Characterization of Dof Transcription Factors and Their Responses to Osmotic Stress in Poplar (Populus trichocarpa)

Han Wang¹, Shicheng Zhao², Yuchi Gao³, Jingli Yang¹*

¹ State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin, Heilongjiang, China, ² School of Pharmacy, Harbin University of Commerce, Harbin, China, ³ Annoroad Gene Technology Co., Ltd, Beijing, China

* yifan85831647@163.com

Abstract

The DNA-binding One Zinc Finger (Dof) genes are ubiquitous in many plant species and are especial transcription regulators that participate in plant growth, development and various procedures, including biotic and abiotic stress reactions. In this study, we identified 41 PtrDof members from Populus trichocarpa genomes and classified them into four groups. The conserved motifs and gene structures of some PtrDof genes belonging to the same subgroup were almost the same. The 41PtrDof genes were dispersed on 18 of the 19 Populus chromosomes. Many key stress- or phytohormone-related cis-elements were discovered in the PtrDof gene promoter regions. Consequently, we undertook expression profiling of the PtrDof genes in leaves and roots in response to osmotic stress and abscisic acid. A total of seven genes (PtrDof14, 16, 25, 27, 28, 37 and 39) in the Populus Dof gene family were consistently upregulated at point in all time in the leaves and roots under osmotic and abscisic acid (ABA) stress. We observed that 12PtrDof genes could be targeted by 15 miRNAs. Moreover, we mapped the cleavage site in PtrDof30 using the 5’RLM-RACE. The results showed that PtrDofs may have a role in resistance to abiotic stress in Populus trichocarpa.

Introduction

Respond to specific signals by gene expression to resist damage and to survive in complicated environments, including specific metabolic and physiological pathways. Abiotic stresses, such as drought treatment and irregular temperature, strongly impact the development and growth of plants and have adverse effects on production and quality. In general, regulation of particular gene transcription has a critical impact on many biological and evolutionary processes in plants, for instance, stress and hormone responses. It is evident that transcription factors (TFs) are essential components that regulate transcriptional rates of their target genes by binding to cis-regulatory elements in the promoters.

The DNA-binding One Zinc Finger (Dof) transcription factor family contains a zinc finger domain, and plays critical roles as plant-specific transcriptional regulators in vital...
processes and functions in higher plants, such as stress response, photosynthetic carbon assimilation, dormancy and seed germination. Dof transcription factors typically contain 200–400 amino acids, among them, there was 52 amino acid with high level of conservation at the N-terminal, which has been considered as a DNA-binding domain. This domain is characterized by the structure of a Cys2/Cys2(C2/C2) zinc finger binding specifically to the cis-regulatory element comprising the common core sequence (AT)/AAAG [1–2]. The DNA-binding domain, which binds DNA and interacts with other proteins, is a vital functional domain [3]. The C-terminus of the Dof proteins contains a transcriptional regulation domain with various functions, including interaction with diverse regulatory proteins and the activation of gene expression [4]. The N- and C-terminal regions of the Dof protein may interact with diverse regulatory proteins or intercept signals to mediate the activation or repression of the target genes [5].

The Dof genes are ubiquitous in many plant species and are plant-specific transcription regulators that are involved in various abiotic processes. The DOF protein (ZmDOF1) first identified in maize plays a role in light-regulated gene expression, but no Dof genes have been isolated from other eukaryotes, such as yeast or humans [5]. In Arabidopsis, some of the well characterized Dof genes were shown to be involved in many plant biological processes. For example the DAG1 genes had the active involvement in seed germination [6]; the CDF1, CDF2, CDF3 and CDF5 genes are associated with photoperiodic regulation of flowering [7–8]; and HPPBF3, COG1 and OBP3, participate in the regulation of phytochrome signaling [9–11]. In rice, OsDofβ regulates the expression of gibberellins, OsDof12 and OsDof23, regulate flowering time and seed expression, respectively, and OsDof24 and OsDof25 are involved in carbon and nitrogen metabolism [12–13]. In tobacco, the Sar8.2b gene can be activated by the Dof transcription factor, which is related in systemic acquired resistance [14]. In sorghum, SbDof genes are associated with the responsiveness to light, hormones and endosperm-specific genes [15]. In maize, Dof1 plays a regulatory role in controlling gene related to carbohydrate metabolism [2]. In wheat, TaDof1 is related to carbon metabolism by increasing the regulation of the C4 pathway [16]. WPBF of wheat is involved in growth and development processes [17]. In addition, Dof genes are involved in physical interactions with other TFs such as bZIP, MYB, and WKRY, implying they have been implicated in the regulation of plant physiological processes.

To date, few studies are concerned with Dof gene family in P. trichocarpa, compared with the comprehensive researches of these genes in other plant species. Moreover, previous research rarely mentioned the responses of Populus Dof genes to drought stress. Therefore, there dose require a comprehensive analysis of the Dof gene family in P. trichocarpa. In this study, 41 Dof genes were systematically studied, along with their gene structures and promoter cis-elements. Their expression profiles in the leaves and roots of Populus under drought stress were examined using heatmap data and gene expression analysis with quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analyses under osmotic and abscisic acid (ABA) treatments. Our study is the first time to provide insight about the role of PtrDof genes in stress response in Populus.

Populus trichocarpa is a valuable forest resource used widely to produce various paper-based and timber products, and has genuine commercial and ecological value. However, recent drought induced forest mortality became more and more serious, especially for Poplar. Thus, identifying PtrDof genes will provide a novel insight for drought stress resistance in Populus.
Materials and Methods

Identification and characteristics of the Dof gene family

Data for the P. trichocarpa genome, including the protein database and the genomic and cDNA libraries, were obtained from the Phytozone v9.1 (http://www.phytozone.net/search.php) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. We searched for the Dof genes of P. trichocarpa using two verified methods. The first method was using the Protein family (Pfam 27.0) database (http://pfam.sanger.ac.uk/), the second method was using the Hidden Markov Model (HMM) profile of the Dof gene family (protein family ID: PF02701). We verified all of the located sequences by additional manual analysis to ensure a zf-Dof domain does exist with the SMART (http://smart.embl-heidelberg.de/) database [18]. All available genes were contrasted with the Dof gene family in PlnTFDB v3.0 (http://plntfdb.bio.unipi.it/v3.0/) to avoid missing genes [19]. Any additional genes were analyzed further. The ExPasy (http://web.expasy.org/protparam/), an online program was used in calculating the molecular weight and isoelectric point (pI) [20]. The subcellular localization of the Dof genes was predicted with WoLF PSORT (http://wolfpsort.org/) [21].

Phylogenetic analysis, exon/intron structure analysis and identification of conserved motifs

Multiple alignments were made using the Clustal X program (version 1.83), on the basis of the protein sequences [22]. The neighbor-joining approach was used to construct unrooted phylogenetic trees in MEGA 5.0 [23]. The exon/intron organizational analyses were revealed with the Gene structure display server (GSDS 2.0, http://gds.cbi.pku.edu.cn/index.php) [24]. Conserved motifs were analyzed with the Multiple Expectation Maximization for Motif Elucidation (MEME) (Version 4.9.1, http://meme.nbcr.net/meme/) [25].

Chromosomal location

The chromosomal locations of genes were mapped by the PopGenIE v3 database (http://www.popgenie.org/) [26]. A physical map was constructed with Adobe Illustrator CS5 (Adobe Systems Incorporated). Genes are separated by five or fewer gene loci with a 100 kb distance were defined as tandem duplicates [27].

Promoter cis-element analysis

We obtained the promoter sequences of all Dof genes from the Phytozone v10.0 database and analyzed them using the cis-element database PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [28], thus predicting and presenting cis-acting regulatory elements.

Gene Ontology (GO) annotation

Blast2GO v3.0 was used to analyze the functional classification of Dof sequences and obtain the details of the annotation results [29]. Genes are described according to three classifications of GO categories: biological processes, molecular functions and cellular components.

ExHeatMap analysis

The PopGenIE v3 database (http://www.popgenie.org/) was used to obtain the expression profiles of Populus Dof genes in leaves and roots under drought stress. Detailed descriptions of Dof gene expression in response to drought stress were downloaded from the exPlot tool at the PopGenIE v3 database. Cluster and java were used to analyze these data.
Plant material and treatment

*P. trichocarpa* (genotype Nisqually-1) was donated by professor Liquan Jiang of North Carolina State University. *P. trichocarpa* was clonally propagated by culture in half-strength Murashige and Skoog medium (1/2 MS, pH 5.8) under long-day conditions (16 h light / 8 h dark) at 25˚C. Before determining the final concentration of mannitol, we performed the preliminary experiment to evaluate the effect of different concentrations of mannitol on plants growth *in vitro*. The results showed that 150 mM mannitol could reduce but not abolish growth of plants *in vitro*. For osmotic stress, plantlets were exposed to 150 mM mannitol. Plants cannot take in water normally due to the high osmotic pressure of mannitol. Thus, the mannitol simulated osmotic stress by increasing osmotic shock. For ABA stress conditions, plantlets were treated with 200 μM ABA. Mannitol was added to 1/2 MS medium at the stated concentrations and then autoclaved (115˚C, 15 min). ABA was filter-sterilized and then mixed into 1/2 MS medium at the stated concentrations. The 150 mM mannitol and 200 μM ABA treatments lasted for 0, 3, 6, 12, or 24 h or 7 d. Young leaves and roots were collected as samples at all time points. Each stress treatment performed with three biological replicates. All samples were quickly frozen in liquid nitrogen and stored at −80˚C until using. Untreated plants were used as controls.

RNA isolation and qRT-PCR verification

Total RNA was extracted using the cetyltrimethylammonium bromide method from roots and leaves [30]. The cDNA synthesized using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. The specific sequences of each gene were based on results from multiple sequence alignment (http://multalin.toulouse.inra.fr/multalin/multalin.html). The specific primers for Dof genes were designed by Primer Premier 5 according to its CDS with following parameters, melting temperatures of 58–62˚C, primer lengths of 18–22 bp and product lengths of 190–210 bp. The primer details are listed in S1 Table.

QRT-PCR was carried out using a TransStart® Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) to determine the transcript levels under stress. Reactions were performed in 20 μL volume containing 10 μL 2xTransStart® Top Green qPCR SuperMix, 1 μL cDNA template, 7 μL ddH₂O, and 2 μL each primer-specific. Three experimental replicates were used for each sample to maintain accuracy. The *P. trichocarpa* Actin1 gene (GenBank ID: XM_002298674) was used as the reference gene [31]. Other specifications, including PCR conditions and relative gene expression calculations we based on previous study [32]. All reactions were carried out under the following PCR conditions: an initial denaturation step of 95˚C for 1 min, a three-step thermal cycling profile of denaturation at 95˚C for 5s, primer annealing at 55˚C for 30 s, and extension at 72˚C for 30 s. Then, an additional step of 80˚C for 1 s was performed to remove primer dimers, followed by plate reading. We used the relative quantification method (2^{-ΔΔCT}) to evaluate relative gene expression between replicates.

Statistical analysis

All the statistical analysis used one-way ANOVA to determine significance. Tukey’s test was analyzed to compare the difference. Significance was defined as * P < 0.05, ** P < 0.01.

miRNA target analysis and target validation by RLM-RACE

PMRD (http://bioinformatics.cau.edu.cn/PMRD) was used to download the mature *P. trichocarpa* miRNA sequences. The Plant Small RNA Target Analysis Server (psRNA Target: http://plantgrn.noble.org/psRNATarget) was used to identify the miRNA target genes in the *PtrDof*
families with default parameters. FirstChoice RLM-RACE Kit (Invitrogen, Thermo Fisher Scientific) was used to perform RLM-RACE and illustrate the predicted targets following the methods researched by Song et al [33]. The gene-specific primers for RLM-RACE are presented in S2 Table. The RLM-RACE products were ligated into the pMD18-T vector (TaKaRa), and sequenced.

Results

Identification of Dof genes in Populus

We used the HMM profile of the Pfam Dof domain (protein family ID: PF02701) to query the Dof genes in the P. trichocarpa genome. All the obtained Dof genes were checked using the SMART database to ensure the presence of the Dof domain. Then, 41 Dof genes were identified and was close to results from various biology analyses which estimated 30, 36, 24, 31 and 54 Dof genes in rice, Arabidopsis, barley, wheat and maize, respectively [34–36].

Molecular masses of the Dofs in P. trichocarpa varied from 17735.9 to 55263.5 Da. The encoded proteins ranged from 159 to 1485 amino acids (aa), with an average of 684 aa. The location of the protein expression levels in the plant cell were predicted with WoLF PSORT. The majority Dof genes were predicated as nuclear proteins, except PtrDof23, which was located in the mitochondrial matrix (Table 1).

Phylogenetic analysis, exon/intron structure analysis and identification of conserved motifs

To examine the phylogenetic relationships, an unrooted phylogenetic tree was drawn (Fig 1A). The 41 PtrDof homologs were separated into four groups (A to D). Subgroup A and D constituted the largest clade with 12 members each, subgroup B contained the fewest PtrDof family members (8 genes), and group C contained 9 members.

To check the structural diversity, we investigated the characterization of exon-intron structure in the genomic DNA sequences of individual PtrDof genes (Fig 1B). The predicted numbers of exons among the PtrDof genes were relatively fewer, varying from one to three, with 19 members having one and 19 with two. Three (PtrDof14, PtrDof39 and PtrDof11) genes had three exons. Furthermore, some PtrDof genes belonging to the same subgroup had similar gene structures, such as introns numbers and extons lengths. For instance, subgroup A genes had one or two introns expect PtrDof33, and subgroup C genes had zero introns with exception of PtrDof17 and PtrDof 4. The majority of three-exon-genes belonged in subgroup A. These similar structural features may be related to their functions in the Populus genome (Fig 1B).

MEME programmer was used to assess PtrDof proteins to characterize motif compositions (Fig 1C). A total of 15 conserved motifs were identified (S3 Table). These motifs are shown in the location of corresponding protein. The majority of related members in the phylogenetic tree had motifs. All genes uniformly contained common motif 1 at the C-terminal region, which was confirmed to be a conserved Dof domain. Moreover, differences in gene composition and motif organization among related PtrDofs members within the same subgroups indicated that these genes may have a divergent function.

Chromosomal location and gene duplication

In silico mapping of the gene loci indicated that the distribution of PtrDof genes span all 19 linkage groups (LGs) in an uneven manner. For example, PtrDof3 was located in the chromosomal scaffold that was not marked in the figure. As shown in Fig 2, the 41 PtrDof genes were
dispersed on 18 of the 19 *Populus* chromosomes (none in chromosome XVIII). Chromosome IV and XI harbored the most (4 of 41) genes. On contrast, only one gene was found on each of chromosome IX, XIII, XVI, XVI I, and XIX. The rest of the chromosomes harbored two or three genes.

**Table 1. The Dof gene family in *Populus trichocarpa*.**

| Gene name | Accession number | NCBI locus ID | Length (aa) | MW (Da) | pI | Localization |
|-----------|------------------|---------------|-------------|---------|----|--------------|
| *PtrDof1* | POPTR_0004s05580 | XM_002305034.2 | 159 | 17735.9 | 9.23 | nucl |
| *PtrDof2* | POPTR_0004s04590 | XM_002305748.1 | 325 | 35553.0 | 9.15 | nucl |
| *PtrDof3* | POPTR_0005s21130 | XM_002307448.2 | 274 | 30875.5 | 5.07 | nucl |
| *PtrDof4* | POPTR_0005s14080 | XM_002306417.1 | 331 | 35465.6 | 9.51 | nucl |
| *PtrDof5* | POPTR_0005s19310 | XM_002307281.1 | 342 | 37004.9 | 8.81 | nucl |
| *PtrDof6* | POPTR_0007s11620 | XM_002307017.2 | 323 | 34168.9 | 8.96 | nucl |
| *PtrDof7* | POPTR_0011s07400 | XM_002316790.2 | 165 | 18292.5 | 8.94 | nucl |
| *PtrDof8* | POPTR_0019s05720 | XM_002325405.2 | 493 | 53501.1 | 5.50 | nucl |
| *PtrDof9* | POPTR_0015s03520 | XM_002322008.2 | 321 | 35235.4 | 7.21 | nucl |
| *PtrDof10* | POPTR_0015s08810 | XM_002321564.1 | 314 | 34797.7 | 6.19 | nucl |
| *PtrDof11* | POPTR_0016s07000 | XM_002322747.1 | 225 | 25219.8 | 6.23 | nucl |
| *PtrDof12* | POPTR_0011s05410 | XM_002327318.1 | 325 | 35711.2 | 9.26 | nucl |
| *PtrDof13* | POPTR_0011s05450 | XM_002377322.1 | 357 | 39200.7 | 8.97 | nucl |
| *PtrDof14* | POPTR_0010s21240 | XM_002378649.1 | 356 | 37530.7 | 9.33 | nucl |
| *PtrDof15* | POPTR_0014s03590 | XM_002374952.1 | 261 | 27530.1 | 5.95 | nucl |
| *PtrDof16* | POPTR_0002s07150 | XM_002300897.2 | 301 | 34180.8 | 4.71 | nucl |
| *PtrDof17* | POPTR_0002s13100 | XM_002386451.1 | 306 | 32229.4 | 5.57 | nucl |
| *PtrDof18* | POPTR_0006s21700 | XM_002309218.1 | 288 | 31912.0 | 6.29 | nucl |
| *PtrDof19* | POPTR_0012s12670 | XM_002317912.2 | 329 | 36234.4 | 6.36 | nucl |
| *PtrDof20* | POPTR_0012s02570 | XM_002317577.2 | 297 | 32791.0 | 7.69 | nucl |
| *PtrDof21* | POPTR_0012s08280 | XM_002318016.1 | 312 | 34205.1 | 6.43 | nucl |
| *PtrDof22* | POPTR_0004s12120 | XM_002305996.2 | 503 | 55067.6 | 5.38 | nucl |
| *PtrDof23* | POPTR_0007s11790 | XM_002310726.2 | 248 | 25507.4 | 8.57 | mito |
| *PtrDof24* | POPTR_0013s06290 | XM_002319159.2 | 1485 | 53797.4 | 6.57 | nucl |
| *PtrDof25* | POPTR_0017s12080 | XM_002323827.2 | 506 | 55263.5 | 5.42 | nucl |
| *PtrDof26* | POPTR_0007s09520 | XM_002380521.1 | 494 | 37080.9 | 8.28 | nucl |
| *PtrDof27* | POPTR_0004s03900 | XM_002305645.2 | 304 | 33910.7 | 8.73 | nucl |
| *PtrDof28* | POPTR_0011s04730 | XM_002316595.2 | 305 | 33893.5 | 8.41 | nucl |
| *PtrDof29* | POPTR_0014s09640 | XM_002320172.2 | 229 | 25083.8 | 9.20 | nucl |
| *PtrDof30* | POPTR_0003s02890 | XM_002385272.1 | 235 | 25146.4 | 8.96 | nucl |
| *PtrDof31* | POPTR_0003s14450 | XM_002303642.2 | 279 | 30688.2 | 8.63 | nucl |
| *PtrDof32* | POPTR_0001s24540 | XM_002299843.1 | 332 | 35591.7 | 9.58 | nucl |
| *PtrDof33* | POPTR_0002s17490 | XM_002301384.2 | 263 | 28908.9 | 8.99 | nucl |
| *PtrDof34* | POPTR_0005s13990 | XM_002306412.2 | 253 | 25995.8 | 8.83 | nucl |
| *PtrDof35* | POPTR_0006s08440 | XM_002308117.2 | 326 | 34632.6 | 9.10 | nucl |
| *PtrDof36* | POPTR_0001s11130 | XM_002299405.1 | 285 | 31392.1 | 8.42 | nucl |
| *PtrDof37* | POPTR_0008s08740 | XM_002379606.1 | 500 | 54067.2 | 6.51 | nucl |
| *PtrDof38* | POPTR_0010s17480 | XM_002316129.2 | 496 | 54199.0 | 6.91 | nucl |
| *PtrDof39* | POPTR_0008s05520 | XM_00231128.2 | 345 | 36903.3 | 9.13 | nucl |
| *PtrDof40* | POPTR_0009s03490 | XM_002314153.1 | 326 | 34651.5 | 9.33 | nucl |
| *PtrDof41* | POPTR_0015s01160 | XM_002321946.2 | 255 | 28121.2 | 8.78 | nucl |

*a* nucl nuclear; mito mitochondrial matrix
doi:10.1371/journal.pone.0170210.t001

PLOS ONE | DOI:10.1371/journal.pone.0170210 January 17, 2017 6 / 19
Researches previously showed that the *Populus* genome has gone through three circles of genome-wide duplication at any rate including multiple segmental duplications, tandem duplications and transposition events in that order [37]. The segmental duplication associated with the salicoid duplication event that happened 65 million years ago promoted the expansion of numerous multigene families [27, 38–40]. We mapped the *PtrDof* genes to the duplicated blocks based on the previous research to check sure the possible relationship between the Dof genes and segmental duplications. The distribution of genes associated with the corresponding duplicate blocks is demonstrated in Fig 2. Approximately 49% (20 of 41) genes were firstly located in duplicated regions. Twelve duplicated genes (*PtrDof1*, 6, 8, 10, 11, 12, 13, 23, 25, 26, 28 and 31) were only contained in one of the blocks and lacked duplicates in the corresponding block. While, eight genes were located outside any duplicated blocks. The segmental duplication also occurred in subgroup C. These results suggest that the *PtrDof* genes likely originated from both segmental and tandem duplications.

Promoter cis-element analysis

Phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and ABA are involved in various processes throughout plants to accommodate abiotic stresses. We identified the putative cis-acting regulatory DNA elements by analyzing the promoter sequences of
all *PtrDof* genes. In the *PtrDof* gene promoter region, many key cis-elements that were related to environmental stress signal responsiveness were identified, such as MBS (MYB binding site, involved in drought-inducibility), HSE (heat stress-responsive element), C-repeat/DRE (cold and dehydration-responsive element), TC-rich repeats (defense and stress-responsive element), LTR (low temperature-responsive element), and W-Box (WRKY binding site, involved in abiotic stress responsiveness). Other key elements included those in phytohormone signaling, such as ABRE (abscisic acid-responsive element), ERE (ethylene-responsive element), TCA-element (salicylic acid-responsive element), CGTCA-motif (MeJA-responsive element), TGACG-motif (MeJA-responsive element), and P-box (gibberellin-responsive element) (S4 Table). Most of the *PtrDof* genes containing cis-elements contained responsiveness to phytohormone signaling and environmental stress signal, whereas *PtrDof18* and *41* contained only three cis-elements (S5 Table).

**Gene Ontology (GO) annotation**

The 41 *PtrDof* genes were classified into biological processes, molecular functions and cellular components by Gene Ontology (GO) using Blast2GO v3.0 (Fig 3, S6 Table). The seven terms...
Fig 3. Gene Ontology (GO) results for *Populus* Dof proteins. GO analysis of 41 *Dofs* sequences predicted for their involvement in biological processes, molecular functions and cellular components. The results are presented as detailed bar diagrams in S6 Table.

doi:10.1371/journal.pone.0170210.g003
of biological processes were defined. The function of all PtrDofs were predicated in the metabolic process, the cellular process and the biological regulation process, followed by the single-organism (~7%) and the multicellular organismal processes and reproduction or developmental process (~5%). Molecular function predictions showed that all PtrDofs were in accordance with organic cyclic compound binding and heterocyclic compound binding. In addition, some PtrDofs were annotated as sequence-specific DNA binding involved in transcription factor activity (~17%). Furthermore, cellular component prediction indicated that four PtrDofs were localized in the membranes (~10%). Only two PtrDofs were organelle-localized and cell-localized (~5%).

**Expression profiles under drought stress**

A majority of land plants encounter environmental stress during their life span and drought is the major environmental stress. In order to characterize the possible roles of PtrDof genes in drought stress, we used the publicly available data to investigate the expression profiles of PtrDof genes responded to drought stress. A detailed description of gene expression was downloaded from the exPlot tool at the PopGenIE v3 database. The data for all the PtrDof genes are shown in S7 Table, except for PtrDof5, 11, 18, and PtrDof3, for which the root has no data. 31 genes were upregulated in leaves and 14 genes were upregulated in roots under drought stress (Fig 4). Ten genes were all upregulated in leaves and roots.

**Expression pattern of PtrDof genes under osmotic and ABA stress**

Numerous PtrDof genes were obtained by heatmap analysis with differences in the expression profiles under drought stress. To verify these results, qRT-PCR was used to study the differential expression of selected genes under osmotic stress. For the leaves, 24 genes were induced, 4 genes were suppressed, and 3 genes were irregular (Fig 5). The results were broadly consistent with the heatmap data, which means the drought-induced genes were all upregulated and the drought-suppressed were all downregulated in leaves. For the roots, 15 genes were induced and 18 genes were suppressed (Figs 6 and 7). A total of 15 genes (PtrDof1, 8, 10, 17, 20, 21, 22, 24, 29, 32, 34, 36, 38, 40 and 41) were significantly downregulated at all time points, while only 8 genes (PtrDof14, 16, 19, 25, 27, 28, 37 and 39) were upregulated at all time points. The expression tendencies were roughly consistent with the exPlot analysis. In addition, PtrDof14, 16, 25, 27, 28, 37 and 39 were all upregulated in the leaves and roots under osmotic stress (Figs 5, 6 and 7).

We got putative cis-acting elements by analyzing the promoter sequences of all PtrDof genes. Twenty-six PtrDof genes harbored ABRE in their promoter region. QRT-PCR was used to analyze the expression profiles under ABA stress (Fig 8). In leaves, 16 genes were upregulated, 8 genes were broadly downregulated and two genes were not influenced by ABA. In roots, 15 genes were upregulated and 11 genes were downregulated. In leaves, 13 genes (PtrDof3, 10, 11, 13, 14, 16, 22, 25, 27, 28, 37, 38, 39 and 40) were upregulated significantly within a short time (24 h), whereas 12 genes (PtrDof1, 6, 10, 11, 13, 14, 16, 25, 27, 28, 37, 39 and 40) were upregulated significantly within a short time (24 h) in roots. However, among these genes, most were also upregulated in the long term (7 d), except PtrDof10 and 40. PtrDof11, 13, 14, 16, 25, 27, 28, 37 and 39 were all upregulated at all points in leaves and roots under ABA. A total of 7 genes (PtrDof14, 16, 25, 27, 28, 37 and 39) in the Populus Dof gene families were upregulated at every time points in the leaves and roots under osmotic and ABA stress (Figs 5, 6, 7 and 8).
Fig 4. Expression profile of *Populus Dof* genes under drought stress. The heatmap was visualized using the exPlot tool in the PopGenIE v2 database. Heatmap showing 41 *Populus Dof* genes in leaves and roots under drought stress.

doi:10.1371/journal.pone.0170210.g004
MiRNA target site prediction and validation

Among the PtrDof genes, 12 were targeted by 15 miRNAs. PtrDof12, PtrDof13 and PtrDof40 were targeted by two miRNAs (S8 Table). PptrDof12 was targeted by ptc-miRf11023-akr and ptc-miRf10540-akr. PptrDof13 was targeted by ptc-miRf10053-akr and ptc-miRf10957-akr. PptrDof40 was targeted by ptc-miRf10540-akr and ptc-miRf11148-akr. The other PptrDofs were only targeted by one miRNA. The sequence analysis of PptrDof30 implied that the 1170–1190 bp region may be the target site of ptc-miR472b. Ptc-miR472b miRNA was previously

![Fig 5. Expression analysis of 31 selected PptrDof genes in leaves under osmotic stress using qRT-PCR.](image)
The relative mRNA abundance of 31 selected PptrDof genes was normalized with respect to the reference gene (Actin1). Error bars represent the standard deviations of three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* P < 0.05, ** P < 0.01).

doi:10.1371/journal.pone.0170210.g005

![Fig 6. Expression analysis of 14 selected PptrDof genes in roots under osmotic stress using qRT-PCR.](image)
The relative mRNA abundance of 33 selected PptrDof genes was normalized with respect to the reference gene (Actin1). These genes were upregulated in roots under drought stress according to the HeatMap. Error bars represent the standard deviations of three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* P < 0.05, ** P < 0.01).

doi:10.1371/journal.pone.0170210.g006
Fig 7. Expression analysis of 19 selected PtrDof genes in roots under osmotic stress using qRT-PCR. The relative mRNA abundance of 19 selected PtrDof genes was normalized with respect to the reference gene (Actin1). These genes were downregulated in roots under drought stress according to the HeatMap. Error bars represent the standard deviations of three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* P < 0.05, ** P < 0.01).

doi:10.1371/journal.pone.0170210.g007

Fig 8. Expression analysis of 26 selected PtrDof genes in leaves and roots under ABA stress qRT-PCR. The relative mRNA abundance of 26 selected PtrDof genes was normalized with respect to the reference gene (Actin1). Error bars represent the standard deviations of three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* P < 0.05, ** P < 0.01).

doi:10.1371/journal.pone.0170210.g008
implicated with stress response [41]. PtrDof30 was confirmed as a real target of miRNA, as all of the 5’ ends of the mRNA fragments mapped to the nucleotide that paired to the tenth nucleotide of each miRNA with higher frequencies than depicted for each pairing oligomer (Fig 9).

**Discussion**

The Dof genes are ubiquitous in many plant species and are specific plant transcription regulators that are involved in various abiotic stress responses. The functional and evolutionary analysis of Dof genes have been preliminary performed in Arabidopsis, rice, soybean, Chinese cabbage, potato, pigeonpea, tomato and cucumber. In this report, we conducted a comprehensive analysis of thePtrDof family in P. trichocarpa to confirm their potential functions in response to osmotic stress.

We identified 41 putative full-lengthPtrDof genes in the P. trichocarpa genome. The number ofPtrDof homologues was similar to that in Arabidopsis and rice [42]. The lengths of these sequences varied, which implied a highly complicacy among thePtrDof genes. Approximately 98% (40 of 41) of thePtrDof genes were identified to be localized in the nuclear, however only one gene was identified as the mitochondrial matrix (Fig 1A). The results were same as a previous analysis of the Dof gene family in cucumber where CsDof proteins were predicted as the nucleus except for CsDof30, which was extracellular [43].

Similar to previous discoveries in rice and Arabidopsis [42], thePtrDof genes had few introns (0–2) in each gene (Fig 1B). The motif analysis showed that motif 1 was uniformly observed in all Dof proteins (Fig 1C), similar to Arabidopsis, rice and tomato. This result indicated that the evolution ofPtrDof transcription factors was conserved in plant development.

With high contribution to genetic novelty, the whole-genome, tandem and segmental duplications are important for genomic expansion, as the fundamental sources of genetic novelty [44–45]. We observed that 78% ofPtrDof genes appeared to contain duplicated regions; however, eight genes were located away the duplicated blocks (Fig 2). The results suggested that partial genomic duplication included rearrangements, which resulted in the loss of a number of genes. The present study found only one pair (PtrDof4/34) with tandem duplication and ten pairs of segmental duplication events inPtrDof genes. This indicated thatPtrDof gene segmental duplication and not tandem duplication is predominantly involved in the evolution of P. trichocarpa. The result was similar to that observed for CsDof duplications in cucumber [43].

The cis-elements play vital parts in the transcriptional regulation of gene expression, controlling phytohormone responses and complicated abiotic stress, to increase the resistance of plants under fluctuating environments. In our report, many cis-elements related to abiotic stress and phytohormone have been identified, including ABRE, MBS, HSE, ERE and TCA-elements (S4 Table). Every gene in the family included three cis-elements mentioned at least (S5 Table). PtrDof18 and 41 have only three cis-elements, which indicated that these genes might not be related to abiotic stress. By comparison, PtrDof11, 16, 19 and 36 have nine cis-elements, which indicated that these genes might be strongly associated with functions under

![Fig 9. Mapping of mRNA cleavage sites confirmed by 5 RLK-RLACE. Arrows indicate the 5’ ends of the mRNA fragments, as identified by cloned 5’ RLM-RACE products, with the frequency of clones shown.](https://doi.org/10.1371/journal.pone.0170210.g009)
different abiotic stresses. According to the expression profiles of the *Populus Dof* genes under various stresses, *PtrDof16, PtrDof19* and *PtrDof36* were upregulated in leaves at some time points after osmotic and ABA treatment. These results were roughly consistent with the promoter analysis.

The Dof family of transcription factors is a major large class of plant-specific factors, which have been identified to be involved in the regulatory networks of plants as very critical roles in response to abiotic stress. The exPlot data indicated that *PtrDofs* have important functions in drought stress. Under osmotic conditions, a total of 7 genes (*PtrDof14, 16, 25, 27, 28, 37 and 39*) in the *Populus Dof* gene family were all upregulated at all time points, including short and long term, in the leaves and roots (Figs 5, 6, 7 and 8). These results further suggest the involvement of these genes in osmotic stress as well as long-term response genes to osmotic stress. An Arabidopsis homolog (*AT5G66940*) of two of the upregulated genes (*PtrDof27 and 28*) was similarly upregulated under drought conditions [46]. Under ABA conditions, *PtrDof27 and 28* and their homolog gene (*AT5G66940*) also have similar responsive expression in the leaves and roots, which were both induced [46]. Moreover, in response to osmotic treatment, osmotic upregulated *PtrDof* members grouped into four subgroups in the leaves. In Chinese cabbage, the 9 *BraDof* genes from different classes were all induced by drought stress in the leaves [4]. However, in Triticum, only two *TaDof* genes from two different clades were significantly upregulated by drought [36]. Probably, the mechanisms of *Dof* genes in response to abiotic stress may be various in different plant species [4]. We found that eight genes were upregulated under osmotic and ABA treatments in roots. The results indicated that *PtrDofs* may be involved in the development of plant roots. A previous study found that *Populus Dofs* were associated with the formation of adventitious roots [47]. Different *PtrDof* genes had different expression pattern under the same conditions. Our research illustrates that *PtrDof* transcription factors act as a vital role resistance to osmotic stress in plants.

The miRNAs act as a vital role during plant development and abiotic stresses-response. We found that 12 *PtrDof* genes were targeted by 15 miRNAs. Each miRNA acts on different genes in most cases. It was indicated that 16 miRNA families could target 13 *CmDOFs* previously and there was no repeat among these miRNAs [43]. The results indicated that each miRNA has its specific regulatory genes and that one miRNA may not simultaneously act on different *Dofs*. Four genes, including *PtrDof12, PtrDof13* and *PtrDof40*, were targeted by two miRNAs, whereas the other nine genes only were targeted by one miRNA. Among the *Dof* gene family in the cucumber, there were three target sites for *CmDOF1* and two target sites for *CmDOF21*, whereas the remaining 11 *CmDOFs* have only one target site [43]. In plants, single or multiple miRNAs are induced to regulate the expression of target genes under various stresses to improve the adaptability of plants [48]. The 12 *PtrDof* target genes belong to four groups of *PtrDofs*. Subgroup D contains the most target genes (four), whereas subgroup B only contains one target gene. Subgroup A and C, respectively, contain four and two target genes. In the cucumber, each subgroup also has a different number of target genes [43]. MiR472 is a miRNA induced by different stresses. The recent study showed that miR472 acts as a negative regulator, preventing an autoimmune response that would have detrimental consequences on plant fitness in Arabidopsis [49], and the targets of miR472 were identified as disease resistance genes in Arabidopsis lyrata [50]. The expression of miR472 plays a role in influencing the expression of their target genes, which are involved in disease resistance in Citrus sinensis [51]. The ptc-miR472b miRNA is regulated by cold stress in Populus [41], but no definite results are available. The expression of miR472 has been shown in the PMRD and the expression in rice was decreased under oxidase stress. The gene expression omnibus of NCBI has reported that miR472 had different expression in an experiment designed by Zhongs et al (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11535). However, to date, the interactions between
miR472 and osmotic stress-related targets have not been reported. Our study provides a new target gene, *PtrDof30*, which is an osmotic-related gene in *P. trichocarpa*. We also confirmed that *PtrDof 30* was a real target of miRNA in plants (Fig 9).

**Conclusions**

Dof-family transcription factors were comprehensively analyzed in the genome of *Populus trichocarpa*. We performed 41 *PtrDof* genes that were classified into four groups. Most genes within the same group had similar gene structures and conserved motifs. *PtrDofs* were dispersed on 18 of the 19 *Populus* chromosomes and likely originated from both tandem and segmental duplications. Promoter *cis*-element analysis indicated that most *PtrDof* genes contain *cis*-elements in response to stress and phytohormones. The HeatMap data for the *PtrDof* genes suggest that they are primarily expressed in leaves and roots. The expression of selected *PtrDof* genes was characterized in response to osmotic and ABA stresses and indicated that *PtrDofs* may be involved in resistance to abiotic stress in *Populus trichocarpa*. In addition, 12 *PtrDof* genes could be the targets of 15 miRNAs and *PtrDof30* was confirmed as a real target of miRNA. The results help to characterize the stress responses of *PtrDof* genes and promote a better understanding of the construction and function of Dofs in *Populus*.

**Supporting Information**

S1 Table. Primers for qRT-PCR of 39 selected *PtrDof* genes. Primers were designed using Primer Premier 5 (F represents a forward primer; R represents a reverse primer).

S2 Table. Primers used for RLM-RACE.

S3 Table. Motif sequences of *PtrDof* genes identified in *P. trichocarpa*.

S4 Table. Abiotic stress and phytohormone related *cis*-elements.

S5 Table. Abiotic stress and phytohormone response elements in *PtrDof* gene promoters.

S6 Table. Details of the Gene Ontology annotation of *PtrDof* sequences.

S7 Table. The detailed data for gene expression under drought stress of *PtrDofs*.

S8 Table. The miRNA target predication of *PtrDof* genes.

**Acknowledgments**

This work was supported by the National Natural Science Foundation of China (31400573) and the Science Fund of Heilongjiang Province (QC2015035).

**Author Contributions**

Conceptualization: JY HW SZ YG.
Data curation: HW SZ.
Formal analysis: HW SZ YG.
Funding acquisition: JY.
Investigation: HW SZ YG.
Methodology: JY HW SZ YG.
Project administration: JY.
Resources: JY.
Software: HW SZ.
Supervision: JY HW.
Validation: JY.
Visualization: HW YG.
Writing – original draft: HW SZ.
Writing – review & editing: JY.

References
1. Umemura Y, Ishiduka T, Yamamoto R, Esaka M. The Dof domain, a zinc finger DNA-binding domain conserved only in higher plants, truly functions as a Cys2/Cys2 Zn finger domain. Plant J. 2004; 37: 741–749. PMID: 14871313
2. Yanagisawa S. Dof domain proteins: plant-specific transcription factors associated with diverse phenomena unique to plants. Plant Cell Physiol. 2004; 45: 386–391. PMID: 15111712
3. Cavalar M, Moller C, Offermann S, Krohn NM, Grasser KD, Peterhansel C. The interaction of DOF transcription factors with nucleosomes depends on the positioning of the binding site and is facilitated by maize HMGB5. Biochemistry-US. 2003; 42: 2149–2157.
4. Ma J, Li MY, Wang F, Tang J, Xiong AS. Genome-wide analysis of Dof family transcription factors and their responses to abiotic stresses in Chinese cabbage. BMC Genomics. 2015; 16: 33–47. doi: 10.1186/s12864-015-1242-9 PMID: 25636232
5. Noguero M, Atif RM, Ochatt S, Thompson RD. The role of the DNA-binding One Zinc Finger (DOF) transcription factor family in plants. Plant Sci. 2013; 209: 32–45. doi: 10.1016/j.plantsci.2013.03.016 PMID: 23759101
6. Papi M, Sabatini S, Bouchez D, Camilleri C, Costantino P, Vittorioso P. Identification and disruption of an Arabidopsis zinc finger gene controlling seed germination. Genes Dev. 2000; 14: 29–33. PMID: 10640273
7. Fornara F, Panigrahi KC, Gissot L, Sauerbrunn N, Ruhl M, Jarillo JA, et al. Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. Dev Cell. 2009; 17: 75–86. doi: 10.1016/j.devcel.2009.06.015 PMID: 19619493
8. Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA. FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. Science. 2005; 309: 293–297. doi: 10.1126/science.1110586 PMID: 16002617
9. Park DH, Lim PO, Kim JS, Cho DS, Hong SH, Nam HG. The Arabidopsis COG1 gene encodes a Dof domain transcription factor and negatively regulates phytochrome signaling. Plant J. 2003; 34: 161–171. PMID: 12694592
10. Tepperman JM, Zhu T, Chang HS, Wang X, Quail PH. Multiple transcription-factor genes are early targets of phytochrome A signaling. P Natl Acad Sci USA. 2001; 98: 9437–9442.
11. Ward JM, Cufre CA, Denzel MA, Neff MM. The Dof transcription factor OBP3 modulates phytochrome and cryptochrome signaling in Arabidopsis. Plant Cell. 2005; 17: 475–485. doi: 10.1105/tpc.104.027722 PMID: 1589636
12. Li D, Yang C, Li X, Gan Q, Zhao X, Zhu L. Functional characterization of rice OsDof12. Planta. 2009; 229: 1159–1169. doi: 10.1007/s00425-009-0893-7 PMID: 19198875
13. Yamamoto MP, Onodera Y, Touno SM, Takaia F. Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. Plant Physiol. 2006; 141: 1694–1707. doi: 10.1104/pp.106.082826 PMID: 16798940

14. Song F, Goodman RM. Cloning and identification of the promoter of the tobacco Sar8.2b gene, a gene involved in systemic acquired resistance. Gene. 2002; 290: 115–124. PMID: 12062806

15. Kushwaha H, Gupta S, Singh VK, Rastogi S, Yadav D. Genome wide identification of Dof transcription factor gene family in sorghum and its comparative phylogenetic analysis with rice and Arabidopsis. Mol Biol Rep. 2011; 38: 5037–5053. doi: 10.1007/s11033-010-0650-9 PMID: 21161392

16. Chen R, Ni Z, Qin Y, Nie X, Lin Z, Dong G, et al. Isolation and characterization of TaDof transcription factor in wheat (Triticum aestivum). DNA Seq. 2005; 16: 358–363. doi: 10.1080/10425170500272940 PMID: 16243726

17. Dong G, Ni Z, Yao Y, Nie X, Sun Q. Wheat Dof transcription factor WPBF interacts with TaQM and activates transcription of an alpha-gladiin gene during wheat seed development. Plant Mol Biol. 2007; 63: 73–84. doi: 10.1007/s11103-006-9073-3 PMID: 17021941

18. Gentilini F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends Biochem Sci. 1998; 23: 403–405. PMID: 9810230

19. Kumar S, Stecher G, Peterson D, Tamura K. MEGA-CC: computing core of molecular evolutionary genetics analysis program for automated and iterative data analysis. Bioinformatics. 2012; 28: 2685–2686. doi: 10.1093/bioinformatics/bts507 PMID: 22923298

20. Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics. 2015; 31: 1296–1297. doi: 10.1093/bioinformatics/btu817 PMID: 25504850

21. Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, et al. PlantCAR E, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 2002; 30: 325–327. PMID: 11752327

22. Libault M, Joshi T, Benedetto VA, Xu D, Udvardi MK, Stacey G. Legume transcription factor genes: what makes legumes so special? Plant Physiol. 2009; 151: 991–1001.
35. Moreno-Risueno MA, Martinez M, Vicente-Carbajosa J, Carbonero P. The family of DOF transcription factors: from green unicellular algae to vascular plants. Mol Genet Genomics. 2007; 277: 379–390. doi: 10.1007/s00438-006-0186-9 PMID: 17180359
36. Shaw LM, McIntyre CL, Gresshoff PM, Xue GP. Members of the Dof transcription factor family in Tritium aestivum are associated with light-mediated gene regulation. Funct Integr Genomic. 2009; 9: 485–498.
37. Hu R, Chi X, Chai G, Kong Y, He G, Wang X, et al. Genome-wide identification, evolutionary expansion, and expression profile of homeodomain-leucine zipper gene family in poplar (Populus trichocarpa). PLoS One. 2012; 7: e31149. doi: 10.1371/journal.pone.0031149 PMID: 22359569
38. Barakat A, Baghouska-Zadworna A, Choi A, Plakkat U, DiLoreto DS, Yellanki P, et al. The cinnamyl alcohol dehydrogenase gene family in Populus: phylogeny, organization, and expression. BMC Plant Biol. 2009; 9: 26–41. doi: 10.1186/1471-2229-9-26 PMID: 19267902
39. Kalluri UC, Difazio SP, Brunner AM, Tuskan GA. Genome-wide analysis of Aux/IAA and ARF gene families in Populus trichocarpa. BMC Plant Biol. 2007; 7: 59–63. doi: 10.1186/1471-2229-7-59 PMID: 17986329
40. Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM. Expansion and diversification of the Populus R2R3-MYB family of transcription factors. Plant Physiol. 2009; 149: 131–193. doi: 10.1104/pp.108.132795 PMID: 19091872
41. Lu S, Sun YH, Chiang VL. Stress-responsive microRNAs in Populus. Plant J. 2008; 55: 131–151. doi: 10.1111/j.1365-313X.2008.03497.x PMID: 18363789
42. Lijavetzky D, Carbonero P, Vicente-Carbajosa J. Genome-wide comparative phylogenetic analysis of the rice and Arabidopsis Dof gene families. BMC Evol Biol. 2003; 3: 17–28. doi: 10.1186/1471-2148-3-17 PMID: 12877745
43. Wen CL, Cheng Q, Zhao L, Mao A, Yang J, Yu S, et al. Identification and characterisation of Dof transcription factors in the cucumber genome. Sci Rep. 2016; 6: 23072–23083. doi: 10.1038/srep23072 PMID: 26979661
44. Moore RC, Purugganan MD. The evolutionary dynamics of plant duplicate genes. Curr Opin Plant Bio. 2005; 8: 122–128.
45. Taylor JS, Raes J. Duplication and divergence: the evolution of new genes and old ideas. Annu Rev Genet. 2004; 38: 615–643. doi: 10.1146/annurev.genet.38.072902.092831 PMID: 15568988
46. He L, Su C, Wang Y, Wei Z. ATDOF5.8 protein is the upstream regulator of ANAC069 and is responsive to abiotic stress. Biochimie. 2015; 110: 17–24. doi: 10.1016/j.biochi.2014.12.017 PMID: 25572919
47. Rigal A, Yordanov YS, Perrone I, Karlberg A, Tisserant E, Bellini C, et al. The AINTEGUMENTA LIKE1 homeotic transcription factor PtAIL1 controls the formation of adventitious root primordia in poplar. Plant Physiol. 2012; 160: 1996–2006. doi: 10.1104/pp.112.204453 PMID: 23077242
48. Lima JC, Loss-Morais G, Margis R. MicroRNAs play critical roles during plant development and in response to abiotic stresses. Genet Mol Biol. 2012; 35: 1069–1077. PMID: 22412556
49. Boccara M, Sarazin A, Theibaud O, Jay F, Voinnet O, Navarro L, et al. The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP- and effector-triggered immunity through the post-transcriptional control of disease resistance genes. PLoS Pathog. 2014; 10: e1003883. doi: 10.1371/journal.ppat.1003883 PMID: 24453975
50. Fahlgren N, Jogdeo S, Kasschau KD, Sullivan CM, Chapman EJ, Laubinger S, et al. MicroRNA gene evolution in Arabidopsis lyrata and Arabidopsis thaliana. Plant Cell. 2010; 22: 1074–1089. doi: 10.1105/tpc.110.073999 PMID: 20407027
51. Lu YB, Yang LT, Qi Y, Li Y, Li Z, Chen YB, et al. Identification of boron-deficiency-responsive microRNAs in Citrus sinensis roots by Illumina sequencing. BMC Plant Biol. 2014; 14: 123. doi: 10.1186/1471-2229-14-123 PMID: 24885979