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Cloning and Functional Analysis of *PtATAF1-1* Transcription Factor Gene of *Populus trichocarpa*

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Abstract The ATAF1 protein is a member of the NAC transcription factor family and plays an important regulatory role in plant development and stress response. In this study, using rapid amplification of cDNA ends, a full-length cDNA of *ATAF1* homolog was cloned from *Populus trichocarpa,* and the corresponding gene was named *PtATAF1-1*. The full-length cDNA sequence of *PtATAF1-1* is 1 242 bp, which consists of 3 exons and 2 introns, and an 873 bp coding region. The multiple amino acid sequences alignment showed that PtATAF1-1 protein contains the conserved NAM domain of NAC family. Transient expressing with poplar protoplast shows PtATAF1-1 protein localization in the nucleus. In addition, with the treatment of N-terminal conserved region of bacterial flagellin (Flg22) and abscisic acid (ABA) altered the expression level of *PtATAF1-1* gene. In this study, the homologous cloning and functional analysis of the *PtATAF1-1* gene were performed, which provided a reference for the further development of the function of *ATAF1* gene in *poplar*.

Keywords *Populus trichocarpa*; *PtATAF1-1* gene; Expression analysis; Subcellular localization

Populus is widely distributed in the middle latitudes of the world, and is one of the important industrial wood and ecological protection tree species in China (An, 2013, Modern Horticulture, (14): 69-71). Poplars have fast growth, strong adaptability and relatively simple reproduction, which have important economic and ecological values (Shao, 2012). Poplar plantation is limited by desertification and salinization, and poplar is often subjected to abiotic and biological stresses such as drought, low temperature and infection of pathogenic microorganisms in the growth process, so it is of practical value to enhance the stress resistance of poplar. At the same time, poplars have full genomic information and complete genetic engineering technology platform, so poplars are also model species for exploring the gene function of woody plants (Wang et al., 2016).

Plants rely on transcription factor (TF) to regulate defense genes, control plant secondary metabolism and respond to hormone signals, so as to cooperate with a large number of genes in response to adversity. Transcription factors can bind specifically to cis-acting elements in upstream promoters of genes to control the expression of target genes (Ao, 2017). According to the sequence specificity of DNA binding domain, transcription factors can be divided into multiple transcription factor families, among which NAC transcription factors with NAM, ATAF1,2 and CUC2 DNA-binding domains are the largest in higher plants (Kasuga et al., 1999). ATAF1 was found to be one of the archetypal NAC transcription factors in *Arabidopsis thaliana* (Souer et al., 1996). *AtATAF1* can be induced to be up-regulate expression under high salt, drought, *Botrytis cinerea* and ABA (Huh et al., 2012). *NCED3* is a regulatory target gene of *ATAF1* and plays a key role in ABA biosynthesis. In plants overexpressed by *AtATAF1*, the transcriptional abundance of *NCED3* and ABA level increased, indicating that *AtATAF1* activates ABA response genes and regulates its biosynthesis (Jensen et al., 2013). Overexpression of *AtATAF1* in *Arabidopsis thaliana* also increased the sensitivity of plants to ABA and salt, thus enhancing drought tolerance of transgenic plants (Liu et al., 2016). However, overexpression of *AtATAF1* can increase plant susceptibility to pathogens such as *Pseudomonas syringe* (Cahill et al., 2018). Therefore, *ATAF1* plays an important role in regulating both biological and abiotic stress in model plant *Arabidopsis thaliana* (Mauch-Mani and Flors, 2009).

In this study, we cloned a *ATAF1* homolog (*PtATAF1-1*) from Poplar, and studied its bioinformation, gene expression pattern and transient expression of poplar protoplast. It is preliminarily speculated that *ATAF1* gene is involved in the response to biological and abiotic stress. This study provides a preliminary theoretical reference for further exploring the function of *ATAF1* gene in poplar.

1 Result and Analysis

1.1 Cloning and sequence analysis of *PtATAF1-1* homolog in *Populus trichocarpa*

Blastp analysis of Arabidopsis AtATAF1 (AT1G01720) amino acid sequences in *Populus trichocarpa* database revealed 8 highly similar proteins, identified as Potri.002G081000, Potri.011G046700, Potri.011G123500, Potri.001G404100, Potri.005G180200, Potri.004G038000, Potri.011G123300, Potri.001G404400, E-Value is 1e-116, 6e-063, 2e-062, 2e-062, 6e-063, 7e-112, 3e-063, 5e-063. Identity is 70.65%, 70.55%, 69.23%, 69.14%, 68.81%, 68.86%, 68.52%, 68.21% respectively. Blastp analysis was performed using these homologous proteins in The Arabidopsis Information Resource (TAIR), it was found that Potri.005G180200 and Potri.002G081000 were most likely to be ATAF1 homologous proteins in poplars. Therefore, they were named PtATAF1-1 and PtATAF1-2, respectively. Under the cultivation conditions of *Populus trichocarpa* in our laboratory, only the full length of *PtATAF1-1* gene sequence can be amplified from cDNA of *Populus trichocarpa* leaves by RT-PCR, so *PtATAF1-1* is only used as the research object in the future.

Rapid amplification of cDNA ends (RACE) was used to amplify 5′ and 3′ ends of *Populus trichocarpa PtATAF1-1*. A full-length cDNA sequence of 1 242 bp, containing an 873 bp ORF encoding 291 amino acids was obtained. It also contains 5′ UTR (97 bp) and 3′ UTR (272 bp) (Figure 1).

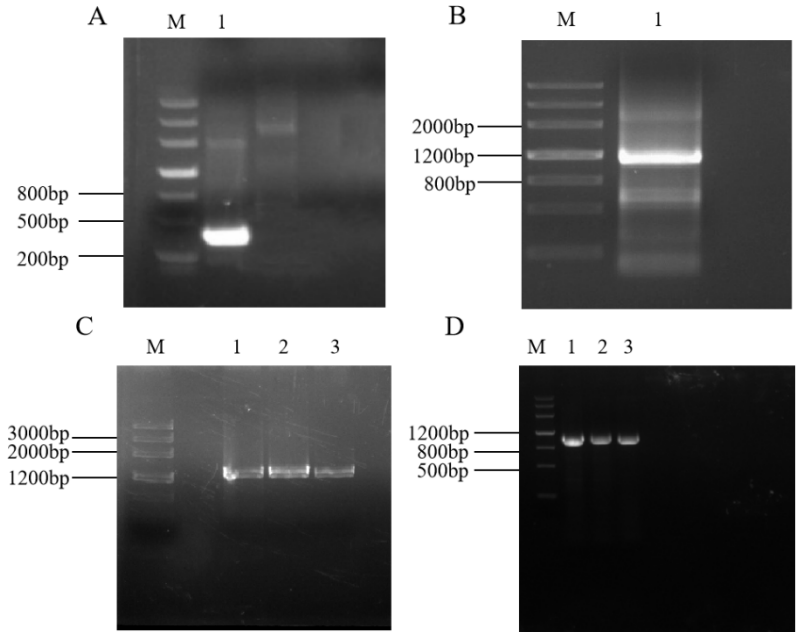


Figure 1 The cloning of *PtATAF1-1* gene

Note: M: MD103 DNA marker; A: 5'RACE amplification products; B: 3'RACE amplification products; C: Full-length gene amplification products; D: Gene coding region ORF amplification product

Blastp comparison was performed on *Populus trichocarpa* PtATAF1-1 in NCBI non-redundant database, and ATAF1 sequences of other 6 representative plants, namely *Populus euphratica* (XP_011027492.1), *Salix Brachista* (KAB5568578.1), *Manihot esculenta* (XP_021604672.1), *Hevea brasiliensis* (XP_021649171.1) *Jatropha curcas* (AEI25534.1) and *Arabidopsis thaliana* (NP_171677.1) were selected for multiple sequence comparison. The comparison results showed that the conserved sequence of NAM (PF02365) was found in the Pfam database, and the amino acid sequences of PtATAF1-1 and other ATAF1 were found to be highly conserved in the NAM domain, indicating that PtATAF1-1 belonged to the NAC family (Figure 2).

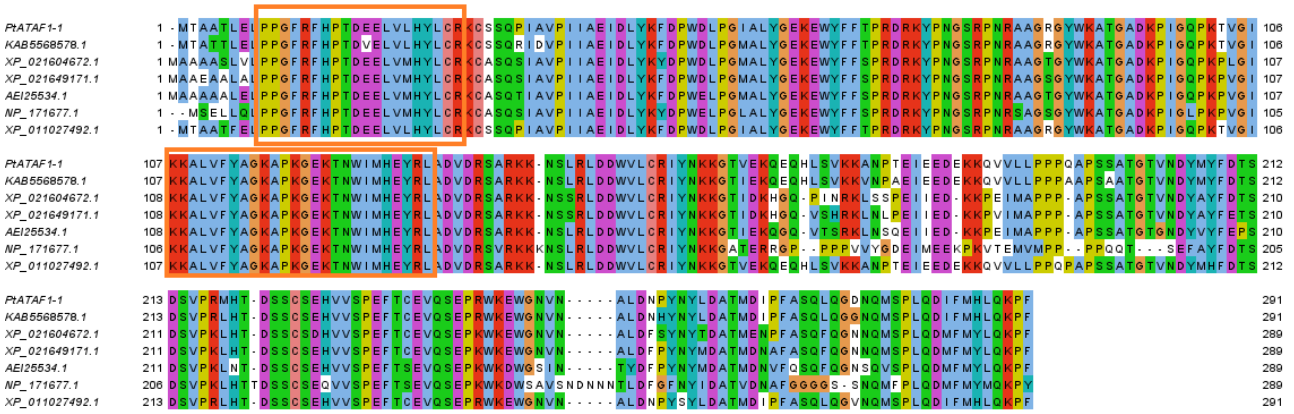
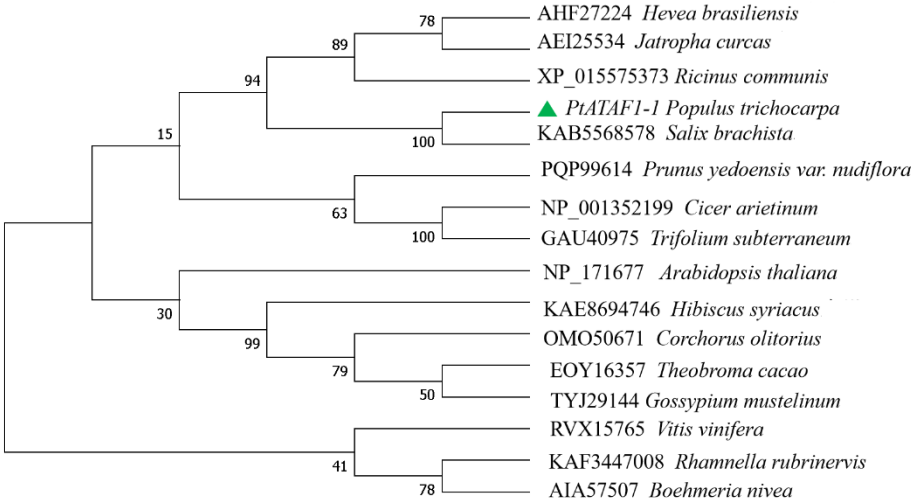


Figure 2 PtATAF1-1 and other plants ATAF1 of multiple sequence alignment
Note: The orange box represents the NAM conservative domain

1.2 Evolutionary analysis of PtATAF1-1 protein

In order to investigate the evolutionary relationship of PtATAF1-1 transcription factor, the amino acid sequences of ATAF1 homologous proteins in 16 species such as *Populus trichocarpa*, *Hevea brasiliensis*, *Jatropha curcas* and *Salix brachista* were used to construct phylogenetic trees. The phylogenetic trees were constructed by MEGA X software. And it turns out, ATAF1 of *Populus trichocarpa* and *Salix brachista* in Salicaceae, and *Ricinus communis*, *Hevea brasiliensis*, and *Jatropha curca* in Euphorbiaceae constituted a highly supported clade (94%), which was consistent with the close relationship between Salicaceae and Euphorbiaceae. However, phylogenetic analysis with *Vitis vinifera* in Vitaceae, *Rhamnella rubrinervis* in



Rhamnaceae, and *Boehmeria nivea* in Urticaceae did not receive more than 50% support (Figure 3).

Figure 3 The Phylogenetic tree of ATAF1 proteins from multiple species
Note: The value on the branch indicates the credibility of the node based on 1000 repeats in Bootstrap verification; The scale indicates the genetic distance

1.3 Physical and chemical properties and protein structure analysis of *PtATAF1-1*

According to the analysis of *PtATAF1-1* gene sequence by ProtParam, the theoretical molecular weight of PtATAF1-1 protein was 33.27 kD, the isoelectric point (pI) was 6.40, and the instability coefficient was 52.88. Among the 291 amino acid residues encoded, the proportion of proline was the largest (8.6%), followed by lysine (7.9%), leucine (7.2%) and alanine (6.9%). The hydrophilicity/hydrophobicity of PtATAF1-1 protein was predicted by ProtScale online analysis website, and the result showed that the average hydrophilicity coefficient was -0.49, suggesting that the protein was a basic hydrophilic protein (Figure 4A). The secondary

structure of PtATAF1-1 was predicted by SOPMA, an online tool of PRABI-GERLAND. The results showed that the secondary structure of PtATAF1-1 had a large amount of random curl, 18.56% α-helix, 11.34% elongation and 2.06% β-rotation (Figure 4B). Using Protein Homology/analogY Recognition Engine V 2.0, a 3d model of PtATAF1-1 Protein was established online and its tertiary structure was predicted (Figure 4C), indicating that the protein was prone to homodimer formation.

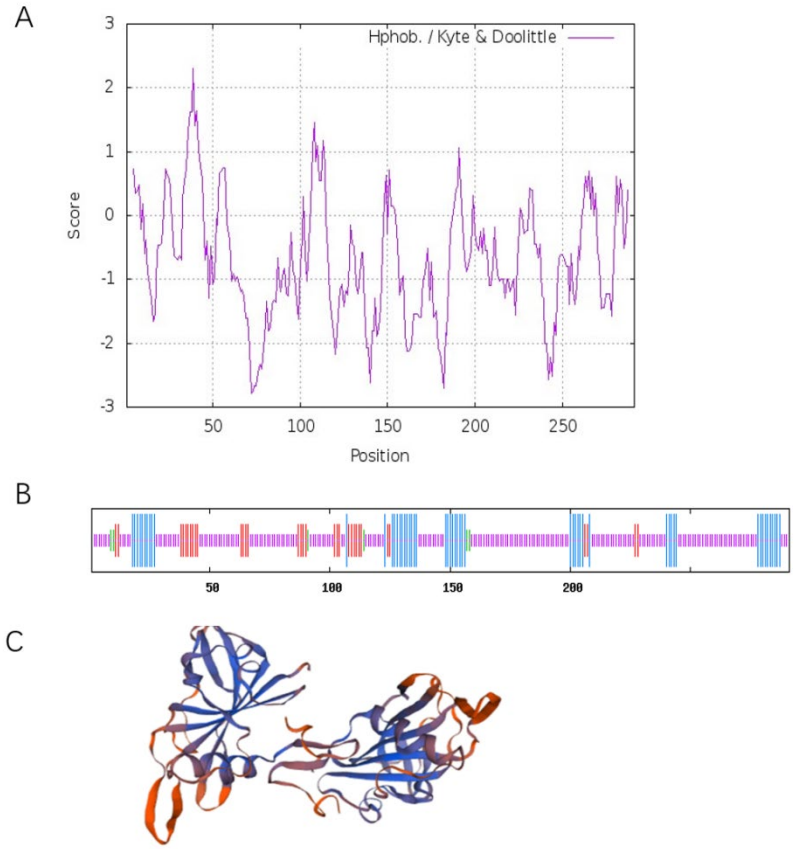


Figure 4 Analysis of physical and chemical properties and protein structure of *PtATAF1-1*

Note: A: Hydrophobicity/phydrophilicity prediction of PtATAF1-1protein: B: Predicted secondary structure of target protein of PtATAF1-1; C: The predicted advanced structure of target protein of PtATAF1-1

1.4 Subcellular localization analysis of PtATAF1-1 protein

The PtATAF1-1-GFP fusion vector was constructed and transient expression of PtATAF1-1 in the protoplast of Populus 895 of Nanjing Forestry University was achieved by PEG mediated method. The localization of PtATAF1-1 in the protoplast was observed by fluorescence microscope with GFP as the reporter gene. The results showed that in the case of single-transformed GFP, green fluorescence existed in the cell membrane and nucleus, while the green fluorescence occurred in the PtATAF1-1-GFP fusion protein only appeared in the nucleus, indicating that PtATAF1-1 protein was a nuclear localization protein, in line with the characteristics of its transcription factor (Figure 5).

1.5 Determination of *PtATAF1-1* gene expression under ABA and Flg22 treatment

The conserved regions of bacterial flagellin N-terminal Flg22 (2 μmol/L) and ABA (0.01 mmol/L) were used to treat tissue culture seedlings of *Populus trichocarpa*. Real-time quantitative PCR was used to detect the expression of *PtATAF1-1* gene. The results showed that after treatment with ABA hormone stress, the expression level of *PtATAF1-1* gene decreased at 1 h, but increased at 12 h and 24 h, and reached the peak at 24 h. Compared with the control group at 12 h and 24 h, the expression level of *PtATAF1-1* gene in ABA treatment group increased. The results showed that the expression level of *PtATAF1-1* gene responded to ABA treatment (Figure 6A). After Flg22 treatment, the expression level of *PtATAF1-1* increased at 1 h and 12 h but decreased

at 24 h. The expression level of *PtATAF1-1* was higher at 1 h, 12 h and 24 h than that of the control group, indicating that the expression level of *PtATAF1-1* responded to Flg22 treatment (Figure 6B). The blank control group was treated with water.

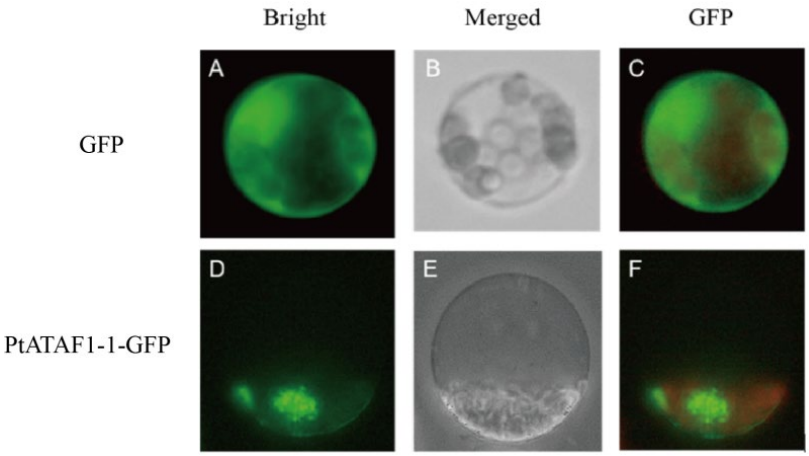


Figure 5 Subcellular localization of PtATAF1-1protein

Note: A~C: Expression of GFP empty vector in *P. trichocarpa* mesophyll protoplasts; D~F: Expression of PtATAF1-1-GFP vector in *P.trichocarpa* mesophyll protoplasts; GFP: Green fluorescence; Bright: Bright field vision; Merged: Overlapped effect

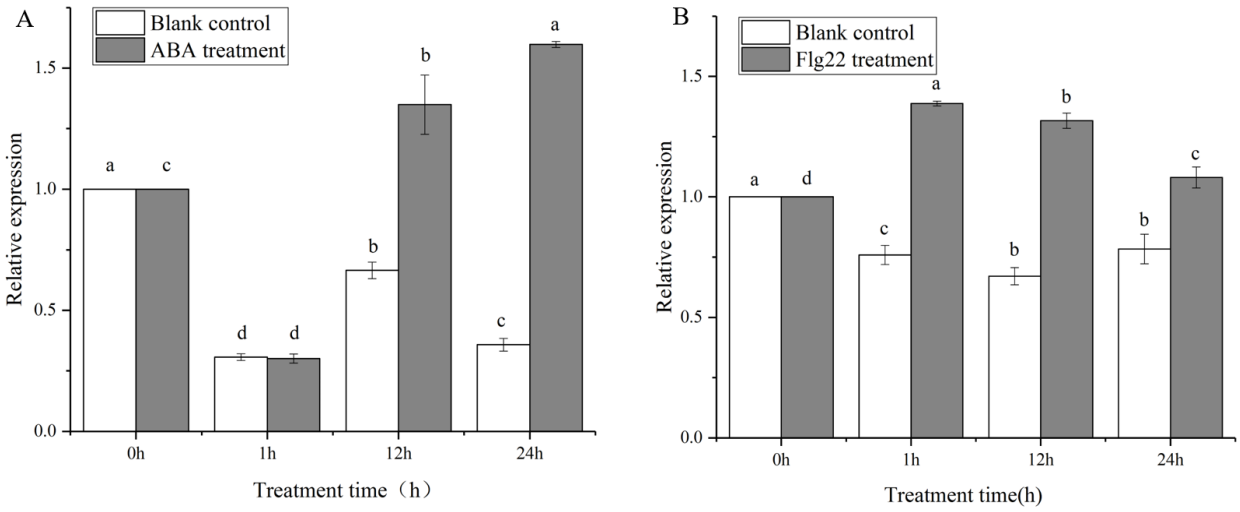


Figure 6 The effects of ABA and Flg22 treatment on the relative expression level of *PtATAF1-1*

Note: A: Expression of *PtATAF1-1* under ABA and control treatment; B: Expression of *PtATAF1-1* under treatment conditions of Flg22 and control treatment; The *EF1a* was used as an internal control for normalization, each experiment was performed three times; Data were analyzed using one-way ANOVA LSD test ($p<0.05$); Different letters indicate significant differences at the $P<0.05$ level; "n.s." indicates no significant differences at the $P<0.05$ level; Data represent the means ±SD of three biological repllicates, error bars represent standard deviation

2 Discussion

ATAF1 is a transcription factor belonging to the plant NAC family, which exists widely in the Plantae. It has been found that *ATAF1* and *ATAF2* genes in *Arabidopsis thaliana* are attacked and induced by pathogens (Zheng et al., 2009). Arabidopsis ATAF1, a NAC transcription factor, is a negative regulator of the defense response of necrotizing fungi and bacterial pathogens. When ATAF1 is silenced, the resistance of plants is enhanced and participates in the regulation of many abiotic stress and necrotrophic pathogen infection responses (Wang et al., 2009; Wu et al., 2009).

In this study, *PtATAF1-1* gene was homologous cloned from *Populus trichocarpa*, and functional domain

analysis of the protein encoded by *PtATAF1-1* gene showed that it had a conserved NAM domain, and phylogenetic tree analysis results proved that the pTATAF1-1 protein did belong to the NAC family. The phylogenetic analysis of *Arabidopsis thaliana* and *Populus trichoderma* does not fully conform to the genetic relationship of the species, and it may be that the evolution of protein sequence is affected by their unique living environment, different growth and development pattern and life cycle, so there may be differences in protein function. The physical and chemical properties and protein structure of PTATAF1-1 were analyzed by bioinformatics, which provided a reference for understanding the function of plant *ATAF1* gene.

Previous studies on subcellular localization of ATAF1 protein showed that ATAFl-GFP fusion protein was verified by transient expression of Arabidopsis *ATAF1* gene in onion epidermal cells by gene bombardment (Lu, 2006, China Agricultural University, pp.55-56). Cucumber NAC transcription factor CsATAF1 was obtained by homologous cloning method, and subcellular localization results showed that CsATAF1 protein was nuclear localization (Wang, 2018). In this study, subcellular localization analysis of 895 Poplar of Nanjing Forestry University showed that pTATAF1-1 protein was also located in the nucleus, which was the same as previous results and also in line with the functional requirements of transcription factors.

Studies have shown that *NCED3* is the regulatory target gene of *ATAF1*. In plants overexpressed by *ATAF1*, the transcription abundance of *NCED3* increases and plays a regulatory role in the biosynthesis of ABA (Jensen et al., 2013). In this study, real-time quantitative PCR was used to analyze the expression pattern of *PtATAF1-1*. *PtATAF1-1* gene was responsive to both ABA and Flg22 treatments, but the effects were different. After ABA treatment, the expression level of *PtATAF1-1* gene decreased first and then increased, and was higher than that of control group at 12 h and 24 h. After treatment with Flg22, the expression level of *PtATAF1-1* gene in *Populus trichocarpa* increased first and then decreased, which were all higher than the control group. It is concluded that *PtATAF1-1* gene may be involved in response to ABA signal and bacterial infection, and has certain functions in these biological processes.

At present, the in-depth study on the function of *PtATAF1-1* gene in *Populus trichocarpa* is not clear. The above results preliminarily analyzed the structure, subcellular localization and expression level of *PtATAF1-1*, providing direct genetic evidence for the role of *PtATAF1-1* as a plant stress regulator.

3 Materials and Methods

3.1 Plant materials and main reagents

The tissue culture seedlings of *Populus trichocarpa* and poplar 895 were subcultured in our laboratory and grew in an incubator with temperature of 22°C/18°C (day/night), light of 16 h/8 h (light/dark) and 50% environmental humidity. Top10 susceptible *Escherichia coli* samples were purchased from Tiangen Biotech, Beijing, China. pMD19-T clone vector was purchased from Dalian TaKaRa Company. RNA extraction kit, PCR product recovery kit, DNA gel recovery kit, plasmid small extraction kit, protoplast separation and transformation related reagents and kits, plasmid small extraction medium quantity kits were purchased from Tiangen Biotech, Beijing, China. The reverse transcription kit was purchased from Promega, Madison, WI, USA. The real-time quantitative kit was purchased from Roche Applied Science, Indianapolis, IN, USA. The expression vector p2GWF7.0 was stored in our laboratory.

3.2 Cloning of PTATAF1-1 gene and amplification of ORF core sequence

Poplar leaves were taken for RNA extraction and fully ground with liquid nitrogen, according to the instructions of Plant RNA extraction Kit RNAprep Pure Plant Kit (Tiangen). Amplify 3′-RACE and 5′-RACE segments using the RACE Kit. Follow the SMARTer® RACE 5′/3′ Kit (TaKaRa) instructions. After gel cutting and recycling, 3′-RACE and 5′-RACE products were connected to pMD19-T vector (TaKaRa), and the reaction procedure of cloned vector connection was 16℃ for 1 h. 2.5 μL of ligand products were used for transformation of Top10 *E. coli*. Positive clones were selected for PCR detection and sequenced to obtain 3′-RACE and 5′-RACE product sequences. Further splicing was performed to obtain the full-length sequence of the gene and

analyze its ORF region. The full-length coding sequence (ORF) of pTATAF1-1 was cloned from the cDNA previously obtained by KOD-Plus (Toyobo, Japan), and a tail was added for another round of PCR extension reaction. PCR product recovery kit was used to recover the amplified fragment and connect it with the pCR8™/GW/TOPO™ entry vector, and then connect it with the expression vector p2GWF7.0 by LR reaction of GATEWAY technology.

Table 1 Primer sequence

Primer name	Primer sequence (5'-3')
PtATAF1-1_3′OUTER	CTATCTCTGCCGTAAATGCTCATC
PtATAF1-1_3′INNER	TGCTGTGCCTATTATTGCTGAAAT
PtATAF1-1_5′OUTER	CAATCGGCTTGTCGGCTCCC
PtATAF1-1_5′INNER	CGATTCGGTCTCGATCCGTTAGGGTACTTC
PtATAF1-1_WHOLE_F	AAGTTTCAAGCGCCGTCACAGC
PtATAF1-1_WHOLE_R	CCAAAATATGAATCCAATTATAACCGC
PtATAF1-1_ORF_F	ATGACGGCGGCAACATTAGAGT
PtATAF1-1_ORF_R	AAACGGCTTCTGCAGGTGCAT
PtATAF1-1_qRT-PCR_F	TATTCCATTTGCGTCTCAGTT
PtATAF1-1_qRT-PCR_R	TTACCGTGCGATTTGCCCTTA

3.3 Bioinformatics analysis of *PtATAF1-1* gene

The RBH method was used to select the *Populus trichocarpa* database for Blastp in phytozome (https://phytozome.jgi.doe.gov), and the genes with high homology were found by sequence comparative analysis. Then, the genes with the highest homology were found by reverse alignment in the TAIR (https://www.arabidopsis.org/Blast/index.jsp), and the *PtATATF1* gene to be cloned was determined by bidirectional alignment. NCBI Blastp was used to retrieve protein similarity, and ATAF1 amino acid sequences of other plants were downloaded. Clustalx2.1 multi-sequence alignment software was used to perform multiple alignment of amino acid sequences and predict their conserved domains. The PF sequence number of NAM was found using Pfam 32.0 (http://pfam.xfam.org/), and the domain of NAM was predicted using the Smart online tool (http://smart.embl.de/). Phylogenetic tree of PtATAF1-1 homologous protein was constructed by MEGA X. The number of Bootstrap cycles using the adjacency method is 1000. ProtParam online tools (https://web.expasy.org/protparam/) preliminary forecast of molecular weight, isoelectric point, unstable factor. The online site (http://life.nthu.edu.tw/~b861625/protscale.html) ProtScale predicts protein hydrophilicity/hydrophobicity. Secondary structure prediction of PtATAF1-1 protein was performed by SOPMA website (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). Online tools Phyre2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict PtATAF1-1 protein tertiary structure.

3.4 Subcellular localization of PtATAF1-1 protein

Referring to the preparation method of poplar protoplasm system (Tan et al., 2013), the protoplasts of tissue culture seedlings of Poplar 895 with good growth at about 6 weeks were extracted by enzymatic hydrolysis. p2GWF7.0-PtATAF1 and positive control plasmid were transformed into protoplasts respectively by PEG mediated method (positive control was empty vector p2GWF7.0 only carrying GFP reporter gene). The localization of PtATAF1 in protoplasts was observed by fluorescence microscope (Axioscope.A1, Carl Zeiss) using GFP as reporter gene.

3.5 qRT-PCR analysis of *PtATAF1-1* gene

Treatment with ABA hormone and Flg22, ABA 0.01 mmol/L, bacterial flagellin short peptide Flg22 2 μmol/L, adding 0.01% Silwet-77 sprayed on leaves, and then harvested leaves at 0 h, 1 h, 12 h, 24 h after treatment. They were immediately frozen in liquid nitrogen and stored at -70℃. The blank control group was treated with H_2O. After growing for 4 weeks, tissue culture seedlings of *Populus trichocarpa* were selected and RNA was

extracted from leaves of *Populus trichocarpa*, and cDNA was synthesized using ordinary reverse transcription kit. The qRT-PCR amplification reaction was carried out using ABI7500 fluorescence quantitative PCR instrument. Using EF1α gene as internal reference, the upstream and downstream primers used in qRT-PCR reaction were PtATAF1-1-qF and PtATAF1-1-qR respectively (Table 1). The PCR program was set as 50℃ for 2 min, 95℃ for 10 min, 95℃ for 15 s, and 60℃ for 1 min, in total, 40 cycles. According to the method of references (Schmittgen and Livak, 2008), the relative quantitative method was used to calculate the relative expression level of the target gene according to the $2^{-\Delta\Delta CT}$ method, and 3 biological replicates were set. IBM SPSS 20.0 was used for one-way anova and multiple comparison of the data. Origin 2017 software was used to draw charts and analyze the significance of differences.

Authors' contributions

PJ is the experimental designer and executor of this study. She has completed data analysis and written the first draft of the paper. WHR participated in experimental design and analysis of experimental results; CQ is the architect and principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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