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Research paper

Identification and characterization of salt-responsive microRNAs in *Populus tomentosa* by high-throughput sequencing

Yuanyuan Ren [1], Lei Chen [1], Yiyun Zhang, Xiangyang Kang, Zhiyi Zhang*,[2], Yanwei Wang*

National Engineering Laboratory for Tree Breeding, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, College of Biological Sciences and Biotechnology, Beijing Forestry University, 100083 Beijing, People's Republic of China

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ABSTRACT

Salt is one of the main environmental factors limiting plant growth and a better understanding of mechanisms of salt stress would aid efforts to bolster plant salt tolerance. MicroRNAs are well known for their important regulatory roles in response to abiotic stress in plants. In this study, high-throughput sequencing was employed to identify miRNAs in *Populus tomentosa* plantlets treated or not with salt (200 mM for 10 h). We found 141 conserved miRNAs belonging to 31 families, 29 non-conserved but previously-known miRNAs belonging to 26 families, and 17 novel miRNAs. Under salt stress, 19 miRNAs belonging to seven conserved miRNA families were significantly downregulated, and two miRNAs belonging to two conserved miRNA families were upregulated. Of seven non-conserved miRNAs with significantly altered expression, five were downregulated and two were upregulated. Furthermore, eight miRNAs were validated by qRT-PCR and their dynamic differential expressions were analyzed. In addition, 269 target genes of identified miRNAs were predicted and categorized by function. These results provide new insights into salt-responsive miRNAs in *Populus*.

1. Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs, approximately 21 nucleotides (nts) in length, that are predominantly derived from intergenic regions and generated from single-stranded precursors with unique hairpin structures [1]. They were initially discovered in *Caenorhabditis elegans* [2] and subsequently found to be widespread in plants [3], animals [4], and some viruses [5]. It is widely accepted that miRNAs play important regulatory roles in many developmental and defense-related processes, such as tissue identity, developmental timing, and environmental stress response [6]. In recent years, increasing evidence has been found that miRNAs are involved in plant responses to a wide variety of abiotic stresses, including high salinity [7–9], drought [10–13], low temperatures [8,14], oxidative stress [15], hypoxic stress [16,17], UV-B radiation [18], and mechanical stress [18,19].

Salt stress is one of the most serious abiotic stresses threatening plant growth. To cope with high salinity stress, plants have evolved multiple gene regulatory profiles involving a broad spectrum of biochemical, cellular, and physiological processes, such as energy metabolism, signal transduction, transcription, protein biosynthesis and decay, membrane trafficking, and photosynthesis [20]. The transcriptional regulation of many miRNAs and genes in response to high salinity stress has been widely reported [21,22]. In *Arabidopsis*, miR393 was strongly upregulated by treatment with 300 mM NaCl [23], and a microarray-based analysis discovered 10 other miRNAs with altered expressions in response to salt treatment [8]. In rice, miR169g was reported to be upregulated during high salinity stress, and analysis of transgenic plants that overexpressed miR393 revealed that they were more sensitive to salt treatment than control plants [24,25]. Recently, the upregulation of miR395, miR398, and miR399 in *Populus tremula* treated with salt was detected using microarrays [7], while miR398 was found to be downregulated in salt-treated *Arabidopsis* [26]. In *Populus trichocarpa*, environmental stimuli including salt stress were shown to influence the expression of a large number of miRNAs [27]. Other research has revealed that miR168, miR169, miR1444, and miR1446 expression levels were altered in salt-shocked *Populus euphratica*

Abbreviations: miRNAs, microRNAs; nts, nucleotides; sRNAs, small RNAs; qRT-PCR, quantitative real-time PCR; miRNA*, microRNA star; S, salt treatments; C, controls.

* Corresponding authors. Tel./fax: +86 010 62336248.
E-mail address: ywwang@bjfu.edu.cn (Y. Wang).

[1] Contributed equally to this work.
[2] Deceased.

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[28]. Further studies on miRNA expression in response to salt stress in plants are still needed, however; this is especially true for white poplar, an important commercial tree species. In addition, there have been few reports on the systemic identification of salt-responsive miRNAs in woody plants at the genome-level using high-throughput sequencing.

Populus tomentosa is an economically important species, widely distributed throughout northern China where it is endemic. It is also commonly used as a model system in transgenosis studies and biomolecular research. Understanding the role of *P. tomentosa* miRNAs in response to salt stress may expand the screening of gene function and regulation in trees, and contribute to more efficient poplar tree breeding. In this study, high-throughput sequencing, which has been widely used for miRNA research [10,12–14,29,30], was used to identify conserved and novel miRNAs of *P. tomentosa*. The altered expression levels of these miRNAs under salt treatment were analyzed and compared with controls, and the potential roles of their target genes were investigated.

2. Material and methods

2.1. Plant treatment and small RNA sequencing

P. tomentosa plantlets were culture-grown on half-strength Murashige–Skoog (MS) medium containing 20 g L^{-1} sucrose and 0.3 mg L^{-1} IBA at 25 °C under a 16/8 h (light/dark) photoperiod. The salt treatment was performed as described [27] with some modifications. After 60 days of growth, plantlets were transferred into 200 mM NaCl liquid culture medium and treated for 10 h. Untreated plantlets were used as controls. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Construction of small RNAs (sRNAs) cDNA libraries was performed as described in Sunkar and Zhu [23]. Briefly, total RNA (20 μg) was separated on a 15% denaturing polyacrylamide gel, and sRNAs of 18–26 nts were recovered. The isolated sRNAs were then sequentially ligated to adapters, reverse transcribed, and amplified by PCR. Finally, Solexa sequencing was employed to sequence the sRNAs at Beijing Genomics Institute (BGI), Shenzhen, China.

2.2. sRNA bioinformatics analysis

Raw sequence reads were converted into clean full-length reads by removing all low-quality reads, adapter reads, contaminants, and reads smaller than 18 nts. The remaining high-quality sRNA sequences were mapped to *Populus* genome sequences obtained from the US Department of Energy Joint Genome Institute (JGI) (http://www.jgi.doe.gov/) using SOAP [31]. sRNA sequences were categorized into classes, including miRNAs, siRNAs, rRNAs, tRNAs, snRNAs, snoRNAs, repeat-associated sRNAs, and degraded tags of exons or introns, and annotated by performing Blastn searches against Rfam (http://www.sanger.ac.uk/Software/Rfam) and NCBI databases (http://www.nabi.nlm.nih.gov/blast/Blast.cgi). Unique miRNA sequences were aligned with known miRNA sequences found in miRBase 17.0 (http://microran.sanger.ac.uk/sequence/index.html/). From the miRNA sequences that could not be annotated, potential novel miRNAs were identified by using MIREAP (http://sourceforge.net/projects/mireap/) and mfold (http://mfold.rna.albany.edu/?q=mfold/) to fold flanking sequences and predict secondary structures [32]. Target predictions were performed using psRNATarget (http://plantgrn.noble.org/psRNATarget/) [33,34]. Based on their location in the *Populus* genome, putative target genes were manually selected from these candidates and subsequently mapped to function by carrying out BLAST searches against the JGI Gene Transcript v2.0 database.

2.3. Expression analysis of miRNAs under salt stress

MiRNA differential expression analyses were based on sequence reads generated from the salt treatment and control libraries. The expressions of miRNA in the two libraries were normalized to obtain the number of miRNAs per million reads [normalized expression = (number of miRNA reads/total number of clean reads) *1,000,000]. Normalized miRNA reads with values less than 1 were excluded from the differential analysis. The remaining normalized reads were used to calculate the change in miRNA expression (fold change = normalized miRNA reads in salt treatment/normalized miRNA reads in control) and *P*-value [35,36]. The *P*-value was obtained according to the calculations as follows:

$$P(x|y) = \left(\frac{N_2}{N_1}\right)\frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y\min|x) = \sum_{y=0}^{y\le y\min} \mathrm{p}(y|x)$$

$$D(y \ge y\max|x) = \sum_{y\ge y\max}^{\infty} \mathrm{p}(y|x)$$

2.4. Quantitative real-time PCR analysis of miRNA expression

Identified *P. tomentosa* miRNAs were experimentally validated using quantitative real-time PCR (qRT-PCR). For the salt treatments, 60-day-old plantlets were treated for 0 h, 2.5 h, 5 h, 7.5 h, and 10 h with 200 mM NaCl. Untreated plantlets were used as controls [27]. qRT-PCR was carried out as previously described [19,37], with 5.8S rRNA used as the endogenous reference. All reactions were run in triplicate for each sample. Validated miRNAs and primers are listed in Table S1. SYBR Green Real-Time PCR Master Mix (Takara, Shiga, Japan) and an ABI 7500 Fast Sequence Detection system were employed to detect and compare miRNA expression levels. The $2^{-\Delta\Delta CT}$ relative quantization method [38] was used to analyze relative changes in gene expression during the qRT-PCR experiments.

3. Results

3.1. Deep sequencing of P. tomentosa sRNAs

A total of 24,625,499 (C) and 24,531,819 (S) raw reads were generated from Solexa Sequencing of the two libraries. After processing, 22,359,561 (C) and 22,970,152 (S) clean reads remained for further analysis. Most of the sRNA sequences obtained from either library were 21–24 nts long (Fig. 1). 21-nt sRNAs were the most numerous, comprising approximately 35% of the total number of sRNAs, with 24-nt sRNAs the second most frequent, averaging approximately 26%. In some plant species, such as *Arabidopsis*, rice, oranges, peanuts, and *Medicago truncatula*, 24-nt sRNAs have been found to be substantially more abundant than 21-nt sRNAs [29,30,39–42]. In contrast, 21-nt sRNAs were more numerous than 24-nt sRNAs in grape, tomato, *Populus balsamifera*, *P. euphratica* and *Pinus* [10,42–44]. These contrasting situations may reflect the fact that 21-nt sRNAs are transcriptionally silenced more frequently in herbs than in trees and vines. These 21-nt sRNAs are often highly-expressed miRNAs and *trans*-acting siRNAs [45] and it should be noted that, in our study, the percentage of known miRNAs matched to the *Populus* genome averaged 34%, nearly the same as the