# PtrWRKY75 overexpression reduces stomatal aperture and improves drought tolerance by salicylic acid- induced ROS accumulation in poplar

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**Abstract**

PtrWRKY75, PtrPAL1, poplar, drought tolerance, reactive oxygen species, salicylic acid, stomatal closure

## INTRODUCTION

Environmental stresses frequently affect plant growth and development. Among the multitudinous adverse factors, drought is a severe environmental factor constraining plant growth and development (Zhu, 2016; Zhu, 2002). Plants have evolved complex mechanisms to cope with drought, including morphological and physiological mechanisms (Zhu, 2002; Bohnert *et al.* , 2006). For example, plants can improve drought resistance by closing their stomata to decrease water loss from leaves under drought stress (Zhu, 2002). The movement (opening and closure) of the stomatal aperture, which is induced by many factors including abscisic acid (ABA), salicylic acid (SA), $CO_2$, reactive oxygen species (ROS), and water status, controls both the influx of $CO_2$ and water loss through transpiration to the atmosphere (Zhao *et al* ., 2015; Ullah *et al* ., 2018). Under drought conditions, ROS accumulate rapidly and act as an important second messenger in stomatal movement (Munemasa *et al.* , 2007), leading to rapid stomatal closure (Maija *et al* ., 2016; Singha*et al* ., 2017) to reduce water loss. Salicylic acid is widely considered to enhance plant defense responses against pathogens (Verma*et al.* , 2016; Lee *et al.* , 2010). It also plays roles in the plant response to abiotic stresses, such as chilling, heat, drought, salt, ultraviolet radiation, and heavy metals (Janda et al., 1999; Senaratna *et al.* , 2000; Munné-Bosch and Peñuelas, 2003; Chini*et al.* , 2004; Hayata *et al.* , 2010). Previous studies have shown that treatment with SA increases tolerance to drought stress (Kang*et al.* , 2012). Under drought conditions, the concentration of SA increases five-fold in the evergreen shrub *Phillyrea angustifolia*(Munné-Bosch and Peñuelas, 2003) and two-fold in barley roots (Bandurska and Stroiński, 2005). In addition, under drought stress, the expression of SA-responsive genes, such as *PATHOGENESIS-RELATED* (*PR* ) genes, are also induced (Miura r *et al.* , 2013; Liu *et al.* , 2013). Salicylic acid induces ROS production in extracellular spaces and subsequently ROS accumulate in guard cells by diffusion (Khokon *et al.* , 2011; Mori *et al.* , 2001). However, the molecular mechanisms related to SA in *Populus* are not fully understood.

The WRKY transcription factor (TF) family is among the most widely studied TFs. Many WRKY TFs are involved in a variety of biotic and abiotic stress responses (Rushton *et al.* , 2010; Parinita *et al.* , 2011; Chen *et al.* , 2012; Jiang *et al.* , 2014).*ABO3 / WRKY63* , *TaWRKY2* , *TaWRKY19* , and*WRKY57* participate

in the response to drought stress in*Arabidopsis* (Ren *et al* ., 2010; Niu *et al* ., 2012; Jiang *et al* ., 2012). *OsWRKY45* in rice and *AtWRKY46*in *Arabidopsis* respond to drought and salt stress by regulating stomatal movement (Qiu, 2008; Ding *et al* ., 2014). Previous research has confirmed that *ZmWRKY* 40 is a positive regulator that improves drought tolerance by regulating stress-related genes and the ROS content in maize (Wang *et al* ., 2018). *AtWRKY75*functions as a positive regulator of flowering as a component of the GA-mediated signaling pathway in *Arabidopsis* (Zhang *et al* ., 2017) and induces leaf senescence by interacting with SA and ROS (Guo *et al* ., 2017). In *Populus, PtrWRKY18* and*PtrWRKY35* promote tolerance the leaf rust pathogen*Melampsora* (Jiang *et al* ., 2017), and *PtrWRKY73* and*PtrWRKY89* play important roles in disease resistance mediated by SA and ROS, as demonstrated by experiments on *Arabidopsis* and transgenic poplar (Duan*et al* ., 2015; Jiang *et al* ., 2014). However, little is known about the role of WRKY TFs in the drought tolerance of*Populus* .

Poplars are among the most adaptable trees and are grown worldwide. They grow rapidly and their wood is used for diverse purposes. Thus, poplars provide substantial economic, ecological, and social benefits. However, poplars have a strong demand for water resources (Monclus *et al.* , 2006; Han *et al.* , 2013). North China is predominantly arid and semi-arid, and drought may negatively affect poplar growth (Tschaplinski *et al.* , 1994). For growth of poplars in such areas, it is important to enhance their drought tolerance. Previous studies have shown that *PtrWRKY75* responds to SA (Jiang *et al* ., 2014), and that an increase in SA content can improve drought tolerance (Kang *et al.* , 2012). To assess the potential biological functions of PtrWRKY75 in tolerance to water stress,*PtrWRKY75*- overexpressing poplars were generated. Our results demonstrate that *PtrWRKY75* responds to exogenous SA treatment by inducing *PAL1* expression. This leads to ROS accumulation in the leaves, which subsequently induces stomatal closure and reduced transpiration, thereby increasing water-use efficiency (WUE) and drought tolerance.

## RESULTS

### Identification and expression pattern analysis of*PtrWRKY75*

A 561-bp *WRKY75* gene encoding 186 amino acids was cloned from*P. trichocarpa* , whose genome has been sequenced (Tuskan *et al.* 2006). Homologous amino acid sequences of WRKY75 proteins in*P. trichocarpa* , *Arabidopsis thaliana* , *Oryza sativa* ,*Zea mays* , and *Vitis vinifera* were obtained from the National Center for Biotechnology Information database (NCBI; https://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed for WRKY75 orthologs from a multiple alignment of the amino acid sequences (Figure 1A). To further analyze the phylogenetic relationships of WRKY TFs in poplar, we constructed phylogenetic tree of 100 members in WRKY TF family in poplar (Figure S1). The phylogenetic reconstruction revealed that PtrWRKY75 is evolutionarily close to AtWRKY75 from *A. thaliana* (61% identity). The multiple sequence alignment revealed that PtrWRKY75 contains a conserved domain WRKYGQK at the N-terminus followed by a basic zinc finger motif (C-$X_5$-C-$X_{23}$-H-$X_1$-H). These analyses confirmed that *PtrWRKY75* belongs to group II of the WRKY TF gene family (Figure 1B) (Eulgem *et al.* 2000).

To determine the subcellular localization of PtrWRKY75, pSuper::PtrWRKY75-eGFP and pSuper::eGFP (as a negative control) fusion proteins were transiently transfected into tobacco (*Nicotiana benthamiana* ) leaves. The pSuper::PtrWRKY75-eGFP fusion protein was localized in the nucleus, as observed under a confocal laser scanning microscope, whereas the pSuper::eGFP fusion protein was distributed throughout the cell without specific localization (Figure 1C). These results suggested that PtrWRKY75 is a transcriptional regulator localized in the nucleus.

Quantitative real-time PCR (qRT-PCR) was applied to detect the tissue-specific abundance of *PtrWRKY7* 5 transcripts in different tissues of*P. trichocarpa* (root, stem, young leaf, mature leaf, and senescent leaf).*PtrWRKY7* 5 transcript levels were higher in mature leaves and senescent leaves than in young leaves and the stem (Figure 1D). To investigate the response of *PtrWRKY7* 5 to water stress, *P. trichocarpa* plants were subjected to drought conditions and the transcript levels of *PtrWRKY7* 5 were quantified by qRT-PCR. The transcript level of *PtrWRKY7* 5 gradually increased and peaked at 6 h of the drought treatment, at a level 12.87-times higher than that in the control, and thereafter decreased (Figure 1E). The *PtrWRKY7* 5

transcript level increased 13.12-fold after 1 h treatment with 5 mM SA (Figure 1F). These results indicated that *PtrWRKY7* 5 is expressed predominantly in the leaf, and is up-regulated by drought stress and SA treatment.

**Identification of *PtrWRKY75*-overexpressing transgenic poplar**

To study the role of *PtrWRKY75* in plants under drought stress, the *pSuper::PtrWRKY75* vector was transformed into wild-type (WT) triploid white poplar (*Populus tomentosa* 'YiXianCiZhu B385') using the leaf disc method, then *PtrWRKY75* -overexpressing transgenic poplar lines ( *WRKY75-OE* ) were generated. Thirteen transgenic lines were verified by PCR using gene-specific primers and qRT-PCR (Figure S2A, B). The levels of *PtrWRKY75* transcripts differed among the transgenic lines. The highest transcript levels were in OE-8 and OE-10 (36.27 and 46.64 times higher, respectively, than those in the other transgenic lines). Therefore, we selected these two lines, hereafter referred to as the OE lines, as experimental materials for subsequent analyses.

*PtrWRKY75* **promotes SA-induced stomatal closure by ROS production**

Recent evidence indicates that SA induces accumulation of ROS and influences stomatal closure in *Arabidopsis* (Borsani *et al.* , 2001; Ullah *et al.* , 2018). To explore the function of *PtrWRKY75* in *Populus* , we measured ROS levels in the OE lines and WT plants. The OE lines and WT were treated with 0 or 0.5 mM SA for 0, 0.5, 1, 2, or 3 h, then their leaves were stained with 3,3'-diaminobenzidine (DAB). Hydrogen peroxide ($H_2O_2$) was visualized as a deep brown product resulting from the reaction of DAB with $H_2O_2$. The leaves of the OE lines were stained a deeper brown color than that of WT leaves under all conditions (Figure 2A). Thus, exogenous SA treatment increased the ROS content in the leaf to higher levels in the OE lines than in WT.

To investigate whether *PtrWRKY75* promotes SA-induced stomatal closure in the OE lines and WT, leaf stomatal movement was observed under a scanning electron microscope. After treatment with exogenous SA for 1 h and 2 h, the stomata closed more rapidly in OE lines than in WT (Figure 2B). The stomatal aperture was much smaller in OE lines than in WT under exogenous SA treatment (Figure 2C). These results suggested that *PtrWRKY75* promotes SA-induced stomatal closure via ROS production.

*PtrWRKY75* **promotes SA biosynthesis by activating *PAL1* transcription**

We speculated that WRKY75 might promote SA biosynthesis during drought. Therefore, we measured the SA content in WT and OE lines under non-stress conditions and dehydration for 4 h. The free SA concentration increased under dehydration treatment, especially in the OE lines, where its peak level was approximately double that in WT (Figure 3A). Next, we explored how WRKY75 promotes SA biosynthesis. On the basis of previous research, we examined the expression level of *SA INDUCTION-DEFICIENT 2* (*SID2* ) (Nawrath and Métraux, 1999) and *PHENYLALANINE AMMONIA LYASE 1* (*PAL1* ) (Cochrane *et al.* , 2004) under non-stress and dehydration conditions. *SID2* and *PAL1* encode crucial enzymes in two SA biosynthesis pathways. The *PtrPAL1* transcript levels were markedly higher in OE lines than in WT, whereas *PtrSID2* transcript levels did not differ between OE lines and WT, or between drought and non-stress conditions (Figure 3B, C). This result suggested that WRKY75 might directly or indirectly induce *PAL1* expression.

To assess whether WRKY75 directly binds to the *PAL1* promoter to regulate its transcription, we performed electrophoretic mobility shift assay (EMSA) experiments. WRKY TFs are known to specifically bind to the W-box (TTGACT) sequence, and we identified one putative W-box sequence in the *PAL1* promoter (Figure 3D). The full-length WRKY75 protein tagged with His (His-WRKY75) was capable of binding to probes containing the W-box, but not to the probe harboring a single nucleic acid mutation (TTAACT) (Figure 3E). These results suggested that WRKY75 can directly bind to the promoter of *PAL1* .

To further investigate the positive regulation of *PAL1* transcription by WRKY75, promoter transactivation assays were conducted in tobacco leaves by transient expression. The *PAL1* promoter was fused with the *β-ΓΛΥ˜ΥPONIΔAΣE* (*GUS* ) reporter gene to generate reporter constructs, and the *35S:WRKY75* construct

was used as the effector (Figure 3F). The results revealed that WRKY75 activated the promoter of *PAL1 in vivo* under both non-stress and dehydration conditions, especially the latter. Taken together, these results indicated that WRKY75 can promote SA production by activating *PAL1* transcription.

### *PtrWRKY75* overexpression enhanced WUE by reducing transpiration

Previous studies have suggested that ROS accumulation in leaves may cause stomatal closure (Song *et al.* , 2014; Sierla *et al.* , 2016), thereby reducing transpiration and improving the WUE of plants (Yoo, C.Y *et al* ., 2009; Yoo, C.Y *et al* ., 2010). The photosynthesis–light curve showed that photosynthesis was similar in the OE lines and WT (Figure 4A). The stomatal conductance ($G_s$) was significantly lower in the OE lines than in WT (Figure 4B). The leaf transpiration rate was about 30% lower in the OE lines than in WT (Figure 4C). As a result, the instantaneous WUE (photosynthesis/transpiration) values of the OE lines were significantly higher than those of WT (Figure 4D). The vapor pressure deficit (VPD) showed no significant difference between the OE lines and WT, indicating that VPD was not a factor in the reduced transpiration rate of the OE lines (Figure 4E). In general, overexpression of *PtrWRKY75* enhanced WUE by reducing the transpiration rate.

### *PtrWRKY75*-overexpressing transgenic poplar showed improved drought tolerance under short-term drought stress

To explore the differences in stress tolerance between OE lines and WT plants, a drought treatment was applied by withholding watering for 7 days. As expected, the leaves of the WT plants were wilted after 7 days of drought, whereas those of the OE lines remained turgid (Figure 5A). During the drought treatment, the net photosynthesis rate,$G_s$, and transpiration rate of WT and OE poplars were measured (Figure 5B–D). The photosynthesis rate of WT plants decreased significantly, with almost no photosynthetic activity recorded on day 7 of the drought treatment, whereas the net photosynthesis rate of the OE lines decreased significantly in the first 4 days and then slowed down, and remained at a certain level at day 7 (Figure 5B). The $G_s$ and transpiration rate in WT and OE lines decreased under drought treatment, but the decline in OE lines was slower than that in WT (Figure 5C, D). In addition, the OE lines maintained a higher leaf relative water content (RWC) than the WT plants under drought (Figure 5E). Leaf water loss, an important parameter for evaluating plant tolerance to water deficit stress, was also examined in the WT and OE lines. The WT plants lost water faster than did the OE lines under dehydration conditions (Figure 5F). Electrical conductivity can reflect the extent of damage to the plasma membrane. After short-term drought stress, the relative electrical conductance (REC) of both WT and the OE lines increased, but to a higher level in WT than in the OE lines, indicating that the leaves of WT suffered greater membrane damage (Figure 5G). Together, these results showed that the OE lines were more tolerant than were WT plants to short-term drought stress.

### *PtrWRKY75*-overexpressing transgenic poplar showed improved drought tolerance under long-term drought stress

To further explore the function of PtrWRKY75 in long-term drought stress, the OE lines and WT were subjected to 35 days of soil RWC at 70% (control) or 20% (drought). On day 35, the OE lines showed better growth than that of WT (Figure 6A). Compared with WT, the OE lines had a higher leaf RWC, indicative of stronger drought tolerance (Figure 6B). The OE lines and WT showed no significant differences in photosynthetic rate in the control (70% soil RWC), but the OE lines had a higher photosynthetic rate under drought conditions (Figure 6C).

To investigate the difference in growth between OE lines and WT, we monitored the plant shoot elongation rate and biomass under well-watered and drought conditions. The shoot elongation rate was significantly higher in OE lines than in WT under 20% RWC, but not significantly different between OE lines and WT under well-watered conditions (Figure 6D). After long-term drought treatment, consistent with the photosynthetic rate, the biomass accumulation was higher in the two OE lines (by 35.003% and 46.32%, respectively) than in WT under low soil RWC conditions, but not significantly different between the OE lines and WT under well-watered conditions (Figure 6E).

The chlorophyll *a* , chlorophyll *b* , and total chlorophyll contents were not significantly different between OE lines and WT under 70% soil RWC, but were significantly higher in the OE lines than in WT under long-term drought conditions (Figure S3A–C). This result indicated that the OE plants showed a better ability to absorb light energy compared with WT, and thus maintained a higher photosynthetic rate under long-term drought conditions. Compared with WT, the OE lines also showed higher maximal photosystem II (PSII) quantum yield ($F_v/F_m$) after long-term drought treatment (Figure S3D).Therefore, overexpression of*PtrWRKY7* 5 was beneficial for plant growth under long-term drought conditions.

*PtrWRKY75* **promotes expression of SA-responsive genes**

Our results indicated that *PtrWRKY7* 5 promotes SA biosynthesis by binding to the promoter of *PtrPAL1* , subsequently promoting ROS accumulation (Figure 2). Consistent with these results, the expression of *PtrPR1* and *PtrPR5* , two widely studied SA-responsive genes (Blanco *et al.* , 2009), was significantly up-regulated in OE plants under dehydration conditions (Figure 7A, B). The results indicated that *PtrWRKY75*increased SA production by inducing *PAL1* transcription, leading to the expression of SA-responsive genes. Thus, our results show that SA-induced ROS production, which leads to stomatal closure (Khokon*et al.* , 2011; Mori*et al.* , 2001), enhances drought tolerance in *Populus* .

## DISCUSSION

Drought severely affects plant growth and development. The WRKY TF family is involved in a multitude of biotic and abiotic stress responses (Rushton *et al.* , 2010; Parinita *et al.* , 2011; Chen*et al.* , 2012; Jiang *et al.* , 2014). A multiple sequence alignment confirmed that PtrWRKY75 contains the highly conserved WRKYGQK domain at the N-terminus homologous to that of AtWRKY75 (Figure 1). Previous studies have shown that *AtWRKY75* accelerates leaf senescence mediated by SA and ROS accumulation in *Arabidopsis*(Guo *et al.* , 2017). However, little was known about the role of WRKY75 in drought tolerance, especially in poplar.

Earlier studies have reported that low concentrations of ROS can serve as signal molecules to regulate stress responses, for example, stomatal closure and the induction of defense gene expression (Desikan *et al.* , 2001; Rizhsky *et al.* , 2002). We observed that*PtrWRKY75* transcription responded to dehydration stress and exogenous SA treatment (Figure 1E, F). Under exogenous SA treatment, the ROS content increased to a greater degree and more rapidly in the OE lines than in WT (Figure 2A). This result is consistent with previous findings that SA promotes ROS production (Khokon *et al.* , 2011; Mori *et al.* , 2001). Reactive oxygen species are a crucial component in the regulation of stomatal closure (Maija *et al.* , 2016; Song *et al.* , 2014) and responses to adverse environmental conditions, such as drought (Carmody *et al.* , 2016). In our study, the stomatal aperture was significantly smaller in the OE lines than in WT after SA treatment (Figure 2C). Other studies have shown that drought stress leads to an increase in the SA concentration, for example, in *Phillyrea angustifolia*(Munné-Bosch and Peñuelas, 2003) and barley roots (Bandurska and Stroiński, 2005). In accordance with these reports, the SA content increased in both WT and OE plants under dehydration, and to a higher level (approximately double) in OE lines than in WT (Figure 3A). The higher expression level of *PtrPAL1* in transgenic plants (Figure 3C), together with the results of the EMSA experiments (Figure 3D) and promoter transactivation assays (Figure 3E), indicate that WRKY75 promotes the production of SA by binding to the promoter of*PtrPAL1* , thereby inducing its expression. These results show that the higher drought tolerance of the transgenic lines overexpressing*PtrWRKY75* results from SA-induced stomatal closure via ROS production.

Stomata play a vital role in water conservation and gas exchange between leaf tissues and the atmosphere. Some 90% of water loss (transpiration) occurs through stomata, and stomatal closure is the first step in water retention by plants under drought stress (Martin-St Paul *et al.* , 2017). Stomatal conductance and transpiration are positively correlated as they are opposite to drought tolerance (Baloch *et al.* , 2011). Compared with WT, the OE poplar lines showed a lower$G_s$ and transpiration rate under well-watered conditions (Figure 4B, C). However, no significant difference in photosynthesis rate was observed between the OE lines and WT under well-watered conditions (Figure 4A), so that the WUE was higher in OE lines

than in WT (Figure 4D). In reality, the photosynthetic rate is not always associated with $G_s$, as indicated by decreases in Rubisco activity (Von Caemmerer *et al.* , 2004; Xu*et al.* , 2010). In the present study, the OE plants showed a lower water loss rate under drought at normal temperature compared with WT plants (Figure 5F). Improvement in the WUE of plants can enhance drought tolerance without penalizing yield under drought conditions (Karaba *et al.* , 2007). Electrolyte leakage, which is symptomatic of plasma membrane damage (Wang *et al.* , 2011; Shi *et al.* , 2013), was higher in WT than in OE lines under drought stress (Figure 5G), suggesting that WT plants suffered greater membrane damage under drought stress. This further supported the conclusion that overexpression of *PtrWRKY75*in poplar enhances its drought tolerance. We observed that drought stress caused reductions in photosynthetic activity and chlorophyll content in all plants, whereas the photosynthetic rate and chlorophyll*a* content were higher in OE lines than in WT under drought stress (Figure 5B, 6C; Figure S3A–C).

A decrease in $G_s$ prevents excessive water loss under drought stress, thereby reducing the water demand of plants (Martin-St Paul *et al.* , 2017). However, it also decreases photosynthesis and biomass accumulation (Tardieu, 2012). Transgenic poplar plants showed no difference in growth and development from the WT plants under well-watered conditions (Figure 6D). Under drought conditions, the OE lines showed an increased growth rate and greater biomass production (Figure 6D,E), which may be attributed to the improved leaf RWC (Figure 6B). Maximal PSII quantum yield ($F_v/F_m$), which reflects the potential maximum light energy conversion efficiency of plants, declines in plants under drought stress (Ke *et al.* , 2016). The OE lines in this study showed higher PSII efficiency than that of the WT (Figure S3D), indicating that there was less damage to chloroplasts in the OE lines. We conclude that *PtrWRKY75* is a promising gene target for increasing the tolerance of poplar to drought stress.

## METHODS

### Plant materials and stress treatments

*Populus trichocarpa* (clone 'Nisqually-1') was used for isolation of*PtrWRKY75* . Plantlets of *P. trichocarpa* were cultured *in vitro* on solid Lloyd and McCown's Woody Plant Basal Salts (WPM) medium or Murashige and Skoog (MS) medium (Song *et al* ., 2006). After plantlet regeneration, the plantlets were transplanted and grown individually in pots containing a mixture of soil and vermiculite (2:1) at 22 °C under a 16 h/8 h (light/dark) photoperiod (150 μmol $m^{-2}$ $s^{-1}$) and 70% relative humidity.

Salicylic acid (5 mM aqueous solution) was applied as a foliar spray onto the leaves. The treated plants were immediately covered with transparent plastic film (Jiang *et al* ., 2014).

For the dehydration treatment, 4-week-old seedlings were removed from the soil and the roots were exposed to air at 50% relative humidity and 25 °C under dim light for 8 h (Ma *et al* ., 2010).

For each experiment, leaves were collected from the third and fifth internodes at different time points and frozen immediately in liquid nitrogen. We also simultaneously collected the following tissues from 2-month-old *P. trichocarpa* plants: young leaf, mature leaf, senescent leaf, stem, and root. The samples were immediately frozen in liquid nitrogen.

Plantlets of triploid white poplar (*P. tomentosa* 'YiXianCiZhu B385') (Zhu *et al* . 1998) were cultured *in vitro* on solid half-strength (1/2) MS medium as previously described (Wang *et al.* , 2016). Leaves were incubated on substrate (pH = 5.8) containing 0.02 mg/L thidiazuron (TDZ), 0.1 mg/L α-naphthalene acetic acid (NAA), and 0.6% (w/v) agar for shoot induction. Adventitious buds elongated on MS medium containing 0.5 mg/L 6-benzylaminopurine (6-BA) and 0.6% (w/v) agar. The regenerated shoots were individually separated from the callus and transferred to rooting medium [1/2 MS medium supplemented with 0.05 mg/L NAA and 0.6% (w/v) agar] (Li *et al.* , 2012). The 4-week-old seedlings were acclimatized in pots and then transferred to a greenhouse at 22 °C under a 16 h/8 h (light/dark) photoperiod and 40%–45% relative humidity.

### cDNA cloning of *PtrWRKY75* from *Populus trichocarpa* and quantitative real-time PCR analysis

Total RNA was extracted from tissue samples using the RN38 EASYspin Plus Plant RNA Kit (Aidlab

Biotech, Beijing, China). A total of 2 μg RNA was used for first-strand reverse transcription using M-MLV Reverse Transcriptase and an oligo (dT) primer (Promega, Madison, WI, USA) following the manufacturer's instructions. The resultant cDNA was used for PCR amplification with gene-specific primers.

Quantitative real-time PCR was performed using the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The reaction mixture for the qRT-PCR analysis comprised 1 μl (˜100 ng) template (the cDNA template diluted ˜10-fold with nuclease-free water), 1 μl (10 μM) forward primer, 1 μl (10 μM) reverse primer, 7 μl RNase-free $ddH_2O$, 12.5 μl SuperReal PreMix Plus, and 2.5 μl ROX Reference Dye in a total volume of 25 μl. Each experiment was based on three biological replicates of each sample and four technical replicates of each biological replicate. The $2^{-[?][?]C\ T}$ method (Schmittgen and Livak, 2008) was used to calculate the relative expression level of *PtrWRKY75* , with *PtrUBQ* employed as the internal control. All primers used are listed in Table S1.

**Phylogenetic tree reconstruction and domain analysis of PtrWRKY75**

The amino acid sequences of *P. trichocarpa* and *Arabidopsis WRKY* genes were obtained from the Plant-TFDB (http://planttfdb.cbi.pku.edu.cn/index.php) and TAIR 9.0 (http://www.Arabidopsis.org/index.jsp) databases. The sequences of*WRKY* genes from other species were used as queries for BLAST searches against the *P. trichocarpa* genome database in the catalogue of WRKY proteins in NCBI. The sequence accession numbers are shown in Table S2-S3. A phylogenetic tree for *WRKY* genes was constructed using the neighbor-joining method with MEGA5. DNAMAN 5 (Lynnon Biosoft Inc., San Ramon, CA, USA) was used to analyze the deduced amino acid sequences of PtrWRKY75 and other WRKY family members in *Arabidopsis* .

**Plasmid construction and transformation**

The*PtrWRKY75*cDNA was cloned into the pSUPER1300(+) vector (containing the enhanced green fluorescent protein (eGFP) gene) under the control of the Super Promoter (Zhao *et al* ., 2011). The hygromycin phosphotransferase of pSUPER1300(+) was used to determine the validity of the positive selection system in the transformation of poplar. The construct was inserted by heat shock into *Agrobacterium tumefaciens* strain GV3101 and then transformed into wild-type triploid white poplar using the leaf disc method (Li et al., 2012; Wang *et al.* , 2016). The leaves of triploid white poplar, which were cut into discs, were pre-cultured on MS medium supplemented with 0.02 mg/L TDZ, 0.1 mg/L NAA, and 0.6% (w/v) agar for 3 days, dipped in the diluted *Agrobacterium* culture for about 15 min and cultured on pre-cultivation medium for an additional 3 days in the dark. Then, the leaf discs were transferred to MS medium (pH 5.8) supplemented with 0.02 mg/L TDZ, 0.1 mg/L NAA, 200 mg/L Timentin, 3 g/L hygromycin phosphotransferase, and 0.6% (w/v) agar for shoot induction and selection. Next, the leaf discs were transferred onto MS medium supplemented with 0.5 mg/L 6-BA, 200 mg/L Timentin, 3 mg/L hygromycin phosphotransferase, and 0.6% (w/v) agar for elongation and selection of adventitious buds. The regenerated shoots were individually separated from the callus and inserted into a selective rooting medium [1/2 MS medium supplemented with 0.05 mg/L NAA, 300 mg/L carbenicillin, 1 g/L hygromycin phosphotransferase, and 0.6% (w/v) agar]. The rooted plantlets were acclimatized in pots and then transferred to a greenhouse at 22 °C under a 16 h/8 h (light/dark) photoperiod and 40%–45% relative humidity.

**Subcellular localization assay**

For the subcellular localization assay, Super:PtrWRKY75-eGFP and Super:eGFP fusion proteins, which were transiently transfected into tobacco leaves as previously described (Cui *et al.* , 2007), were observed under a confocal laser scanning microscope (DMI6000 CS; Leica, Wetzlar, Germany).

**Molecular verification**

The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from non-transgenic plants (WT) and transgenic OE lines (Arseneau *et al.* , 2017). We confirmed the transformation lines by PCR using the combination of a forward primer for *PtrWRKY75* and a reverse primer for eGFP

(Table S1). Quantitative real-time PCR was used to determine the expression levels of *PtrWRKY75* in the transgenic lines and WT using the afore-mentioned procedure. The primers used were identical to those used for the qRT-PCR assay listed in Table S1.

**Short-term and long-term drought experiments**

Thirty WT and 60 *oxPtrWRKY75* (30 *oxPtrWRKY75-8* and 30*oxPtrWRKY75-10* ) two-month-old plants were used as experimental materials. For the short-term drought experiment, plants in pots (150 cm width and 135 cm height) were subjected to drought by withholding watering for 7 days. During this period, net $CO_2$ assimilation, $G_s$, and transpiration were measured daily. For the long-term drought experiment, plants were grown in pots (150 cm width and 135 cm height) with 70% soil RWC (no stress) or 20% soil RWC (severe water stress), which was achieved by withholding water for 35 days (Wang *et al.* , 2016). The soil RWC was measured daily. The height of each plant was measured every 10 days. After 35 days, the photosynthesis rate, leaf RWC, chlorophyll content, and plant aboveground biomass were measured.

**Physiological analysis**

Net $CO_2$ assimilation ($A$) ,$G_s$, and transpiration ($E$) and VPD were measured in the sixth to ninth leaves of WT and *oxPtrWRKY75*plants using an infrared gas analysis system (LI-COR 6400, Lincoln, NE, USA) as previously described (Wang *et al.* , 2016). Eighteen plants were analyzed (six per line). The LI-COR 6400 infrared gas analysis system was used to measure light and $CO_2$ curves in fully expanded leaves of plants grown in a greenhouse for 2 months under normal conditions. Light curves were measured at photosynthetically active radiation intensities of 1800, 1500, 1200, 1000, 800, 600, 400, 200, 150, 100, 80, 50, 20, and 0 μmol/$m^2$/s with 450 μmol/mol external $CO_2$.

Chlorophylls were extracted from detached leaves of WT and*oxPtrWRKY75* plants with 80% acetone. A UV/visible spectrophotometer (YHB-061; GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used to measure absorbance at 663 and 645 nm, and then chlorophyll contents were calculated as described elsewhere (Lichtenthaler, 1987; Wang*et al.* , 2016).

Chlorophyll fluorescence parameters were measured using a Dual-PAM-100 measuring system (Walz Heinz GmbH, Effeltrich, Germany) after 15 min of dark adaptation for each plant.

To measure biomass, the aboveground parts of plants were collected, killed by heating at 105 °C for 15 min, and then dried at 65 °C to constant weight and weighed.

**Detection of ROS**

Histochemical staining was used to detect ROS in leaf tissue as described previously (Fryer *et al.* , 2002). The leaves of 2-month-old WT and OE lines were sprayed with SA. At 0, 0.5, 1, or 3 h after spraying with SA, the leaves were stained with DAB solution (1 mg/ml DAB-HCl, pH 3.8; Sigma-Aldrich) in the dark for 8 h at 37 °C. The stained leaves were immersed in 70% alcohol to remove chlorophyll and then observed and photographed.

**Stomatal aperture treatment**

Leaves were detached from 2-month-old WT and *oxPtrWRKY75* plants, perforated at the same position on both sides of the main vein with a perforator, and immersed under light in stomata-opening solution containing 0.01 M KCl, 0.5 M $CaCl_2$, and 0.1 M MES-KOH for 2 h. The leaves were soaked in aqueous SA solution (0.5 mM) for 0, 1, or 2 h, as described by Wang (2016). The fixed samples were immediately frozen in liquid nitrogen and stored in a -80 degC Ultra-low Freeze Dryer (Biosafer-18A, Jiangsu, China (Mainland)) and fully dried for 24 h using a vacuum freeze dryer. The stomata were examined under a scanning electron microscope (Hitachi S-3400N, Tokyo, Japan).

**Analysis of SA content**

Two-month-old WT and OE lines were used as experimental materials. The leaves of plants grown under well-watered conditions and dehydration for 4 h were collected and immediately frozen in liquid nitrogen.

The leaves were ground into powder in liquid nitrogen, and 50 mg powder was used for quantitative free-SA measurements by high-performance liquid chromatography–tandem mass spectrometry (Pan *et al.* , 2010).

**Electrophoretic mobility shift assay**

The *PtrWRKY75* cDNA was cloned into the PET28a vector, then the construct was inserted by heat shock into *Escherichia coli* strain BL21. Production of the His-PtrWRKY75 fusion protein was induced by 0.2 Mm isopropyl β-D-1-thiogalactopyranoside.

Oligonucleotide probes (*PAL1* W-box: TTGACC) were synthesized by Sangon (Beijing, China) and labeled with biotin using an EMSA Probe Biotin Labeling Kit (GS008, Beyotime Biotechnology, Jiangsu, China). For the mutated probe, the single mutation site was located in the core sequence of the W-box (changed from TGAC to TAAC). The EMSA was performed using a Chemiluminescent EMSA kit (GS009, Beyotime Biotechnology, Beijing, China) in accordance with the manufacturer's instructions. Briefly, biotin-labeled probes were incubated with the fusion proteins in binding buffer for 30 min at room temperature (20–25 °C). The reaction products were transferred to 4% polyacrylamide gel, then electrophoresed in 0.5× Tris-borate-EDTA (TBE) buffer for 60 min. All oligonucleotide sequences are listed in Table S2.

**Transient expression assays**

For *ProPAL1:GUS* vector construction, an approximately 2000-bp promoter fragment of *PAL1* was amplified by gene-specific primers (Table S1) and cloned into the pCAMBIA-1301 vector. To generate*Pro35S:WRKY75* , the *WRKY75* cDNA fragment was amplified and inserted into the pCAMBIA-1301 vector. The *35S:PtrWRKY75*construct was used as an effector. Leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* cells containing the effector and reporter using the agroinfiltration method (Yang *et al.* , 2000). Activity of GUS was measured using a previously described method (Jefferson et al., 1987), which monitored the cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide by hydrolysis to produce the fluorescent 4-methylumbelliferone. Protein concentrations were measured as described by Bradford (1976).

**Relative water content and relative electrical conductance assay**

For leaf RWC measurement, the leaves were quickly removed from the plant and weighed to record leaf fresh weight (FW). The leaves were then incubated in distilled water for 24 h and weighed to obtain the leaf turgid weight (TW). The leaves were dried to constant weight in a 65 °C oven and the dry weight (DW) was recorded. The leaf RWC was calculated as (FW - DW)/ (TW - DW) x100 (Wang *et al.* , 2016).

For determination of leaf REC, the leaves were detached at 0 and 7 days under drought stress and REC was measured as previously described with a DDS-307 Conductivity Meter (Leici, Shanghai, China) (Shi *et al.* , 2013).

**Statistical analysis**

All data were subjected to analysis of variance using SPSS software (IBM Corporation, Armonk, NY, USA). Student's $t$ -tests were used to determine the significance of differences between means. Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test to identify statistically significant means ($^*P < 0.05$; $^{**}P < 0.01$).

## LEGENDS TO FIGURE

**Figure 1 *PtrWRKY75* of *Populus trichocarpa.***

(A) Phylogenetic relationships among PtrWRKY75 and WRKY proteins from other plant species.

(B) Multiple alignment of amino acid sequences of PtrWRKY75 and other plant WRKY proteins.

(C) Subcellular localization of PtrWRKY75 protein in tobacco leaf cells.

(D) Transcript levels of *PtrWRKY75* in various organs. Total RNA was isolated from the root, stem, young leaf, mature leaf, and senescent leaf of poplar plants(normalized against internal control *UBQ* ). Data are means +- SE ($n = 6$).

(E) Quantitative real-time PCR (qRT-PCR) analysis of *PtrWRKY75*transcript levels under dehydration stress (normalized against internal control *UBQ* ). Data are means +- SE ($n = 6$).

(F) qRT–PCR analysis of *PtrWRKY75* transcripts levels in response to exogenous salicylic acid treatment (normalized against internal control *UBQ* ). Data are means +- SE ($n = 6$).

**Figure 2 *PtrWRKY75* promotes salicylic acid (SA)-induced stomatal closure via reactive oxygen species (ROS) accumulation.**

(A) DAB staining to visualize ROS content in leaves of wild-type (WT) and *PtrWRKY75* -overexpressing (OE) lines in response to 0.5 mM SA treatment for 0 h, 0.5 h, 1 h, 2 h, and 3 h. Scale bars = 2 cm. Data are means +- SE ($n = 6$).

(B) SA-induced stomatal closure in leaves of WT and OE lines. Leaves were detached and immersed in stomata-opening solution for 2 h under light, then treated with 0.5 mM SA for 1 or 2 h. Scale bars = 10 μm. Data are means ± SE ($n = 60$).

(C) Stomatal aperture observed at 0, 1, and 2 h of SA treatment by scanning electron microscopy. Data are means ± SE ($n = 60$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{**}P < 0.01$.

**Figure 3 *PtrWRKY75* promotes salicylic acid (SA) biosynthesis by activating *PtrPAL1* transcription.**

(A) Free SA levels in mature leaves from 35-day-old plants under non-stress and dehydration conditions. Data are means ± SE ($n = 3$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{*}P$ [?] 0.05;$^{**}P < 0.01$.

(B) Quantitative real-time PCR (qRT-PCR) analysis of transcript levels of *PtrSID2* under non-stress and dehydration conditions. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test.

(C) qRT-PCR analysis of *PtrPAL1* transcript levels under non-stress and dehydration conditions. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{*}P$ [?] 0.05; $^{**}P < 0.01$.

(D) Locations of putative WRKY75 binding sites in *PAL1* promoter.

(E) EMSA analysis of binding of recombinant WRKY75 protein to*PAL1* promoter. Hot probe is biotin-labeled, and hot mProbe contains a single nucleic acid mutation from TGAC to TAAC.

(F) Promoter activity analysis of SA biosynthetic gene *PtrPAL1*using *PtrWRKY75* as effector under non-stress and dehydration conditions. Promoter of *PAL1* was isolated from *P. trichocarpa* genome. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{**}P < 0.01$.

**Figure 4 Gas exchange analysis of *OxPtrWRKY75* plants showing higher instantaneous water-use efficiency (WUE) as a result of reduced transpiration and stomatal conductance.**

(A) Net photosynthesis rate–light curve. Data are means +- SE ($n$= 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test.

(B) Stomatal conductance–light curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{*}P$ [?] 0.05; $^{**}P < 0.01$.

(C) Transpiration–light curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $*P$[?] 0.05; $**P < 0.01$.

(D) Instantaneous WUE–intercellular $CO_2$ concentration curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $*P$ [?] 0.05; $**P< 0.01$.

(E) Vapor pressure deficit–light curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test.

**Figure 5 Overexpression of *PtrWRKY75* enhanced drought tolerance under short-term drought conditions.**

(A) Morphological differences between wild-type (WT) and*PtrWRKY75* -overexpressing (OE) lines in short-term drought experiments. Scale bars =10 cm. Data are means +- SE ($n = 6$).

(B) Net photosynthesis rate–drought duration curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(C) Stomatal conductance–drought duration curve. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(D) Transpiration–drought duration curve. Data are means +- SE ($n= 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $*P$ [?] 0.05; $**P < 0.01$.

(E) Leaf relative water content under non-stress and drought conditions. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(F) Water loss from detached leaves. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $*P$ [?] 0.05; $**P < 0.01$.

(G) Leaf relative electrical conductance under non-stress and drought conditions. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

**Figure 6 Overexpression of *PtrWRKY75* enhanced drought tolerance under long-term drought conditions.**

(A) Morphological differences between wild-type (WT) and*PtrWRKY75* -overexpressing (OE) lines in long-term drought experiments. Scale bars = 10 cm. Data are means +- SE ($n = 6$).

(B) Leaf relative water content under different water conditions. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(C) Photosynthetic rate. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(D) Whole plant biomass. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $**P < 0.01$.

(E) Stem elongation rate of WT and OE lines under 70% soil RWC and 20% soil RWC. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range

test. Asterisks denote significant differences: $^{**}P < 0.01$.

**Figure 7 Relative gene expression in response to salicylic acid and reactive oxygen species under non-stress and dehydration conditions.**

Quantitative real-time PCR (qRT-PCR) analysis of transcript levels of*PtrPR1* (A) and *PtrPR5* (B) in WT and*PtrWRKY75* -overexpressing (OE) lines. Data are means +- SE ($n = 6$). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. Asterisks denote significant differences: $^{**}P < 0.01$.

**Supplementary information**

**Figure S1.** Phylogenetic tree of WRKY transcription factor family in poplar.

**Figure S2.** Analysis of *PtrWRKY75* overexpression in transgenic poplar plants.

**Figure S3.** Physiological and photosynthesis parameters of wild-type and transgenic plants under long-term drought conditions.

**Table S1.** Primer and oligonucleotide sequences used in this study.

**Table S2.** Accession numbers for WRKY proteins in various plant species.

**Table S3.** WRKY gene family in *Populus.*

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**Competing financial interests**

Authors declare no competing financial interests.

**Author contributions**

YZ, WLY, and XLX conceived and designed the research. YZ performed the research. YYZ, DZ, XLT, XH, WHD and ZL participated in the experiments. CS contributed analytic tools. YZ, YYZ, DZ, XLT, and ZL analyzed the experimental data. YZ wrote the manuscript. YYZ, DZ, XLT, and ZL contributed to writing the manuscript. All authors discussed the results and approved the final manuscript.

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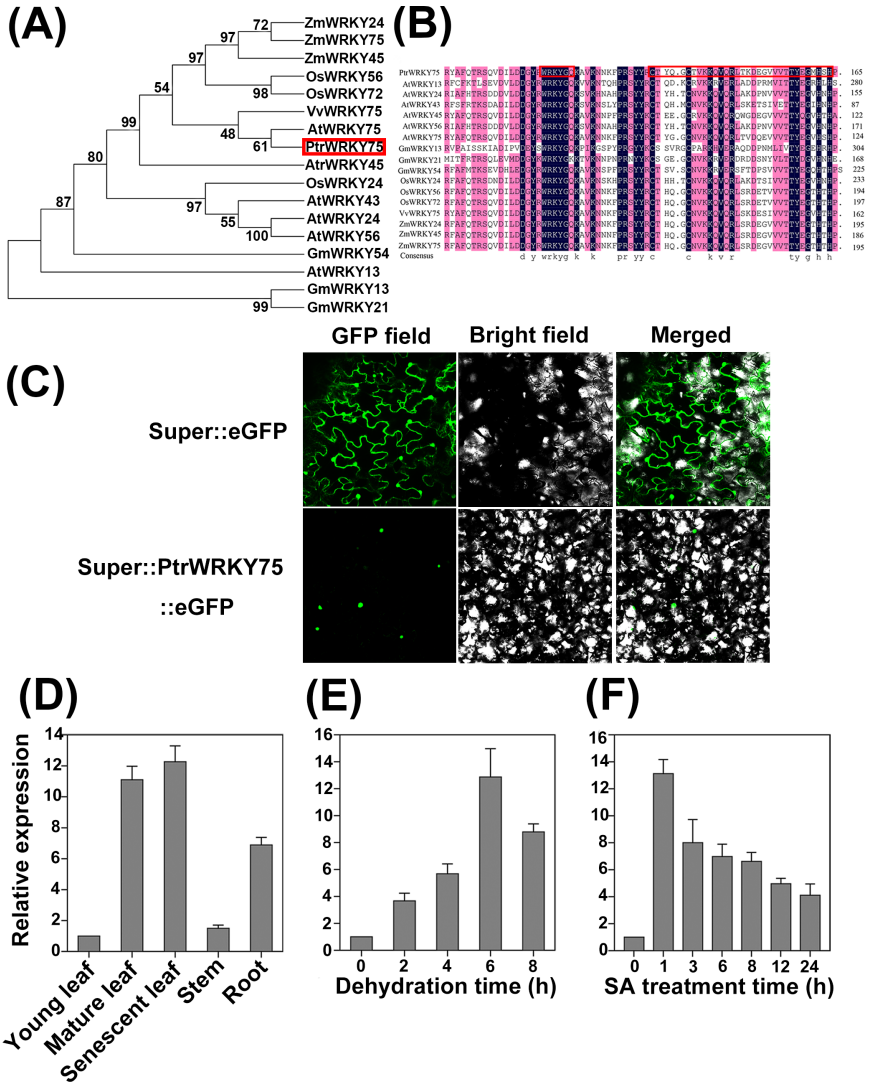


**Figure 1** ***PtrWRKY75*** **of Populus trichocarpa.**

**(A) Phylogenetic relationships among PtrWRKY75 and WRKY proteins from other plant species.**

**(B) Multiple alignment of amino acid sequences of PtrWRKY75 and other plant WRKY proteins.**

**(C) Subcellular localization of the PtrWRKY75 protein in tobacco leaf cells.**

**(D) Total RNA was isolated from the root, stem, young leaf, mature leaf, and senescent leaf of poplar plants. The expression levels were normalized using the internal control UBQ. Data are means± SE (*n* = 6).**

**(E) Quantitative real-time PCR (qRT-PCR) analysis of *PtrWRKY75* transcripts in response to dehydration. The expression levels were normalized using the internal control UBQ. Data are means ± SE (*n* = 6).**

**(F) qRT–PCR analysis of *PtrWRKY75* transcripts in response to exogenous salicylic acid treatment. The expression levels were normalized using the internal control UBQ. Data are means ± SE (*n* = 6).**

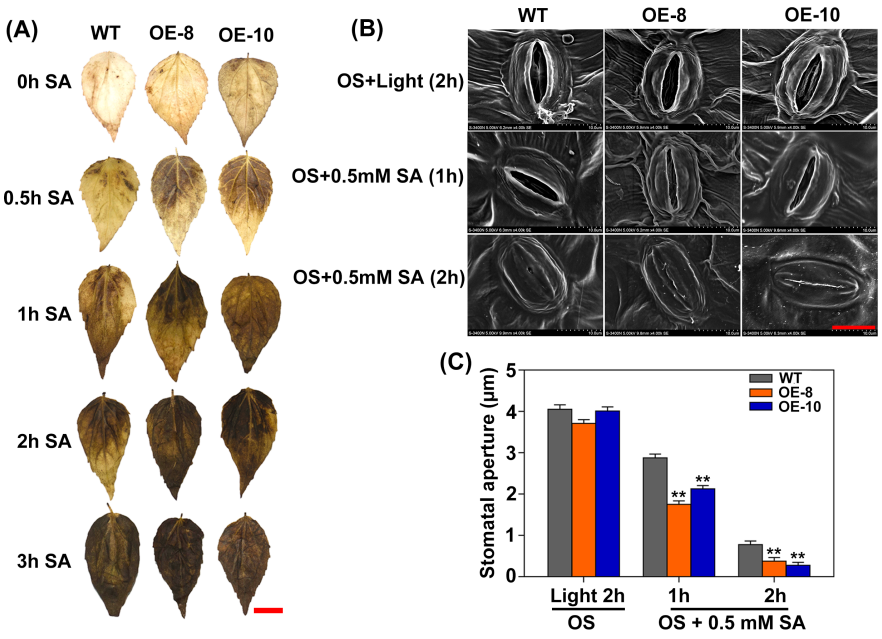


Figure 2 *PtrWRKY75* promotes salicylic acid (SA)-induced stomatal closure via reactive oxygen species (ROS) accumulation.
(A) DAB staining to visualize ROS content in wild-type (WT) and transgenic poplar leaves in response to 0.5 mM SA treatment for 0h, 0.5h, 1h, 2h and 3h.Scale bars = 2 cm. Data are means ± SE (*n* = 6).
(B) SA-induced stomatal closure in leaves of the WT and transgenic lines. Leaves were detached and immersedunder light in stomata-opening solution for 2 h, then treated with 0.5 mM SA for 1 or 2 h. Scale bars = 10 μm. Data are means ± SE (*n* = 60).
(C) Stomatal aperture observed at 0, 1, and 2 h of SA treatment by scanning electron microscopy. Data are means ± SE (*n* = 60). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

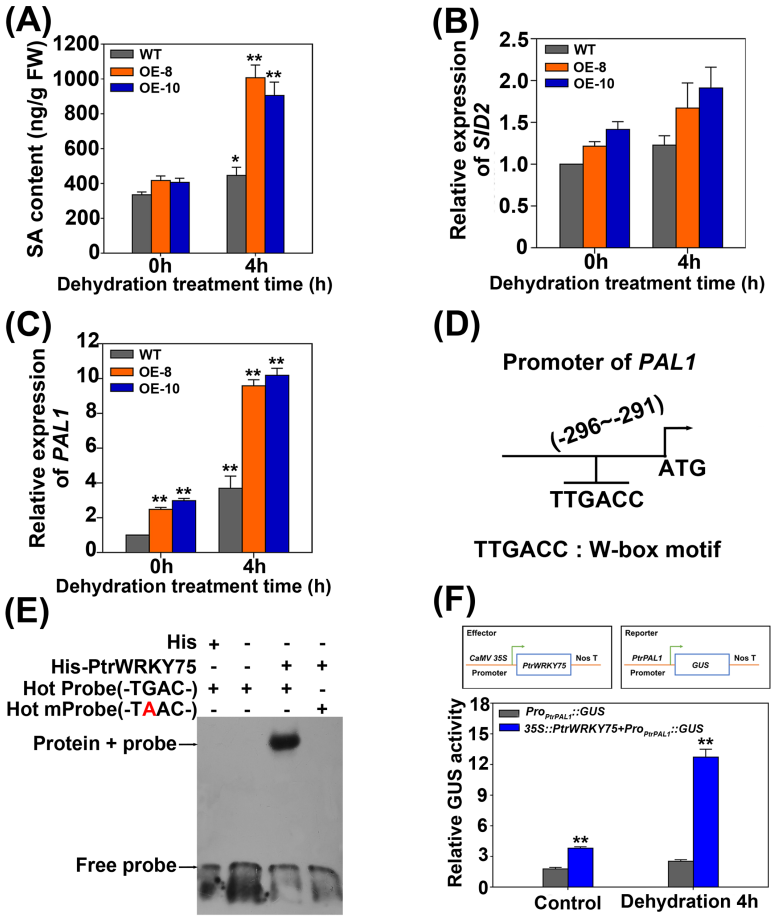


Figure 3 *PtrWRKY75* promotes salicylic acid (SA) biosynthesis by activating *PtrPAL1* transcription.

(A) Measurement of free SA levels in mature leaves from 35-day-old plants under non-stress and dehydration conditions. Data are means ± SE ($n$ = 3). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(B) Quantitative real-time PCR (qRT-PCR) analysis of transcript levels of *PtrSID2* under non-stress and dehydration conditions. The expression levels were normalized using the internal control UBQ.Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test.

(C) qRT-PCR analysis of transcript levels of *PtrPAL1* under non-stress and dehydration conditions. The expression levels were normalized using the internal control UBQ.Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(D) Schematic diagram indicating the locations of putative WRKY75 binding sites in the promoter of *PAL1*.

(E) EMSA analysis of binding of the recombinant WRKY75 protein to the promoter of *PAL1*. The hot Probe is biotin-labeled, and the hot mProbe contains a single nucleic acid mutation from TGAC to TAAC.

(F) Promoter activity analysis of the SA biosynthetic gene *PtrPAL1* using *PtrWRKY75* as the effector under non-stress and dehydration conditions. The promoter of *PAL1* was isolated from the P. trichocarpa genome. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

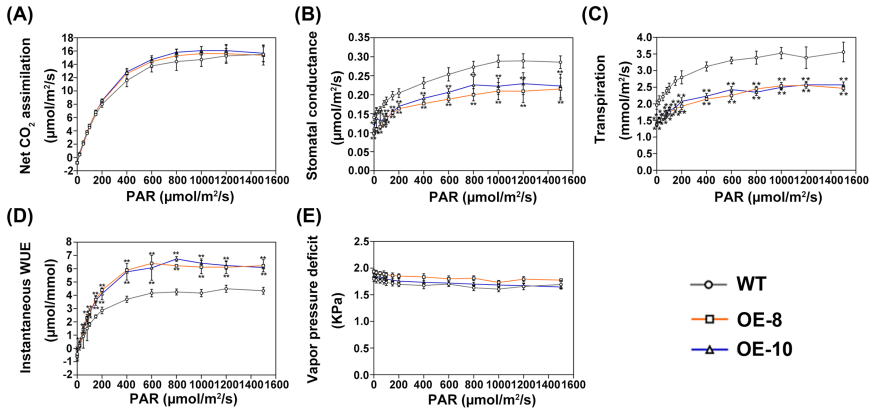


Figure 4 Gas exchange analysis of *OxPtrWRKY75* plants showed higher instantaneous water-use efficiency (WUE) by reducing transpiration and stomatal conductance.

(A) Net photosynthesis rate–light curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysisof variancefollowed by Duncan's multiple range test.

(B) Stomatal conductance–light curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(C) Transpiration–light curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(D) Instantaneous WUE–intercellular CO2 concentration curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(E) Vapor pressure deficit–light curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test.

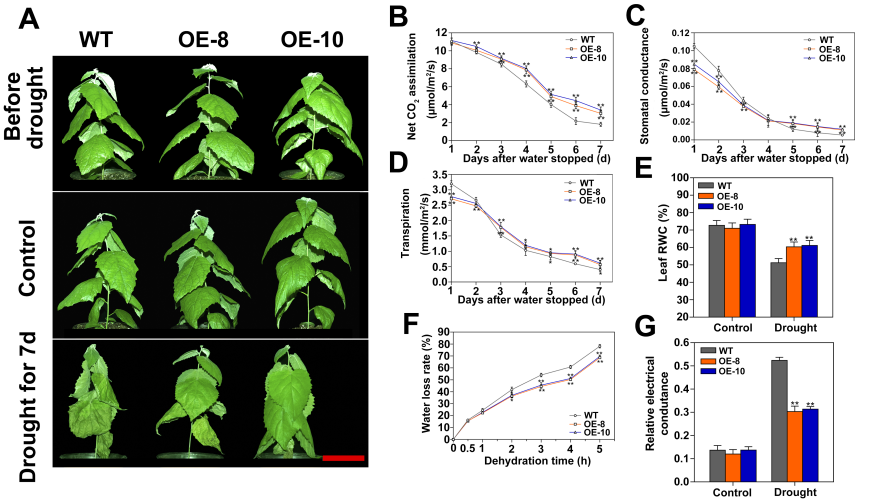


Figure 5 Overexpression of *PtrWRKY75* enhanced drought tolerance under short-term drought conditions.

(A) Morphological differences of plants in short-term drought experiments. Scale bars =10 cm. Data are means ± SE ($n$ = 6).

(B) Net photosynthesis rate–drought duration curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

(C) Stomatal conductance–drought duration curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

(D) Transpiration–drought duration curve. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(E) Leaf relative water content under non-stress and drought conditions. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

(F) Water loss from detached leaves. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{*}P \leq 0.05$; $^{**}P < 0.01$.

(G) Leaf relative electrical conductance under non-stress and drought conditions. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

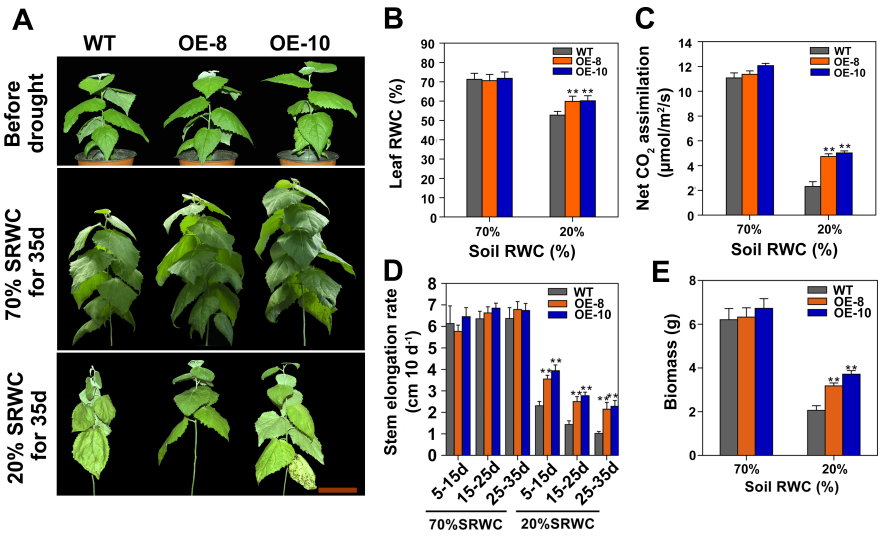


Figure 6 Overexpression of *PtrWRKY75* enhanced drought tolerance under long-term drought conditions.
(A) Morphological differences of plants in long-term drought experiments. Scale bars = 10 cm. Data are means ± SE ($n$ = 6).
(B) Leaf relative water content under different water conditions. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.
(C) Measurement of photosynthetic rate. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.
(D) Whole plant biomass. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.
(E) Stem elongation rate of wild-type and transgenic plants under 70% soil RWC and 20% soil RWC. Data are means ± SE ($n$ = 6). Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.

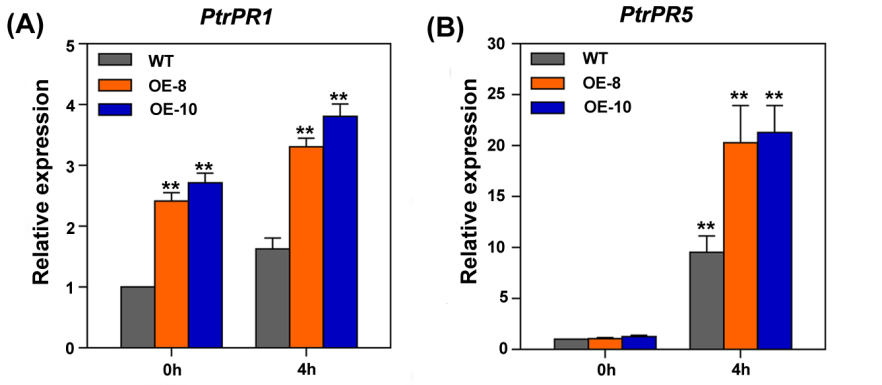


Figure 7 Relative gene expression in response to salicylic acid and reactive oxygen species under non-stress and dehydration conditions. Quantitative real-time PCR (qRT-PCR) analysis of transcript levels of *PtrPR1* (A) and *PtrPR5* (B) in WT and *OxPtrWRKY75* plants. Data are means ± SE ($n$ = 6). The expression levels were normalized using the internal control UBQData were analyzed using one-way analysis of variance followed by Duncan's multiple range test, asterisks denote significant differences: $^{**}P < 0.01$.