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Multi-Omics Analysis of Gene and microRNA Expression in Diploid and Autotetraploid Poplar under Drought Stress by Transcriptome, microRNA, and Degradome Sequencing

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Abstract: Drought-induced forest death has become a global phenomenon, which is hindering the development of sustainable forestry. Polyploidy breeding has been considered as an effective method of genetic improvement for tree stress resistance. However, the response mechanisms of tetraploid poplars to drought stress are unclear. In this study, based on high-throughput sequencing of transcriptome, small RNA, and degradome for these samples, which selected three genotypes of tetraploid poplars and their counterpart diploids for drought stress and rewatering trial in the experiment, we performed multi-omics analyses to investigate the distinction in drought resistance between tetraploid and diploid. A total of 3391 differentially expressed genes (DEGs) were found from the Dro-Di vs. CK-Di, 3753 DEGs from the Re-Di vs. Dro-Di, 3857 DEGs from the Dro-Te vs. CK-Te, and 4177 DEGs from the Re-Te vs. Dro-Te. Of the above DEGs, 1646 common-DEGs were identified significantly related to drought-stress response, 2034 common-DEGs related to rewater response, 158 and 114 common-DEGs showed opposite expression patterns between diploid and tetraploid, implying that these DEGs might play important roles in response to drought stress as a result of differences in ploidy. Additionally, 586 known miRNAs and 72 novel miRNAs were identified through analysis of 18 small RNA libraries, among which eight common-miRNAs were significantly related to drought-stress response, and four were related to rewater response. The degradome sequencing analysis revealed that 154 target transcripts for 24 drought-stress-associated differentially expressed miRNAs (DEmiRs), and 90 for 12 rewatering-associated DEmiRs were identified in the tetraploid based on both degradome and TargetFinder analyses. These findings provide valuable information for further functional characterization of genes and miRNAs in response to drought stress in *Populus* polyploidy, and potentially contribute to drought-resistant breeding of polypoid in the future.

Keywords: tetraploid; polyploidy; drought; transcriptome; microRNA; degradome

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1. Introduction

The issue of drought is becoming increasingly serious against the background of global warming, and drought-induced forest death has become a global phenomenon, which is hindering the development of sustainable forestry [1,2]. Forest trees have evolved various mechanisms to control their stress response, including morphological adaptation, physiological and biochemical regulation, and gene expression regulation [3,4]. In particular, polyploidization results in changes in chromosome number and structure, and further alters the expression of phenotypic and functional traits, which make polyploids more resistant and adaptable to harsh environmental conditions than diploids [5]. This is commonly found in high-altitude and climate-change regions [6]. For instance, it is shown

that tetraploids have greater tolerance to drought stress than their diploid counterparts in species such as cassava [7], trifoliate orange [8], *Ficus carica* [9], *Paulownia* [10], *Populus ussuriensis* [11], *Betula* [12], and apple [13]. Thus, there is mounting evidence that polyploid breeding is an effective method of tree genetic improvement for resistance to drought stress.

Poplars are a highly adaptable species with a wide distribution, and their timber is commonly used for woodcraft, plywood, pulp and paper, and biofuel production [14–16]. As one of the centers of origin for natural poplars, China has abundant germplasm resources of *Populus* spp. (Section Tacamahaca). However, due to the effects of global warming, such as changes in precipitation patterns, extensive degradation and mortality of poplars have been occurring in the "Three-North" Shelterbelt [17]. Polyploid breeding in poplars has shown great potential for the development of the "Three-North" Shelterbelt, due to a combination of ploidy advantage and heterozygosity advantage. Several key technical problems of poplar polyploid induction technology have been solved in recent years, including 2n pollen induction, protoplast fusion, and chromosome doubling in somatic or zygote cells. These offer new opportunities to create poplar varieties through genetic manipulation and selection for the purpose of improvement in growth rate, wood quality, and stress resistance [18]. For example, *Populus tomentosa* triploids have shown better tolerance to drought stress and resistance to disease than diploid ones, making them more suitable for extreme mountain conditions [19]. A previous study has shown that *Populus* tetraploids exhibit slower vegetative growth [20], but their tolerance and stress response to drought stress remain unknown. It is well known that plants respond to drought stress by regulating gene expression at the transcriptional and post-transcriptional levels to enhance their tolerance [21]. In poplar, recent studies have pinpointed crucial genes that actively react to drought stress [22–24], found that miRNAs play vital roles in regulating drought stress gene expression in poplar by mediating translational inhibition of their mRNA or target RNA cleavage [25,26], and identified miRNAs and their target genes that are responsive to drought stress using computational methods for genome-wide analysis in *P. trichocarpa* [27].

In the past few years, breakthroughs in molecular biology technology have made it possible to perform multiple-omics analyses that can help us explore the molecular mechanisms of stress resistance in plants. To better comprehend how tetraploid poplars respond to drought stress, this study selected three genotypes of tetraploid poplars and their counterpart diploids for drought stress and rewatering trial, and the first multi-omics analyses of transcriptome, small RNA, and degradome were conducted to uncover the potential mechanisms that account for the variance in drought resistance between tetraploid and diploid. The findings provide a molecular foundation for studying drought tolerance and conducting functional genomic researches in poplars.

2. Materials and Methods

2.1. Plant Material and Treatment

Three diploid (2n = 2x = 38; Di) full-sib progeny genotypes [(*P. pseudo-simonii* × *P. nigra* 'Zheyin3#') × (*P.* × *beijingensis*)] and their autotetraploid (2n = 4x = 76; Te) clones were involved in the drought and rewatering trial. The autotetraploids were obtained by in vitro leaf explants of these diploids with colchicine treatment. In April 2018, the cuttings of these autotetraploid and diploid clones were planted in pots (diameter: 27 cm upper, 24 cm lower; height: 27 cm) filled with a mixed media consisting of clay, sand, and peat (1:1:1 in volume) in the greenhouse of Beijing Forestry University. They were watered periodically to keep the media's relative water content at 75% until the start of the drought treatment on August, 2018 when they were four months old.

Nine plants of each clone were randomly divided equally into three groups, arranged randomly as control (CK), drought treatment (Dro), and rewatering after water stress (Re). Based on the pre-experiment, it was observed that significant morphological changes and the relative water content dropped to less than 20% after 10 days of drought stress, and drought-stressed plants recovered three days after rewatering. Leaves of the plants were

thus sampled separately from Dro after 10 days, from CK at the same time as Dro, and from Re after rewatering of three days in the experiment, and a total of 54 leaf samples were collected. These samples were promptly frozen in liquid nitrogen, and stored at −80 °C.

2.2. Construction and Sequencing of Transcriptome, sRNA, and Degradome Libraries

These samples were ground on ice, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. The quantity and purity of the total RNA was assessed with NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA), and the integrity of the total RNA was assessed by Agilent 2100. For each ploidy type, equal amounts of total RNAs extracted from three genotypes were mixed together as a sample for each treatment. A total of 18 samples were obtained, and were separately used for construction of mRNA-Seq and small RNA libraries. A degradome library was constructed for each ploidy with the RNA from the three biological replicates under three different treatments (CK, Dro, and Re) pooled in equal amounts, according to the method described by German et al. [28] and Ma et al. [29] with minor modifications. Subsequently, all the libraries were applied for sequencing on the Illumina Hiseq 2000/2500 (LC Sciences, Hangzhou, China).

2.3. Analysis of Transcriptome Sequencing Data

The clean reads were aligned to the reference genome of *Populus trichocarpa* using Hisat2 tool [30]. The expression levels of the mRNAs were determined with StringTie, calculating fragments per kilobase of gene per million mapped reads (FPKM). The mean FPKMs from three biological replicates were conducted for differential expression analyses. Differentially expressed genes and mRNAs were identified based on log2 (fold change) >1 or log2 (fold change) < −1 and with statistical significance (p-value < 0.05) using the R package.

2.4. Analysis of Small RNA Sequencing and miRNA Identification

An in-house program, ACGT101-miR (LC Sciences, Houston, TX, USA), was used to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats from raw reads. Unique sequences within the length range of 18~25 nucleotides were then aligned against species-specific miRNA precursor sequences available in the miRBase 22.0 database (http://www.mirbase.org/; 1 July 2019), specifically targeting *Populus trichocarpa*. This alignment was performed using BLAST searches to identify both known and novel miRNAs. Differentially expressed miRNAs were identified on the basis of normalized deep-sequencing levels in tetraploid and diploid under different treatments using a t-test and ANOVA with significance thresholds of 0.01 and 0.05, respectively.

2.5. Analysis of Degradome Sequencing Data and miRNA Targets Identification

The candidate targets were identified in the degradome sequences for both known and novel miRNAs using two software tools: the ACGT101-DEG program (LC Sciences, Houston, TX, USA) and the CleaveLand 3.0 software package [31]. Meanwhile, the miRNAs identified were aligned to unique reads from the degradome sequence, and these alignments were scored according to established criteria for plant miRNA/target pairings [32]. Specifically, alignments with a score of no more than four, and the presence of at least one raw read at the cleavage site, were considered as candidate target transcripts. Given the abundance of the resulting mRNA tags in relation to the overall degradome reads matching the target [33], the identified target genes were categorized into five groups: 0, 1, 2, 3, and 4.

Futhermore, the annotation of the candidate target genes was carried out using the GO (http://www.geneontology.org/; 1 July 2019) and KEGG (http://www.genome.jp/kegg/; 1 July 2019) pathway databases. This allowed for a more comprehensive understanding of the functional roles and pathways associated with these target genes.

2.6. Verification of RNA-seq Data by qRT-PCR Analysis

To validate the RNA-Seq results, eight common-DEGs from diploid and tetraploid poplars were selected to evaluate with qRT-PCR. The template strands for qRT-PCR were prepared using the FastKing cDNA Kit (KR116 TIANGEN, Beijing, China). The 7500 Fast system was utilized for both the PCR process and analysis of the fluorescence signal, with three technical replicates performed per gene [34]. A list of all the primers used for the qRT-PCR are presented in Table S1.

3. Results

3.1. mRNA Expression Profiling

A total of 775.1 million raw reads were generated from 18 transcriptome libraries accounting for approximately 96.3 G raw bases, and the ratio of valid bases is above 94%. After removing the adaptors and low-quality sequences, the valid reads ranged from 30.9 million to 69.3 million per library. Over 26.9 million total mapped reads were obtained in each library by alignment of the clean reads to the reference genome, and the ratio of the total mapped reads to the total clean reads varied from 85.77% to 88.24% for all 18 libraries (Table S2). These demonstrated that the sequencing quality was high.

Genome-wide RNA-sequencing analyses showed that 3391 differentially expressed genes (DEGs) (1414 up-regulated, 1977 down-regulated) were obtained from the Dro-Di vs. CK-Di; 3857 DEGs (1864 up-regulated and 1993 down-regulated) from Dro-Te vs. CK-Te (Figure 1); and 3753 DEGs (2144 up-regulated, 1609 down-regulated) from Re-Di vs. Dro-Di; and 4177 (2184 up-regulated, 1993 down-regulated) from Re-Te vs. Dro-Te (Figure 2). Of the above DEGs, 1646 common-DEGs were identified related to drought-stress response, with 791 (48%) up-regulated and 697 (42%) down-regulated in both diploid and tetraploid. Annotation through GO and KEGG analysis showed that these common-DEGs were mainly involved in biosynthetic process, carbohydrate metabolism, lipid metabolism, energy metabolism, nucleotide metabolism, signal transduction, transmembrane transport, and a wide variety of enzyme activities (Figure 3). There were 2034 common-DEGs identified as related to rewater response, of which 887 (43%) were up-regulated and 1033 (50%) were down-regulated in both diploid and tetraploid. These common-DEGs were mainly correlated with metabolic processes such as amino acids, lipids, carbohydrates, cofactors, and vitamins, biosynthesis of secondary metabolites, signal transduction, transmembrane transport, and various enzyme activities (Figure 4). These indicated that the common-DEGs related to drought stress and rewater response were mostly the same in both ploidies.

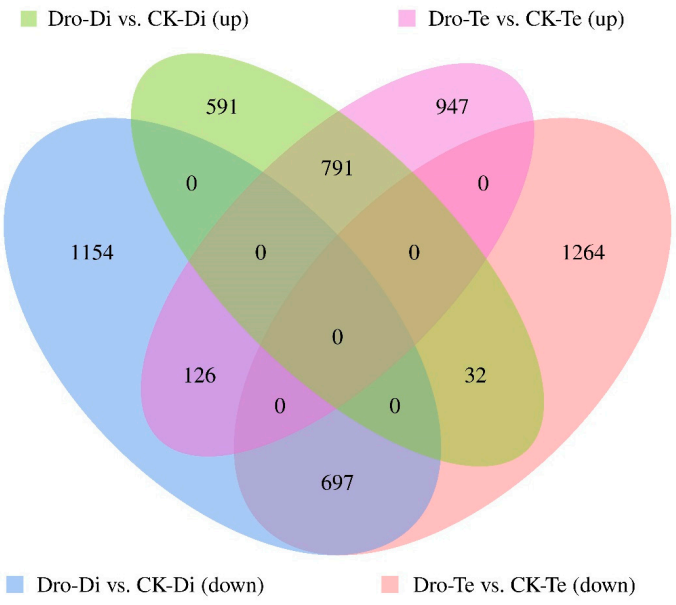


Figure 1. Gene expression profile changes between tetraploid and diploid during drought condition.

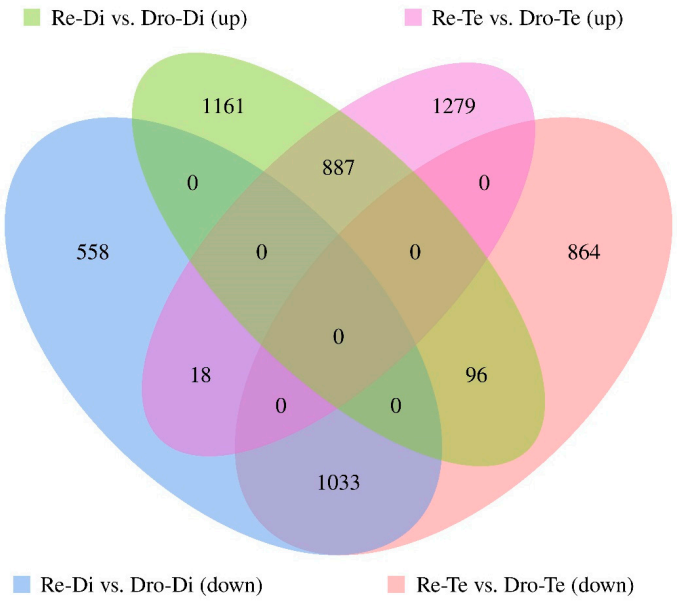
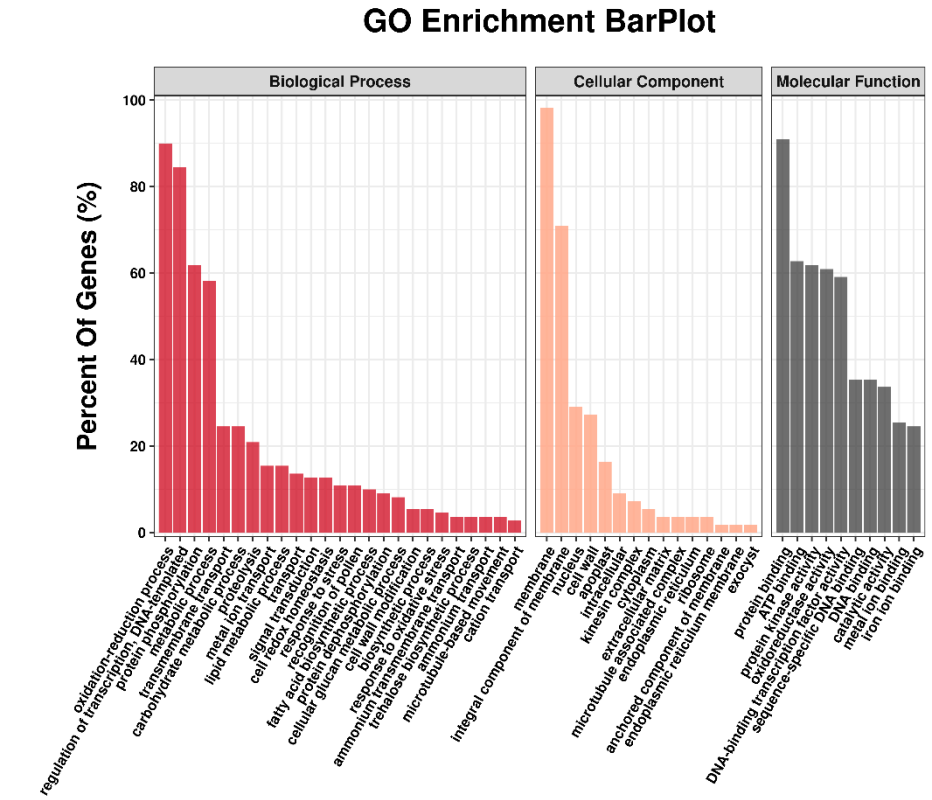


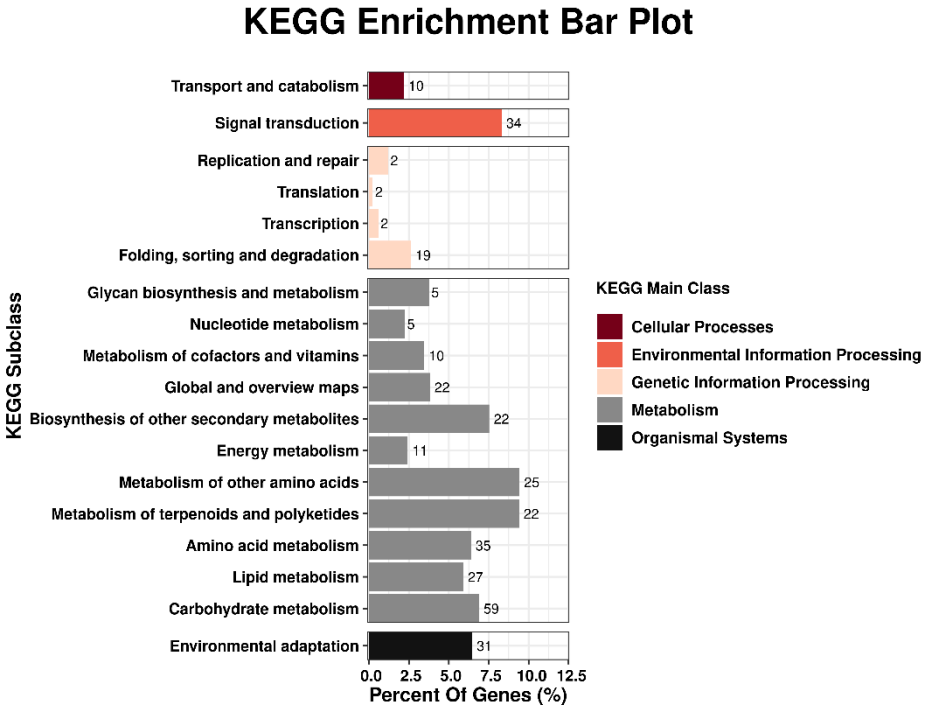
Figure 2. Gene expression profile changes between tetraploid and diploid during recovery condition.

There were 158 and 114 common-DEGs identified as related to responses to drought stress and rewatering, respectively. These DEGs all showed opposite expression patterns between diploid and tetraploid, implying that these DEGs might play an important role in response to drought stress as a result of differences in ploidies (Tables S3 and S4). Six transcription factor families were found to be involved in transcriptional regulation in response to drought stress in tetraploid poplars compared with diploid ones. Up-regulated expression genes contained the NAC transcription factor gene *NST1*, C3H transcription factor gene *OZF2*, MYB transcription factor gene *MYB52*, and TALE transcription factor gene *BLH11*; and down-regulated expression genes consisted of the C3H transcription factor gene *KHZ2*, GRF transcription factor gene *GRF7*, and G2-like transcription factor gene *AT3G10760*. After rewatering, five transcription factor families in tetraploid were involved in transcription regulation in response to water stimulation, including: the ZF-HD transcription factor gene *HB34*, which was up-regulated; and the C3H transcription factor gene *OZF2*, the WRKY transcription factor gene *WRKY70*, the B3 transcription factor gene *NGA4*, and the NAC transcription factor gene *NAC090*, which were down-regulated. Compared with diploid, protein kinases were activated in tetraploid, as seen in the cell-mediated signaling pathway, which also involves protein phosphorylation, up-regulating the expressions of *CIPK21*, *NCRK*, *MDIS1*, *MIK2*, *AT1G35710*, and downregulating those of *AT1G33940*. After rewatering, the expression of *AT1G33940* was up-regulated, and the expressions of *AT1G56130*, *CRK25*, *BIR1*, *NILR1*, *LECRK-S.5*, *CRK10*, *RK3*, *RLK1*, *LRK10L1.2*, *AT4G27290* and *AT1G51790* were down-regulated in tetraploid.

Many plant hormones have been identified as signaling molecules involved in the regulation of physiological processes in plants under environmental stress. Under drought stress, four up-regulated expression genes were identified in tetraploid poplars compared with diploid ones, including: *SAG12*, which increases cytokinin biosynthesis in response to senescence; *IRX1*, a positive regulator involved in the abscisic acid biosynthesis process; and *SYD* and *ILL6*, both of which are involved in the regulation of the jasmonic acid-mediated signaling pathway. Meanwhile, three down-regulated expression genes were identified related to drought-stress response, including: the linking protein *DAF*; *LOX2*, which responds to the biosynthesis of jasmonic acid; and *LHW*, a growth hormone-responsive transcription protein. After rewatering, down-regulated expression of genes such as: *AT5G08350*, which induces the expression of abscisic acid; *ACS6*, which responds to ethylene biosynthesis; *JAV1*, which regulates the jasmonic acid-mediated signaling pathway; and *ALD1*, which regulates the salicylic acid-mediated signaling pathway.

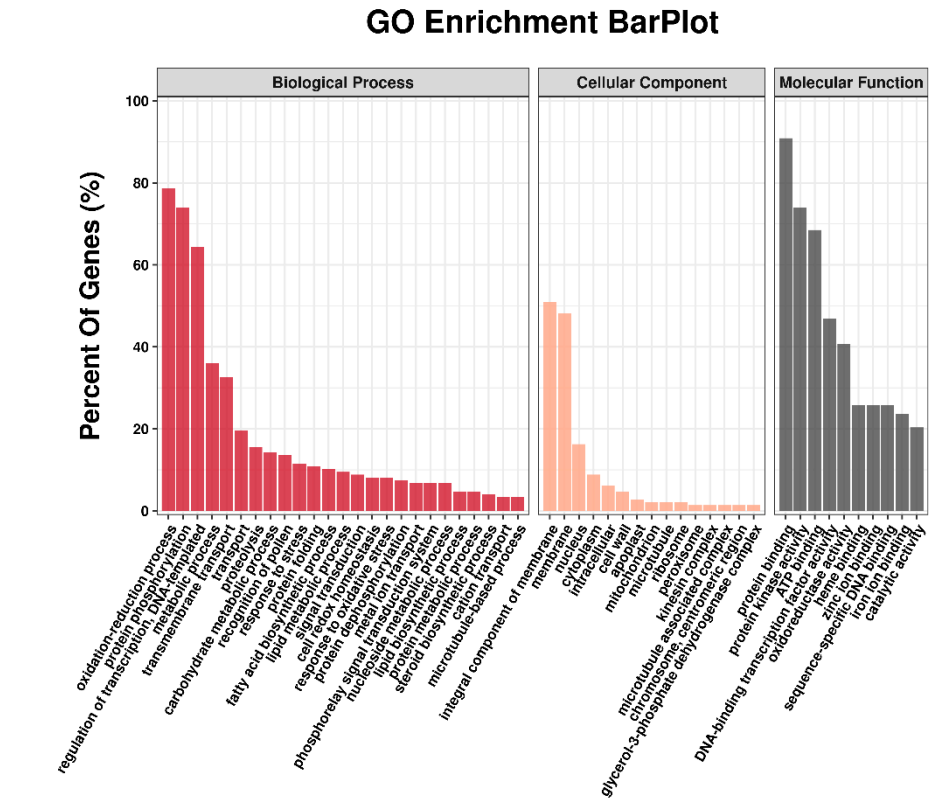


(**A**)

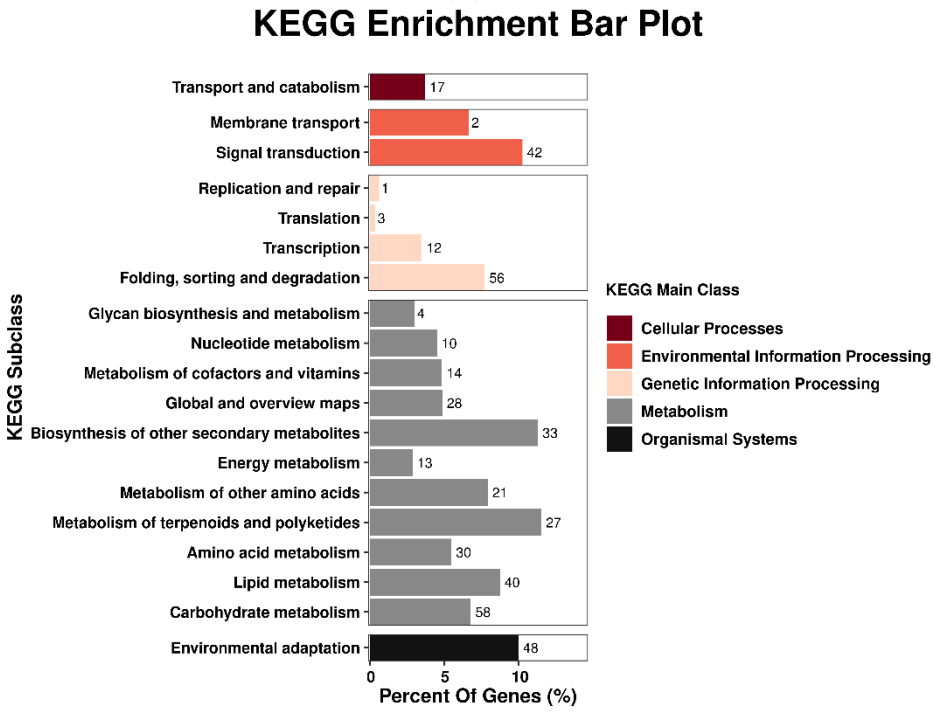


(**B**)

Figure 3. The GO enrichment (**A**) and Classification of KEGG pathway (**B**) analysis of co-expressed co-DEGs on the compared tetraploid with diploid during drought conditions.



(**A**)



(**B**)

Figure 4. The GO enrichment (**A**) and Classification of KEGG pathway (**B**) analysis of co-expressed co-DEGs on the compared tetraploid with diploid during recovery condition.

Transmembrane transport plays a significant role in drought tolerance. In tetraploid poplars: up-regulated genes encoding amino acid transporter proteins *CAT6* and *LAX3*, nucleotide/sugar transporter family protein *UXT1*, phosphate transmembrane transporter protein *PHT4*, and transmembrane transporter *AT2G16990* and *ABCB21*; and down-regulated expression genes consisted of transmembrane transporter *AT1G65720*, *AT1G53035*, and *AT5G16740*, compared with diploid poplars. After rewatering, the expressions of transmembrane transporter *AT1G65720* was up-regulated, and the expressions of the transmembrane amino acid transporter protein *AT1G47670*, the osmotic protein *OSM34*, and the ion transmembrane transporter *VDAC2* were down-regulated in tetraploid.

Calcium-binding proteins are a class of calcium receptor proteins widely distributed in eukaryotic cells that regulate several important physiological functions in cells by mediating Ca^{2+} signaling. Under drought stress, the calcium-mediated signaling gene *IQD10* and the calcium-binding protein gene *RBOHE* were up-regulated in poplar tetraploids; after rewatering, the calcium-mediated signaling genes *GLR2.7* and *CML11* were down-regulated. Other key pathway proteins (*TUA2*) involved in response to drought stress were up-regulated in poplar tetraploids.

It was shown from the qRT-PCR results that of eight genes (Potri.002G030900, Potri.004G059600, Potri.011G153300, Potri.006G209200, Potri.012G067600, Potri.013G090300, Potri.015G033600, Potri.017G082900) in four comparisons mentioned above, only Potri.002G030900 (*BLH11*) in Dro-Di vs. CK-Di comparison, Potri.015G033600 (*MYB52*) in Re-Di vs. Dro-Di comparison and Potri.017G082900 (*HB34*) in Re-Te vs. Dro-Te comparison expressed inconsistently with those from the RNA-Seq results (Figure 5), indicating that the transcriptome sequencing data are reliable.

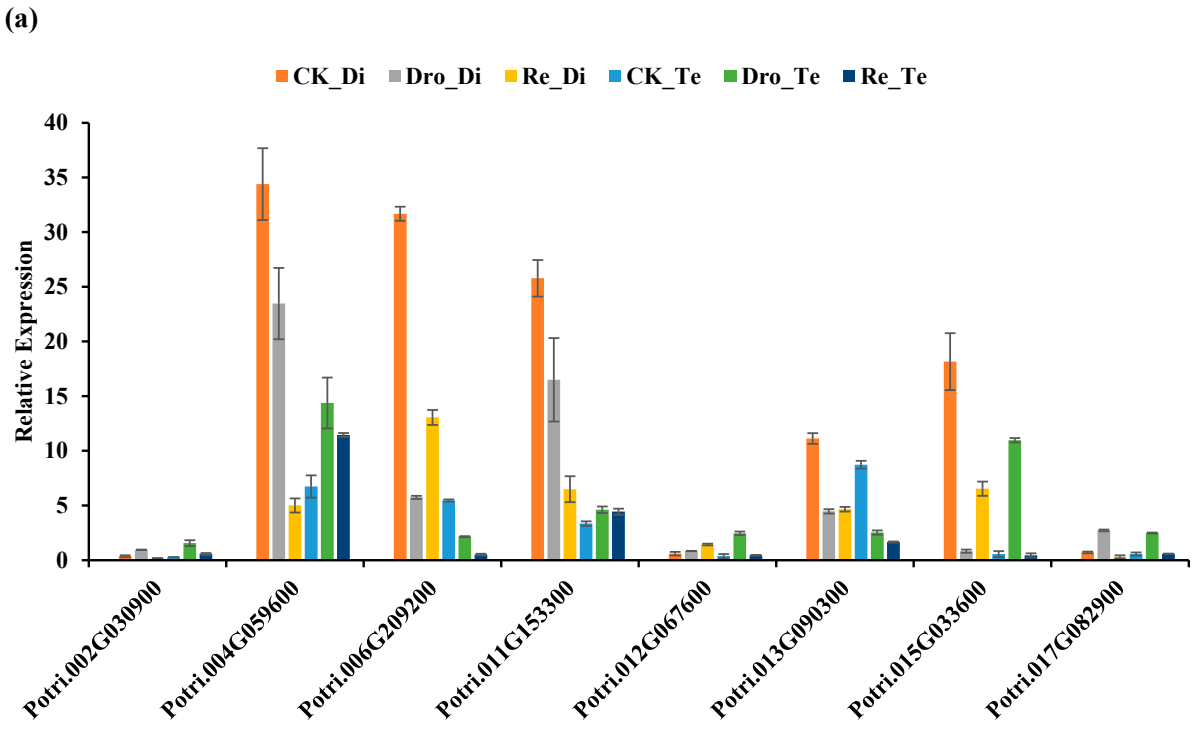


Figure 5. *Cont.*

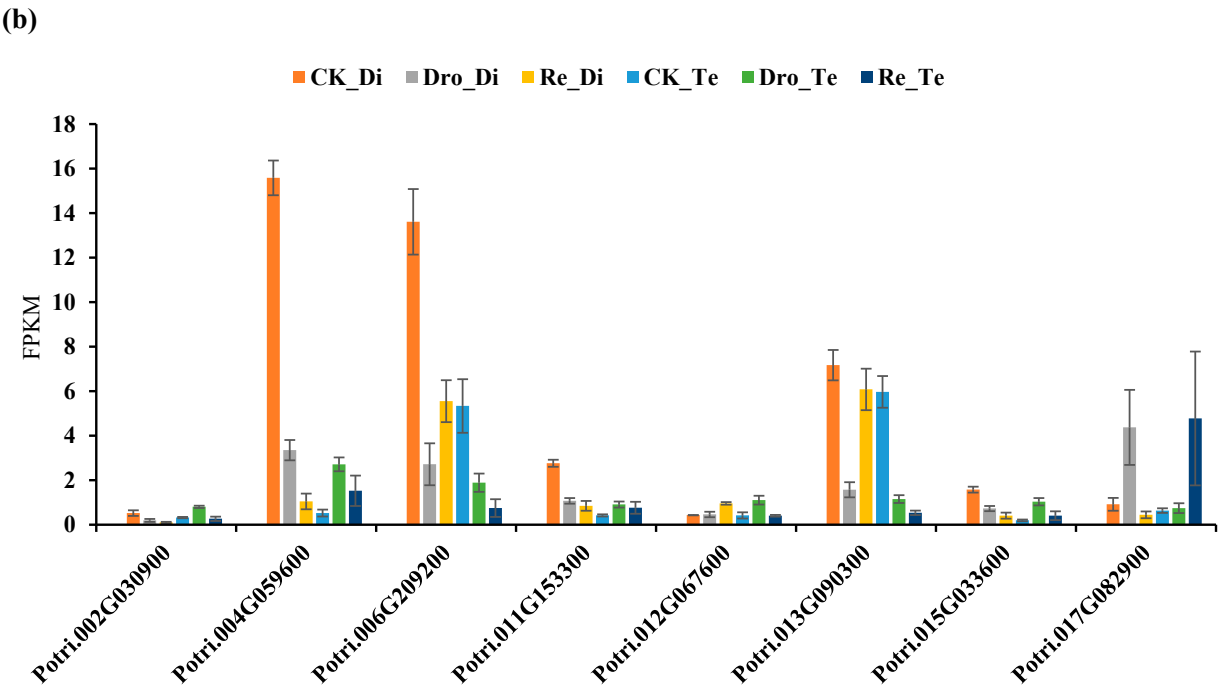


Figure 5. qRT-PCR verification of RNA-Seq analysis of gene expression. (**a**) the relative expression level of RNAs measured by RT-qPCR; "Relative expression" represents the results of RT-qPCR validation; (**b**) the expression profiles of eight genes by RNA sequencing; RPKM: Reads Per Kilobase per Million mapped read.

3.2. MicroRNA Sequencing Analysis

To elucidate miRNAs from diploid and tetraploid poplars under drought and rewatering conditions, 18 small RNA libraries were constructed and sequenced, obtained averages of 13,666,421 (CK-Di) and 12,499,445 (CK-Te) raw readers were obtained on average from plants of diploid and tetraploid under control, 15,895,868 (Dro-Di) and 13,338,498 (Dro-Te) from those under drought stress, 14,337,480 (Re-Di) and 11,326,364 (Re-Te) from those under rewatering, respectively. After removing the low-quality reads and adaptor sequences, obtained were 9,044,347 (CK-Di), 9,194,328 (CK-Te), 11,420,007 (Dro-Di), 10,153,020 (Dro-Te), 10,425,640 (Re-Di), and 8,744,623 (Re-Te) unique reads (Table S5). The length distribution of small RNAs ranged from 18 to 25 nucleotides in the diploid and tetraploid library, and the majority of sRNAs contained 24 and 21 nucleotides, consistent with the cleavage features of Dicer enzymes (Figure S1). The 24- and 21-nucleotide-long miRNAs accounted for 31.79–51.17% and 14.87–20.56%, respectively, which is consistent with the abundances reported previously in *Populus* [35]. Totally, 658 miRNAs were obtained from sequencing in 18 libraries, including 586 known miRNAs and 72 novel miRNAs, and the number of known and novel miRNAs was different between both ploidy levels. These miRNAs were expressed significantly based on fold change (≥ 1 or ≤ -1) and p-value (<0.05) criteria. miRNAs identified in different comparisons are shown in Table 1. There were 61 miRNAs differentially expressed in Dro-Te vs. CK-Te comparison, including 58 conserved miRNAs (34 up-regulated and 24 down-regulated) and three novel miRNAs (one up-regulated and two down-regulated). In Re-Te vs. Dro-Te comparison, 43 miRNAs were differentially expressed, including 34 conserved miRNAs (17 up-regulated and 17 down-regulated) and nine novel miRNAs (seven up-regulated and two down-regulated). In diploid, 30 miRNAs were differentially expressed in Dro-Di vs. CK-Di comparison, including 25 conserved miRNAs (eight up-regulated and 17 down-regulated) and five novel miRNAs (three up-regulated and two down-regulated); 69 miRNAs were differentially expressed in Re-Di vs. Dro-Di, including 63 conserved miRNAs (11 up-regulated and 52 down-regulated) and six novel miRNAs (two up-regulated and four down-regulated).

Table 1. The different expressed miRNA among different comparisons.

Comparison	Known miRNA		Novel miRNA		Total
	Up	Down	Up	Down	
Dro-Di vs. CK-Di	8	17	3	2	35
Re-Di vs. Dro-Di	11	52	2	4	69
Dro-Te vs. CK-Te	34	24	1	2	61
Re-Te vs. Dro-Te	17	17	7	2	43

Moreover, eight common-miRNAs were differentially expressed in Dro-Te vs. CK-Te and Dro-Di vs. CK-Di comparison, the expression changes of four miRNAs (one up-regulated and three down-regulated) were similar in diploid and tetraploid under drought treatment, and the remainder of four miRNAs (three up-regulated in tetraploid but down-regulated in diploid and one down-regulated in tetraploid) showed opposite expression patterns. Four common-miRNAs were differentially expressed in Re-Te vs. Dro-Te and Re-Di vs. Dro-Di comparison, the expression changes of three miRNAs (down-regulated) were similar in diploid and tetraploid after rewatering treatment, and the remainder of one miRNA (up-regulated in diploid but down-regulated in tetraploid) showed opposite expression patterns.

In Dro-Te vs. CK-Te and Re-Te vs. Dro-Te comparison: 10 miRNAs were differentially expressed (Table 2), which were drought stress-specific miRNAs in tetraploid. ptc-MIR477c-p3_1ss16TC was consistently up-regulated and ptc-miR399i_L+1_1ss22AT was consistently down-regulated under drought stress and rewatering treatments; seven miRNAs (ptc-MIR156j-p3, rco-MIR156h-p3, ptc-miR169ac_R-2_1ss16TA, PC-3p-19149_647, ptc-MIR169k-p3, gra-MIR7486j-p5_2ss9TG17CG, and gra-MIR7486j-p3_2ss9TG17CG) were up-regulated during drought stress treatment but down-regulated after rewatering treatment, and the ptc-MIR481b-p5_1ss1CT was down-regulated during drought treatment but up-regulated after rewater treatment in tetraploid.

Table 2. The differently co-expressed miRNA in tetraploid during drought stress and recovery phase.

miRNA	Dro-Te vs. CK-Te	Re-Te vs. Dro-Te
ptc-MIR156j-p3	up	down
rco-MIR156h-p3	up	down
gra-MIR7486j-p5_2ss9TG17CG	up	down
gra-MIR7486j-p3_2ss9TG17CG	up	down
ptc-miR399i_L+1_1ss22AT	down	down
ptc-MIR481b-p5_1ss1CT	down	up
ptc-miR169ac_R-2_1ss16TA	up	down
ptc-MIR169k-p3	up	down
ptc-MIR477c-p3_1ss16TC	up	up
PC-3p-19149_647	up	down

3.3. Determination of miRNAs-Target Genes by Degradome Analysis

In order to identify the target genes of the miRNAs and predict their functions, two degradome libraries (Di and Te) were constructed from pooled RNA samples about the tissues with control, drought stress and rewatering treatments (Table 3). A total of 16,418,609 (Di), 23,532,098 (Te) raw reads were generated, with 7,699,368 (Di), 5,654,938 (Te) unique raw reads and 14,573,489 (Di), 10,863,582 (Te) transcript mapped reads, of which 52,596 and 51,046 covered transcripts were obtained. After processing and analysis with CleaveLand 3.0, 238 miRNAs (including 222 conserved and 16 novel miRNAs) with 905 target transcripts were verified to form 1624 miRNA–target pairs in the Te library, and 274 miRNAs (including 257 conserved and 17 novel miRNAs) with 1144 target transcripts were verified to form 2040 miRNA–target pairs in the Di library. These cleaved targets were classified into five categories (0–4) according to the relative abundance of the degradome tags at the target sites. In the Te library, 337, 80, 630, 48, and 529 target transcripts were classified into

categories 0, 1, 2, 3 and 4, respectively. In the Di library, 509, 24, 777, 83 and 647 target transcripts were classified into categories 0, 1, 2, 3 and 4, respectively (Figure 6).

Table 3. Summary of degradome sequencing in *Populus* tetraploid and diploid.

Sample	Di		Te	
	Number	Ratio	Number	Ratio
Raw Reads	23,659,860	/	16,514,363	/
reads < 15nt after removing 3 adaptor	127,762	0.54%	95,754	0.58%
Mappable Reads	23,532,098	99.46%	16,418,609	99.42%
Unique Raw Reads	7,699,368	/	5,654,938	/
Unique reads < 15 nt after removing 3 adaptor	58,802	0.76%	43,558	0.77%
Unique Mappable Reads	7,640,566	99.24%	5,611,380	99.23%
Transcript Mapped Reads	14,573,489	61.60%	10,863,582	65.78%
Unique Transcript Mapped Reads	4,688,516	60.89%	3,642,364	64.41%
Number of input Transcript	63,498	/	63,498	/
Number of Coverd Transcript	52,596	82.83%	51,046	80.39%

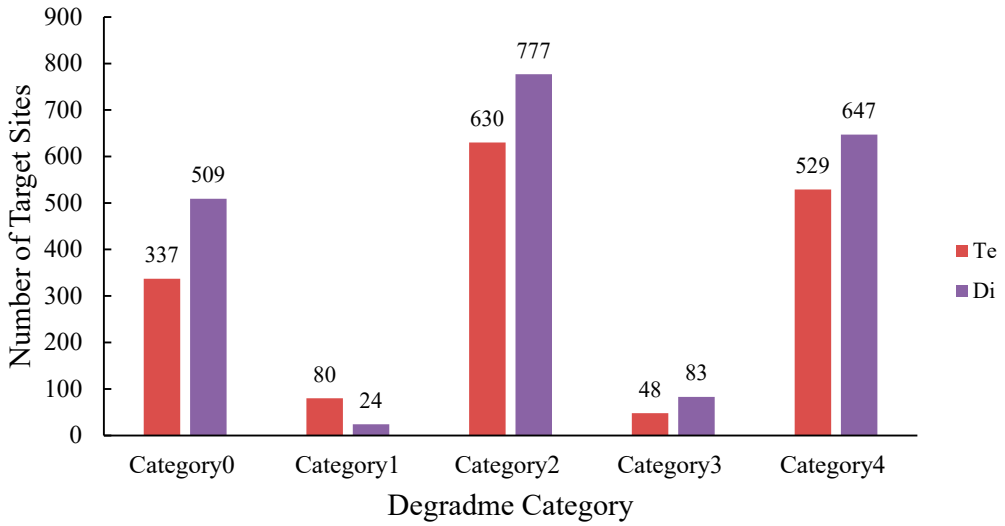


Figure 6. Te and Di Degradome libraries category and target genes number.

In total, 586 transcripts were predicted as potential targets of the 50 drought stress-associated DEmiRs in tetraploid poplars with the TargetFinder software (https://github.com/carringtonlab/TargetFinder; 27 September 2023), including 154 target transcripts for 24 DEmiRs co-identified in the degradome and the TargetFinder analysis. Moreover, 678 transcripts were predicted as potential targets of the 30 rewatering-associated DEmiRs in tetraploid poplars through the TargetFinder software, including 90 target transcripts for the 12 DEmiRs co-identified in the degradome and TargetFinder analysis. Three DEmiRs co-expressed in tetraploids and diploids were identified through the degradome analysis, and seven target genes (Potri.015G098900.3, Potri.015G098900.1, Potri.015G098900.2, Potri.003G169400.2, Potri.003G169400.1, Potri.014G057800.1, Potri.001G058600.1) were regulated by gma-miR156a_R+1, which has been identified to encode squamosa promoter-binding protein (SBP) transcription factors; ptc-miR2111a targeted Potri.001G331500.4, Potri.001G331500.3, Potri.001G331500.1, Potri.001G331500.2 encoding kelch repeat-containing F-box family protein; three target genes (Potri.012G039000.2, Potri.012G039000.5, Potri.012G039000.7) were regulated by ptc-MIR395k-p5 have been identified to encode glutamate decarboxylase (Table 4). Only one of the DEmiRs (PC-3p-19149_647) was co-expressed during drought-stress and rewatering process in tetraploid poplars through degradome analysis, targeted Potri.018G100700.5 encoding amidase.

Table 4. The target genes of differently expressed miRANs in response to *Populus* tetraploid under drought stress, recovery phase predicted by degradome sequencing.

miRNA	Transcript	Annotation
gma-miR156a_R+1	Potri.015G098900.3	SPL13A
	Potri.015G098900.1	SPL13A
	Potri.015G098900.2	SPL13A
	Potri.003G169400.2	SPL13B
	Potri.003G169400.1	SPL13B
	Potri.014G057800.1	SPL10
	Potri.001G058600.1	SPL13B
ptc-miR2111a	Potri.001G331500.4	
	Potri.001G331500.3	Galactose oxidase/kelch
	Potri.001G331500.1	repeat superfamily protein
	Potri.001G331500.2	
ptc-MIR395k-p5	Potri.012G039000.2	
	Potri.012G039000.5	glutamate decarboxylase-like
	Potri.012G039000.7	
PC-3p-19149_647	Potri.018G100700.5	amidase

To further understand the function of the target genes, the GO term was used to classify the function of their targets. In the Dro-Te vs. CK-Te comparison, 154 target genes were classified into 11 biological processes, 13 molecular functions and three cellular components. The most target numbers (80%) in the biological process were "regulation of transcription and DNA-dependent", "signal transduction", and "response to hormone". "nucleus", "intracellular", and "ribosome" were constituted in the cellular components category. Of the molecular function categories, the binding of DNA, protein, ADP, and ATP constituted the most abundant group, followed by "protein kinase activity" (Table S6).

In Re-Te vs. Dro-Te comparison, 90 target genes were classified into 12 biological processes, 25 molecular functions, and four cellular components. The target genes in the biological process were involved mainly in "protein phosphorylation", "prolyl-tRNA aminoacylation", and "tRNA aminoacylation for protein translation". "cytoplasm", "membrane", "myosin complex", and "intracellular" were constituted in the cellular components category. Of the molecular function categories, "protein binding" and "ATP binding" constituted the most abundant group, followed by "protein kinase activity", "proline-tRNA ligase activity", "aminoacyl-tRNA ligase activity", and "ionotropic glutamate receptor activity" (Table S7).

The KEGG pathway analysis showed that nine KEGG pathways were obtained in the Dro-Te vs. CK-Te comparison, among which "Butanoate metabolism", "Taurine and hypotaurine metabolism", "beta-Alanine metabolism", "Alanine, aspartate and glutamate metabolism", and "Ribosome" were significant (Table S8). Four KEGG pathways were obtained in the Dro-Te vs. CK-Te comparison, including "Aminoacyl-tRNA biosynthesis" and "RNA transport" were significantly pathways (Table S9).

4. Discussion

Numerous studies have reported that polyploidization might notably promote plant tolerance to drought stress [36,37], which may lead to better breeding and improvement strategies. However, the molecular mechanism for increased tolerance to drought in poplar polyploids remain unclear. Therefore, it is valuable to explore the key gene related to drought stress at the transcriptional level and post-transcriptional level, and in turn obtain deep mechanistic insights for breeders.

4.1. The Role of Transcription Factors in the Adaptive Responses to Drought Stress and Rewatering

When plants are exposed to drought stress, transcription factors play an important role in the transcriptional regulation [38,39]. In this review, compared with diploid, six families of transcription factors including *NAC*, *C3H*, *MYB*, *TALE*, *GRF* and *G2-like* that were involved in the transcriptional regulation in the response of the tetraploid to drought stress,

and five families of transcription factors including *ZF-HD*, *C3H*, *WRKY*, *B3*, *NAC* that were involved in the transcriptional regulation in the response of the tetraploid to rewatering. Members of the C3H [40], MYB [41], NAC [42], WRKY [43], GRF [44], ZF-HD [45], and G2-like [46] families have been verified to have regulatory effects on the stress responses of the plant. The *NAC* transcription factors are not only involved in plant growth processes such as cell formation, growth and development, and organ senescence, but also play a vital regulatory role in response to abiotic stresses [47–50]. A number of studies have shown that the NAC gene *ATAF1*, *DgNAC1*, *TaNAC2*, *OsNAC1*, and *GmNAC11* can regulate the growth of Arabidopsis [51], Chrysanthemum [52], wheat [53], rice [54], and soybean [55] for drought tolerance. *MYB* transcription factors play a critical role for adaptation to abiotic stresses by regulating the expression of downstream gene [56]. He et al. [57] found that *MYB* transcription factors were involved in the regulation of *Haloxylon ammodendron* in response to drought stress and water stimulation. An et al. [58] studied the response to drought stress and water stimulation of kenaf and found that the *MYB* transcription factor was up-regulated under drought stress and down-regulated after rewatering treatment. In this review, the *MYB* transcription factor plays a significant role in the negative regulation effect in response to water stimulation of the tetraploid. This paper is in agreement with previous work on the regulatory expression of *MYB* transcription factors in response to the water stimulation response, but there are some differences in the response to drought stress. Many members of *MYB* family have been shown to enhance drought tolerance in plants [59], whereas the regulated expression of MYB transcription factors in response to drought stress was not identified in this study, probably because many transcription factors are difficult to detect when they overlap in function and are expressed at very low levels. In conclusion, the plant response to drought stress and water stimulation was regulated by a multiple and complex signaling network, in which a series of transcription factors were involved in the response to stress, forming a coordinated and mutually repressive regulatory network rather than a single transcription factor.

4.2. miRNA Involved in the Responses to Drought Stress and Rewatering

As an important factor regulating gene expression in plants, microRNA has an irreplaceable role in the regulatory network of stress response. In recent years, with the development of high-throughput technologies, several studies have shown that miRNAs have emerged as important modulators in the regulation and control of drought stress in *Ammopiptanthus mongolicus* [60], tea [61,62], mulberry [63,64], *Paulownia* [65,66], *Populus tomentosa* [67], *Populus euphratica* [27], and *Populus trichocarpa* [68]. Analyzing from the differential expression patterns of miRNAs, there are some miRNAs that are regulated in response to drought stress in tetraploid *Populus*, such as the miR395 and miR169 family members. miR395, as a class of miRNAs associated with nutrient-deficient stress in plants, takes part in the assimilation and metabolism of sulfate and also the responses to heavy metal stress [69,70]. Recent studies indicated that miR395 is involved in responses to abiotic stress [71], and was found to significantly upregulate responses to drought stress in tobacco, soybean, rice, and maize [72]. In this study, miR395 targets glutamate decarboxylase, which is involved in the transport of the proline pathway. The low expression levels of miR395 cause overexpression of the *GAD* gene, which would lead to the accumulation of proline translocation to increase plant tolerance to stress. In general agreement with the results of previous studies [73], we inferred that miR395 and its targets' feedback regulation plays a key role in responses of poplar polyploid to drought stress. miR169 is an important miRNA that responds to drought and its post-transcriptional level regulates the expression of the NF-YA transcription factors in plants. Several studies have shown that miR169 is down-regulated, whereas its target gene overexpressed in *Arabidopsis* drought responses [74], and miR169 negatively regulates stomatal movement in response to drought in tomatoes [75]. In this study, the miR169-mediated target gene regulation is involved in the response to drought stress in poplar polyploid. This result is in general agreement with previous studies [76] that down-regulation of miR169 leads to overexpression of target genes to

increase plant tolerance to drought, indicating that miR169 can play an important role in stress regulation. These results indicate that the miRNAs may respond with differentially regulated mechanisms during drought stress and rewatering treatment among different ploidies in poplar, and these specifically expressed miRNAs may be the key regulators.

4.3. Protein Phosphorylation Involved in the Responses to Drought Stress and Rewatering

Plants have gradually evolved a wide range of response mechanisms to external stimuli under adversity stress, including the perception of external stimuli, the transmission of stress signals, the recognition and transmission of stress signals by the appropriate receptors, and the expression of relevant resistance genes [77]. Protein phosphorylation–dephosphorylation plays a very important regulatory role during the signaling and response process, which is mainly catalyzed by protein kinase and protein phosphatase [78]. Environmental factors such as water deficit and moisture saturation could stimulate the production of intracellular chemical signaling and protein phosphorylation processes [79]. Protein phosphorylation modifications have been found to play an important role in response to drought stress in species such as Arabidopsis [80], canola [81], rice [82], and alfalfa [83]. In the present study, the GO enrichment of differentially expressed genes in different ploidy during drought stress and rewatering repair was significantly analyzed, and a large number of genes related to redox processes, protein phosphorylation, metabolic processes, transmembrane transport, protein phosphorylation, and carbohydrate metabolic processes were differentially expressed. The KEGG pathways responsible for the differential enrichment of genes were mainly related to phytohormone signal transduction, plant–pathogen interaction, etc. During drought stress, tetraploid poplars used down-regulation of protein phosphorylation for stress response; then in rewatering repair, tetraploid poplars used up-regulation of protein phosphorylation and down-regulation of protein dephosphorylation for stress response.

5. Conclusions

In summary, a total of 1646 common-DEGs were identified as significantly related to the drought-stress response, and 2034 were related to the rewatering response; eight common-miRNAs were related to the drought-stress response and four were related to the rewatering response. The degradome sequencing analysis identified 154 target transcripts for 24 differentially expressed miRNAs (DEmiRs) associated with drought stress, and 90 target transcripts for 12 DEmiRs associated with rewatering in the tetraploid were identified in both the degradome analysis and the TargetFinder analysis. When tetraploid poplars are subjected to drought stress, the genes encoding calcium receptor proteins are notably down-regulated at the transcriptome level, and thus mediate Ca^{2+} signaling and regulate several important physiological functions in the cell, including encoding plant hormones, transcription factor regulation, transmembrane transport, and protein phosphorylation. The regulation of miRNAs occurs at the post-transcriptional level, the feedback regulatory mechanism of *miR395* and its target gene GAD plays an important role in regulating drought stress in tetraploid poplars. Furthermore, miRNAs regulate abscisic acid (ABA), thus activating the protein kinase pathways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14112268/s1, Supplemental Figure S1: Size distribution of small RNA; Supplemental Table S1: Primer sequences used in this study; Supplemental Table S2: The statistic of transcriptome sequence quality pre- and post-processing; Supplemental Table S3: The opposite expression co-DEGs on the compared tetraploid with diploid during drought condition; Supplemental Table S4: The opposite expression co-DEGs on the compared tetraploid with diploid during recovery condition; Supplemental Table S5: Read abundance of various classes of sRNAs sequences in eighteen libranes; Supplemental Table S6: The GO enrichment of these target genes in Dro-Te vs. CK-Te comparison; Supplemental Table S7: The GO enrichment of these target genes in Re-Te vs. Dro-Te comparison; Supplemental Table S8: Classification of KEGG pathway analysis of these target genes in Dro-Te vs. CK-Te comparison; Supplemental Table S9: Classification of KEGG pathway analysis of these target genes in Re-Te vs. Dro-Te comparison.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are not publicly available due the data also form part of an ongoing study but are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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