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RESEARCH REPORT

The transformation of energy metabolism and Endoplasmic Reticulum stress regulation in Pacific oyster *Crassostrea gigas* under air exposure

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Abstract

The Pacific oyster *Crassostrea gigas* is an important species living in the intertidal zones. It is of great significance to study the mechanism of oysters to adapt air exposure. In the present study, weighted gene co-expression network analysis (WGCNA) with the transcriptome data of gills and adductor muscle was conducted to investigate the metabolic transformation of *C. gigas* under air exposure. GO enrichment of modules specifically expressed in adductor muscle of oysters exposed to air for five, seven and nine days revealed the phased expression of respiratory chain, protein turnover and lipid metabolism, indicating the conversion of energy metabolism. During air exposure, “respiratory chain” and “ribosome biogenesis” were enriched in the muscle on the fifth day, suggesting that glycogen metabolism was dominant in the early stages of air exposure. On the seventh day, many terms about the regulation of proteolysis were enriched, indicating that carbohydrates were not be able to meet the metabolic needs in the oyster adductor muscle, and proteins began to be degraded for energy supply. The processes related to lipid metabolism were enriched on the ninth day. The extremely high glycogen content of *C. gigas* allowed it to maintain a basic metabolic activity for a long time with a conservative compensation strategy. GO and KEGG enrichments of the modules sensitive to air exposure in gills were mainly involved in “response to endoplasmic reticulum stress”, “Endoplasmic Reticulum (ER) to Golgi transport vesicle membrane” and “protein processing in endoplasmic reticulum”. It revealed that the mechanism of oyster adapting to air exposure was a complex regulatory network depending on the ER. Hub gene network and PPI network analyses found that some transcription factors containing zinc finger domains regulated the biochemical reactions for stress adaptation, indicating that the ER, as a regulatory element sensitive to external stress, could regulate apoptosis, autophagy and protein degradation in gills of *C. gigas* under air exposure. These results would provide new insights into the adaptation of *C. gigas* to air exposure in terms of energy metabolism and homeostasis.

Key Words: *Crassostrea gigas*; air exposure; WGCNA; transcriptome; ER stress; energy metabolism

Introduction

The Pacific oyster *Crassostrea gigas* is a world-wide marine bivalve distributing in the intertidal zones. It can adapt to a wide range of environmental stresses such as air exposure, salinity, temperature, acidification, and anoxia (Zhang *et al*., 2015), and has become an ideal model to study the molecular adapt mechanisms of marine molluscs to environmental stresses, especially the air exposure. Air exposure is a common stress to the species living in the intertidal zones, which is also considered as a complex stress consisting of hypoxia, drought, heat, and solar radiation. As a complex environmental stress, air exposure can influence various aspects of *C. gigas* such as metabolism, immune system and DNA repair. So far, there have been many studies on the response of

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oysters to air exposure. For instance, calnexin (CNX) and calreticulin (CRT) were proved to function as endoplasmic reticulum (ER) chaperones in *C. gigas* to adapt to air exposure (Kawabe and Yokoyama, 2010). Upregulated glucose concentration in serum induced the synthesis of interleukin-17 and inflammatory response during air exposure (Xin *et al*., 2016). Serotonin could modulate apoptosis and redox during air exposure in *C. gigas* (Dong *et al*., 2017). Most of the previous studies were focused on functional verification of a key gene or gene family in a process under air exposure. The rapid development of omics provides systematic analysis for the comprehensive understanding of the powerful adaptability of oysters to the complex environment stress.

Omics is a science of collective characterization and quantification of pools of biological molecules. So far, transcriptome and proteome have been applied to investigate the response of oysters against stresses such as temperature, salinity and pesticides (Epelboin *et al*., 2015; Zhao *et al*., 2016; Yang *et al*., 2017). Transcriptome data from *C. gigas* exposed to air for eleven days have been collected and analyzed, and IAPs are found to be highly expressed in the gills (Zhang *et al*., 2012). However, the responses and the underlying mechanisms of oysters against air exposure cannot be described simply by the changes of a single gene or gene family. Weighted gene co-expression network analysis (WGCNA) is a system biology network algorithm based on the concept of a scale-free network to analyze a few highly connected nodes participating in a very large number of metabolic reactions (Zhao *et al*., 2010). WGCNA is an ideal systematic biology method to describe the correlation patterns among genes across microarray or RNA-seq samples (Langfelder and Horvath, 2008). This systematical biology method has been used to study the response upon complex conditions in different species (Carlson *et al*., 2006; Gargalovic *et al*., 2006; Horvath *et al*., 2006; Dong and Horvath, 2007). In oysters, WGCNA was used to study the adaptability of various environmental stresses. For instance, FAAs metabolism associated modules were identified in oyster *C. gigas* by WGCNA analysis with gill transcriptome in response to different salinity (Zhao *et al*., 2016). It was also employed to verify the activating function of TNF under different neurotransmitter stimulation (Liu *et al*., 2016). The increasing reports demonstrated that WGCNA was an advantaged tool to find the core regulatory networks and key genes in complex processes from multi-samples.

In the present study, a gene co-expression network was constructed to comprehensively understand the response and regulation strategies of Pacific oyster *C. gigas* against air exposure. The air exposure related modules were identified, and several modules of them were found to be involved in energy metabolism and homeostasis. Hub gene network and protein-protein interaction network were constructed to reveal the possible mutual control relationships between these modules. These genes and modules found in the present study provided informative clues to understand the mechanisms of homeostasis and energy allocation in oysters to adapt a wide range of environmental stresses.

Materials and Methods

RNA extraction and quantitative real-time PCR

About 120 Pacific oysters, collected from Zhangzidao aquaculture farm in Dalian, China in September 2017, were acclimatized in aerated seawater feeding with microalgae at 20 ± 1°C for one week. Subsequently, they were placed into a dry box and kept at room temperature of 20 ± 1°C for air exposure. Nine oysters were sampled to collect the gills and adductor muscle at 10:00 every day. To reduce individual variations and improve result reliability, the tissues from three individuals were pooled together as one sample, and there were three replicates for subsequent RNA extraction in each group. On the 8th day, most of the remaining oysters died, and the oysters under air exposure for 0-7 days (72 in total) were collected for the follow-up experiments. The total RNA was extracted by using Trizol reagent (Invitrogen) and reverse transcribed using a cDNA synthesis kit (TransGen). NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13-like (LOC105341417) and hormone-sensitive lipase (LOC105345018) were selected as target genes for qRT-PCR analysis. Primers of target genes and the internal reference gene (oyster Elongation Factor, GenBank accession No. NM_001305313) were designed by NCBI Primer-BLAST (Table 1). qRT-PCR was performed with the ABI7500 fast Real-Time Detection System (Applied Biosystems, USA) using SYBR Green fluorescent. The relative expression was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1 Primers used in this paper

Primers	sequence (5'-3')
P1 (LOC105341417-RT-F)	TGTCTGGCCTGGTCCAATTT
P2 (LOC105341417-RT-R)	TGCTCGAAGTAAGCTCCCTG
P3 (LOC105345018-RT-F)	AACTCCCAGCTGTGAACTCG
P4 (LOC105345018-RT-R)	GGGTCTCTCCAGTCCATCCT
P5 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG
P6 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT

Transcriptome data acquisition

The raw transcriptome data, the RNA-seq data of gills and adductor muscle from adult oysters *Crassostrea gigas* under air exposure for 0, 1, 3, 5, 7, 9, 10 and 11 days were obtained from the Sequence Read Archive (SRA) (Leinonen *et al*., 2011) database with the accession number SRX093464 and SRX093475–SRX093489 from the first paper concerning the oyster genome sequencing (Zhang *et al*., 2012). These RNA-seq data were derived from Illumina HiSeq 2000 single-ended 49bp sequencing.

Data preprocessing and mapping to the genome

The SRA files were transferred to fastq files by SRA toolkit v2.8.2. FastQC v0.11.5 was used to control the quality of raw data. The genome data and gene annotation files of *C. gigas* were downloaded from NCBI RefSeq database (ftp://ftp.ncbi.nih.gov/genomes/Crassostrea_gigas). The filtered raw data were aligned to the genome using HISAT2 v2.0.5 (Pertea *et al*., 2016). The RPKM (Reads Per Kilobase per Million mapped reads) was quantified by StringTie v1.3.3b with the parameter "-e -B" (Pertea *et al*., 2015). The quantified expression matrix was filtered by an R package ballgown with a variance across samples less than one (Frazee *et al*., 2015).

Weighted gene co-expression network analysis

The weighted gene co-expression network was constructed with an R package WGCNA (Langfelder and Horvath, 2008). Sixteen samples were clustered to detect outliers according to the tutorials of the package. The soft thresholding power β was detected with the criterion of approximate scale-free topology by the function *pickSoftThreshold*. All raw reads from gills and adductor muscle transcriptomes of *C. gigas* were mapped to oyster genome. The scale-free topology model fit and the mean connectivity of the network were evaluated over a range of the soft threshold power β. To minimize effects of noise and spurious associations, the adjacency was transformed into Topological Overlap Matrix (TOM) to calculate the corresponding dissimilarity. Then a hierarchical clustering tree (dendrogram) of genes was produced by TOM. The branch of the tree was cut to identify modules by function *cutreeDynamic* with the minimum cluster size 30.

Identification of air exposure related modules

Module eigengene (ME) of a module was considered as a representative of a module's gene expression profile. The ME value (*E*) was calculated and a heatmap was drawn to visualize the express pattern of each module. The genes in the modules were annotated by a comprehensive annotation software suite Trinotate v3.0.1 to predicate their potential biological roles in the response to air exposure (Haas *et al*., 2013). The mRNA sequences of *C. gigas* were aligned to SwissProtKB database by BLAST v2.6.0 (Altschul *et al*., 1990). The HMMER v3.1 (Finn *et al*., 2011) was used to identify protein domains from Pfam-A database. The results of Gene Ontology (GO) (Ashburner *et al*., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2012) annotation terms were extracted by in-house Perl scripts and imported into an R package AnnotationForge (Carlson and Pages, 2017) to make an OrgDB organism annotation object. The R package clusterProfiler (Yu *et al*., 2012) was employed to analyze the GO and KEGG enrichments for all modules under hypergeometric distribution with p-value < 0.05 and q-value < 0.05 (Benjamini-Hochberg method).

Table 2 Statistics of reads and mapping rate of RNA-seq

Samples	Total reads	%Mappable
dried_gills_0d	15775296	72.65%
dried_gills_1d	17218677	73.16%
dried_gills_3d	18281681	74.85%
dried_gills_5d	16430916	73.56%
dried_gills_7d	16612191	74.39%
dried_gills_9d	17289062	75.29%
dried_gills_10d	18058657	75.09%
dried_gills_11d	19406328	71.46%
dried_muscle_0d	18829273	82.05%
dried_muscle_1d	15603618	80.63%
dried_muscle_3d	18384113	79.48%
dried_muscle_5d	15359448	80.45%
dried_muscle_7d	16114873	81.08%
dried_muscle_9d	16739672	80.86%
dried_muscle_10d	15281286	81.08%
dried_muscle_11d	16702190	79.52%

Network construction and visualization

The top 5% genes with the highest connectivity in each module were defined as hub genes. The hub gene network was visualized by Cytoscape v3.6.1. The gene list was mapped to the *Strongylocentrotus purpuratus* (purple sea urchin) proteome (source from STRING-db "7668.protein.sequences.v10.5") by BLAST v2.6.0 with the parameter "-evalue 1e-5 -max_target_seqs 1 -outfmt 6" to obtain the best match of orthologous genes. The corresponding gene list was imported into STRING database (https://string-db.org) to generate PPI network (Szklarczyk *et al*., 2015). The network was then imported into Cytoscape for functional annotation, manual curation, and grouping.

Results

Identification of air exposure related modules

All raw reads from gill and adductor muscle transcriptomes of *C. gigas* were mapped to oyster genome (Table 2). Of all the 33790 genes, 15139 genes with a variance less than one across samples

were filtered to reduce the range of genes that need to be analyzed (Table S1). The sample clustering analysis revealed that there was no obvious outlier (Fig. S1). The adjacencies of the network were calculated using the soft thresholding power $\beta = 5$ (Fig. S2). The number of genes per module was listed in Table 3 and a heatmap was drawn to visualize the MEs of all twenty-one modules (Fig. 1). These modules were roughly divided into several categories: (I) specifically expressed in a sample (green, yellow, lightcyan, orange, etc.); (II) specifically expressed during a certain period of time (tan, magenta, darkred, royalblue, and lightgreen); (III) gradually changed over time (brown); (IV) tissue-specific expression (blue). Air exposure related modules were defined as the modules specifically expressed at a certain stage of air exposure or significantly correlated with air exposure time. After the gene expression was normalized, the gene clusters specifically expressed in tissues were effectively filtered by the co-expression network of the dual-tissue samples under air exposure to greatly reduce the possibility of background noise. Eight modules (green, yellow, lightcyan, midnightblue, lightyellow, greenyellow, salmon, grey60) were expressed differently at different air exposure time in gills. Other six modules (black, red, cyan, darkgrey, darkturquoise, orange) exhibited periodic expression patterns on the 0, 5, 7, 9, 10 and 11th day in adductor muscle. The ME of brown module increased in a time dependent manner during the exposure period, indicating that the expressions of these genes in oyster gills were highly correlated with exposure time. The above 14 modules were initially identified as air exposure related modules for further analysis.

Table 3 List of module size

Module	Gene Numbers
black	330
blue	13088
brown	1593
cyan	111
darkgrey	38
darkred	68
darkturquoise	62
green	847
greenyellow	145
grey60	98
lightcyan	106
lightgreen	94
lightyellow	74
magenta	446
midnightblue	107
orange	34
red	355
royalblue	73
salmon	132
tan	138
yellow	712

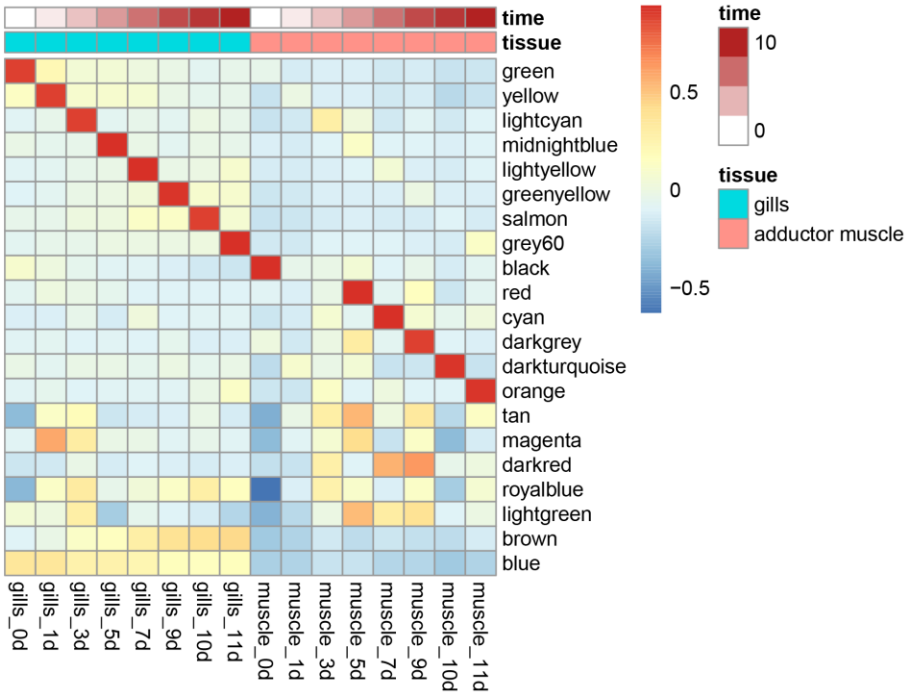

Fig. 1 Module Eigengenes heatmap of 21 modules. The x-axis represents different samples, the y-axis represents the name of each module, and the color shade of the patch represents Module eigengene (ME) values. Each row represents a module's general expression trend

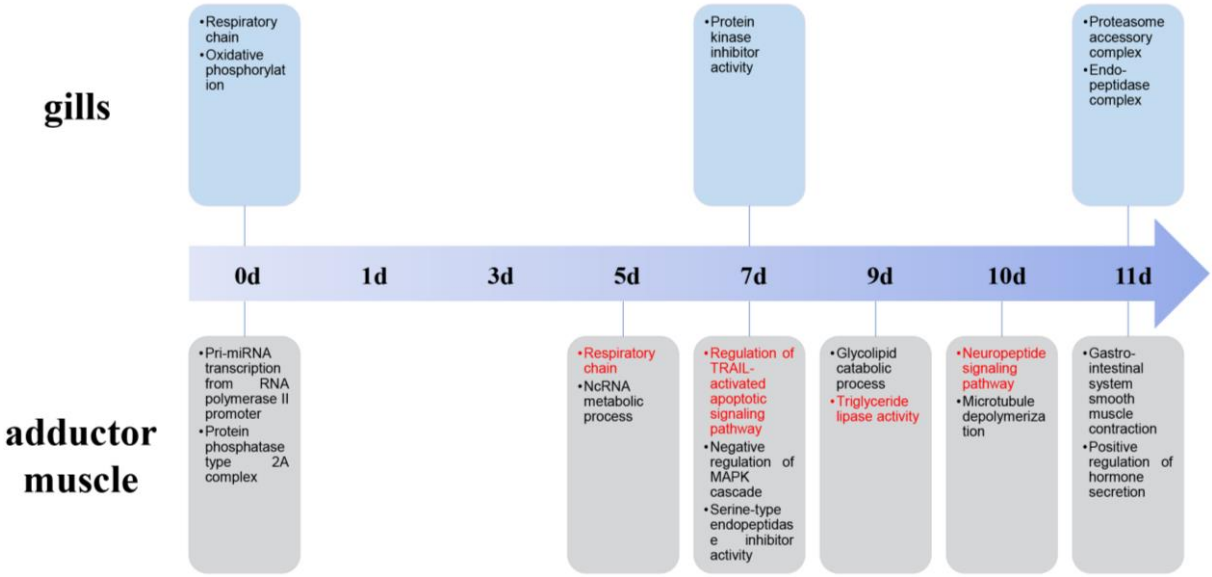

Fig. 2 Important GO terms enriched in different duration of air exposure. A schematic of important GO entries enriched in gills and adductor muscle of *C. gigas* under air exposure for 0-11 days. GO terms mentioned in the text were marked red. See Table S2 for more details.

Functional enrichment of air exposure related modules

GO and KEGG pathways were enriched for all the modules to gain a comprehensive understanding of the biochemical reactions in oysters under air exposure. Fourteen modules specifically expressed in different duration of air exposure were first analyzed (Fig. 2), and GO terms enriched on the fifth, seventh and ninth day displayed a clear trend of convert from glucose metabolism to lipid metabolism. On the fifth day (Fig. 3a), “respiratory chain” and “ribosome biogenesis” were enriched in the adductor muscle, suggesting that glycogen metabolism was dominant in the early stages of air exposure (Fig. 3b). On the seventh day, many terms were enriched in the regulation of proteolysis, indicating that the carbohydrates could not meet the metabolic needs in oyster adductor muscle, and proteins began to be degraded for energy supply (Fig. 3c). The TRAIL-mediated apoptotic pathway was also enriched on the seventh day, indicating that the apoptotic pathway was initiated in this period. The processes related to lipid metabolism were enriched on the ninth day.

The brown module was a special module whose ME value increased with the time of air exposure in gills. GO enrichment reveled that the gene clusters sensitive to exposure time in the brown module (Table S2) were mainly involved in “autophagy”, “small GTPase mediated signal transduction”, “response to endoplasmic reticulum stress”, and “ER to Golgi transport vesicle membrane”. “Protein processing in endoplasmic reticulum”, “SNARE interactions in vesicular transport” and “Mitophagy – animal” were enriched pathways in KEGG database (Fig. 5a).

The mRNA expression pattern of energy metabolism related genes

The expression levels of NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13-like (*Cg*NDUFA13, NCBI gene ID: LOC105341417, Fig. 4a) and hormone-sensitive lipase (*Cg*HSL, NCBI gene ID: LOC105345018, Fig.4b) in oyster adductor muscle were examined under air exposure. The expression level of *Cg*NDUFA13 gradually increased in the first three days of air exposure, reached a maximum on the third day, and then gradually decreased. The expression level of *Cg*HSL displayed a similar trend, but peaked on the fourth day.

Hub gene connectivity and protein-protein interaction network of brown module

To further clarify the biological significance of the brown module, a hub gene co-expression network and a protein-protein interaction (PPI) network were established. The top 5% genes with the highest connectivity in the module were chosen as hub genes (Fig. 6a, Table S3). “Serine/threonine-protein kinase 17A” (LOC105324979) was the center of the network and several hub genes were found to involve in ERAD pathway. For the lack of oyster protein interaction data, the oyster genes were mapped to the genome of purple sea urchin *S. purpuratus*, a model species of marine invertebrates, to characterize protein interactions of these genes (Fig. 6b). The enrichment *p*-value of PPI was 0.0329 (< 0.05), indicating that the proteins in the same network reacted more frequently with each other than with a random set of other proteins. This network was centered by some transcription factors containing

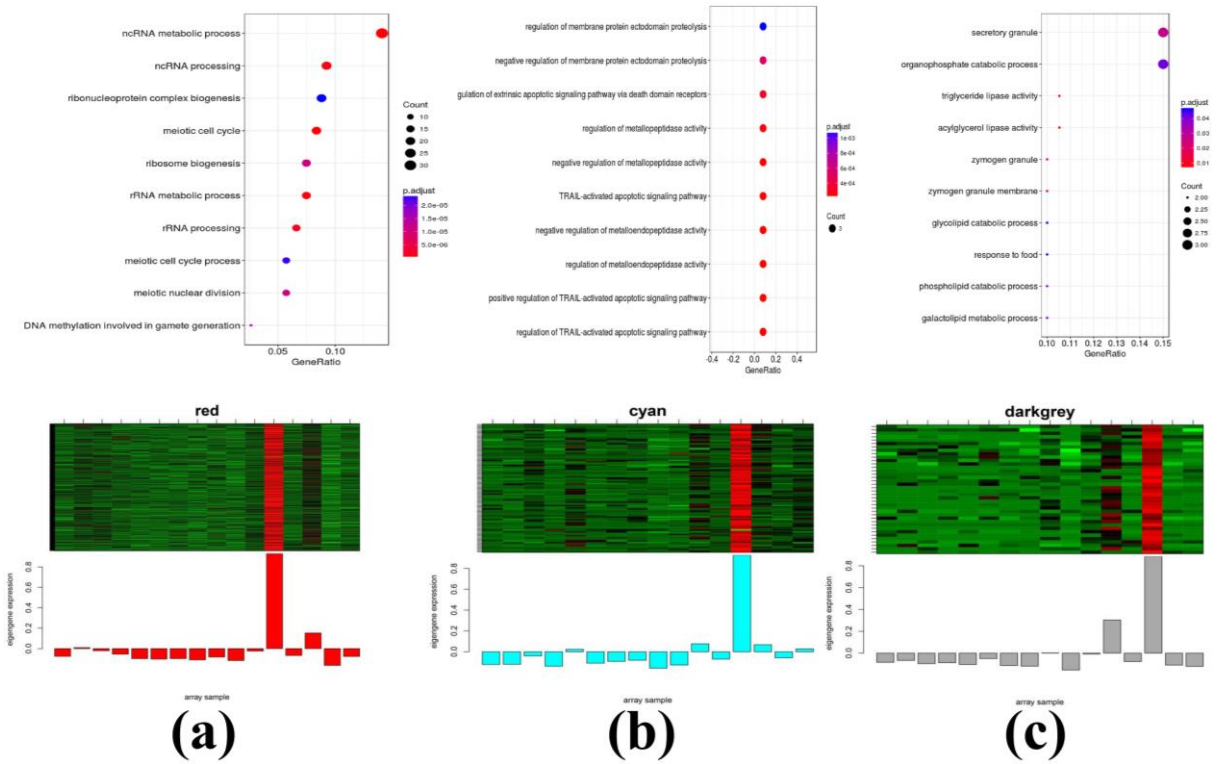

Fig. 3 Gene expression pattern and GO enrichment of three energy metabolism related modules. GO enrichment dotplot, expression heatmap and ME barplot of red (a), cyan (b), and darkgrey (c) module. The x-axis of dotplots represents the proportion of genes enriched to the total number of genes in the entry. The size of the dots represents the number of genes enriched. The x-axis of heatmaps and barplots represents different samples, from left to right: the oyster gills and adductor muscle under air exposure for 0, 1, 3, 5, 7, 9, 10, 11 days respectively. The y-axis of heatmaps represents module genes. The color shade of the heatmap represents the FKPM of module genes increasing from green to red. The y-axis of barplots represents the MEs

C2H2 type zinc finger which was involved in the regulation of endocytosis, RNA transport, and ubiquitin mediated proteolysis (Table S3). KEGG pathway enrichment showed that the PPI network was mainly composed of endoplasmic reticulum-mediated N-glycosylation, endocytosis, and ERAD pathways. The centered gene LOC583350 was annotated as an E3 ubiquitin-protein ligase MIB2.

Discussion

Transcriptomes have been mainly analyzed to find differentially expressed genes (DEGs), which are limited to be applied directly to time-series expression data due to the differences in sampling rates and variations in the timing of biological processes (Bar-Joseph *et al.*, 2003). WGCNA is an ideal tool for merging genes with approximate expression patterns into modules, so that all the gene expressions can be separated into different subsets, and the key pathways can be revealed for time-series data. In the present study, a weighted gene co-expression network was constructed with the RNA-seq data to reveal the adaptation mechanism of *C. gigas* to air exposure. The genes expressed in the various periods of air exposure were grouped into different modules in the ME heatmap. The biological functions of these modules and the biochemical reactions occurred during the various stages of air exposure could provide helpful clues to understand the possible mechanisms of adaptability of oysters to the environmental stresses.

It has been demonstrated that all the organisms have different strategies to deal with various stresses by mediating the energy metabolism to meet the increasing maintenance costs. In an energy-based stress classification, the overall scope of the values of an environmental factor experienced by an organism is divided into several ecologically and physiologically relevant ranges: optimal range, pejus range, pessimum range, and lethal range (Pörtner and Farrell, 2008). The judgment of the range conversion is mainly based on the proportion of the basic maintenance costs to the total energy expenditure of the organism. Base maintenance costs refer to the maintenance of key cellular processes (*e.g.*, protein turnover, ion balance, acid-base regulation, and anabolism of substances) and the basic activity costs of living organisms (*e.g.*, circulation and excretion) (Sokolova *et al.*, 2012). Adductor muscle has been considered as one of the important energy storage organs in *C. gigas* which provides the protein and energy for gametogenesis

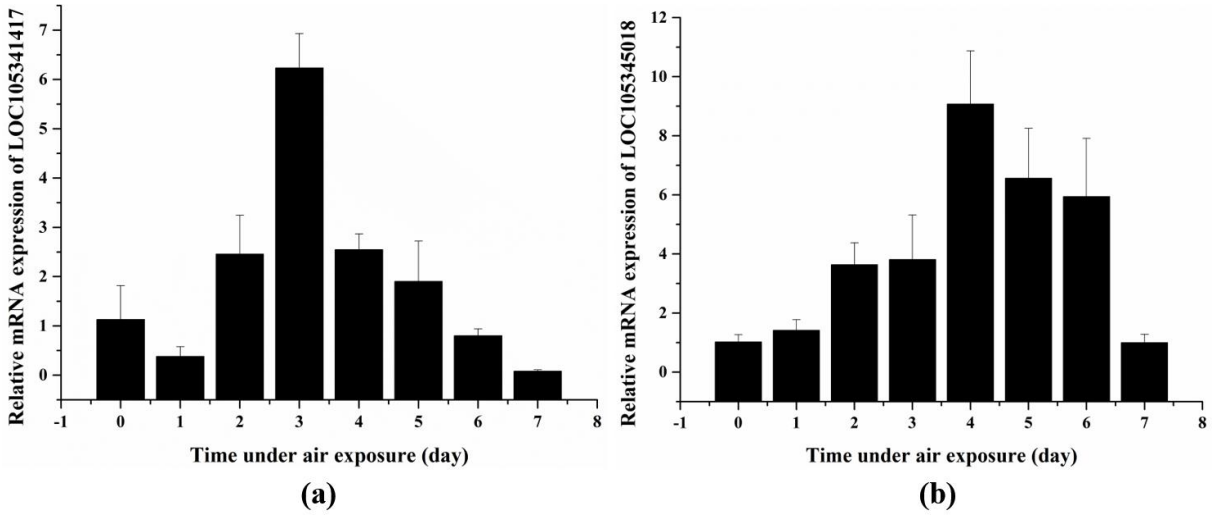

Fig. 4 The mRNA expression patterns of energy metabolism related genes. The mRNA transcripts of NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13-like (a) and hormone-sensitive lipase (b) in oyster adductor muscle were detected by real-time PCR at 0, 1, 2, 3, 4, 5, 6 and 7 day after air exposure. Each value was shown as mean ± S.D. (N = 3)

and reproduction (Berthelin *et al*., 2000; Li *et al*., 2006). In the present study, the modules specifically expressed at the fifth, seventh and ninth day in the adductor muscle indicated the changes of energy allocation strategies and the response range during the exposure process (Fig. 3). On the fifth day (red module), “respiratory chain” and “ribosome biogenesis” were enriched in the adductor muscle. As a rule, the exposures to pejus and pessimum stresses cause metabolic and ATP turnover acceleration to compensate additional energy expenses for increased physiological activity, cellular maintenance, and damage repair (Sokolova *et al*., 2012). In the present study, the increasing expression of *Cg*NUDFA13 (Fig. 4a) demonstrated an increase of cellular energy demands for basal maintenance. The increased maintenance costs exacerbated the consumption of nutrients (especially glycogen). In general, glycogen makes up 20–40% of the oyster’s dry weight. As the main flavor component, the glycogen content is critical to oyster quality (Li *et al*., 2017). The rich glycogen content of *C. gigas* can provide sufficient energy to maintain pressure from pejus to pessimun, and endow oysters the ability to adapt to prolonged air exposure. On the seventh day (cyan module), many terms were enriched in the regulation of proteolysis and TRAIL-mediated apoptotic pathway, suggesting the metabolic compensation in pejus stress. The activation of TRAIL-mediated apoptotic pathway in the present study proved the similar transfer of energy metabolism strategy for maintenance and repair in the middle stage of response against air exposure. The increased protein turnover, negative regulation of proteolysis, and activation of the apoptotic pathway suggested that the metabolic strategy of *C. gigas* turned into conservation and compensation to reduce negative impact of stress. On the ninth day (darkgrey module), enrichment of lipid metabolism-related processes indicated that prolonged hunger consuming glycogen made energy insufficient to maintain basic physiological activity. The up-regulated expression of *Cg*HSL, functioning in hydrolyzing triglyceride, was also detected in the oyster muscles under air exposure by qRT-PCR (Fig. 4b). The representative signals of cell death, such as “neuropeptide signaling pathway” and “microtubule depolymerization” (Linden *et al*., 2005; Oropesa-Ávila *et al*., 2013) were enriched on the tenth day (darkturquoise module). The enrichment of neuropeptide signaling pathway suggested that the lethal pressure at this time exceeded the tolerance range of oysters, and death was irreversible.

ER is responsible for folding and processing of nearly all polypeptides destined for secretion and plays a major role in maintaining cell homeostasis (Frakes and Dillin, 2017). It has been reported that there is a significant response to endoplasmic reticulum stress and endoplasmic reticulum associated degradation (ERAD) in gills of *C. gigas* under lead (Pb) stimulation (Meng *et al*., 2018) and heat stress (Yang *et al*., 2017). ER stress could be induced by a wide range of cellular environments and events, including increased levels of protein synthesis, impaired ubiquitination and proteasomal degradation, deficient autophagy, energy deprivation, excess or limitation of nutrients, dysregulated calcium levels, redox homeostasis, inflammatory challenges, and hypoxia (Wang and Kaufman, 2016). Gills, as the respiratory and filtration organ of *C. gigas*, is the first line in contact with seawater (Fabioux *et al*., 2015), which could dynamically adjust production of secreted proteins in response to environmental insult and nutrient availability (Zhang *et al*., 2014). In the present study, the ME of brown module increased gradually with the exposure time. This module was consisted of two clusters in the expression heatmap (Fig. 5c). The expression of one

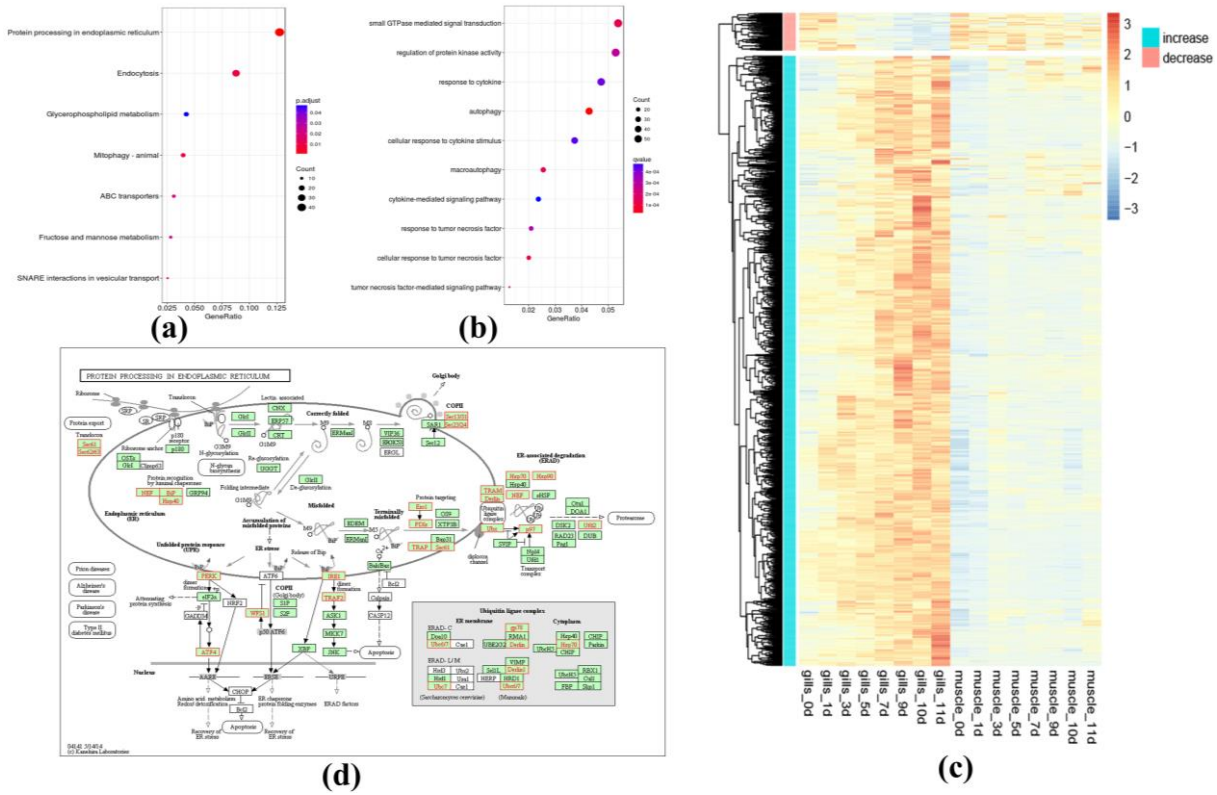

Fig. 5 Gene expression pattern and function enrichment of brown module. KEGG (a) and GO (b) enrichment dotplot of endoplasmic reticulum related module. The color of the dots represents adjust p-value. (c): Expression heatmap of brown module genes, these genes can be divided into two clusters: the expression level gradually increases and decreases in gills over time. (d): KEGG pathway of “Protein processing in endoplasmic reticulum” (crg:04141). Genes in brown module were marked red

cluster increased over time and the other one decreased over time. Their correlations with air exposure time indicated that the modules were the most important modules for oysters to adapt to air exposure. KEGG enrichment showed that protein processing in endoplasmic reticulum was enriched in brown module (Fig. 5b). It has been reported that ER-related pathways function in the adaption of oysters to various environmental stresses, such as high temperature, heavy metal ions, and diesel (Zhu *et al.*, 2016; Meng *et al.*, 2018; Müller *et al.*, 2018). ER stress related pathways were suggested to be inseparable for oysters to adapt to long time air exposure. ERAD pathway related genes and part of ER stress element (*e.g.*, PERK, ATF4, TRAF2) were significantly enriched in brown module (Fig. 5d). ER stress has been reported to activate PERK pathway and regulate autophagy gene transcriptional program in response to amino acid starvation under short stress. For the prolonged stress, the response of ER stress changes from promoting cellular survival to committing the cell to a apoptosis pathway mediated by TRAF2 (Rozpędek *et al.*, 2016). A hub gene network and a PPI network were constructed to further clarify the possible expression regulation and protein interactions of the brown module (Fig. 6). The highly connected hub genes in a module work together synergistically and play important roles in the biological processes (Yuan *et al.*, 2017). The centered gene in the hub gene network was serine/threonine-protein kinase 17A, which had been reported to be a member of the death-associated protein (DAP) kinase-related apoptosis-inducing protein kinase family encoding an autophosphorylated nuclear protein with a protein kinase domain in humans (Lawrie *et al.*, 2009). Meanwhile, many ERAD-related genes were identified from the hub genes, indicating that this module was an endoplasmic reticulum-associated protein-mediated degradation and apoptosis regulatory network. A PPI network was further constructed to clarify the specific control mechanism of this complex control module. In PPI network, the autophagy and apoptosis related pathways regulated by ER were obviously clustered in brown module. The center of the network was a group of transcription factors rich in C2H2 zinc finger domains to regulate various processes, such as ubiquitination, purine metabolism, Jak-STAT signaling, and RNA transport. On pejus press, the ubiquitination pathway is activated to degrade the misfolded protein resulted from oxidative damage and energy deficiency. On pessimun press, the apoptosis is induced to avoid more damage caused

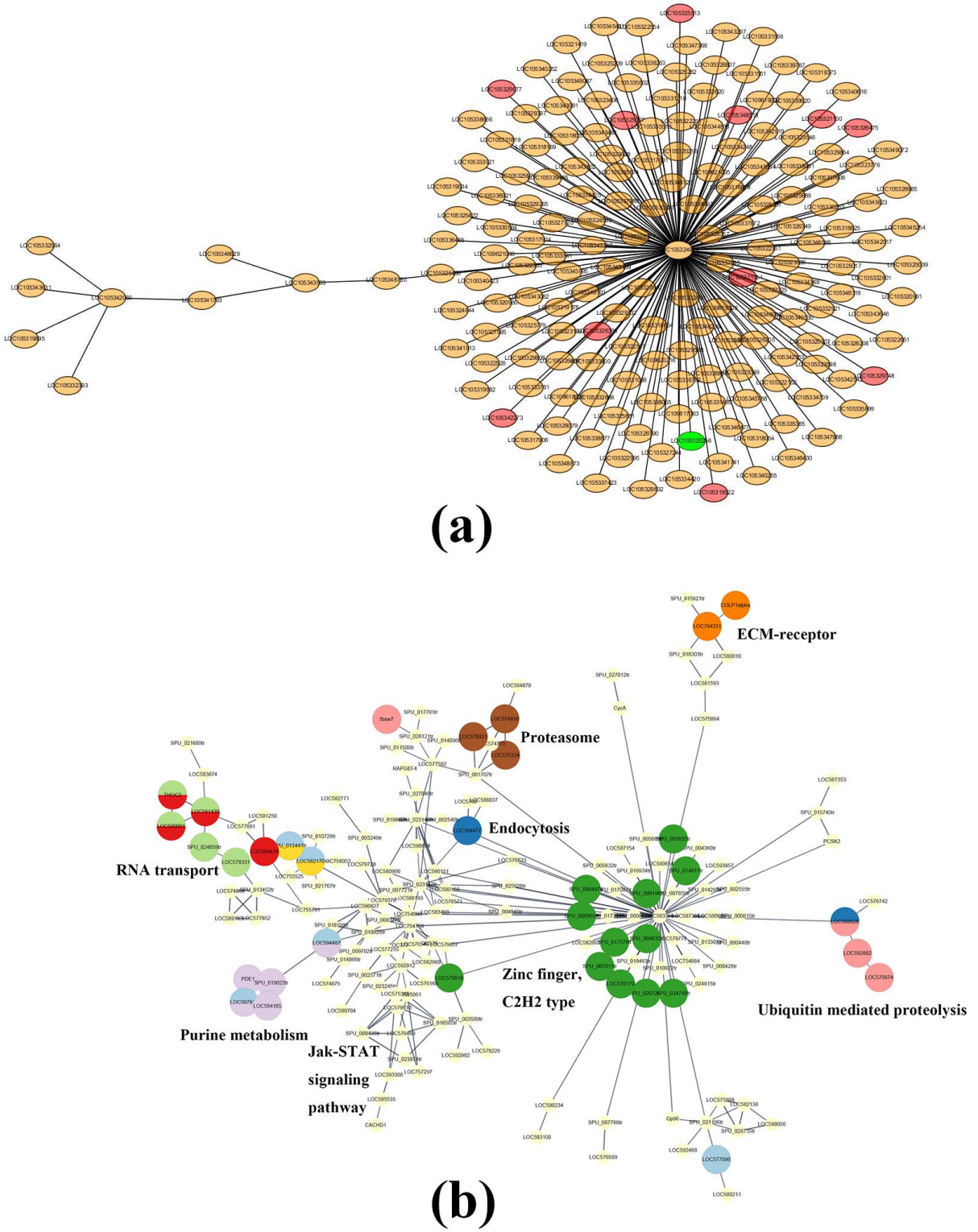

Fig. 6 Hub genes connectivity network and protein-protein interaction network of brown module. (a): Hub genes connectivity network. Network visualization of the interactions between top 5% highest connectivity genes in brown module. Each node represents a gene, which is labeled with NCBI ID. Green nodes represent IAPs. Red nodes represent genes in ERAD pathway. See Table S2 for the genelist of hub genes. (b): Protein-protein interaction network. All genes were aligned to the sea urchin by BLAST to find the best match. Edges with the interaction confidence < 0.8 were filtered. Nodes information and the STRING enrichment of colored nodes can be found in Table S3

by the inflammatory reaction. The regulation strategy of the transfer from damage repair to apoptosis according to the press greatly reduced the basic maintenance cost of *C. gigas*, which was suspected to be the key mechanism of *C. gigas* to adapt to harsh environments. The results indicated that this module was an ER associated protein mediated complex regulatory network. Oyster cells relied on ER as a receptor for environmental stress to dynamically regulate various homeostasis-maintaining pathways (such as autophagy and apoptosis) through ER stress and ERAD pathways to maintain the homeostasis in oyster gills.

In conclusion, gene co-expression network analysis of transcriptome data indicated that *C. gigas* had evolved a set of air exposure adaptation mechanisms based on energy metabolism and regulation endoplasmic reticulum-related pathways. Rich glycogen content provided sufficient energy to maintain stress induced damage in a pejus range. Apoptotic pathway was activated when the stress increased to pessimum, and the overall energy strategy was changed from compensation to conservation. As a sensitive receptor for the external environment, ER in the gill cell could adjust the strategy of homeostasis according to the magnitude of the environmental pressure, and rationally allocate the energy to meet the requirement for damage repair, transcriptional regulation and apoptosis.

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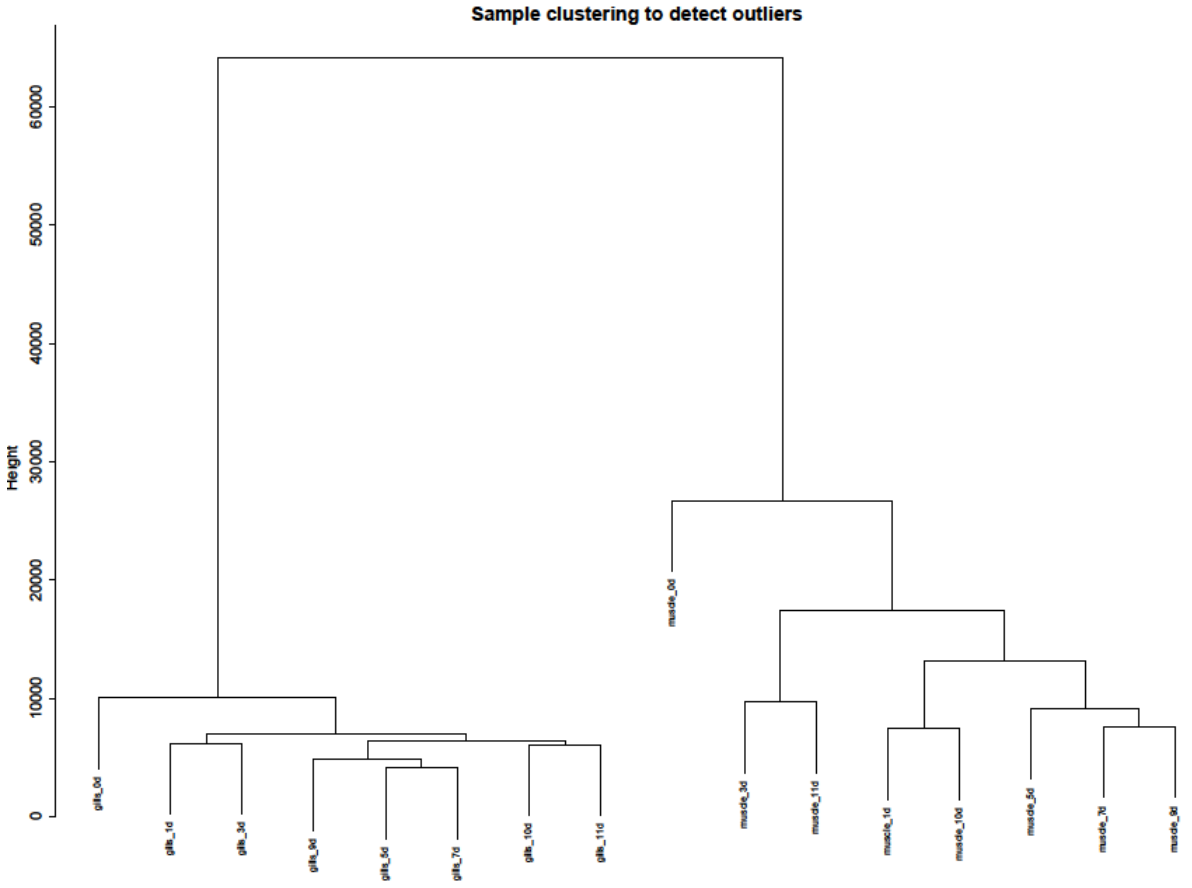

Fig. S1 Sample clustering to detect outliers. R function hclust was used to detect sample outliers (Euclidean distance). Samples were grouped into two clusters according to the tissues. No obvious outliers were found.

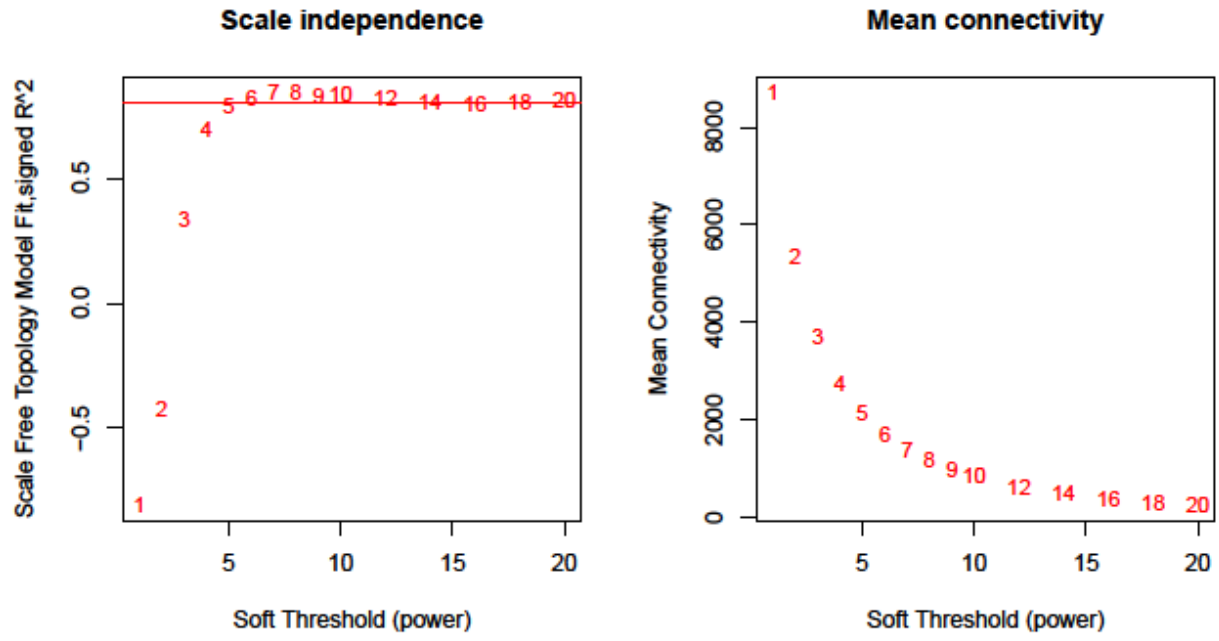

Fig. S2 Scale independence. A plot of the fit for the scale-free topology model and the average connectivity under different soft powers. Generally, the minimum softpower with a scale free topology fit larger than 0.8 was chosen.