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Expression Analysis of the NAC Transcription Factor Family of *Populus* in Response to Salt Stress

Shengji Wang [1], Juanjuan Huang [1], Xingdou Wang [1], Hui Dang [1], Tingbo Jiang [2] and Youzhi Han [1,*]

[1] College of Forestry, Shanxi Agricultural University, Taigu 030801, China
[2] State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, 51 Hexing Road, Harbin 150040, China
* Correspondence: hanyouzhi@sxau.edu.cn or hyzsilv@163.com; Tel.: +86-0354-6288329

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Abstract: Research Highlights: Sequence phylogeny, genome organisation, gene structure, conserved motifs, promoter cis-element and expression profiling of poplar *NACs* related to salt stress were detected. In addition, expression of two salt-induced *NACs* was analysed. Background and Objectives: NAC transcription factor (TF) proteins are involved in a wide range of functions during plant development and stress-related endurance processes. To understand the function of *Populus* NAC TFs in salt stress tolerance, we characterised the structure and expression profile of a total of 289 *NAC* members. Materials and Methods: Sequence phylogeny, genome organisation, gene structure, motif composition and promoter cis-element were detected using bioinformatics. The expression pattern of *Populus* NAC TFs under salt stress was also detected using RNA-Seq and RT-qPCR. Results: Synteny analysis showed that 46 and 37 *Populus NAC* genes were involved in whole-genome duplication and tandem duplication events, respectively. The expression pattern of *Populus* NAC TFs under salt stress showed the expression of the 289 *PtNACs* of 84K poplar was induced. Similar expression trends of *NACs* were found in *Populus simonii* × *P. nigra* T. S. Hwang et Liang and *Arabidopsis thaliana* (L.) Heynh. Conclusions: The correlation analysis showed that the expression of two differentially expressed NAC genes *PtNAC024* and *PtNAC182* was significantly associated with most of the 63 differentially expressed genes tested. The expression of *PtNAC024* and *PtNAC182* in different tissues was also analysed in silico and different expression patterns were found. Together, this study provides a solid basis to explore stress-related NAC TF functions in *Populus* salt tolerance and development.

Keywords: NAC; transcription factor; salt stress; gene express; *Populus*

1. Introduction

Gene expression regulation at the level of transcription can directly or indirectly influence many biological processes in plants, such as cellular morphogenesis, metabolic and physiological balance, signalling transduction and stress responses [1]. Plant growth and development is affected on differential gene expression and is controlled by transcription factors (TFs) acting as switches of regulatory cascades depending on the cell type [2]. Furthermore, the alterations of transcriptional regulators gene expression is becoming a major source of the diversity and change during the evolution process of plants [3]. TFs are proteins that regulate gene expression by binding to specific DNA elements located in gene promoters and/or introns [1,4]. Therefore, the identification and function characterisation of TFs is essential for understanding transcriptional regulatory networks [5,6]. In plants, with the development of bioinformatics and the bioinformatics database, a total of 320,370 TFs from 165 species have been identified [7]. The number of TFs proteins of *Arabidopsis thaliana* (L.) Heynh. is more than 2296, about 9.2% of its estimated total number of genes [7,8]. As for *Populus*, the number

of TFs is about 4287 accounting for 9.5% of its genome and the proportion is similar to that of *Arabidopsis thaliana* [7,9,10].

NAC family proteins with a consensus sequence known as the NAC domain (NAM, ATAF1, ATAF2 and CUC2) are plant-specific TFs represented by ~105 genes in *Arabidopsis* [11], ~140 in rice [12] and ~163 in *Populus* genomes [6]. The conserved NAC domain is always located in the N-terminal region of NAC proteins and divided into the five A–E subdomains [11,13,14]. The NAC domains have been implicated in nuclear localisation, DNA binding and formation of homodimers or heterodimers with other NAC proteins [15–19]. In contrast, the C-terminal region of NAC proteins is generally not conserved and confers transcriptional activation diversity [5,11–13,18,20–25]. NAC proteins have been reported to participate in a wide range of plant development processes, including floral organ morphogenesis [26,27], lateral root development [28,29], shoot apical meristem and branching development [13,30,31], xylogenesis and fiber formation in vascular plants [20,21,25,32,33]. In addition, numerous NAC proteins play crucial roles in plant abiotic stress and defence responses, including responses to drought, salinity, cold, mechanical wounding and viral infection [22,34–38]. In *Arabidopsis*, ANAC019 was identified as a positive regulator of abscisic acid (ABA) signalling, conferring ABA-hypersensitivity when ectopically expressed in plants [19]. NTL8, a membrane-bound NAC TF induced by high salinity mediates salt regulation in *Arabidopsis* seed germination via the gibberellin acid (GA) pathway, primarily independently of ABA [39]. Its expression is increased by the GA biosynthetic inhibitor paclobutrazol (PAC) and is repressed by GA. NTL8 activity is also regulated by the controlled proteolytic release of the membrane-bound NTL8 form. Its release from the membranes is activated by PAC and high salinity. Interestingly, *ATAF1* cloned from *Arabidopsis* is repressed by necrotrophic fungal and bacterial pathogens and acts as a negative regulator during the defence responses process [40]. Expression of *ATAF1* is down-regulated after infection with *Botrytis cinerea* or *Pseudomonas syringae* pv. tomato or after treatment with salicylic acid (SA), jasmonic acid and 1-amino cyclopropane-1-carboxylic acid (the precursor of ethylene biosynthesis). Transgenic plants that overexpress the *ATAF1* gene (*ATAF1-OE*) show increased susceptibility while those expressing an *ATAF1* chimeric repressor construct (*ATAF1-SRDX*) exhibit enhanced resistance to *P. syringae* pv. tomato DC3000, *B. cinerea* and *Alternaria brassicicola*. In *ATAF1-OE* plants, SA-induced expression of pathogenesis-related genes and disease resistance against *P. syringae* pv. tomato DC3000 was partially suppressed. Another gene, *SNAC1*, plays an important role in the rising drought stress tolerance [35]. Plants expressing *SNAC1* display significantly enhanced tolerance to drought and salinity in multiple generations and their leaves contain higher levels of water and chlorophyll, as compared to the wild type.

Although quite a few NAC TFs have been functionally characterised in model plants such as *Arabidopsis* and rice, the functions of most NAC proteins remain unknown [6]. Especially in *Populus*, the typical model of tree species, there are only very limited reports on the salt stress tolerance characterisation of NAC TFs. Movahedi et al. (2015) reported that the *CarNAC3* and *CarNAC6* salinity and drought tolerant genes from *Cicer arietinum* play a significant role in improving drought and salt tolerance when expressed in poplar [37]. Among poplars, the 84K (*Populus alba* × *P. glandulosa*) poplar is known by foresters to be relatively resistant to water stress, low temperature, diseases and insects. As the main afforestation species in North China, the 84K poplar is of great ecological importance [36]. Shen et al. (2009) carried out a genome-wide informatics survey on plant NAC TFs and identified a total of 148 NAC TFs from *Populus* [41]. However, their report only pertains to the sequence phylogeny analysis [41]. Hu et al. (2010) performed a relatively comprehensive analysis of the *Populus* NAC gene family and identified 163 NAC TFs [6]. In this article, we have used a genome-wide approach taking advantage of the functional diversity of the NAC proteins to dissect structure–function aspects of NAC TF modularity. This approach revealed an expanded NAC family with a total of 289 members. We performed a detailed analysis including sequence phylogeny, genome organisation, gene structure, conserved motifs, promoter *cis*-element and expression profiling of genes underlying salt stress. Noteworthy, we found that two NAC genes (*PtNAC024* and *PtNAC182*)

were significantly induced by salt stress and showed different tissue-specific expression patterns. The function and expression patterns of these two genes will be further characterised with respect to poplar growth and stress endurance in our future studies. Taken together, this will provide a basis to explore stress-related NAC TF members and clarify their response to abiotic stress in *Populus*.

2. Materials and Methods

2.1. Database Search and Phylogenetic Analysis of NAC Proteins

A systematic search was performed for NAC TFs in *Populus* using PlantTFDB (Version 4.0, http://planttfdb.cbi.pku.edu.cn/family.php?sp=Ptr&fam=NAC) [7]. The NAM domain in each *Populus* NAC protein was further manually confirmed using the InterProScan programme (http://www.ebi.ac.uk/interpro/). The sequences of *Arabidopsis* NAC proteins were searched and downloaded from the *Arabidopsis* genome TAIR 9.0 (http://www.Arabidopsis.org/index.jsp). Multiple sequence alignments of NAC proteins were performed using Clustal X 1.83. Unrooted phylogenetic trees were constructed with MEGA 7.0.21 using the Neighbour Joining (NJ) method and the bootstrap test carried out with 2100 iterations [42]. Pairwise gap deletion mode was used to ensure that the more divergent C-terminal domains could contribute to the topology of the NJ tree.

2.2. Chromosome Location and Synteny Analysis of NAC Genes

Genes on genome were located using the TBtools with the "Map Genes On Genome From Sequence Files" methods (http://www.tbtools.com/). The subject sequences were downloaded from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ptrichocarpa). Intra-species gene synteny and collinearity were detected by MCScanX using default parameters considering pBLAST $\leq 1 \times 10^{-5}$. Links of colinear blocks between sets of Linkage Groups (LGs)/chromosomes are shown by circle plot [43].

2.3. Gene Structure and Conserved Motifs Analysis of NAC Genes

Toolbox for Biologist (TBtools V0.664445552, http://www.tbtools.com/) was used to illustrate the gene structure of individual NAC transcripts by comparison with their corresponding genomic DNA sequences from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ptrichocarpa) [44]. Conserved motifs in 289 *Populus* NAC proteins were detected using the programme MEME version 5.0.2 [45]. MEME was run with the following parameters: Any number of repetitions, 15 maximum number of motifs and between six and 50 residues for the optimum motif widths. Structural motif annotation was performed using the SMART and Pfam databases [46,47].

2.4. Promoter Cis-Element Analysis

Promoter sequences (2 kb upstream of the translation start site) of *Populus* NACs were blasted and obtained from the Phytozome v12.1 database. The *cis*-elements prediction and location in promoters was performed using the PlantCRAE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) programme [48].

2.5. Gene Ontology Annotation

Functional grouping and annotation of *Populus* NACs were analysed using the programme Blast2GO v 5.2 [49]. The default blast annotation configurations were used in our study according to the manual (http://docs.blast2go.com/user-manual/). Genes are presented in terms related to three levels of Gene Ontology (GO) classification as follows: Biological processes, molecular functions and cellular components.

2.6. Plant Materials and Stress Treatment

Twigs cut from 84K poplar (*P. alba* × *P. glandulosa*) with the same genetic background were grown in the Murashige and Skoog (MS) medium for one month under conditions of 60%–70% relative humidity, 16/8-h light/dark cycle and an average temperature of 25 °C, and then transferred to hydroponic cultivation for one month under similar conditions. After that, strong and healthy strain with similar state were divided into two groups and treated with 0.15 M salt (S1, S2, S3, S4) or regular water (W1, W2, W3, W4) for 24 h, respectively. Four clones in the same tested group served as biological replicates. Secondary leaves from each of the replicates (eight samples) were collected, frozen immediately in liquid nitrogen and stored at −70 °C for RNA isolation. RNA extraction was processed according to the protocol presented in our former study [50].

2.7. Expression Characterization of NACs Using RNA-Seq

Total RNA of each sample was shipped to the GENEWIZ Company (https://www.genewiz.com/) for library construction and RNA-Seq using the Illumina HiSeq 2500 platform. Sequencing library construction and RNA-Seq data analysis were performed as in our former studies. Gene expression profiling by RNA-Seq is a powerful method to identify the molecules involved in environmental stress endurance. Gene expression was reported as fragments per kilo-base transcript per million mapped reads (FPKM). Identification of differentially expressed genes (DEGs) was performed in this study. The overall expression in the salt-treated (S1, S2, S3, S4) and the control (W1, W2, W3, W4) plants was compared using the software Pop's Pipes (http://sys.bio.mtu.edu/) and degeR was chosen to identify the DEGs. The false discovery rate was controlled at 0.05 for multiple tests correction in the Pop's Pipes processing. The fold change (FC) of each gene was the log transformation (base 2) of the specific value (S-tested/W-control) of FPKM. If $FC > 0$, the gene is up-regulated, and if $FC < 0$, the gene is down-regulated.

2.8. Expression Validation with RNA-Seq in Other Plant Species

P. simonii × *P. nigra* T. S. Hwang et Liang and *Arabidopsis thaliana* were also used as materials and treated with salt to validate the expression of genes in response to salt stress. The methods used to cultivate and treat *P. simonii* × *P. nigra* were similar to those for the 84K poplar used in this study. Two biological replicates (A1 and A2) of *P. simonii* × *P. nigra* were treated with 0.15 M NaCl and the other two replicates (B1 and B2) cultivated in regular water were used as the control. For *Arabidopsis thaliana* (Columbia-0) cultivation, wild-type seeds were surface-sterilised and sown on plates containing 1/2 MS medium solidified with 0.2% (*w*/*v*) phytoagar according to our former study [50]. After nine days, seedlings at two-leaves stage were transferred into soil mix pots and placed in 8/16-h light/dark (short-day) photoperiod conditions for two weeks. Each two biological replicates of strong and healthy *Arabidopsis* plants were treated with 0.15 M NaCl (C1 and C2) or water (D1 and D2) for 24 h. All the samples were then shipped to the GENEWIZ Company for RNA-Seq analysis.

2.9. Reverse Transcription-Quantitative Polymerase Chain Reaction Assay

To quantify the expression level of two important putative NAC DEGs, *PtNAC024* and *PtNAC182*, in 84K poplar under both the salt stress treatment (0.15 M NaCl) and the control (water) conditions, leaf tissues were used for RT-qPCR. RT-qPCR was performed as in our previous study [51]. Two house-keeping genes, Actin and EF1, were used as internal control genes [52]. The primers for *PtNAC024* and *PtNAC182* used for RT-qPCR are presented in Table S1. The relative expression level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method, defined as: $\Delta\Delta C_t = (C_{t\text{-target}} - C_{t\text{-control}})_2 - (C_{t\text{-target}} - C_{t\text{-control}})_1$.

2.10. Tissue-Specific Expression Pattern in Silico

In silico, the expression patterns in different tissues of *PtNAC024* and *PtNAC182* were detected using the exImage tool of the PopGenIE V3 database (http://popgenie.org/). The related data can be directly downloaded using the accession numbers of genes from PopGenIE.

2.11. Statistical Analysis

Single variable analysis was used to compare the gene expression between the samples exposed to salt stress and the controls using the *t*-test. We applied unsupervised clustering analysis to identify the DEGs with similar expression profiles. We also used the correlation analysis to detect the relationship among different DEGs. All statistical analyses were conducted using R v3.3.1 (http://cran.r-project.org/).

3. Results

3.1. Identification and Phylogenetic Analysis of Poplar NAC TFs

In this study, we identified 289 NAC transcripts of the 171 nonredundant genes in *Populus* with the PlantTFDB (Table S1). They were designed as PtNAC001–PtNAC289 following the nomenclature proposed in the previous study. All of them (except PtNAC289) encode proteins of 123–698 amino acids in lengths, molecular weights between 14,836.8 and 77,446.2 Da and isoelectric point values varying from 4.0667 to 11.3163. In most cases, two or more PtNACs were hit for every ortholog in *Arabidopsis* (Table S1). The detail information of PtNACs is shown in Tables S1 and S2, including TF_ID and similarities to their *Arabidopsis* orthologs as well as their complementary DNA and protein sequences.

Homology studies of plant TFs can give insights into their functions. To examine the phylogenetic relationships among PtNACs, unrooted trees were constructed with alignments of the full-length protein sequences (Figure 1). The tree produced by the three algorithms NJ, ME and MP were largely comparable with only minor modifications at interior branches (data not shown). Therefore, only the NJ phylogenetic tree was subjected to further analysis in our study. PtNACs were divided into 18 subfamilies and were designed as NAC-a to NAC-r according to Hu et al. [6]. Some of these subfamilies contain two or three subgroups (Figure 1 and Figure S1), but none of the PtNACs belonged to the subfamily NAC-p. Moreover, the phylogenetic tree constructed with the conserved N-terminal NAC domains A–E using the same algorithm was largely consistent with the phylogenetic analyses performed with the full-length protein sequences (Figure S2). In addition, other evidences support the reliability of the subfamily classification including gene structure, motif composition and expression patterns, as described below.

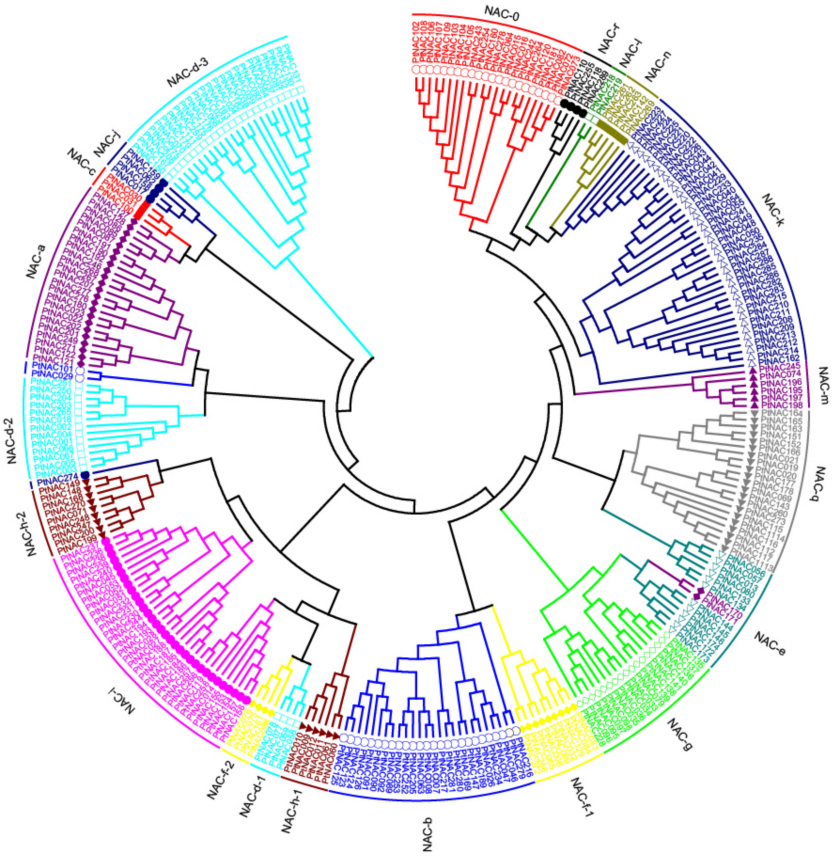

Figure 1. Multiple alignment and phylogenetic analysis of *Populus* NAC proteins. The full-length amino acid sequences of 289 *Populus* NAC proteins were aligned using Clustal X 1.83 and the phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) 7.0.21 with the NJ (Neighbor-Joining) method. Each subfamily is indicated in a specific colour.

3.2. Chromosome Location and Synteny Analysis of NAC Genes

In silico, 287 of the 289 (99.3%) *PtNACs* were mapped to the 19 LGs, while only *PtNAC278* and *PtNAC289* remained as yet unmapped scaffold_141 and scaffold_80, respectively (Figure 2). The distributions of *PtNACs* genes across the LGs appeared to be non-random (Figure 2). There were 28 and 27 *PtNACs* mapped on LG I and LG II, respectively. In contrast, LG VIII, LG XVI and LG XVIII each encompassed no more than 10 *PtNACs*. In addition, a clustering phenomenon of *PtNACs* was found on several LGs, especially on those with large numbers of *NAC* genes (Figure 2). For instance, 16 *PtNACs* were cluster localised on a 3.0 Mb segment on LG II, and more than 20 *PtNACs* were arranged in a cluster localised to a 5.0 Mb segment on LG XIV. The *PtNACs* were also mapped to the duplicated blocks established in the previous studies to determine the impacts of segmental duplication event on the *Populus NAC* genes. A total of 215 out of 287 (75.3%) *Populus NAC* genes were mapped on 29 of 36 identified duplicated blocks related to the recent salicoid duplication event (Figure S3). Among the 29 block pairs, 12 block pairs only harboured *NAC* genes on one of the blocks and lacked the corresponding duplicates. In contrast, the remaining 72 *NAC* genes were located outside of any duplicated blocks (Figure S3).

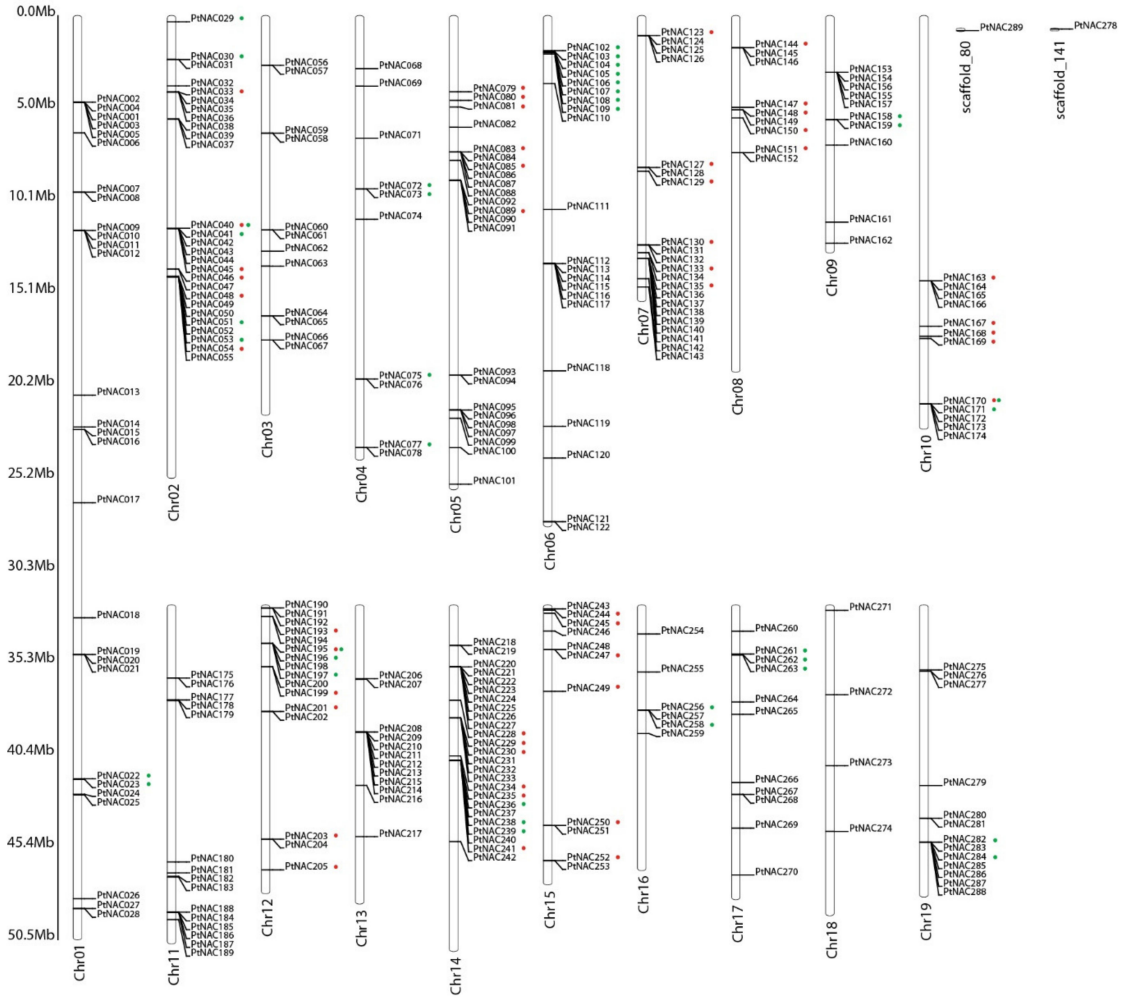

Figure 2. Chromosome locations of *Populus NAC* genes. The 287 *NAC* genes are mapped to the 19 LGs (Linkage-Groups) and the other two genes reside on scaffold_80 and scaffold_141. Whole-genome wide or segmental duplicated genes are labelled with red colour dots. The green dots indicate the genes are involved in tandem duplicates. The scales represent the distance of chromosomes.

Synteny analysis of the 171 nonredundant *Populus NAC* family genes was performed using the programme MCScanX and 46 collinear genes were found (Figure 3, Table S3). These collinear genes considered as resulting from a whole-genome duplication event (indicated in red colour dots) are mainly located on eight LGs of *Populus* (Figure 2, Table S3). The tandem duplications might have an impact on the expansion of the *Populus NAC* gene family. In our study, 37 *Populus NAC* genes related to tandem duplications were identified and are indicated in green colour dots (Figure 2, Table S3). They are distributed on 11 of the 19 LGs (Figure 2).

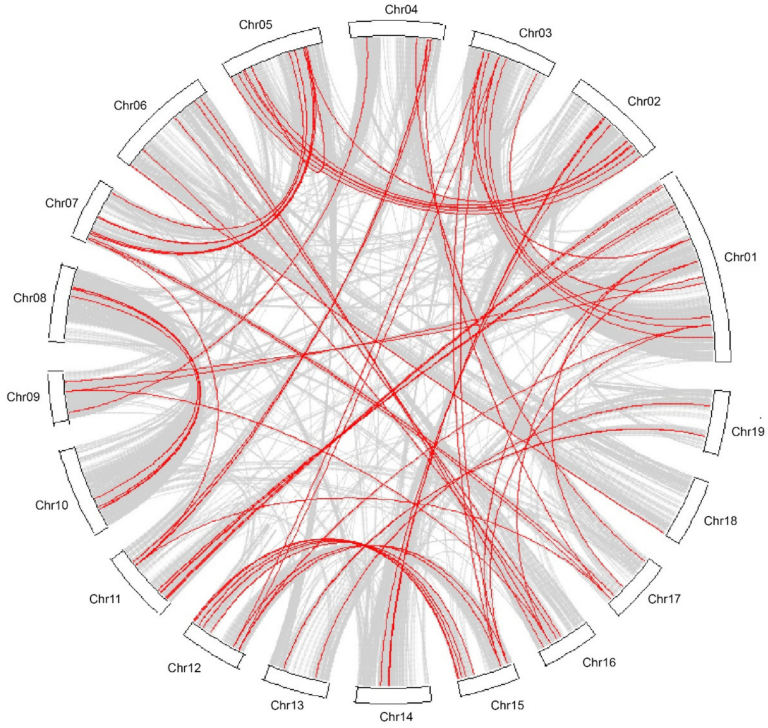

Figure 3. Duplicated blocks in *Populus* genome. The syntenic relationships among 19 *Populus* LGs are detected using the MCScanX programme. The colinear blocks of *Populus* genome are shown in grey connecting lines and the colinear blocks of *NAC* genes are marked by red connecting lines.

3.3. Gene Structure and Conserved Motifs Analysis of NAC Genes

Gene structures comprising UTR, CDS (coding sequence) and introns of individual genes were compared to investigate the structural diversity of *PtNACs*. In general, most related members in the same subfamily shared similar exon/intron structures in terms of intron numbers and exon length (Figure S4c). For instance, genes in Subfamily-o all had no intron with the exception of *PtNAC015*, *PtNAC016*, *PtNAC072*, *PtNAC073* and *PtNAC160*, which possessed only one intron, while most of the genes in Subfamily-d harboured two introns. In contrast, genes in Subfamily-b, Subfamily-k and Subfamily-q appeared to have a significantly variable structure organisation with a large number of exon/intron structure variants. However, we found that although the structure of NAC genes varied significantly, the intron was highly conserved (Figure S4c).

To further reveal the diversity of PtNACs, 15 conserved motifs were predicted using the programme MEME. Details of the 15 putative motifs are shown in an additional file (Table S4). The biological functions of most of the putative motifs remain unknown as we searched against the SMART and Pfam database. As shown in previous studies, most NAC proteins encompass the A to E subdomains in the N-terminal amino acid sequences that confer DNA-binding activities. In this study, motif-1, motif-6, motif-2, motif-3 and motif-7 representing the NAC subdomains A to E, respectively, were found in most of the *Populus* NAC proteins, and a small number of NAC proteins did not harbour all of these five motifs in their corresponding DNA-binding domains (Figure S4a, Table S4). Some conserved motifs still could be found, even though the C-terminal sequences of NAC proteins were divergent. We also found that most members in the phylogenetic tree had common motif compositions, suggesting functional similarities among *Populus* NAC proteins within the same subtree (Figure S4a). Noticeably, some specific motifs were present in NAC specific subfamilies, such as motif-10 for Subfamily-a and motif-11 for Subfamily-q. However, whether these motifs confer unique molecular functions to NACs remains to be further investigated.

3.4. Promoter Cis-Element Analysis

To investigate putative *cis*-acting regulatory elements in the promoter region of *Populus NAC* genes, we collected 15 experimentally characterised regulatory sites from published studies (Table S5). We found that the promoters of *PtNACs* harboured multiple *cis*-acting regulatory elements involved in plant defence and stress responsiveness, such as ABRE, CGTCA-motif, ERE, MBS, TC-rich repeats, TCA-element and W-box (Table S6). The 289 *PtNACs* all contained *cis*-elements related to phytohormone or stress signal responsiveness, but the kinds and numbers of *cis*-elements in different promoters varied. For instance, *PtNAC009*, *PtNAC010*, *PtNAC011* and *PtNAC012* had all of the 15 *cis*-elements except for the P-box motif, whereas *PtNAC272* had only three *cis*-elements (ARE, MBS and MYC) in the corresponding promoter sequences (Table S6). In addition, the number of the same *cis*-element also varied in different promoters of *Populus NACs* (data not shown).

3.5. Gene Ontology Annotation

The biological processes, molecular functions and cellular components of *Populus* PtNACs were determined using the programme Blast2GO v 5.2 based on the GO terms. Results showed that PtNACs are involved in diverse biological processes. Most PtNACs (267) are predicted to function in the biosynthetic, cellular metabolic and nitrogen compound metabolic processes; 15 PtNACs (PtNAC280, PtNAC217, PtNAC004, PtNAC005, PtNAC002, PtNAC003, PtNAC066, PtNAC165, PtNAC001, PtNAC067, PtNAC166, PtNAC152, PtNAC163, PtNAC164, PtNAC151) participate in the stress response process (Figure 4a, Table S7). Molecular function prediction found 265 PtNACs annotated as heterocyclic compound binding or organic cyclic compound binding, whereas 14 (PtNAC280, PtNAC182, PtNAC206, PtNAC217, PtNAC158, PtNAC159, PtNAC024, PtNAC255, PtNAC025, PtNAC179, PtNAC110, PtNAC275, PtNAC062, PtNAC183) and three (PtNAC204, PtNAC203, PtNAC250) PtNACs were respectively involved in the TF activity and oxidoreductase activity, which may be related to plant stress endurance (Figure 4b, Table S7). In addition, cellular component prediction indicated that *Populus* PtNACs were mainly localised in intracellular organelle, intracellular part, intracellular and membrane-bounded organelle (Figure 4c, Table S7).

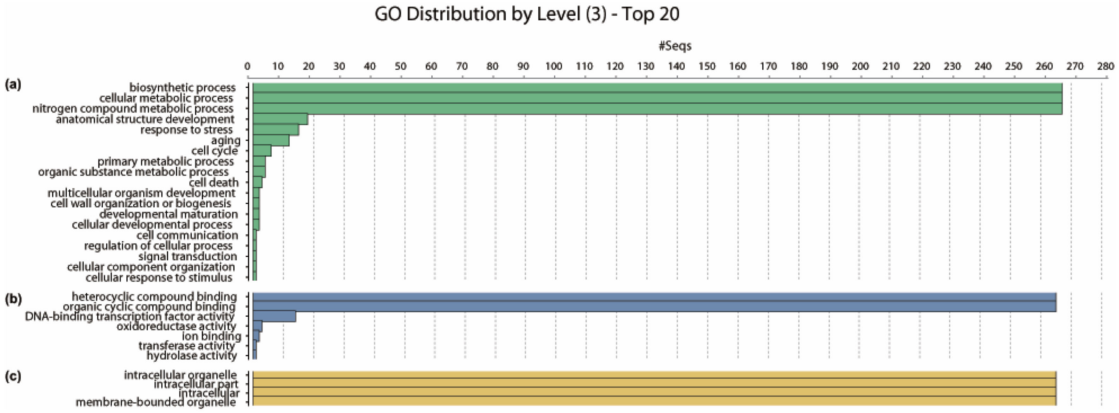

Figure 4. GO (gene ontology) annotation of *Populus NAC* genes. GO analysis of 289 *Populus NAC* genes sequences predicted for their involvement in biological processes (**a**), molecular functions (**b**) and cellular components (**c**).

3.6. Expression Characterization of NACs Under Salt Stress

The expression levels of the 73,013 genes of 84K poplar found in this study were detected using RNA-Seq. Results showed that the overall expression of 73,013 poplar genes was suppressed by salt stress. The FPKM of samples exposed to salt stress (S1, S2, S3, S4) were lower than those of the control (W1, W2, W3, W4) (Figure 5a). On the contrary, the expression levels of the 289 *PtNACs* from 84K poplar under salt stress condition were higher than the control (Figure 5b), which suggests that *NACs* play positive roles in poplar salt stress endurance. Consistent with this, similar expression trends of *NACs* were found in *P. simonii* × *P. nigra* (Figure 5c) and *Arabidopsis thalian* (Figure 5d). However, the differences among the samples do not reach a significant level (Figure S5), which may be due to the exposure time of the tested materials not being long enough.

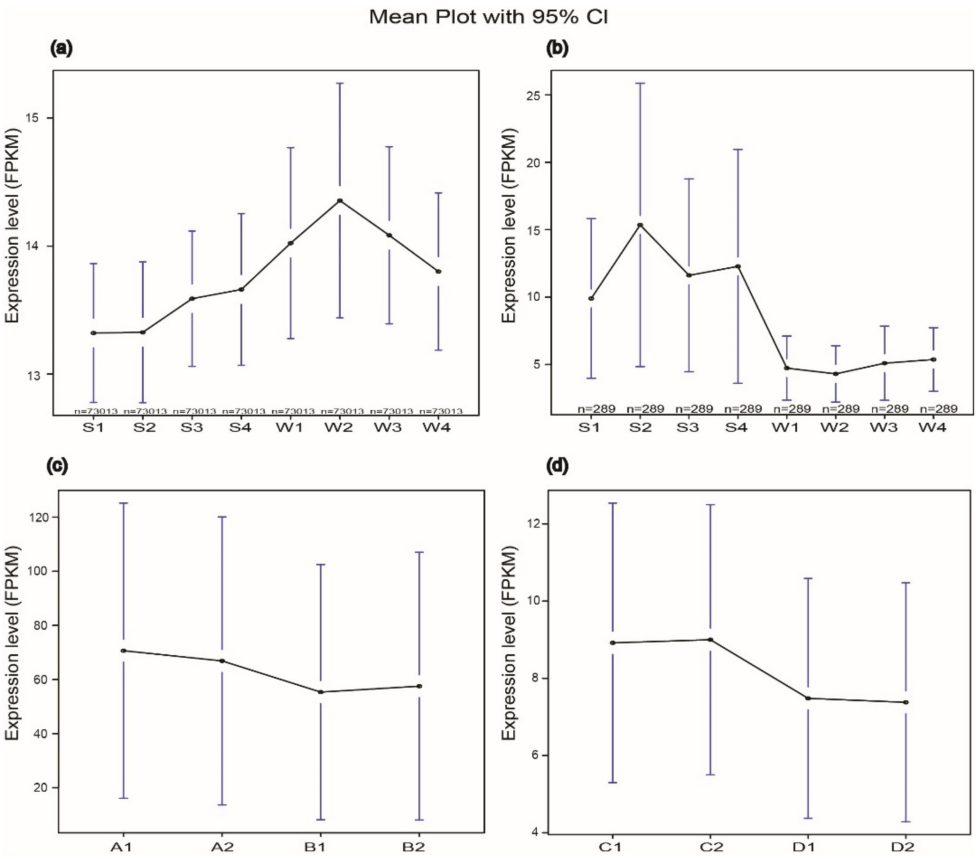

Figure 5. Expression level of *Populus* genes under salt stress detected by RNA-Seq. The expression is measured as FPKM (fragments per kilo-base transcript per million mapped reads) based on the Illumina HiSeq platform. A1, A2, C1, C2, S1, S2, S3 and S4 were treated with NaCl. B1, B2, D1, D2, W1, W2, W3 and W4 were controls treated with regular water. The overall expression of all the genes in 84K poplar (**a**). The overall expression of *NACs* in 84K poplar (**b**), *P. simonii* × *P. nigra* T. S. Hwang et Liang (**c**) and *Arabidopsis thaliana* (L.) Heynh. (**d**).

DEGs (differentially expressed genes) were detected using RNA-Seq data between the salt-treated samples and the control of 84K poplar. A total of 692 DEGs were identified in comparison of S1 and W1 (comparison group S1_W1), and 496 to 553 DEGs in the comparison groups S2_W2, S3_W3 and S4_W4 (Figure 6a). The number of up-regulated DEGs related to salt stress was generally similar to that of the down-regulated DEGs (Figure 6c). However, only 63 DEGs overlapped among the tested comparison groups (Figure 6b). DEGs in the poplar NAC family were also analysed in this study. Five *NAC* DEGs were found in the comparison group S3_W3 (Figure 6b). Two common differentially expressed *NAC* DEGs (*PtNAC024* and *PtNAC182*) were found in groups S1_W1, S3_W3 and S4_W4,

and no differentially expressed *NAC* was found in the comparison group S2_S2 (Figure 6b,d). Both *PtNAC024* and *PtNAC182* were up-regulated and retained to explore further their molecular functions in salt stress (Figure 6d). In addition, the expression patterns with $\log_2$FPKM of the above 63 DEGs and the two differentially expressed *NAC* DEGs in eight different samples (S1, S2, S3, S4, W1, W2, W3, W4) were characterised using Heatmap. For these 65 genes, the number of up-regulated genes was twice that of the down-regulated genes (Figure 7). The correlation analysis showed that the expressions of *PtNAC024* and *PtNAC182* were significantly associated with most of the 63 DEGs (Figure S6).

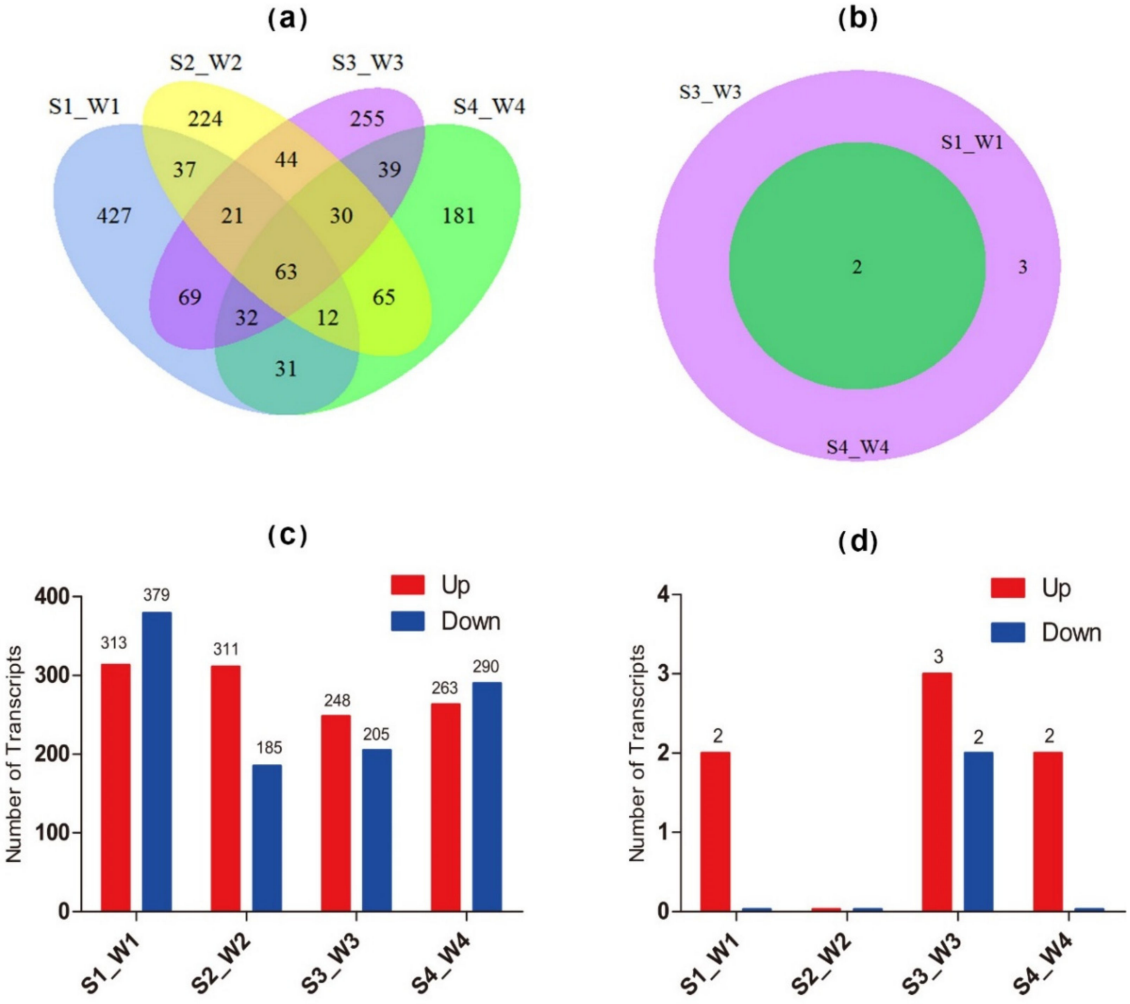

Figure 6. Number of stress-related differentially expressed genes (DEGs) in *Populus*. (**a**) Veen diagram analysis of DEGs of *Populus* in response to salt stress. (**b**) Veen diagram of DEGs of the NAC transcription factor (TF) family of *Populus* in response to salt stress. (**c**) Up/down-regulated DEGs of *Populus* in response to salt stress. (**d**) Up/down-regulated DEGs of the NAC TF family of *Populus* in response to salt stress. S1, S2, S3 and S4 were treated with NaCl. W1, W2, W3 and W4 were controls treated with regular water.

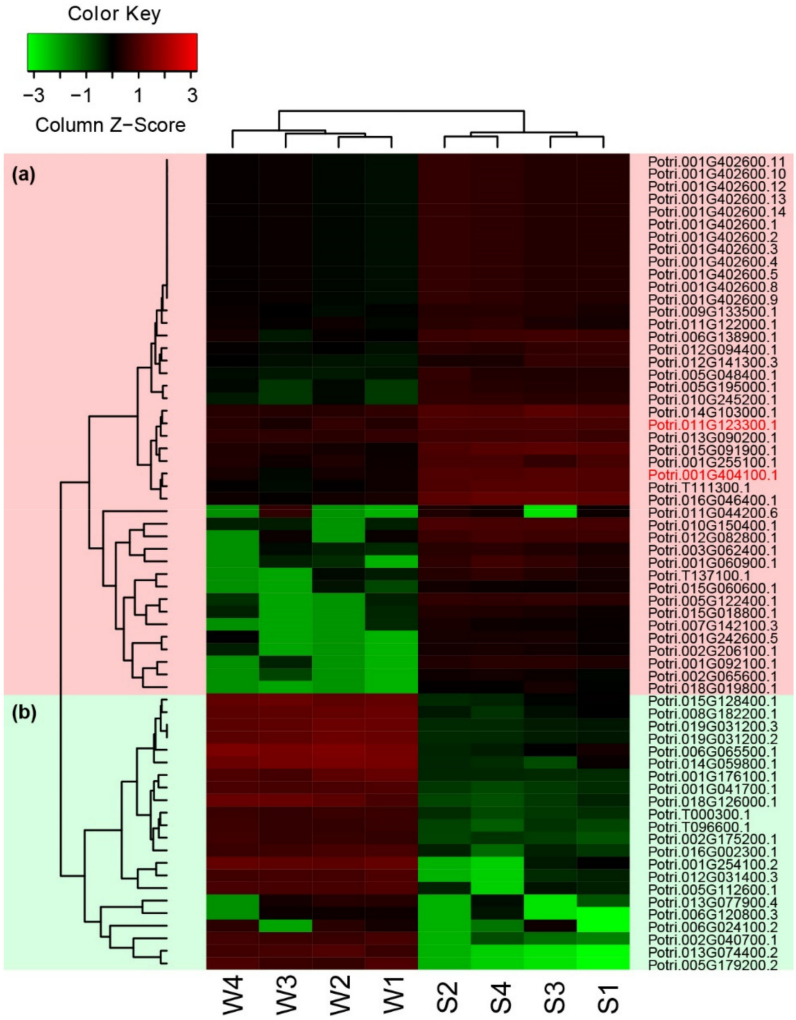

Figure 7. Heatmap and clustering of the 63 DEGs and two putative differentially expressed *NACs*, *PtNAC024* and *PtNAC182*, based on RNA-Seq in different samples exposed to salt stress. (**a**) Up-regulated genes are indicated with a pink background. (**b**) Down-regulated genes are indicated with an aqua background. The expression is measured as the log transformation (base 2) of the specific value (S-tested/W-control) of FPKM (fragments per kilo-base transcript per million mapped reads). The expression values were standardised by columns. Green indicates low expression and red denotes high expression. S1, S2, S3 and S4 were treated with NaCl. W1, W2, W3 and W4 were controls treated with regular water. *PtNAC024* and *PtNAC182* are marked with red font.

The expression patterns of the two differentially expressed *NACs* in the other plant species were also detected based on the RNA-Seq data in this study. In 84K poplar, the expression levels of both *PtNAC024* and *PtNAC182* after exposure to salt stress were significantly higher than under regular water conditions (Figure 8a). Similar trends were found in *P. simonii* × *P. nigra*. *PtNAC024* and *PtNAC182* of *P. simonii* × *P. nigra* were induced by salt stress, but the differences between the tested samples and the control were not significant (Figure 8b). It's worth noting that *Arabidopsis thalian* carries a single homolog gene for *PtNAC024* and *PtNAC182*, *AT4G27410.2*, which was also significantly induced by salt stress (Figure 8c). To cross-validate the expression changes of *PtNAC024* and *PtNAC182* in response to salt stress challenge, we examined the available RT-qPCR data from 84K poplar seedlings treated with NaCl and compared them to controls. Similar to the RNA-Seq data, the RT-qPCR results indicated that *PtNAC024* and *PtNAC182* were highly induced by salt stress (Figure 8d). The relative expression of *PtNAC024* under salt stress condition is about 3.8 times higher than under the control condition, and for *PtNAC182* about 8.2 times compared to the control (Figure 8d).

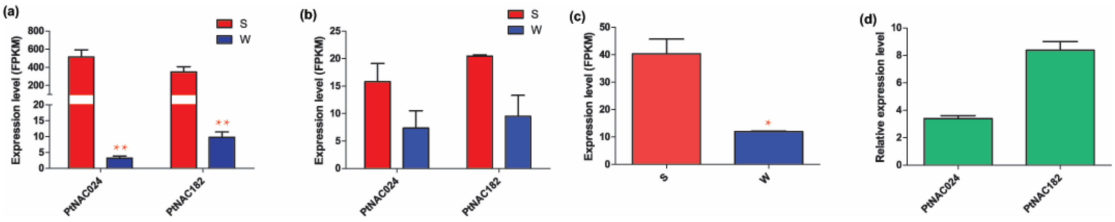

Figure 8. Expression analysis of differentially expressed *NACs* in response to salt stress based on RT-qPCR of 84K poplar and RNA-Seq of *Populus* and *Arabidopsis*. (**a**) Expression of *PtNAC024* and *PtNAC182* of 84K poplar based on RNA-Seq. (**b**) Expression of *PtNAC024* and *PtNAC182* of *P. simonii* × *P. nigra* based on RNA-Seq. (**c**) Expression of the gene homolog of *PtNAC024* and *PtNAC182* in *Arabidopsis* (*AT4G27410.2*) based on RNA-Seq. (**d**) Relative expression of *PtNAC024* and *PtNAC182* of 84K poplar based on RT-qPCR. The expression level based on RNA-Seq was measured as FPKM and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, defined as: $\Delta\Delta C_t = (C_{t\text{-target}} - C_{t\text{-control}})_2 - (C_{t\text{-target}} - C_{t\text{-control}})_1$. S indicates samples treated with NaCl and W the controls treated with regular water. *Stars* represent *t*-test results of gene expression after salt treatment compared to that of the controls. * indicates a significant difference at $p < 0.05$ and ** at $p < 0.01$.

3.7. Tissue-Specific Expression Pattern In Silico

In silico, the expressions of *PtNAC024* and *PtNAC182* in different tissues were analysed using the exImage tool. Both *PtNAC024* and *PtNAC182* were highly expressed in mature leaves, followed by the roots and finally the nodes (Figure 9). However, the expression of *PtNAC024* in internode tissues is relatively higher than that in young leaves (Figure 9a), while the opposite is true for *PtNAC182* (Figure 9b). Similar trends were also found in the internode and node tissues (Figure 9). These results may suggest that *PtNAC024* and *PtNAC182* are also involved in the synthesis of lignin and fibrin, respectively.

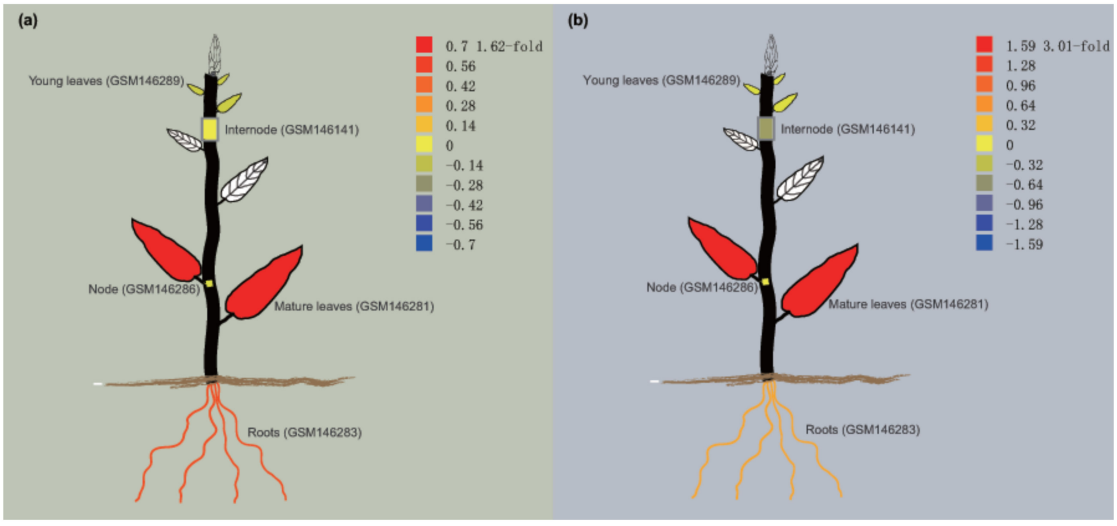

Figure 9. Tissue-specific expression pattern of *PtNAC024* and *PtNAC182* of *Populus* in silico. (**a**) Expression pattern of *PtNAC024*. (**b**) Expression pattern of *PtNAC182*. The related data were directly downloaded using the accession numbers of genes from PopGenIE and detected using the exImage tool of the PopGenIE V3 database (http://popgenie.org/). Blue indicates low expression and red denotes high expression.

4. Discussion

The NAC domain TFs are plant-specific TFs [5,11]. In a former published report, a total of 163 *NAC* genes were identified in *Populus* by searching the HMM domain [6]. In this study, a total of

289 NAC transcripts of 171 nonredundant genes were obtained and analysed from the PlantTFDB database, which identifies TFs using the Joint Genome Institute Ptri version 3.0 [7]. The transcripts were designed as PtNAC001–PtNAC289 for the convenience of analysis and identification (Table S1). These 171 genes covered 85.27% (139/163) of previously reported NAC TF genes of *Populus* (Table S1). The difference in the numbers of *PtNACs* may be due to different analysis methods, requiring further exploration. The homology sequence analysis found that two or more *Populus* NAC TFs are hit with one ortholog in *Arabidopsis*, which is consistent with the findings of former studies [6]. The phylogenetic tree constructed with the full-length protein sequences divided the 289 PtNACs into 18 subfamilies according to the methods used in former studies, but no PtNACs belonged to the subfamily NAC-p [6]. Although the bootstrap values were somewhat low due to the large number of sequences, which was also the case in previous studies [53–55], more significant bootstrap values in the distal branches allowed us to group the NAC proteins into distinct families (Figure S1). Phylogenetic analyses of the conserved N-terminal NAC domains A–E showed that the clusters were largely consistent with those of the full-length protein sequences, suggesting that the conserved NAC domains may play primary roles in NAC TF structures and functions [11,14].

It has been speculated that *Populus* has undergone at least three gene duplication events during the evolutionary process [9,56]. The genome duplication provides more copies of the genes, making it possible for poplar to gain new functions [55,57,58]. To detect the relationship between segmental duplication events and NAC TFs, we mapped *PtNACs* to the duplicated blocks and found that 75.3% *PtNACs* were mapped on 29 block pairs and 12 block pairs lacked corresponding duplicates [9], suggesting that dynamic changes have occurred following the segmental duplication events and some genes were lost (Figure S3). In addition to segmental duplications, whole-genome duplication and tandem duplication events might also have an impact on the expansion of the *Populus* NAC TF family [55,57,58]. The synteny analysis showed that 46 *PtNACs* related with the whole-genome duplication and 37 *PtNACs* involved in tandem duplications were mainly distributed on 14 of 19 LGs (Figure 2). It is intriguing that *PtNAC040*, *PtNAC170* and *PtNAC195* were detected in both whole-genome and tandem duplications. However, whether the duplicated *NACs* impact genetic redundancy or have evolved into divergent functions remains to be further study.

Although phylogenetic analysis provides important information for candidate genes selection, it cannot alone unequivocally indicate gene function [41]. For this reason, we combined phylogenetic grouping, gene structure, conserved motif identification, promoter *cis*-element and induction/tissue expression pattern. In this study, members of the same subfamily shared similar gene structure and conserved motif compositions. For instance, genes in Subfamily-q have the specific motif motif-11 and a large number of exon/intron structure variants (Figure S4). We also investigated putative *cis*-acting elements in the promoter region of *PtNACs* and found that *PtNAC009*, *PtNAC010*, *PtNAC011* and *PtNAC012* distributed in LG I of the same subfamily, Subfamily-f, contain 14 of 15 stress-related *cis*-elements (Figure 1, Figure 2, Table S6). GO annotation found that 15 *PtNACs* are involved in the stress response process and three participate in the oxidoreductase activity. It is interesting that 10 (*PtNAC001*, *PtNAC002*, *PtNAC003*, *PtNAC004*, *PtNAC005*, *PtNAC066*, *PtNAC067*, *PtNAC203*, *PtNAC204*, *PtNAC250*) of these 18 stress-related genes are located in Subfamily-d and six (*PtNAC151*, *PtNAC152*, *PtNAC163*, *PtNAC164*, *PtNAC165*, *PtNAC166*) in Subfamily-q (Figure 1, Figure 4, Table S7). The expression of *PtNACs* under salt stress was also characterised in this study and two DEGs, *PtNAC024* and *PtNAC182*, were selected for further stress-related study. *PtNAC024* and *PtNAC182* are located in the Subfamily-d, consistent with the GO analysis predicting that members in Subfamily-d play roles in the stress response process. In addition, both *PtNAC024* and *PtNAC182* along with *AT4G27410.2*, the homolog gene in *Arabidopsis thalian*, were significantly induced by salt stress [59]. Tissue-specific expression analysis in silico showed that *PtNAC024* and *PtNAC182* were also involved in *Populus* synthesis of lignin and fibrin. Taken together, these findings indicate that *Populus* NAC TFs play important roles in plant stress endurance and development. The function and expression patterns

in poplar using overexpression and RNAi of these two genes will be determined in our future study. We are looking forward to further explore the molecular functions of PtNACs.

5. Conclusions

In conclusion, we uncovered the salt stress-related expression of a total of 289 *Populus* NAC TFs in this study. The phylogenetic relationship analysis found that members in the same subfamily share a common gene structure, motif composition and promoter *cis*-elements, suggesting functional similarities among *Populus* NAC proteins within the same subtree. The expression pattern of *Populus NACs* under salt stress showed that *PtNACs* expression of 84K poplar, *P. simonii* × *P. nigra* and *Arabidopsis thalian* was induced by salt stress. In addition, two differentially expressed *NAC* genes (*PtNAC024* and *PtNAC182*) induced by salt stress were found using edgeR. The correlation analysis showed that the expressions of *PtNAC024* and *PtNAC182* were significantly associated with most of the tested 63 DEGs under salt stress condition. The tissue-specific expression analysis found that *PtNAC024* and *PtNAC182* were differentially expressed in the internode, node and young leaf tissues. These findings support that NAC TF members play important functions in the *Populus* salt tolerance and tissue development.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/8/688/s1, Table S1: NAC TFs of *Populus*. Table S2: Amino acids and cDNA sequences of the 289 *Populus* NAC genes identified in this study. The detailed information, including amino acid lengths, molecular weights and isoelectric points (PI) are also shown. Table S3: Synteny analysis of 171 *Populus* NACs. Intra-species gene synteny and collinearity were detected by MCScanX using default parameters considering pBLAST $\leq 1 \times 10^{-5}$ [43]. Table S4: Sequence logos of the putative conserved motifs of NAC proteins. Conserved motifs and the sequence logos were generated using the MEME and TBtools programmes. Information of the discovered motifs is shown in the excel table. Motif-1 represents the NAM sub-domain A, motif-6 represents the NAM sub-domain B, motif-2 represents the NAM sub-domain C, motif-3 represents the NAM sub-domain D and motif-7 represents the NAM sub-domain E. Table S5: Phytohormone and abiotic stress-related *cis*-elements. Table S6: Abiotic stress-related and phytohormone response elements in 289 PtNACs promoters. Table S7: Details of the Gene Ontology annotation of 289 PtNAC sequences. Figure S1: Phylogenetic tree of 17 subfamilies of 289 *Populus* NAC proteins. The full-length amino acid sequences of each subfamily NAC proteins were aligned using Clustal X 1.83 and the phylogenetic tree was constructed using MEGA 7.0.21 with the NJ method. The numbers at nodes indicate the percentage bootstrap scores and only bootstrap values higher than 50% are shown. Figure S2: Phylogenetic tree of N-terminus of *Populus* NAC proteins. The Phylogenetic tree was constructed using MEGA 7.0.21 with the NJ method after alignment of the conserved N-terminus domain of 289 *Populus* NAC proteins. Only the topology is presented. Figure S3: Genome-wide duplication of *Populus*. The schematic diagram of genome-wide chromosome organisation arisen from the salicoid-specific genome duplication 65 Ma in *Populus* was adapted from Tuskan et al. [9]. Segmental duplicated homologous blocks are indicated with the same colour. Only the duplicated regions containing NAC genes are connected with lines in shaded colours. The diagram to the left uses the same colour coding and further illustrates the chimeric nature of most linkage groups. Figure S4: Conserved motifs, phylogenetic relationships and gene structure of *Populus* NAC genes. (a) Conserved motifs of *Populus* NAC proteins were discovered using the MEME programme and are shown by colourful boxes. The black lines represent the non-conserved sequences and the detailed information of each motif is presented in ESM_6. (b) Multiple alignments of the full-length sequences of *Populus* NAC proteins were performed using the programme Clustal X 1.83 and the phylogenetic tree was constructed using MEGA. The percentage bootstrap scores higher than 50% are indicated on the nodes. The 18 phylogenetic subfamilies are designed as a to r. (c) The UTR/CDS structures of Populus NAC genes were detected using the programme TBtools. UTR and CDS are represented by green and yellow boxes, respectively. The black lines represent the introns of each NAC gene. The sizes of UTR, CDS and introns can be estimated using the scale at the bottom. Figure S5: Significance analysis of the expression level of *Populus* genes under salt stress detected by RNA-Seq. (a) The overall expression of all the genes of 84K poplar. (b) Overall expression of NACs of 84K poplar. (c) Overall expression of NACs of *P. simonii* × *P. nigra*. (d) Overall expression of NACs of *Arabidopsis*. The 95% family-wise confidence level was measured. The X-coordinate indicates the differences in mean levels (FPKM) of groups. S1, S2, S3 and S4 were treated with NaCl. W1, W2, W3 and W4 were controls treated with regular water. Figure S6: Correlation analysis of the expression level of the 63 DEGs and two putative differentially expressed NACs, *PtNAC024* and *PtNAC182*, based on RNA-Seq in different samples exposed to salt stress. *Red* indicates poor correlation and *blue* denotes high correlation. Statistical analyses were conducted using R v3.3.1 (http://cran.r-project.org/).

Author Contributions: Conceptualization, S.W. and Y.H.; Methodology, S.W.; Software, S.W.; Validation, J.H. and X.W.; Investigation, J.H. and X.W.; Resources, T.J.; Data curation, H.D.; Writing—Original draft preparation, J.H. and X.W.; Writing—Review and editing, S.W.; Supervision and project administration, Y.H.; Funding acquisition, S.W.

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