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Identification and Target Prediction of MicroRNAs in *Ulmus pumila* L. Seedling Roots under Salt Stress by High-Throughput Sequencing

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Abstract: MicroRNAs (miRNAs) are a class of endogenous small RNAs with important roles in plant growth, development, and environmental stress responses. *Ulmus pumila* L., a deciduous broadleaved tree species of northern temperate regions, is widely distributed in central and northern Asia and has important economic and ecological value. With the spread and aggravation of soil salinization, salt stress has become a major abiotic stress affecting the normal growth and development of *U. pumila.* However, the influence of salt stress on *U. pumila* miRNA expression has not been investigated. To identify miRNAs and predict their target mRNA genes under salt stress, three small RNA libraries were generated and sequenced from roots of *U. pumila* seedlings treated with various concentrations of NaCl corresponding to no salt stress, light short-term salt stress, and medium-heavy long-term salt stress. Integrative analysis identified 254 conserved miRNAs representing 29 families and 49 novel miRNAs; 232 potential targets of the miRNAs were also predicted. Expression profiling of miRNAs between libraries was performed, and the expression of six miRNAs was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Our findings provide an overview of potential miRNAs and corresponding targets involved in regulating *U. pumila* salt defense responses. These results lay the foundation for further research into molecular mechanisms involved in salt stress resistance in *U. pumila* and other Ulmaceae species.

Keywords: *Ulmus pumila* L.; MicroRNA; MicroRNA target; salt stress; high-throughput sequencing; qRT-PCR

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding, single-stranded RNAs approximately 22 nt long that are widely present and evolutionarily conserved in eukaryotes [1]. They modulate gene expression by guiding target mRNA cleavage or translational repression in plants and animals [2,3]. Evidence exists that miRNAs play crucial roles in many biological and metabolic processes in higher plants, such as growth, development, and responses to biotic and abiotic stresses [4–8] including salt stress [9]. For instance, transgenic plants overexpressing *osa-miR319a* exhibited enhanced salt tolerance associated with increased leaf wax content and water retention but reduced sodium uptake [10]. Conversely, transgenic rice and *Arabidopsis thaliana* (L.) Heynh. plants overexpressing *osa-miR393* were more sensitive to salt and alkali treatment compared with wild-type plants [11], while overexpression of *miR402* accelerated *A. thaliana* seed germination and seedling growth under salt stress conditions [12].

Ulmus pumila L. is naturally distributed from northern subtropical to cold temperate zones. As a major tree species used for timber, shelter, food, medicine, fodder, and ecological protection

in East Asia, *U. pumila* is economically and ecologically important [13–16]. Several ecological and pharmaceutical studies of *U. pumila* have been previously carried out, including investigations of the phenological responses of *U. pumila* to climate change in the temperate zone of China [14], the effects of sand burial on survival and growth of *U. pumila* seedlings [13], adipogenesis inhibition by *U. pumila* extracts [17], and the inhibition of clinically isolated antibiotic-resistant bacteria by *U. pumila* [16]. A few molecular biological studies of *U. pumila* have also been performed, such as the isolation and characterization of *U. pumila* microsatellite markers [18] and research on reactive oxygen species-provoked mitochondria-dependent cell death during ageing of *U. pumila* seeds [19]. However, neither the genome nor transcriptome of *U. pumila* has been published, and only 209 *U. pumila* DNA and RNA sequences have been deposited in the National Center for Biotechnology Information (NCBI) database (until October 2016).

Salt stress is one of the major abiotic stresses limiting plant growth and development [20]. With the spread and aggravation of soil salinization [21], salt stress has become a major abiotic stress affecting *U. pumila* growth and factors such as its landscape value, timber yield, and role in ecological protection. In recent years, several groups have studied the physiology and biochemistry of *U. pumila* under these conditions [22,23], but none have investigated *U. pumila* miRNAs under salt stress. High-throughput sequencing of small RNAs associated with transcriptome sequences is an effective way to identify miRNAs and their targets.

The aim of this study was to identify *U. pumila* miRNAs and predict their target mRNA genes, and to provide an overview of potential miRNAs and their corresponding targets involved in the regulation of salt defense responses in *U. pumila*. In the present study, we consequently performed high-throughput sequencing of three small-RNA sequencing libraries constructed from roots of *U. pumila* seedlings treated with different salt stresses. Comparison of the resulting *U. pumila* transcriptome sequences allowed us to identify miRNAs and predict their targets. Expression profiles of miRNAs between the different libraries were also analyzed. The generated data (254 conserved miRNAs, 49 novel miRNAs, 232 potential miRNA targets, and miRNA differential expression profiles) will provide new insights into salt stress response regulation in *U. pumila* and facilitate the improvement of salt resistance of *U. pumila* and other Ulmaceae species.

2. Materials and Methods

2.1. Plant Materials and Salt Stress Treatment

U. pumila seeds produced by one maternal parent are half sibs family, and the pollen source was relatively fixed in our Experimental Base (Dongying, China), Research Center of Saline and Alkali Land of State Forestry Administration, which previously showed smaller salt-tolerant differences [23]. Seeds were washed thoroughly with water and then sown in peat pots filled with equal amounts of perlite and vermiculite in a greenhouse with a 14 h light/10 h dark photoperiod under 25/18 °C day/night temperatures and a relative humidity of 70%–75%. Five days after germination, seedlings were irrigated with half-strength Hoagland's culture liquid; this was repeated every seven days. Approximately 30 days after germination, three salt stress treatments were implemented: (1) culturing of seedlings under normal conditions without NaCl stress (CK); (2) addition of 50 mM NaCl to a final concentration of 50 mM followed by culturing with salt (50 mM) for 6 h (light salt stress for a short time, LSS); and (3) gradual stepwise acclimation over four days to a final NaCl concentration of 200 mM (from 50 mM to 100 mM, then 150 mM, and finally 200 mM NaCl), followed by culturing with 200 mM salt for 24 h (medium-heavy salt stress for a long time, MSL) (Table 1) [24,25].

After treatment, uniformly developed seedling roots were collected from each treatment and immediately frozen in liquid nitrogen before being stored at −80 °C for small RNA library construction and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays.

Table 1. Experimental design and sample collection of salt-treated 30-day-old seedlings.

Sample Names	The Time Points of Salt Treatment and Sample Collection					
	−102 h	**−72 h**	**−48 h**	**−24 h**	**−6 h**	**0 h**
CK (0)						sample collection
LSS (50 mM)					50 mM	sample collection
MSL (200 mM)	50 mM	50 mM	50 mM	50 mM		sample collection

CK, without salt stress; LSS, light salt stress for a short time; MSL, medium-heavy salt stress for a long time.

2.2. Small RNA Library Construction and Sequencing

Three seedling roots from each treatment were mixed evenly and ground to a powder under liquid nitrogen using a mortar and pestle; 100 mg of the powder was then added to a 2.0-mL sterile Eppendorf tube. After the addition of 1 mL TRIzol reagent (Invitrogen, CA, USA), the contents were homogenized for 3 min, and then total RNA was extracted according to the TRIzol manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels and integrity was assessed using a bioanalyzer (Agilent 2100, CA, USA; RNA Integrity Number $\geq$ 7.5). The purified RNAs were used to construct three small RNA libraries (CK, LSS, and MSL). Sequencing libraries were generated using a NEBNext multiplex small RNA library preparation kit (NEB, MA, USA) following the manufacturer's recommendations. Index codes were added to attribute sequences to each sample. Briefly, a NEB 3′ SR Adaptor was directly specifically ligated to the 3′ ends of miRNA, small interfering (si)RNA, and piwi-interacting (pi)RNA. SR RT Primer was then hybridized to an excess of 3′ SR Adaptor to transform the single-stranded DNA adaptor into a double-stranded DNA molecule. The 5′ end adaptors were ligated to the 5′ ends of miRNA, siRNA, and piRNA, then first-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H^-). PCR amplification was performed using LongAmp *Taq* 2× Master Mix, Illumina SR Primer (Illumina, CA, USA), and the index (X) primer. PCR products were purified on an 8% polyacrylamide gel (at 100 V for 80 min), and DNA fragments corresponding to 140–160 bp (the length of small noncoding RNA plus the 3′ and 5′ adaptors) were recovered and dissolved in 8 µL elution buffer. Library quality was assessed on an Agilent Bioanalyzer 2100 system using DNA high-sensitivity chips. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq SR Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. The prepared libraries were then sequenced on an Illumina Hiseq 2500 platform at the Novogene Company (Beijing, China). The small-RNA sequencing data used in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE84937.

2.3. Identification of Conserved and Novel miRNAs

After removing reads containing poly-N, with 5′ adaptor contaminants, without 3′ adaptors or the insert tag, containing poly-A, T, G, C, and low-quality reads from the raw data, unique sequences of 18–30 nt were used for further analysis. First, small RNA tags with fewer than two mismatches were mapped to the *U. pumila* transcriptome in the Sequence Read Archive (SRA) database (SRP080724) using Bowtie-0.12.9 (main parameters: −v 1 −k 1) [26]. Second, the mapped small RNA tags were queried against non-coding RNAs (Ribosomal RNA (rRNA), Transfer RNA (tRNA), Small nuclear RNA (snRNA), and Small nucleolar RNA (snoRNA)) from GenBank and Rfam databases with no mismatches allowed using Bowtie-0.12.9 (main parameters: −v 0 −k 1). Any small RNA sequences with matches to these sequences were removed from the downstream analysis. Third, the remaining mapped small RNA tags were used to search conserved miRNAs in miRBase 21.0 with a maximum of two mismatches allowed using a modification of the software package mirdeep2 [27] and srna-tools-cli to obtain potential miRNAs and draw secondary structures [28]. Finally, the mapped small RNA tags that were not annotated in the previous steps were used to predict novel miRNAs.

These predictions were accomplished by exploring the secondary structure, Dicer cleavage site, and minimum free energy of the unannotated small RNA tags using available software packages miREvo [29] and mirdeep2 [27]. A potential novel miRNA precursor was required to meet the following criteria: (1) the formation of a characteristic stem-loop structure; (2) a miRNA sequence length of 20–24 nt; (3) a minimum free energy for the miRNA precursor of <-20 kcal·mol^{-1}; and (4) the existence of at least 16 common base pairs between sense and antisense miRNAs, no more than four bulges, and a maximum asymmetry of four bases between sense and antisense miRNA duplexes [30].

2.4. Prediction of miRNA Target Genes

Sequence complementarity between a miRNA and its target mRNA offers a fundamental advantage for computational analysis [31]. Target predictions were performed using psRobot_tar in psRobot [32] and through alignment with the *U. pumila* transcriptome (SRA database, SRP080724). The following criteria were then used to identify potential miRNA targets: (1) no more than four mismatches between the mature miRNA and its potential target site; (2) no more than one mismatch at nucleotide positions 1–9; (3) no more than two consecutive mismatches; and (4) no mismatches at positions 10 and 11 [30,33,34]. The extracted sequences, corresponding to *U. pumila* putative miRNA targets, were used in BLASTx searches against non-redundant protein sequences from the NCBI database to identify potential protein-coding genes in *U. pumila*. Similarities with an e-value lower than $1 \times e^{-10}$ were considered consistent hits.

2.5. Expression Analysis of miRNAs between Libraries

Expression levels of miRNA families as well as conserved and novel individual miRNAs were estimated as TPM (transcript per million) [35]. Expression profiles of miRNAs between different libraries and *p*-values were obtained using the DEGseq R package [36]. Comparisons of expression levels of each miRNA family and individual miRNA in LSS, MSL, and CK libraries was performed using the following formula:

$$fold\ change = log_2(LSS\ or\ MSL/CK)$$

2.6. Verification of miRNAs by qRT-PCR

Small RNAs were extracted using a small RNA extraction kit (BioTek, Beijing, China), according to the manufacturer's instructions. Small RNAs from each sample were then reverse-transcribed using a miRcute miRNA First-Strand cDNA Synthesis kit (Tiangen, Beijing, China) following the manufacturer's instructions. Forward primers for the miRNAs were designed based on the mature miRNA sequences, and the reverse primer was provided in the kit. 5.8S rRNA and U6 were used as endogenous internal reference genes for the miRNAs. qRT-PCR primers are listed in Table S1. Three biological replicates were run for each sample. qRT-PCR amplifications were performed on an ABI7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) using SYBR FAST qPCR Kit Universal Master Mix (2×) (Kapa Biosystems, Wilmington, MA, USA). Reaction conditions were the following: an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A dissociation curve was obtained by heating the amplicon from 60 to 95 °C. Technical repetitions were performed three times for each sample. Following qRT-PCR data collection, fold changes were calculated by the $2^{-\Delta\Delta C}{}_{T}$ method [37].

3. Results

3.1. Deep Sequencing of U. pumila Small RNAs

Three small RNA libraries were constructed and sequenced using total RNAs from *U. pumila* roots under three treatment conditions: CK, without salt stress; LSS, light salt stress for a short time; and MSL, medium-heavy salt stress for a long time. This process generated approximately 11 million, 8 million, and 9 million raw reads from the CK, LSS, and MSL libraries, respectively. After removing

adaptor sequences, low-quality tags, and reads shorter than 18 nt or longer than 30 nt, we obtained approximately 8 million, 6 million, and 7 million clean reads from the corresponding three libraries. A total of 4,175,143 reads (748,789 unique), 2,953,860 reads (602,673 unique), and 3,893,883 reads (676,645 unique) from the three respective libraries were subsequently mapped to the *U. pumila* transcriptome sequence. These mapped small RNAs comprised rRNAs, tRNAs, snRNAs, snoRNAs, known miRNAs, and unannotated small RNAs (Table 2). Among the mapped small RNAs of the three libraries, 5.24%–7.06% were additionally found to be known miRNAs and 75.28%–76.40% were unannotated small RNAs. The high proportion of small RNAs that could not be annotated suggests that some unknown sRNAs remain to be discovered in *U. pumila*. The unannotated small RNAs were used to predict novel miRNAs.

Table 2. Overview of small RNA reads from three small RNA libraries.

Category	CK		LSS		MSL	
	Total Small RNAs (%)	**Unique Small RNAs (%)**	**Total Small RNAs (%)**	**Unique Small RNAs (%)**	**Total Small RNAs (%)**	**Unique Small RNAs (%)**
Raw reads	11,073,886		8,469,247		9,159,614	
Clean reads (18–30 nt)	7,655,641	3,162,254	6,291,043	2,930,417	7,153,780	2,877,526
Clean reads mapping to transcriptome of *Ulmus pumila* L.	4,175,143 (100%)	748,789 (100%)	2,953,860 (100%)	602,673 (100%)	3,893,883 (100%)	676,645 (100%)
rRNA, snRNA, snoRNA, and tRNA	802,856 (19.23%)	44,528 (5.95%)	527,510 (17.86%)	38,240 (6.35%)	644,135 (16.54%)	37,383 (5.52%)
Known miRNA	218,706 (5.24%)	824 (0.11%)	202,718 (6.86%)	820 (0.14%)	274,876 (7.06%)	774 (0.11%)
Unannotated	3,153,581 (75.53%)	703,437 (93.94%)	2,223,632 (75.28%)	563,613 (93.52%)	2,974,872 (76.40%)	638,488 (94.36%)

CK, without salt stress; LSS, light salt stress for a short time; MSL, medium-heavy salt stress for a long time.

Most small RNAs in the three libraries were 20–24 nt in size. The most abundant small RNA sequences were 24 nt long (24.97%–30.07%), followed by 21 nt (12.59%–13.98%) and 22 nt (11.00%–12.33%) (Figure 1). These sizes and proportions are consistent with typical small RNA distributions in angiosperms such as cotton [7], *Lycium chinense* Mill. [28], *Punica granatum* L. [38], and potato [39].

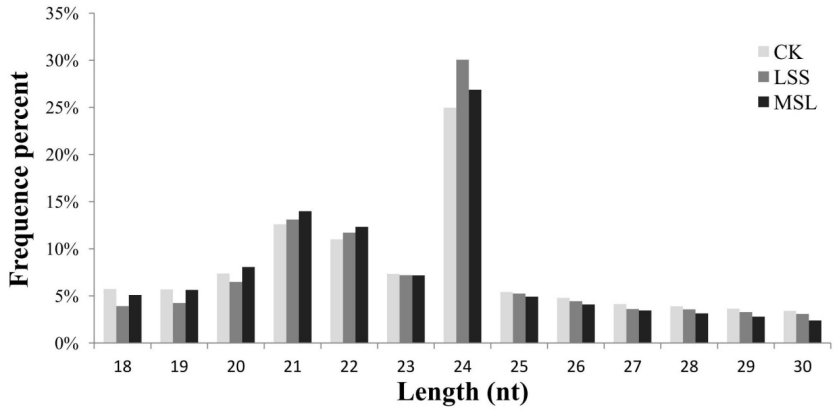

Figure 1. Small RNA length distribution in *Ulmus pumila* L.. CK, without salt stress; LSS, light salt stress for a short time; and MSL, medium-heavy salt stress for a long time.

3.2. Identification of Conserved miRNAs in U. pumila

To identify conserved miRNAs in *U. pumila*, small RNA sequences were aligned with known mature plant miRNAs in the miRBase database (release 21.0, June 2014), with transcriptome sequences

of *U. pumila* used to find inverted repeats and stem-loop structures. In total, 254 conserved miRNAs were identified in the three libraries (Table S2). The criteria used to assign these miRNAs to distinct families were described by Meyers [40], and the classification of miRNA families referenced the miRbase database. After a comprehensive analysis, 217 miRNAs have been classified into 29 miRNA families, while 37 miRNAs have not been classified. The number of members of each miRNA family was also analyzed (Figure 2A), which revealed that most families contained more than one member, with only seven miRNA families having a single member. MIR159 was the largest family with 31 members, while MIR171, MIR156, MIR166, MIR167, and MIR399 possessed 24, 20, 17, 12, and 13 members, respectively. Only a few members were identified for MIR319, MIR394, MIR477, and MIR482 families. The size of a miRNA family may be indicative of its function [41]. Different miRNA families also displayed drastically different expression levels (Figure 2B), with some miRNA families having low expression while others had high expression. Notably, MIR159, MIR162, MIR166, MIR167, MIR482, and MIR858 families were highly abundant in the three libraries, while others, such as MIR169, MIR319, MIR395, MIR477, and MIR1511, were much less abundant.

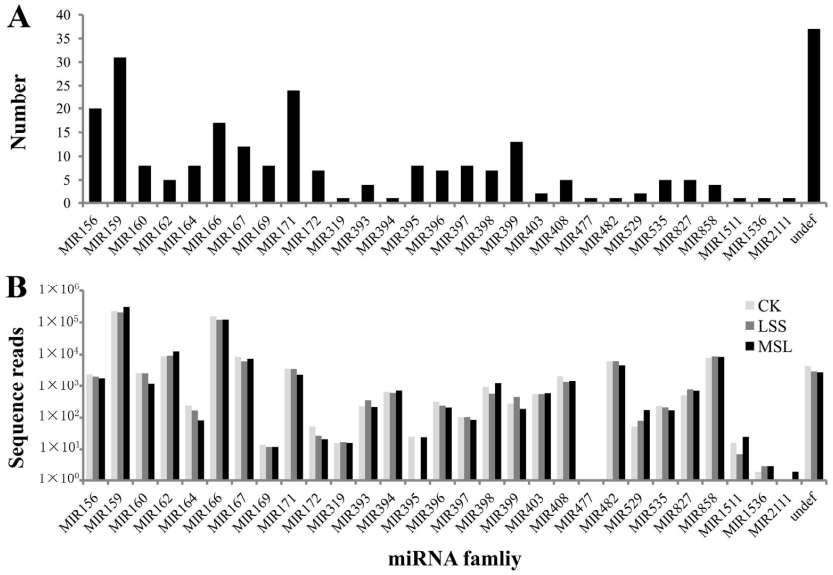

Figure 2. Number and abundance of conserved miRNAs for each miRNA family. (**A**) Number of conserved miRNAs for each miRNA family; (**B**) Abundance of conserved miRNAs for each miRNA family. CK, without salt stress; LSS, light salt stress for a short time; and MSL, medium-heavy salt stress for a long time; Undef, conserved miRNAs that have not been classified into miRNA families in the miRbase database.

3.3. Discovery of Novel miRNA in U. pumila

Novel miRNAs were predicted on the basis of the characteristics of the hairpin structure of their precursor, a feature that distinguishes them from other endogenous small RNAs [40,42]. A total of 49 novel miRNAs were identified, of which 15 had corresponding miRNAs (Table S3). Among these miRNAs, 46 were detected in at least two independent small RNA libraries, and 38 were detected in all three. The novel mature miRNA sequences were 20–24 nt long; approximately half of them were 21 nt in length. We also found that the novel miRNAs were less abundant than the conserved miRNAs. These candidate novel miRNAs are likely to be new miRNAs or new members of known miRNA families in *U. pumila*.

3.4. Prediction of Potential miRNAs Targets in U. pumila

To understand the functions and potential regulatory roles of the novel and conserved miRNAs identified in this study, putative targets were predicted using the criteria and methods described above.

In total, 232 target genes of *U. pumila* miRNAs were predicted (Table S4). In addition, 49 target genes were predicted for MIR156, while MIR159, MIR172, and MIR396 possessed 34, 16, and 14 target genes, respectively. Only a few target genes were predicted for MIR162, MIR166, MIR171, MIR394, MIR398, MIR535, and MIR1511 families. A number of upu-miRNAs (including upu-miR156/157, 159, 164, 397, and 398) were predicted to target known genes that play roles in plant responses to salt stress. These included *SQUAMOSA-PROMOTER BINDING-LIKE* (*SPL*) [43], *MYB* [44], *NAM/ATAF/CUC* (*NAC*) [45], several laccase genes [46], and copper/zinc superoxide dismutase genes [30].

3.5. miRNA Expression Profiles Between Libraries

To explore miRNA expression patterns under salt stress, the normalized expression of miRNA families in the CK library was compared with that in the LSS and MSL libraries, and their fold-changes were calculated. Compared with expressions in CK, 14 miRNA families showed an up-regulated tendency and 13 miRNA families showed a down-regulated tendency in LSS. Of these, five miRNA families (MIR399, MIR827, MIR393, MIR529, and MIR1536) were highly expressed and five miRNA families (MIR395, MIR1511, MIR172, MIR398, and MIR408) were weakly expressed (Figure 3A). In MSL, 15 miRNA families exhibited an up-regulated tendency and 12 miRNA families showed a down-regulated tendency compared with CK. Of these, seven miRNA families (MIR529, MIR1511, MIR1536, MIR162, MIR827, MIR398, and MIR159) were highly expressed and three (MIR164, MIR172, and MIR160) were weakly expressed (Figure 3B). The expression profiles of individual conserved miRNAs and novel miRNAs are shown in Tables S2 and S3. The expression patterns of these miRNAs suggest that they may play important roles in *U. pumila* under salt stress conditions.

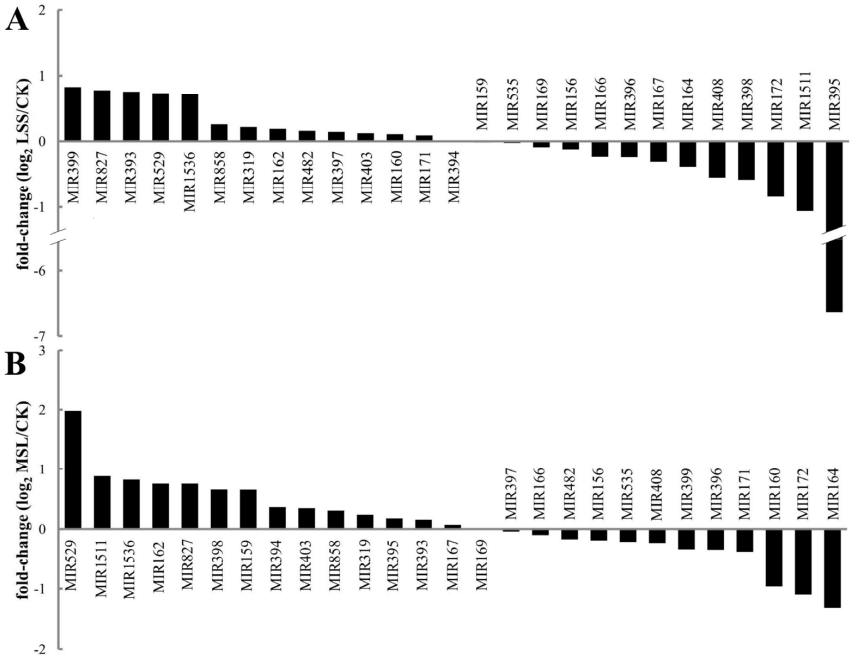

Figure 3. Expression profiles of miRNA families generated via deep sequencing. (**A**) Expression profiles of miRNA families between LSS (light salt stress for a short time) and CK (without salt stress) libraries; (**B**) Expression profiles of miRNA families between MSL (medium-heavy salt stress for a long time) and CK libraries.

3.6. Validation of Deep Sequencing Results by qRT-PCR

qRT-PCR, a reliable method to detect and measure miRNA expression levels, was used to experimentally validate the sequencing data [6,7]. In this study, six miRNAs (four conserved and two novel) were selected for validation. qRT-PCR results revealed similar expression patterns to the

sequencing results (Figure 4) and confirmed the observed changes in miRNA expression in response to salt stress. For example, upu-miR399n expression showed a 2.08-fold decline under medium-heavy salt stress for a long time (Table S2), and a similar result was reflected in the qRT-PCR results, where an approximately two-fold reduction in expression was observed (Figure 4B).

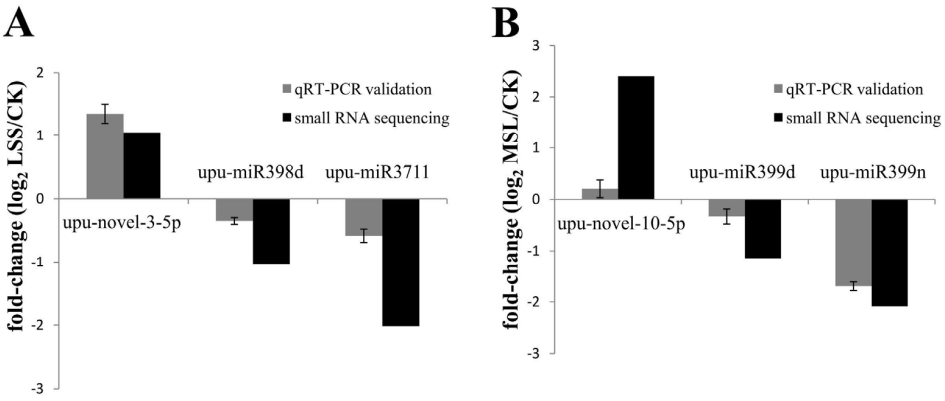

Figure 4. qRT-PCR validation of differentially expressed miRNAs in *Ulmus pumila* L. roots under salt stress. (**A**) Relative expression analysis of miRNAs by qRT-PCR analysis and high-throughput sequencing between LSS (light salt stress for a short time) and CK (without salt stress) libraries; (**B**) Relative expression analysis of miRNAs by qRT-PCR analysis and high-throughput sequencing between MSL (medium-heavy salt stress for a long time) and CK libraries. Error bars indicate standard deviations.

4. Discussion

miRNAs are a class of non-coding small RNAs that play crucial roles in many developmental processes and in responses to various abiotic stresses in plants [3,4]. Many efforts have therefore been made to discover and identify miRNAs from different plant species. Traditional methods to identify miRNAs, such as Sanger sequencing and computational prediction, are slow and complicated. Moreover, such techniques do not always detect non-conserved or species-specific miRNAs that may accumulate at lower levels [30]. High-throughput sequencing technologies provide a rapid and more efficient approach for identifying novel species-specific or low-abundance miRNAs and for profiling populations of small RNAs at different plant developmental stages and under various environmental conditions [6,38].

Since its first use in the model species *A. thaliana* [47], high-throughput sequencing has contributed greatly to miRNA discovery and has been successfully applied to many different species, including peanut [48], barley [49], peach [50], moso bamboo [51], rubber tree [52], *Nicotiana benthamiana* Domin. [53], and *Eucheuma denticulatum* (N.L.Burman) F.S.Collins & A.B.Hervey [54]. To date, 6547 hairpin sequences and 7956 mature sequences have been identified from 66 species of angiosperms. These sequences have been deposited in the miRBase database (miRBase 21.0); however, sequence information for *U. pumila* miRNAs and indeed miRNAs of Ulmaceae plants is entirely absent from miRBase.

In the present study, we obtained tens of millions of small RNA sequences from salt stressed seedling roots of *U. pumila*. The small RNA sequences were aligned with Viridiplantae miRNAs in miRBase, enabling the identification of 254 conserved miRNAs representing 29 families. This result indicated that the majority of these miRNAs, already known from *A. thaliana* and other plant species, were detectable in *U. pumila* (Table 3). We also found that MIR159 was the largest miRNA family, with 31 members. In *A. thaliana*, the MIR159 family includes only three miRNAs, while the number of MIR159 family members in *Populus trichocarpa* Torr. & Gray, *Malus domestica* Borkh., *Oryza sativa* L., and *Sorghum bicolor* (L.) Moench is five, three, six, and two, respectively. MIR166, MIR171, and MIR399 had 17, 24 and 13 members, respectively, which are higher numbers than in other analyzed

plant species (Table 3). Although these large miRNA families were usually expressed in *U. pumila* at relatively high levels (Table S2), some low-abundance miRNA families, such as MIR1536 and MIR2111, were also detected. A total of 49 novel miRNAs were identified. We also observed 15 pairs of sense and antisense miRNAs, with the antisense miRNA sequence usually paired to the corresponding miRNA with two overhanging 3′ nucleotides (Table S3). This observation indicates that the pairs of novel sense and antisense miRNAs originated from DCL1 processing, providing further evidence that they are true miRNAs [47,55,56].

Table 3. miRNA family members in *Ulmus pumila* L. and other plant species according to miRBase 21.0, and *U. pumila* miRNA family predicted targets.

miRNA family	*Ulmus pumila* L. *	*Arabidopsis thaliana* (L.) Heynh.	*Populus trichocarpa* Torr. & Gray	*Malus domestica* Borkh.	*Oryza sativa* L.	*Sorghum bicolor* (L.) Moench	Predicted Targets #
MIR156	20	14	12	29	12	9	49
MIR159	31	3	5	3	6	2	34
MIR160	8	3	8	5	6	6	1
MIR162	5	2	2	2	2	1	2
MIR164	8	3	6	6	6	5	3
MIR166	17	9	17	9	13	11	2
MIR167	12	4	8	10	10	9	3
MIR169	8	14	33	6	18	17	6
MIR171	24	4	13	15	9	11	2
MIR172	7	5	9	15	4	6	16
MIR319	1	3	9	3	2	2	2
MIR393	4	5	3	6	2	2	0
MIR394	1	2	2	2	1	2	2
MIR395	8	6	11	9	25	12	12
MIR396	7	2	7	7	8	5	14
MIR397	8	2	3	2	3	1	5
MIR398	7	3	3	3	6	1	2
MIR399	13	6	10	10	11	11	9
MIR403	2	1	4	2	0	0	0
MIR408	5	1	1	4	1	1	4
MIR477	1	0	4	2	0	0	0
MIR482	1	0	4	4	0	0	4
MIR529	2	0	0	0	2	1	11
MIR535	5	0	0	4	1	0	2
MIR827	5	1	1	1	1	0	0
MIR858	4	2	0	1	0	0	6
MIR1511	1	0	0	1	0	0	2
MIR1536	1	0	0	0	0	0	0
MIR2111	1	2	2	2	0	0	0

* number of miRNA family members for *U. pumila* according to our study data; # number of *U. pumila* miRNA family predicted targets.

Because of the unavailability of *U. pumila* genome sequences and the limited number of DNA and RNA sequences in public databases, a mRNA transcriptome (poly(A) enrichment of the mRNA) from *U. pumila* seedlings under different salt treatments was sequenced as a reference (SRA Database, SRP080724). Transcriptomes, however, cannot capture true pre-miRNA sequences because the latter are very short and devoid of poly-A tails; consequently, the sequences mapped by miRNA were likely pri-miRNAs [57]. Based on the hairpin structural characteristics of pre-miRNA, pre-miRNA sequences could subsequently be obtained from pri-miRNA sequences using bioinformatics approaches [27,28].

To help understand the functions of miRNAs in the regulation of salt defense responses in *U. pumila*, a total of 232 target genes were predicted, and miRNA expression patterns between different libraries were analyzed. Unlike most miRNA families, MIR393, MIR403, and MIR2111 had no predicted target genes. Many predicted miRNA targets are functional genes that play crucial roles in plant defense against different abiotic stresses. For instance, the copper/zinc superoxide dismutase gene, a predicted target of upu-miR398, is involved in plant response to abiotic stresses [58]. The MIR398 family tended

to be down-regulated in LSS compared with CK, consistent with previous studies in *A. thaliana* and *Caragana intermedia* Kuang et H. C. Fu where miR398 expression was inhibited under salt stress [30,59]. The phosphoinositide phosphatase gene was predicted to be a target of upu-miR399, a result that has been verified in other plants [60,61]. The MIR399 family showed a down-regulated tendency in MSL compared with CK, consistent with previous research on *Brassica napus* L. in which miR399 expression was inhibited under salt stress [62]. Finally, the expressions of upu-miR398d and upu-miR399d were validated by qRT-PCR. These results suggest that the target mRNAs (stress tolerance-related genes) may be modulated by the different expression of their corresponding miRNAs in *U. pumila* during adaptation to salt stress.

This is the first report on *U. pumila* miRNAs and their target genes under salt stress. Our findings provide an overview of potential miRNAs and corresponding targets involved in the regulation of salt defense responses in *U. pumila*. Future investigations using supplementary experimental methods to verify the targets and determine the regulatory gene network underlying differential miRNA expression are required to provide novel information about the regulatory network of *U. pumila* under salt stress.

5. Conclusions

In this study, we used small RNA high-throughput sequencing and transcriptome sequences of *U. pumila* to identify 254 conserved miRNAs representing 29 families as well as 49 novel miRNAs and 232 potential miRNA targets. Expression profiling of miRNAs between libraries was performed, with the expressions of six miRNAs validated by qRT-PCR. This study provides data that can be used for further research into the molecular mechanisms involved in salt stress resistance of *U. pumila* and other Ulmaceae species.

Supplementary Materials: The following is available online at www.mdpi.com/1999-4907/7/12/318/s1. Table S1: qRT-PCR-validated miRNAs and their primers, Table S2: Conserved miRNAs identified in *Ulmus pumila* L., Table S3: Novel miRNAs identified in *Ulmus pumila* L., Table S4: Identification of putative targets for *Ulmus pumila* L. miRNAs.

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