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Identification and Characterization of Salt-Responsive MicroRNAs in *Taxodium hybrid* 'Zhongshanshan 405' by High-Throughput Sequencing

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Abstract: MicroRNAs (miRNAs) are a type of noncoding RNA participating in the post-transcriptional regulation of gene expression that regulates plant responses to salt stress. Small RNA sequencing was performed in this study to discover the miRNAs responding to salt stress in *Taxodium hybrid* 'Zhongshanshan 405', which is tolerant to salinity stress. A total of 52 miRNAs were found to be differentially expressed. The target genes were enriched with gene ontology (GO), including protein phosphorylation, cellular response to stimulus, signal transduction, ATP and ADP binding, showing that miRNAs may play key roles in regulating the tolerance to salt stress in *T. hybrid* 'Zhongshanshan 405'. Notably, a G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*) regulated by novel_77 and novel_2 miRNAs and a mitogen-activated protein kinase kinase kinase (*MAPKKK*) regulated by novel_41 miRNA were discovered under both short- and long-term salt treatments and can be selected for future research. This result provides new insights into the regulatory functions of miRNAs in the salt response of *T. hybrid* 'Zhongshanshan 405'.

Keywords: microRNAs; taxodium; high-throughput sequencing; salt stress

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1. Introduction

Salt stress can seriously endanger plant growth and development. About one fifth of agricultural lands and half of the croplands in the world suffer from salt stress [1]. High salinity can also lead to secondary stresses such as oxidative stress and nutritional imbalance, leading to cell damage, growth inhibition and crop yield reduction [2]. *Taxodium* is an excellent wetland species and important landscape plant living in river and coastal floodplains [3]. It has a long lifespan, is relatively free from pest problems, is plentiful in its natural environment and is generally tolerant to flooding, salt and hurricanes [4]. To combine the best characteristics of superior parents, different species of *Taxodium* were undertaken to produce hybrids called *Taxodium hybrids* 'Zhongshanshan' (hereafter referred to as *T. hybrid*), excellent woody plants for the afforestation of wetland and coastal areas in southeastern China [5], where they currently play an important role in the water system and coastal floodplains areas [6]. In addition, previous studies have shown that *T. hybrid* is tolerant to salinity stress [7,8]. To analyze the genetic basis of this salt tolerance, RNA-Seq and the analysis of differentially expressed genes in *T. hybrid* subjected to salt stress have been performed. Those studies indicated that genes related to transport, signal transductions and genes of unknown function were involved in salt tolerance [8].

miRNAs are endogenous short (21–24 nucleotides) and non-coding RNAs, which are important in post-transcriptional gene regulation through mRNA degradation or inhibition of mRNA translation [9]. miRNAs play significant roles in the regulation of many biological processes, including stress responses in plants [10]. The expression of plant miRNAs can be altered in response to several abiotic stress stimuli, such as drought, salinity, extreme

temperatures and others [11,12]. In response to a high salt environment, miRNA regulates changes in gene expression involved in a wide range of biological processes, including signal transduction [13,14]. Next-generation sequencing (NGS) technologies have generated extensive sequencing data for detecting salt-sensitive miRNAs in different plant species [13,15]. Many miRNAs and genes responding to salt stress have been studied at the level of transcription with these technologies [13,16] and indicate that plant responses to salt treatment may be determined by miRNA-directed gene regulation. Understanding the role of *T. hybrid* miRNAs under salt stress will help identify the genes involved and provide insights into the regulatory mechanism underlying salt tolerance in *Taxodium*, thereby providing a basis for more effective plant breeding.

High-throughput sequencing technology was used in this study to identify differentially expressed miRNAs of *T. hybrid* under high salt stress conditions. To investigate the underlying mechanism of the miRNA-mediated regulation of gene expression under a salt environment, the potential target genes of differentially expressed miRNAs were analyzed through Gene Ontology (GO) enrichment and pairs of miRNAs and target genes with opposite expression patterns in such comparisons were chosen for analysis of regulation mechanism. This study helps to study the potential regulatory mechanism of miRNA-mediated responses to salt stress in *T. hybrid*. The specific miRNAs in *T. hybrid* can be used to breed salt-tolerant plants growing on marginal lands.

2. Materials and Methods

2.1. Plant Materials

The process of plant growth and high salt treatments were consistent with the previous method of Yu [8]. Briefly, *T. hybrid* 'Zhongshanshan 405' were planted in plastic pots in a ventilated greenhouse. After one year, the plantlets of uniform growth were carefully removed from the soil to avoid injury, their roots were washed with tap water and groups of seedlings were placed in 1/2 Hoagland solution. After 1 week, seedlings were transferred to containers with 0, 100 or 200 mM NaCl solutions in 1/2 Hoagland solution.

After the seedlings were subjected to salt stress for different times, the total roots were harvested, frozen in liquid nitrogen and stored at −80 °C until analysis. Four sample types were taken for sequencing, negative control (0 mM NaCl treated for 1 h) (T1), 100 mM NaCl treated for 1 h (T2), 200 mM NaCl treated for 1 h (T3) or 24 h (T4). Three biological replicates of each sample type were used.

2.2. Small RNA Library Construction and High-Throughput Sequencing

Total RNA was extracted with Trizol reagent following the manufacturer's protocol (Takara Bio Inc., Otsu, Japan). RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentrations were tested with a Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorimeter (Life Technologies, CA, USA). RNA integrity was measured with the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries of small RNAs were generated from 3 μg RNA of each sample with NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs, Inc., Ipswich, MA, USA) as per the manufacturer's instructions, and index codes were added to attribute sequences to each sample. Index-coded samples were clustered on a cBot Cluster Generation System with TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA). The resulting library preparations were sequenced on an Illumina Hiseq 2500 platform to generate 50 bp single-end reads after cluster generation.

2.3. Data Filtering and Mapping Reads

Clean data were obtained by deleting reads that contained poly-N, poly nucleotides, 5′ adapter contaminants, missing the 3′ adapter or insert tag and low-quality reads by custom perl and python scripts. The length distribution of the clean reads was then sorted to analyze the composition of the sRNA data, and the sRNAs of 18–30 nt were kept for

further analyses. The small RNA tags were mapped to reference sequences by Bowtie (bowtie-0.12.9, Baltimore, MD, USA) without mismatch to analyze their expression and distribution on the reference [8].

2.4. Identification of Known MicroRNAs and Novel MicroRNAs

To map each unique small RNA to only one annotation, we followed the following priority rule: known miRNA > rRNA > tRNA > snRNA > snoRNA > repeat > gene > NAT-siRNA > gene > novel miRNA > ta-siRNA. Taking miRBase20.0 as a reference, we used modified software mirdeep2 [17] and srna-tools-cli to obtain potential miRNAs and to draw the secondary structures. miREvo and mirdeep2 were integrated to predict novel miRNAs [17,18].

2.5. Analyzing sRNA Expression

TPM (transcript per million) was calculated to show miRNA expression levels by the following criteria: normalized expression = mapped readcount/Total reads * 1,000,000 [3]; differential expression analysis was performed with the DESeq R package (1.8.3, European Molecular Biology Laboratory Heidelberg, Germany); the Benjamini and Hochberg method was selected for adjusting *p*-values and an adjusted *p*-value of 0.05 was set as the default threshold for significantly differential expression.

2.6. Target Prediction

Predicting the target gene of miRNA was performed by psRobot_tar in psRobot [19] for plants, using the following parameters: penalty score threshold: 3.0; five prime boundary of essential sequence: 1; three prime boundary of essential sequence: 31; maximal number of permitted gaps: 0; position after which with gaps permitted: 1. GOseq-based Wallenius non-central hyper-geometric distribution [20], which could adjust for gene length bias, was implemented for GO-enrichment analysis.

2.7. Validation of miRNAs' Expression by Real-Time Quantitative PCR (QRT-PCR)

To validate the high-throughput sequencing, six miRNAs with differential expression patterns were randomly selected and tested with qRT-PCR. RNA was reverse-transcribed to cDNA with the miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China) with the addition of 5 pmol of forward and reverse primers in a final volume of 20 µL (Table S1). The reaction system was constructed using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), ROX plus (Takara) according to recommendations of the manufacturer. qRT-PCR conditions were set as follows: 95 °C 30 s; 40 cycles of 95 °C 5 s, 60 °C 40 s; followed by a melt curve. Each transcript abundance of miRNA was normalized relative to U6 snRNA [21], and the relative miRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method. There were three biological replicates per sample and three technical replicates per reaction. Statistical analysis was performed by one-way ANOVA with the least significant difference (LSD) test using the statistical program SPSS 21.0 (IBM-SPSS Inc, Chicago, IL, USA) for Windows. Values were considered statistically different when $p < 0.05$. All figures were represented by OriginPro 9.1 software (Microcal, Northampton, MA, USA).

3. Results

3.1. Deep-Sequencing of sRNAs

To identify miRNAs involved in the regulation of the salt resistance of *T. hybrid*, high-throughput sequencing technique was used to construct libraries from the treated samples. Over 10 million reads were generated from the initial libraries, and after filtering out adapter sequences and removing low quality sequences, over 93.90% remained as clean reads (Table 1). The sequences lengths ranged from 18–30 nt, with 21–24 nt small RNAs highly enriched.

Table 1. Summary of filtered data produced by small RNA sequencing.

Sample	Total Reads	N% > 10%	Low Quality	5 Adapter Contamine	3 Adapter Null or Insert Null	with ployA/T/G/C	Clean Reads
T1-1	13,212,250 (100.00%)	34 (0.00%)	10,535 (0.08%)	10,746 (0.08%)	419,301 (3.17%)	11,549 (0.09%)	12,760,085 (96.58%)
T1-2	14,554,682 (100.00%)	34 (0.00%)	10,988 (0.08%)	15,718 (0.11%)	422,272 (2.90%)	16,198 (0.11%)	14,089,472 (96.80%)
T1-3	11,047,511 (100.00%)	33 (0.00%)	6373 (0.06%)	13,511 (0.12%)	371,044 (3.36%)	11,949 (0.11%)	10,644,601 (96.35%)
T2-1	10,616,411 (100.00%)	21 (0.00%)	4386 (0.04%)	17,123 (0.16%)	269,497 (2.54%)	19,629 (0.18%)	10,305,755 (97.07%)
T2-2	14,511,597 (100.00%)	33 (0.00%)	7570 (0.05%)	20,556 (0.14%)	450,817 (3.11%)	20,197 (0.14%)	14,012,424 (96.56%)
T2-3	13,778,931 (100.00%)	32 (0.00%)	7794 (0.06%)	17,722 (0.13%)	784,585 (5.69%)	30,837 (0.22%)	12,937,961 (93.90%)
T3-1	12,036,280 (100.00%)	28 (0.00%)	7326 (0.06%)	8488 (0.07%)	369,020 (3.07%)	18,987 (0.16%)	11,632,431 (96.64%)
T3-2	10,291,894 (100.00%)	10 (0.00%)	4139 (0.04%)	11,050 (0.11%)	455,849 (4.43%)	12,135 (0.12%)	9,808,711 (95.31%)
T3-3	11,345,585 (100.00%)	26 (0.00%)	4628 (0.04%)	6330 (0.06%)	348,991 (3.08%)	7011 (0.06%)	10,978,599 (96.77%)
T4-1	11,909,066 (100.00%)	36 (0.00%)	5711 (0.05%)	8714 (0.07%)	407,633 (3.42%)	10,874 (0.09%)	11,476,098 (96.36%)
T4-2	12,833,399 (100.00%)	32 (0.00%)	6067 (0.05%)	15,353 (0.12%)	519,695 (4.05%)	8185 (0.06%)	12,284,067 (95.72%)
T4-3	12,115,728 (100.00%)	35 (0.00%)	8663 (0.07%)	9867 (0.08%)	474,255 (3.91%)	14,847 (0.12%)	11,608,061 (95.81%)

3.2. Identification of Known miRNAs and Novel miRNAs

To identify miRNAs in *T. hybrid*, the mapped sRNA library was compared to known plant miRNAs in the miRBase 20.0 database (www.mirbase.org, accessed on 19 September 2022). A total of 49 known plant miRNAs were identified. In addition, 98 candidate novel miRNAs were predicted based on their secondary structure (Table 2). At the same time, 155 miRNA hairpins and 68 star miRNAs were identified (Table 2).

Table 2. Quantity of the known miRNAs and predicted novel miRNAs in *T. hybrid*.

	Types	Total	T1-1	T1-2	T1-3	T2-1	T2-2	T2-3	T3-1	T3-2	T3-3	T4-1	T4-2	T4-3
Mapped known miRNAs	Mature	49	36	43	39	31	30	29	40	26	29	19	31	26
	Hairpin	55	38	48	44	34	34	32	43	31	33	22	36	28
Mapped novel miRNAs	Mature	98	88	88	83	77	74	72	80	62	77	57	73	70
	Hairpin	100	92	93	88	84	80	79	85	67	83	66	81	76
	Star	68	44	42	40	37	25	30	39	17	40	16	32	18

Notes: Mature referred to the miRNA mature body on the alignment; Hairpin referred to the miRNA precursor on the alignment; Star referred to the number of miRNA reads matched to the 3.3. Differentially Expressed miRNAs between Salt-Treated Samples and Control

To identify differentially expressed miRNAs, their expression levels were normalized, clustered and are presented in a heatmap in Figure 1A. The results showed that the expressions of some miRNAs were upregulated after salt treatments, while some other miRNAs were higher expressed in the control group (T1) and the short-term and low

concentration of salt treatment (T2) than in the high concentration (T3) and long-term (T4) of salt treatments. Among them, 14, 14 and 21 miRNAs were differentially expressed relative to the T1 (control) in T2, T3 and T4, respectively. In addition, one, five and six miRNAs were shared by T2 vs. T1 and T3 vs. T1, T2 vs. T1 and T4 vs. T1, and T3 vs. T1 and T4 vs. T1, respectively, and one and one miRNAs were shared by the group of T2 vs. T1, T3 vs. T2, T4 vs. T2 and the group of T3 vs. T1, T3 vs. T2, T4 vs. T3 (Figure 1B). T4 vs. T3 represented a comparison between long- and short- term salt treatments, and 11 miRNAs were differentially expressed in this comparison group. These are listed in Table S2.

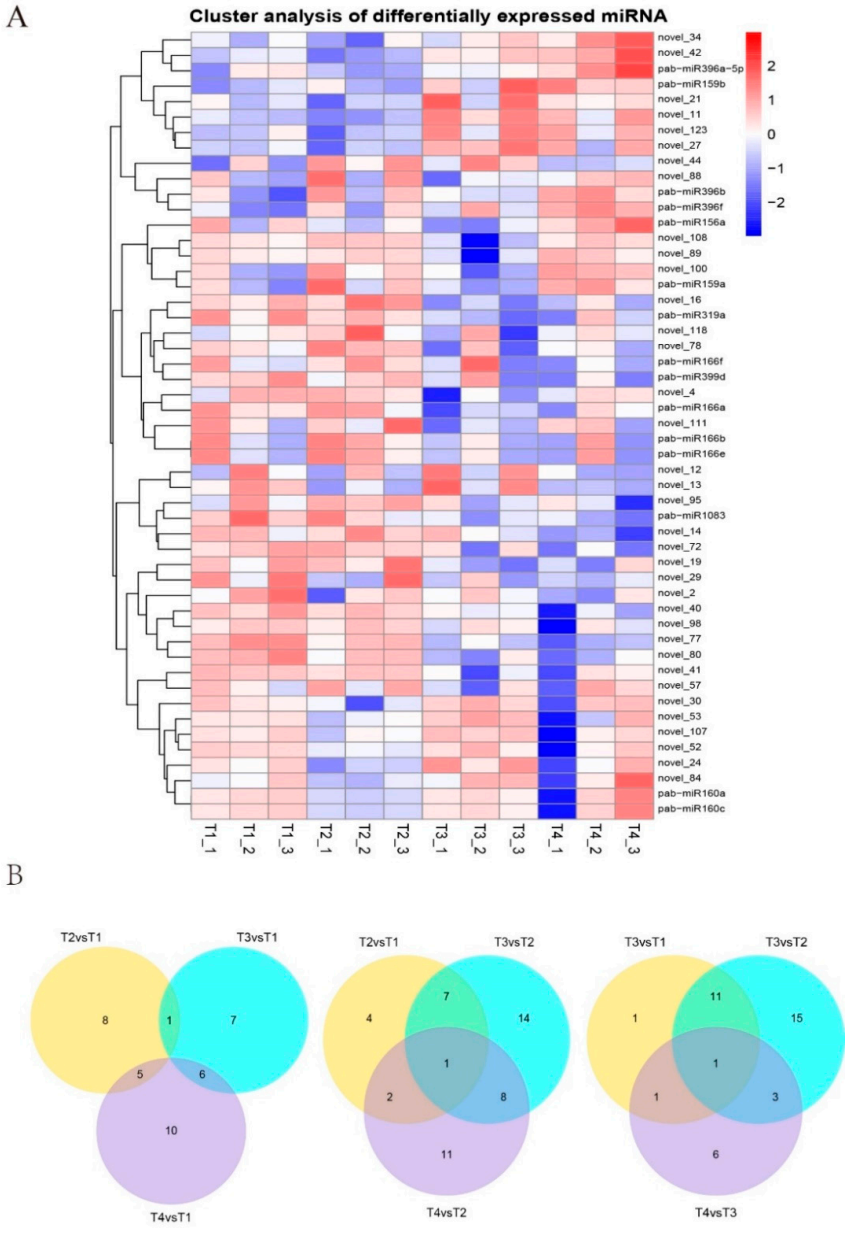

Figure 1. Expression analysis of miRNAs. (**A**) Cluster analysis of relative differential expression of 52 miRNAs. The bar represents the scale of the expression abundance for each miRNA ($\log_{10}$(TPM + 1)), where, relative to control levels, red represents miRNA with high expression and blue represents miRNA with low expression; T1-1-T1-3 indicated three biological replicates of T1 sample (control), T2-1-T2-3 indicated three biological replicates of T2 sample (100 mM NaCl, 1 h), T3-1-T3-3 indicated three biological replicates of T3 sample (200 mM NaCl, 1 h), T4-1-T4-3 indicated three biological replicates of T4 sample (200 mM NaCl, 24 h). (**B**) Differentially expressed miRNAs among different salt treatments presented by VENN analysis.

3.3. Target Prediction for Known and Novel miRNAs

A total of 8737 potential target genes were predicted from the transcripts of *T. hybrid* libraries (Files S1 and S2). A gene ontology enrichment analysis for each group of target genes is presented in Figure 2. Most target genes were enriched in a biological process, such as ADP binding, adenyl ribonucleotide binding, ATPase activity and others, while fewer genes were enriched in a molecular function and the fewest genes were enriched in cellular components, indicating the important roles of binding, catalytic, transporter activity and others in response to salt treatments. Subsequently, pairs of miRNAs and target genes with opposite expression patterns in such comparisons were chosen based on the analysis of transcriptome [8], such as the zinc finger CCCH domain-containing protein and glycosyltransferase family protein and others. Under short-term and low concentration of salt treatment (T1 vs. T2), related proteins such as polyprotein, TIR-NBS-LRR protein and others may be regulated by miRNAs, while when the treatment concentration increased (T1 vs. T3 and T2 vs. T3), some kinases and transcription factors, which may be regulated by miRNAs, became involved in response to high salinity. Compared with short-term treatment, prolonged salt treatment (T1 vs. T4 and T3 vs. T4) also stimulated a number of miRNAs related to protein kinase, transport and energy synthesis. Among them, a G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*) and a mitogen-activated protein kinase kinase kinase (*MAPKKK*) were discovered in both T1 vs. T3 and T1 vs. T4 comparisons. These can now be selected for future research (Table 3).

Table 3. Identified pairs of miRNAs and target genes with opposite expression patterns under salt treatment.

	miRNA	Target	Expectation	Target Accessibility	Target Description	Inhibition	Multiplicity
T1 vs. T2	novel_118	CL16860Contig1	4.5	18.248	Putative polyprotein	Cleavage	1
	novel_13	CL15Contig4	4.5	20.381	Probable disease resistance protein	Translation	1
	novel_16	T3_Unigene_BMK.33047	4	18.583	Putative truncated TIR-NBS-LRR protein	Translation	1
	novel_52	CL10744Contig1	4.5	10.073	Zinc finger CCCH domain-containing protein 35	Translation	1
	novel_78	CL25843Contig1	4.5	12.165	Probable nucleoredoxin 1	Translation	1
	miR160a	CL8543Contig1	4	5.964	Chaperone protein dnaJ 11	Translation	1
	miR396b	CL5428Contig1	3.5	22.527	Glycosyltransferase family protein 2	Cleavage	1
T1 vs. T3	novel_123	CL27539Contig1	4.5	13.132	RNA-binding protein 25	Cleavage	1
	novel_21	CL18504Contig1	4	24.442	TMV resistance protein N	Translation	1
	novel_41	CL2111Contig1	5	12.456	Mitogen-activated protein kinase kinase kinase	Cleavage	1
	novel_4	CL1685Contig1	4.5	12.818	Ethylene-responsive transcription factor RAP2-13	Cleavage	1
	novel_77	T2_Unigene_BMK.14386	4.5	16.719	G-type lectin S-receptor-like serine/threonine-protein kinase	Cleavage	1
	miR156a	CL14355Contig1	4	18.821	RNA and export factor-binding protein 2	Cleavage	1
	miR319a	CL11314Contig1	4	17.74	Beta-amylase 1 isoform 1	Cleavage	1
T1 vs. T4	novel_100	CL24684Contig1	4.5	17.879	ATP synthase subunit	Cleavage	1
	novel_13	CL4989Contig1	4	20.269	Salicylate O-methyltransferase	Cleavage	1
	novel_14	CL1013Contig1	3.5	18.997	Probable LRR receptor-like serine/threonine-protein kinase	Cleavage	1
	novel_29	CL11748Contig1	2	18.906	Glycerol-3-phosphate 2-O-acyltransferase 6	Translation	1

Table 3. *Cont.*

	miRNA	Target	Expectation	Target Accessibility	Target Description	Inhibition	Multiplicity
T1 vs. T4	novel_2	CL14285Contig1	4	16.055	G-type lectin S-receptor-like serine/threonine-protein kinase	Translation	1
	novel_40	CL1182Contig1	5	18.447	Disease resistance RPP13-like protein 4	Translation	1
	novel_41	CL2111Contig1	5	12.456	Mitogen-activated protein kinase kinase kinase	Cleavage	1
	novel_42	CL1146Contig1	2.5	13.999	F-box/LRR-repeat protein 17	Cleavage	1
	novel_77	CL1110Contig2	4	19.906	Cysteine-rich receptor-like protein kinase	Cleavage	1
	novel_98	CL13461Contig1	4	14.38	Homeobox-leucine zipper protein ATHB-13	Cleavage	1
	miR159a	CL12428Contig1	3	16.021	Chlorophyll a-b binding protein 7	Translation	1
	miR396a-5p	CL10009Contig1	5	20.004	DNA replication licensing factor mcm5	Cleavage	1
	miR396f	CL2465Contig1	4	18.647	U-box domain-containing protein 12	Cleavage	1
	miR399d	CL1025Contig1	5	20.819	Tonoplast dicarboxylate transporter	Cleavage	1
T2 vs. T3	novel_100	CL772Contig3	4.5	13.205	Transcription factor MYB59	Cleavage	1
	novel_108	CL805Contig1	4	14.766	LRR receptor-like serine/threonine-protein kinase	Translation	1
	novel_111	CL1347Contig2	3.5	14.988	Subtilisin-like protease	Cleavage	1
	novel_123	CL14243Contig1	4.5	12.566	Trehalose-phosphatase	Translation	1
	novel_16	CL22Contig4	5	21.524	TMV resistance protein	Translation	1
	novel_24	CL228Contig1	2.5	20.215	TMV resistance protein	Cleavage	1
	novel_30	CL14581Contig1	4.5	19.087	Xyloglucan endotransglucosy-lase/hydrolase	Translation	1
	novel_41	CL23589Contig1	4.5	13.732	Chaperone protein dnaJ	Cleavage	1
	novel_52	T3_Unigene_BMK.32994	4.5	23.774	Protein LURP-one-related	Cleavage	1
	novel_77	T2_Unigene_BMK.14386	4.5	16.719	G-type lectin S-receptor-like serine/threonine-protein kinase	Cleavage	1
	novel_78	CL10767Contig1	5	17.257	BON1-associated protein	Translation	1
	novel_88	CL15264Contig1	4	14.363	Cysteine-rich receptor-like protein kinase	Translation	1
	novel_89	T3_Unigene_BMK.16696	3	17.651	Squamosa promoter-binding-like protein	Cleavage	1
	pab-miR159a	CL2378Contig1	3	16.57	Cinnamoyl CoA reductase	Translation	1
	pab-miR319a	CL26045Contig1	4	17.17	Disease resistance protein RPS2	Cleavage	1
T3 vs. T4	novel_108	CL1146Contig1	4	13.459	F-box/LRR-repeat protein 17	Translation	1
	novel_13	CL4989Contig1	4	20.269	Salicylate O-methyltransferase	Cleavage	1
	novel_14	CL1013Contig1	3.5	18.997	Probable LRR receptor-like serine/threonine-protein kinase	Cleavage	1
	miR156a	CL24146Contig1	3	15.592	Probable LRR receptor-like serine/threonine-protein kinase	Cleavage	1
	miR159a	CL12428Contig1	3	16.021	Chlorophyll a-b binding protein 7	Translation	1
	miR396a-5p	CL10009Contig1	5	20.004	DNA replication licensing factor mcm5	Cleavage	1
	miR396b	CL2465Contig1	4.5	18.647	U-box domain-containing protein 12	Cleavage	1
	miR396f	CL13812Contig1	4.5	16.915	Potassium transporter 1	Translation	1

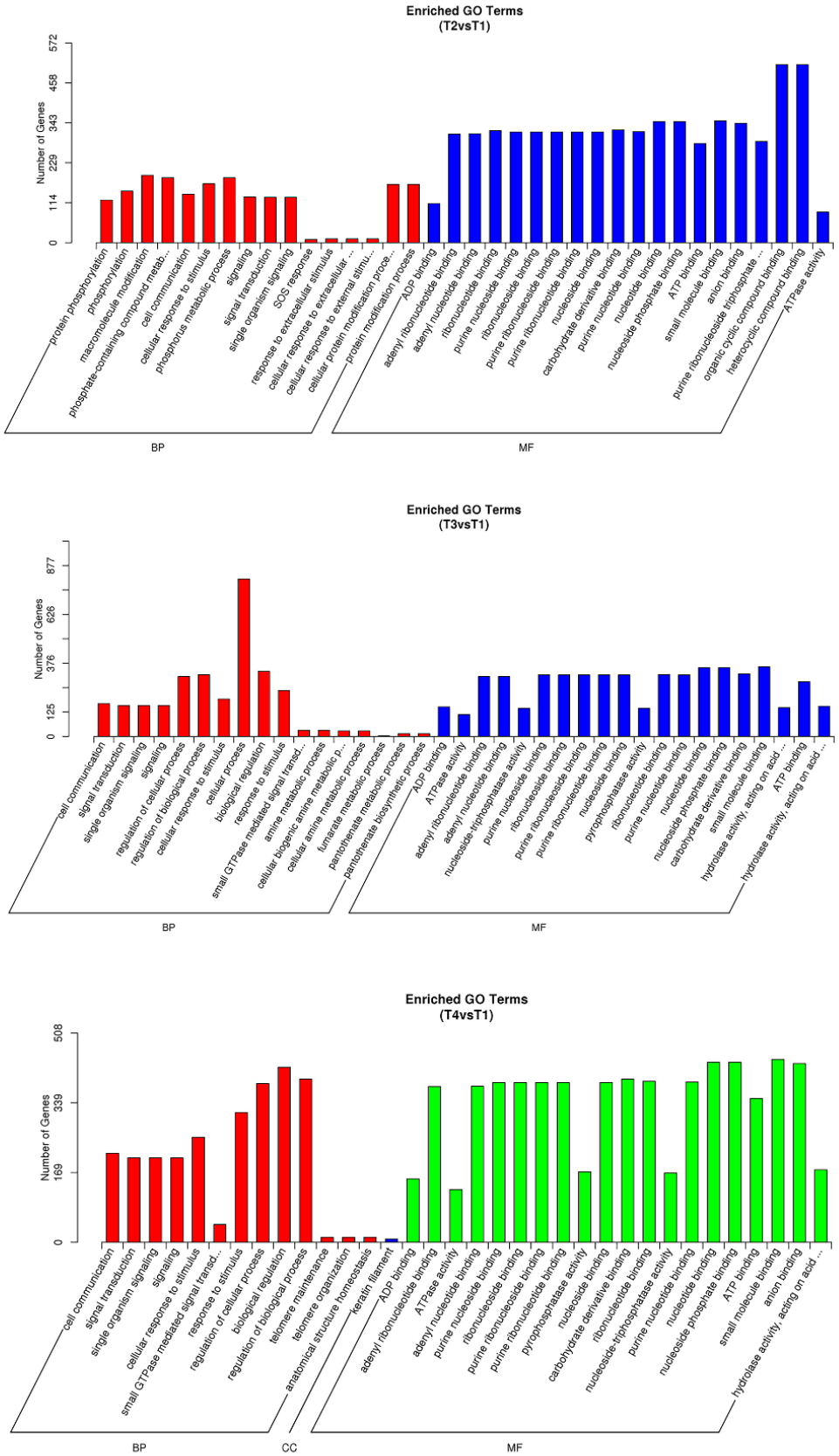

Figure 2. Gene ontology (GO) classifications of putative targets of differentially expressed miRNAs in *T. hybrid* subjected to salt stress in T2 vs. T1, T3 vs. T1 and T4 vs. T1, respectively. BP—biological process; MF—molecular function; CC—cellular component.

3.4. QRT-PCR Validation

We randomly chose six differentially expressed miRNAs and analyzed the relative changes in expression by qRT-PCR (Table S1). The expression data of the six miRNAs are presented in Figure 3 and File S3. The results showed that the expression patterns of most miRNAs tested with qRT-PCR were similar with the sequencing data (Figure 3). Additionally, it was proved again that differentially expressed miRNAs in this study responded differently to low and high concentration, short- and long-term salt treatments.

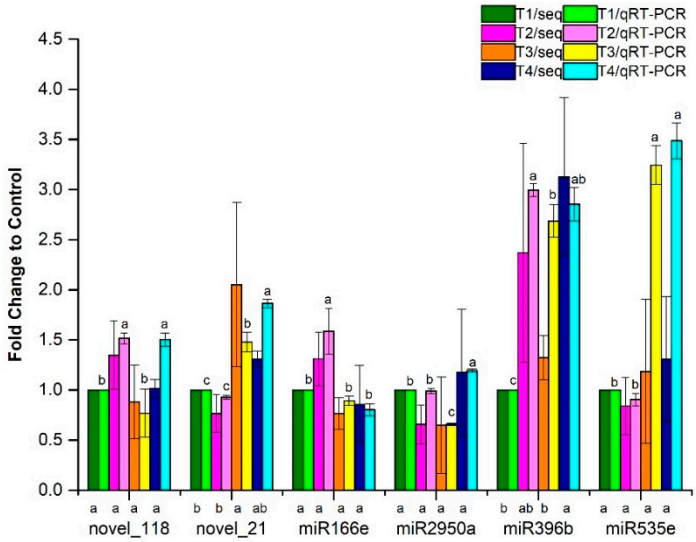

Figure 3. Validation of six miRNAs by qRT-PCR. T1-T4/seq indicated relative expression level of miRNAs calculated based on TPM and the significance was indicated below the bar chart, T1-T4/seq indicated relative expression level of miRNAs calculated based on $2^{-\Delta\Delta Ct}$, and different lowercase letters indicating statistically significant difference were indicated above the bar chart.

4. Discussion

miRNAs play an important role in the regulation of plant growth and development [10,11]. The sRNA transcriptome is complex and significantly different in different plant species and organs [22]. Changes in miRNA expression profiles in several plant species under salt-stress conditions have been reported [23–25]. In a previous study, RNA-Seq had been performed to analyze changes in the transcriptome of T. hybrid roots treated with NaCl in order to describe the genetic basis of salt tolerance [8]. Here, we have specifically monitored miRNAs' expression in *T. hybrid* subjected to short- (1 h) and long-term (24 h) salt treatments.

A total of 49 known plant miRNAs and 98 candidate novel miRNAs were identified from sRNA-Seq libraries. Among them, a total of 52 miRNAs exhibited altered expression in response to salt stress. Most target genes were enriched in biological process, while fewer genes were enriched in molecular function and the fewest genes were enriched in cellular components. The majority of the predicted target genes of miRNAs with altered expression were protein-coding genes involved in protein phosphorylation, cellular response to stimuli, signal transduction, ADP binding, ATP binding, ribonucleoside binding and others, suggesting that *T. hybrid* may rapidly alter these functions under salt stress, which is consistent with previous reports [26–28]. For example, the putative target gene related with protein phosphorylation was reported to interact with the salt-inducible TaMIRs, suggesting that it could be involved in the mediation of salt response in wheat [26]. An miRNome analysis also showed that response to stimuli was the main GO feature of miRNA-targeted genes in the wheat-root response to salt stress [27]. GO-enrichment analysis showed that the main function of the target genes of salinity stress-responsive miRNAs in wild emmer wheat was the binding of molecules, such as ATP binding, ADP binding and others [28].

In our study, several miRNA and target genes showed opposite expression under salt stress; the results showed that kinases and transcription factors, which may be regulated by miRNAs, were mainly involved in response to high salinity. Additionally, prolonged salt treatment stimulated a number of miRNA related to transport and energy synthesis. *GsSRK* and *MAPKKK* were indicated as potential target genes of differentially mobilized miRNAs in both the T1 vs. T3 and the T1 vs. T4 comparisons. The *GsSRK* in soybean has been shown to be induced by salt stress and to improve plant tolerance to salt stress when heterologously expressed in *Arabidopsis* [29]. In this study, novel_77 and novel_2, which putatively target *GsSRK*, both showed different expression patterns under salt treatments. This is similar to that observed for miR535c and a target gene *GsSRK*, which were differentially expressed in response to high salinity in banana roots [30]. Mitogen-activated protein kinase (MAPK) cascades participate in salt-stress signaling responses in plants [31]. Upstream signals activate the *MAPKKK*, which eventually causes the activation of the specific MAP kinases and in turn phosphorylates various downstream targets and regulates the stress responses of organisms [32]. *MAPKKK* genes were induced by salt in *Arabidopsis* and negatively regulated salt tolerance [33,34]. In addition, 23 *MAPKKK* genes were predicted to be targeted by 11 miRNAs in barley [35]. One novel miRNA identified in this study, novel_41, potentially interacts with *MAPKKK*, and this interaction may play an important role in the *T. hybrid* response to high salinity environments. These results indicated that *GsSRK* and *MAPKKK* may be regulated by miRNA in response to and participate in improving salt tolerance in *T. hybrids.* The specific regulatory mechanisms and functions need to be further studied and verified.

5. Conclusions

In conclusion, the discovery of microRNAs responding to salt stress provide an extensive perspective about salt tolerance in *T. hybrid* 'zhongshanshan405'. Sequencing and qRT-PCR validation indicated that some miRNAs exhibited distinct expression patterns under different salt treatments. The prediction and annotation of miRNA-mediated target genes provided favorable information for future gene function studies, which will provide new information about factors that regulate salt tolerance in *T. hybrid* 'Zhongshanshan405'. Taken together, our study provides valuable information for further identification of the function of miRNAs related to salt tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/f13101685/s1, Table S1: The list of miRNA primers used for qRT-PCR; Table S2: The number of differentially expressed miRNAs; File S1: annotate; File S2: sequences; File S3: data.

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