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Identification and characterization of salt-tolerance relative miRNAs in *Procambarus clarkii* by high-throughput sequencing

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Abstract

Procambarus clarkii is one of the important economic species in China and has been served as tasty food in recent years after being introduced to Nanjing. Significant problems of environment factors, such as salinity, pH and temperature, especially salinity, has the potential to result in significant economic losses in many crayfish-producing farms in China. miRNAs are a kind of ~ 22 nucleotide small non coding RNAs which were encoded by plants, animals and some viruses with functions in RNA silencing or post-transcription regulation. We constructed four sRNA library of *P. clarkia* from different tissues and treatments by using high-throughput sequencing technology. A total of 101 conserved miRNAs and two novel pre-miRNAs were identified and RT-qPCR were further performed to confirm existence of part of identified miRNAs. A genome wide expression profile of salt-tolerance miRNAs was proved and three miRNAs were further validated by RT-qPCR with dynamic response to different salinity stages. The study of miRNAs in *P. clarkia* can help us better understanding the role of miRNAs in salt-tolerance in *P. clarkia*.

Keywords: miRNA, Crayfish, *Procambarus clarkia*, High-throughput sequencing, Salt tolerance

Background

Procambarus clarkii is the most cosmopolitan crayfish around the world. In some countries, *P. clarkii* is a species of great commercial interest [1]. This crayfish is one of the important economic species in China and has been served as tasty food in recent years after being introduced to Nanjing, China from Japan in 1929 [2]. *P. clarkia* can tolerate extreme and polluted environments and served as an indicator of metal pollution in numerous studies of aquatic environments [3]. Generally, *P. clarkia* has great resistance to diseases in natural environments. Nevertheless, the present sustainability and healthy development of the crayfish aquaculture are at risk due to significant problems of environment factors, such as salinity, pH and temperature. These factors, especially salinity, has the potential to result in significant economic losses in many crayfish-producing farms in China. Under these circumstances, an investigation into the mechanisms of salt-tolerance of *P. clarkii* might be beneficial to the management of crayfish farming.

MicroRNAs (miRNAs) are a kind of ~ 22 nucleotide small non coding RNAs which were encoded by plants, animals and some viruses [4–6]. miRNAs have great functions in RNA silencing or post-transcription regulation via base-pairing with complementary sequences in mRNAs [7]. miRNAs are abundant in many cell types [8, 9] and can regulate nearly 60% of genes in mammals [10, 11]. A total of 10,000 different miRNAs had been identified and reported in miRBase for all species [12]. Expression profile of miRNAs changed a lot in pathological state or environmental factor stimulation. Previous studies have reported the miRNA profiles in *P. clarkia*, for example, Wang et al. and Du et al. identified the miRNAs in gills, intestines and lymph organs of *P. clarkia* infected with white spot syndrome virus [13–15]. Ou et al. screened the miRNAs potentially related to immunity against Spiroplasma eriocheiris infection in *P. clarkia* [16]. However, the miRNA profiles in *P. clarkia* under environmental factor stimulation have never been reported. This study of

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miRNAs in *P. clarkia* can help us better understanding the role of miRNAs in salt-tolerance in *P. clarkia*.

In this study, we used high-throughput sequencing technology and bioinformatics analysis to identify conserved and novel miRNAs in *P. clarkii*. In addition, the possible salt-tolerance relative miRNAs of *P. clarkii* were analyzed.

Results

High-throughput sequencing of *P. clarkia* small RNAs

To investigate the existence of miRNAs in *P. clarkia*, high-throughput sequencing technology was employed. Hearts and Gills of *P. clarkia* from fresh or salt water were collected for analysis. A total of 92,125,122 (heart of *P. clarkia* from fresh water, FW-H), 91,146,311 (gill of *P. clarkia* from fresh water, FW-G), 101,323,891 (heart of *P. clarkia* from salt water, SW-H) and 92,819,109 (gill of *P. clarkia* from salt water, SW-G) raw reads were generated from four libraries. After removing low quality reads and mask adapters, 83,152,532 (FW-H), 81,298,133 (FW-G), 83,946,234 (SW-H) and 79,341,871 (SW-G) clean reads were remained for further analysis. Among them, a majority was 20–24 nt in length (~ 97% in average) and the length distribution peaked at 22 nt in length (~ 74% in average) (Fig. 1). These results suggest that these 20–24 nt small RNA sequences are dominant classes of small non-coding RNAs in *P. clarkia*, implying an enrichment of miRNA in the small RNA library of *P. clarkia*.

Identification of conserved miRNAs in *P. clarkia*

To identify conserved miRNAs in *P. clarkia*, four sRNA libraries were aligned to known mature miRNA sequences collected in miRBase v19.0 [12] with a tolerance of only one mismatch. We detected 71, 88, 68 and 88 miRNAs in FW-H, FW-G, SW-H, SW-G sRNA libraries, respectively. Venn diagram analysis showed that 55 miRNAs can be identified in these sRNA libraries and FW-G sample had the most number of specific expressed miRNAs (Fig. 2a). Expression level of common miRNAs were then used for hierarchic clustering of miRNA expression in four samples. The result showed that FW-H and FW-G have similar miRNA profile while SW-H and SW-G have similar one, indicating that the environment have more influence to miRNA profile (Fig. 2b). Pearson correction analysis were also applied to judge the similarity between four samples by the copy number of individual miRNAs. As shown in scatter plot comparing FW-G to FW-H and SW-G to SW-H, miRNA profiles from different tissue in the same environment have a certain similarity (pearson correlation coefficient between 0.6 to 0.8) (Fig. 2c). miRNA profiles of hearts of *P. clarkia* from salt water (SW-H) and fresh water (FW-H) have high pearson correlation coefficient 0.9792, suggesting that salinity have nearly no effects on miRNA abundances in hearts. However, miRNA profiles showed great differences in gills of *P. clarkia* from salt water (SW-G) and fresh water (FW-G) with pearson correlation coefficient 0.4643 (Fig. 2c).

A stem-loop reverse transcription polymerase chain-reaction (RT-PCR) assay was adapted to detect mature miRNA existence in *P. clarkia*. Nine mature miRNAs (miR-1, miR-2a, miR-275, let-7a-3p, miR-184, miR-71, miR-8-3p, miR-276 and miR-279), which was top expressed in deep sequencing result, were chosen for further validation. As shown in Fig. 3a, these nine mature miRNAs were clearly expressed in *P. clarkia* as detected by semi-quantitative RT-PCR analysis with 30 cycles. Quantitative RT-PCR (RT-qPCR) assays were then used to detect these miRNA abundances in different tissues. RNA samples of neuro, gill, muscle and heart samples were collected from *P. clarkia*. Among these nine miRNAs, miR-2a and miR-275 were nearly undetectable in these four tissues. miR-1 was highly expressed in muscle and

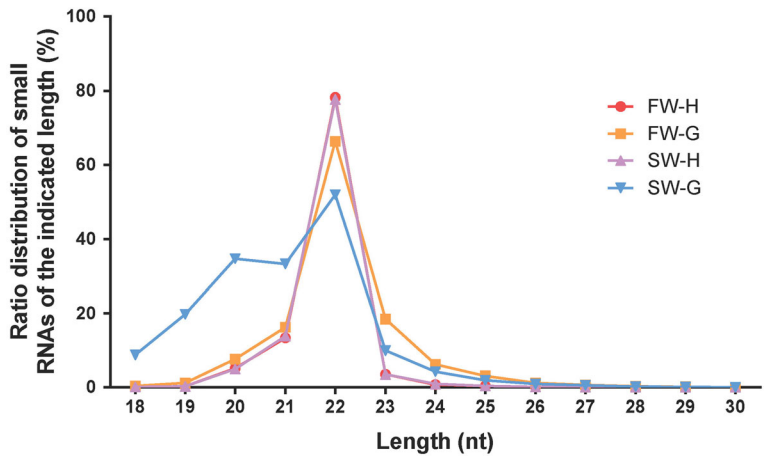

Fig. 1 Length Distribution of small RNA libraries. Heart of *P. clarkia* from fresh water, FW-H; gill of *P. clarkia* from fresh water, FW-G; heart of *P. clarkia* from salt water, SW-H; gill of *P. clarkia* from salt water, SW-G

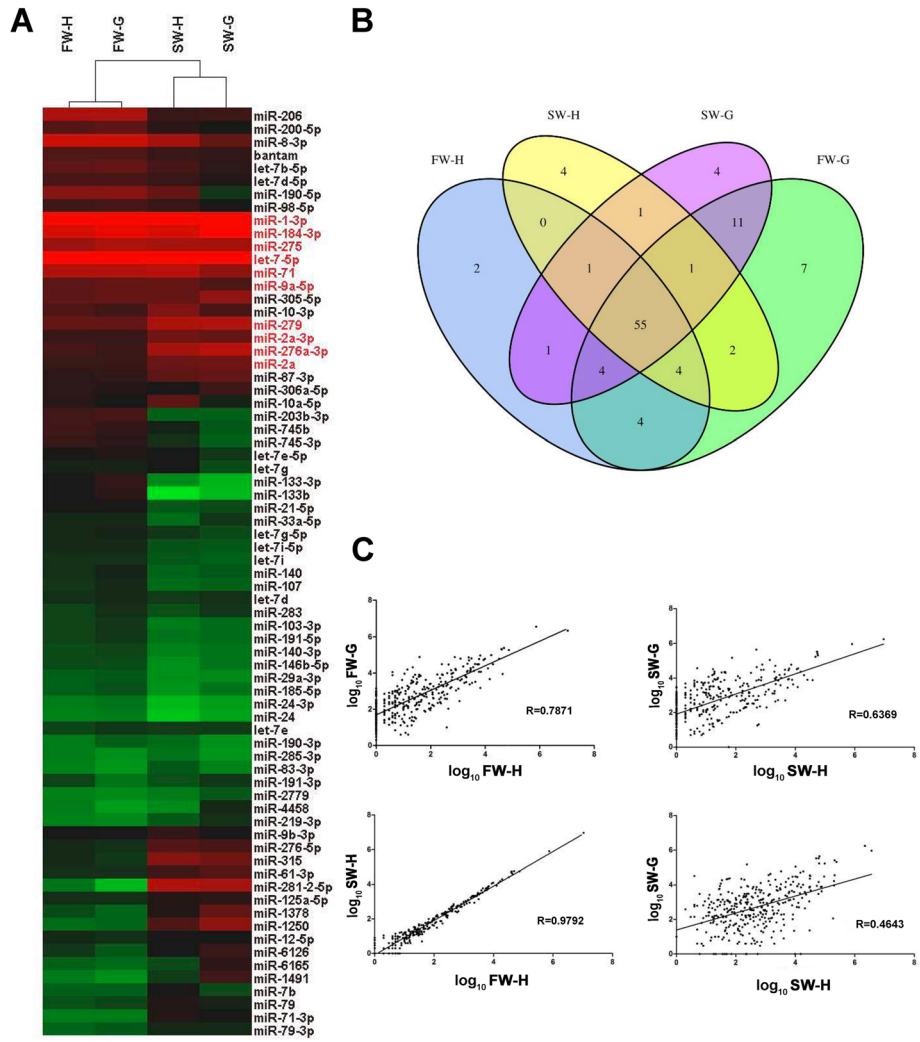

Fig. 2 Characterization of conserved miRNAs of *P. clarkia*. **a** hierarchic clustering analysis of miRNA profiles in FW-H, FW-G, SW-H and SW-G. **b** Venn diagram analysis of miRNA species in FW-H, FW-G, SW-H and SW-G. **c** Pearson correlation scatter plots of miRNA profiles in FW-H, FW-G, SW-H and SW-G

heart. miR-184 was neuro specific miRNA. And miR-276 and miR-279 were gill specific miRNAs (Fig. 3b).

Identification of novel miRNAs in *P. clarkia*

To identify the unique miRNAs in *P. clarkia*, the unclassified sequences were further analyzed to predict novel miRNA candidates. Expressed Sequence Tags (ESTs) of *P. clarkia* were collected from NCBI GenBank database [17]. After aligning the remained sRNA reads in sRNA libraries, MIREAP was then used to predicted candidate miRNA precursors and mature miRNAs. Twenty-three pre-miRNA candidates were found for further analysis. MiPred were applied for figuring out pseudo pre-miRNAs in these pre-miRNA candidates [18]. Only two miRNA candidates (Pcl-s1 and Pcl-s2) and four mature candidates were remained at last (Fig. 4a). A stem-loop RT-PCR assay was also used to detect these four novel mature miRNAs in *P. clarkia*. Figure 4b showed that these four mature miRNAs were clearly expressed in *P. clarkia* and mature miRNAs from Pcl-s1 had higher expression level than those from Pcl-s2 by semi-quantitative RT-PCR analysis with 30 cycles. RT-qPCR assays were also used to check two novel pre-miRNA levels. As shown in Fig. 4c, Pcl-s1 also have higher level than Pcl-s2 in neuro, gill, muscle and heart.

Analysis of miRNA involved in salt tolerance in *P. clarkia*

As previously descripted, miRNA profiles of gills of *P. clarkia* from fresh water and salt water had great difference. It was easy to understand that gills were directly get in touch with environments. To figure out salt-tolerance relative miRNAs in *P. clarkia*, the relative abundances of FW-G and SW-G

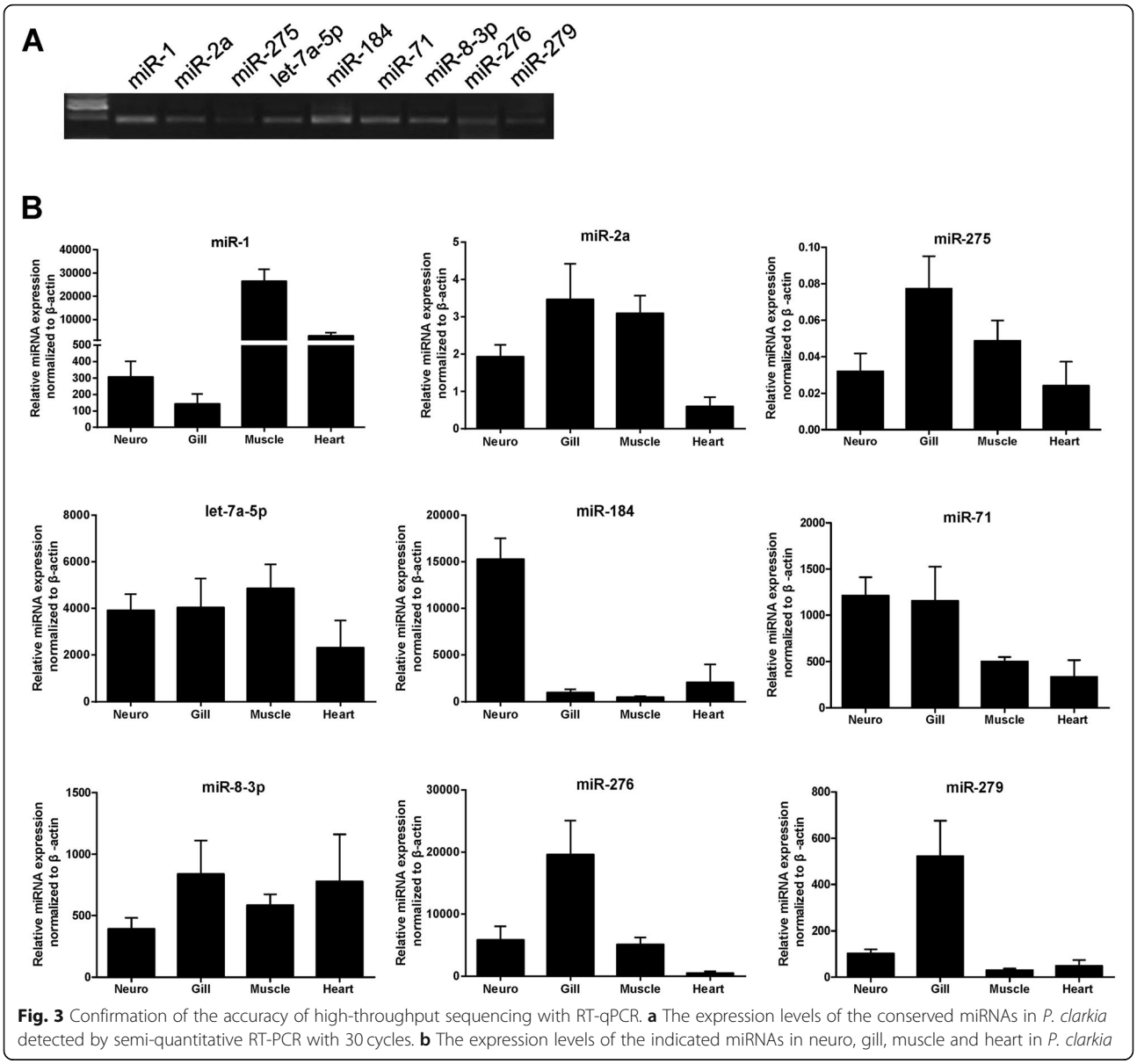

Fig. 3 Confirmation of the accuracy of high-throughput sequencing with RT-qPCR. **a** The expression levels of the conserved miRNAs in *P. clarkia* detected by semi-quantitative RT-PCR with 30 cycles. **b** The expression levels of the indicated miRNAs in neuro, gill, muscle and heart in *P. clarkia*

miRNAs were compared. RT-qPCR assays were applied to quantify 9 mature miRNAs' expression level in FW-G and SW-G (Fig. 5a). Among 9 miRNAs, only miR-275 was up-regulated and miR-276, let-7a-5p, miR-71 and miR-184 were downregulated in SW-G. Considering expression level, miR-276, let-7a-5p and miR-71 were chosen for further analysis. To confirm the expression of three miRNAs and detect their dynamic response to salt stress at different treatment stage, four groups of *P. clarkia* were kept in water with different salinity (0‰, 2‰, 4‰, 6‰) for 1 week. Gills were then collected for RNA extraction. We then detected miRNA abundances in these four groups of RNA samples. As shown in Fig. 5, expression level of miR-276 (Fig. 5b), let-7a-5p (Fig. 5c) and miR-71 (Fig. 5d) were decreased with the increase of salinity in water. These results showed that miR-276, let-7a-5p and miR-71 were salt-tolerance relative miRNAs. To further understand the potential functions of these miRNAs, we conducted bioinformatics analysis. Gene Ontology (GO) analysis showed that the high-enrichment GO terms targeted by the miRNAs included transcriptional activator activity, protein kinase activity and so on (Fig. 5e). The above result is beneficial for further research in the potential mechanism for the role of miRNAs in salt-tolerance in *P. clarkia.*

Discussion

In this study, we used high-throughput sequencing technology to identify potential miRNAs of *P. clarkia*. We

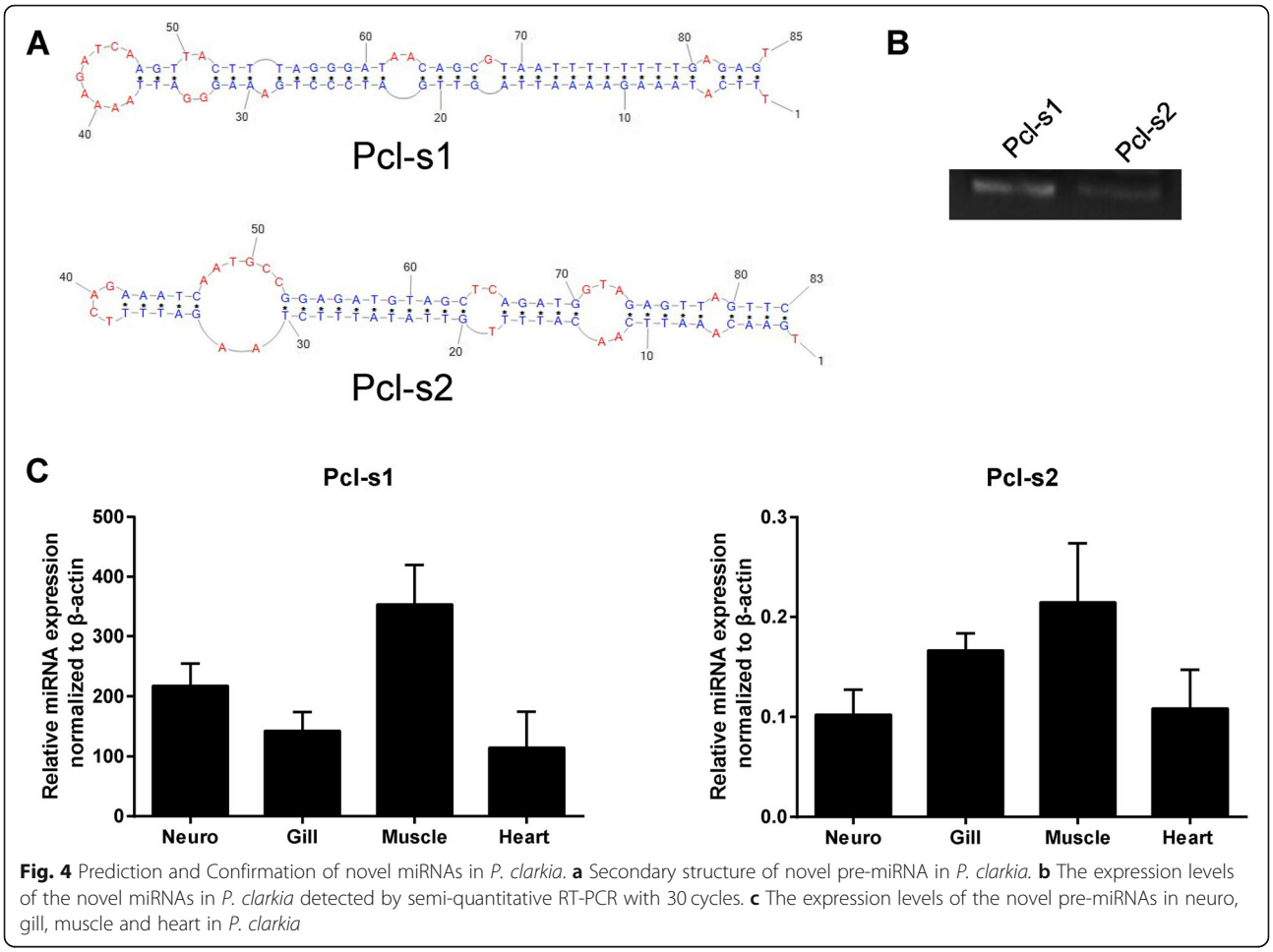

Fig. 4 Prediction and Confirmation of novel miRNAs in *P. clarkia*. **a** Secondary structure of novel pre-miRNA in *P. clarkia*. **b** The expression levels of the novel miRNAs in *P. clarkia* detected by semi-quantitative RT-PCR with 30 cycles. **c** The expression levels of the novel pre-miRNAs in neuro, gill, muscle and heart in *P. clarkia*

used SOAP (http://soap.genomics.org.cn) [19, 20] to align sRNA reads in our libraries to known miRNA in miRBase v19. If we can find perfect match sequences of one miRNAs in our libraries, certain miRNAs were thought to be existed in *P. clarkia*. However, whole genome of *P. clarkia* was not reported yet, it was hard to prove that the sequences we detected were from *P. clarkia* or contaminations. Although we used semi-quantitative RT-PCR and RT-qPCR to further prove the reliability of part identified miRNAs. More experiments should be done to prove the existence of miRNAs in *P. clarkia.* Two novel pre-miRNAs were also identified using ESTs in this study. ESTs can't reflect whole genome of *P. clarkia*, more novel pre-miRNAs could be found if we got whole genome of *P. clarkia*.

Among 9 miRNAs detected by RT-qPCR assays, miR-276, let-7a-5p and miR-71 showed negative correlation with salt-tolerance. For transcriptome of *P. clarkia* was not sequenced yet, we cannot search possible target genes of these miRNAs as we usually do. Previous study reported that Na^+-K^+-ATPase was mainly located in crustacean gill and its activities had positive correlation with salinity level [21, 22]. The identified salt-tolerance relative miRNAs may play roles in Na^+-K^+-ATPase pathways. Further researches can be deployed to study the relationship between miRNA and Na^+-K^+-ATPase activities.

We have proven that salinity level has effects on miRNA profile of *P. clarkia* in this study. Other factors can also have effects on miRNA profile of *P. clarkia.* Emodin diet and white spot syndrome virus have been proven that can influence miRNA abundances of *P. clarkia* and miRNAs played important roles in immunity, RNA transport and other important biological progresses [15, 23]. The study of miRNAs in *P. clarkia* contributes a better understanding of miRNA function in crayfish.

Conclusions

We constructed four sRNA library of *P. clarkia* from different tissues and treatments by using high-throughput sequencing technology. A total of 101 conserved miRNAs and two novel pre-miRNAs were identified and RT-qPCR were further performed to confirm existence

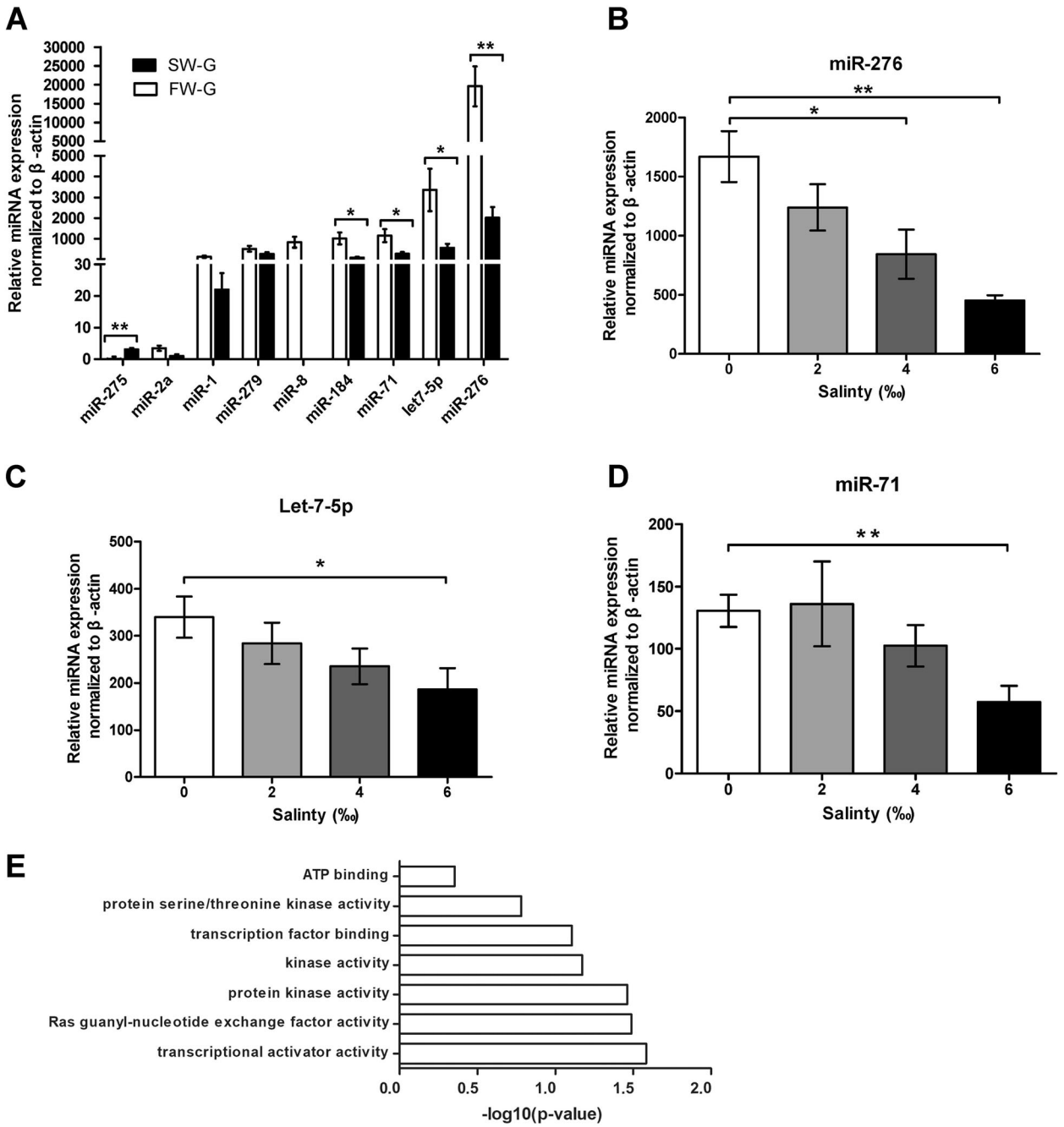

Fig. 5 Identification of salt-tolerance relative miRNAs in *P. clarkia*. **a** Comparison of nine conserved miRNAs expression level in FW-G and SW-G. **b**-**d** RT-qPCR assays of miR-276 (**b**), let-7a-5p (**c**) and miR-71 (**d**) levels in *P. clarkia* gills with different salinity stages. **e** The most enriched GO molecular functions of the miRNAs. GO, molecular function for all miRNA targets. $^{*}P < 0.05$, $^{**}P < 0.01$

of part of identified miRNAs. A genome wide expression profile of salt-tolerance miRNAs was proved and three miRNAs were further validated by RT-qPCR with dynamic response to different salinity stages. High-throughput sequencing provides an opportunity to analyze salt-tolerance relative miRNAs in *P. clarkia*, which will help to unravel new components of salt stress pathways and gain new insights into gene function and regulation in crayfish.

Methods

Experimental animal collection and RNA isolation

All sampels of *P. clarkia* were bought from local market. Total RNA was isolated using TRIzol Reagent

(Invitrogen) according to the manufacturer's instructions.

High-throughput sequencing

The high-throughput sequencing was conducted by using Illumina Genome Analyzer IIx according to the manufacture's protocols. Small RNA molecules (18-30 nt) were purified from total RNA using PAGE gel and were then used for library preparation according Illumina TruSeq Small RNA Sample Preparation Guide. Briefly, after ligation of 3′ and 5′ adapters to their both ends, the RNA samples were then amplified by using adapter primers for 17 cycles. The PCR products (around 147 bp) were isolated from agarose gels and directly used for cluster generation. Small RNA library was then sequenced using Illumina Genome Analyzer IIx. CASAVA 1.5 was used to get raw sequencing data from the image files generated by the machines. Quality control of raw sequencing data was performed by fastx-toolkit. After filtering the low-quality reads and trimming 3′ adaptor sequencing and removing 5′ adaptor and polyA contaminations, clean reads were processed for following analysis.

In silico analysis

To identify known miRNAs *P. clarkii*, high-throughput sequencing reads were aligned against all known miRNA precursors and mature miRNAs present in miRBase database with SOAP [19, 20]. Sequences not matched in above databases were remained for further analysis.

To identify novel miRNAs in *P. clarkii*, EST sequences of *P. clarkia* were collected from GenBank database in NCBI. SOAP was also used to align remained sequences to ESTs. MIREAP is used to identify genuine miRNAs from 4 constructed small RNA libraries combining miRNA biogenesis, sequencing depth and structural features. All pre-miRNA candidates were subjected to MiPred to filter out pseudo-pre-miRNAs. True pre-miRNA candidates were used for further analysis.

Quantitative RT-PCR assays

Quantitative RT-PCR was performed using TaqMan miRNA probes (Applied Biosystems, Froster City, CA, USA) using an ABI-7300 PCR machine according to the manufacturer's instructions. The amount of RNA input was o.5μg. Relative expression of miRNAs in tissues was determined after normalization to β-actin mRNA levels.

Abbreviations

miRNA: microRNA

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FFJ conceived and designed the experiments. YLS and FFJ participated in the bioinformatic analysis, experiments and drafted the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Competing interests

The authors declare that they have no conflict of interests.

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