the highest connectivity in the network were defined as hub genes (Yang et al. 2014). We defined the hub genes in a given module by the intramodular connectivity (Kwithin), which measures a gene's connectivity in the specific module.

Identification of Salinity Stress-Related Modules

To identify modules that are significantly associated with the salinity, we first calculated the module eigengene using all genes in each module, and then correlate eigengenes with salinity treatments and free amino acids (FAAs) contents. The only modules with P values <0.05 were considered as salinity stress-related modules. To characterize those modules, GO and KEGG pathway enrichment analysis of the annotated genes in each salinity stress-related module using GSEABase package (Morgan et al. 2008). GO terms and KEGG pathways with P values <0.05 were considered to be significantly more enriched.

Hub Genes Selection and Visualization

Highly connected intramodular hub genes may be more biologically significant than hub genes in global network (Zhou

et al. 2014). Based on the module sizes, we chose the top 2 % of the genes with the highest connectivity in the module as hub genes. In addition, the differential expressed genes with high connectivity would be selected as genes of interest. Co-expression patterns and interactions of hub genes were visualized using Cytoscape (Shannon et al. 2003).

Results

Construction of Gene Co-Expression Network

In the study, the transcriptome profiling of Zhang et al. (2012a) was performed using the high-throughput sequencing technologies for Pacific oysters that were sampled at different salinity (5, 10, 15, 20, 25, 30, and 40 ‰). Firstly, we clustered these samples and removed the salt 5 as an outlier (Fig. S1) and the left samples used the blockwise Consensus Modules R function to carry out network construction. To ensure that the network was biologically relevant, the scale-free topology model fit and the mean connectivity of the network was evaluated over a range of the soft threshold power β (β = 9, Fig. S2). All 25,463 coding sequences were parsed into 22 gene modules, with module size ranging from 128 to 12,942 (Table 1).

Identification of Salinity Stress-Related Modules

The eigengene-trait correlation analysis showed that brown module was significantly positively correlated with salinity stress and almost all the FAAs contents (except glutamate) after multiple testing correction (Fig. 1). In addition, pink module was correlated with taurine; lightyellow correlated with glycine, alanine, and proline; and cyan and royalblue correlated with glutamate. These five modules were conducted by over-representation analysis of DEGs using a hypergeometric test (Table 2) (Fig. 2). Among them, all the five modules showed differential expression at different salinity: brown module responded at salt 10 and salt 40; pink module responded at hypo-salinity (salt 10 and salt 15); cyan module responded at salt 20 and salt 40; lightyellow module responded at salt 40; and royalblue module responded at salt 25 and salt 40. Combining the results that four modules correlated with different FAAs contents, it may imply that different FAAs play a dominant role at different salinity.

Functional Annotation of Salinity Stress-Related Modules

To identify modular features with their biological roles response to salinity, functional annotations of the salinity stress-related modules were performed on the basis of their gene composition using GSEABase software (Table 3). A detailed functional enrichment of GO annotations in these modules was provided in Table S1 with P value <0.05. According to functional annotations, brown module was significantly enriched with genes functioning in "cellular metabolic process," "primary metabolic process," "catalytic activity," "oxidoreductase activity," and so on. These biological activities were closely associated with metabolic process of *C. gigas*. Pink module was closely associated with "biological regulation," and lightyellow module was involved in "ATP metabolic process," "actin filament organization," and "peptide metabolic process." Royalblue and cyan modules were related to "glucide metabolism" and "macromolecule biosynthesis," respectively.

The pathway annotations were assigned to KEGG database (http://www.genome.jp/kegg/). Pathways enrichment analysis (Table 4) showed that seleno compound metabolism, methane metabolism, biosynthesis of amino acids, and glycine, serine, and threonine metabolism were top enriched in brown module. "Cysteine and methionine metabolism" was enriched in both brown module and pink module. It was also found that "oxidative phosphorylation" and "protein export" were enriched in lightyellow module, "starch and sucrose metabolism" and "flavone and flavonol biosynthesis" were enriched in royalblue module, and "ribosome" was enriched in cyan module. To investigate the interactions between KEGG pathways and show the systematic osmoregulation pathways, we extracted the genes in brown module and in enriched KEGG pathways (Table S2), and constructed a sketch of the hypothetical pathways network that are significantly correlated to osmoregulation (Fig. 3). In this framework, *C. gigas* response to salinity was initiated upon the sensing of ion via cell membrane receptors. The signals subsequently

Table 1 List of module size

Module	Gene No.	Module	Gene No.	Module	Gene No.	Module	Gene No.
Turquoise	12,942	Blue	2306	Brown	1718	Yellow	1115
Green	936	Red	808	Black	765	Pink	643
Magenta	582	Purple	534	Greenyellow	474	Tan	445
Salmon	363	Cyan	318	Midnightblue	302	Lightcyan	240
Grey60	238	Lightgreen	161	Lightyellow	160	Royalblue	151
Darkred	134	Darkgreen	128				

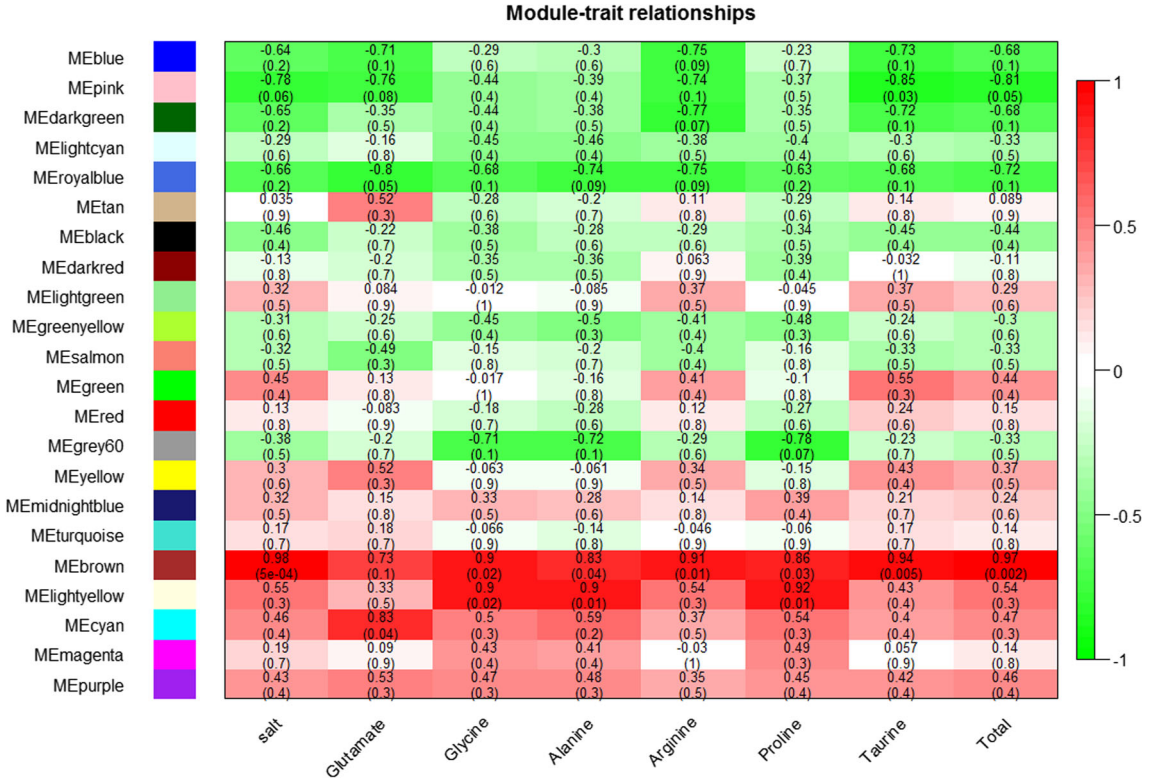

Fig. 1 Module-trait associations. Each *row* corresponds to a module eigengene, column to a trait. Each cell contains the corresponding correlation and *p* value. The table is color-coded by correlation according to the color legend

transmitted down through the AMPK signaling pathways and triggered the production of a series of FAAs as the end results. In parallel, energy metabolism was activated to provide energy.

Hub Genes Selections

In network biology, a hub gene is a good representative of a module. All the top 2 % hub genes of each salinity stress-related module were searched against the genome of *C. gigas*. Their correlation were visualized in Fig. 2, and the related information of hub genes were listed in Table S3. For instance, brown module, as the only module significantly correlated with salinity, has huntingtin-interacting protein 1 (HIP1), involved in cytoskeleton, Protein iolS, Betaine aldehyde dehydrogenase (BADH); 2-aminoethanethiol dioxygenase (ADO) and eosinophil peroxidase (EPX), involved in oxidation-reduction process; and myosin-Vb, organic cation transporter protein (OCT), and metal transporter CNNM2 involved in transport and so on.

Discussion

In this study, we constructed a gene co-expression network of *C. gigas* in response to different salinity, and identify salinity stress-related modules by transcriptome data of gills using WGCNA analysis. Importantly, we used the data from the same article to avoid batch effects when constructing the networks from expression data from multiple profiling techniques. Although differential expression analysis had been used to study the oyster transcriptome of euryhaline adaptation, gene co-expression network analysis focuses on the strong correlation patterns between the genes (Miller et al. 2008).

WGCNA considers not only the correlation between two genes but also the connectivity of their shared neighbors across the whole network. Hierarchical average linkage clustering based on topological overlap matrix (TOM) was used to genes with highly similar co-expression patterns into modules. Based on the cluster result, salt 5 was identified as an outlier. In the previous analysis, salt 5 was considered to be special with previous principal component analysis (PCA) of transcriptomes (Zhang et al. 2012a). This results indicated that 5 ‰ may exceed the range of oyster salinity tolerance. For increasing the confidence of results, data of the other six groups were used to perform the gene co-expression network analysis. As a result, we identified 22 highly correlated modules, and only five modules were significantly correlated with salinity based on the results of gene significance and module membership.

The most interested module is the brown module, which is the only one module positively correlated to salinity and almost all FAAs contents. It consisted of 1718 genes, of which there are

Table 2 Over-representation analysis of differentially expressed genes in four stress-responsive modules

Module	Gene No.	Hypergeometric *P* value				
		Salt 10	Salt 15	Salt 20	Salt 25	Salt 40
Pink	643	0**	0**	0.994195	0.960947	0.956752
Lightyellow	160	1	0.902007	0.532703	1	0.001158*
Cyan	318	0.998413	1	3.13E-06**	0.447157	0.014929
Royalblue	151	1	1	0.501779	0.00018**	3.68E-07**

*$P < 0.01$ and **$P < 0.001$

165 DEGs. As the previous studies reported, osmolytes play a primary role in the osmotic activities of the osmoconformer, such as the large amount of nitrogenous solutes, amino acids, and inorganic ions (Berger and Kharazova 1997; Pierce 1982; Yancey et al. 1982). Genes in these enriched GO terms might be correlated with the metabolism and biosynthesis of the osmolytes. Based on the results of KEGG pathways enrichment, "selenocompound metabolism," "biosynthesis of amino acids," and "glycine, serine, and threonine metabolism" were enriched, which verified amino acids playing important roles in osmoregulation in *C. gigas* again. In parallel, the hypothetical pathways network showed that signals transmitted down by AMPK signaling pathways and triggered the production of a series of FAAs. The other enriched pathway "methane metabolism" belongs to "energy metabolism," which took part in oxidation to CO_2 for energy source for energy supply. Functional annotations and enrichments found that "cellular metabolic process," "nucleoside monophosphate biosynthetic process," and "single-organism biosynthetic process" were the top three enriched GO terms. As listed in Table S1, "nitrogen compound metabolic process," "cellular amino acid metabolic process," "RNA metabolic process," "oxidation-reduction process," "protein dephosphorylation," and others were involved in GO-enrichment analysis. This proved that it is a complex cellular process in response to osmotic stress. FAAs and amine were the important participants in osmotic equilibrium. Other biological process might help the biosynthesis, metabolic and transport of FAAs and amine, and signal transduction.

Hub genes were considered a good representative of a module in network biology. HIP1 contains clathrin-binding domains and actin-binding TALIN homology domains, and its domains can assist clathrin-mediated endocytosis (Ross 2012). Comparative genomic analyses indicated that HIP1 was similar with a protein involved in organization of cortical

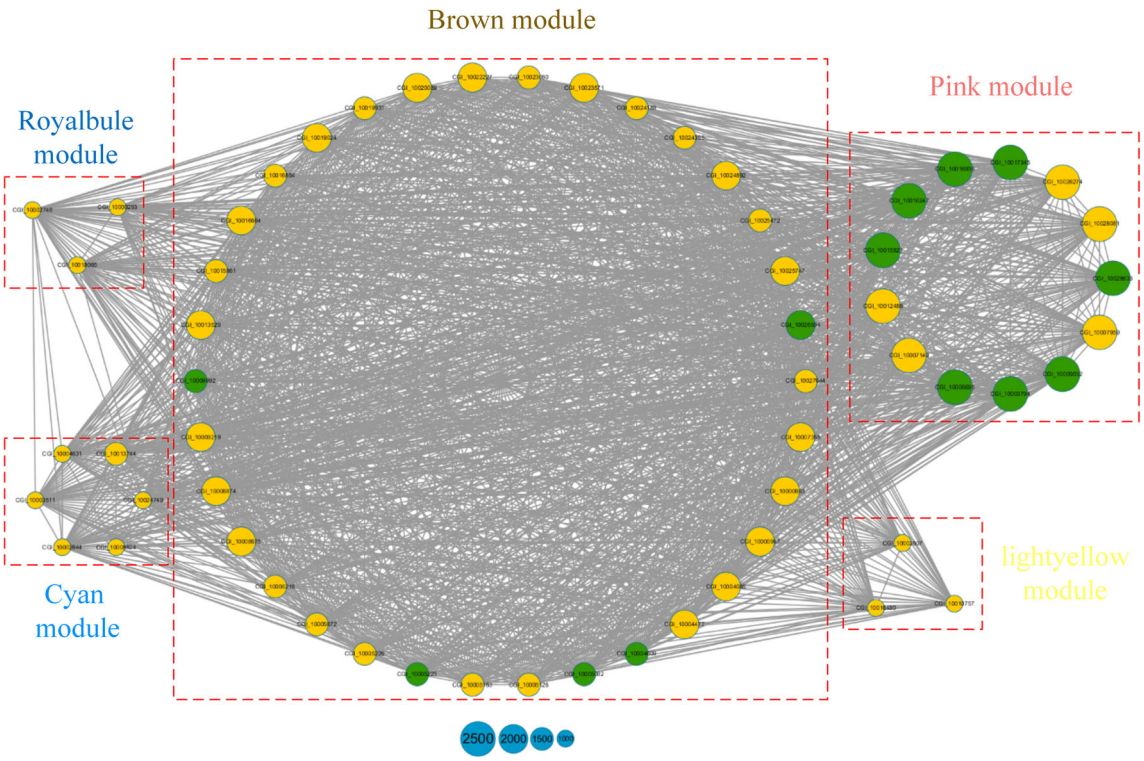

Fig. 2 Network visualization of interactions between hub genes in five stress-responsive modules. Each node represents a gene, which is labeled with sequence ID. *Green nodes* represent DEGs. Genes in the same *rectangle* belong to the same module. Node size represents total connectivity (ktotal)

Table 3 Top-rank functional annotations enriched in salinity responsive modules

Module	Category	GOID	Term	*P* value
Brown	Biological process	GO:0044237	Cellular metabolic process	1.61E-05
	Biological process	GO:0009124	Nucleoside monophosphate biosynthetic process	0.000242
	Biological process	GO:0044711	Single-organism biosynthetic process	0.000393
Pink	Biological process	GO:0006355	Regulation of transcription, DNA-templated	0.000696
	Biological process	GO:2001141	Regulation of RNA biosynthetic process	0.000696
	Biological process	GO:0051252	Regulation of RNA metabolic process	0.000719
Lightyellow	Biological process	GO:0046034	ATP metabolic process	0.006377
	Biological process	GO:0007015	Actin filament organization	0.007764
	Biological process	GO:0006518	Peptide metabolic process	0.010207
Royalblue	Biological process	GO:0044723	Single-organism carbohydrate metabolic process	0.011084
	Biological process	GO:0019318	Hexose metabolic process	0.021978
	Biological process	GO:0005996	Monosaccharide metabolic process	0.022793
Cyan	Biological process	GO:0034645	Cellular macromolecule biosynthetic process	1.14E-05
	Biological process	GO:0071704	Organic substance metabolic process	1.24E-05
	Biological process	GO:0009059	Macromolecule biosynthetic process	1.33E-05

actin cytoskeleton in yeast (Hackam et al. 2000). However, the function of HIP1 has been studied for Huntington's disease; it was not known completely, especially in invertebrates. HIP1, as the hub gene with max connectivity, might be involved in amino acids transport and assembly of cytoskeleton. TRIM2 was also found to be under positive selection in adaptation to hypo-osmotic environment in a previous study (Zhao et al. 2014). Myosin-Vb is annotated as transmembrane transport in mammals. Apparently, myosin-Vb should participate in the transport of osmolytes to ensure osmotic equilibrium. As

Table 4 Top enriched pathways in salinity stress-responsive modules

Module	Map	Pathway name	Total entities	Overlap	*P* value
Brown	00450	Selenocompound metabolism	12	6	0.000365
	00680	Methane metabolism	21	8	0.000371
	01230	Biosynthesis of amino acids	62	15	0.000421
	00270	Cysteine and methionine metabolism	37	9	0.005789
	00260	Glycine, serine and threonine metabolism	40	9	0.009904
Pink	05200	Pathways in cancer	163	16	0.000707
	00270	Cysteine and methionine metabolism	37	6	0.003198
	04512	ECM-receptor interaction	27	5	0.003914
	03050	Proteasome	39	6	0.004201
	04060	Cytokine-cytokine receptor interaction	18	4	0.004989
Lightyellow	00190	Oxidative phosphorylation	67	4	0.002382
	03060	Protein export	22	2	0.014986
Royalblue	00500	Starch and sucrose metabolism	28	2	0.015524
	00944	Flavone and flavonol biosynthesis	3	1	0.020606
Cyan	03010	Ribosome	120	6	0.000653

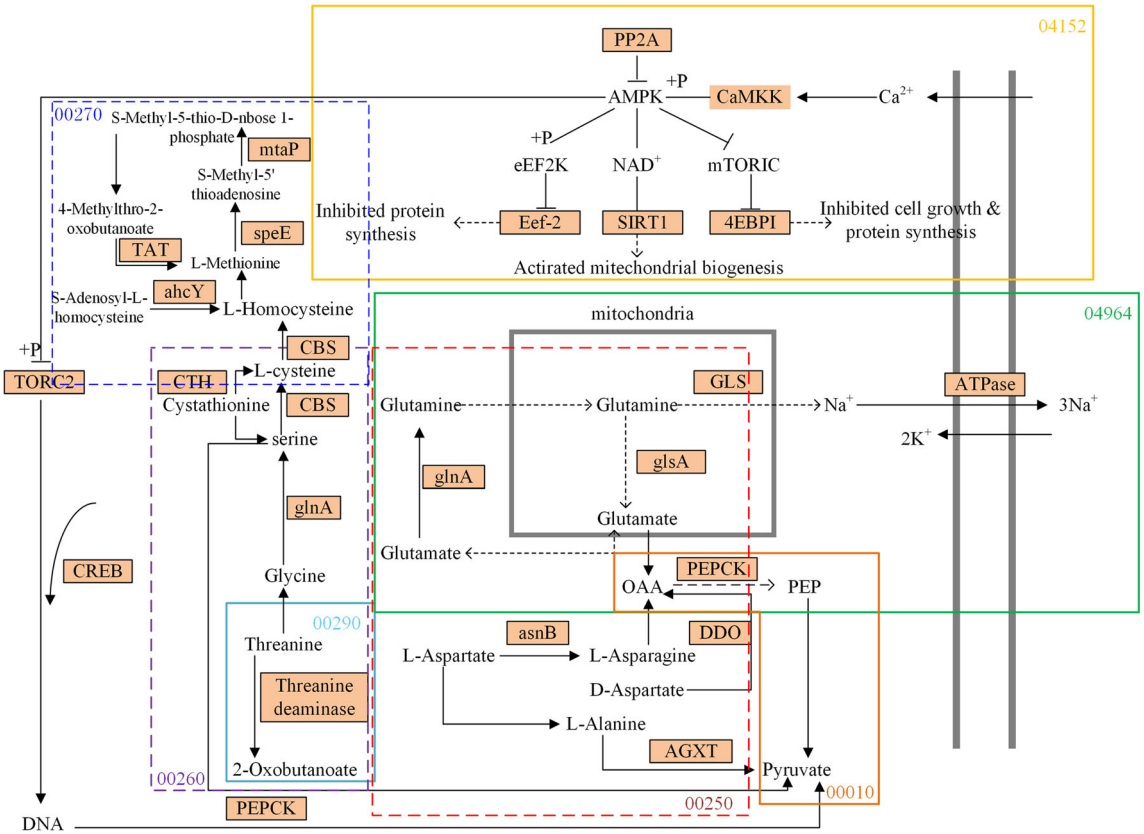

Fig. 3 Overview of salinity-related enriched pathways of genes in brown modules during *Crassostrea gigas* response to salinity stress. Only pathway components interacted directly with genes in brown modules (shown in *orange text boxes*) are presented within each enriched KEGG pathway (framed in *orthogonal polygons*). The numbers in orthogonal polygons represent the KEGG ID, *00010*: glycolysis/gluconeogenesis; *00250*: alanine, aspartate, and glutamate metabolism; *00260*: glycine, serine, and threonine metabolism; *00270*: cysteine and methionine metabolism; *00290*: valine, leucine, and isoleucine biosynthesis; *04152*: AMPK signaling pathway; *04964*: proximal tubule bicarbonate reclamation

is reported, mammalian TRIM2 and TRIM3 are more similar to each other, and both predominantly expressed in brain compartments and interact with myosin-Vb (El-Husseini and Vincent 1999; Meroni 2012). In this study, TRIM2 has been found to co-express with myosin-Vb which might be implicated in osmolytes transport and regulation in *C. gigas*. ADO is the key enzyme in the pathway, taurine and hypotaurine metabolism, and was important in the conversion of cysteamine to hypotaurine (Coloso et al. 2006; Goto et al. 2001). This suggested that ADO might participate in FAAs biosynthesis for osmoregulation in *C. gigas*.

Among the top 2 % hub genes in brown module, there are five DEGs, and of which three DEGs are annotated. EPX differentially expressed in both salt 10 and salt 15 significantly and was annotated as "oxidation-reduction process." Besides EPX, there were several genes among the top 2 % hub genes of brown module participating in oxidation reduction. Changes in temperature, oxygen levels, and salinity can cause the stress in natural and artificial conditions via induction of disbalance between reactive oxygen species (ROS) production and elimination (Lushchak 2011). The downregulation of EPX in hypo-osmotic conditions may represent a protective strategy to reduce cellular stress and decrease the ROS. Similar changes in oxidation reduction associated genes have been observed in crabs and Japanese eels exposed to osmotic stress (Paital and Chainy 2010; Tse et al. 2013). Compared with the results of differentially expressed genes (Meng et al. 2013), ROS signal transduction pathways were identified as one of salt stress signal transduction pathway.

Pink module was negatively correlated with taurine significantly. Taurine is well known that during adaptation to environmental salinity, levels of taurine in invertebrate and amphibian tissues will change to maintain osmotic equilibrium (Thurston et al. 1980). In the Pacific oyster, taurine, as the primary osmolyte, accounts for approximately 80 % of the total amino-acid content (Hosoi et al. 2003). Considered the enriched pathways, "ECM-receptor interaction" and "cytokine-cytokine receptor interaction" belonged to "signaling molecules and interaction" module and "proteasome" participated in signal transduction pathways and stress signaling and so on. It implicated that pink module might be involved in signal transduction. "Cysteine and methionine metabolism"

was enriched in both pink and brown modules. In bivalves, taurine metabolism occurs mainly via synthesis from cysteine and a high-affinity transport system. "Cysteine and methionine metabolism" manipulated the content of the sulfur-containing amino acid to play important roles in taurine metabolism. Only two hub genes in pink module had been annotated: cytochrome P450 2G1 (CYP2G1) and innexin unc-9 (UNC-9). Cytochromes P450 family are membrane proteins and require a source of electrons from an electron transfer chain to function (Nelson and Nebert 2011). CYP2G1 was found olfactory specific in the rabbit and have distinct substrate specificities against endogenous compounds or nasal toxicants (Maïbèche-Coisne et al. 2002; Maïbèche-Coisne et al. 2005). This gene may take part in the sense of osmotic pressure in *C. gigas*, which were also identified as salt stress effectors under low osmotic stress (Meng et al. 2013). While UNC-9 regulated gap junctions to connect and communicate between two cells. In mammals' central nervous system, taurine was a neurotransmitter (Wu and Prentice 2010). Taken together, taurine may be both an important osmolyte and a signal factor or key molecule regulating signal transduction.

Lightyellow module was positively correlated with glycine, alanine, and proline significantly. Based on the result of GO and KEGG enrichment, lightyellow module was involved in "ATP metabolic process," "actin filament organization," and "protein export." The hub genes in lightyellow module were integral component of membrane. Neuronal acetylcholine receptor subunit beta-3 (CHRNB3) binds acetylcholine and then leads to opening of an ion-conducting channel across the plasma membrane (Kormelink and Luyten 1997). Sodium-dependent phosphate transporter 1 (SLC17A1) is known to co-transport sodium and phosphate, with a capacity to also transport organic anions (Sreedharan et al. 2010). The results showed that genes in this module increased energy metabolism and altered membrane fluidity, which initiated transport of organic and inorganic osmolytes.

Cyan and royalblue modules were correlated with glutamate. In cyan module, histone-lysine *N*-methyltransferase SETD8-A (SETD8-A) is involved in methyltransferase activity. SETD8 was found to be conserved in zebrafish and mammals and take part in epidermal differentiation to help epithelium survive in a hypotonic external environment (Thompson 2011). SETD8-A was probably conserved between oyster and zebrafish to ensure the balance between internal environments and external environments. Deoxyribonucleoside 5′-monophos phate *N*-glycosidase (DNMP) was another target gene of c-Myc, as the same as SETD8, and involved in epidermal differentiation (Driskell et al. 2012; Ghiorghi et al. 2007). According to the GO and KEGG enrichment analysis, royalblue module was enriched in carbohydrate metabolism. The function of glutamate was similar with alanine to form ammonia to regulate the osmotic pressure. While glutamate also participated in carbohydrate metabolism (Brosnan 2000). We speculated that during the salinity stress, cells might increase energy demand to compensate for osmoregulation.

Based on the significant correlation between certain modules and certain FAAs, the over-representation analysis of DEGs at different osmotic stress was conducted in four salinity-related modules (pink, lightyellow, cyan, and royalblue). Salt 10 and salt 15 were enriched with DEGs in pink module, indicating that pink module responded specifically at extreme hypo-osmotic conditions, where taurine might be the primary osmolyte to maintain osmotic equilibrium. Owing to the negative correlation between pink module and taurine content, the genes in pink module may be related to inhibition of taurine biosynthesis. Lightyellow module responded specifically at salt 40. Alanine was involved predominantly in reductive amination reactions to transiently produce the ammonia for osmotic equilibrium with glutamate. It was reported that ammonia changed little with an abrupt decrease in salinity but increased with an abrupt increase in salinity (Livingstone et al. 1979). Alanine, glycine, and proline were found to be accumulated, and the activity of the cilia may be relatively insensitive to changes in cytoplasmic osmolality during hyperosmotic volume regulation in mussels (Deaton 2001). It indicated that amino acids export and ammonia production were the primary reaction in response to hyperosmotic stress. Cyan module responded specifically at salinity 20. Ribosome was enriched in this module. One characteristic shared by the DEGs in this module is that all genes were upregulated at salinity 20. It was found that repressed protein synthesis may be a self-protection mechanism against heat stress that is commonly existed in various animals (Fu et al. 2014). It indicated that salinity 20 may not be an acute stress to *C. gigas*. DEGs were enriched in salt 25 and salt 40 in royalblue module, which suggested that energy metabolism was the highlight process during moderate salinity stress.

Meng et al. (2013) confirmed the important roles of FAAs for oyster low salinity adaptation using physiological experiment and differential gene expression analyses, and described the salt stress signal transduction network in oyster. Compared with these results, organic substances such as FAAs and quaternary amines were verified to play dominant roles in oysters in our analyses. Organic osmolytes are able to provide osmotic bulk under high osmotic stress without the direct physiological trade-off that inorganic ions would have (Yancey 2005). Therefore, organic osmolytes have an advantage over inorganic ions in the study. In differential genes expression analyses, ROS signal transduction pathways were identified to be induced by osmotic stress, which was confirmed by gene co-expression network analysis. Meanwhile, the hypothetical pathways network indicated that salt stress induced cytosolic Ca^{2+} oscillations and

excited AMPK signal pathway. Furthermore, amine was also found to be an important osmolyte in response to high salinity.

Collectively, we postulate that *C. gigas* might have triggered several cellular processes to acclimate to osmotic stress. Amino acids and ammonia are the primary regulated factor to maintain osmotic equilibrium accompanying ion transport. Genes participating in oxidation reduction were possibly to decrease production of ROS that can potentially cause damage to all cellular components. ROS signal transduction pathways and AMPK signal pathway were identified to participate in the oyster's responses. We speculate that different osmolytes regulated osmotic pressure in different salinity. At low salinity (salt 10, salt 15), genes related to regulation of transcription were enriched that might indicate that DNA transcription was active in response to hypo-osmotic stress, and signal transduction and cellular communication were also active. Meanwhile, sulfur amino acid metabolic process was enriched, thereby assisting in regulation of taurine contents for isotonicity. At moderate low salinity (salt 20, salt 25), cellular macromolecule biosynthesis and carbohydrate metabolism were enriched. Energy supply and cell differentiation were triggered that indicated salt 20 and salt 25 were the relatively suitable salinity. At moderate high salinity (salt 40), ammonia was the primary osmolyte for osmotic equilibrium assisting with glycine and proline. Overall, our study provides the first systematic insights into molecular mechanisms underlying the physiological changes of *C. gigas* in response to salinity stress.

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Author Contributions XZ carried out the molecular genetic studies, participated in the data analysis, and drafted the manuscript. HY participated in the data analysis. LK participated in the design of the study and performed the statistical analysis. QL conceived of the study, participated in experimental design and coordination, and contributed to t the manuscript preparation. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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