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ORIGINAL PAPER

Identification of microRNA-size sRNAs Related to Salt Tolerance in *Spirulina platensis*

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Abstract microRNAs (miRNAs) are endogenous non-coding small RNAs (sRNA) that play important regulatory functions in growth, development, and environmental stress response of eukaryotes. Currently, miRNA-size sRNA (msRNAs) was discovered in several prokaryotes through deep sequencing. However, no data are available on whether msRNAs exist in cyanobacteria and whether they regulate biological tolerance to salt stress. In this study, three small RNA libraries were constructed from control (0.02 M NaCl), medium- (0.3 M NaCl), and high-salt treatments (0.5 M NaCl) of *Spirulina platensis*. After sequencing using a high-throughput Illumina Solexa system, nine msRNAs with msRNA* were identified, and 21 candidate msRNAs showed significantly differential expression under salt-stress conditions. Seven of the selected msRNAs presented the consistent expression trends when compared with their deep sequencing results as verified by quantitative real-time polymerase chain reaction (qRT-PCR), demonstrating that the expression analyses for msRNAs according to small RNA sequencing data were reliable. Through computational identification, 33 target genes were predicted for 12 msRNAs in *S. platensis*; Gene Ontology (GO) and KEGG enrichment analyses revealed that the putative target genes were grouped into the categories of proteolysis, protein homotrimerization, glycosylation, ubiquinone biosynthetic process, DNA restriction-modification system, and polysaccharide catabolic process. Using proteomic analysis, two target proteins (glyceraldehyde-3-phosphate dehydrogenase and forkhead-associated (FHA) domain-containing protein) were differentially expressed under salt-stress conditions, which might be regulated by msRNAs. Our study demonstrates that msRNAs exist in *S. platensis*, and these msRNAs may have an important role in salt-stress responses; however, their functional significance requires further investigation.

Keywords *Spirulina platensis* · msRNA · sRNA · Salt stress · Deep sequencing

Introduction

microRNAs (miRNAs) are small (18~25 nt) non-coding RNAs, which negatively regulate their target gene expression through mRNAs cleavage or repression of translation in plants and animals (Valencia-Sanchez et al. 2006). The miRNAs are involved in all major cellular events via inhibitory mechanisms such as growth, development, environmental stress response, and signal transduction, as well as response to pathogen invasion (Zhang et al. 2006; Inui et al. 2010; Feng et al. 2013). Non-coding small RNAs (sRNAs) have long been recognized in bacteria, varying in size between 50 and 250 nt in length (Livny and Waldor 2007). Like miRNAs, sRNAs usually act as a post-transcriptional regulator by interacting with the target transcripts in a variety of ways to modulate stabilities of target mRNAs and conformation of RNAs (Kang et al. 2013). Bacterial sRNAs are often bound to the RNA chaperone protein Hfq to stabilize their folding (Gottesman 2004); they target not only the 5′ ends but also the 3′ ends, the internal part of RNAs, combinations within

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the transcripts, and even proteins (Lee and Hong 2012). Recently, bacterial sRNAs of comparable size to eukaryotic miRNAs have received considerable attention. Increasing evidence indicates the existence of a class of miRNA-like RNAs in cells other than plants and animals. Certain fungi were shown to contain miRNA-size, endogenous small interfering RNAs (siRNAs) (Zhou et al. 2012). Zhao et al. (2007) discovered that the unicellular green alga *Chlamydomonas* contains a variety of sRNAs including miRNAs and phased siRNAs, as well as other siRNAs originating from intergenic regions, sense and antisense strands of protein-coding genes, and repeat regions. Lee and Hong (2012) suggested the term "msRNA" (miRNA-size sRNA) and identified a number of msRNAs in *Streptococcus mutans*. At the same time, msRNAs were discovered in the model bacterium (*Escherichia coli*) by deep sequencing with the inference that msRNAs were evolutionarily well conserved from bacteria to animals (Kang et al. 2013).

In cyanobacteria, Voss et al. (2007) detected Yfr1, an abundant sRNA between 54 and 69 nt in size in all lineages of cyanobacteria. Researchers also observed that Yfr1 was required for growth under various stress conditions, such as oxidative stress and high-salt stress (Nakamura et al. 2007). Otherwise, orthologues of the Hfq protein were annotated in cyanobacterial genomes, which are structurally conserved but exhibit divergent RNA binding sites (Schuergers et al. 2014). The fact that cyanobacteria contain sRNAs and Hfq raises the question of whether msRNAs also exist in cyanobacteria. In order to probe the diversity of sRNAs pathways in cyanobacteria, we turned to *Spirulina platensis*, a representative species of cyanobacteria with well-understood haploid genetics that occupies a unique niche in the evolution of cyanobacteria. Previous studies found that this organism possesses some unusual and valuable physiological characteristics, such as alkali and salt tolerance. Importantly, it can grow at salt concentrations ~1.5-fold higher than sea water (Pinero Estrada et al. 2001; Chamorro et al. 2002), allowing it to often dominate in aquatic environments with high alkalinity and high salt. Srisuk et al. (2010) identified 334 predicted non-coding RNA (ncRNA) loci in *S. platensis* by computational method, among which 129 ncRNA loci were matched with known ncRNAs, including the mir-598 sequences. Additionally, through calculating the interaction scores between each ncRNA and all protein-coding sequences by IntaRNA, a list of predicted targets was acquired for these ncRNAs in *S. platensis*. However, it remains unknown whether *S. platensis* encodes endogenous msRNAs and whether such msRNAs have a regulatory role in gene expression. Identification of comprehensive sets of msRNAs in *S. platensis* is a critical first step toward facilitating our understanding of regulatory mechanisms or networks. Based on Solexa/Illumina small RNA deep sequencing technology and computational prediction, we aim to extend *S. platensis* msRNA studies to reveal the response of *S. platensis* to salt stress, to predict the target genes of identified msRNAs, and to analyze the functions of the target genes. After analyzing the proteome profile of *S. platensis* using 2D gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), the differentially expressed proteins that maybe regulated by msRNAs were verified. For the first time, our findings reveal the existence of several msRNAs in a filamentous prokaryotic organism and also provide a rich resource for studying the roles of sRNAs in prokaryotic cell biology and the evolutionary transition from prokaryotes to eukaryotic plants. This study enhances our understanding of the environment-adapting ability of cyanobacteria and provides basic clues to further clarify the response mechanisms to environmental stress.

Materials and Methods

S. platensis Strain and Culture Conditions

S. platensis-FACHB350, sourced from the Chinese freshwater algal library (http://algae.ihb.ac.cn/), was cultured in 0.02 M NaCl Zarrouk medium at 30 °C, light intensity of 8 klx and 75 % humidity (Richmond 1986). Within the logarithmic growth period, *S. platensis*-FACHB350 cells were transferred into fresh Zarrouk medium and cultivated for 7 days. The fresh Zarrouk medium was fortified with 0.02, 0.3, and 0.5 M NaCl, which represented control, medium-salt (MS), and high-salt (HS) treatments, respectively. The harvested cells were rinsed three times with phosphate-based buffer and then centrifuged at 8500 g for 5 min with ultrapure water to completely remove NaCl. The cells for total RNA and protein extraction were stored at −80 °C prior to analysis.

RNA and Protein Extraction

The filaments were collected, and the total RNA was extracted from *S. platensis*-FACHB350 cultured in control (0.02 M), MS, and HS treatments using RNeasy Plant Mini Kit (Qiagen, Germany) and treated with DNase I using RNase-Free Dnase Set (Qiagen, Germany). The RNA quality was measured by the Nanodrop method and should meet minimum requirements of $OD_{260}/OD_{280}>1.8$ and $OD_{260}/OD_{230}>1.5$. Proteins from control and HS treatment cells were extracted according to our previously report protocols (Wang et al. 2013). The protein concentration of samples was determined using a DC-kit protein assay (Bio-Rad, USA) (Hongsthong et al. 2007). Finally, aliquots of these RNA and protein samples were immediately frozen in liquid nitrogen and stored at −70 °C for later use.

Small RNA Library Construction and Sequencing

The total RNA from control, MS, and HS treatment samples were used to prepare the small RNA sequencing libraries using an Illumina Small RNA Sample Prep Kit (Illumina, San Diego, USA). The 3′-adaptor and 5′-adaptor were ligated to the aforementioned RNA with T4 RNA ligase (Promega, Madison, WI). The ligated RNA was reversely transcribed to single-stranded complementary DNAs (cDNAs) using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) using the RT primers recommended by Illumina. The cDNAs were amplified with Pfx DNA polymerase (Invitrogen) in 15 cycles of PCR using Illumina's sRNA primers set. PCR products were run on 6 % TBE polyacrylamide gel, and a slice of gel containing fragments <147 bp in length was excised. This fraction was eluted, precipitated, and quantified by Nanodrop method (Thermo Scientific, Rockford, IL). The sample concentration was adjusted to 10 nM in the sequencing reaction. After library preparation, small RNA sequencing was undertaken using the Illumina Genome Analysis II (Illumina, San Diego, USA).

Identification of new msRNAs in *S. platensis*

Mature miRNAs are a class of molecular small non-coding RNA about 22 nt in length. The genes encoding miRNAs are much longer than the processed mature miRNA molecule. In plant, miRNAs are transcribed by RNA polymerase II as large RNA precursors (pri-miRNAs), then the RNase III enzyme DICER-LIKE 1 (DCL1) cleaves the flanks of pri-miRNA to the precursor miRNA (pre-miRNA), which folded into imperfect stem-loop structures. The miRNA* was originated from the same hairpin structure (pri- and pre-miRNA) of the miRNA, and it was complementary to the "main" miRNA.

Raw reads were processed using Illumina's Genome Analyzer Pipeline software (San Diego, CA, USA) and then were subjected to a series of data filtration steps to analyze the sequencing data using the ACGT101-miR V4.2 software package (LC Sciences, Houston, TX, USA). Analyses included clipping adapters, trimming low-quality reads, and removing reads either shorter than 17 nt or longer than 25 nt. The remaining unique reads were then aligned with the reference database: *Arthrospira platensis* NIES-39 genome (http://www.ncbi.nlm.nih.gov/nuccore/AP011615.1). Sequences with a perfect match to the genome were retained for further analysis. Sequences matching non-coding RNAs including rRNA, tRNA, and other sRNAs in the Rfam (http://www.sanger.ac.uk/Software/Rfam) were removed. *Spirulina* is a kind of lower plants, and it can carry on the photosynthesis. To explore whether msRNAs were evolutionarily conserved from S*pirulina* to plants, the remaining unique sequences were aligned with known miRNAs from *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Arabidopsis*, *Brassica napus*, *Manihot esculenta*, *Cucumis melo*, *Glycine max*, and other plant species downloaded from miRBase 21.0 (http://www.mirbase.org) with a maximum of two allowed mismatches. To predict the secondary structure of pre-msRNA, RNA folding algorithm software UNAfold (http://www.ibridgenetwork.org/rpi/unafold) was used for generating RNA hairpin structure from their flanking sequences and calculating the minimum free energy (Maiti et al. 2010).

To identify msRNAs, all sRNA reads with non-annotation information in the genome were first subjected to a secondary structural study. As shown in Fig. S1, only sequences that met the following criteria were considered as potential msRNAs in *S. platensis* : (1) number of nucleotides in one bulge in stem ≤12; (2) number of base pairs in the stem region of the predicted hairpin ≥16; (3) free energy cutoff ≤ −15 kcal/mol; (4) length of hairpin (up and down stems+terminal loop) ≥50 nt; (5) length of hairpin loop ≤350 nt; (6) number of nucleotides in one bulge in mature region ≤4; (7) number of biased errors in one bulge in mature region ≤2; (8) number of biased bulges in mature region ≤2; (9) number of errors in mature region ≤4; (10) number of base pairs in the mature region of the predicted hairpin ≥12; and (11) percentage of mature in stem ≥80 % (Xu et al. 2014). The pairing bases in mature miRNA sequences are ≥12, and the occupied area is ≥80 % in stem, i.e., allowing ≤20 % of the bases in the ring.

Differential Expression Analysis of msRNAs Under Salt Stress

The frequency of msRNAs from three libraries was normalized to one million using the total clean reads of msRNAs in each sample (normalized expression=actual msRNA count / total count of clean reads×1,000,000). If the normalized read count of a given msRNA was zero, the expression value was set to 0.0010 for further analysis. Differentially expressed genes were identified using Fisher's exact test and Chi square (2 × 2) test. The fold change among the control, MS, and HS treatment libraries was calculated as fold change (MS/C)= $\log_2$(MS/C) and fold change (HS/C)=$\log_2$(HS/C). The fold changes >1 or < −1 and with $p \leq 0.01$ were considered to be differentially expressed in response to salt stress.

Analysis of msRNAs Expression Using qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) for mature msRNAs was performed to validate the sequencing results. We used All-in-One™ miRNA qRT detection system (Genecopoeia) followed by SYBR Green PCR (Bio-Rad) analysis to validate and measure the expression of the selected differentially expressed msRNAs under salt stress. As shown in Table S1, the specific primers for seven selected msRNAs and 16S rRNA were designed and synthesized by Sunny Biotech (Shanghai). The expression of each msRNA gene

was determined from three biological replicates, and each biological replicate included three technical replicates in qRT-PCR experiments. Pearson correlation coefficient (PCC) was used to quantify the degree of relevance between quantitative PCR (qPCR) and high-throughput sequencing results. To authenticate the amplicon of the msRNAs, all the real-time PCR products were cloned into PMD18-T simple vector and the sequence was determined by Sunny Biotech (Shanghai).

Prediction of Potential msRNAs Target Genes in *S. platensis*

Target finder was used to predict potential target mRNAs in a sequence database using a plant-based scoring metric (http://carringtonlab.org/resources/targetfinder). Due to the limited number of accessible *S. platensis*-FACHB350 mRNAs, all mRNA sequences of *A. platensis* NIES-39 in GeneBank (http://www.ncbi.nlm.nih.gov/genbank) were chosen as a target gene database. A penalty score criterion was introduced according to the alignment between the msRNAs and its potential target as previously described (Fahlgren et al. 2007). Penalties were assessed for mismatches, bulges, and gaps (+1 per position) and G:U wobble pairs (+0.5 per position). Penalties were doubled if the mismatch, bulge, gap, or G:U pair occurred at positions 2 to 13 relative to the end of the msRNA. Only one single-nt bulge or single-nt gap was allowed. Based on a reference set of predicted msRNA targets, only predicted targets with scores of less than 4 were acceptable matches. Enrichment analysis of the predicted target genes was conducted with Gene Ontology (GO) (http://www.geneontology.org/) and KEGG pathway (http://www.genome.jp/kegg/) at Bonferroni-corrected p values ≤ 0.05.

2-DE Verification of msRNA Targets

For 1D gel electrophoresis, three technical replicates were established for control and HS treatment. In a preliminary experiment, the protein isoelectric points (p*I*) for most *Spirulina* species were found to be pH 4–7 by means of dry strips in the pH range of 3–10. Therefore, maximum separation was obtained using the pH 4–7 dry strips. First-dimensional isoelectric focusing (IEF) based on the p*I* value of each protein was performed as described by Blum et al. (1987). After completing IEF, the proteins on the gels were stained with a silver-based color development system using Gelcode (The Upjohn Co. Kalamazoo, Michigan). Protein spots were scanned using a UNAX Powerlook 2100XL (Bio-Rad, Hercules, CA) and analyzed by ImageMaster 2D platinum 5.0 (Amersham Bioscience, Piscataway, NY). In image analysis, the TwoClassDif (RVM-T test) method with a cutoff p value ≤ 0.05 and FDR ≤ 0.05 was applied to analyze the differentially expressed proteins in control and HS treatments. Only those with reproducible and significant changes were considered to be differentially expressed protein spots. The significant differentially expressed protein spots were judged using the criterion >3-fold upregulation or downregulation.

Differentially Expressed Proteins by MALDI-TOF/MS Analysis

The differentially expressed protein spots were manually selected and excised. The peptide samples were analyzed using a MALDI-TOF/MS proteomics analyzer (Bruker Daltonics, Bremen, Germany) following our group's previous protocols (Wang et al. 2013). Data were searched on the Internet using a Mascot search engine (Matrix Science Ltd., London, UK) against all entries in the NCBI nr database, which contains accessible public protein sequences (http://www.ncbi.nlm.nih.gov/nuccore/NZ_ACSK 01001820.1/ *A. platensis* str. Paraca NZ_ACSK0100182 and http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABYK00000000.1/ *Arthrospira maxima* CS-328). Only significant hits, as defined by a MASCOT probability analysis ($p \leq 0.05$), were accepted. If the theoretical values for MW and p*I*, which were identified by peptide mass fingerprinting (PMF) and MALDI-TOF/MS analysis, were consistent with the actual values analyzed by ImageMaster 2D platinum 5.0 in gel, we regarded this protein spot as a positive match. The matched peptides for this protein were blasted to the protein database nr (ftp://ftp.ncbi.nih.gov/blast/db/) and Swiss-Prot (ftp://ftp.uniprot.org/pub/databases/uniprot datafiles_by_format/fasta/) in order to obtain the protein functional annotation information with the highest sequence similarity. Subsequently, we acquired information on the ortholog of gene products classification of gene functions in cells by COG (ftp://ftp.ncbi.nih.gov/pub/COG/) (E-value < 0.00001) functional classification. These differentially expressed proteins were grouped by their respective metabolic pathways using COG software online. Finally, we compared the functions of differentially expressed proteins with the prediction target genes for msRNAs.

Verification of the Transcription Level of Target Genes by qRT-PCR

qRT-PCR was used to verify the transcription level of predicted targets, which were also identified by proteomic technology. RNA was reverse-transcribed using the All-in-One™ First-Strand cDNA Synthesis kit (GeneCopoeia, USA). The chromosomal 16S rRNA was used as a reference gene, and the specific primers of target genes were designed (Table S2). qPCR was conducted by CFX manager 3.10 software (Bio-Rad) with All-in-One™ qPCR Mix (GeneCopoeia).

Results

sRNA Deep Sequencing in *S. platensis*

Through sRNA sequencing of *S. platensis*, we acquired 10,229,325, 9,326,145 and 7,508,874 sequences from the control, MS, and HS treatments, respectively. After removal of adapter and junk reads, 7,497,482 (representing 1,208,904 unique sequences); 4,919,552 (representing 897,957 unique sequences); and 7,696,811 (representing 1,258,460 unique sequences) clean reads in the 17 to 25 nt size range were obtained from the three groups, respectively (Table 1). The acquired sRNA sequences were successfully mapped to the reference genome sequences. After comparing to the Rfam database (http://rfam.sanger.ac.uk/), rRNA, tRNA, and other non-coding RNAs were removed (Table 1). This resulted in a total of 1,076,247 (control); 794,373 (MS); and 1,070,799 (HS) unique mappable reads.

We analyzed the unique size distribution patterns of small RNAs in *S. platensis*-FACHB350 (Fig. 1). The majority of unique sRNA reads ranged from 20 to 24 nt in length, accounting for 69.46 % (control) and 68.05 % (MS) of the unique reads in both control and MS libraries, among which the 24-nt sRNAs were the most abundant. For the HS library, the majority of the sRNAs was in the size range of 18 to 21 nt, with 20 nt having the highest abundance. The abundance of sRNAs in the range 17 to 21 nt was relatively higher in the HS library compared to the control and MS libraries. In contrast, the relative abundance of sRNAs, ranged from 22 to 25 nt size in the HS library, was significantly lower than those in control and MS libraries (Fig. 1). These results indicated that sRNAs in the 17 to 21 nt size range might play an important role in *S. platensis*'s response to salt stress.

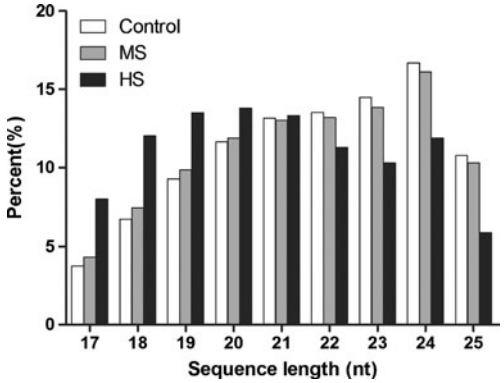


Fig. 1 The size distribution of the unique sRNA in control and salt-treated (MS and HS) libraries from *S. platensis*

Identification and Characteristics of Potential *S. platensis* msRNAs

To identify msRNAs in *S. platensis*, the remaining mappable reads were compared to the known plant miRNAs in miRBase 21.0 with a maximum of three mismatches allowed. The msRNAs and their extended sequences (flanking sequences) were analyzed for hairpin structural prediction and classification (Table S3). None of the known miRNAs were identified in the three libraries, possibly because the known miRNA sequences in miRBase were all identified from eukaryotic organisms, while *S. platensis* is a prokaryotic cyanobacterium. Even for eukaryotic algae, there were a limited number of miRNA sequences reported in miRBase.

The ability of the miRNAs flanking sequences to fold back into a stem-loop precursor was applied for differentiating candidate miRNAs from other sRNAs. By mapping all unique sRNA sequences to the *A. platensis* NIES-39 genome and predicting the hairpin structures for their flanking sequences, 728, 842, and 967 candidate msRNAs were found in the control, MS, and HS treatments, respectively. These msRNA precursor sequences were filtered with the criteria mentioned

Table 1 Summary of standard data analysis

Category	Control		MS		HS	
	Sequences	Unique sequences	Sequences	Unique sequences	Sequences	Unique sequences
Raw reads	10,229,325	2,104,927	6,841,430	1,590,064	12,739,854	2,231,282
3ADT and length (sequence <17,>25 nt) filter	2,621,479	846,560	1,826,822	655,016	4,714,236	906,584
Junk reads	110,364	49,463	95,056	37,091	128,807	66,238
Mappable to other RNAs (Rfam: rRNA, tRNA, and other RNA species)	1,227,643	78,861	726,039	59,648	1,298,981	82,002
Mappable to Repbase	355,252	65,486	264,004	54,018	832,739	128,676
Mappable reads	6,024,463	1,076,247	4,005,622	794,373	6,019,573	1,070,799

Mappable reads the resulting sequences when the raw reads were passed through a series of the digital filters by Illumina Genome Analyzer Pipeline software and the ACGT101-miR program, *MS* medium-concentration (0.3 M) salt-stress treatment, *HS* high-concentration (0.5 M) salt-stress treatment, 3ADT 3′ adapter

above along with two additional criteria: discovering msRNA and miRNA*-like msRNA* in the same sequencing library or discovering msRNA candidates among multiple and independent libraries. After filtering, we discovered 51 msRNA precursor sequences with two msRNA mature sequences located in the 5′ and 3′ ends of the precursor and 731 precursor sequences with only one msRNA mature sequence located in the 5′ or 3′ end of the precursor.

Moreover, of these potential msRNAs, nine unique msRNA sequences were identified with miRNA*-like msRNA* sequences (see Fig. 2), and all these paired to their corresponding msRNAs with ≤2 nt 3′ overhangs, which was similar to recent annotation criteria for plant miRNAs (Mao et al. 2012). The length of the msRNAs ranged from 17 to 24 nt, and the length of msRNA precursors varied from 53 to 226 nt with an average of 128.3 nt. The average minimum free energy (MFE) value was −61.42 kcal mol^{-1}, which is close to land plant msRNA precursors in the range of −101.7 to −23.4 kcal mol^{-1}. The secondary structures of the nine msRNA precursors are shown in Fig. 2. The expression levels of msRNAs and their msRNAs* varied significantly between the control, MS, and HS libraries (Table 2), demonstrating the complexity of generation and expression under salt stress in *S. platensis*.

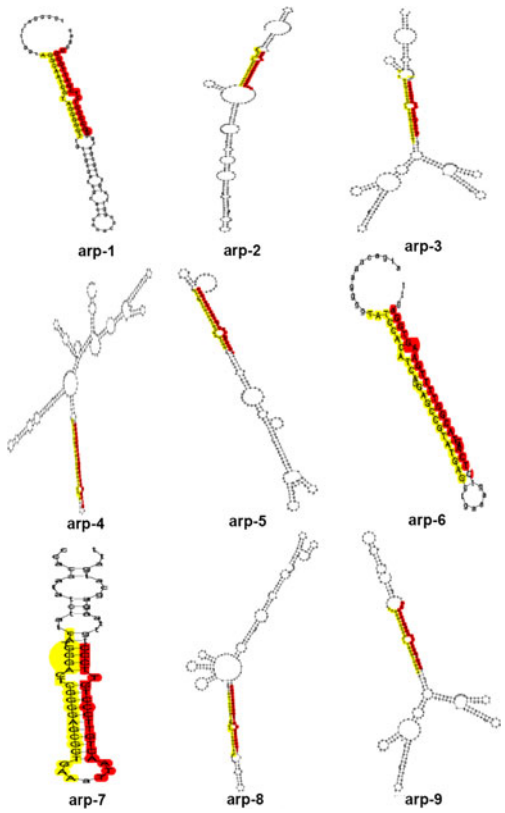


Fig. 2 Predicted secondary RNA structures formed by validated msRNAs (*red*) and putative msRNA* (*yellow*)

Identification of Salt-Responsive msRNAs in *S. platensis*

To discover salt-stress responses of msRNAs in *S. platensis*, differential expression analyses of msRNAs were performed among the control, MS, and HS libraries. Twenty-one msRNAs were found to be differentially expressed between the MS treatment and control or the HS treatment and control (Table 3). Among them, arp-10 and arp-20 showed consistent upregulation; arp-14 and arp-27 showed upregulation under both MS and HS treatments, but the expression changes in the HS library were smaller than in the MS library; arp-17 was upregulated under MS treatment but downregulated under HS treatment. The expression levels for both nine msRNAs were upregulated under MS treatment and HS treatment, respectively (Table 3). The msRNAs with the greatest changes in expression level were arp-14, arp-15, arp-23, and arp-27, while they were not detected in the control library, suggesting induction of these msRNAs under salt stress. The detailed characteristics of these 21 msRNAs are listed in Table S4. As shown in Fig. 3, the cluster analysis of 21 msRNAs revealed that these differentially regulated msRNAs might play an important role in the response of *S. platensis* to salt stress.

To validate these msRNAs expression changes, seven msRNAs were randomly selected for qRT-PCR analyses. The amplification curve and melting curve showed that these seven msRNAs were all specifically amplified (Fig. S2). All msRNAs presented consistent expression trends when compared with their deep sequencing results (Fig. 4). As shown in Fig. S3, the Pearson correlation coefficient value was 0.842, indicating the strong correlation between qPCR and small RNA sequencing results and the reliable expression analysis for msRNAs based on small RNA sequencing data. The sequencing results of seven clones proved that the nucleotides were completely consistent with the msRNA sequences (Fig. S4).

Identification and Annotation of Targets for *S. platensis* msRNAs

Through target finder, a total of 33 target genes for 12 msRNAs were successfully identified (Table 4). Except for arp-19, 22, 27, and 28, the other eight msRNAs all have two or more than two target genes. In contrast, TPR domain protein was regulated by two msRNAs (arp-18 and arp-23), which was similar to the previous results for plants (Fahlgren et al. 2007; Mao et al. 2012; Li et al. 2013). Function annotations of the identified targets in CyanoBase were found to be diverse and mainly involved in transcription and translation, such

Table 2 Detailed information for the msRNAs with msRNAs* identified from *S. platensis*

msRNA	Sequence	Arm	LM	LP	MFE (kcal/mol)	Normalized reads		
						Control	MS	HS
arp-1	AGGCAATCGTAGGGGGA	5′	17	74	−38.8	46.6008	36.9504	15.3582
arp-1*	GTCCCCCTCTCGATTGCCT	3′	19	74	−38.8	17.4753	23.5139	20.9430
arp-2	AGCCCGTCGGAACTTTCCCT	5′	20	226	−85.8	0.8322	0.0010	1.3962
arp-2*	TAAGGGAAAGTTCCTCGG	3′	18	226	−85.8	0.8322	2.7993	2.7924
arp-3	TTGAGTCCCGTTTGCGTCGGAGT	5′	23	193	−76.2	23.3004	25.1935	11.1696
arp-3*	TATCGATCGCATTCGGGACTCCCT	3′	24	193	−76.2	24.5486	12.8767	16.0563
arp-4	ATCTGGTAGTCGGGTTGATA	5′	20	58	−101.7	0.41608	7.8380	1.7453
arp-4*	GTTAAAATCCGACTACTAGG	3′	20	58	−101.7	13.3145	27.4329	12.2168
arp-5	GTTGATCGTCATGGGGGCGG	5′	20	197	−49.6	1.248236	1.679565	1.04715
arp-5*	AGCCGGTTTCTGATGATGATGAC	3′	23	197	−49.6	0.0010	0.0010	0.34905
arp-6	TATCCACATCAGGAGCCGTATGAG	5′	24	53	−23.4	1.2482	0.0010	2.0943
arp-6*	CTCAAGTACGGTTTTGAAGTGG	3′	22	53	−23.4	2.0803	0.0010	1.7453
arp-7	ATGGGACTCGGGGAGCGGTGAA	5′	22	66	−27.2	7.48941	15.1161	0.6981
arp-7*	TTAACTGTTCCCTGTTCCC	3′	19	66	−27.2	4.9929	11.7570	8.02815
arp-8	GATCGCGTCAGGCTTAGGTAGC	5′	22	75	−79.7	5.4090	1.1197	3.4905
arp-8*	AACCTAGACTGCGCGATCGCC	3′	21	75	−79.7	2.4965	1.1197	2.4434
arp-9	TTGAGTCCCGTTTGCGTCGGA	5′	21	213	−70.4	20.8039	11.7570	8.3772
arp-9*	TCGATCGCATTCGGGACTCCCT	3′	22	213	−70.4	8.7376	9.51754	16.0563

Arm the location of msRNA sequence, *LM* length of the mature msRNA, *LP* length of the msRNA precursor, *MFE* minimal folding free energy, *Normalized reads* actual msRNA count/total count of clean reads × 1,000,000

*star miRNA

as RNA polymerase beta subunit, Rho termination factor, N-terminal domain protein, ParB-like partition protein, tRNA-i(6)A37 modification enzyme MiaB, translation elongation factor EF-P, and *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase. Moreover, some transcripts were involved in inorganic ion transport and metabolism, such as calcium-transporting ATPase 1, putative ferredoxin–nitrite reductase, putative iron ABC transporter ATP-binding protein, sulfate ABC transport substrate protein, and ABC transporter permease protein. The remaining targets had various functions such as carbohydrate transport and metabolism, amino acid transport and metabolism, cell wall/membrane/envelope biogenesis, and defense mechanisms.

Enrichment Analysis of the Putative Target Genes of Host msRNAs

To better understand the pathways in which host msRNAs were involved, the putative target genes of the differentially expressed host msRNAs were subjected to GO analysis and KEGG pathway analysis. GO enrichment analysis based on biological process showed that the five predicted target genes of *S. platensis* were clustered into seven GO terms, which were associated with positive regulation of proteolysis, protein homotrimerization, protein O-linked glycosylation, multicellular organism development, ubiquinone biosynthetic process, DNA restriction-modification system, and polysaccharide catabolic process (Table S5). Interestingly, all the genes involved in these seven GO terms were targeted by upregulated msRNAs.

Similar to GO analysis, KEGG pathway analysis showed that the putative target genes predicted in *S. platensis* were grouped into three pathways, which were involved in purine metabolism, histidine metabolism, and RNA polymerase.

2-DE Profiles of Total Proteins and MALDI-TOF/MS Identification of Differentially Expressed Proteins

The differentially expressed protein profiles of *S. platensis* from the 0.5 M NaCl treatments and control (0.02 M) were analyzed by 2-DE and ImageMaster 2D platinum 5.0 software. There were 18 protein spots with more than threefold differential expression under salt-stress treatment (0.5 M NaCl), among which 13 proteins were upregulated, while 5 proteins were downregulated (Fig. 5a). In addition, 22 proteins were solely present in the 0.5 M NaCl treatment, while 15 proteins were solely present in the control (Fig. 5b).

In order to acquire more information on the differentially expressed proteins, 55 differentially expressed protein spots

Table 3 The salt-responsive msRNAs identified from control, MS, and HS treatments

msRNA	Normalized reads			Fold change log_2(MS/C)	Fold change log_2(HS/C)	*p* value MS/C	*p* value HS/C	Sig MS/C	Sig HS/C	Expression (MS/C)	Expression (HS/C)
	Control	MS	HS								
arp-4	0.416079	7.837971	1.74525	4.235551	2.068504	0.0041	–	*	–	Up	–
arp-10	27.87726	57.66507	80.97959	1.048609	1.53847	0.00154	1.82e−06	*	**	Up	Up
arp-11	7.489414	11.1971	25.8297	0.5802	1.786106	–	0.0027	–	*	–	Up
arp-12	48.26511	113.0907	55.14989	1.228428	0.192377	4.79e−07	–	**	–	Up	–
arp-13	29.1255	62.70377	39.79169	1.106269	0.450184	0.000563	–	**	–	Up	–
arp-14	0.0010	17.91536	13.2639	14.12891	13.69522	2.58e−05	0.000414		**	Up	Up
arp-15	0.0010	2.23942	12.5658	11.12891	13.61721		0.000589		**	–	Up
arp-16	0.832157	8.957681	2.7924	3.428198	1.746578	0.009933		*	–	Up	–
arp-17	3.328628	17.91536	1.3962	2.428197	−1.25342	0.001705	0.000168	*	**	Up	Down
arp-18	7.489414	15.67594	24.4335	1.065627	1.705936		0.004725	–	*	–	Up
arp-19	2.91255	3.35913	16.7544	0.205805	2.524185		0.002835	–	*		Up
arp-20	9.985885	42.54898	79.58339	2.091162	2.994505	8.55e−06	1.56e−12	**	**	Up	Up
arp-21	6.657257	4.47884	27.924	−0.5718	2.068506		0.00059	–	**	–	Up
arp-22	193.0604	231.78	521.1316	0.263704	1.432595		9.02e−31	–	**	–	Up
arp-23	0.0010	7.837971	4.188599	12.93626	12.03225	0.005389		*	–	Up	–
arp-24	3.328628	14.55623	5.235749	2.128637	0.653468	0.008548		*	–	Up	–
arp-25	11.6502	12.31681	30.0183	0.080274	1.365488		0.008125	–	*	–	Up
arp-26	10.81804	8.957681	37.69739	−0.27224	1.801026		0.000267	–	**	–	Up
arp-27	0.0010	12.31681	8.377199	13.58834	13.03225	0.000486	0.00502	**	*	Up	Up
arp-28	19.97177	59.34464	38.39549	1.571156	0.942975	1.24e−05		**	–	Up	–
arp-29	22.88432	27.4329	65.27234	0.261548	1.512112		2.36e−05	–	**	–	Up

The expression of msRNA was qualified by small RNA deep sequencing

C control, *MS* medium-concentration salt-stress treatment, *HS* high-concentration salt-stress treatment, *Sig* significance level, – no data available because the relevant data failed in passing the significance level at the $p<0.01$ level; the fold change (*MS/C* and *HS/C*) >1 or < −1 and $p<0.01$

*$p<0.01$; **$p<0.001$

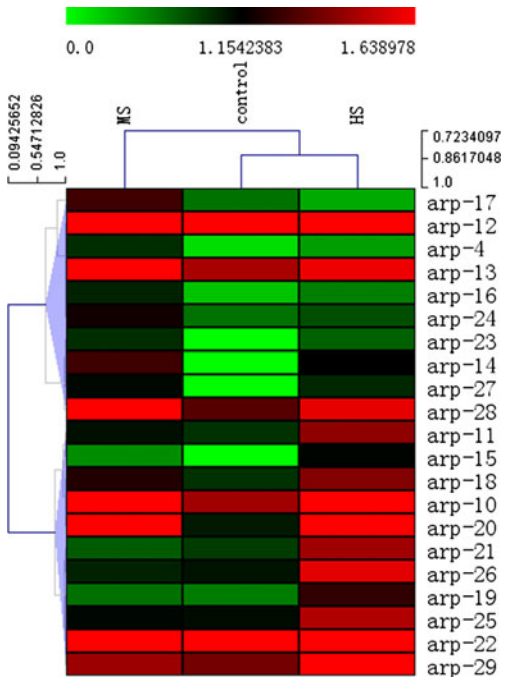


Fig. 3 Clustering analysis of 21 msRNAs under different salt concentrations

were identified using MALDI-TOF/MS analysis. We performed COG function prediction and classified these 55 positive proteins into 14 functional categories, which were mainly involved in carbohydrate transport and metabolism, cell envelope biogenesis, outer membrane, posttranslational modification, protein turnover, chaperones, translation, ribosomal structure, and biogenesis (Fig. S5). The detailed information on the 55 differentially expressed proteins is summarized in Tables 5 and 6.

Analysis and Comparison of the Targets for msRNAs and Differentially Expressed Proteins

In total, we identified 21 salt-responsive msRNAs, among which 33 target genes corresponding to 12 msRNAs were predicted. Through functional annotation in CyanoBase, we found these 33 target genes mainly participating in transcription and translation, which were generally consistent with the COG classification of differentially expressed proteins, even though not the same gene. Importantly, two protein spots, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and forkhead-associated (FHA) domain-containing protein, were

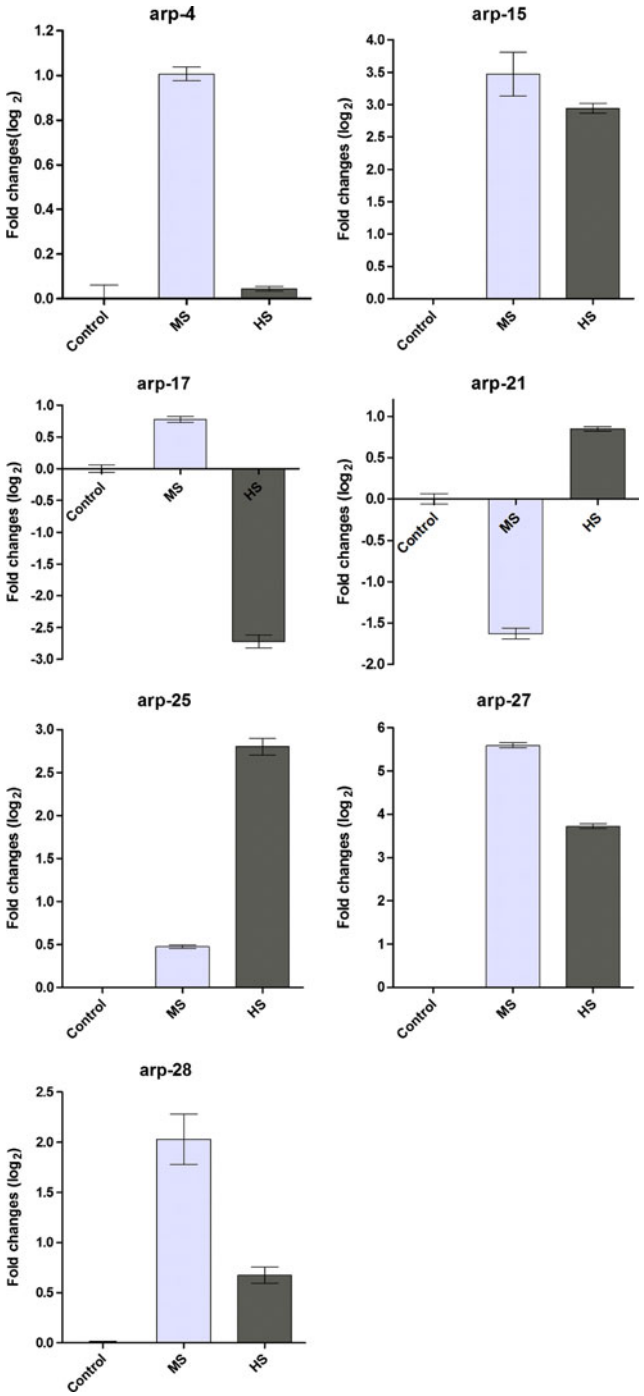


Fig. 4 Expression of seven randomly selected msRNAs by qRT-PCR. (1) The amount of expression was normalized to the level of 16 s rRNA. (2) The normalized msRNA levels in control were arbitrarily set to 1

predicted as the targets of arp-12 and arp-27, respectively. GAPDH decreased by *ca*. 3.2-fold in the HS treatment when compared to the control, and FHA was only presented in the HS treatment. Using qRT-PCR, the amplification curve and melting curve showed that the two genes were all specifically amplified (Fig. S6). The transcription levels of GAPDH and FHA were all decreased under 0.5 M NaCl stress (Fig. 6). The expression level of arp-12 and arp-27 was upregulated in the HS treatment, so a reverse expression between msRNAs and their target genes was observed. The transcription level of GAPDH was basically similar to its translation level found in proteomic experiment. While for FHA, it was inconsistent between transcription and translation levels.

In order to further verify the action sites between arp-27 or arp-12 and their target genes, we designed the specific primers of GAPDH and FHA on the basis of genome sequence of *A. platensis* and cloned the two full-length genes using *S. platensis*-FACHB350 DNA as a template (see Fig. S7). Through mapping of arp-27 and arp-12 action target sites, the two genes are fully and incompletely complementary with their target mRNA-encoding region, respectively (see Fig. 7).

Discussion

Deep sequencing has been widely used to rigorously identify miRNAs (or sRNA) in plants, animals, and bacteria; and it provides valuable information on miRNAs-mediated regulatory networks in stress tolerance. Recently, msRNAs were discovered in bacteria [12,14], while no reports were concerned with msRNAs in *S. platensis*. Herein, we found 728, 842, and 967 candidate msRNAs in the control, MS, and HS treatments, respectively. Among these candidate msRNAs, 9 of them with msRNAs* and 21 of them showed the significantly differential expression between control and MS treatments or control and HS treatments. To the best of our knowledge, so far there are no reports concerned with such sRNAs being identified in cyanobacteria. Srisuk et al. reported that mir-598 existed in *S. platensis* by comparative genomics approach (Srisuk et al. 2010). However in our study, the mature sequence of mir-598 was not found; maybe this sRNA was just similar to the sequence of mir-598, but not a kind of pre-miRNAs.

In *S. platensis*-FACHB350, the size distribution patterns of sRNAs were quite different in plants. In plants, RNase III enzyme DCL1, which is homologous to animal Dicer, is required for miRNA maturation (Kurihara and Watanabe 2004; Blevins et al. 2006). DCL1 is localized in the nucleus and can make both the first pair of cuts made by Drosha and the second pair of cuts made by animal Dicer. Until now, no reports were concerned with such a DCL1 existing in the prokaryotic cell. Because *S. platensis* is a prokaryote, there probably exists another enzyme, which has different shear mechanisms of msRNAs.

Because the *S. platensis*-FACHB350 genome was not currently available, we used the *A. platensis* NIES-39 genome,

Table 4 Annotation of target genes

msRNA	Target gene ID	Score	Range	Target annotation	Function category
arp-12	15166803	4	194–210	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate transport and metabolism
	15165012	4	50–66	Transglutaminase-like domain	Amino acid transport and metabolism
	15166480	4	1064–1080	Putative ferredoxin–nitrite reductase	Inorganic ion transport and metabolism
	15166602	4	447–463	Alpha-amylase	Carbohydrate transport and metabolism
arp-15	15161491	1	1157–1173	tRNA-i(6)A37 modification enzyme MiaB	Translation, ribosomal structure and biogenesis
	15165953	2	504–520	ParB-like partition protein	Transcription
	15166522	4	485–501	Heme O synthase	Posttranslational modification, protein turnover, chaperones
arp-16	15161677	0	417–434	Imidazoleglycerol-phosphate dehydratase	Amino acid transport and metabolism
	15162755	2	712–729	Probable 5′-nucleotidase	Nucleotide transport and metabolism
	15165705	3	369–385	Translation elongation factor EF-P	Translation, ribosomal structure and biogenesis
arp-17	15166858	3	521–541	Putative zinc metalloprotease	Cell wall/membrane/envelope biogenesis
	15164396	4	625–644	Calcium-transporting ATPase 1	Inorganic ion transport and metabolism
	15164907	4	1968–1987	Putative helicase	No assignment
	15161655	4	535–554	*S*-adenosylmethionine:tRNA ribosyltransferase-isomerase	Translation, ribosomal structure, and biogenesis
	15165384	4	15–35	Putative peptidase	No assignment
	15160708	4	822–840	Malic enzyme	Energy production and conversion
arp-18	15161907	4	730–747	Putative sugar kinase	Multiple categories
	15163246	4	775–792	Pyridine nucleotide-disulfide oxidoreductase	General function prediction only
	15163785	4	901–918	TPR domain protein	No assignment
	15161434	4	974–990	Putative iron ABC transporter ATP-binding protein	Inorganic ion transport and metabolism
arp-19	15165053	0	375–393	Putative sulfate ABC transport substrate protein	Inorganic ion transport and metabolism
arp-22	15164274	4	19–36	Hypothetical protein	No assignment
arp-23	15163605	3	484–503	Putative ABC transporter permease protein	Multiple categories
	15162161	4	316–336	TPR domain protein	No assignment
arp-26	15167151	2	903–920	RNA polymerase beta subunit	Transcription
	15164531	3	384–400	Putative methyltransferase	Replication, recombination, and repair
	15166609	4	308–325	Acetolactate synthase	Multiple categories
arp-27	15166489	0	1047–1065	FHA domain-containing protein	No assignment
arp-28	15166971	0	1179–1197	Photosystem II CP47 protein	No assignment
arp-29	15167180	4	674–690	Type I restriction-modification system R subunit	Defense mechanisms
	15164118	4	812–828	Hypothetical protein	No assignment
	15164596	4	141–157	Rho termination factor, N-terminal domain protein	Translation

Target gene accession the gene ID blast to Nr database, *Score criterion* explained in the "Method and materials" section, *Range* the transcript site of target gene, *Function category* the function category of target gene in CyanoBase

which limits the discovery of new msRNAs to those existing in *A. platensis* NIES-39 genome. Another concern was whether our newly identified msRNAs are truly new msRNAs or just false signals which randomly map to the reference genome. To address this issue, strict criteria were applied to msRNAs identification: Firstly, it should not exist mismatch mapping between the msRNA and the reference genome; and secondly, msRNAs could be discovered in at least two libraries (Li et al. 2013). Based on these criteria, we discovered that arp-14, arp-15, arp-23, and arp-27 were induced by salt stress, indicating that these four msRNAs might play an important role in preventing *S. platensis* from salt-stress damage.

Knowledge of the target function for the identified *S. platensis* msRNAs provides insight into the important functions and regulations of msRNAs in tolerant mechanisms of *S. platensis*. Due to the limited number of accessible *S. platensis* mRNAs, all mRNAs of *A. platensis* NIES-39 in GeneBank were chosen as a target gene database, in which not all of the *S. platensis* msRNAs were found to have detectable targets in this study. The lack of a complete mRNAs database for *S. platensis* limits the comprehensive identification of targets. From this analysis, 12 candidate msRNAs were found to have target genes in *S. platensis*, which were involved in various biological processes such as transcription, translation, inorganic ion transport and metabolism, carbohydrate

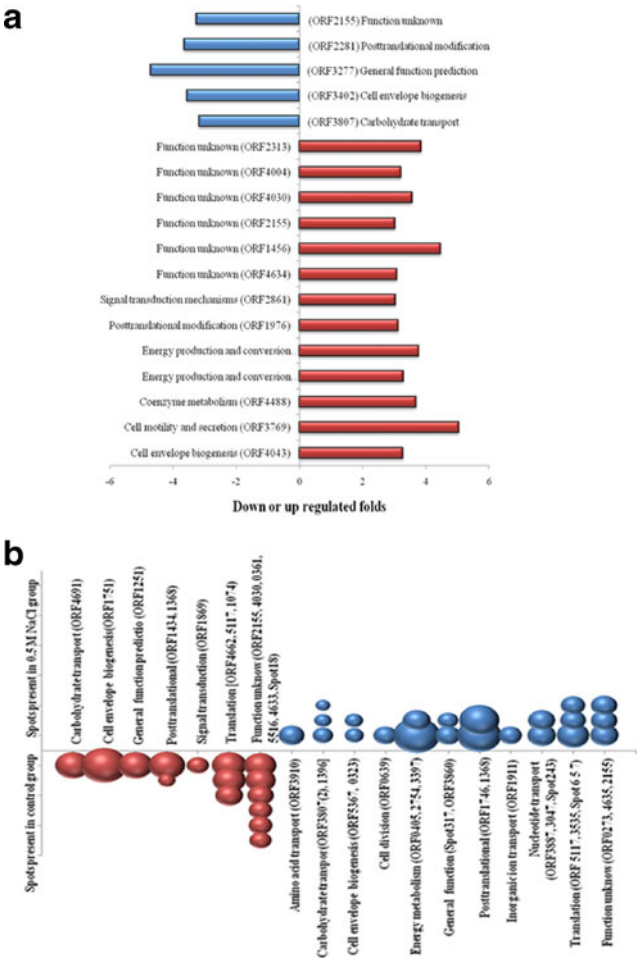


Fig. 5 The differential protein points between medium-salt treatment (0.5 M) and control group (0.02 M). **a** Fold change (>3.0-fold) for the differential protein points. **b** Protein spots showing absent or present difference. *Vol%* indicates the relative protein expression abundance calculated by the ratio of volume of each protein spot to volume of total protein spots in the whole gel

transport and metabolism, and defense mechanisms. These identified biological processes were similar to those found by COG functional classification of differentially expressed proteins determined by proteomic analysis.

Based on identification by proteomic analysis, the concentration of GAPDH was decreased by 3.2-fold in the HS treatment compared to the control. The transcription level of GAPDH was also decreased under salt stress. These results were inconsistent with the expression changes of arp-12, which was upregulated under HS treatment compared to control. There might existed a reverse expression between msRNAs and their targets, which was in agreement with the mechanism by which miRNA can restrain post-transcriptional gene expression by promoting mRNA degradation or inhibiting protein translation.

Through qPCR analysis, the transcription level of FHA domain-containing protein was also decreased under salt stress. However, FHA domain containing protein was only present in the HS treatment by proteomic experiment, which is in accordance with the expression trend of arp-27. The inconsistency between transcription and translation levels may be influenced by many factors. For the different biological species, their miRNA regulating mechanisms are different. As for plant, the action mode of miRNA is similar to that of siRNA. miRNA is interacted with its target mRNA-encoding specific sites in a mode of fully or nearly complementary pairing and degrade the target mRNA (Yu et al. 2015). For animals, the majority of miR does not degrade mRNA showing non-regulation of target gene at transcriptional level but can inhibit translational level by means of incompletely complementary pairing with 3′-UTR sequence and of inhibiting translational initiation factor or poly(A) tail function (Belver et al. 2011; Macfarlane et al. 2010). So far, little data are available on miRNA discovery and its regulating mechanism in *S. platensis*, and the fact that the genetic characterization of miRNA in *Spirulina* is similar to plants, animals, or bacteria is still unclear. The other possible reasons on the inconsistency between translation and transcription levels of genes can be referred to the literatures (Wang et al. 2013).

GAPDH is a highly conserved glycolytic enzyme that catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate, which plays an important role in the carbon economy of cyanobacteria (Tamoi et al. 2005). Many studies indicated that the accumulation of GAPDH increased under different stress conditions. For example, Guo et al. found that the GAPDH gene *OsGAPC3* was overexpressed in 200 mM NaCl treatment to alleviate salt toxicity in rice (Guo et al. 1997). While in creeping bentgrass, GAPDH in cytosol was increased, but GAPDH in chloroplast was decreased under salinity stress (Xu et al. 2010). However, such GAPDH form was not found in cyanobacteria. We found the GAPDH was decreased under 0.5 M NaCl treatment, suggesting that salinity tolerance in *S. platensis* may affect the maintenance of glycolytic activity. Otherwise, our findings provided another possibility that GAPDH was targeted by arp-12 in response to salt stress. It has been well established that calcium-transporting ATPase may be involved in Na^+ homeostasis in cyanobacterium through counteraction of Na^+ ATPase; disruptions of a putative Ca^{2+}-ATPase and a gene cluster encoding a putative Na^+-ATPase subunit also cause high NaCl sensitivity in *Synechocystis* sp. PCC 6803 (Wang et al. 2002). Researchers reported that miR4376 could regulate the expression of an autoinhibited Ca^{2+}-ATPase in tomato (*Solanum lycopersicum*) ACA10, which played a critical role in tomato reproductive growth (Wang et al. 2011). ABC transporter ATP-binding proteins are transmembrane proteins that utilize the energy of ATP binding and hydrolysis to carry out certain biological processes including

Table 5 The MALDI-TOF/MS analytical results of differential protein spots (>3-fold) between 0.5 M NaCl treatment and control (0.02 M)

Spot (0.02 M)	Spot (0.5 M)	ORF	Accession number	Gene product (best hit in NCBI nr database)	Theoretical/Experimental		Matched peptides	Cov (%)	MASCOT score	E-value	Fold changes
					MW	p*I*					
Carbohydrate transport and metabolism											
N489	M542	ORF3807	ZP_03274253	Glyceraldehyde-3-phosphate dehydrogenase, type I [*Arthrospira maxima* CS-328]	36,534/37,135	6.07/4.48	12	52	135	3.3e−08	−3.17
Cell envelope biogenesis, outer membrane											
N139	M134	ORF4043	ZP_03274588	dTDP-4-dehydrorhamnose 3,5-epimerase [*Arthrospira maxima* CS-328]	20,785/22,132	5.00/5.24	13	70	138	1.7e−08	+3.27
N809	M858	ORF3402	ZP_03272127	Glycoside hydrolase family 25 [*Arthrospira maxima* CS-328]	50,658/50,551	5.22/6.16	10	20	88	0.0015	−3.55
Cell motility and secretion/intracellular trafficking and secretion											
N526	M582	ORF3769	ZP_06381070	Twitching motility protein [*Arthrospira* str. Paraca]	40,645/38,664	8.22/6.78	25	51	152	6.6e−10	+5.04
Coenzyme metabolism											
N202	M198	ORF4488	YP_001516744	Pterin-4-alpha-carbinolamine dehydratase [*Acaryochloris marina* MBIC11017]	10,179/25,864	5.42/5.52	7	59	80	0.0096	+3.68
Energy production and conversion											
N109	M93	ORF2739	ZP_03274295	ATP synthase F0, B subunit [*Arthrospira maxima* CS-328]	19,538/17,970	5.14/5.04	13	59	114	4.2e−06	+3.28
N145	M140	ORF2740	ZP_06381970	F0F1 ATP synthase subunit delta [*Arthrospira* str. Paraca]	20,108/22,132	6.15/6.79	15	53	116	2.6e−06	+3.77
General function prediction only											
N701	M718	ORF3277	ZP_03275759	FAD-dependent pyridine nucleotide-disulfide oxidoreductase [*Arthrospira maxima* CS-328]	47,147/42,726	6.67/6.49	6	20	78	0.018	−4.70
Posttranslational modification, protein turnover, chaperones											
N275	M305	ORF2281	ZP_06380867	Peptidyl-prolyl *cis-trans* isomerase, cyclophilin type [*Arthrospira* str. Paraca]	23,921/28,931	4.74/4.49	6	36	75	0.036	−3.65
N922	M1011	ORF1976	ZP_03275215	Chaperonin GroEL [*Arthrospira maxima* CS-328]	58,209/64,271	5.00/4.69	32	56	190	1e−13	+3.13
Signal transduction mechanisms											
N199	M196	ORF2861	ZP_06382415	Stress protein [*Arthrospira* str. Paraca]	22,195/25,745	4.93/5.19	23	93	148	1.7e−09	+3.04
Function unknown											
N94	M78	ORF4634	ZP_03271568	Phycocyanin, alpha subunit [*Arthrospira maxima* CS-328]	17,703/17,228	5.82/5.99	11	74	78	0.015	+3.08
N176	M178	ORF1456	ZP_06380822	Pentapeptide repeat-containing protein [*Arthrospira* str. Paraca]	19,860/25,627	5.13/5.42	12	47	77	0.021	+4.47
N342	M394	ORF2155	ZP_06382427	Phycobilisome linker polypeptide [*Arthrospira* str. Paraca]	29,450/31,199	9.25/5.68	15	55	218	1.6e−16	−3.26
N398	M435	ORF2155	ZP_06382427	Phycobilisome linker polypeptide [*Arthrospira* str. Paraca]	29,450/33,050	9.25/6.70	30	71	269	1.3e−21	+3.02
N500	M548	ORF4030	ZP_03276569	Gas vesicle protein GvpC [*Arthrospira maxima* CS-328]	32,452/37,662	9.22/6.83	39	76	310	1e−25	+3.56
N514	M560	ORF4004	ZP_06381784	Peptidoglycan binding domain-containing protein [*Arthrospira* str. Paraca]	39,737/38,196	6.32/6.77	17	46	196	2.6e−14	+3.21
N740	M813	ORF2313	ZP_06384820	Type III effector Hrp-dependent outers [*Arthrospira* str. Paraca]	34,715/45,116	4.97/5.70	16	56	116	2.6e−05	+3.85

Table 6 The MS analytical results of the sole present protein spots in 0.5 M NaCl treatment or control (0.02 M)

Spot (0.02 M)	Spot (0.5 M)	ORF	Accession NO.	Gene product (best hit in NCBI nr database)	Theoretical/ experimental		Matched Peptides	Cov (%)	MASCOT Score	E-value	Vol‰
					MW	p*I*					
Amino acid transport and metabolism / Cell envelope biogenesis, outer membrane											
	M463	ORF3910	ZP_06382240	Dihydrodipicolinate synthase [*Arthrospira* str. Paraca]	30,869/34,837	5.20/5.80	22	51	141	8.3e−09	0.28
Carbohydrate transport and metabolism											
	M595	ORF3807	ZP_03274253	Glyceraldehyde-3-phosphate dehydrogenase, type I [*Arthrospira maxima* CS-328]	36,534/39,635	6.07/5.26	24	61	110	0.00001	0.03
	M681	ORF3807	ZP_03274253	Glyceraldehyde-3-phosphate dehydrogenase, type I [*Arthrospira maxima* CS-328]	36,534/41,796	6.07/6.71	23	54	164	4.2e−11	0.17
	M966	ORF1396	ZP_06380414	Phosphoglucomutase [*Arthrospira* str. Paraca]	59,997/61,933	5.05/5.12	17	43	93	0.0005	0.11
N928		ORF4691	ZP_06382051	Transketolase [*Arthrospira* str. Paraca]	72,979/64,761	5.78/5.75	21	45	204	4.1e−15	0.51
Cell envelope biogenesis, outer membrane											
	M522	ORF5367	ZP_06382090	Glycosyl transferase family protein [*Arthrospira* str. Paraca]	35,242/36,895	5.98/6.59	10	31	76	0.023	0.15
Cell envelope biogenesis, outer membrane / Carbohydrate transport and metabolism											
	M528	ORF0323	ZP_06381275	Dtdp-glucose 4,6-dehydratase [*Arthrospira* str. Paraca]	35,782/37,239	6.08/6.86	20	58	140	1e−08	0.13
N251		ORF1751	ZP_06381424	NAD-dependent epimerase/dehydratase [*Arthrospira* str. Paraca]	23,630/28,078	5.15/5.75	12	70	184	4.1e−13	0.89
Cell division and chromosome partitioning											
	M125	ORF0639	ZP_02928939	FHA domain-containing protein [*Verrucomicrobium spinosum* DSM 4136]	170,844/21,007	5.09/5.86	27	19	84	0.0041	0.22
Energy production and conversion											
	M371	ORF2754	ZP_06385149	NADH dehydrogenase subunit B [*Arthrospira* str. Paraca]	27,251/32,011	6.75/6.69	16	48	191	8.3e−14	0.17
	M868	ORF3397	ZP_06382626	Aldehyde dehydrogenase [*Arthrospira* str. Paraca]	48,847/52,543	5.68/6.02	16	36	131	8.3e−08	0.31
General function prediction only											
	M317		ZP_03130156	Conserved hypothetical protein [*Chthoniobacter flavus* Ellin428]	73,654/30,716	6.98/4.60	17	27	77	0.019	0.17
	M1012	ORF3860	ZP_06382439	Carbonate dehydratase [*Arthrospira* str. Paraca]	59,145/69,350	8.66/5.61	27	47	197	2.1e−14	0.25
N934		ORF1251	ZP_06381540	Hypothetical protein aplap_07632 [*Arthrospira* str. Paraca]	67,407/65,336	5.66/6.20	19	42	166	2.6e−11	0.52
Posttranslational modification, protein turnover, chaperones											
	M113	ORF1746	ZP_06383547	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen [*Arthrospira* str. Paraca]	16,988/19,692	5.07/4.99	8	71	108	1.7e−05	0.67
	M123	ORF1368	ZP_03273475	Redoxin domain protein [*Arthrospira maxima* CS-328]	19,780/20,671	4.88/4.26	10	52	87	0.002	0.81
N156		ORF1368	ZP_03273475	Redoxin domain protein [*Arthrospira maxima* CS-328]	19,780/23,444	4.88/6.35	10	49	125	3.3e−07	0.13
N805		ORF1434	ZP_06380920	Chaperonin groel [*Arthrospira* str. Paraca]	57,425/50,615	5.00/4.47	24	50	271	8.2e−22	0.56
Inorganic ion transport and metabolism											
	M137	ORF1911	ZP_06383116	Adenylylsulfate kinase [*Arthrospira* str. Paraca]	19,897/22,320	5.22/5.46	16	79	200	1e−14	0.22
Nucleotide transport and metabolism											
	M148	ORF3887	ZP_06380517	Bifunctional pyrimidine regulatory protein pyrR uracil phosphoribosyltransferase [*Arthrospira* str. Paraca]	19,867/23,752	5.10/5.29	22	92	227	2.1e−17	0.34
	M243		ZP_03275636	Adenylate kinase [*Arthrospira maxima* CS-328]	21,863/28,121	5.22/5.54	14	82	94	0.00043	0.30

Table 6 (continued)

Spot (0.02 M)	Spot (0.5 M)	ORF	Accession NO.	Gene product (best hit in NCBI nr database)	Theoretical/ experimental		Matched Peptides	Cov (%)	MASCOT Score	E-value	Vol‰
					MW	p*I*					
Signal transduction mechanisms											
N555		ORF1869	YP_002485086	Multisensor signal transduction histidine kinase [*Cyanothece* sp. PCC 7425]	85,539/39,515	5.95/4.29	11	12	74	0.044	0.19
Translation, ribosomal structure and biogenesis											
	M166	ORF5117	ZP_06381795	Ribosome recycling factor [*Arthrospira* str. Paraca]	20,263/24,928	5.71/5.92	12	63	145	3.3e−09	0.25
	M657		ZP_05756741	RNA polymerase ECF-type sigma factor [*Bacteroides* sp. D2]	10,349/41,207	6.26/5.20	8	98	84	0.0042	0.44
	M836	ORF3535	ZP_06385129	Elongation factor Tu [*Arthrospira* str. Paraca]	42,538/49,960	5.08/5.32	33	85	260	1e−20	0.26
N210		ORF5117	ZP_06381795	Ribosome recycling factor [*Arthrospira* str. Paraca]	20,263/26,103	5.71/6.12	10	59	140	1e−08	0.44
N283		ORF4662	ZP_03271593	Sigma 54 modulation protein/ribosomal protein S30EA [*Arthrospira maxima* CS-328]	23,973/29,221	7.03/6.22	9	58	94	0.00044	0.49
N377		ORF1074	ZP_06380559	Elongation factor TS [*Arthrospira* str. Paraca]	24,626/32,194	5.74/6.12	7	53	75	0.034	0.31
Function unknown											
	M175	ORF0273	ZP_06384609	Hypothetical protein aplap_23383 [*Arthrospira* str. Paraca]	20,229/25,353	5.93/5.71	9	49	121	8.3e−07	0.33
	M252	ORF4635	ABD64607	Phycocyanin beta chain [*Arthrospira*]	18,506/28,381	5.19/4.58	11	55	98	0.00016	0.28
	M421	ORF2155	ZP_06382427	Phycobilisome linker polypeptide [*Arthrospira* str. Paraca]	29,450/33,199	9.25/6.63	34	83	340	1e−28	0.28
N18			YP_003289399	Hypothetical protein Rmar_0102 [*Rhodothermus marinus* DSM 4252]	25,840/28,842	7.88/6.13	7	31	74	0.04	0.23
N65		ORF4030	ZP_06384870	Hypothetical protein aplap_24737 [*Arthrospira* str. Paraca]	17,324/15,867	5.96/6.23	18	86	245	3.3e−19	0.38
N339		ORF0361	ZP_06384062	Peptidase S8 and S53 subtilisin kexin sedolisin [*Arthrospira* str. Paraca]	44,238/31,219	4.59/4.65	16	44	200	1e−14	0.28
N385		ORF2155	ZP_06382427	Phycobilisome linker polypeptide [*Arthrospira* str. Paraca]	29,450/32,400	9.25/5.26	23	67	269	1.3e−21	0.43
N423		ORF4633	ABV01983	Cpch [*Arthrospira* Sp-16]	30,852/33,929	7.82/6.29	25	60	295	3.3e−24	0.19
N424		ORF5516	ZP_03272395	Conserved hypothetical protein [*Arthrospira maxima* CS-328]	41,116/34,016	6.04/6.08	13	50	205	3.3e−15	0.23

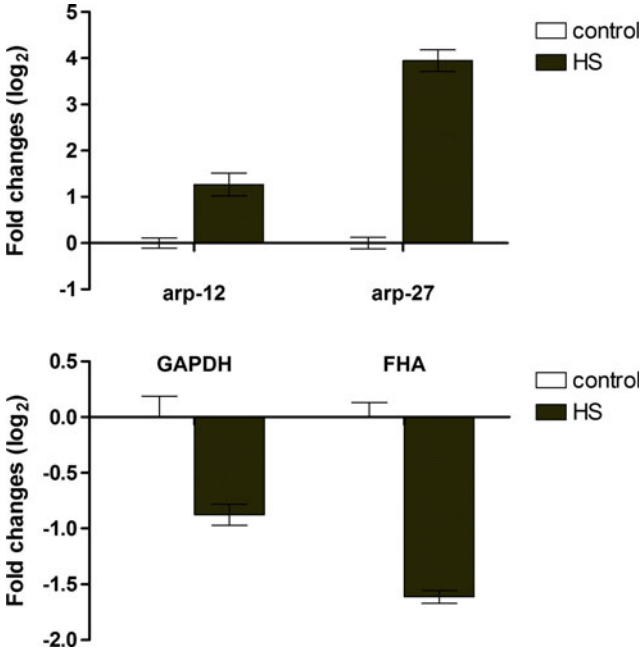


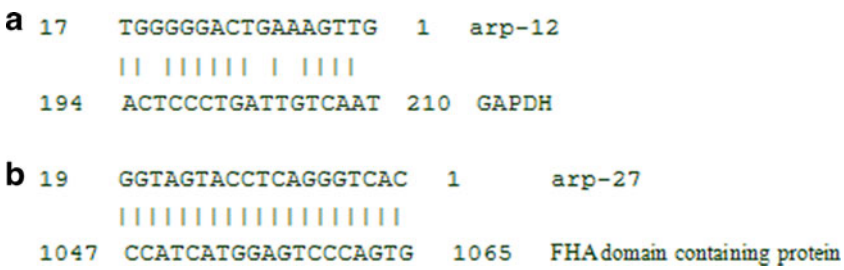
Fig. 6 The transcription levels of GAPDH and FHA domain-containing protein

translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair (Davidson et al. 2008). The G subfamily ABC transporter (OsABCG5) was involved in accumulation of essential and non-essential minerals, the Na/K ratio, and salt tolerance in *Oryza sativa* L. (Matsuda et al. 2014). Small nuclear RNA-derived microRNA hsa-miR-1291 could modulate cellular drug disposition through direct targeting of ABC transporter ABCC1 (Pan et al. 2013). ABC transporter, as the target of miRNAs in rice seedlings and radish, was involved in plant response to cadmium stress (Xu et al. 2013; Tang et al. 2014). Photosystem II (PSII) is the first protein complex in the light-dependent reactions of oxygenic photosynthesis, which is located in the thylakoid membrane of plants, algae, and cyanobacteria. Salt stress resulted in a significant decrease in PSII electron transport activity and PSII-mediated oxygen evolution activity in *S. platensis* (Zhang et al. 2010). Western blot analysis revealed that the inhibition in PSII activity was due to a 40 % loss of a thylakoid membrane protein, most prominently, a dramatic diminishment of the 47-kDa chlorophyll protein (CP) and 94-kDa protein. The changes in 47- and 94-kDa proteins lead to decreased energy transfer from light-harvesting antenna to PSII (Sudhir et al. 2005). $NADP^+$-dependent malic enzyme (NADP-ME) catalyzes the oxidative decarboxylation of malate to pyruvate, concomitantly releasing CO_2 in cyanobacteria. NADP-ME in plant functions in many different pathways, and expression of the gene encoding cytosolic NADP-ME in *O. sativa* L and *Aloe vera* L. was induced by salt stress (Sun et al. 2003; Liu et al. 2007). NADP-ME activities in leaves and roots of rice also increased in response to NaCl (Cheng and Long 2007; Liu et al. 2007).

GO and KEGG pathway analyses showed that the predicted target genes were involved in diverse biological processes, including positive regulation of proteolysis, protein homotrimerization, protein O-linked glycosylation, multicellular organismal development, ubiquinone biosynthetic process, DNA restriction-modification system, and polysaccharide catabolic process. It is of note that all the genes involved in these seven GO terms were targeted by upregulated msRNAs. Since msRNAs function to suppress gene expression, this phenomenon implies that the genes associated with these cellular pathways are probably downregulated in response to salt stress.

In summary, a total of nine msRNAs were identified with msRNAs* and 21 candidate msRNAs were differentially expressed among the control, MS, and HS treatments. Thirty-three targets were predicated by bioinformatics, and some target transcripts were predicated to code salt-responsive proteins or enzymes. Among the 33 targets, two were verified by proteome analysis. qRT-PCR was also used to validate the differential expression of msRNAs and target genes under NaCl stress. The transcription level of GAPDH and FHA exhibited an inverse correlation with arp-12 and arp-27. Although the functional significance of the revealed msRNAs in *S. platensis* requires further investigation, our study suggests that the existence of msRNAs may not be limited to eukaryotes and they can be profiled in prokaryotes by deep sequencing. These studies of msRNAs in *S. platensis* could enhance our understanding of the mechanisms on protecting cyanobacteria from environment stress.

Fig. 7 Matching sites of arp-27 and arp-12 with their target genes



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