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Conserved ERF54-mediated activation of *ERD7* enhances drought resilience in *Populus*

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Abstract

Understanding the conserved transcriptional regulatory mechanisms underlying drought stress responses across diverse *Populus* species is critical for enhancing the drought resilience of woody plants. In this study, we conducted a comparative transcriptomic analysis of five *Populus* taxa, which revealed *PtrERF54*, an ethylene response factor (ERF), as a core drought-inducible transcription factor consistently upregulated under drought conditions. Weighted gene co-expression network analysis further revealed *PtrERD7* as a candidate downstream gene co-expressed with *PtrERF54*. To elucidate the functional role of *PtrERF54*, we generated overexpression lines for *PtrERD7* and *PtrERF54* in the hybrid poplar (*Populus alba* × *P. glandulosa* '84K'). Both *PtrERF54*- and *PtrERD7*-overexpressing (OE) plants (*PtrERF54-OE* and *PtrERD7-OE*, respectively) exhibited significantly enhanced drought tolerance, as evidenced by reduced leaf wilting, lower relative electrical conductivity, elevated antioxidant enzyme activity, and improved photosynthetic performance under drought stress conditions. Notably, *PtrERF54-OE* lines maintained reduced stomatal conductance and transpiration rates without compromising photosynthetic carbon assimilation, thereby increasing instantaneous water-use efficiency. Yeast one-hybrid and ChIP-qPCR assays demonstrated that PtrERF54 directly binds to the *PtrERD7* promoter, while dual-luciferase assays showed that PtrERF54 activates *PtrERD7* transcription. Collectively, our results establish a conserved PtrERF54–*PtrERD7* transcriptional module that modulates drought responses in *Populus*, providing novel insights into the genetic basis of water deficit adaptation and offering candidate targets for breeding drought-resilient trees.

Keywords: *PtrERF54*, *PtrERD7*, Drought stress, Poplar, Weighted gene co-expression network analysis

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Introduction

Poplars (*Populus* spp.) are fast-growing trees widely used for timber production and ecological restoration, providing substantial economic and environmental benefits[1,2]. However, poplar is a relatively water-demanding species and highly sensitive to soil water deficits. Consequently, drought is a major environmental factor limiting growth and productivity. Prolonged drought disrupts physiological and metabolic processes, impairs root water uptake, induces stomatal closure, reduces photosynthetic capacity, and suppresses plant growth. Sustained water deficit also causes leaf wilting and chlorosis, accelerates senescence, and triggers premature abscission, reducing wood yield and quality. Considering the increasing frequency and intensity of drought events under global climate change, it is critical to elucidate the mechanisms underlying drought responses in trees and develop drought-tolerant poplar varieties.

The ethylene response factor (ERF) family, a major subfamily of the AP2/ERF superfamily, encodes key transcriptional regulators that recognize and bind to GCC-box *cis*-elements via a conserved AP2/ERF domain of 60–70 amino acids, thereby modulating downstream target gene expression. ERFs play important roles in plant drought responses by regulating hormone signaling[3], anthocyanin accumulation[4], osmotic adjustment[5], antioxidant defenses[6], root hair development[7], stomatal behavior[8], and stress-responsive gene expression[9]. In woody plants, several ERFs have been implicated in drought adaptation. For example, drought-induced PtoERF15 upregulates *PtoMYC2b*, activating secondary wall and xylem vessel-related genes, thereby inducing adaptive morphological changes in xylem vessels under drought stress[10]. *Pal*ERF2 rapidly triggers stomatal closure and enhances drought tolerance by directly activating the positive regulator of stomatal closure, *PalRD20*, and repressing the ABA pathway inhibitor *PalSAG113*[11]. *PtaERF194* enhances drought tolerance by improving WUE, limiting water loss, and maintaining high water potential. It is also involved in ABA signal transduction and ROS scavenging[12]. Together, these findings indicate that ERFs constitute important components of drought-responsive regulatory networks in *Populus*.

In *Arabidopsis thaliana*, the homolog of *Populus PtrERF54*, *AtERF16* (*AT*5G21960), has been functionally characterized as a downstream TF in the ethylene-signaling pathway. Studies have shown that *AtERF16* negatively regulates the expression of *RESISTANCE TO POWDERY MILDEW 8.1* (*RPW8.1*), thereby preventing excessive activation of cell death and immune responses. Loss-of-function mutations in *AtERF16* enhance disease resistance[13]. Another study has revealed that *AT*5G21960 binds to a carbohydrate metabolic signal-responsive element (CMSRE-1) in the promoters of sweet potato storage protein genes, where it plays an essential role in the sugar-responsive expression of sporamin and β-amylase genes[14]. Collectively, these studies established that *ERF16*-related TFs serve as versatile regulators of metabolic, hormonal, and stress signalling. However, the functional roles and regulatory mechanisms of their homologs in woody plants, particularly those under drought stress, remain largely unexplored.

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The early responsive to dehydration (ERD) genes are defined by their rapid activation under drought stress and are widely used as markers of stress responses. To date, 16 ERD genes (*AtERD1–AtERD16*) have been annotated in *A. thaliana*[15]. Among these, *AtERD1* is induced by dehydration but acts independently of the ABA signaling pathway[16], whereas *AtERD15* is negatively regulated by ABA signaling to prevent hyperactive stress responses[15]. *AtERD5* encodes a precursor of proline dehydrogenase (oxidase), which is regulated at the level of mRNA accumulation in both dehydrated and rehydrated plants[17]. *Arabidopsis AtERD7* (*AT*2G17840) encodes a lipid droplet-localized protein; loss of its function results in altered membrane lipid composition and reduced membrane fluidity under cold stress, indicating its critical role in membrane remodeling during stress responses[18,19]. Moreover, *AtERD7* accumulates under various stress conditions and plays essential roles in both stress regulation and development. A triple mutant lacking *AtERD7* and its homologs exhibits embryonic lethality[19]. Despite these advances in elucidating *ERD7* functions, the upstream transcriptional regulatory mechanisms governing *ERD7* expression in response to environmental signals remain elusive.

To address these knowledge gaps, we performed a comparative transcriptomic analysis across five *Populus* taxa and integrated weighted gene co-expression network analysis (WGCNA) to identify a conserved drought-responsive TF, PtrERF54, and its potential downstream target, *PtrERD7*. Subsequently, using molecular interaction assays, we demonstrated that *Ptr*ERF54 acts as a direct upstream transcriptional activator of *PtrERD7* and further evaluated the functional relevance of these two genes in drought adaptation by generating stable poplar overexpression lines. Collectively, the findings of this study establish the PtrERF54–*PtrERD7* module as a key regulator of drought resistance in poplar, providing new theoretical insights and valuable genetic resources for drought-tolerant breeding in tree species.

Materials and methods

Plant materials and growth environment

In this study, we used two poplar genotypes: *P. trichocarpa* (genotype 'Nisqually-1') and the hybrid *P. alba* × *P. glandulosa* '84K'. For *P. trichocarpa*, tissue culture seedlings were subcultured on hormone-free Murashige and Skoog medium, with the pH adjusted to 5.78–5.83. The medium was supplemented with 30 g L^{-1} sucrose and solidified using 0.6% (w/v) agar. For the '84K' hybrid, plantlets were rooted in Woody Plant Medium containing the same concentrations of sucrose (30 g L^{-1}) and agar (0.6% w/v), with the pH adjusted to 5.78–5.83. Both genotypes were cultivated in a controlled-environment chamber at 23–25 °C under a 16-h light/8-h dark photoperiod.

Twelve plants of each species, *Populus tomentosa*, *P. trichocarpa*, and *P. deltoides*, were grown in a 1:1:1 (v/v/v) mix of vermiculite, nutrient soil, and turfy soil in a greenhouse for 2 months (16 h light/8 h dark, 20–24 °C, 40%–50% relative humidity). After 2 months of acclimation, plants of each species were divided into drought and control groups (n = 6 per group). Before the treatments, the soil in all pots was saturated with water and allowed to drain for 2 h. The drought-treated group was then subjected to water deprivation for 6 d, whereas the control group received regular watering. On day 6, leaves from the same position were collected, immediately frozen in liquid nitrogen, and stored at −80 °C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using three independent biological replicates, each comprising four technical replicates, to ensure data reliability. The SYBR Premix Dimer Eraser (Perfect Real Time) (TaKaRa, Tokyo, Japan) was used for all reactions, and *18S* served as an internal control to normalize the data[20]. Gene expression levels were quantified using the comparative $2^{-\Delta\Delta C_T}$ method, as described by Livak & Schmittgen[21]. Primers for the target genes were designed using the DNAMAN 10 software (Lynnon Biosoft, Quebec, QC, Canada). The primer sequences are listed in Supplementary Table S1.

Gene cloning and plant transformation

The coding sequences (CDSs) of *PtrERF54* and *PtrERD7*, excluding the termination codon, were isolated from *P. trichocarpa*. These sequences were cloned into the *pBI121-EGFP-1* vector under the *CaMV35S* promoter (35S), yielding constructs *35S::PtrERF54-EGFP-1* and *35S::PtrERD7-EGFP-1*. Primers used for amplification are listed in Supplementary Table S1. Each recombinant plasmid was then transformed into the *Agrobacterium tumefaciens* strain GV3101. These constructs were subsequently transformed into '84K' poplar *via* an *Agrobacterium*-mediated method following the protocol of Liu et al.[22].

Yeast one-hybrid (Y1H) assay

A 2,000 bp promoter sequence upstream of the *PtrERD7* start codon was obtained from the *P. trichocarpa* genome and analyzed for putative ERF-binding motifs using PlantTFDB (https://planttfdb.gao-lab.org/). To examine interactions between PtrERF54 and the promoter of *PtrERD7*, the promoter fragments were cloned into the pBait-AbAi vector using *Sac*I and *Xho*I restriction sites. Concurrently, the CDS of *PtrERF54* was amplified and inserted into the pGADT7 vector using the *Eco*RI and *Xho*I restriction sites to obtain the construct pGADT7-*PtrERF54*. The Y1H assay was performed using the Matchmaker Gold Y1H Library Screening System (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's instructions[23].

Transient expression assay in *Nicotiana benthamiana* leaves

For the transient expression assays, the full-length CDS of *PtrERF54* was amplified and cloned into the pGreen II 62-SK vector using the *Bam*HI and *Sal*I restriction sites to generate the effector construct. For the reporter construct, the promoter fragments of *PtrERD7* were inserted into the *Sal*I and *Bam*HI sites of the pGreen II 0800-LUC vector. *A. tumefaciens* GV3101 cultures carrying the effector and reporter plasmids were mixed at a 1:1 ratio, and the resulting mixture was co-infiltrated into the abaxial side of *N. benthamiana* leaves. After 72 h of incubation in a growth chamber, the infiltrated leaf discs were harvested and treated with 1 mM D-Luciferin, Potassium Salt (BN11009; Biorigin, Beijing, China). After 5 min of dark adaptation, firefly (LUC) and Renilla luciferase activities were measured using the Dual-Luciferase Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Luminescence images were captured using the NightOWL II LB983 *in vivo* imaging system (Berthold Technologies, Bad Wildbad, Germany).

Chromatin immunoprecipitation qPCR (ChIP-qPCR)

ChIP-qPCR was performed to examine the *in vivo* binding of *PtrERF54* to the *PtrERD7* promoter. The *PtrERF54*-GFP overexpression poplar lines and GFP-expressing transgenic control lines were used for this assay. Leaf tissues were crosslinked with 1% formaldehyde, and chromatin was sheared to 300–500 bp fragments. Immunoprecipitation was performed using an anti-GFP antibody (AE119; ABclonal, Wuhan, China). After DNA purification, qPCR was performed using primers that specifically amplify the *PtrERD7* promoter region containing the predicted AP2/ERF-binding motif.

Gas exchange analyses

Photosynthetic parameters were measured using a Li-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA). The measurements were performed on the ninth to the eleventh fully expanded leaves of *PtrERF54*-overexpressing (*PtrERF54-OE*) and wild-type (WT) poplar plants grown in a greenhouse under well-watered conditions for 1.5 months. Light and carbon dioxide (CO_2) response curves were obtained on the same day. The measured parameters included net photosynthetic rate (*Pn*), stomatal conductance (*Gs*), transpiration rate (*Tr*), and instantaneous WUE (iWUE = *Pn*/*Tr*).

The light response curves were measured under the photosynthetically active radiation (PAR) levels of 1,500, 1,200, 1,000, 800, 600, 400, 200, 150, 100, 50, and 0 μmol m^{-2} s^{-1}, with ambient CO_2 concentrations maintained at 450 μmol mol^{-1} (controlled by the Li-6400 system). The CO_2 response curves were determined under external CO_2 concentrations of 1,800, 1,500, 1,250, 1,000, 800, 600, 400, 300, 200, 150, 100, 50, and 0 μmol mol^{-1}, with PAR maintained at 800 μmol m^{-2} s^{-1}.

For WT and *PtrERD7-OE* poplar plants, the *Pn* and *Tr* values were measured on the sixth to the eighth fully expanded leaves using the Li-6400 portable photosynthesis system under a PAR value of 800 μmol m^{-2} s^{-1} and a CO_2 concentration of 400 μmol mol^{-1}, between 09:00 and 11:00 a.m.

Drought tolerance experiment

To evaluate drought tolerance, the *PtrERF54-OE*, *PtrERD7*-overexpressing (*PtrERD7-OE*), and WT poplar lines were subjected to short-term drought stress. One-month-old tissue-cultured *PtrERF54-OE* (*OE3, OE5*), *PtrERD7-OE* (*OE6, OE11*), and WT plants with uniform growth and consistent developmental stages (approximately 9 cm in height) were selected. Six individuals from each line were transplanted into plastic pots (10 × 10 × 10 cm) filled with an equal-volume mix of vermiculite, nutrient soil, and turfy soil, and then cultivated in a controlled greenhouse environment at 20–25 °C with a 16-h/8-h light/dark photoperiod. The plants were grown for 3 months until they reached a height of approximately 35 cm.

Before drought treatment, all plants were grown under well-watered conditions, with relative soil moisture content (RSMC) maintained at 70% by daily weighing and replenishing water loss[24,25]. In the drought-treated group, watering was withheld, causing RSMC to gradually decrease from 70%, whereas all other conditions remained unchanged. After 6 days, RSMC reached approximately 25% (ψ_{Soil} = −1.25 MPa). RSMC was calculated as (fresh weight − dry weight)/(saturated water weight − dry weight) × 100%[24]. Soil water potential was measured using a PSYPRO Water Potential System with C-52 sample chambers (Wescor Inc., Logan, UT, USA). Phenotypic changes were recorded using a digital camera, and leaf samples were collected before and after the drought stress treatments to measure leaf relative water content (RWC), relative electrical conductivity (REC), and the activities of peroxidase (POD) and superoxide dismutase (SOD).

Physiological index analyses

Approximately 0.1 g of leaf tissue was homogenized with the corresponding extraction buffer on ice. The homogenate was centrifuged at 8,000 × *g* for 10 min at 4 °C, and the resulting supernatant was kept on ice for later enzyme activity assays. Enzymatic activity was measured using commercial assay kits (POD Kit, BC0095; SOD Kit, BC5165; Beijing Solarbio Science and Technology Co. Ltd, Beijing, China). POD activity was determined spectrophotometrically at 470 nm, and SOD activity was measured at 450 nm. Each assay was performed in 12 replicates (three biological replicates × four technical replicates).

REC and leaf RWC were measured as previously described[26]. For the REC assay, leaf samples (0.1 g fresh weight) were excised and placed in 4-mL centrifuge tubes containing 3 mL of distilled water. The initial electrical conductivity (V_1) was measured after incubation at 37 °C for 4 h, followed by boiling for 10 min. After cooling to room temperature, the final electrical conductivity (V_2) was measured. REC was calculated as the ratio of V_1 to V_2 ($V_1/V_2 \times 100\%$). RWC was determined using leaf samples excised from the same nodal position on each plant. The fresh weight (M_1) was recorded, and the leaves were submerged in distilled water for 5–6 h until fully water-saturated. After saturation, excess surface water was removed by blotting, and the turgid weight (M_2) was determined. The samples were oven-dried at 105 °C for 30 min, further dried at 65 °C until constant weight, and then the dry weight (M_3) was measured. Leaf RWC was calculated as $(M_1 - M_3)/(M_2 - M_3) \times 100\%$. Twelve replicates (three biological replicates × four technical replicates) were used.

Photosynthetic performance was assessed using a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany). Before the measurements, all plants were dark-adapted for 15 min. The maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) and non-photochemical quenching (NPQ) were determined according to the protocol described by Li et al.[27].

Phylogenetic and promoter *cis*-element analysis

Full-length ERF protein sequences were aligned using MUSCLE, implemented in MEGA[28]. A phylogenetic tree was constructed using the neighbor-joining method.

The 2,000 bp genomic sequences upstream of the translation start site of each ERF gene were extracted from the *P. trichocarpa* reference genome (V3.1) and used as putative promoter sequences. *Cis*-acting regulatory elements within these promoter regions were identified using the PlantCARE database[29]. The identified elements were classified according to their annotated functions, and their distributions among ERF genes were summarized and visualized using TBtools[30].

Transcriptome data processing

We obtained transcriptome data for five *Populus* taxa, *P. tomentosa*, *P. trichocarpa*, *P. alba* × *P. davidiana*, *P. davidiana*, and *P. deltoides*, from the NCBI Sequence Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra). The data were downloaded in FASTQ format using the SRA Toolkit and mapped to the *P. trichocarpa* genome (V3.1) using HISAT[31] with default options. In total, 55 RNA-seq samples were collected, encompassing 27,644 genes (Supplementary Table S2). To ensure consistency across different samples

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and conditions, transcript expression levels were normalized by calculating *Z*-scores based on transcripts per million (TPM).

Gene Ontology enrichment analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed using TBtools v2.452[30]. The go-basic.obo ontology file was retrieved from the Gene Ontology database (http://geneontology.org/) through TBtools. All genes with GO annotations in the *Populus trichocarpa* reference annotation v3.1 available from the Phytozome platform[32,33] were used as the background gene set. Significantly enriched GO terms were identified using a threshold of $p < 0.05$.

WGCNA using the WGCNA package in R

WGCNA was performed to identify genes co-expressed with *PtrERF54* and explore *PtrERF54*-associated downstream pathways under drought stress. As *PtrERF54* has been prioritized as a conserved drought-induced *ERF* gene across the five *Populus* taxa, WGCNA was used for *PtrERF54*-centered co-expression analysis rather than for its initial identification. The RNA-seq datasets from the five *Populus* taxa were used to construct the gene network. Genes with a maximum TPM value < 1 across all samples were removed before analysis. The remaining genes were used to construct a signed weighted co-expression network using the WGCNA package in R, following the method described by Langfelder & Horvath[34]. A soft thresholding power of 10 was selected for network construction. The blockwiseModules function was used to automatically detect transcriptional modules using dynamic tree cutting. The minimum module size was set to 30 genes, and modules with highly similar eigengene expression profiles were merged using a threshold of 0.25. Other parameters were maintained at their default settings, and after module detection, the weighted co-expression network was exported as an edge table using an edge weight threshold of 0.10. Genes directly connected to *PtrERF54* in this network were extracted to construct a *PtrERF54*-centered co-expression network. Candidate downstream genes were further prioritized based on their network connections with *PtrERF54*, drought-responsive expression consistency across *Populus* species, functional annotation related to dehydration or stress responses, and the presence of AP2/ERF-related *cis*-elements in their promoter regions.

Statistical analyses

Statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) and SPSS v25 (IBM Corp., Armonk, NY, USA). All data are presented as means ± standard error. Relative gene expression levels were compared using a one-way analysis of variance (ANOVA) followed by the least significant difference post hoc test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$). Before performing ANOVA, the assumptions of normality and homogeneity of variance were verified.

Results

The ERF family participates in the drought stress response in *Populus*

To explore the involvement of the ERF family in drought responses, we first analyzed the promoter regions of 175 *ERF* genes in the *P. trichocarpa* genome. The *cis*-element enrichment analysis revealed that *ERF* promoters are significantly enriched for salinity-, drought-, and wounding stress-related motifs, as well as motifs responsive to hormone-related pathways, including those of ABA, auxin, and JA (Supplementary Fig. S1). Collectively, these findings suggest that ERFs are widely involved in stress-responsive regulatory networks.

To elucidate the transcriptional regulatory programs underlying drought responses in *Populus*, we compared the drought-induced differential expression profiles across the five *Populus* taxa: *P. tomentosa*, *P. trichocarpa*, *P. alba* × *P. davidiana*, *P. davidiana*, and *P. deltoides*. Under drought stress, we identified 11,227, 2,992, 2,156, 2,007, and 3,340 differentially expressed genes (DEGs) in these five species, respectively (Fig. 1a). Among these, 4,737 genes were differentially expressed in at least two species (Fig. 1a). A functional enrichment analysis of these shared DEGs indicated a significant

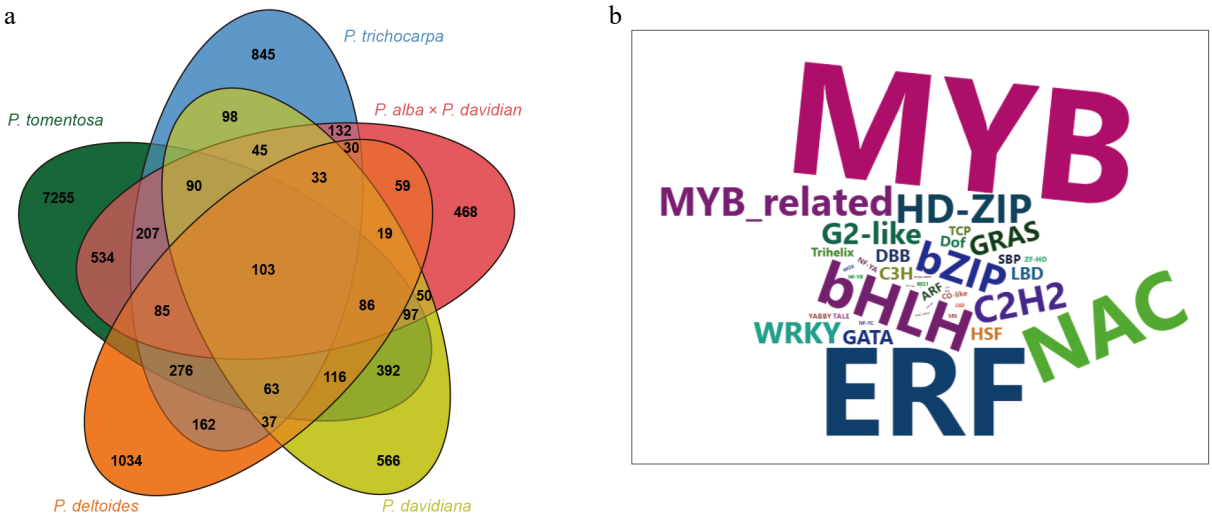

Fig. 1 Ethylene-responsive factors (ERFs) are key transcription factors involved in drought stress responses in *Populus*. (a) Venn diagram showing the overlap of differentially expressed genes (DEGs) under drought stress among five *Populus* taxa: *P. tomentosa*, *P. trichocarpa*, *P. alba* × *P. davidiana*, *P. davidiana*, and *P. deltoides*. (b) Word cloud showing all differentially expressed transcription factors (TFs). Font size reflects the frequency of occurrence, with larger fonts denoting higher representation across species.

over-representation of multiple biological processes and molecular functions, with strong enrichment of catalytic, oxidoreductase, protein kinase, and TF activities (Supplementary Table S3). The enriched biological processes included cell wall modification, protein phosphorylation, cell recognition, carbohydrate metabolism, cell division, response to abiotic stimuli, and defense responses (Supplementary Table S3).

TFs play pivotal roles in drought responses by reprogramming stress-induced gene expression. Here, we identified 369 drought-responsive TFs spanning 40 families (Supplementary Table S4), highlighting the complexity of the regulatory networks activated in *Populus* under water-deficit conditions. Among these, the myeloblastosis (MYB) and ERF families were significantly enriched (Fig. 1b). ERF TFs are well-established regulators of abiotic stress responses, including drought responses, largely by regulating the expression of stress-responsive genes[35]. The *ERF* genes used in this study were assigned based on their phylogenetic classification within the *Populus* ERF family. Among the 175 ERF family members in *Populus*, 50 were differentially expressed under drought stress in at least two poplar species (Supplementary Table S5). Moreover, these genes did not exhibit any preferential distribution across the different branches of the ERF phylogenetic tree (Supplementary Fig. S2). Notably, three genes, *Potri.002G141200* (*ERF37*), *Potri.018G038100* (*ERF54*), and *Potri.016G126100* (*ERF156*), were differentially expressed in all five poplar species (Supplementary Fig. S2; Supplementary Table S5), indicating that these genes play broad roles in drought stress response in poplars. Among these, *ERF54* exhibited the strongest differential expression, making it a prime candidate for further investigation.

Construction of the co-expression networks of *ERF54* under drought stress in *Populus*

To further delineate ERF-mediated transcriptional regulatory networks under drought stress in *Populus*, we performed WGCNA using drought-stress transcriptomes from the five *Populus* taxa. Genes with a maximum TPM < 1 were excluded, and the remaining 26,022 genes were clustered into 25 co-expression modules, each designated with a distinct color (Supplementary Fig. S3). Using *ERF54* as a hub gene, we identified genes whose expression patterns were significantly correlated with its expression pattern and constructed an *ERF54*-centered co-expression network (Fig. 2a). To further validate the stability of the co-expression relationships across different poplar species, we conducted RT-qPCR analysis of *ERF54* and its top three co-expressed genes in *P. tomentosa*, *P. trichocarpa*, and *P. deltoides* (Fig. 2b–e). The results demonstrated that *ERF54* homologs were significantly upregulated under drought stress in all three species (Fig. 2b). Similarly, the homologs of three co-expressed genes, *Potri.004G174100* (*ERD7*), *Potri.006G260100* (*WAKL*), and *Potri.008G159400* (*APG1*), exhibited expression patterns consistent with those of *ERF54* under drought stress (Fig. 2c–e). *ERD7* exhibited the highest correlation with *ERF54* (R = 0.89; p = 2.17 × 10^{-19}) and showed the most significant changes under drought stress.

Based on multiple lines of evidence, *ERD7* was selected for further functional characterization. First, *ERD7* exhibited the strongest co-expression relationship with *ERF54*, showing a high correlation coefficient in the *ERF54*-centered WGCNA network (Fig. 2a). Second, the drought-induced expression pattern of *ERD7* was highly consistent with that of *ERF54* across the representative *Populus* species, as further validated by RT-qPCR (Fig. 2b, c). Third, the functional annotation of *ERD7* is directly relevant to drought stress, as *ERD*

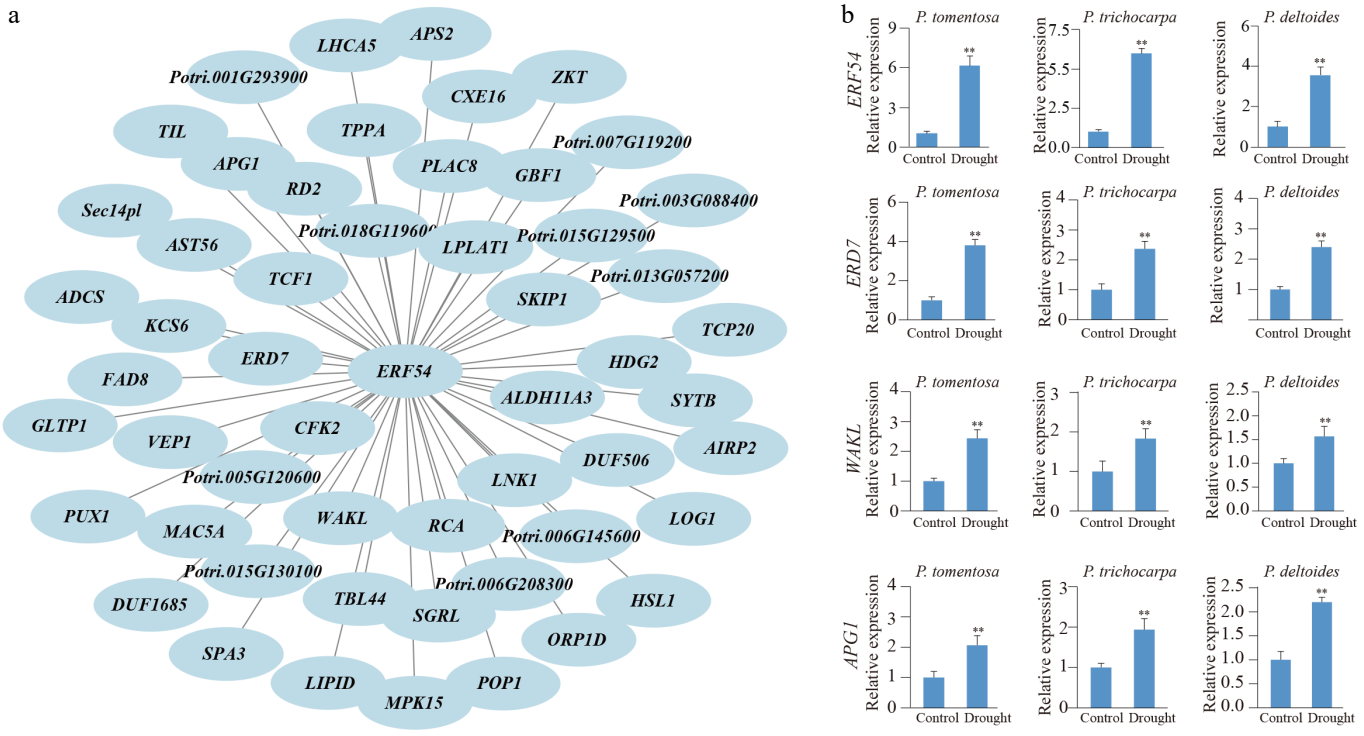

Fig. 2 Co-expression network centered on *ERF54* and expression validation across *Populus* species. (a) Drought-responsive gene co-expression network centered on *ERF54*, constructed using weighted gene co-expression network analysis across five *Populus* taxa. Nodes represent genes, and edges indicate significant co-expression relationships. (b)–(e) Reverse transcription-quantitative polymerase chain reaction validation of *ERF54* and selected co-expressed genes (*ERD7*, *WAKL*, and *APG1*) in *P. tomentosa*, *P. trichocarpa*, and *P. deltoides* under drought stress. Data represent means ± SE (n = 3 biological replicates, each with four technical replicates). Asterisks denote significant differences compared with the control: ** p < 0.01.

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genes are widely recognized as early dehydration-responsive genes involved in plant stress adaptation. Finally, the promoter sequence analysis revealed putative AP2/ERF-related binding motifs in the *ERD7* promoter (Supplementary Fig. S4). Taken together, these results support the selection of *ERD7* for subsequent functional characterization.

PtrERF54 overexpression improves iWUE by reducing transpiration

To explore the biological functions of *ERF54* in poplar, we cloned *ERF54* from *P. trichocarpa* and designated it *PtrERF54*. Subsequently, we generated transgenic '84K' (*P. alba* × *P. glandulosa*) plants overexpressing *PtrERF54* under the control of the *CaMV35S* promoter (*PtrERF54-OE*). The RT-qPCR analysis revealed variable *PtrERF54* transcript accumulation in transgenic lines. Among them, *PtrERF54-OE3* and *PtrERF54-OE5* exhibited the highest expression levels, with 9.86-fold and 8.85-fold increases relative to those in the WT control, respectively (Supplementary Fig. S5); therefore, they were selected for subsequent analyses.

To further elucidate the physiological basis of enhanced drought tolerance in the transgenic lines, we measured leaf gas-exchange parameters using the Li-6400 portable photosynthesis system under controlled CO_2 concentration and PAR. Both CO_2- and light-response curves revealed that *PtrERF54-OE* plants maintained net photosynthetic rates (*Pn*) comparable to those of WT plants (Fig. 3a, e). In contrast, stomatal conductance (*Gs*) and transpiration rate (*Tr*) were consistently lower in the *PtrERF54-OE* lines than in WT plants under both CO_2- and light-saturating conditions (Fig. 3b, c, f, and g). As photosynthetic carbon assimilation remained largely unchanged, the reduction in *Tr* led to a significant increase in iWUE in *PtrERF54-OE* lines (Fig. 3d, h).

PtrERF54 overexpression enhances drought tolerance in *Populus*

To assess the effects of *PtrERF54* overexpression on drought tolerance, *PtrERF54-OE* (*OE3* and *OE5*) and WT poplars were grown under identical conditions and subjected to drought stress, followed by a 2-day re-watering period. By day 6 of the drought treatment, the WT plants exhibited severe wilting, whereas the transgenic lines exhibited only mild wilting symptoms (Fig. 4a). After re-watering, the WT plants recovered poorly, whereas *PtrERF54-OE* lines largely regained their normal morphology (Fig. 4a). The physiological measurements were consistent with phenotypic observations. Following drought stress, the *PtrERF54-OE* plants maintained significantly higher leaf RWC than did WT plants, indicating improved water retention (Fig. 4b). REC, an indicator of cell membrane integrity, increased under drought stress in both WT and *PtrERF54-OE* plants; however, the increase was substantially lower in *PtrERF54-OE* plants, with REC values ranging from 24.8% to 27.9%, compared with 52.6% in WT plants (Fig. 4c). Moreover, the *PtrERF54-OE* lines exhibited a higher maximum photochemical efficiency of PSII (F_v/F_m) and increased NPQ under drought conditions, suggesting better maintenance of photosynthetic performance and enhanced photoprotective capacity (Fig. 4d, e). To further evaluate oxidative stress responses, we quantified the activity of the major antioxidant enzymes POD and SOD. Under drought stress, both POD and SOD activities were significantly higher in *PtrERF54-OE* lines than in the WT plants (Fig. 4f, g).

PtrERF54 binds to the *PtrERD7* promoter and activates its transcription

The RT-qPCR analysis confirmed the significant upregulation of *PtrERD7* in *PtrERF54-OE* lines compared with that in WT lines, consistent with the WGCNA results (Fig. 5a). To investigate whether *PtrERF54* directly regulates *PtrERD7*, we analyzed the *PtrERD7* promoter sequence and identified putative *PtrERF54*-specific binding motifs (Supplementary Fig. S4). Subsequently, Y1H assays were performed to validate protein–DNA interactions. These results demonstrated that *PtrERF54* directly binds to the *PtrERD7* promoter. Yeast co-transformed with pGADT7-*PtrERF54* and *pAbAi-PtrERD7pro* grew on selective medium (SD/−Leu) supplemented with 200 ng mL^{-1} aureobasidin A (AbA), confirming this binding interaction (Fig. 5b). The ChIP-qPCR analysis revealed a significant enrichment of the *PtrERD7* promoter fragment harboring the putative

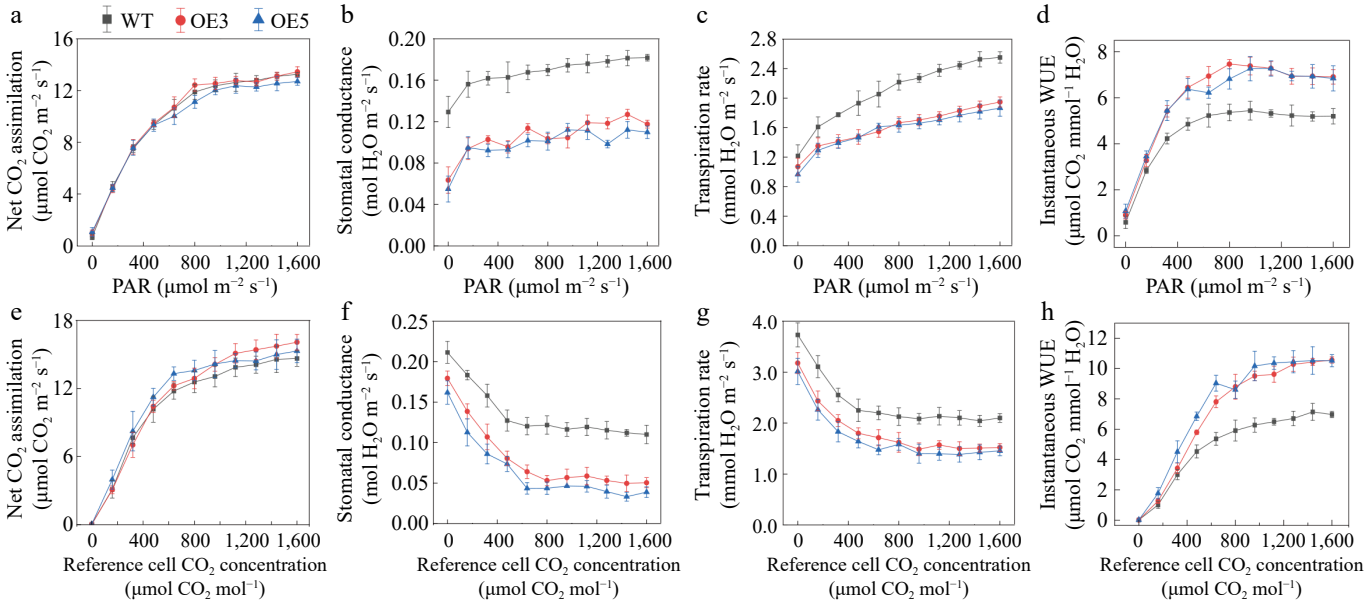

Fig. 3 *PtrERF54* overexpression improves iWUE. Light response curves of (a) net photosynthetic rate (*Pn*), (b) stomatal conductance (*Gs*), (c) transpiration rate (*Tr*), and (d) instantaneous water-use efficiency (iWUE). CO_2 response curves of (e) *Pn*, (f) *Gs*, (g) *Tr*, and (h) iWUE. Data represent means ± SE (n = 3 biological replicates, each with three technical replicates).

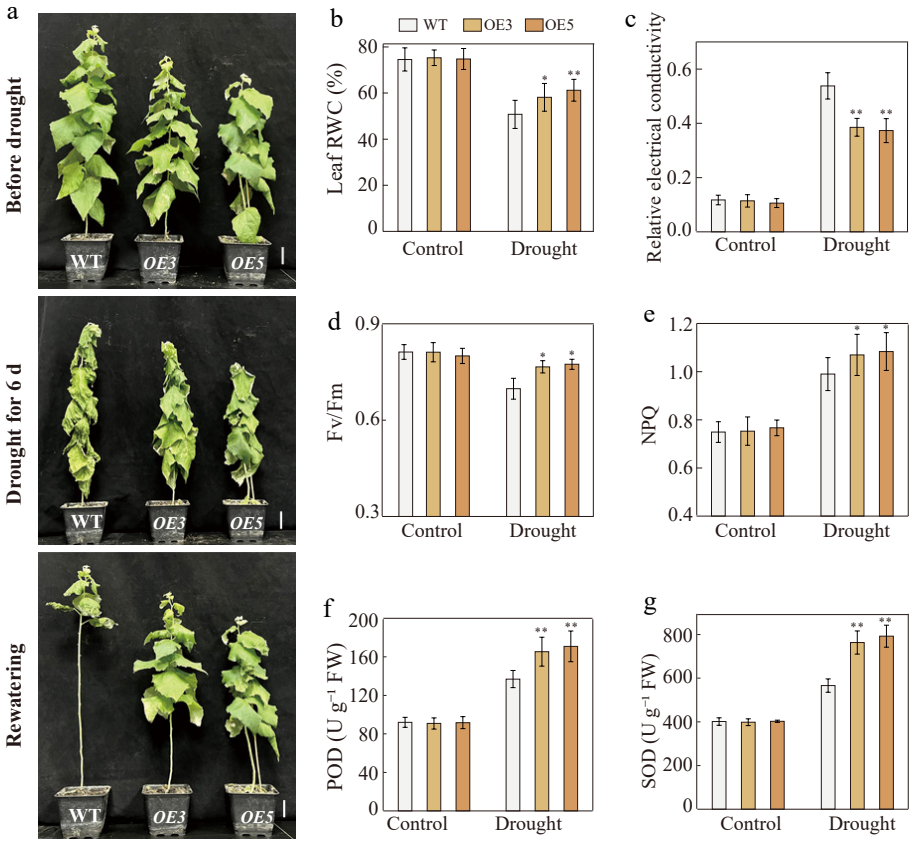

Fig. 4 *PtrERF54* enhances drought tolerance in poplar. (a) Morphological comparison between wild-type (WT) and *PtrERF54*-overexpressing (*PtrERF54-OE*) poplar lines following drought stress. Scale bar = 5 cm. (b) Leaf relative water content (RWC) and (c) relative electrical conductivity (REC), indicating water status and membrane integrity. (d) Maximum photochemical efficiency of photosystem II (F_v/F_m) and (e) non-photochemical quenching (NPQ) under drought stress. Enzymatic activities of (f) peroxidase (POD) and (g) superoxide dismutase (SOD) as indicators of antioxidant responses. Data represent means ± SE (n = 3 biological replicates, each with four technical replicates). Asterisks denote significant differences compared with the control: * $p < 0.05$; ** $p < 0.01$.

ERF-binding site in the *PtrERF54*-GFP transgenic lines relative to that in the GFP-only control, confirming the *in vivo* binding of PtrERF54 to the *PtrERD7* promoter (Fig. 5c). To further assess the regulatory effects of PtrERF54 on *PtrERD7* expression, we performed a dual-luciferase reporter assay in *N. benthamiana*. The *PtrERD7* promoter fragments were cloned into the pGreen II 0800-LUC vector and co-infiltrated with the *35S::PtrERF54* effector construct into tobacco leaves (Fig. 5d). After 72 h, the luciferase activity was significantly enhanced in the presence of *PtrERF54*, indicating that PtrERF54 activates *PtrERD7* transcription (Fig. 5e). Taken together, these results demonstrate that *Ptr*ERF54 directly binds to the *PtrERD7* promoter and acts as a transcriptional activator, indicating that *PtrERD7* is a downstream target of *Ptr*ERF54 in the drought-response regulatory network of poplars.

PtrERD7 overexpression improves drought tolerance in *Populus*

To assess the effects of *PtrERD7* overexpression on drought tolerance, *PtrERD7-OE* (*OE6* and *OE11*) and WT poplars were grown under identical conditions and subjected to drought stress, followed by a 2-day re-watering period. By day 6 of the drought treatment, WT plants exhibited pronounced wilting and partial shoot tip desiccation, whereas the *PtrERD7-OE* lines displayed only mild wilting symptoms (Fig. 6a). After 2 d of re-watering, WT plants recovered poorly, whereas *PtrERD7-OE* plants substantially recovered their normal morphology (Fig. 6a). After drought stress, the *PtrERD7-OE* lines maintained significantly higher RWC than WT plants, indicating a superior water-retention capacity (Fig. 6b). REC increased in both genotypes under drought stress but was significantly lower in the transgenic lines (~34.61%) than in WT plants (~52.55%), indicating reduced membrane damage (Fig. 6c). Under drought stress, *Pn* decreased in both genotypes; however, the *PtrERD7-OE* lines maintained significantly higher *Pn* than WT plants (Fig. 6d). Similarly, *Tr* decreased in all plants under drought stress; however, the decline was more pronounced in the *PtrERD7-OE* lines. By day 6, *Tr* in *OE6* and *OE11* was reduced by approximately 39% and 51%, respectively, relative to WT plants (Fig. 6e). To further investigate their oxidative stress responses, we measured the activities of POD and SOD. Both POD and SOD activities were significantly elevated in the *PtrERD7-OE* lines under drought conditions compared with those in WT plants (Fig. 6f, g). Collectively, these results demonstrate that the overexpression of *PtrERD7* enhances drought tolerance in poplar by mitigating oxidative stress, reducing membrane damage, and maintaining higher photosynthetic activity while reducing transpiration rates relative to WT plants under water-deficit conditions.

Discussion

Drought stress is a major limiting factor for the growth and productivity of *Populus* species, highlighting the importance of identifying core regulatory modules that can be leveraged to

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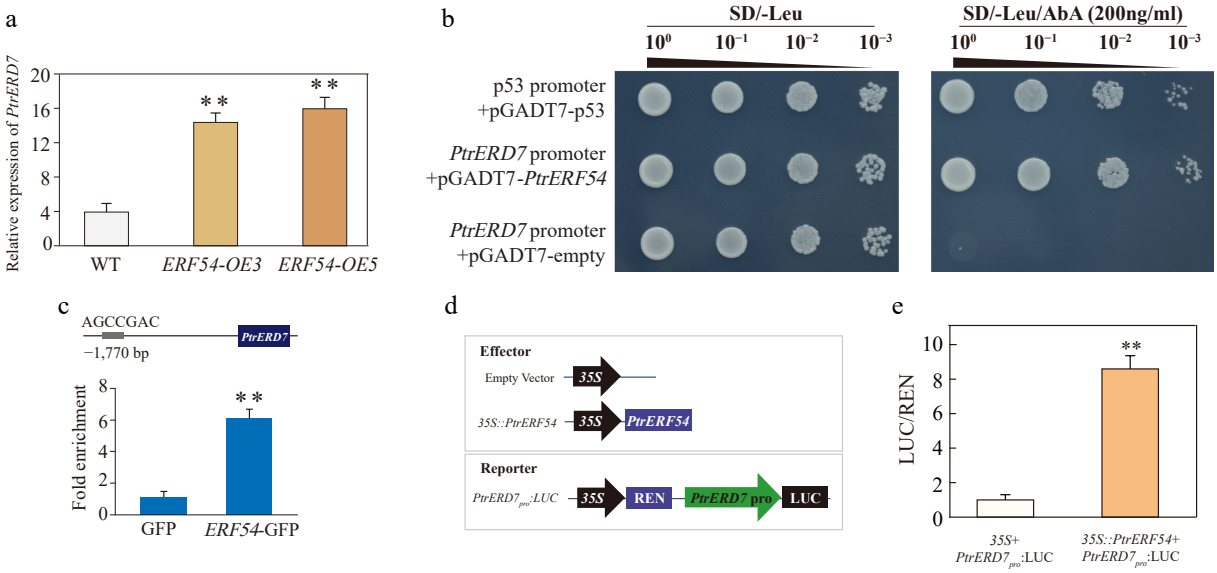

Fig. 5 *PtrERF54* binds to the *PtrERD7* promoter and regulates its expression. (a) Relative expression level of *PtrERD7* in wild-type (WT) and *PtrERF54*-overexpressing *(PtrERF54-OE)* poplar lines as determined by RT-qPCR. (b) Yeast one-hybrid assay showing that *PtrERF54* binds to the *PtrERD7* promoter. Yeast strains co-transformed with pGADT7-*PtrERF54* and pAbAi-*PtrERD7* pro grew on selective medium (SD/-Leu) supplemented with 200 ng mL^{-1} aureobasidin A (AbA). (c) ChIP-qPCR analysis showing the enrichment of the *PtrERD7* promoter fragment containing the predicted AP2/ERF-binding motif by *PtrERF54*-GFP. GFP-only transgenic plants served as the negative control. (d) Schematic representation of the constructs used in the dual-luciferase reporter assay, including the effector and reporter constructs. (e) A dual-luciferase reporter assay was used to assess transactivation of the *PtrERD7* promoter by *PtrERF54* in *N. benthamiana* leaves. Effector and reporter constructs were co-infiltrated, and luciferase (LUC) activity was measured 72 h later. Values were normalized to REN (Renilla luciferase) activity, with the empty effector vector serving as the control. Data represent means ± SE (n = 3 biological replicates, each with four technical replicates). Asterisks denote significant differences compared with the control: ** $p < 0.01$.

improve drought resistance[26,36,37]. Previous studies have established that ERFs function not only in plant development[38,39] but also in regulating responses to biotic[40,41] and abiotic stresses[10,42–44]. Consistent with these observations, our comparative transcriptomic analysis across the five *Populus* taxa (*P. tomentosa*, *P. trichocarpa*, *P. alba* × *P. davidiana*, *P. davidiana*, and *P. deltoides*) highlighted the involvement of the ERF family in drought adaptation (Fig. 1). The promoter analysis of 175 ERF genes in *P. trichocarpa* revealed a significant enrichment of *cis*-acting elements associated with stress- and hormone-responsive pathways (Supplementary Fig. S1), supporting a broad regulatory role for ERFs in abiotic stress signaling. Among drought-responsive ERFs, *ERF54* exhibited the most pronounced and consistent induction across the five species (Supplementary Fig. S2), which prompted us to prioritize it for functional characterization.

To assess the ability of *PtrERF54-OE* plants to mitigate drought-induced photosynthetic inhibition, we compared the gas-exchange parameters between *PtrERF54-OE* and WT plants. *PtrERF54-OE* plants exhibited significantly reduced *Gs* and *Tr* values compared with WT plants. Notably, this decline did not affect *Pn*, resulting in a substantially enhanced iWUE (Fig. 3). Although sustained low *Gs* typically impedes CO_2 diffusion into leaves, thereby limiting photosynthesis, the maintained *Pn* in *PtrERF54-OE* plants may suggest a compensatory mechanism. We hypothesized that this compensation might involve an elevated Rubisco activation state or enhanced carboxylation efficiency, thereby sustaining efficient carbon fixation despite limited intercellular CO_2 availability[27].

The analysis of chlorophyll fluorescence parameters provided further physiological insights (Fig. 4d, e). The maximum photochemical efficiency of PSII (F_v/F_m), which typically declines with increasing stress severity, was significantly higher in *PtrERF54-OE* plants than in WT plants. Concurrently, NPQ, a key indicator of photoprotective heat dissipation, was higher in the transgenic lines under drought stress than in WT plants. The increase in NPQ serves as a photoprotective mechanism, reflecting the plant's capacity to dissipate excess light energy as heat and protect the photosynthetic apparatus from excess light exposure. This process represents a conserved strategy that prioritizes survival[45,46]. We attributed the higher F_v/F_m to the superior antioxidant capacity and water status of *PtrERF54-OE* plants, which collectively minimized photo-oxidative damage to PSII reaction centers. Higher NPQ suggests that the *PtrERF54-OE* plants rapidly activate photoprotection, leading to elevated NPQ, which facilitates the efficient dissipation of excess light energy, thereby effectively minimizing PSII photodamage and preserving a high F_v/F_m ratio. Taken together, these findings demonstrate that *PtrERF54* overexpression confers enhanced cellular protection and optimizes photochemical efficiency under drought stress.

Furthermore, the overexpression of either *PtrERF54* or *PtrERD7* significantly enhanced drought tolerance in poplars. Under drought stress, both transgenic lines (*PtrERF54-OE* and *PtrERD7-OE*) exhibited higher leaf RWC, elevated activities of antioxidant enzymes (POD and SOD), and lower REC than those of WT plants (Figs 4, 6). These results indicate that both *PtrERF54* and *PtrERD7* effectively maintain cellular homeostasis under drought stress by improving water retention and activating the antioxidant system, thereby protecting the structural and functional integrity of cell membranes[25,47]. Under drought stress, the *PtrERD7-OE* lines also exhibited higher *Pn* and lower *Tr* (Fig. 6d, e). *PtrERD7* overexpression may help maintain photosynthetic performance by preserving the functionality of the photosynthetic apparatus while reducing water loss, resulting in a substantial improvement in iWUE.

The comparative transcriptomic analysis revealed that *ERF54* was consistently induced under drought stress in all five tested species (*P. tomentosa*, *P. trichocarpa*, *P. alba* × *P. davidiana*, *P. davidiana*, and *P. deltoides*) (Supplementary Fig. S2). The co-expression relationship

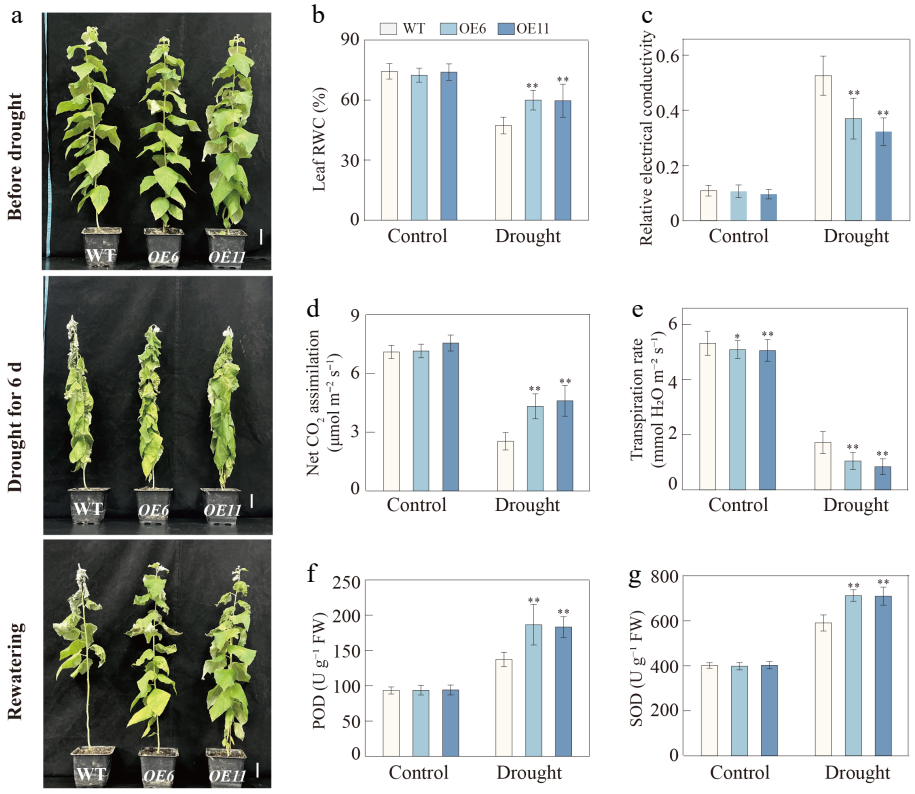

Fig. 6 Overexpression of *PtrERD7* enhances drought tolerance in poplar. (a) Morphological comparison between wild-type (WT) and *PtrERD7*-overexpressing (*PtrERD7-OE*) poplar lines after drought treatment. Scale bar = 5 cm. (b) Leaf relative water content (RWC) and (c) relative electrical conductivity, indicating water retention capacity and membrane integrity, respectively. (d) Net photosynthetic rate and (e) transpiration rate under drought conditions. Activities of the antioxidant enzymes (f) peroxidase (POD) and (g) superoxide dismutase (SOD) in response to drought stress. Data represent means ± SE (n = 3 biological replicates, each with four technical replicates). Asterisks denote significant differences: * $p < 0.05$; ** $p < 0.01$.

between *ERF54* and *ERD7* was validated by RT-qPCR in three representative species, which showed a consistent upregulation pattern (Fig. 2b–e). Although functional validation was performed in the hybrid '84K' poplar (*P. alba* × *P. glandulosa*), a widely used model genotype for poplar genetic transformation, the *ERF54* and *ERD7* homologs in this genotype shared high sequence identity with the corresponding genes from the five analyzed species. The direct binding of PtrERF54 to the *PtrERD7* promoter (Fig. 5; Supplementary Fig. S4), together with the drought-tolerant phenotypes observed in *PtrERF54-OE* and *PtrERD7-OE* lines, suggests that this regulatory module functions similarly across different poplar genetic backgrounds. Therefore, we propose that the PtrERF54–*PtrERD7* module represents an evolutionarily conserved regulatory axis for drought response in the genus *Populus*.

Although our experiments demonstrated that the PtrERF54–*PtrERD7* module enhances drought tolerance in poplar under controlled conditions, field environments are characterized by recurrent drought cycles, soil heterogeneity, and combined stresses, such as heat, nutrient deficiency, and pathogen attack. In this study, the *PtrERF54-OE* lines exhibited reduced *Gs* and *Tr* without compromising *Pn*, leading to improved iWUE, a trait that may contribute to improved performance under fluctuating precipitation (Fig. 3). The enhanced antioxidant capacity and membrane integrity may contribute to improved tolerance under variable field conditions (Fig. 4).

Notably, in this study, overexpression lines were generated only for *PtrERF54* and *PtrERD7*. Although these lines clearly demonstrated the positive roles of both genes in drought tolerance, loss-of-function evidence remains lacking. Future studies employing CRISPR–Cas9-mediated gene knockout or knockdown approaches in poplar are essential to further validate the functional necessity of the PtrERF54–*PtrERD7* module in drought responses. In addition, we noted that *PtrERF54* overexpression led to a moderate reduction in plant growth under normal watering conditions, suggesting that it may be worthwhile to explore stress-inducible or tissue-specific promoters to uncouple drought-tolerance benefits from growth penalties. Alternatively, fine-tuning the expression levels of these genes via promoter editing using the CRISPR–Cas9 system, or moving the functional module directly into elite poplar cultivars, could help accelerate the molecular breeding of drought-tolerant poplars.

Conclusions

Our findings reveal a PtrERF54–*PtrERD7* transcriptional module that enhances drought resilience in poplar by improving iWUE, strengthening antioxidant defenses, and maintaining membrane integrity, thereby providing mechanistic insights into how ERF TFs orchestrate drought responses in woody plants. The conserved drought-inducible expression of *ERF54* and *ERD7* across diverse poplar species suggests that this module represents an evolutionarily conserved regulatory axis for adaptation to water deficit within the genus *Populus*. Future work employing CRISPR–Cas9-mediated knockout lines and stress-inducible promoter strategies will be essential to validate gene function and mitigate potential growth-defense trade-offs.

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Author contributions

The authors confirm their contributions to the paper as follows: study conception: Li P, Sang YL; data curation: Niu MX, Dong WC; experiments: Zhang ZC, Lu JX, Meng XR; data analysis and manuscript preparation: Niu MX, Dong WC, Li P; manuscript revision: Niu MX, Li P. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The data were obtained from publicly available resources.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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