The identification and characteristics of salinity-related microRNAs in gills of *Portunus trituberculatus*

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate gene expression in organisms. To understand the underlying mechanisms behind the molecular response of the crab to low salt-stress, high-throughput Illumina/Solexa deep sequencing technology was used to investigate the expression profiles of miRNAs under low salinity challenged. Two mixed RNA pool libraries of gill tissues from low salinity challenged (LC) and the control groups (NC) were sequenced on the Illumina platform. A total of 6,166,057 and 7,032,973 high-quality reads were obtained from the NC and LC libraries, respectively. Sixty-seven miRNAs consisting of 16 known and 51 novel ones were identified, among which, 12 miRNAs were differentially expressed in LC compared to NC. Thirty-four of the target genes predicted were differentially expressed in the opposite direction to the miRNAs, which were involved in crucial processes related to osmoregulation by gene ontology (GO) functional enrichment analysis, such as anion transport processes (GO:0006820) and chitin metabolic process (GO:0006030). These results provide a basis for further investigation of the miRNA-modulating networks in osmoregulation of *Portunus trituberculatus*.

Keywords *Portunus trituberculatus* · MicroRNA · Salinity · Osmoregulation

Background

Portunus trituberculatus (Crustacea: Decapoda: Brachyura), often known as the swimming crab, is the most important economic crab species and fishery resource in China (Yu et al. 2003). According to China's Ministry of Agriculture, production of the crab reached 99,580 t in 2012, which was valued at more than AU\$ 2.7 billion.

Salinity is an important abiotic factors that influence the distribution, abundance, physiology, and well-being of crustaceans (Romano and Zeng 2012), which is also an important factor during artificial propagation (Xu and Liu 2011). When the environmental osmolality is lower than that of the hemolymph, it is termed “hypo-osmotic” and crustaceans must compensate for ion loss from their hemolymph via hyperosmoregulation (Rainbow and Black 2001; Romano and Zeng 2012). Throughout the prolonged culture period, the swimming crab often experiences low salinity stress due to heavy rainfalls, which could have significant impacts on farm productivity. Therefore, the identification and characterization of the genes and the regulatory factors involved in hyperosmoregulation are now essential for increasing the production and the efficiency of selective breeding programs for this important species. With recent advances in genomic sequencing and transcriptome mapping (microarray and high-throughput sequencing), some salt-related genes and regulatory factors have been identified in *P. trituberculatus* on a large scale at the genome-wide level (Lv et al. 2013; Xu and Liu 2011). Nevertheless, the molecular basis of tolerance to salinity stress has not yet been identified.

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MicroRNAs (miRNAs) are 20–22 nt non-coding RNAs that play an important role in the regulation of many biological processes, such as signal transduction, organ development, cellular proliferation, apoptosis, oncogenesis, and immunity (Ma et al. 2012; Zhou et al. 2014; Nilsen 2007; Yang et al. 2013; Pushpavalli et al. 2014). In animals, miRNAs regulate their target genes through mRNA destabilization and translational inhibition (Bushati and Cohen 2007). So far, miRNAs have been identified in a few aquatic crustacean species, and these were involved in regulation of oocyte meiosis (Song et al. 2014), adaptive immunity (Li et al. 2013; Ou et al. 2012; Zeng et al. 2015), and drug response (Valenzuela-Miranda et al. 2015). Previous reports have shown that some plant miRNAs respond to salinity stress and that some miRNA targets are salt stress-related genes (Xie et al. 2014; Peng et al. 2014; Frazier et al. 2011). This suggests that miRNAs play important roles in plant salt stress responses. However, little is known regarding crustacean miRNA expression profiles in response to salinity stress, and their roles in osmoregulation remain unclear.

High-throughput next generation sequencing technology is an efficient method for discovering genes in an unbiased manner [7–10]. Measurement of miRNA expression levels and elucidation of the regulatory relationship between miRNA and its corresponding mRNA target are important to clarify pathways and biological processes including biotic and abiotic stress responses (Rao et al. 2014). Transcriptome analyses of *P. trituberculatus* growth (Lv et al. 2015) and responses to salinity challenged (Lv et al. 2013) by high-throughput next-generation sequencing technology have been reported; however, the study or experimental identification of miRNAs has been no performed.

In this study, we examined two miRNA transcriptomes from the gills of *P. trituberculatus* under normal conditions and low salinity stress using the Illumina/Solexa deep sequencing technology. Then, we elucidated their cellular miRNAs and the genes profiles related to hyperosmoregulation (Fig. 1). These results provide a basis for further investigation of miRNA-modulating networks underlying the osmoregulation of *P. trituberculatus*.

Materials and methods

Ethics statement

P. trituberculatus is not an endangered or protected species. All animal work has been conducted according to the relevant national and international guidelines. No specific permissions are required to work with invertebrates in China. Similarly, no specific permissions are required for the collection of *P. trituberculatus* from sample sites because they were not collected from protected areas of land.

Salinity challenge experiment and sample preparation

Eighty-day-old swimming crabs were collected from a local farm in Qingdao, China (5.62–11.66 g in body weight). All the samples were acclimated in the laboratory environment (33 ppt, 18 °C) for 7 days before the experiment. The crabs were divided into two groups (90 crabs per group) and acclimated in non-challenged (NC, 33 ppt) and low salinity challenged (LC, 5 ppt) at 18 °C, respectively. The gills from nine crabs were collected after 10 days, and all samples were stored in RNeasy (Ambion) at 4 °C over night and then at –20 °C.

The construction and deep sequencing of small RNA libraries

Total RNA was isolated from each sample by TRIzol (Invitrogen, CA, USA). RNA degradation and contamination was monitored on 1 % agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then, all samples were pooled in equal amounts in each group to generate two mixed sample. A total of 3 µg pooling samples was used as input material for the small RNA sample preparations. All samples had RIN values above 8. Sequencing libraries were generated using an IlluminaTruSeq™ Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's recommendations. The libraries were sequenced on an Illumina HiSeq 2000 platform and 100-bp single-end reads were generated.

miRNA identification

Raw data (raw reads) in the fastq format were first processed through our self-written Perl and Python scripts (Novogene Bioinformatics Institute). In this step, clean data (clean reads) were processed by getting rid of reads with low-quality reads, containing ploy-N, with 5' primer contaminants, without 3' primers or the insert tag, and with ploy A or T or G or C from the raw data. At the same time, Q20, Q30, and GC-content of the raw data were calculated. Then, the length of sRNA tags within a certain range from clean reads was remained for all the downstream analysis.

All the clean reads were searched against Rfam (<http://rfam.sanger.ac.uk/>) database for annotation. The reads annotated as tRNA, rRNA, snoRNA and snRNA were discarded. Considering that there was no published genome information of *P. trituberculatus* available, the remainder small RNA reads were compared to the known miRNA from all metazoan species in the miRBase 20.0 (<http://www>).

Fig. 1 Schematic of Illumina deep sequencing and analysis

mirbase.org/) to identify conserved miRNAs. Only perfect matches were considered as conserved miRNAs. Reads that were not aligned to the miRBase database were used to predict novel miRNAs. The miREvo and mirdeep2 (Friedlander et al. 2012) software were used for novel miRNA prediction by exploring the secondary structure, the Dicer cleavage site, and the minimum free energy of the former unannotated small RNA tags that could be mapped to reference sequences.

Target gene prediction and analyses

As there was no published genome information on *P. trituberculatus*, we tried to extract the 3'-UTR and 5'-UTR from the *P. trituberculatus* transcriptome that has been published (Lv et al. 2013). The sequences of transcriptome containing the extracted 3'-UTR and 5'-UTR were considered as candidate gene databases for target gene prediction. MiRanda-3.3 (Enright et al. 2003) software was used to predict the target genes of identified miRNAs. The predicted target genes were aligned by blastx, and then gene ontology (GO) analysis were performed on the target genes by GOEAST (Zheng and Wang 2008).

Differential expression of miRNA

miRNA expression levels were estimated by TPM (transcript per million) through the following criteria (Zhou et al. 2010). Differential expression analysis of two samples was performed using the DEGseq (2010) R package. *P*-value was adjusted using q value (Storey and Tibshirani 2003). *q*value <0.01 and $|\log_2(\text{foldchange})| > 1$ were set as the threshold for significantly differential expression.

Quantitative real-time PCR assay

MiRNAs expression levels were assayed by quantitative real-time PCR (qPCR) using a SYBR primescryptTM miRNA RT-PCR kit (TaKaRa) (Song et al. 2014). Total RNAs extracted from the gills were reverse transcribed using a stem-looped antisense primer. The resultant cDNA was submitted to the amplification of mature miRNAs using a miRNA specific primer and a universal primer. U6 snRNA gene was employed as an endogenous control. The primers for miRNAs and U6 snRNA are listed in Table 1. The qPCR was conducted in 15 µl reaction volumes containing 300 nM of each primer and cDNA derived from 0.1 µg of total RNA. Cycling

Table 1 The primers for miRNAs real-time RT-PCR

Primer names	Primer sequences (5'-3')
U6-F	CATATACTAAAATTGGAACGATACAG
U6-R	AACGCTTCACGATTTTTCGT
miR-2788b	CAATGCCCTTGGAATCCC
novel_1	AGTTACAGAGATCGTGGAAGA
novel_9	GCTGTAGGAATATCAAGAGAGT
miR-750b	CCAGATCTAACTCTCCAGCTCA
novel_42	TTGGTCATAGAATTCCTCCGT
novel_61	AGTTCGTTCTCCCTGGA
novel_2	GTTCCACACTCCAGGCACTG
novel_3	TCTTTGGTGATCTAGCTGTATGA
novel_28	AGGGGTGTGCAACAAATTATTTT
novel_32	TCGGGCTCTGTTGTGGTGTGT
novel_63	CCCGAGGCCACACTGTCTGCAG
novel_70	CGGCACACTCAGTACCAGTA
miR-2788a	CAATGCCCTTGGAATCCCAAAAT
novel_54	ACCTGATTGACTCCCTGGCC
novel_67	GTGGTTTGTGAGGACTCTGAGAGG

parameters were 95 °C for 1 min, and followed by 40 cycles of 95 °C for 35 s and 60 °C for 30 s. All reactions were run in triplicate. The expression of miRNAs was normalized against U6 snRNA levels. Significant differences were examined by paired *t*-test and *p* value < 0.05 was considered to be statistically significant.

Result

Solexa sequencing of small RNAs

After removal of the 5' and 3' adapters, polluted reads and reads smaller than 18 nucleotides (nt), a total of 7,032,973 clean reads, consisting of 153,814,547 nt with an average GC content of 51.18 % were obtained from the LC sample, while 6,166,057 clean reads, consisting of 136,575,094 nt with an average GC content of 51.03 % were obtained from the NC sample (Table 2). 77.39 % of the reads were 21–23 nt in length with a peak at 22 nt (Fig. 2). A total of 2,656,713 of the resulting unique small RNAs were annotated by the RFam 10.1 database in the LC sample while 2,899,003 of the resulting unique small RNAs were annotated in the NC sample. 2,103,956 and 2,501,788 reads of rRNA, tRNA, snoRNA, snRNAs, and other reads were annotated in LC and NC respectively, and then removed from the following analysis (Table 3).

Table 2 Statistics of microRNA transcriptome output sequencing

Samples	LC	NC
Total raw reads	10,983,455	8,819,069
Total clean reads	7,032,973	6,166,057
Total clean nucleotides (nt)	153,814,547	136,575,094
Q20 percentage	99.28	98.63
GC percentage	51.18	51.03

The discovery of known and novel miRNAs

A total of 16 known miRNAs, belonging to eight families were obtained from LC and NC, which exhibited variable abundance. MiR-750b was the most accumulated miRNA with a total of 349,452 reads detected between the two libraries (220,568 reads in LC and 128,884 in NC), while miR-2788a (20 reads in NC and no reads in LC) were the least accumulated miRNA in the two libraries (Table 4). The results of the family analysis showed that mir-317, mir-2788 and mir-305 were only found in arthropod species (Table S1S1).

Altogether, we obtained 51 predicted novel miRNAs. The novel miRNAs also displayed unequal expression levels between the two libraries (Table 4). Novel_5 was the most accumulated miRNA with a total of 20,307 reads detected between the two libraries (13,211 reads in LC and 7,096 in NC), while novel_40 and novel_36 (three reads in NC and no reads in LC) were the least abundant miRNA in the two libraries.

We matched the miRNAs of *P. trituberculatus* against the miRNAs of the Chinese mitten crab, *Eriocheir sinensis*, another important economic aquaculture species in China, and 85.7 % (12 out of 16) of the known miRNAs were matched, whereas none of the novel miRNAs were found among the miRNAs from *E. sinensis*.

miRNA differential expression analysis

A comparison of miRNA expressions showed that a total of 12 miRNA were differentially expressed after low salinity stress ($q_value < 0.005$ & $|\log_2(\text{fold change})| > 1$). Six were up-regulated and six were down-regulated between LC and NC (Table 4). Novel_61 ($\log_2(\text{Fold_change}) = 4.9663$) and miR-2788b ($\log_2(\text{Fold_change}) = -7.7936$) were the most up- or down-regulated miRNAs in LC vs. NC respectively. Among the 12 miRNAs, novel_1, novel_9 and miR-2788b were the most abundant miRNAs with 56,145, 5,892, and 5,128 reads detected between the two libraries, respectively (Table 4). Fifteen miRNA (4 known miRNAs and 11 novel miRNAs) were selected to confirm the expression pattern by stem-loop RT-qPCR assays. The results showed that there was a general consistency (86.7 %) between the quantitative assay and deep sequencing analysis for the fifteen miRNAs in terms

Fig. 2 Length distribution of small RNAs

of directions of regulation and significance (Ou et al. 2012) (Table 5).

Targets prediction and go enrichment analysis

A total of 94,511 transcriptome unigene of *P. trituberculatus* (Lv et al. 2013) were used to identify miRNA targets. The results show that 67 miRNA had 4702 affiliated target genes (70 target genes / miRNA), among which 12 differentially expressed miRNAs had 2051 affiliated targets (171 target genes / miRNA). GO enrichment analysis demonstrated that these targets were involved in many biological processes including regulation of cellular process (GO:0050794), cellular response to stimulus (GO:0051716), transport (GO:0006810), cell communication (GO:0007154), protein modification (GO:0036211), signal transduction (GO:0007165), phosphorylation (GO:0016310), etc. (Table S2).

To increase efficiency to discover true target genes, we filtered the targets showing differential expression in the opposite direction compared to miRNA. In total, 34 of the target genes predicted by miRanda had an opposite differential

expression to the miRNAs, which included sodium-independent sulfate anion transporter, sodium-dependent phosphate transporter 1-B, S-adenosylmethionine synthase, RNA-directed DNA polymerase from mobile element jockey, ribonuclease H1, protein henna, polypeptide N-acetylgalactosaminyltransferase 2, phosphoethanolamine N-methyltransferase 1, chorion peroxidase, carbohydrate sulfotransferase 3, etc. (Table 6). Biological processes of the 34 genes were further analyzed according to GO functional enrichment analysis, which were involved in six biological processes. The main processes involving unigenes were anion transport (GO:0006820, two unigenes), chitin metabolic process (GO:0006030, two unigenes), alpha-amino acid metabolic process (GO:1901605, two unigenes), intracellular protein transport (GO:0006886, three unigenes), organic substance transport (GO:0071702, four unigenes), and cytoskeleton organization (GO:0007010, two unigenes) (Fig 3).

Discussion

Although miRNAs have been researched extensively in recent years, no systematic study has been reported for *P. trituberculatus*, which is one of the most economically important mariculture species in China. In this study, we combined high-throughput Illumina sequencing technology with bioinformatic methods to efficiently identify and systematically analyze the miRNA response to low salinity stress in *P. trituberculatus*. The miRNA sequencing pipeline yielded 6,166,057 and 7,032,973 clean reads in the NC and LC libraries, respectively. The peak size was 22 nt, which was consistent with the results for *Triops cancriformis* (Ikeda et al. 2015), *E. sinensis* (Song et al. 2014), and *Scylla paramamosain* (Li et al. 2013). This suggests that 22 nt may be a typical size for crustacean miRNAs.

Table 3 Annotation of small RNAs

Types	LC	NC
known_miRNA	397,081	250,542
rRNA	299	295
tRNA	5	4
snRNA	27	35
snoRNA	69	50
repeat	2,287	2,670
novel_miRNA	155,676	146,673
other	2,101,269	2,498,734
total	2,656,713	2,899,003

Table 4 miRNA expression in LC and NC

Type	miRNA	Sequence	LC	NC	log2.Fold_change.	p value	q value	Different expression
Known miRNA	miR-190	AGAU AUGUUUGAU AUUCUUGG	3602	1738	0.36	1.5E-71	2.9E-71	No
Known miRNA	miR-2788a	CAAUGCCCUUGGAAAUCCCAAU	0	20	-6.66	9.0E-13	1.2E-12	Yes
Known miRNA	miR-2788b	CAAUGCCCUUGGAAAUCCC	37	5091	-7.79	0.0E+00	0.0E+00	Yes
Known miRNA	miR-281a	UGUCAUGGAGUUGCUCUCUUU	86006	56476	-0.08	4.6E-45	7.2E-45	No
Known miRNA	miR-281b	AAGAGAGCUAUCCGUCGACAGUACU	927	851	-0.57	2.1E-17	3.0E-17	No
Known miRNA	miR-281c	AAGAGAGCUAUCCGUCGACAGU	30504	23961	-0.34	1.5E-105	3.2E-105	No
Known miRNA	miR-305a	AUUGUACUUCUACAGGUGCUCU	12013	7081	0.07	2.1E-52	3.4E-52	No
Known miRNA	miR-305b	AUUGUACUUCUACAGGUGCUCGG	20436	13941	-0.14	1.3E-01	7.3E-02	No
Known miRNA	miR-305c	AUUGUACUUCUACAGGUGCUC	12013	7081	0.07	2.1E-52	3.4E-52	No
Known miRNA	miR-317a	UGAACACAGCUGGUGUAUCUCAGU	33584	20252	0.04	3.8E-110	8.5E-110	No
Known miRNA	miR-317b	UGAACACAGCUGGUGUAUAUCAGU	79	34	0.53	7.0E-04	5.9E-04	No
Known miRNA	miR-750a	CCAGAUCUAAACUCUCCAUAUGACG	334	131	0.66	3.7E-16	4.9E-16	No
Known miRNA	miR-750b	CCAGAUCUAAACUCUCCAGCUCA	220568	128884	0.09	0.0E+00	0.0E+00	No
Known miRNA	miR-7a	UGGAAGACUAGUGAUUUUGUUGU	10764	7053	-0.08	1.1E-06	1.2E-06	No
Known miRNA	miR-7b	UGGAAGACUAGUGAUUUUGUUGUUC	10767	7058	-0.08	1.4E-06	1.5E-06	No
Known miRNA	miR-9	UCUUUGGUGAUCUAGCUGUAUGA	28293	15943	0.14	7.2E-199	3.0E-198	No
Novel miRNA	novel_1	AGUUACAGAGAUCGUGGAAAGA	9686	46459	-2.95	0.0E+00	0.0E+00	Yes
Novel miRNA	novel_10	CUUACGACCGCCUAGCACGGU	2221	782	0.82	2.4E-131	7.9E-131	No
Novel miRNA	novel_11	CAAGAAAUCACUAAUCCUCCUA	1773	743	0.57	8.0E-64	1.5E-63	No
Novel miRNA	novel_12	ACUAAGAAUCAAACAUUAUAUCA	1940	1124	0.10	2.0E-11	2.4E-11	No
Novel miRNA	novel_14	AGUUGGAAGUGGGGAUCUCCGGCA	983	625	-0.04	2.2E-02	1.4E-02	No
Novel miRNA	novel_15	CUGUUGAGCUUGACUCUAGU	311	214	-0.15	9.6E-01	4.4E-01	No
Novel miRNA	novel_16	UUCCACACUCCAGGCACUG	256	143	0.15	3.5E-03	2.8E-03	No
Novel miRNA	novel_17	CCAUGGAACCACUAGUGCACC	207	95	0.43	1.5E-06	1.5E-06	No
Novel miRNA	novel_2	GUUCCACACUCCAGGCACUG	35580	19762	0.16	4.3E-287	2.1E-286	No
Novel miRNA	novel_20	CUUUCUCCUCUCCACUUUCUCG	25	5	1.63	3.7E-05	3.4E-05	No
Novel miRNA	novel_21	UAAGUGGGAUCGGUAUGCUCUAAU	19	6	0.97	1.1E-02	7.8E-03	No
Novel miRNA	novel_26	UCCAUUUUCACGGAGUCCUGU	10	2	1.63	9.0E-03	6.7E-03	No
Novel miRNA	novel_27	UUGUAUGUGAAACAAUACCAU	10	5	0.31	3.9E-01	2.1E-01	No
Novel miRNA	novel_28	AGGGGUGUGCAACAAUUAUUUU	5	0	3.97	4.5E-03	3.4E-03	Yes
Novel miRNA	novel_30	UGGGGAAAGAUUUGGAGGAACU	4	6	-1.27	8.7E-02	5.1E-02	No
Novel miRNA	novel_31	CAUCCCUAAUCAGUCUGUCUCU	4	5	-1.01	2.1E-01	1.1E-01	No
Novel miRNA	novel_32	UCGGGCCUCUGUUGUGGUGUGU	5	0	3.97	4.5E-03	3.4E-03	Yes
Novel miRNA	novel_36	UGAAUCAAAGGAGAUGUAGCA	3	0	NA	NA	NA	No
Novel miRNA	novel_37	UGACGUGAAGUGAGAGGCUACA	2	2	-0.69	6.0E-01	3.0E-01	No
Novel miRNA	novel_39	UUUAACAAGAUUUCAGUUGCAU	3	3	-0.69	5.2E-01	2.7E-01	No
Novel miRNA	novel_40	AGUGCACACGCUGGCUGGGAGU	3	0	NA	NA	NA	No
Novel miRNA	novel_42	UUGGUCAUAGAAUCCUCCGU	17	2	2.40	2.1E-05	2.0E-05	Yes
Novel miRNA	novel_46	UGUGGGACCCGAGUACCGCCGA	29	20	-0.15	1.0E+00	4.5E-01	No
Novel miRNA	novel_47	UGUCCAGUUGGCUCCAGAGA	30	12	0.63	1.8E-02	1.2E-02	No
Novel miRNA	novel_48	CUGCUUGCUGAGGGGAGGGA	516	904	-1.50	2.6E-130	7.6E-130	No
Novel miRNA	novel_5	AAAUAUCAGCUGGUAAAUUUGG	13211	7096	0.21	5.2E-136	1.9E-135	No
Novel miRNA	novel_51	AAACAUCUUGAGAACUUGGA	15	12	-0.37	5.9E-01	2.9E-01	No
Novel miRNA	novel_52	UAGGCCUUUGGAUCGUCAA	5	4	-0.37	7.6E-01	3.6E-01	No
Novel miRNA	novel_53	GUGGCCAUACUCUAUGCACAUC	242	78	0.94	5.7E-19	8.4E-19	No
Novel miRNA	novel_54	ACCUGAUUGACUCCCUUGGCC	0	2	-3.33	4.9E-02	3.0E-02	Yes
Novel miRNA	novel_55	GUACAGUGGAACCAUGUGUGCUU	0	1	NA	NA	NA	No
Novel miRNA	novel_56	AGAGGAGGCCUGGACACCU	7	5	-0.20	9.3E-01	4.3E-01	No
Novel miRNA	novel_58	GCAGCAGUGUCUCUGCCCU	17	20	-0.92	2.2E-02	1.4E-02	No
Novel miRNA	novel_59	GUUUUAGCGAAUUUUCUGGGU	4	8	-1.69	1.2E-02	8.2E-03	No

Table 4 (continued)

Type	miRNA	Sequence	LC	NC	log2.Fold_change.	p value	q value	Different expression
Novel miRNA	novel_6	CAUCACAGUGAUAGUACCUUACU	10329	5450	0.23	1.3E-120	3.4E-120	No
Novel miRNA	novel_61	AGUUCGUUCCUCCUGGA	10	0	4.97	2.7E-05	2.5E-05	Yes
Novel miRNA	novel_62	GGUGUAGUGGAAGUUAUUGG	42	38	-0.54	8.8E-02	5.1E-02	No
Novel miRNA	novel_63	CCCAGGCCACACUGUCCUGCAG	8	0	4.64	2.0E-04	1.7E-04	Yes
Novel miRNA	novel_64	UGAAUUCUUACACGAUGAGUGC	28	9	0.95	2.4E-03	2.0E-03	No
Novel miRNA	novel_66	CUUUGAAGGUGUGUUGAACCCC	3	3	-0.69	5.2E-01	2.7E-01	No
Novel miRNA	novel_67	GUGGUUUGUCAGGACUCUGAGAGG	1	8	-3.69	4.0E-05	3.6E-05	Yes
Novel miRNA	novel_68	AUGUAUGGUAUGGAUGUGACUGCA	11	11	-0.69	2.2E-01	1.2E-01	No
Novel miRNA	novel_7	UCCUGAGACCCUUCUUGUGA	7720	4957	-0.05	1.3E-08	1.5E-08	No
Novel miRNA	novel_70	CGGCACACUCAGUACCAGUA	4	0	3.64	1.3E-02	9.1E-03	Yes
Novel miRNA	novel_71	UUGCACCUGAUCCAGGAUUUCU	5	4	-0.37	7.6E-01	3.6E-01	No
Novel miRNA	novel_75	AUCUUGGUCGUUUUGCAAAUG	27	19	-0.18	9.3E-01	4.3E-01	No
Novel miRNA	novel_76	UGUGACGUCAUCGUGGUACUCUU	6	2	0.90	1.8E-01	9.8E-02	No
Novel miRNA	novel_77	GUGUUCUGACUGGGGGCUGA	6	1	1.90	2.7E-02	1.7E-02	No
Novel miRNA	novel_78	CUGGAACGCAUUUUUAUC	2	4	-1.69	7.4E-02	4.5E-02	No
Novel miRNA	novel_8	UUUACGACCGUUUAGCACGGU	5786	2158	0.73	3.5E-293	2.1E-292	No
Novel miRNA	novel_9	GCUGUAGGAAUAUCAAGAGAGU	1070	4822	-2.86	0.0E+00	0.0E+00	Yes

A total of 67 miRNAs were identified in this study, among which, 76.1 % (51 out of 67) were novel miRNAs. A similar result was found in *E. sinensis* (71.2 % were novel miRNA) (Song et al. 2014). The results suggest that the basis for the study of miRNA is weak in aquatic crustacean animals. So far, only 52 crustacean miRNAs have been listed in the miRBase, which accounted for 0.21 % of the total (24,521)

(Kozomara and Griffiths-Jones 2013). We matched *P. trituberculatus* miRNAs against *E. sinensis* miRNAs (Song et al. 2014; Ou et al. 2012), another important economic aquaculture species in China. A total of 85.7 % (12 out of 16) of the known miRNAs could be matched with the miRNAs from *E. sinensis*, whereas none of the novel miRNAs matched the miRNAs from *E. sinensis*. The results indicate that *P. trituberculatus*

Table 5 Gene expression levels of 15 selected miRNAs for the comparison of non-challenged and low salinity-challenged groups according to Illumina sequence and qPCR analysis

Gene	qPCR LC vs. NC log2 (fold change)	Illumina sequence LC vs. NC log2 (fold change)
novel_54	-1.22*	-3.3337*
novel_67	-0.49	-3.6893*
novel_9	-1.86*	-2.8613*
novel_1	-1.81*	-2.9513*
miR-2788a	-3.78*	-6.6556*
miR-2788b	-6.64*	-7.7936*
novel_32	1.50*	3.9663*
novel_70	1.38*	3.6444*
novel_28	1.32*	3.9663*
novel_63	1.78*	4.6444*
novel_42	1.64*	2.3982*
novel_61	0.15	4.9663*
miR-750b	0.55	0.08585
novel_2	0.42	0.15904
miR-9	0.16	0.13823

*Significant difference between salinity-challenged group and control group ($p < 0.05$)

Table 6 The predicted targets of the differentially expressed miRNAs

Type	miRNA ID	Putative target	Annotation
Down	novel_1	comp72508_c0	Phenylalanine hydroxylase
		comp73104_c0	Chitin-binding peritrophin-A
		comp77164_c0	Zinc finger protein ZPR1
Down	novel_25	comp44245_c0	S-Adenosylmethionine synthase
Down	novel_54	comp71288_c0	Calcineurin B homologous protein 1
Down	novel_67	comp70423_c0	Anaphylotoxin-like domain
		comp71288_c0	Calcineurin B homologous protein 1
		comp77164_c0	Zinc finger protein ZPR1
Down	novel_9	comp55772_c0	Sodium-independent sulfate anion transporter
		comp72508_c0	Phenylalanine hydroxylase
Down	miR-2788b	comp44245_c0	S-Adenosylmethionine synthase
Up	novel_28	comp38756_c0	Collagen alpha-1(IV) chain
		comp43033_c0	–
		comp67982_c0	Alcohol acetyltransferase
		comp70501_c0	Transmembrane protein 70 homolog, mitochondrial
		comp72677_c0	Outer dense fiber protein 3
		comp74429_c0	Microtubule-associated protein TAU
		comp76458_c0	Polypeptide N-acetylgalactosaminyltransferase 2
		comp76540_c0	Egl nine homolog 1
Up	novel_32	comp70501_c0	Transmembrane protein 70 homolog, mitochondrial
		comp75735_c1	–
Up	novel_42	comp66615_c0	Sodium-dependent phosphate transporter 1-B
		comp70501_c0	Transmembrane protein 70 homolog, mitochondrial
		comp72399_c0	RNA polymerase II, large subunit/ameloblastin precursor
		comp74211_c0	Carbohydrate sulfotransferase 3
		comp77007_c0	2-Hydroxyacylsphingosine 1-beta-galactosyltransferase
Up	novel_61	comp55240_c0	Phosphoethanolamine N-methyltransferase 1
		comp76538_c0	Compound eye opsin BCRH2
Up	novel_63	comp44799_c0	Arrestin homolog
		comp66615_c0	Sodium-dependent phosphate transporter 1-B
		comp68146_c0	Cuticlin-1
		comp70501_c0	Transmembrane protein 70 homolog, mitochondrial
		comp74011_c0	Helix-loop-helix protein delilah
		comp74806_c0	Splicing coactivator SRm160/300
Up	novel_70	comp44799_c0	Arrestin homolog
		comp55240_c0	Phosphoethanolamine N-methyltransferase 1
		comp65138_c0	Ribonuclease H1
		comp66513_c0	–
		comp75339_c0	Filamin-A
		comp76267_c0	Chorion peroxidase
		comp76440_c0	Villin headpiece domain
		comp76744_c0	Chitin-binding peritrophin-A /cytochrome oxidase c subunit
		comp76758_c2	Apolipoprotein III precursor (apoLp-III)
		comp77517_c0	RNA-directed DNA polymerase from mobile element jockey

miRNAs have high homologies to miRNAs from *E. sinensis*, but there are also many *P. trituberculatus*-specific miRNAs.

Many miRNAs are highly conserved among organisms, and they probably have relatively conservative and important roles in regulating basic cellular pathways

Fig. 3 GO enrichment analysis of the differentially expressed targets genes

in both lower and higher organisms (Glazov et al. 2008). In this study, mir-317, mir-2788, and mir-305 of *P. trituberculatus* specifically existed in the arthropod. The mir-317 influences reproductive responses by females to male sex peptide (Fricke et al. 2014) and was involved in cell cycle control in association with G2/M cyclin B in *Drosophila* (Pushpavalli et al. 2014). miR-305 regulates the Notch and insulin pathways in the intestinal stem cells, which are required for adaptive homeostasis in the *Drosophila* gut (Foronda et al. 2014). mir-2788a plays a potential role in butterfly wing development in *Heliconius* (Surridge et al. 2011). Previous research indicated that mir-317, mir-2788, and mir-305 may have similar roles in *P. trituberculatus*, but this needs further verification.

Comparison of gene expression among the different treatment groups in the current experiment was helpful for identification of candidate miRNAs underlying responses to salinity stress in *P. trituberculatus*. In this study, we detected 12 miRNAs differentially expressed between the comparison of LC vs NC (six upregulated and six downregulated). Identification of targets improves our understanding of the physiological functions of these differentially expressed miRNAs. As miRNA binding to 3'-UTR of target gene generally caused destabilization and degradation of mRNA (Guo et al. 2010), for increasing the strength to discover true target genes, we chose to narrow down potential targets to those showing differential expression in the opposite direction compared to miRNA. In total, 34 target genes were identified, among which, S-adenosylmethionine synthetase (SAM) was the only predicted target of the only known differentially expressed miRNA mir-2788. SAM is also known as

methionine adenosyltransferase (MAT), catalyzes the formation of S-adenosylmethionine by joining methionine and ATP (Horikawa et al. 1990), and plays an important role in plant responses to salt, drought, alkali, and flood stresses (Kamal et al. 2012; Chen et al. 2014). SAM is also a methyl donor, which controls gene expression by DNA methylation (Reytor et al. 2009). The mir-2788 miRNA was the only potential regulatory miRNA of SAM and was differentially expressed between LC and NC in this study, which indicated that it may play an important role in hyper-osmoregulation by regulating the expression of SAM.

Interestingly, many other genes encoding biological enzymes other than SAM were predicted as targets of differentially expressed miRNAs. These accounted for 38.2 % of the total predicted targets (13 out of 34). Enzymes are macromolecular biological catalysts that can increase the reaction rate by lowering the activation energy. They are able to catalyze more than 5000 biochemical reactions (Schomburg et al. 2013), and some can make reactions of substrates to products occur millions of times faster. Orotidine 5'-phosphate decarboxylase is an extreme example, which catalyzes a reaction that would otherwise take millions of years to occur in milliseconds (Radzicka and Wolfenden 1995; Callahan and Miller 2007). Four out of 13 genes encoding biological enzymes have been reported to play an important role in salinity adaptation. These are SAM [29–32], phosphoethanolamine N-methyltransferase 1 (Wu et al. 2007), cytochrome oxidase c (Zikos et al. 2014; Tine et al. 2011), and calcineurin B (Chen et al. 2015; Gu et al. 2008). Other enzyme genes have functions in anoxic response (alcohol acetyltransferase) (Kwast et al. 2002), disease responses (phenylalanine hydroxylase and polypeptide N-acetylgalactosaminyltransferase 2)

(Kaufman 1999) and stress responses (RNA polymerase II) (Bourbonnais et al. 2001). These results indicate that miRNA play important roles in responding to low salinity stress through a series of enzymatic reactions that are highly efficient and have a low energy consumption. However, the hypothesis needs further confirmation.

Six GO biological processes, which help *P. trituberculatus* to adapt to low salinity environments, were significantly enriched. The top two processes based on the enrichment level (*p* value), were anion transport (GO:0006820) and chitin metabolic process (GO:0006030). It is worth noting that the two processes were also shown to be enriched in the transcriptome analysis of *P. trituberculatus* when responding to salinity stress (Lv et al. 2013). Anion transport (GO:0006820) is the directed movement of anions within or between cells by transporters or pores. It is important in “compensatory process” adopted by crustaceans for osmoregulation and predominately occurs in gills (Péqueux 1995; Rainbow and Black 2001). The sodium-independent sulfate anion transporter and sodium-dependent phosphate transporter 1-B were involved in this process. Previous study showed that the two genes were also important osmoregulation factors in *E. sinensis* (Hui et al. 2014). Peritrophins, which are tightly associated with the peritrophic membrane (PM) or chitin fibrils, were first isolated from insect PMs. They protect insects from invasion microorganisms and stimulate digestion (Loongyai et al. 2007). Peritrophins are also involved in anti-bacterial innate immunity in crabs (Huang et al. 2015); however, their role of osmoregulation has not been reported. In this study, two peritrophin-like genes containing chitin binding peritrophin-A domains were the predicted targets of two differentially expressed miRNAs (novel_1 and novel_70) enriched in the chitin metabolic process (GO:0006030). These results indicated that anion transport and the chitin metabolic process play important roles in osmoregulation; moreover, these processes were controlled by miRNAs according to regulate specific target genes.

Conclusion

This study is the first systematic analysis of miRNAs in *P. trituberculatus* by high-throughput Illumina sequencing technology. Sixty-seven miRNAs were identified, among which, 12 miRNAs were related to low salinity stress. Thirty-four of the target genes predicted were differentially expressed in the opposite way to the miRNAs, which were enriched in six important GO biological processes related to osmoregulation, such as anion transport and the chitin metabolic process. The results provide a basis for further investigation of miRNA-modulated osmoregulation networks in *P. trituberculatus*.

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