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PwuWRKY48 Confers Drought Tolerance in *Populus wulianensis*

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Abstract: *Populus wulianensis* mainly grows in hilly and sloped areas and has strong resistance to adversity. Previous transcriptome studies have shown that a *WRKY* gene *PwuWRKY48* is expression-induced under drought stress. In this study, we aim to characterize the gene's structure and investigate its role in plant drought resistance. The results show that PwuWRKY48 (1113 bp) belongs to a class IIc WRKY subfamily and it was determined as a nuclear localization protein. The gene promoter region contains a variety of cis-elements in relation to stress resistance. Under drought stress, *PwuWRKY48* was expression-induced in leaves and stems, 29.7 and 16.6 times those before treatment, respectively. Overexpressing *PwuWRKY48* lines were associated with increased activities of peroxidase (POD) and superoxide dismutase (SOD), 2.5 and 1.6 times higher than those of the wild type. While malondialdehyde content (MDA), superoxide anion radical ($O_2^{\cdot-}$), and relative conductivity were decreased by 20%, 30%, and 21.3%, proline and chlorophyll contents increased by 37.5% and 11.2%, respectively. This indicates that *PwuWRKY48* efficiently improved the drought tolerance of transgenic plants. *PwuWRKY48* can be used as a gene resource for molecular breeding of plant drought resistance.

Keywords: *Populus wulianensis*; WRKY transcription factor; drought stress; transformation

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1. Introduction

WRKY is a kind of transcription factor that functions via regulating the expression level of target genes. It is widely involved in plant growth and development as well as in adversity resistance processes [1,2]. For example, overexpressing *Pyrus betulaefolia PbrWRKY53* in *Nicotiana tabacum* L. increased the expression level of *PbrNCED1* and caused vitamin C accumulation [3], while knockdown of *PbrWRKY53* down-regulated the expression of *PbrNCED1* and reduced drought tolerance. *Phyllostachys edulis* PheWRKY86 binds to the W-box element in the promoter region of *NCED1* to enhance the drought tolerance of plants [4]. PtrWRKY75 from *Populus trichocarpa* regulates the expression of *PAL1* and promotes SA biosynthesis, which increases the accumulation of reactive oxygen species (ROS), reduces stomatal pore size, and enhances drought resistance [5]. CRK5, a cysteine-rich receptor-like protein kinase in *Arabidopsis thaliana*, is involved in ABA signaling and is jointly negatively regulated by *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* [6]. PtrWRKY19 could inhibit the transcription of *PtoC4H2* and negatively regulate the formation of the secondary wall of the pulp in *P. trichocarpa* [7].

The expression of transcription factors is also regulated by other proteins and hormones. For example, the *PeWRKY41* promoter contains several cis-elements associated

with stress resistance [8], resulting in the increased expression of *PeWRKY41* under ABA, ethylene, MeJA, salicylic acid, and NaCl stresses. Therefore, it is of great significance to analyze the composition of the transcription factor genes and their promoter elements and to understand the regulation process and gene network that determine their functions.

Populus wulianensis S. B. Liang and X. W. Li is a tree mainly distributed in barren habitats, displaying strong drought resistance [9,10]. Wild populations of *P. wulianensis* are only distributed on Kunyu Mountain, Zhaohu Mountain, and Jiuxian Mountain in Shandong, China [11]. The number of surviving individuals of this endemic species has been declining because of many threats, which mainly include anthropogenic activities, habitat destruction, and climate change. This species has also been listed in the provincial rare and endangered conservation list in Shandong Province [12]. A previous transcriptome analysis (GEO accession # GSE 252898) showed a *WRKY* gene significantly enhanced under drought stress in *P. wulianensis*. Sequence alignment revealed it to be homologous to *A. thaliana AtWRKY48*, and it was then named *PwuWRKY48*. In this paper, we aim to investigate the expression model under drought stress and the effects of associated regulatory elements on the promoter and further evaluate its contribution to plant resistance against drought stress through genetic transformation. These results might provide a gene resource for a drought-resistant breeding program and offer more ideas for conservation and utilization of *P. wulianensis* germplasm.

2. Materials and Methods

2.1. Plant Materials and Drought Treatment

Newly extracted shoots of *P. wulianensis* with buds in the spring were used as explants and inserted in the bud induction medium (1/2 MS (NH_4NO_3) + 0.05 mg/L naphthalene acetic acid (NAA) + 0.4 mg/L 6-benzylaminopurine (6-BA)) [13]. The differentiated buds were inserted in 1/2 MS rooting medium supplemented with 0.05 mg/L NAA. The culture conditions were 6000–8000 lux light intensity, 16/8 h of light/dark cycle, 25 °C, and relative humidity of 60%–70%.

One-month-old *P. wulianensis* seedlings were transplanted into pans (7 cm × 7 cm × 7.5 cm) with light substrate (peat:perlite:vermiculite = 3:1:1) and grown in the greenhouse (6000 lux light intensity, 16/8 h of light/dark cycle, 25 °C). Water irrigation was provided once every three days. After one month of growth, the water irrigation was removed for 10 days as a drought treatment. The regular watering was used as a control. The soil was sampled for moisture measurement during the drought stress according to the formula [(fresh soil sample weight − dried soil sample weight)/fresh soil sample weight]. The stems, leaves, and root tissues were also harvested on each day of drought treatment for RNA isolation and physiological index tests.

2.2. Gene Cloning and Vector Construction

The total RNA from *P. wulianensis* leaves was extracted by a modified hexadecyl trimethyl ammonium bromide (CTAB) method [14]. The 1st-strand cDNA was synthesized using the PrimeScript™ IV 1st Strand cDNA Synthesis Mix Kit (Takara, Dalian, China). Based on the genomic data (https://phytozome-next.jgi.doe.gov/, accessed on 12 September 2020) of *P. trichocarpa*, *PtrWRKY48*-specific primers (PtrWRKY48F: 5′-TCACACTCTCACAATGCT-3′: PtrWRKY48R: 5′-ATGTGAACTGGGATCGTCT-3′) were designed. RT-PCR was performed using PrimeSTAR® Max DNA Polymerase (TaKaRa, Dalian, China). The PCR products were recovered and ligated to the pMD19-T™ vector with a Cloning Kit (Takara, Dalian, China) and transformed into *Escherichia coli* for sequencing.

For construction of the recombinant expression vector, a pair of primers (PwuWRKY48LCF: 5′-CCCAAGCTTATGAATGAAATGAAGGAGAATAAT-3′: PwuWRKY48LCR: 5′-TGCTCTAGACTACTCCTCTTTAGGCTCCT-3′) were further designed at both ends of the *PwuWRKY48* gene with *HindIII* and *XbaI* cleavage sites, respectively. The full-length gene fragment of *PwuWRKY48* was amplified and recovered. Both the gene fragment and the purified expression vector PEZR-(K)-LC plasmid were digested with *HindIII* and *XbaI* and ligated

with T4-DNA ligase (Takara, Dalian, China) (Figure 1), then transformed into *E. coli*. The recombinant plasmid was extracted and transformed into *Agrobacterium tumefaciens* GV3101. The positive clones were identified and used for the subsequent genetic transformation.

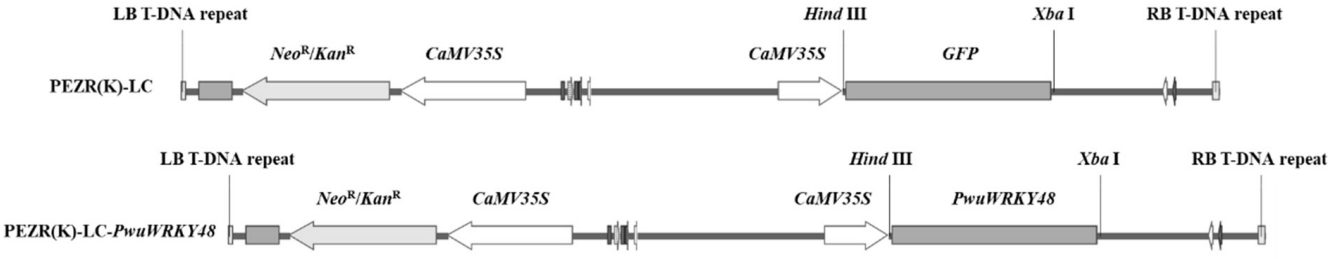

Figure 1. Structure of *PwuWRKY48* overexpression vector.

2.3. Gene Characterization and Phylogenetic Analysis

To explore the evolutionary relationship of PwuWRKY48, six WRKY members were selected from each subgroup (I, IIa–e, III) of the WRKY family in *A. thaliana* and *P. trichocarpa* and phylogenetic analysis was performed. The amino acid sequences of the WRKY genes were obtained from the Phytozome database (http://www.phytozome.com, accessed on 31 October 2023). Multiple sequence alignment was conducted using ClustalW. 2.0.11 A phylogenetic tree was constructed using MEGA 10 with the maximum likelihood method, 1000 repetitions of bootstrap tests, and a JTT matrix-based model. The sequence comparison figure was generated by use of DNAMAN 9.0 software. The theoretical isoelectric point, molecular weight, and hydrophilicity/hydrophobicity of PwuWRKY48 protein were calculated using ProtParam online software (https://web.expasy.org/protparam/, accessed on 31 October 2023).

2.4. Expression Analysis of PwuWRKY48

The total RNA was extracted from each sample of *P. wulianensis* during drought treatment and reverse transcribed using PrimeScript™ RT Master Mix (Perfect Real Time) (TaKaRa, Dalian, China). cDNA was used as a template for quantitative real-time PCR (qRT-PCR) analysis, and each sample was tested three times. The relative expression of genes was calculated using the $2^{-\Delta\Delta CT}$ method [15]. The significance of expression difference between the drought-treated sample and control was evaluated using a one-way ANOVA model. Two pairs of fluorescent quantitative primers were designed for *PwuWRKY48* (qPwuWRKY48F: 5′-CAAGGAGTTATTATCGTTGC-3′; qPwuWRKY48R: 5′-TGGATGTATGTGTTGACCT-3′) and reference gene *Actin7* (qActinF: 5′-CGTACAACTGGT ATTGTGT-3′; qActinR: 5′-CTCAGTCAGAATCTTCAT-3′).

2.5. Subcellular Localization of PwuWRKY48

The specific primers were designed for the gene fragment of *PwuWRKY48* without termination codon (pNCPwuWRKY48F: 5′-AGTGGTCTCTGTCCAGTCCTATGAATGAAATG AAGGAG-3′; pNCPwuWRKY48R: 5′-GGTCTCAGCAGACCACAAGTCTCCTCTTTAGGC TCC-3′). The product was integrated into the 5′ end of the GFP coding region of the transient expression vector pNC-Amp-GFP-C [16] to construct a *35S*::*PwuWRKY48*::*GFP* fusion vector by seamless cloning technology.

Tobacco leaf protoplasts were extracted [17]. A PEG-mediated method [18] was used to transfer GFP-fusion vector into the protoplasts. Leaf protoplasts were observed with an Olympus FV1000 (Olympus, Japan) confocal microscope. GFP fluorescence was observed with 473 nm laser excitation and emission between 487 nm and 521 nm, and chloroplast autofluorescence was observed with 559 nm laser excitation and emission between 606 nm and 673 nm.

2.6. Promoter Cloning and Cis-Acting Element Identification

Based on the genome database of *P. trichocarpa*, specific primer pairs (ProPwuWRKY48F: 5′-TCACACTCTCACAATGCT-3′: ProPwuWRKY48R: 5′-ATGTGAACTGGGATCGTCT-3′) upstream of the *PwuWRKY48* gene (approximately 1000 bp from the start codon) were designed. The promoter fragment was amplified with the genomic DNA of *P. wulianensis*, cloned, and sequenced. The cis-elements in the promoter region were predicted using the online software PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 12 October 2023).

2.7. Gene Transformation

The positive agrobacterium strains with *PwuWRKY48* plasmid were inoculated into YEB medium solution and cultured until OD600 reached 0.5–0.6. After centrifugation, the strains were collected and resuspended in 1/2(NH_4NO_3) MS liquid medium with 100 µmol/L acetosyringone (β-AS). The healthy *P. wulianensis* leaves were harvested and cut 3–4 times along the main leaf veins, and then soaked in the positive strain solution for 10 min. They were then moved onto solid 1/2(NH_4NO_3) MS medium with 100 µmol/L β-AS in the dark. After 3 days of co-culture, the leaves were transferred onto differentiation medium (1/2(NH_4NO_3) MS + 0.5 mg/L 6-BA+ 0.05 mg/L NAA + 100 mg/L kanamycin + 300 mg/L timentin). The regenerated buds were transferred on rooting medium (1/2 MS + 0.05 mg/LNAA + 100 mg/L kanamycin + 300 mg/L timentin). The induced resistant rooted seedlings were taken from the leaves and cultured again for amplification of leaf-induced adventitious shoots, while the rooted seedlings were transplanted for refining, and after one month of greenhouse culture, the leaves of rooting seedlings were collected for DNA extraction with a M5 Hiper Plant Genomic DNA Kit (Mei5bio, Beijing, China). After PCR testing for *PwuWRKY48*, the positive plants were used for total RNA isolation, which were then used for expression testing via qRT-PCR.

2.8. Drought Resistance Evaluation of the Transgenic Plants

The overexpressing *PwuWRKY48* poplar lines and wild type were vegetatively propagated. The identical clonal plants were transplanted into pans with light substrate (peat:perlite:vermiculite = 3:1:1). After two months of regular management, the poplar plants had water deficiency for 10 d (regular watering was used as a control). The leaves were then harvested for the physiological index tests.

Chlorophyll content of the plants was determined using a chlorophyll meter (SPAD-502-PLUS, Konica Minolta, Japan) [19]. Leaf-staining experiments were carried out with nitrotetrazolium chloride blue (NBT), 3,3′-diaminobenzidine (DAB), and Evans blue, respectively [20]. H_2O_2 content, superoxide dismutase (SOD) activity, peroxidase (POD) activity, proline content, $O_2^{\cdot -}$ content, and malondialdehyde (MDA) content were measured using Physiological Indicator Determination Kits (Solarbio Science and Technology, Beijing, China). Relative conductivity was determined using a conductivity meter (S230-K, Mettler Toledo, China) [21]. Three replicates for each line/treatment were analyzed. IBM SPSS Statistics 26 software was used for multiple comparisons between the transgenic line and the wild type at significant difference level $p = 0.05$.

3. Results

3.1. Expression Pattern of PwuWRKY48 Gene in Drought Treatment

Soil moisture was measured during the drought treatment. The separate values were 68.6% (0 d), 65.2% (1 d), 63.2% (2 d), 60.4% (3 d), 57.3% (4 d), 53.8% (5 d), 48.6% (6 d), 40.5% (7 d), 33.2% (8 d), 25.1% (9 d), and 20.8% (10 d). One-month-old *P. wulianensis* plants grew well in the earlier days of the drought treatment, but the leaves started to wilt at 8 d, and severe wilting occurred at 10 d (Figure 2).

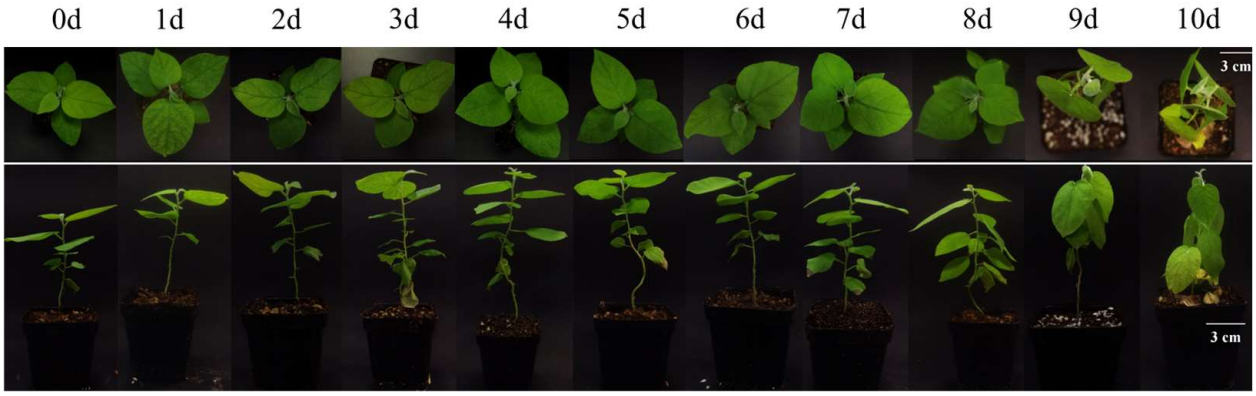

Figure 2. Phenotype performance of *P. wulianensis* plants in drought treatment (0 d–10 d). The upper row displays an aerial view of entire plant, and the lower shows the state of the plant.

Physiological index tests also showed a great change in the late days of drought treatment compared with that in the earlier days (0–5 days). For example, the relative electrical conductivity of plants changed slightly, 0.56-fold higher at 5 d than that at 0 d [(5 d − 0 d)/0 d]. It increased significantly from 8 d, 2.4-fold higher at 10 d than that at 0 d (Figure 3A). Meanwhile, SOD activity displayed a similar trend, 0.1-fold higher at 5 d than that at 0 d, but 4.1-fold higher at 10 d than that at 0 d (Figure 3B). *P. wulianensis* sustained drought injury from 7 d.

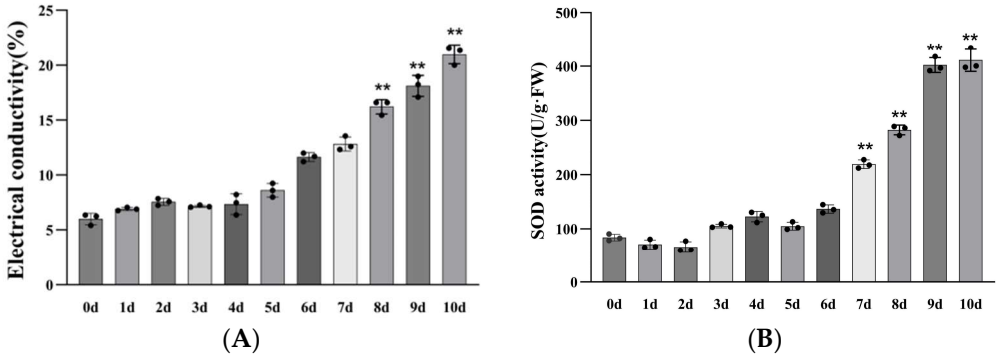

Figure 3. Performance of relative electrical conductivity (**A**) and SOD activity (**B**) in *P. wulianensis* under drought treatment. Three biological replicates were conducted. The error bars represent standard deviation. Asterisks indicate significant differences between the data at the treatment day (1 d−10 d) and control (0 d) (*t* test, $p < 0.05$).

The PCR tests showed that *PwuWRKY48* was expressed in all tissues of stems, roots, and leaves of *P. wulianensis* (Figure 4A) under normal growth conditions. It had the highest expression value in roots, 1.8 to 4.3-fold higher than those in the stems and leaves, respectively. In the early days of water control (0 d–5 d), the *PwuWRKY48* expression model displayed first an upward and then a downward trend in the roots. It reached the peak at 1 d and 2 d of stress treatment, both 2.0-fold higher than those at 0 d (Figure 4B). In the later stress treatment (6 d–10 d), *PwuWRKY48* expression displayed a continuous increase. Additionally, *PwuWRKY48* had relatively low expression in the stems in the early days of water control, then gradually increasing later. It reached its peak at 10 d with an expression level 17.6-fold higher than that at 0 d (Figure 4C). In leaves, *PwuWRKY48* had relatively low expression in the early days of water control, then increasing sharply at 8 d. It reached a peak at 9 d with an expression level 28.7-fold higher than that at 0 d, and then gradually decreased (Figure 4D). In summary, *P. wulianensi* roots are sensitive to water deficiency, while leaves and stems were somewhat insensitive until real drought stress started.

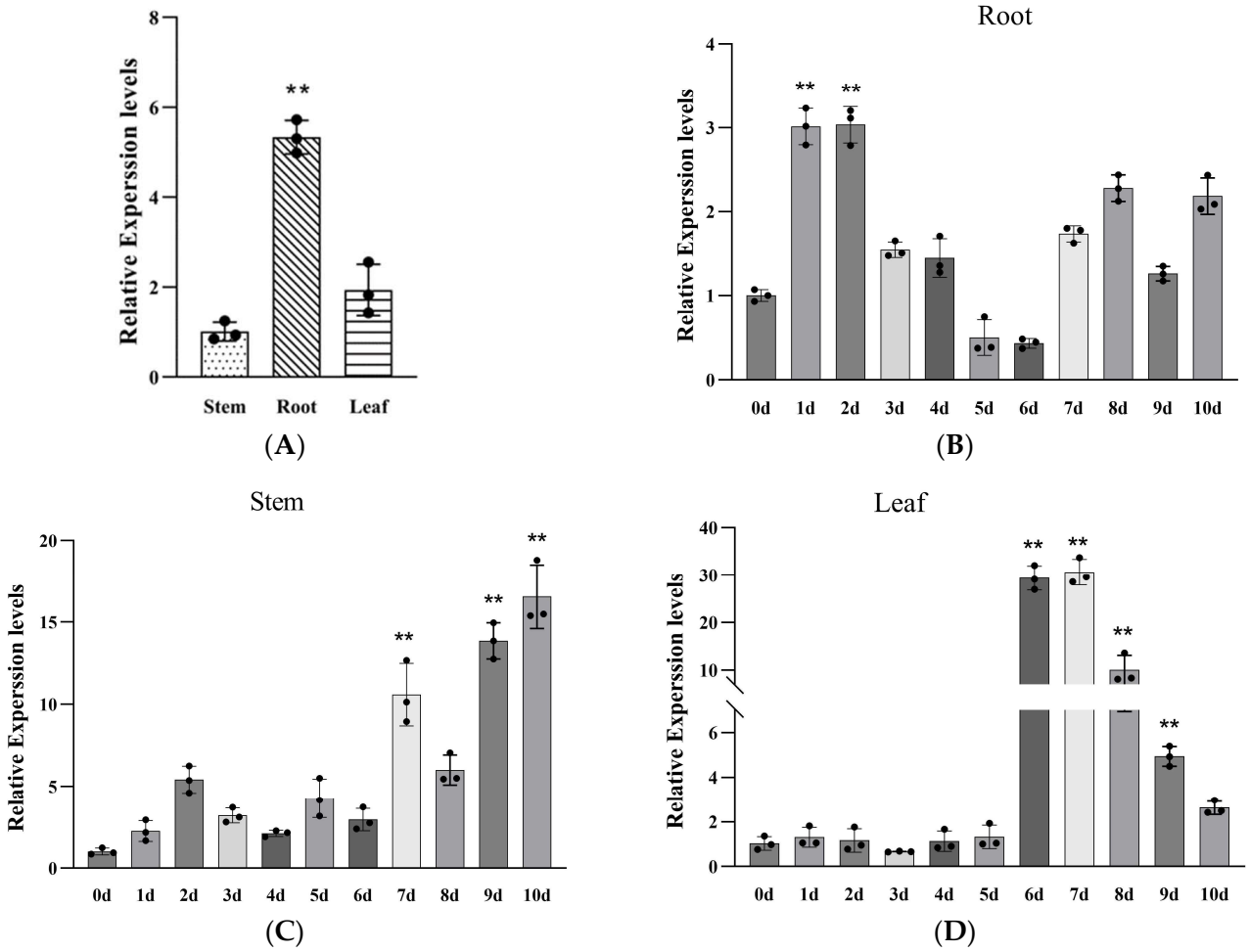

Figure 4. Expression pattern of *PwuWRKY48*. (**A**) *PwuWRKY48* expression under normal growth conditions. (**B**–**D**) *PwuWRKY48* expression in roots (**B**), stems (**C**), and leaves (**D**) in response to drought stress. Three biological replicates were used. Error bars indicate the standard deviation. Statistical analysis was performed using one-way ANOVA. The asterisks indicate significant differences between the treatment group and the pre-treatment (0 d) (*t* test, $p < 0.05$).

Comprehensively, the phenotype/physiological performance and *PwuWRKY48* expression pattern of *P. wulianensis* under drought treatment were positively associated, indicating that *PwuWRKY48* was involved in plant drought resistance.

3.2. Characterization of PwuWRKY48 Gene and Homologous Analysis

A full-length cDNA fragment of *PwuWRKY48* was cloned from *P.wulianensis* using RT-PCR, with a coding region of 1113 bp (370 amino acids) (GenBank accession # PP101018) (Figure 5). ExPaSy analysis showed that the PwuWRKY48 protein has molecular formula $C_{1785}H_{2803}N_{503}O_{584}S_{14}$ with a relative molecular weight of 41.11 kD, theoretical isoelectric point 6.41, and hydrophilic/hydrophobic GRAVY value of −0.743, indicating it to be a hydrophobic protein.

A phylogenetic tree was constructed with the WRKY members in *A. thaliana* and poplar and PwuWRKY48. As shown in Figure 6A, the PwuWRKY48 protein belongs to the WRKY IIc subfamily and contains a subfamily-specific WRKYGQK heptapeptide sequence and a $Cx_4Cx_{23}HxH$ zinc-finger domain (Figure 6B).

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1       ATGAATGAAATGAAGGAGAATAATAATATTAGTAGCGTGTGCACAAATTCCATGGAGAAAAGAGAAGAGAAGAAAGAGCTAACAAGAACA
1        M  N  E  M  K  E  N  N  N  I  S  S  V  C  T  N  S  M  E  K  R  E  E  K  K  E  L  T  R  T

91      ACAATATTAAAGAGAGAGAATTTCGATATGGCAAATTCTTCCTTCATTACACCACCACCAGCAACAACAACAAGTGATATTGATCGGATG
31       T  I  L  K  R  E  N  F  D  M  A  N  S  S  F  I  T  P  P  P  A  T   T  T  S  D  I  D  R  M

181     CCAAGTGTTTATGCTTTATCAACAACAGCAACACCACCACCACCATTAAATAGCATCTTTGACATGATGCCATGTGATATTATTGGTGGC
61       P  S  V  Y  A  L  S  T  T  A  T  P  P  P  P  L  N  S  I  F  D  M  M  P  C  D  I  I  G  G

271     GATCAAAAGGCTGGGTCTTTAGGATTTATAGATTTGCTTGATATCAACCAAGATTTTGGCACCGCTGCTTCTTTATTCGATTGGTTTGCT
91       D  Q  K  A  G  S  L  G  F  I  D  L  L  D  I  N  Q  D  F  G  T  A  A  S  L  F  D  W  F  A

361     CAAAATCCGATTGTGGGGTCTCAGCAGCAACAAACTTTTGTCCCATCGCCGGCATCAACTCTACCGGAGACATCAGAGGTTTTGAATAAC
121      Q  N  P  I  V  G  S  Q  Q  Q  Q  T  F  V  P  S  P  A  S  T  L  P  E  T  S  E  V  L  N  N

451     CCAGCAACTCCAAATTCATCAGCTTCTATCTCTTCCTCTTCAAATGAAGCAGGAAATGATGCTTTCCAACAAGTTAAAACTGGAGATCAA
151      P  A  T  P  N  S  S  A  S  I  S  S  S  S  N  E  A  G  N  D  A  F  Q  Q  V  K  T  G  D  Q

541     GAAGAAGAGCAGGATCACGACAAGACCAAGAAACAGTTGAAACCGAAAAAGAAGATCCAAAAAAGGCAAAGAGAGCCGAGATTTGCTTTC
181      E  E  E  Q  D  H  D  K  T  K  K  Q  L  K  P  K  K  K  I  Q  K  R  Q  R  E  P  R  F  A  F

631     ATGACAAAGAGTGAAGTCGATCACCTAGATGATGGGTTTAGGTGGAGGAAGTACGGCCAAAAAGCTGTGAAAAACAGCCCCTATCCAAGG
211      M  T  K  S  E  V  D  H  L  D  D  G  F  R  W  R  K  Y  G  Q  K  A  V  K  N  S  P  Y  P  R

721     AGTTATTATCGTTGCACTAGTGCAGGATGTGGTGTGAAGAAGAGAGTGGAGAGATCATCTGATGATTCATCTATAGTTGTTACAACATAC
241      S  Y  Y  R  C  T  S  A  G  C  G  V  K  K  R  V  E  R  S  S  D  D  S  S  I  V  V  T  T  Y

811     GAAGGTCAACACATACATCCAAGCCCAATAACTCCTAGAGGAAGTATTGGAATTTTGACTGACTCAACTGGTTTTGGTGCTGCAACATCA
271      E  G  Q  H  I  H  P  S  P  I  T  P  R  G  S  I  G  I  L  T  D  S  T  G  F  G  A  A  T  S

901     TCTTTTGTTATTCCACAAACTCAGTATCTACAACATGCCTACATGTATAGCTCATCACCTTCTTTGAACATTACCACAAGTAATACTAGT
301      S  F  V  I  P  Q  T  Q  Y  L  Q  H  A  Y  M  Y  S  S  S  P  S  L  N  I  T  T  S  N  T  S

991     TTTAGTCCCACCTTTTCTTTTCACCAGAGAAGGTCGGATAGTCCTGCTTCTTTGCTTAGAGACCATGGCCTTCTTCAGGATATTGTGCCC
331      F  S  P  T  F  S  F  H  Q  R  R  S  D  S  P  A  S  L  L  R  D  H  G  L  L  Q  D  I  V  P

1081    TCCCAGATGAGAAAGGAGCCTAAAGAGGAGTAG
361      S  Q  M  R  K  E  P  K  E  E  *
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Figure 5. Nucleotide sequence and amino acid sequence of PwuWRKY48. The WRKY domains are identified by the black line. The conserved WRKYGQK motif and zinc finger motif are indicated by red and blue triangles, respectively. A predicted nuclear localization signal is framed in red.

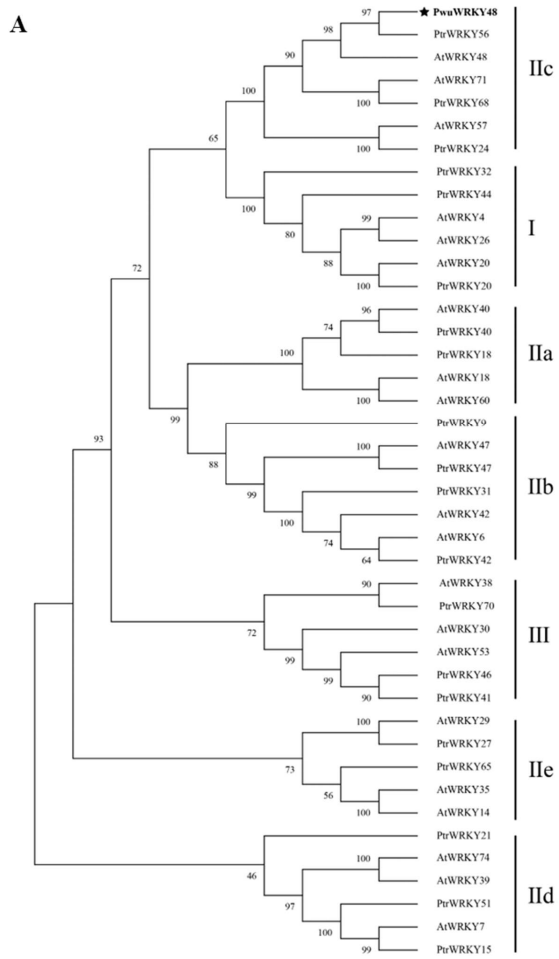

Figure 6. *Cont.*

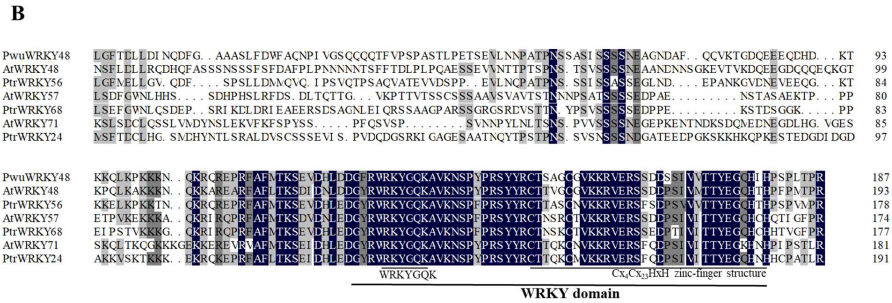

Figure 6. Phylogenetic tree (**A**) and multiple sequence alignment (IIc) (**B**) of WRKY proteins. Multiple sequence alignment was conducted using ClustalW. The pentagram is indicated the target protein (PwuWRKY48). Light to dark colors are indicated by low to high similarity. A phylogenetic tree was constructed using MEGA-X with the maximum likelihood method, 1000 repetitions of bootstrap tests, and JTT matrix-based model. The figure was generated by use of DNAMAN 9.0 software. PtrWRKY56, Potri.008G094000.1; AtWRKY48, AT5G49520.1; AtWRKY71, AT1G29860.1; PtrWRKY68, Potri.004G007500.1; AtWRKY57, AT1G69310.1; AtWRKY24, AT5G41570.1; PtrWRKY32, Potri.006G184800.1; PtrWRKY44, Potri.006G133200.2; AtWRKY4, AT1G13960.1; AtWRKY26, AT5G07100.1; AtWRKY20, AT4G26640.1; PtrWRKY20, Potri.011G087900.1; AtWRKY40, AT1G80840.1; PtrWRKY40, Potri.001G044500.1; PtrWRKY18, Potri.006G263600.1; AtWRKY60, AT2G25000.1; PtrWRKY9, Potri.001G208600.1; AtWRKY47, AT4G01720.1; PtrWRKY47, Potri.002G186600.2; PtrWRKY31, Potri.002G228400.3; AtWRKY42, AT4G04450.1; AtWRKY6, AT1G62300.1; PtrWRKY42, Potri.011G007800.2; AtWRKY38, AT5G22570.1; PtrWRKY70, Potri.016G137900.1; AtWRKY30, AT5G24110.1; AtWRKY53, AT4G23810.1; PtrWRKY46, Potri.002G168700.1; PtrWRKY41, Potri.001G092900.1; AtWRKY29, AT4G23550.1; PtrWRKY27, Potri.017G149000.1; PtrWRKY65, Potri.011G070100.1; AtWRKY35, AT2G34830.1; AtWRKY14, AT1G30650.1; PtrWRKY21, Potri.003G111900.2; AtWRKY74, AT5G28650.1; AtWRKY39, AT3G04670.1; PtrWRKY51, Potri.018G139300.1; AtWRKY7, AT4G24240.1; PtrWRKY15, Potri.007G047400.1.

3.3. Identification of the Regulation Elements on PwuWRKY48 Promoter

A promoter fragment of *PwuWRKY48* with a length of 1208 bp (GenBank accession # PP101019) was cloned using *P. wulianensis* DNA as a template (Figure 7). The cis-acting elements in the sequence were determined and showed it contained typical TATA-box and CAAT-box, as well as some adversity-responsive elements such as MYB, ARE, ABRE, CGTCA-motif, and TGACG-motif (Figure 7) and some light-response elements such as GATA-motif, Box 4 and G-Box. Accordingly, *PwuWRKY48* expression is in response to light signaling and abiotic stresses.



Figure 7. *PwuWRKY48* promoter sequence. The black line highlights sequences indicating the major cis-acting elements annotated in the promoter.

3.4. Subcellular Localization of PwuWRKY48

The 35S::WRKY48-GFP fusion vector and 35S::GFP empty control, respectively, were transformed into tobacco protoplasts using PEG-mediated transformation. The fluorescence signal showed that the WRKY48-GFP protein was only addressed in the nucleus, but the fluorescent signal of the control covered the whole cell (Figure 8). This indicates that PwuWRKY48 is a nuclear localization protein and might function as a regulator by binding to the promoter region of the target genes.

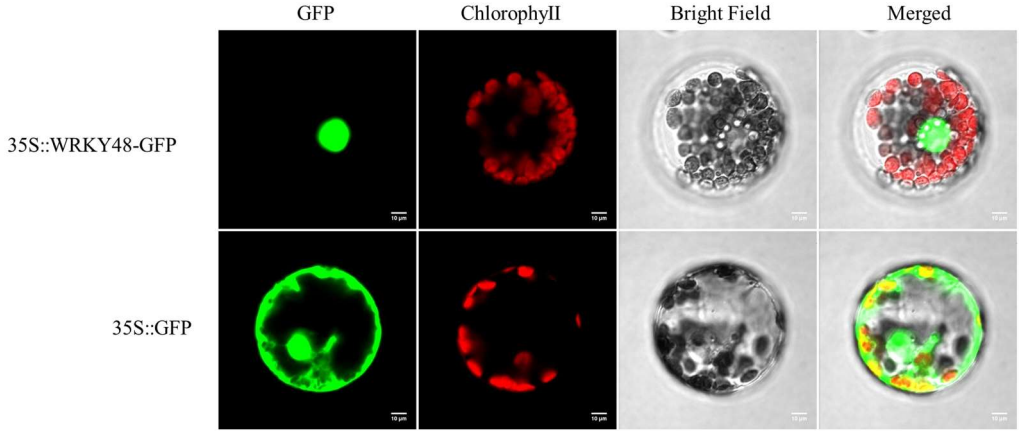

Figure 8. Subcellular localization of PwuWRKY48. GFP fluorescence, chlorophyll, brightfield, and the combined field (merged GFP fluorescence, chlorophyll, and brightfield) were observed for the fusion protein (WRKY48-GFP) (**upper**) and control (GFP) (**lower**), respectively.

3.5. Drought Resistance Analysis of P. wulianensis Plants with PwuWRKY48 Overexpression

Transformation of *PwuWRKY48* was conducted. Based on DNA tests, seven positive lines (OE1–OE7) were obtained. Gene expression assay showed that OE2, OE3, and OE5 had high expression of *PwuWRKY48*, 40.2, 70.6, and 91.4 times higher than the wild type, respectively (Figure 9). These three lines were selected for the subsequent resistance evaluation.

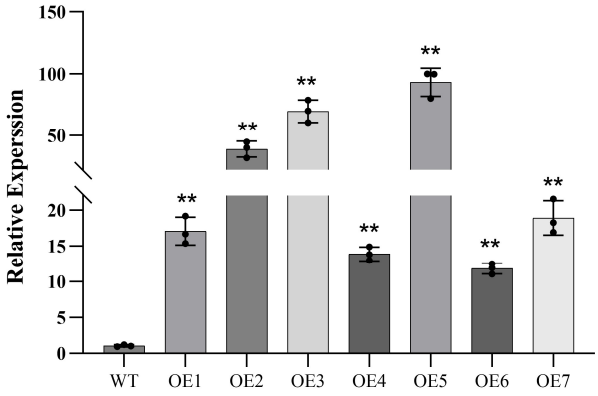

Figure 9. Expression tests of *PwuWRKY48* in transgenic poplar. Three biological replicates were used. The error bars represent standard deviation. Asterisks indicate significant differences between transgenic lines and wild-type lines (*t* test, $p < 0.05$). WT, wild-type poplar; OE1-OE7, transgenic lines.

The three OE lines were vegetatively propagated. After 2 months of transplanting, three plants from each line with consistent growth were subjected to drought treatment. The results showed that there was no significant morphological difference between the transgenic and wild-type plants under regular growth conditions (Figure 10). At 8 d of drought stress, most leaves of the wild type wilted, while the OE lines grew better with a few basal leaves wilting slightly.

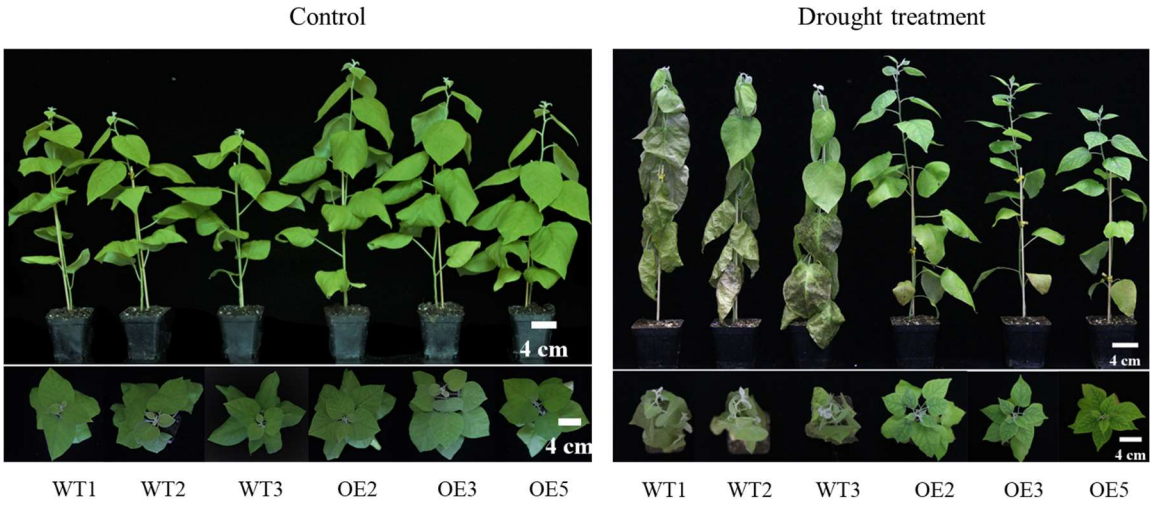

Figure 10. Phenotypes of *PwuWRKY48*-overexpressing lines (OE2, OE3, OE5) and wild type (WT1–3) under regular conditions (control) (**left**) and drought stress (**right**).

Evans blue staining assays showed that the coloration of the wild-type and OE leaves was similar under regular conditions (Figure 11). However, the coloring area of wild type leaves increased significantly at 8 d of drought stress, while the leaves' coloring in OE lines increased a little compared to that before drought treatment. Obviously, OE lines had better cell activity and more drought tolerance than the wild type.

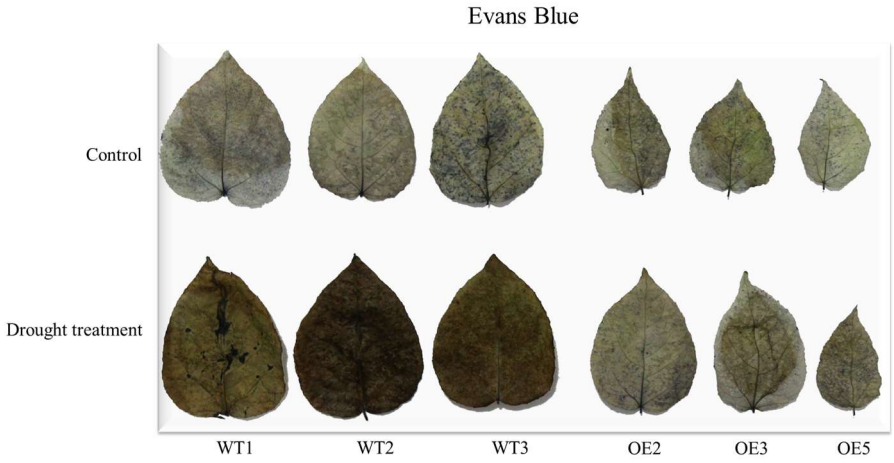

Figure 11. Evans blue staining of *PwuWRKY48*-overexpressing lines (OE2, OE3, OE5) and wild type (WT1–3) under regular conditions (control) and drought stress.

Physiological index tests showed that no significant difference was present between the wild-type and OE lines under regular conditions. Along with the drought treatment, all the indexes changed significantly. Among them, the conductivity in the wild type was increased by 28.5% at 8 d of drought stress compared with that at 0 d [(8 d − 0 d)/0 d] and 7.2% in the OE lines (Figure 12A). Meanwhile, MDA content in the wild type increased 1.3-fold and increased 1.0-fold in the OE lines (Figure 12B). Proline content in the wild type increased 1.5-fold and increased 3.2-fold in the OE lines (Figure 12C). Chlorophyll content in the wild type reduced by 17.3% and reduced by 6.2% in the OE lines. Obviously, the OE lines were more tolerant to drought stress than the wild type (Figure 12D).

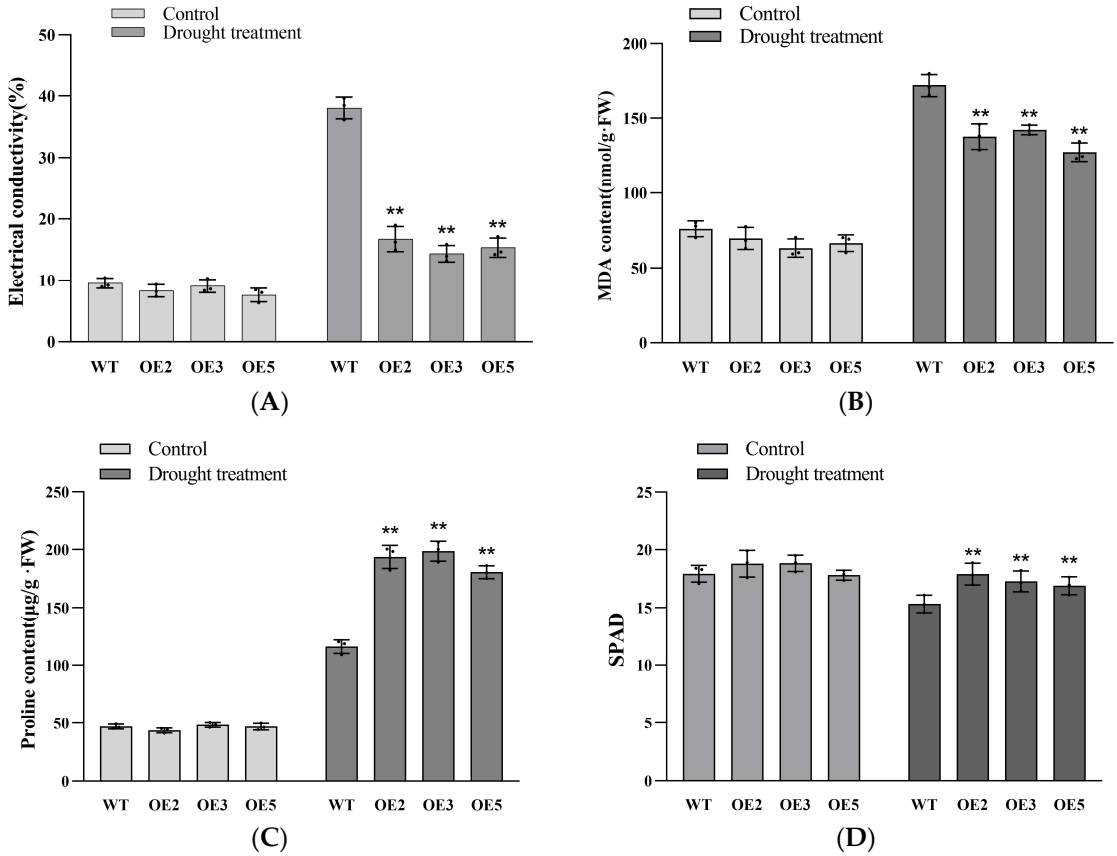

Figure 12. Performance of the physiological indexes under drought stress. (**A**) Relative conductivity; (**B**) malondialdehyde content (MDA); (**C**) proline content; (**D**) chlorophyll relative value. Three biological replicates were used in the experiment. Error bars indicate the standard deviation. The asterisks indicate significant differences between the transgenic and wild-type lines under drought treatment (*t* test, $p < 0.05$).

NBT and DAB staining of plant leaves showed that the wild-type and OE lines performed similarly under regular growth conditions. At 8 d of drought stress, all the lines colored more deeply. Of them, the wild type exhibited significant changes while the OE lines changed a little (Figure 13A). Further measurement of $O_2^{\cdot -}$ content showed that all were at a low level and no significant difference was found between the wild-type and OE lines under regular growth conditions, but $O_2^{\cdot -}$ content increased significantly under drought stress in all lines. That in OE lines increased less, 0.7-fold in comparison with the wild type at 8 d of drought stress (Figure 13B). Similarly, SOD and POD activities in both wild-type and OE lines were maintained at low levels under normal conditions and no significant differences were found between them. However, under drought stress, SOD and POD activities increased in all lines. SOD activity was 2.5-fold higher and POD activity was 1.6-fold higher in OE lines than the wild type at 8 d of drought stress (Figure 13C,D). These results reveal that overexpressing *PwuWRKY48* could significantly reduce $O_2^{\cdot -}$ accumulation in leaves under drought stress, which is beneficial for regular metabolic activity in cells.

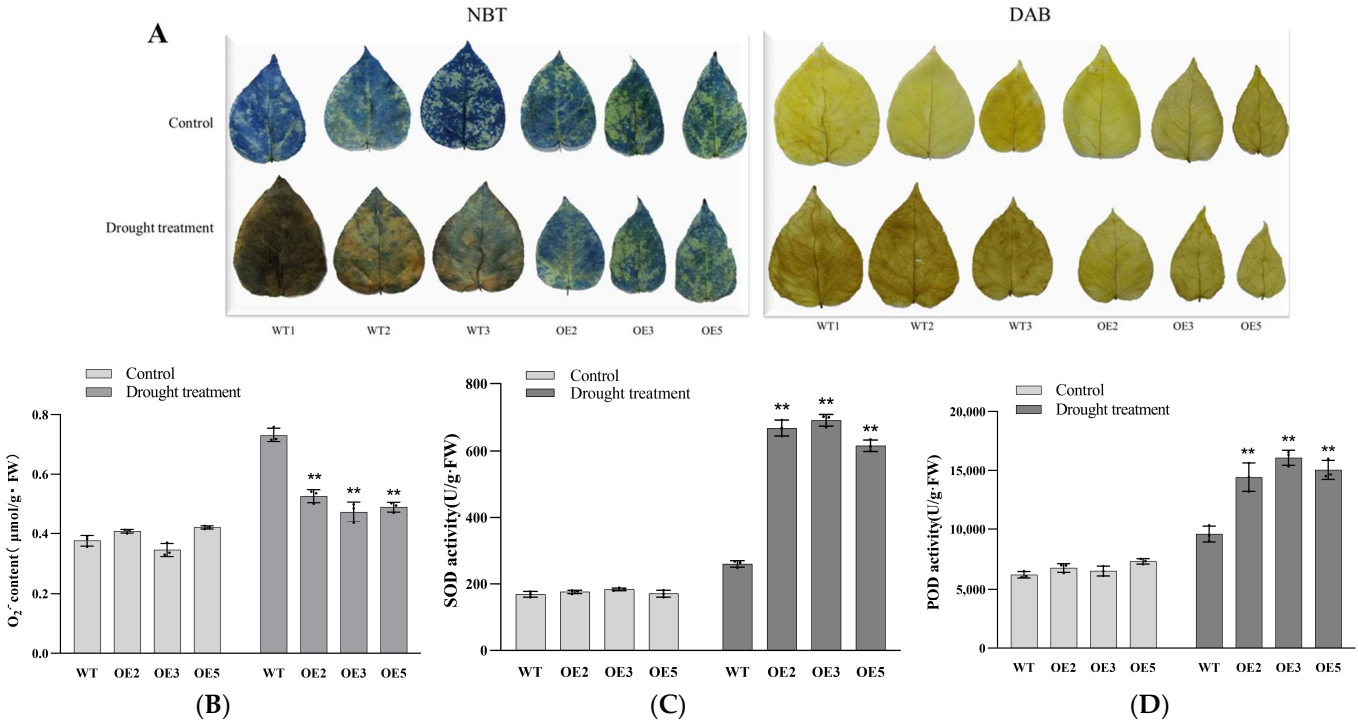

Figure 13. Evaluation of ROS-scavenging capacity. (**A**) NBT and DAB staining of leaves under regular conditions (control) and at 8 d of drought stress. (**B**) $O_2^{\cdot-}$ content; (**C**) SOD activity; (**D**) POD activity. Three biological replicates were used in the experiment. Error bars indicate the standard deviation. The asterisks indicate significant differences between the transgenic and wild-type lines (*t* test, $p < 0.05$).

4. Discussion

4.1. Cis-Regulation of WRKY Expression

WRKY is a large gene family in the plant genome and is involved in plant growth and development and adversity resistance. Each *WRKY* gene has its specific expression pattern in tissues and organs and under abiotic stress. For example, *IbWRKY2* is more highly expressed in the leaves of *Ipomoea batatas* than that in the stems, hairy roots, fibrous roots, or storage roots [22]. *HbWRKY1* is more highly expressed in the latex of *Hevea brasiliensis* than in the bark, leaves, flowers, or roots [23]. Salt stress could promote the transcription of *AcWRKY29*, *AcWRKY40*, *AcWRKY48*, *AcWRKY55*, *AcWRKY95*, and *AcWRKY96* in *Actinidia chinensis*, while it decreased the transcription of *AcWRKY38* [24]. RNA-seq results revealed that 61 *PtrWRKYs* were subjected to biotic and abiotic stresses such as *Marssonina brunnea*, wounding, cold, and salinity. They were also expressed in different tissues including roots, stems, and leaves [25]. Furthermore, 33 *SiWRKYs* and 26 *SiWRKYs* in *Sesamum indicum* were determined in response to flooding and drought, respectively [26]. In this study, *PwuWRKY48* was more likely to be expressed in roots but less in stems. Possibly, the variable expression pattern of *WRKY* genes is due to its upstream regulators, which bind to the promoter region of the *WRKY* genes and regulate their expression level. Thus, it is important to understand the regulatory elements affecting *WRKY* promoters.

Several studies have analyzed the regulatory elements of *WRKY* gene promoters. For example, *PtrWRKYs* were widely involved in stress response in *P. trichocarpa*, in which the promoter region contains various elements associated with stress and defense such as TCA-element, CGTCA-motif, EIRE, ELI-box3, Box-S, WUN motif, ABRE and W-box [25]. The tomato fruit ripening regulator SlRIN was able to bind to the CArG cis-acting element in the promoter region of the *SlWRKY35* gene, up-regulate its expression, and enhance carotenoid accumulation [27]. Under non-oxidative conditions, WRKY25 can bind to an element W-box in the *WRKY53* promoter and act as a positive regulator of *WRKY53* expression,

whereas oxidative conditions inhibit the regulatory role of *WRKY25* [28]. In this study, we obtained a 1208 bp promoter fragment of *PwuWRKY48*, containing multiple adversity-related elements MBS, ARE, ABRE, CGTCA-motif, and TGACG-motif, as well as many elements involved in the light-responsive GATA-motif, Box 4, and G-Box. Accordingly, *PwuWRKY48* was subjected to various abiotic stress responses such as light signal, drought, and anaerobiosis. These elements coincided with the expression pattern of PwuWRKY48 in stems and leaves under drought stress. It was expected that the associated elements would be identified and the regulatory mechanism for *PwuWRKY48* could be understood through yeast one-hybridization and other techniques.

4.2. PwuWRKY48 Gene Advanced Drought Tolerance in Plants via Regulating Target Gene Expression

Several studies have reported that the *WRKY* genes are widely involved in stress resistance via regulating target gene expression [1]. RNA-seq was assayed for *Populus yunnanensis* plants with *PyWRKY48* overexpression under cadmium stress. Analysis of differentially expressed genes (compared to the wild type) showed that the *PyWRKY48* gene could upregulate the expression of the cell wall protein PaGRP, the antioxidant enzyme PaPER, and the heavy metal-related protein PaPHOS. These genes are greatly involved in tolerance to cadmium [29]. WRKY46 is able to bind to a specific W-box region on the *VITL1* (vacuolariron transporter like1) promoter and repress *VITL1* transcription, which regulates the uptake and translocation of iron in plants under iron deficiency conditions [30]. WRKY7/WRKY11/WRKY17 can inhibit the transcription of *bZIP28* by binding to the W-box element in the *bZIP28* promoter, and decreased the resistance *of A. thaliana* to *Pseudomonas syringae* [31]. Overexpressing *AtWRKY40* in *A. thaliana* can bind to the W-box of the promoter region of ABA signaling regulator genes (*ABF4*, *ABI4*, *ABI5*, and *MYB2*) and repress their expression [32]. In this study, we obtained *PwuWRKY48*-overexpressing lines. POD and SOD activity of the plants were significantly increased under drought stress compared with those of the wild type (Figure 12C,D), while $O_2^{\cdot -}$ and MDA contents were significantly reduced (Figure 12B). This indicates that *PwuWRKY48* could reduce the membrane damage caused by ROS. Further detection of target genes is necessary to ascertain the regulatory network of PwuWRKY48 in cells and to understand the mechanism against drought stress.

4.3. WRKY Genes Exhibited Various Resistance to Abiotic Stress

Gene transformation is an efficient way to evaluate the contribution of the genes in stress resistance. *CbWRKY27* was overexpressed in *Catalpa bungei.* Under salt stress, $O_2^{\cdot -}$ content in the overexpressing line was greatly increased, 1.31-fold compared with the wild-type plant. Meanwhile, H_2O_2 content was reduced to 63.50%, MDA content increased by 17.90%–25.25%, and POD and SOD activity decreased by 52.74% and 18.10%, respectively [33]. *MuWRKY3* overexpression in *Arachis hypogaea* L. showed a 3.5 to 2-fold decrease in MDA content, 1.5- to 2.5-fold increase in proline content, 3–5-fold increase in SOD activity, 3–7-fold increase in APX activity, and 2.3- to 4.4-fold increase in total soluble sugar content compared with wild-type plants under drought stress [34]. In this study, the overexpression of *PwuWRKY48* in *P. wulianensis* under drought stress resulted in increases in POD activity and SOD activity, 2.5- and 1.6-fold higher than the wild type, while MDA and $O_2^{\cdot -}$ content were 0.2-and 0.3-fold lower, proline and chlorophyll content were 37.5% and 11.2% higher, and relative conductivity was 21.3% lower. This indicates that *PwuWRKY48* exhibits strong drought resistance and can be used as a gene resource for molecular breeding programs in plant drought resistance. The various contribution of each *WRKY* gene to stress resistance is possibly due to their differences in gene sequence and protein structure. More efforts are required in the future to reveal the molecular basis of *WRKY* genes' function.

5. Conclusions

The *P. wulianensis* transcription factor *PwuWRKY48* promoter involves multiple adversity-induced inducible elements, which showed a significant up-regulation trend under drought stress and positively regulated the drought tolerance of poplar by increasing the scavenging capacity for ROS, promoting the content of proline, and inhibiting the accumulation of MDA substances. It can be used as a genetic resource for molecular breeding of drought tolerance in plants.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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