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Identification and analysis of microRNAs in *Botryococcus braunii* using high-throughput sequencing

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ABSTRACT: MicroRNAs (miRNAs) play important regulatory roles in the growth and development of organisms. The colonial green microalga *Botryococcus braunii* is an oil-rich algae and little is known about its miRNAs and their target genes. Here we constructed and sequenced a small RNA library for *B. braunii* using the HiSeq 2000 deep sequencing method. In total, we identified 42 known miRNA families and 14 novel miRNAs from *B. braunii* via sequence alignment and secondary structure prediction. Quantitative real-time PCR analysis indicated that the majority of miRNAs were effective and credible. Gene ontology analysis showed that most of the targets of miRNAs were classified as being involved in metabolic and cellular processes, gene expression regulation and stress/defense functions. Our findings provide the first large-scale identification and characterization of *B. braunii* miRNAs and their potential target genes. This study could lead to further identification of *B. braunii* miRNAs and enhance our understanding of their regulatory mechanisms in diverse biological and metabolic processes.

KEY WORDS: *Botryococcus braunii* · MicroRNAs · High-throughput sequencing · Target genes

INTRODUCTION

MicroRNAs (miRNAs) are small non-protein-coding RNAs with a length of approximately 22 nucleotides (nt), which have emerged as important regulators of genes at both transcriptional and post-transcriptional levels through translational repression, mRNA degradation and chromatin modification (Czech & Hannon 2011, Prakash et al. 2016). To date, numerous miRNAs have been identified from various plant species (Lu et al. 2008, Pantaleo et al. 2010, Jin et al. 2013, Ferdous et al. 2015, Pantaleo et al. 2016), and a few reports have been published recently to describe the structure and function of miRNA from algae, such as *Chlamydomonas reinhardtii* (Zhao et al. 2007, Valli et al. 2016), *Thalassiosira pseudonana* (Norden-Krichmar et al. 2011), *Phaeodactylum tricornutum* (Huang et al. 2011), *Porphyra yezoensis* (Liang et al. 2010) and *Porphyridium purpureum* (Gao et al. 2016).

Microalgae have the potential to generate significant quantities of biomass and oil suitable for conversion to biodiesel. The colonial green microalga *Botryococcus braunii* has a remarkable ability to produce high levels of liquid hydrocarbons (Banerjee et al. 2002, Metzger & Largeau 2005). In previous studies, *B. braunii* was classified into 3 races (A, B and L) based on hydrocarbon structures (Kawachi et al. 2012, Yoshimura et al. 2013), and the genome and transcriptome of *B. braunii* were sequenced and analyzed (Weiss et al. 2011, Ioki et al. 2012a,b). To date, no systematic studies have been conducted on small RNA in this microalga. Thus, the aim of our study was to isolate miRNAs from *B. braunii* and (1) to establish a small RNA library of *B. braunii*, (2) to obtain sequences and information of miRNAs in *B. braunii*, and (3) to identify novel miRNAs in *B. braunii* using bioinformatics methods. The results will help us to identify the miRNA-based regulatory system of this microalga.

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MATERIALS AND METHODS

Strains and culture conditions

Botryococcus braunii was obtained from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences, and propagated photoautotrophically in 1 l Erlenmeyer flasks containing 400 ml of modified Chu-13 medium (Largeau et al. 1980). The flasks were placed in a 25 ± 1°C illuminated incubator (Jiangnan Instrument Factory) for 14 d under a 14:10 h light:dark photoperiod and a light density of 50 ± 5 µE m^{-2} s^{-1}. The microalgal cells were then collected and cleaned with sterilized water. After being dried with hygroscopic filter paper, the samples were immediately frozen in liquid nitrogen and stored at −80°C before use.

Construction and sequencing of a small RNA library

Total RNA used for small RNA library construction was extracted from frozen *B. braunii* cells using Trizol (Invitrogen, Life Technologies) according to the manufacturer's instructions. Briefly, the frozen cells were re-suspended in 3 ml of Trizol reagent. Total RNA was then extracted and purified with the standard Trizol/RNeasy column procedure. Following purification, total RNAs were digested with DNase and quantified by spectrophotometry. Total RNAs were then separated by size fractionation on denaturing 15% polyacrylamide gels. Fragments of 18 to 28 nt were gel-purified and then ligated to a 5′-adaptor and a 3′-adaptor. After reverse transcription, a 12-cycle PCR reaction was performed, and the products were sequenced by the Beijing Genomics Institute (BGI) using the HiSeq 2000 deep sequencing method.

Small RNA analysis and miRNA identification

For analysis of small RNAs in *B. braunii*, unique small RNAs were compared and aligned with the sequences of non-coding RNAs (rRNA, tRNA, snRNA, snoRNA) available in Rfam (www.sanger.ac.uk/software/Rfam) (Griffiths-Jones et al. 2005) and in the GenBank noncoding RNA database (www.ncbi.nlm.nih.gov/) using blastn with an e-value of 0.01 as the cutoff. In addition, all sequences were searched for conserved miRNAs of *B. braunii* using the plant mature miRNAs from miRBase (release 19.0) allowing for 2 mismatches and 3 gaps (Griffiths-Jones et al. 2008).

Prediction of novel miRNAs

Fold-back structures in miRNA precursors can be used to predict novel miRNAs (Allen et al. 2005). In this study, the Mireap program, developed by the BGI, was used to predict the novel miRNAs in *B. braunii*. The adopted strategy was as follows: (1) candidate miRNA sites were screened out from breakpoints defined by mapping of the small RNAs, (2) a minimal stringent criterion was used to select miRNA candidates and (3) the RNA secondary structure was checked with the Mfold program (Zuker 2003).

Prediction of miRNA targets

The miRanda program (www.microrna.org/) was used to detect potential target sites for the *B. braunii* candidate miRNA sequences. The parameters employed were as follows: match score $S \geq 90$, target duplex free energy $\Delta G \leq -20$ kcal mol^{-1} and scaling parameter = 2. The miRNA-target duplexes were then checked manually according to rules suggested by John et al. (2004) and Huang et al. (2011).

Quantitative real-time PCR analysis

Ten miRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) analysis. TaqMan miRNA assays were designed and ordered from Applied Biosystems. Each TaqMan miRNA assay includes a specific RT primer, forward and reverse primers and the TaqMan probe. Specific primers were designed according to the method of Chen et al. (2005) and synthesized by Sangon Biotechnology (Shanghai, China). Primers used in this study are listed in Table 1. qRT-PCR was performed according to the manufacturer's protocol and run on an ABI 7300 machine (Applied Biosystems) with thermal cycling parameters at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min according to the manufacturer's protocol. qRT-PCR reactions were run in triplicate with 2 biological replicates. The U6 snRNA was selected as a reference gene for normalization. A relative quantitative method ($\Delta\Delta C_t$) was used to evaluate relative expression levels of different miRNAs in *B. braunii*.

Table 1. Primers used for quantitative real-time PCR of miRNAs from *Botryococcus braunii*

MicroRNA	Primer	Primer sequence (5′-3′)
miR1	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCCTCA
	Forward	ATGGTTCGTGGGAGGGGTGAGAGGGGGTGAGG
miR3	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCCTCT
	Forward	ATGGTTCGTGGGGAGGGGAAGAGGGGAAGAGG
miR4	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCTCTACC
	Forward	ATGGTTCGTGGGGAGGGGTAGAGGGGTAGAGG
miR6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAACTACCA
	Forward	ATGGTTCGTGGGGTAGTTGGTAGTTGGTAGTT
miR7	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCCCCCCT
	Forward	ATGGTTCGTGGGGGGAGAGGGGAGAGGGGGGA
miR8	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCTCTC
	Forward	ATGGTTCGTGGGAGGGGGAGAGGGGGAGAGG
miR9	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCTCTC
	Forward	ATGGTTCGTGGGAGGGGGAGAGGGGAGAGG
miR10	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCCCTC
	Forward	ATGGTTCGTGGGGGGAGAGGGGGGAGAGGG
miR12	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCCTCTC
	Forward	ATGGTTCGTGGGAGGGGGAGAGGGGGAGAGG
miR13	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCCCCATC
	Forward	ATGGTTCGTGGGGGAAGGAGGGGGAGATGG
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAATATGGAAC
	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
Universal reverse		GCAGGGTCCGAGGTATTC

RESULTS AND DISCUSSION

Sequencing and analysis of the small RNA library

A small RNA library was established using Solexa technology to identify candidate miRNAs in *Botryococcus braunii*. In total, we obtained 10 020 143 sequence reads from the library. After filtering out low-quality tags, trimming adaptors and cleaning up shortages and contamination formed by adaptor–adaptor ligation, we obtained 9 786 842 (98.08 %) clean reads, representing 2 005 344 unique sequences. Among the clean reads, 131 905 (1.35 %) were similar to known miRNAs, and 6 602 059 (67.46 %) were unannotated small RNA, suggesting that small RNA in *B. braunii* has not been investigated extensively in previous studies. Thus, our study has potential to discover more miRNA genes. The rest of the sequences were other types of RNA, including tRNA, rRNA, snRNA, snoRNA and other non-coding RNAs. The numbers and proportions of different categories of small RNAs are shown in Table 2.

As an important feature, size profile has often been used to distinguish miRNA from other small RNAs in previous studies. Most mature miRNAs with known functions commonly have a length of 20–24 nt (Wei et al. 2009). In this study, the length distribution pattern of the reads was analyzed and is presented in Fig. 1, showing that the majority of small RNAs in *B. braunii* library were 21 and 20 nt in size, accounting for 11.40 and 11.39 % of the total reads, respectively (Fig. 1), followed by 19 nt (10.59 %), 22 nt (10.17 %), 23 nt (9.57 %), and 24 nt (8.87 %). This distribution pattern

Table 2. Distribution of small RNAs among different categories in *Botryococcus braunii*

Category	Unique RNAs		Total RNAs	
	Number	Percent	Number	Percent
Total	2005344	100.00	9786842	100.00
exon_antisense	121	0.01	440	0.00
exon_sense	364	0.02	947	0.01
intron_antisense	1217	0.06	9609	0.1
intron_sense	1203	0.06	12689	0.13
miRNA	14887	0.74	131905	1.35
rRNA	110727	5.52	1444330	14.76
repeat	53	0.00	89	0.00
snRNA	1381	0.07	27088	0.28
snoRNA	175	0.01	546	0.01
tRNA	40923	2.04	1557140	15.91
Non-annotated sRNAs	1834293	91.47	6602059	67.46

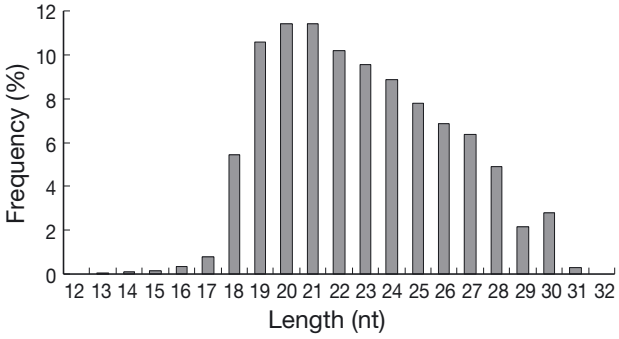

Fig. 1. Lengths of unique small RNA sequences in *Botryococcus braunii*. The occurrences of each unique sequence read were counted to reflect the relative expression level, and only small RNA sequences in the range of 12 to 32 nt were considered

is highly consistent with the typical size of miRNA from Dicer digestion products and previous other algal small RNA sequencing using Solexa technology, such as *Chlamydomonas reinhardtii* (Zhao et al. 2007, Valli et al. 2016), *Porphyra yezoensis* (Liang et al. 2010), *Phaeodactylum tricornutum* (Huang et al. 2011) and *Porphyridium purpureum* (Gao et al. 2016).

Identification of known miRNAs in *B. braunii*

To identify the known miRNAs in *B. braunii*, the small RNA sequences were compared with the known plant miRNAs in miRBase 19.0 (www.mirbase.org/). Among the 9 786 842 sequences, 14 887 unique sequences were orthologs of known miRNAs from other plant species previously deposited in miRBase (version 19.0). Allowing 1 or 2 mismatches between sequences, these miRNAs represented 42 known miRNA families (Table 3). Solexa sequencing technology has the ability to generate millions of small RNA sequences, which could provide a resource with information on the abundance of various miRNA families and even distinguish between different members of a given family (Ruan et al. 2009). Thus, as a powerful tool, Solexa sequencing technology is often used to estimate the expression profiles of miRNA genes. In this study, the frequencies of known miRNA families in the sequenced library varied from 6 (miR164a) to 41 735 (miR408a-5p), indicating that expression varies significantly among different miRNA families. Among them, 13 miRNA families had more than 1000 sequence counts, suggesting these miRNAs were highly expressed in *B. braunii*. miR408a-5p, miR3948, and miR6426a were the most frequently expressed miRNA, with 41 735, 34 938, and 30 629 copies, respectively. Moreover, 15 miRNA families had 100 to 1000 sequence counts, and others had fewer than 100 sequence counts.

Table 3. Putative miRNA families represented in the *Botryococcus braunii* miRNA pool

miRNA family	Count	Sequence
miR157a	798	TTGACAGAAGATAGAGAGCAC
miR6180	1886	AGGGGCTAGAAAGAGGGCAG
miR6426a	30629	GTGGACGAAACGGAAGTGAGA
miR167a	313	TGAAGCTGCCAGCATGATCTA
miR408a-5p	41735	CAGGGGAGCAGGGGAGCAG
miR166a	157	TCGGACCAGGCTTCATTCCCC
miR158a	172	TCCCAAATGTAGACAAAGCA
miR5519	2399	TGGCAGACGCTACGGACTTAA
miR5742	492	CCACATAAGGGTCGTTGTA
miR5076	609	GAAATGGTGTAGCGGAGCAGGATT
miR5077	162	GAATCGCGTCGGGGTCACCA
miR3948	34938	GGGAGTAGGGAGTAGGGAGTAGGGAG
miR535a	48	TGACAACGAGAGAGAGCACGC
miR6164a	442	TCCAAAAGTGTAACGGAGG
miR1115-5p	710	TGAGGTAAGAGCATTTGGTGG
miR844-5p	742	AGTAGGACTAGGAGATGCT
miR394b-3p	5953	AGGTGGGGATGACGTCAAGT
miR168a	50	TCGCTTGGTGCAGGTCGGGAC
miR397a	39	TCATTGAGTGCAGCGTTGATGT
miR169h	831	GGCGACATACTGGCTCATT
miR6478	56	GCCGCCTTAGCTCAGATGGTT
miR161-5p.1	15	TTGAAAGTGACTACATCGGGG
miR165a	18	TCGGACCAGGCTTCATCCCCC
miR854a	1058	GTTGAGGATGGGGGGAGGG
miR5026	11	ACTCATAAGATCGTGACACGT
miR6173	1374	TAGCCGTAAACGATGGATAC
miR390a	17	AAGCTCAGGAGGGATAGCGCC
miR5501	6234	ACTTGTGGCTAGGGGTGAA
miR5568c-3p	101	ACTACGAGTGTGGAACGGAGG
miR894	23	GTTTCACGTCGGGTTCACCA
miR5656	73	ACTTGAAGTAGACGATTTGGATT
miR173-5p	9	TTCGCTTGCAGAGAGAAATCAC
miR841b-5p	4843	ACAGGCAGTGGAAACTGAA
miR5503	1408	TTCGGATTGTCAGAGGCACT
miR164a	6	TGGAGAAGCAGGGCACGTGCA
miR4414a-5p	427	AGCTGCTGACTGTATGGTTGT
miR916	1088	CAAGAGGTCGTCGGTTCGAATCC
miR2876-5p	366	AATGGTGGCTGCGACTGTTTA
miR5673	5805	GCGGAACTGGCGGAAGACAT
miR482-5p	51	AGGGAGTGAAGGAGCGCCT
miR6300	13	GGTGGTTGTAGTATAGCGGT
miR3633a-5p	846	GGAATGGATGGTGTAGCG

Table 4. Predicted novel miRNAs in *Botryococcus braunii*

Name	Count	Sequence	Length (nt)	Precursor length (nt)	Folding energy (kcal mol^{-1})
miR1	19	AGGGGTGAGAGGGGGTGAGGGGG	23	188	−90.6
miR2	19	AGGGGTGAGAGGGGGTGAGGGGG	23	284	−148.2
miR3	10	GAGGGGAAGAGGGGAAGAGGGGG	23	241	−122.6
miR4	14	GAGGGGTAGAGGGGTAGAGG	20	340	−139.8
miR5	9	GGGAGAGGGGGGAGAGGGGGG	21	278	−144.5
miR6	11	GTAGTTGGTAGTTGGTAGTT	20	148	−53.7
miR7	8	GGGAGAGGGGAGAGGGGGGA	20	207	−72.0
miR8	124	AGGGGGAGAGGGGGAGAGGGG	21	189	−98.2
miR9	10	AGGGGGAGAGGGGAGAGGGG	20	263	−108.6
miR10	18	GGGAGAGGGGGGAGAGGGGGG	21	224	−98.0
miR11	8	GAGGAGGTGAGGAGGTGAGG	20	311	−125.0
miR12	124	AGGGGGAGAGGGGGAGAGGGG	21	151	−77.2
miR13	1106	GGAAGGAGGGGGAGATGGGGA	21	88	−23.4
miR14	6	TACCAGTTACCAGTTACCAG	20	342	−113.9

Predicted novel miRNAs in *B. braunii*

One of the important features that distinguishes miRNA from other small RNAs is the ability of miRNA flanking sequences to fold back into a hairpin structure (Ambros et al. 2003). Our search revealed that a total of 14 small RNAs met our criteria according to Allen et al. (2005) and were considered predicted novel miRNAs (Table 4). The lengths of these novel candidates

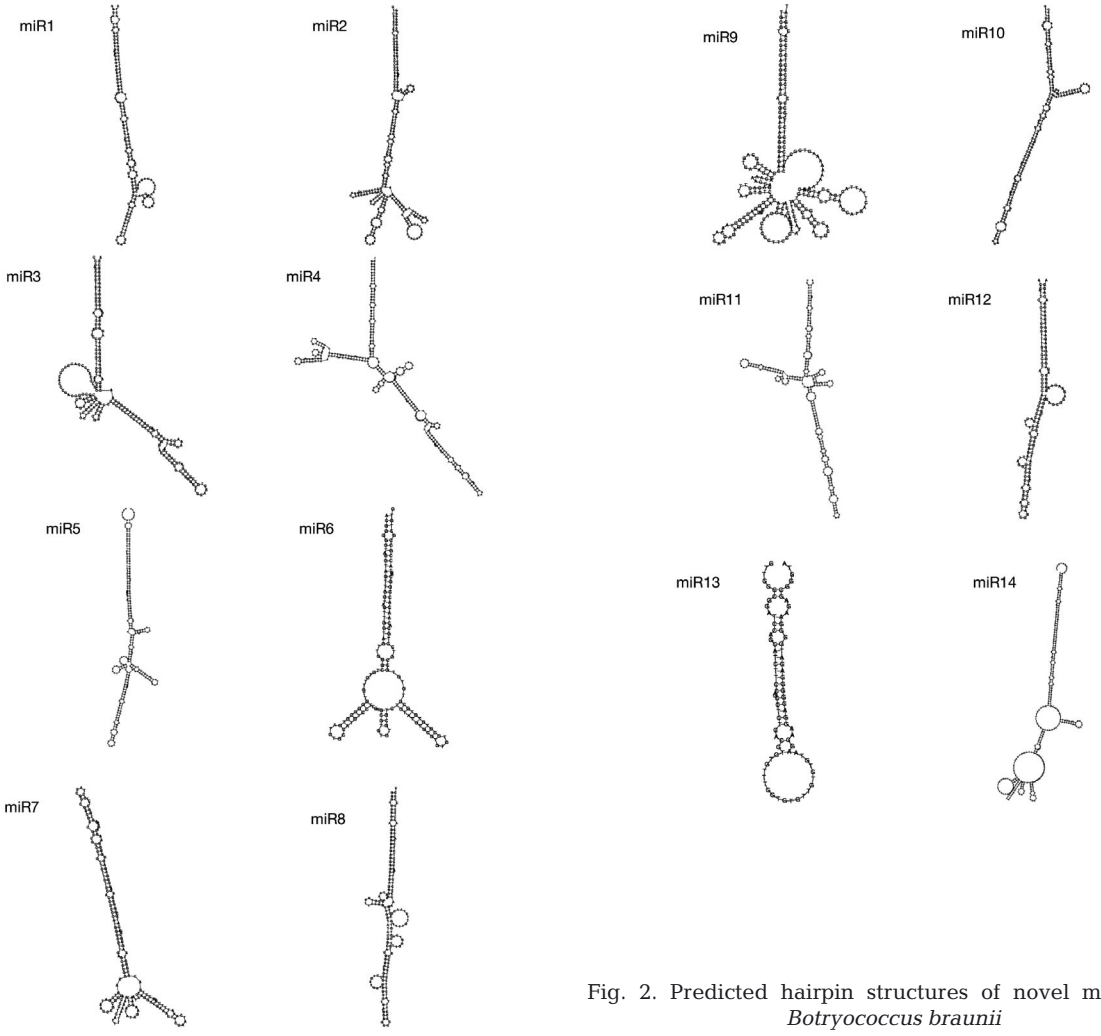

Fig. 2. Predicted hairpin structures of novel miRNAs in *Botryococcus braunii*

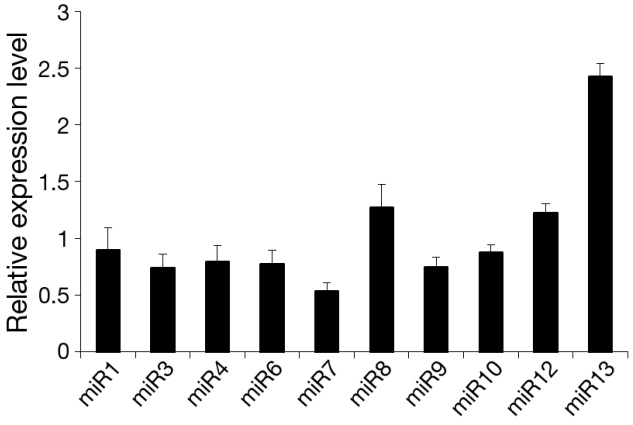

Fig. 3. Quantitative real-time PCR (qRT-PCR) validation and expression analysis of miRNAs in *Botryococcus braunii*. Data are mean ± SD. qRT-PCR reactions were run in triplicate with 2 biological replicates

Validation of novel miRNAs in *B. braunii*

To validate the predicted novel miRNAs, 10 miRNAs were randomly selected for qRT-PCR assays which were performed to examine whether the miRNAs were expressed in *B. braunii*. Fig. 3 shows that all 10 miRNAs were expressed in the samples of *B. braunii*, which was attributed to the conservation of miRNA families. The expression level of miR13 was relatively higher than that of the other 9 miRNAs, which was in agreement with the Solexa sequencing data (Table 4). These findings were also in line with those reported by Gao et al. (2016), who found that miRNAs are relatively conserved in different species. Moreover, these results suggest that a majority of the miRNAs identified in *B. braunii* are effective and credible.

ranged from 20 to 23 nt, and precursors of these novel miRNAs had negative folding free energies ranging from −148.2 to −23.4 kcal mol^{-1}, with an average of about −101.1 kcal mol^{-1} according to Mfold, which was much lower than terrestrial plant miRNA precursors (−71.0 kcal mol^{-1} in rice, −59.5 kcal mol^{-1} in *Arabidopsis* and −72.4 kcal mol^{-1} in wheat; Yao et al. 2007). The predicted hairpin structures (Fig. 2) for the precursors of these novel miRNAs required 88 to 342 nt, and a majority of the identified miRNA precursors (71.4%) required 148 to 284 nt, which was more than what has been observed in other plants (Zhang et al. 2006). Moreover, the predicted novel miRNAs exhibited much lower expression levels. This finding was in line with the notion that the expression level of non-conserved miRNAs is lower than that of conserved miRNAs. Each novel miRNA family had only 1 member, and only the miR13 family had more than 1000 sequence reads. The majority of novel miRNA families had fewer than 20 reads. The low abundance of novel miRNAs might suggest a specific role for these miRNAs under various growth conditions, under biotic or abiotic stress, or during developmental stages (Zhao et al. 2010, Song et al. 2015).

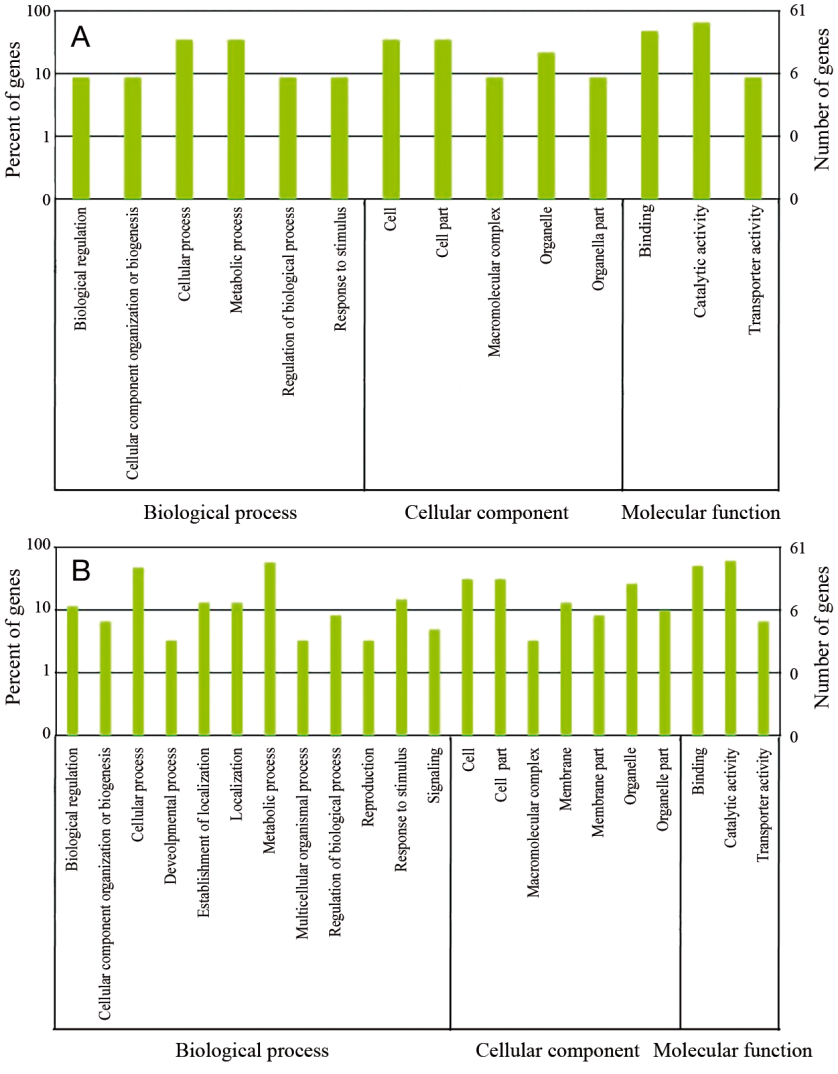

Fig. 4. Gene categories and distribution of (A) known miRNA targets and (B) novel miRNA targets in *Botryococcus braunii*

Prediction of miRNA targets in *B. braunii*

In plants, miRNAs are involved in regulating a variety of biological processes, such as development, signal transduction, protein degradation, response to environmental stress and pathogen invasion (Lu et al. 2008). Their target sites have been shown to be primarily located in the coding regions. As expected, most miRNA target sites in *B. braunii* were located in the coding regions, and the putative target genes appear to be involved in a wide variety of biological processes (Fig. 4). Of these predicted targets, most were classified as being involved in metabolic and cellular processes, gene expression regulation, and stress/defense functions. Many metabolism networks have also been found, including lipid metabolism, amino acid metabolism, carbohydrate metabolism, energy metabolism, and nitrogen metabolism (Chi et al. 2011).

CONCLUSIONS

This is the first report of genome-wide identification of miRNAs in *Botryococcus braunii* using a high-throughput Solexa sequencing strategy. We have identified 42 known miRNA families and 14 novel miRNAs from *B. braunii*, suggesting that a significant number of novel microRNAs remain to be discovered and characterized. Additionally, we also predicted putative targets for these miRNAs. These results may help to improve our understanding of regulatory mechanisms of miRNAs in biological and metabolic processes of *B. braunii*.

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