Identification of drought-responsive miRNAs in *Hippophae tibetana* using high-throughput sequencing

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Received: 9 March 2019 / Accepted: 30 December 2019 / Published online: 21 January 2020
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Abstract

MicroRNAs (miRNAs) play an important role in abiotic stress response in plants. However, the total miRNA profiles (miRNome) and drought-responsive miRNAs in *H. tibetana* have not been identified. In this study, we present the first report on the miRNome profiles of *H. tibetana* by high-throughput sequencing technology. 116 known and 4 predicted novel miRNAs were all identified in six *H. tibetana* samples. Moreover, to reveal the drought-responsive miRNAs in *H. tibetana*, we compared the miRNA profiles of *H. tibetana* grown under water sufficiency and drought stress. The results showed that 39 known miRNAs were up-regulated, while 34 miRNAs were downregulated under drought stress. Moreover, the expression of two novel miRNAs (novel_mir_24 and novel_mir_87) showed notable changes in response to drought stress. The target genes of these differentially expressed miRNAs were mainly enriched in cellular process, metabolic process, cell part, and response to stimulus. The identified drought-responsive miRNAs might be used for improving drought tolerance in *H. tibetana* and other plateau plants.

Keywords *Hippophae tibetana* · MicroRNA · High-throughput sequencing · Drought-responsive miRNAs

Introduction

MicroRNAs (miRNAs) are ~21 nucleotide (nt) long, endogenous non-coding molecules which show their functions as an essential regulatory component in eukaryotes (Xie et al. 2010). In plants, miRNAs can regulate the expression of target genes encoding various transcription factors, thereby affecting plant growth, development and physiological functions (Jones-Rhoades et al. 2006; Rogers and Chen 2013). For example, miR156 overexpression in *Arabidopsis thaliana* caused a moderate delay in flowering and a severe decrease in apical dominance (Schwab et al. 2005).

Recently, increasing number of miRNAs have been found to be related to abiotic stress responses, such as cold, drought, salinity, and nutrient deprivation (Sunkar et al. 2012). For example, Li et al. (2008) found that miR169 is related to drought stress in *A. thaliana*. Mutum et al. (2016) identified a series of novel miRNAs from drought-tolerant rice variety Nagina 22. In addition, the expression of 3 conserved and 25 predicted miRNAs showed significant changes in response to cold stress in rice (Zhang et al. 2009). Accompanied with the rapid development of deep sequencing and bioinformatics analysis, total miRNA profile (miRNome) has been measured and predicted in several model plants, such as *Arabidopsis*, rice and poplar (Fahlgren et al. 2007; Sunkar et al. 2008; Chen and Cao 2015). miRNAs in plants are highly conserved (Jones-Rhoades et al. 2006). Moreover, the miRBase database (<http://www.mirbase.org/>) for miRNAs identification has been well developed (Kozomara and Griffiths-Jones 2014). Therefore, potential miRNAs in non-model plants may be identified.

Most crop plants grow in a suboptimal environment with multiple abiotic stresses, such as drought, cold, heat, and salinity, which will prevent them from gaining potential production (Atkinson and Urwin 2012; Chaudhary and Sharma 2015). Amongst these stresses, drought stress is a typical

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-2045-5>) contains supplementary material, which is available to authorized users.

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environmental factor that can affect plant growth (Shinozaki and Yamaguchi-Shinozaki 2007). Therefore, studies on the mechanisms of drought resistance in plants are crucial. *Hippophae tibetana* is a small, dioecious wind-pollinated shrub endemic to the cold and arid regions of the Tibetan–Qinghai Plateau. As one of the highly drought-resistant and cold-resistant plants, the *H. tibetana* genome is likely to hide some genes or miRNAs associated with stress response, which may be useful for enhancing the tolerance of other plants to abiotic stresses in the future. However, no miRNAs analysis has been performed on *H. tibetana*. To date, the potential miRNAs responsive to drought stress have not been identified in *H. tibetana*.

As mentioned above, miRNAs play an important role in abiotic stress response in plants. In this study, high-throughput pyrosequencing technology was applied to obtain the miRNome profiles of *H. tibetana* from two habitats and identify the potential miRNAs related to drought stress in *H. tibetana*. The results will provide an idea of the miRNAs importance in drought stress in *H. tibetana* and contribute to its stress-resistant breeding.

Materials and methods

Plant material

Two sets of *H. tibetana* (HT for abbreviation) were collected from different places with two habitats in the Qinghai–Tibet Plateau (Sichuan Province, China). The first set of samples named HT-1, HT-2 and HT-3 were mainly collected from sandy land. The relative soil moisture content (RSMC) values of HT-1, HT-2 and HT-3 were 12%, 13% and 10%, respectively. The second set of samples including HT-4, HT-5 and HT-6 were from the river valley. Their RSMC values were 70%, 74% and 73%, respectively. Plant materials used for RNA extraction and sequencing were from fresh leaves of *H. tibetana*. After collection, all six samples were frozen in liquid nitrogen and then stored at -80°C until further use.

Small RNA library preparation and sequencing

Total RNA was extracted from each tissue sample using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's protocols. RNA purity was examined using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA, USA).

A total of 3 μg RNA per sample was used as the input material for the small RNA library. Sequencing libraries were generated using NEBNext Multiplex Small RNA

Library Prep Set for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. In brief, NEB 3' SR adaptors were ligated to the 3' end of small RNA. After the 3' ligation reaction, the SR RT Primer was hybridized to the 3' SR adaptor transforming the single-stranded DNA adaptor into a double-stranded DNA molecule. Moreover, the 5' end adapter was ligated to the 5' ends and first-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H). Thereafter, PCR amplification was performed using LongAmp Taq 2X Master Mix, SR Primer for Illumina and an index (X) primer. PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). After quality assessment, DNA fragments 140–160 bp in length were recovered and dissolved in 8 μl of elution buffer. Small RNA sequencing was carried out by Beijing Genomics Institute (BGI) (Shenzhen, Guangdong, China) using the high-throughput pyrosequencing technology developed by Illumina (Song et al. 2010). The sequencing data were deposited in the NCBI Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/Traces/sra/>) under Accession Number SRR5514888, SRR5514889, SRR5514890, SRR5514891, SRR5514892, and SRR5514893 for HT-1 to HT-6, respectively.

Bioinformatic analysis of sequencing data

After sequencing, clean reads were obtained by removing the reads containing ploy A, with 5' adapter contaminants, without 3' adapter and the insert tag, low-quality reads, and reads shorter than 18 nucleotides from the raw data. On the other hand, Q_{20} , Q_{30} , and GC content of the raw data were calculated. Then, a certain range of length from clean reads was considered significant and further analyzed. To remove tags originating from protein-coding genes, repeat sequences, ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), the small RNA tags were mapped to the RepeatMasker and Rfam databases (<http://rfam.sanger.ac.uk/>), and the mapped tags were ruled out. The remaining small RNA tags were used to search for known miRNAs. miRBase database (<http://www.mirbase.org/>) was used as a reference, whereas modified software mirdeep2 (Friedlander et al. 2012) and srna-tools-cli (Moxon et al. 2008) were used to identify potential miRNA and draw the secondary structures. Custom scripts were used to obtain the miRNA counts and base bias on the first position of the identified miRNA with certain length and on each position of all identified miRNAs, respectively.

The characteristic hairpin structure of miRNA precursors can be used to predict novel miRNAs. The remaining unannotated small RNA reads were analyzed by an integrated combination of miREvo and mirdeep2 software (Friedlander

et al. 2012; Wen et al. 2012) to predict potential novel miRNAs by exploring the hairpin structure, the Dicer cleavage sites and the minimum free energy.

Differential expression analysis of miRNAs under the drought stress

The expression level of each miRNA was normalized by Transcripts Per Kilobase Million (RPM, (reads Count*1,000,000)/total miRNA reads count in each sample) (Zhou et al. 2010). Differential expression analysis of the two groups was performed using DESeq R package (1.8.3). The *p* values were adjusted using the Benjamini and Hochberg method. The corrected *p* value of 0.05 was set as the threshold for significantly differential expression by default.

Target prediction, gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

The target genes of the differentially expressed miRNAs were predicted using psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) and psRobot (Wu et al. 2012) for plants. GO enrichment analysis was used for the target gene candidates of the differentially expressed miRNAs ('target gene candidates' in the following). It was implemented using the Goseq R packages based Wallenius non-central hyper-geometric distribution (<http://bioinf.wehi.edu.au/software/goseq/>), which can adjust for gene length bias. KEGG (Kanehisa et al. 2008) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, organism and ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>). In this study, we used KOBAS software to test the statistical enrichment of the target gene candidates in the KEGG pathways (Xie et al. 2011).

Quantitative real-time PCR (qRT-PCR) validation of differentially expressed miRNAs

To confirm the deep sequencing results, qRT-PCR validation was performed to examine the levels of differentially expressed miRNAs. Five miRNAs (three conserved and two novel miRNAs) that were up-regulated under drought stress were selected for qRT-PCR validation. The RNA extraction process was the same as presented above. The first-strand cDNA is synthesized using RevertAid™ First-Strand cDNA Synthesis kit (Thermo Scientific, MA, USA) following the protocols with specific stem-loop primer (Supplementary Table S7). The reaction was 20-μl system containing 2.5 μl of 1:5 diluted cDNA, 10 μl of 2× SuperReal PreMix Plus

(Tiangen, Beijing, China), 0.4 μl ROX, 4.1 μl RNase-Free ddH₂O and 3 μl the forward and the reverse primers (5 μM). It was performed at 95 °C for 15 min; followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 40 s. RNase-free water replacing of cDNA template was used as negative control. U6 small nuclear RNA was used as the internal control for normalization (Zhang et al. 2016). The qRT-PCR assay was carried out in ABI 7500 Fast Real-Time PCR (Thermo Scientific, MA, USA) and analyzed with 7500 SDS 2.0 software.

Results

Overview of the miRNome data of *H. tibetana*

In this study, we chose two habitats of *H. tibetana*, with each habitat comprising three samples. The first set of samples containing HT-1, HT-2 and HT-3 grew in sandy land far away from the water source, whereas the second set of samples including HT-4, HT-5 and HT-6 were from the river valley near the water source. After filtering low-quality reads, a total of 26471337, 20373444, 29072173, 19999092, 20697959, and 21972110 high-quality reads were obtained from the six samples termed HT-1, HT-2, HT-3, HT-4, HT-5, and HT-6, respectively (Table 1). Furthermore, 26204665, 20054824, 28527784, 19459367, 20350479, and 21484416 clean reads remained after removing reads containing poly A, reads with 5' adapter contaminants, reads without the 3' adapter and insert tag, and reads shorter than 18 nt. These clean data accounted for 98.99%, 98.44%, 98.13%, 97.30%, 98.32%, and 97.78% of the total reads, respectively (Table 1), suggesting good sequencing quality. Length distributions of the miRNome for the six samples are illustrated in Supplementary Fig. S1. In all samples, the majority of small RNAs were 21 and 24 nt in size, and these values were consistent with findings of several other studies (Morin et al. 2008; Moxon et al. 2008). Moreover, the distributions of miRNA, rRNA, snRNA, snoRNA, and tRNA differed amongst the clean reads obtained (Supplementary Table S1). More than 98% unique and 55% redundant reads in the six samples were unannotated, which was consistent with the observations in strawberry (Ge et al. 2013), indicating that much work is still needed to annotate the small RNAs of *H. tibetana* in the future.

Differential expression of conserved miRNAs between the two habitats

To identify the conserved miRNAs from the miRNome data, the miRBase database (<http://www.mirbase.org/>) was utilized (Kozomara and Griffiths-Jones 2014). After mapping analysis, a total of 277, 237, 294, 222, 234, and

Table 1 Overview of the miRNome data in six *H. tibetana* samples

Type	HT-1		HT-2		HT-3		HT-4		HT-5		HT-6	
	Count	%	Count	%	Count	%	Count	%	Count	%	Count	%
Total reads	26,576,463		20,475,057		29,185,352		20,109,117		20,820,787		22,084,706	
High quality	26,471,337	100	20,373,444	100	29,072,173	100	19,999,092	100	20,697,959	100	21,972,110	100
3' adapter null	9,594	0.04	14,340	0.07	7742	0.03	9727	0.05	16,524	0.08	16,674	0.08
Insert null	3705	0.01	5277	0.03	7915	0.03	8725	0.04	6464	0.03	14,596	0.07
5' adapter contaminants	204,559	0.77	254,743	1.25	304,770	1.05	373,232	1.87	286,756	1.39	372,538	1.70
Smaller than 18 nt	39,085	0.15	38,388	0.19	216,188	0.74	144,102	0.72	32,286	0.16	78,167	0.36
Poly A	9729	0.04	5872	0.03	7774	0.03	3939	0.02	5450	0.03	5719	0.03
Clean reads	26,204,665	98.99	20,054,824	98.44	28,527,784	98.13	19,459,367	97.3	20,350,479	98.32	21,484,416	97.78

269 conserved miRNAs were identified from HT-1, HT-2, HT-3, HT-4, HT-5, and HT-6, respectively (Supplementary Table S2). Interestingly, miR166, miR167, miR168, miR156, miR172, miR408, and miR535 families were highly expressed in the six *H. tibetana* samples. These miRNA families were known and well-conserved in various plants (Barik et al. 2014). Amongst them, miR172 was found to be related to the regulation of flowering time and floral patterning in model plant *Arabidopsis* (Zhu and Helliwell 2011). Another study found that miR156 and miR172 can regulate developmental timing in *Arabidopsis* (Wu et al. 2009). Venn diagram analysis was carried out to investigate the similarities and/or differences in miRNAs amongst the different samples (Fig. 1). Figure 1a shows that 149 unique miRNAs were shared for the three *H. tibetana* samples from sandy land characterized by severe water shortage (i.e., HT-1, HT-2 and HT-3). Moreover, 135 unique miRNAs (Fig. 1b) were identified in the three *H. tibetana* samples from the river valley characterized by water sufficiency (i.e., HT-4, HT-5 and HT-6). Meanwhile, 116 miRNAs (Fig. 1c) were shared by HT-1, HT-2, HT-3, HT-4, HT-5 and HT-6, whereas only 33 and 19 miRNAs were specific to HT-1, HT-2 and HT-3 (HT123) and HT-4, HT-5 and HT-6 (HT456), respectively.

To reveal the drought-responsive miRNAs in *H. tibetana*, we focused on the shared 116 miRNAs to investigate whether there were differences in their expression levels in HT123 and HT456. The results are shown in Supplementary Table S3. It was found that only 43 conserved miRNAs were unchanged, whereas 73 miRNAs were different between HT123 and HT456 (reads of each miRNA were normalized by TPM and using a cutoff of > 1.2 fold-change). Amongst the 73 differentially expressed miRNAs, 34 miRNAs were up-regulated in HT456, whereas the remaining 39 miRNAs showed higher expression levels in HT123 than in HT456 (Table 2). These results indicated that habitats could affect the expression levels of miRNAs in *H. tibetana*, and some miRNAs may be involved in the drought stress response in *H. tibetana*.

Moreover, the target genes of the differentially expressed miRNAs were predicted using psRNATarget. A total of 1004 target genes were predicted for the regulated miRNAs. GO enrichment analysis was performed to further investigate the potential role of miRNAs in response to drought stress in *H. tibetana*. The results showed that most target genes were enriched in cellular process, metabolic process, cell part, organelle, response to stimulus, single-organism process, binding, and catalytic activity (Fig. 2 and Supplementary Table S8). Notably, the enrichment item 'response to stimulus' may be directly related to drought stress in *H. tibetana* because of long-term water deficiency for HT-1, HT-2 and HT-3. On the other hand, KEGG pathway analysis showed that up to 60 pathways ($p < 0.05$) were different between the two groups of *H. tibetana* samples (Supplementary

Fig. 1 Distribution of the identified miRNAs in different *H. tibetana* samples based on venn plot analysis. HT123 (i.e., HT-1, HT-2 and HT-3) refers to the three samples from the sandy land, while HT456 (i.e., HT-4, HT-5 and HT-6) from the river valley

Table S4), such as ‘ko03008: Ribosome biogenesis in eukaryotes’, ‘ko03018: RNA degradation’, ‘ko00030: Pentose phosphate pathway’, ‘ko00900: Terpenoid backbone biosynthesis’, and ‘ko00910: Nitrogen metabolism’.

Prediction of novel miRNAs and their differential expression in response to drought stress

We predicted the miRNA candidates from the miRNome data using the miRNA prediction tools (Friedlander et al. 2012; Wen et al. 2012) (Supplementary Table S5). Totally, we predicted 31, 36, 49, 39, 29, and 27 novel miRNAs in HT-1, HT-2, HT-3, HT-4, HT-5, and HT-6, respectively. Venn diagram analysis showed that six novel miRNAs were shared by HT-1, HT-2 and HT-3, whereas eight novel miRNAs were shared by HT-4, HT-5 and HT-6. By contrast, four novel miRNAs were commonly identified in the six samples (Supplementary Table S6 and Fig. S2). The predicted structures of these novel miRNAs are illustrated in Fig. 3. Interestingly, for these four shared miRNAs, two of them, novel_mir_24 and novel_mir_87, showed significant abundance differences between HT123 and HT456, which were 2.1 and 1.5-fold changes, respectively (Supplementary Table S6). These results indicated that their functions may be related to the drought stress response. In addition, novel_mir_80 and novel_mir_75 were unique for HT123. Although no information is available for these novel miRNAs, their potential correlation with the drought stress response is worthy of further investigation.

Validation of miRNA expression by qRT-PCR

To validate the reality of the potential drought-responsive miRNAs from the miRNome data, qRT-PCR was performed using the RNA samples of HT-1, HT-2, HT-3, HT-4, HT-5 and HT-6 (primers used are listed in Supplementary

Table S7). Five miRNAs (three conserved and two novel miRNAs) that were up-regulated under drought stress were selected for qRT-PCR. Compared with the samples from the river valley (HT-4, HT-5 and HT-6), the expression of the five miRNAs in the drought-stressed samples (HT-1, HT-2 and HT-3) had a similar tendency between small RNA sequencing and qRT-PCR analysis (Fig. 4). The results indicated that deep sequencing is a reliable and effective method for quantifying miRNAs expression abundance in *H. tibetana*.

Discussion

Hippophae tibetana is a typical drought-tolerant plant, which is widely grown in the water shortage locations of the Qinghai–Tibet Plateau. Studies on the drought resistance mechanism of *H. tibetana* are important for its artificial cultivation, maintenance of soil erosion and improvement in the ecological environment of the Qinghai–Tibet Plateau. However, to date, the mechanism of drought tolerance for *H. tibetana* has not been fully elucidated.

Previous studies have shown that miRNAs play an important role in adapting to environmental stress in plants (Zhang et al. 2009; Li et al. 2008). However, to date, no report has revealed the role of miRNAs in *H. tibetana* adaptation to arid environments. In this study, small RNA sequencing technology was applied to investigate the similarities and/or differences in miRNAs amongst different *H. tibetana* samples. It was found that the expression levels of about 64% shared miRNAs differed in the two habitat samples of *H. tibetana*. On the other hand, some miRNAs were also found to be unique in drought habitat. These findings indicated that growth environment could affect the miRNA profiles of *H. tibetana*, and some miRNAs may play an important role in response to drought stress in *H. tibetana*.

Table 2 The conserved miRNAs with different expression levels between HT-1, HT-2, HT-3 (HT123) and HT-4, HT-5, HT-6 (HT456)

miRNA name	Sequence	Normalized expression in HT123	Normalized expression in HT456	Fold change (HT123/HT456)
HT-miR397a	TCATTGAGTGCAGCGTTGATG	3452.75	499.87	6.91
HT-miR398b-3p	TATGTGTTCTCAGGTCGCCC	73.18	12.19	6.01
HT-miR398b	TGTGTTCTCAGGTCGCCCCTG	482.82	89.57	5.39
HT-miR408-5p	ACAGGGAAGATGCAGAGCATG	9765.15	2223.79	4.39
HT-miR3623-3p	TGGTCTGGATGAATTTGGCTA	27.34	7.04	3.88
HT-miR3515	AATGTAGAAAAATAAACGGAGTAT	163.51	46.28	3.53
HT-miR774b-5p	TGAGATCAGAGATATGGGGTT	6.08	1.97	3.09
HT-miR2619b-5p	ATGAAGTTTGATTGTTTGGCA	96.85	38.65	2.51
HT-miR482c-5p	GGAATGGGCGGATTGGGATG	3602.69	1609.86	2.24
HT-miR6157	TGGTAGAAGTAGTATTTGAAA	47.15	23.51	2.01
HT-miR415	TCAGAGCAGAAACAGAGACAC	10.22	5.25	1.95
HT-miR390a-3p	CGCTATCCATCCTGAGTTTCC	25.40	14.67	1.73
HT-miR477i	ACTCTCCCTTAAGGCTTCCGG	259.02	151.24	1.71
HT-miR5227	TGAAAGAAGAAGAGATGCTGAA	552.57	329.81	1.68
HT-miR853-5p	CTCTATCTTGTCCTGTTGGCA	7.20	4.58	1.57
HT-miR5077	TTCACGTCGGGTTACCA	174.86	113.07	1.55
HT-miR5161	TATCGGATCAGATGAGTATA	73.77	48.63	1.52
HT-miR3513-3p	TTGATTCGTAGAAATTGGTAT	7.49	4.99	1.50
HT-miR902l-3p	AGAAGAATCTGCAACTACTCC	3.20	2.19	1.46
HT-miR5072	CGTTCGCCAGCGGAGTCGCCA	8.11	5.58	1.45
HT-miR5669	CAATTGGAGATAGTGAAGTGGTC	488.36	336.43	1.45
HT-miR2111a-5p	TAATCTGCATCCTGAGGTTTA	19.10	13.22	1.44
HT-miR164a	TGGAGAAGCAGGGCACGTGCA	376.38	264.36	1.42
HT-miR482a-3p	TTCCCAATGCCGCCATTCCGA	470.08	330.37	1.42
HT-miR7782-3p	TCCTGCTCTGATACCATGTAGA	55.33	39.04	1.42
HT-miR4385	AATCGATGTAGAGAAGGATGT	67.15	47.99	1.40
HT-miR6466-5p	TCAGTGGTAGAGCATTTGACTGCA	27.30	19.54	1.40
HT-miR160a	TGCCTGGCTCCCTGTATGCCA	22.21	16.00	1.39
HT-miR6204	AGGAGAATAATATGATGATCTTGA	108.40	79.85	1.36
HT-miR828a	TCTTGCTCAAATGAGTATTTCCA	1.88	1.41	1.33
HT-miR1511-3p	ACCTGGCTCTGATACCATGAAGAA	40.64	30.60	1.33
HT-miR403	TTAGATTACGCACAAACTCG	202.83	152.81	1.33
HT-miR535-3p	GTGCTCTTCCTCGTTGTCAT	208.32	157.34	1.32
HT-miR5260	TTTGATTGTGAGAGATGGCTT	56.75	43.18	1.31
HT-miR165a-3p	TCGGACCAGGCTTTCCCC	72.18	55.27	1.31
HT-miR168a-3p	CCCGCCTTGCATCAACTGAAT	632.66	491.31	1.29
HT-miR8051-5p	TGTGAATAAGTGATTGTCTGA	81.52	66.77	1.22
HT-miR6269	AGTATGGTAGAAAGAAAA	4.08	3.33	1.22
HT-miR7783-3p	AGGCTCTGATACCATGTGAAGAGT	58.37	48.67	1.20
HT-miR894	GTTTCACGTCGGGTTACCA	1222.68	1485.23	0.82
HT-miR393b-5p	TCCAAAGGGATCGCATTGATCT	41.81	50.96	0.82
HT-miR7532a	GAACAGCCTCTGGTCGATGG	159.22	194.70	0.82
HT-miR162a	TCGATAAACCTCTGCATCCAG	153.26	188.10	0.81
HT-miR1310	AGGCATCGGGGGCGCAACGCC	46.65	58.30	0.80
HT-miR1027a	TTTTATCATCTCTTCCAATAA	1.22	1.54	0.79
HT-miR6478	CCGACCTTAGCTCAGTTGGC	125.53	158.91	0.79
HT-miR393-3p	ATCATGCGATCCCTTGGGAAT	20.80	26.44	0.79
HT-miR8125	CAGGAAAGAATGGTGGTG	17.82	22.79	0.78

Table 2 (continued)

miRNA name	Sequence	Normalized expression in HT123	Normalized expression in HT456	Fold change (HT123/HT456)
HT-miR171b-3p	CGAGCCGAATCAATATCACTC	763.61	996.60	0.77
HT-miR3447-3p	TTTGAGTAGTTTGATTAGAA	319.08	417.66	0.76
HT-miR845b-5p	TAAGATTGGTATCAGAGCATG	45.21	59.25	0.76
HT-miR912	TGGATTGCTTCCAGCCGGCA	130.35	170.94	0.76
HT-miR6196	AGGACGAGGAAGTTGAAGAGA	71.87	95.71	0.75
HT-miR159-3p	CCTTTGGATTGAAGGGAGCT	16.13	21.53	0.75
HT-miR5813	ACAGCAGGACGGTGGTCATGGA	119.30	161.26	0.74
HT-miR2916	TGGGGGCTCGAAGACGATCAG	198.01	268.05	0.74
HT-miR535a	TGACAACGAGAGAGAGCACGC	19,540.96	27,117.81	0.72
HT-miR5021	TGAGAAGGTAGAAGAAAGAA	18.12	25.23	0.72
HT-miR5721	AAAGAGTGGAGGAGAAATGGA	86.67	123.45	0.70
HT-miR156f-3p	TGACAGAAGAGAGTGAGCAC	31.78	45.37	0.70
HT-miR1432-5p	ATCAGGAGAGATGACACCGACA	2.22	3.21	0.69
HT-miR5226	TTGTACAAATTGGAGGGTTC	4.18	6.04	0.69
HT-miR5368	AGGGACAGTCTCAGGTAGA	38.17	55.70	0.69
HT-miR8154	AGGGGAAGAGAGACTGAAGAGGGA	2.06	3.12	0.66
HT-miR5807	AGGCGGTTGGACAGTATGTGGC	68.04	104.36	0.65
HT-miR157a	TTGACAGAAGATAGAGAGCAC	1474.99	2271.30	0.65
HT-miR156a	GCTCACTTCTCTTTCTGTCAGT	14,075.99	23,001.15	0.61
HT-miR8155	CGTAACCTGGCTCTGATACCA	11.07	19.41	0.57
HT-miR159c	TTTGGATTGAAGGGAGCTCC	555.18	1019.10	0.54
HT-miR167f-3p	CAGATCATGTGGCAGTTTCAT	1686.96	3130.44	0.54
HT-miR1873	TACATGGTATCAGAGCAGGAAA	31.67	60.80	0.52
HT-miR399b	TGCCAAAGGAGAGTTGCCCTG	30.21	66.08	0.46
HT-miR5298b	TGAATTGGAGAGTATGAAGATGAA	4.52	19.00	0.24

Fig. 2 Gene Ontology enrichment analysis to the target genes of differentially expressed miRNAs between HT123 (HT-1, HT-2 and HT-3) and HT456 (HT-4, HT-5 and HT-6). The horizontal axis listed

the processes including biological process, cellular component, and molecular function. The vertical axis listed the numbers of target genes in each process and the according percentage

Fig. 3 The structures of the predicted novel miRNAs in six *H. tibetana* samples

To determine potential drought-responsive miRNAs in *H. tibetana*, we investigated the differentially expressed miRNAs in HT123 and HT456 using high-throughput sequencing technology. Finally, we found some key miRNAs associated with drought stress response in *H. tibetana*, such as HT-miR1511-3p, HT-miR1432-5p, HT-miR403, HT-miR6269, HT-miR482c-5p, HT-miR159c, HT-miR393b-5p, HT-miR828a, and HT-miR167f-3p. Some of them were verified by qRT-PCR method. These findings are in good agreement with previous observations in other plants. For example, miR1511-3p and miR6269 were found to be up-regulated after drought treatment in tomato (Candar-Cakir et al. 2016). Cheah et al. (2015) compared the expression profiles of miRNAs in three drought-treated rice varieties by high-throughput sequencing, and miR1432-5p was found to be downregulated in drought-tolerant varieties. In addition, miR403 and miR828 were found to be associated with drought stress in cowpea for the first time (Barrera-Figueroa et al. 2011). Besides, miR159 and miR482 were found to be significantly induced under drought stress in

wheat (Akdogan et al. 2016). Apart from those drought-responsive miRNAs reported previously in other plants, several miRNAs (e.g., HT-miR397a, HT-miR3623-3p, HT-miR3515, HT-miR774b-5p, HT-miR2619b-5p, HT-miR6157, HT-miR390a-3p, and HT-miR415) were also found to be associated with drought stress for the first time in our study. In addition, we found that the expression levels of two novel miRNAs (novel_mir_24 and novel_mir_87) were significantly higher in HT123 than those in HT456, which indicated that their functions may be related to the drought stress response in *H. tibetana*. However, further studies are needed to verify these inferences by over-expressing or knocking-down these miRNAs in *H. tibetana*.

In this study, the miRNome profiling of *H. tibetana* was reported for the first time using high-throughput sequencing technology. More importantly, some conserved and novel drought-associated miRNAs were identified in *H. tibetana*. These results will help us understand the molecular mechanisms of *H. tibetana* adaptation to arid environments, and provide a valuable resource of miRNAs for potential use in

Fig. 4 Validation and comparison of the expression of five selected miRNAs in HT123 (drought stress group) and HT456 (control group) by small RNA sequencing (a) and qRT-PCR (b) analysis. The

expression level in HT456 was set as 1. For qRT-PCR analysis, U6 was used as the internal control. The values indicate means of three biological replicates $\pm$ standard deviation

improving drought tolerance in *H. tibetana* and other plateau plants.

Acknowledgements The authors gratefully acknowledge the financial support from National Natural Science Foundation of China (No. 81473428) and the National Key Research and Development Program of China (No. 2017YFC1703900).

Compliance with ethical standards

Conflict of interest The authors have declared there was no conflict of interest.

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