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RESEARCH PAPER

WUSCHEL-related homeobox gene *PagWOX11/12a* responds to drought stress by enhancing root elongation and biomass growth in poplar

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Abstract

In plants, a large root system improves the uptake of water and nutrients, and is important for responding to drought stress. The poplar WUSCHEL-related homeobox (WOX) transcription factor promotes adventitious rooting, but its regulation of root growth in response to drought stress remains elusive. In this study, we found that *PagWOX11/12a* from hybrid poplar 84K (*Populus alba*×*Populus glandulosa*) is expressed predominantly in the roots and is strongly induced by drought stress. Compared with non-transgenic 84K plants, transgenic poplar plants overexpressing *PagWOX11/12a* displayed increased root biomass and enhanced drought tolerance, while opposite phenotypes were observed for PagWOX11/12a dominant repression plants. PagWOX11/12a functions as a nuclear transcriptional activator with a transactivation domain at the C-terminus. In addition, PagERF35 was found to specifically bind to a dehydration-responsive element (DRE) within the *PagWOX11/12a* promoter and activate *PagWOX11/12a* gene expression. These results indicate that PagERF35 may activate *PagWOX11/12a* expression in response to drought stress by promoting root elongation and biomass, thereby increasing drought tolerance of poplar.

Keywords: Drought resistance, *PagERF35*, *PagWOX11/12a*, *Populus alba*×*P. glandulosa*, root elongation, root biomass

Introduction

Plants are constantly subjected to various abiotic stresses throughout their life cycles. To maintain adequate growth under multifarious environmental conditions, plants undergo various cellular, molecular, physiological, and biochemical changes by altering the expression of stress-related genes (Yin *et al.*, 2005; Chaves *et al.*, 2009). Among these abiotic stresses, drought stress has been highlighted as the most severe abiotic stressor that affects plant growth and development (Josine *et al.*, 2011). As such, plants have evolved a variety of strategies to coordinate their growth and drought resistance.

Plant roots provide a critical link in the soil–plant–air continuum to fine-tune the water and nutrient permeability and play a prime role in perceiving dehydration stress signals and transferring them to the shoots (Li *et al.*, 2009; Hamanishi and Campbell, 2011). Therefore, it is generally believed that root system architecture is a pivotal determinant of water use efficiency under different drought conditions (Coudert *et al.*,

2010). Studies have shown that a number of key root morphological traits, including root length, root density, and root diameter, are positively associated with drought resistance in several plant species (Hund *et al.*, 2009; Henry *et al.*, 2011; Comas *et al.*, 2013), and the related genes have been shown to activate downstream drought-responsive genes in the regulation of root architecture (Miyazawa *et al.*, 2012; Iwata *et al.*, 2013; Dash *et al.*, 2017). For instance, overexpression of the Arabidopsis *AVP1* or *HARDY* gene led to increased root biomass and better performance of transgenic plants under drought conditions (Li *et al.*, 2005; Karaba *et al.*, 2007). In rice, *Deeper rooting 1* (*Dro1*), a quantitative trait locus (QTL) for deep rooting, plays an important role in deep rooting ability and was shown to increase drought tolerance by causing plants to absorb water from relatively deep soil layers under upland field conditions (Uga *et al.*, 2011, 2013). In poplar, *PtabZIP1L*, a homolog of Arabidopsis *bZIP1*, is a positive modulator of lateral root (LR) development and enhances drought resistance (Dash *et al.*, 2017). In addition, overexpression of the *AP2/ERF* family members *OsERF137* (Ambavaram *et al.*, 2014), *OsERF71* (Lee *et al.*, 2016), or *OsERF48* (Jung *et al.*, 2017) in rice results in vigorous root growth, improving grain yields under drought conditions.

The WUSCHEL-related homeobox (WOX) family, a novel plant-specific transcription factor family, plays crucial roles in key developmental processes, including stem cell maintenance, embryonic patterning, and organ formation (Van der Graaff *et al.*, 2009). Interestingly, some of these WOXs are involved in regulating cell division and differentiation during root formation and growth in several plant species (Zhao *et al.*, 2009; Cho *et al.*, 2013; Alvarez *et al.*, 2015; Zhou *et al.*, 2017). In Arabidopsis, AtWOX11 and AtWOX12 are involved in the first step of cell fate transition from leaf procambia or nearby parenchyma cells to root founder cells during *de novo* root organogenesis (J. Liu *et al.*, 2014). In rice, *OsWOX11* is an auxin- and cytokinin-responsive gene, and transgenic plants overexpressing *OsWOX11* displayed increased root biomass by activating crown root emergence and growth (Zhao *et al.*, 2009). *OsWOX3A* is involved in the development of both LRs and root hairs by regulating the expression of auxin transport genes (Yoo *et al.*, 2013). Similarly, poplar *WOX11* was found to be expressed during adventitious rooting, and transgenic poplar plants overexpressing *WOX11* exhibited an increase in the number of adventitious roots on the cuttings (B. Liu *et al.*, 2014; Xu *et al.*, 2015). Although it has been reported that several rice *WOX* genes (*OsWOX5*, *OsWOX9B*, *OsWOX11*, *OsWOX12A*, and *OsWOX12B*) and cotton *WOX* genes (*GhWOX10*, *GhWOX12*, *GhWOX13a*, and *GhWOX13b*) are responsive to drought and/or salt stress (Cheng *et al.*, 2014; Yang *et al.*, 2017), how these *WOX* genes are involved in the alteration of the root system in response to abiotic stress remains unclear.

In this work, a member of the *WOX* gene family, *PagWOX11/12a*, was cloned from hybrid poplar 84K, and its overexpression led to increased root biomass and enhanced tolerance to drought stress in transgenic poplar plants. In addition, *PagWOX11/12a* expression was activated by PagERF35, a member of the ERF transcription factor family. These results suggest that *PagWOX11/12a* can modulate the response of the poplar root system to drought stress via the stress-responsive protein PagERF35.

Materials and methods

Plant materials and growth conditions

Hybrid poplar (*Populus alba*×*P. glandulosa* cv. '84K') was used for gene cloning, expression analysis, and genetic transformation. All poplar plants were grown on half-strength Murashige and Skoog (1/2 MS) medium (pH 5.8–6.0) that consisted of 30 g l^{-1} sucrose, 5 g l^{-1} agar, 0.05 mg l^{-1} indolebutyric acid (IBA), and 0.02 mg l^{-1} naphthylacetic acid (NAA) in a phytotron (25 °C temperature, 16/8 h light/dark photoperiod, and 50–60% relative humidity).

Arabidopsis thaliana [ecotype Columbia (Col-0)] seeds were surface sterilized and then sown onto 1/2 MS solid medium that consisted of 0.8% agar. One-week-old seedlings were transplanted into pots filled with soil and perlite in a growth chamber (22 °C temperature, 16/8 h light/dark photoperiod, and 70–75% relative humidity), grown for 20 d, and then used for transcriptional activation analysis.

Gene cloning, vector construction, and plant transformation

The 1.5 kb promoter fragment and *PagWOX11/12a* coding region were amplified from the genomic DNA and cDNA of 84K poplar plants, respectively, via specific primers (see Supplementary Table S1 at *JXB* online). The promoter fragment was first cloned into the pDONR207 vector and then into pMDC164 to drive the expression of β-glucuronidase (*GUS*). The full coding sequence (CDS) of *PagWOX11/12a* or the CDS with the SRDX repression sequence was first cloned into the pDONR207 vector and then into the pMDC32 binary vector under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter to generate overexpression and suppression constructs. All binary constructs were generated via BP and LR Gateway cloning (Invitrogen, Thermo Fisher Scientific), verified by sequencing, and transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium*-mediated transformation of leaf discs was performed as described previously (B. Liu *et al.*, 2014). The positively transformed plants were selected on 1/2 MS medium containing 3 mg l^{-1} hygromycin and verified by PCR-based amplification of genomic DNA and quantitative reverse transcription–PCR (qRT–PCR) of the transgene.

Drought stress assay

To evaluate the growth status under drought stress conditions, shoot segments ~3 cm in length with 2–3 young leaves were cut from sterilized plants and cultivated on 1/2 MS solid medium for 4 weeks. The plants were transplanted to plastic pots with a mixture of soil and sand (3:1), grown in a greenhouse for 20 d, and subsequently subjected to drought stress as described by Dash *et al.* (2017), with minor modifications. The plants were watered 2–3 times per week, with fertilizer added on the last watering (10 d before the start of drought treatment), after which the soil water content was maintained at 25–35% of field capacity (FC) for 20 d for drought treatment; FC was maintained at ≥70% of the amount of the control. To maintain the soil water content at the corresponding FC, the pots were weighed daily and watered as needed. Moreover, 4-week-old plants were hydroponically cultivated in Hoagland's nutrient solution for 10 d at 23–25 °C with a 16/8 h light/dark photoperiod and then transferred into Hoagland's nutrient solution containing 10% (w/v) polyethylene glycol (PEG) 6000 (PEG-simulated drought stress) for 20 d. The solution was changed every 3 d. The plant heights were measured periodically after the beginning of the treatment, and biomass measurements were performed after 20 d of treatment. The dry weights of the roots and shoots were measured after incubation in an oven at 80 °C for 72 h. The experiments involving soil cultivation or hydroponics were performed three times, and 10 plants were used for each genotype per experiment.

RNA isolation and qRT–PCR analysis

For tissue-specific gene expression analysis, root, stem, and leaf tissues of 4-week-old 84K plants grown in 1/2 MS solid medium were collected and frozen immediately in liquid nitrogen. The plants were subjected to a solution of 10% PEG6000 for 0, 3, 6, 9, 12, and 24 h for drought treatment or to fresh water as a control. Root tissues were harvested at each time point and immediately frozen in liquid nitrogen. The treatment experiments were repeated three times, and 10 plants were used each time.

Total RNA was extracted from the tissues, frozen in liquid nitrogen using the RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany), and digested with RNase-free DNase I (Sigma, Santa Clara, CA, USA). Then, 500 ng of RNA was used to synthesize first-strand cDNA with the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). qRT–PCR was performed in a Roche Light Cycler 480 (Roche Applied Science, Penzberg, Germany) in conjunction with the SYBR Premix Ex Taq™ Kit (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. The relative expression levels of target genes relative to the reference gene *PagActin* were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Three biological replicates with four technical repeats were performed for each sample. The primers used for qRT–PCR are listed in Supplementary Table S1.

Histochemical GUS staining

Transgenic 84K poplar plants expressing *GUS* under the control of the *PagWOX11/12a* promoter ($Pro_{PagWOX11/12a}$*::GUS*) were cultivated on 1/2 MS solid medium for 4 weeks. To detect drought-induced expression, the $Pro_{PagWOX11/12a}$*::GUS* plants were subjected to 10% PEG6000 for 3 h and then transferred to the staining solution. GUS staining was performed according to the procedure described by B. Liu *et al.* (2014). The chlorophyll-free stained plants were observed under an Olympus SZX16 microscope. Three independent $Pro_{PagWOX11/12a}$*::GUS* plants were used for staining and imaging.

Subcellular localization and transactivation activity assays

The CDS of *PagWOX11/12a* (without a stop codon) was cloned into the pBI121 vector containing the green fluorescent protein (GFP) reporter gene driven by the CaMV 35S promoter. The fusion construct (PagWOX11/12a–GFP) and the control vector (GFP) were separately introduced into live onion epidermal cells using the particle bombardment method. The nuclei were stained with DAPI and visualized by a confocal laser scanning microscope as described by Wang *et al.* (2017).

To investigate the transactivation activity of PagWOX11/12a, the CDS of *PagWOX11/12a* and fragments of *PagWOX11/12a* encoding the N-terminus (amino acids 1–128) or the C-terminus (amino acids129–255) were amplified (Supplementary Table S1) and fused to the GAL4 DNA-binding domain within the pGBKT7 vector (Clontech, Palo Alto, CA, USA). The three constructs and the empty pGBKT7 vector (negative control) were individually transformed into yeast strain Y2HGold cells (Clontech). All the transformants were plated on SD medium lacking tryptophan (Trp) (SD/−Trp) supplemented with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and cultured at 30 °C for 2–3 d, and then underwent a β-galactosidase assay. The experiment was repeated three times.

Yeast one-hybrid (Y1H) assays

A dehydration-responsive element (DRE) was found in the promoter of *PagWOX11/12a*, suggesting that *PagWOX11/12a* may be regulated by stress-related transcription factor(s). A Y1H assay was carried out using the Matchmaker™ Gold Yeast One-Hybrid System (Clontech) to identify the regulator of *PagWOX11/12a*. Three tandem copies of the DRE 'ACCGAGA' were inserted into the pHIS2 vector as the reporter construct to screen an 84K poplar cDNA library. To investigate the interactions between the DRE and candidate regulator, we mutated the DRE core motif 'ACCGAGA' to 'ACGCAGA' (mDRE), and the interactions between the DRE or mDRE sequences and the candidate were investigated using the Y1H assay. To further confirm these interactions, fragments of the *PagWOX11/12a* promoter (288 bp) containing the DRE motif (Pro) or the corresponding mutated fragment containing the mDRE motif (mPro) were cloned into pHIS2 reporter vectors, and the interactions were evaluated using the Y1H assay. The DNA–protein interactions were evaluated according to the growth ability of the co-transformants on SD medium lacking leucine (Leu) and tryptophan (Trp) (SD/−Leu/−Trp, DDO) and SD medium without leucine (Leu), tryptophan (Trp), and histidine (His) (SD/−Leu/−Trp/−His, TDO) containing 3-amino-1,2,4-triazole (3-AT). All primers used for the DNA cloning and mutagenesis are listed in Supplementary Table S1.

Transient expression assays

To further evaluate the transactivation activity of PagERF35 (the regulator identified using a Y1H system) towards *PagWOX11/12a*, a dual reporter system that included an F reporter, an R reporter, and effector constructs was constructed. For the F reporter construct, three tandem copies of the DRE or mDRE, the promoter fragment of *PagWOX11/12a* containing the DRE motif (Pro), or the corresponding mutated fragment (mPro) used in the Y1H assay were inserted upstream of the firefly luciferase (*LUC*) gene containing the minimal TATA box (Promega). For the R reporter construct, the *GUS* gene in the pBI221 vector (Clontech) was replaced with the *Renilla* luciferase gene (Promega) and used as an internal control. For the effector construct, the *GUS* gene in the pBI221 vector was replaced with the *PagERF35* gene in the same manner as that above. Preparation of Arabidopsis mesophyll protoplasts and transient transfection of the reporter and effector constructs were performed according to the PEG-mediated DNA transformation method (Zhao *et al.*, 2006; Riazunnisa *et al.*, 2007). A 6 μg aliquot of the F reporter, 1 μg of the R reporter, and 6 μg of the effector plasmid were used for each co-transformation. The *Renilla* and firefly luciferase activities were quantified according to the dual-luciferase reporter assay system protocol (Promega). Each experiment was independently repeated three times.

ChIP assays

To further confirm whether the PagERF35 protein can regulate the expression of *PagWOX11/12a*, the CDS of *PagERF35* without the termination codon was fused to the N-terminus of GFP under the control of the CaMV 35S promoter within the pBI121 vector. The recombinant vector (PagERF35–GFP) was introduced into *A. tumefaciens* strain GV3101, which was then transiently transformed into 4-week-old 84K plants cultured in liquid 1/2 MS medium as described previously (Ji *et al.*, 2014), with minor modifications. In brief, the plants were soaked in a solution [1/2 MS medium+5% (w/v) sucrose+100 μM acetosyringone+0.8 OD_{600} *A. tumefaciens*+0.01% (w/v) Tween-20, pH 5.8] and then shaken at 130 rpm at 25 °C. After 6 h of cultivation, the plants were transferred to 1/2 MS solid medium for 48 h. The roots of transgenic plants were then used for ChIP assays following the method of Li *et al.* (2014). The sonicated chromatin was immunoprecipitated with anti-GFP antibody (ChIP) or immunoprecipitated without the anti-GFP antibody (Mock). qPCR was used to study the fold enrichment of the studied promoter fragments with the following program: 95 °C for 30 s, followed by 45 cycles of 94 °C for 5 s, 60 °C for 30 s, dissociation at 95 °C for 5 s, and then 60 °C for 60 s. The PCR products were detected via 1.2% agarose gel electrophoresis. The DNA sequence of *PagActin* was used as an internal control. To ensure that the *PagERF35-GFP* gene could be expressed in transiently transformed poplar plants, the pCAMBIA1301 vector with the *GUS* reporter gene driven by the CaMV 35S promoter was used as a positive control for the transient transformation, and the expression of the *GFP* gene in the roots of transiently transformed poplar plants was checked by semi-quantitative RT–PCR. All of the primers used are shown in Supplementary Table S1. Three independent biological replicates were performed for the ChIP assay.

Statistical analyses

All the data are presented as the mean ±SDs and were subjected to inferential statistical analysis using Student's *t*-test. The differences between

two groups of data for comparisons were evaluated as statistically significant (*P<0.05).

Results

Isolation and characterization of PagWOX11/12a

From 84K poplar plants, we cloned *PagWOX11/12a*, which was 768 bp long and encoded a 255 amino acid protein, and sequence alignment showed that PagWOX11/12a shared high amino acid sequence similarity with the product of *PtrWOX11/12a* (Potri.013G066900) of *Populus trichocarpa* and *PtoWOX11/12a* (AHL29320.1) of *Populus tomentosa*. All of these species contained a highly conserved homeobox domain of 61 amino acids within their N-terminal region. PagWOX11/12a was clustered with PtWOX11/12a and PeWOX11 according to phylogenetic analysis (Supplementary Fig. S1).

Expression patterns of the PagWOX11/12a *gene*

qRT–PCR was performed to investigate the expression profiles of the *PagWOX11/12a* gene in the root, stem, and leaf tissues. The results showed that *PagWOX11/12a* was expressed predominantly in the roots (Fig. 1A), and the expression was strongly induced by drought stress, increasing up to nearly 5-fold at 3 h after treatment (Fig. 1B). The GUS construct driven by the *PagWOX11/12a* promoter was introduced into poplar plants ($Pro_{PagWOX11/12a}$::*GUS*), and GUS staining showed that *PagWOX11/12a* was mainly expressed in the root tips (Fig. 1C). The *PagWOX11/12a* promoter sequence analysis indicated a number of stress-related *cis*-elements, including DREs (Supplementary Fig. S2), which suggests that *PagWOX11/12a* is possibly involved in the drought stress response process. GUS staining of $Pro_{PagWOX11/12a}$::*GUS* plants revealed that *PagWOX11/12a* was expressed strongly in the main roots (MRs) and LRs treated with PEG for 3 h (Fig. 1C; Supplementary Fig. S2). The GUS activities of $Pro_{PagWOX11/12a}$::*GUS* plants significantly increased by >13-fold in the roots under drought stress (Fig. 1D). Taken together, these results indicate that *PagWOX11/12a* is mainly expressed in the roots and can be strongly induced by drought.

PagWOX11/12a *promotes root growth in transgenic poplar plants*

To further determine the roles of *PagWOX11/12a* in relation to root growth in poplar, overexpression (OE) and dominant repression (DR) transgenic *PagWOX11/12a* poplar plants were generated, and the resulting altered gene expression was confirmed by qRT–PCR (Supplementary Fig. S3). Two representative OE lines (OE1 and OE3) with high *PagWOX11/12a* expression levels and two DR lines (DR7 and DR9) with low expression levels were selected for further analysis. MR and LR development was significantly different between 3-week-old transgenic and 84K plants cultivated in 1/2 MS solid medium (Fig. 2A). Compared with that of the 84K plants, MR number in OE plants was significantly greater but not statistically significant in DR plants. In addition, the MRs were markedly longer in OE plants than in 84K plants but shorter in DR plants (Fig. 2B, C). The LR numbers in OE and DR lines were obviously lower than those in the 84K plants. However, LR length significantly increased for the OE plants compared with the 84K plants but dramatically decreased for the DR plants (Fig. 2D, E).

PagWOX11/12a *alleviates the inhibition of root and shoot growth under drought conditions*

We next investigated whether *PagWOX11/12a* is involved in regulating root elongation and biomass during drought stress. Four-week-old plants were cultivated in soil for 40 d under normal conditions, and OE plants were larger than

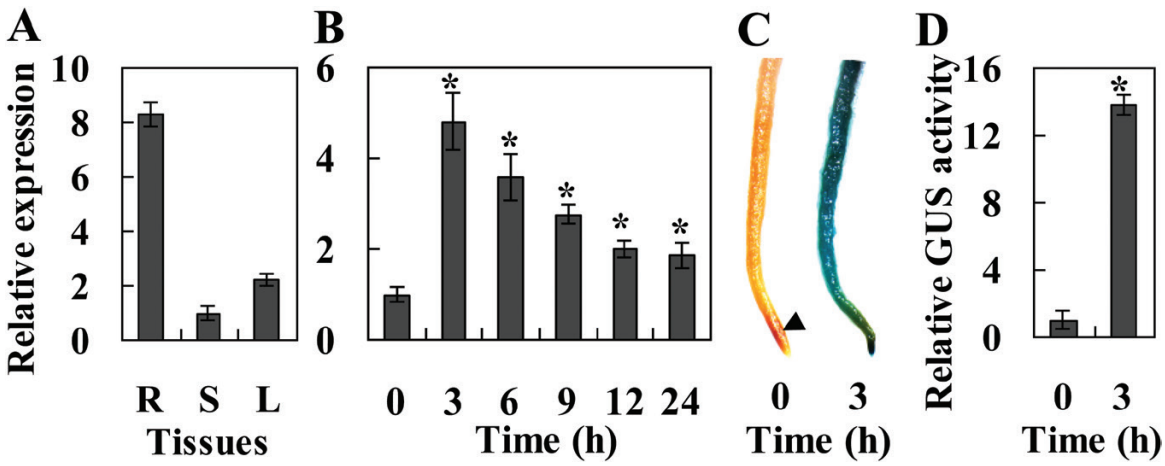

Fig. 1. Expression patterns of *PagWOX11/12a* in poplar. (A, B) qRT–PCR analysis of *PagWOX11/12a* expression in different tissues and total roots of poplar grown under PEG treatment for the indicated times. The *PagActin* gene was used as an internal control. (C, D) Histological staining and GUS activity of the root tips of $Pro_{PagWOX11/12a}$::*GUS* transgenic poplar plants under drought treatment for 3 h. The values under normal conditions were normalized to 1. The error bars represent ±SD from three biological repeats, and the asterisks indicate significant differences with respect to the values for 0 h (*P<0.05).

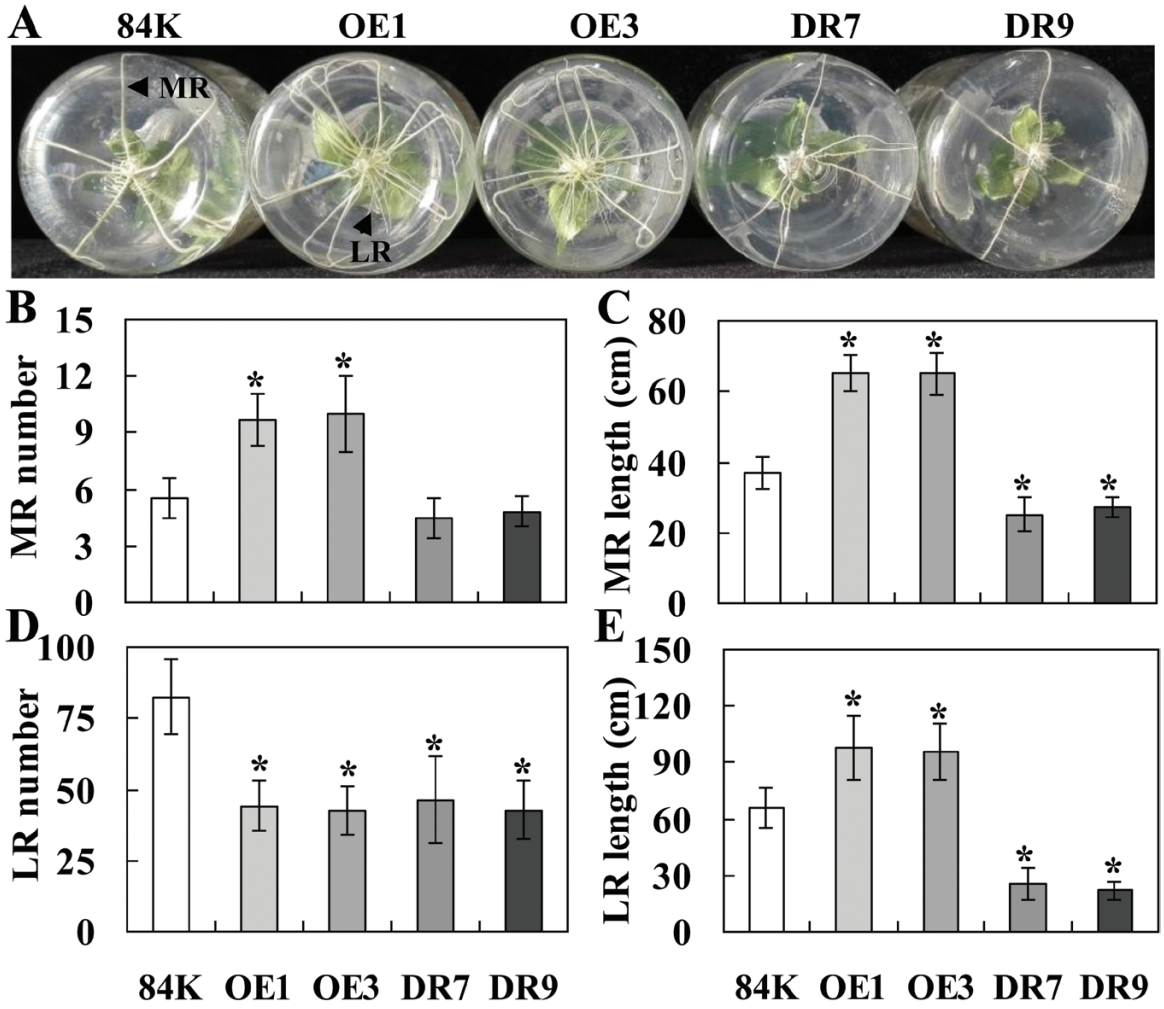

Fig. 2. Root development in *PagWOX11/12a* transgenic poplar plants. (A) Roots of 84K and transgenic (OE and DR) plants. (B–E) Average MR/LR numbers (B, D) and MR/LR lengths (C, E) of 84K and transgenic plants. The cutting-propagated plants were cultivated on 1/2 MS solid medium for 3 weeks. All the MR/LR numbers and lengths for individual plants were measured. The error bars represent ±SD from multiple biological replicates, and the asterisks indicate significant differences between 84K plants and transgenic plants (*P<0.05).

84K plants. In contrast, compared with that of the 84K plants, the shoot and root growth of DR plants was dramatically reduced (Fig. 3A). The stem height, and shoot and root biomass were higher in OE plants but lower in DR plants than in 84K plants (Fig. 3C–E). After 20 d of drought treatment, although some older leaves of transgenic and 84K plants started to wilt and fall off, compared with the 84K and DR plants, the OE plants were less stressed (Fig. 3B). Stem height was significantly greater in OE plants compared with 84K plants, leading to markedly high shoot biomass. Conversely, compared with the other types of plants, the DR poplar plants exhibited notably dwarf and slender phenotypes and significantly reduced stem height and biomass accumulation. Remarkably, the stem height and biomass accumulation were decreased to a greater extent in 84K and DR plants than for the OE plants under drought treatment (Fig. 3C–E). Consistent with these results, the 84K and DR plants appeared more stressed than did the OE plants under hydroponic conditions afforded by Hoagland's nutrient solution supplemented with PEG6000 (Fig. 4A). Compared with those of the 84K plants, the stem height and biomass of the OE and DR plants were significantly greater and lower, respectively (Fig. 4B–D). These results suggest that *PagWOX11/12a* overexpression can increase drought tolerance in transgenic poplar plants.

PagWOX11/12a is a nuclear protein and has transcriptional activation activity

To examine the subcellular localization of PagWOX11/12a, the fusion protein PagWOX11/12a–GFP driven by the 35S promoter was generated and transformed into live onion epidermal cells. GFP fluorescence (positive control) was distributed throughout the cell, and PagWOX11/12a–GFP was exclusively observed in the nucleus (Fig. 5A). This indicates that PagWOX11/12a is localized in the nucleus.

To determine the transcriptional activation ability of PagWOX11/12a, the full CDS and the N-terminal and C-terminal fragments of *PagWOX11/12a* were separately transformed into Y2HGold yeast cells (Fig. 5B). All transformants harboring the recombinant vectors or pGBKT7 control vectors grew well on SD/−Trp medium, indicating

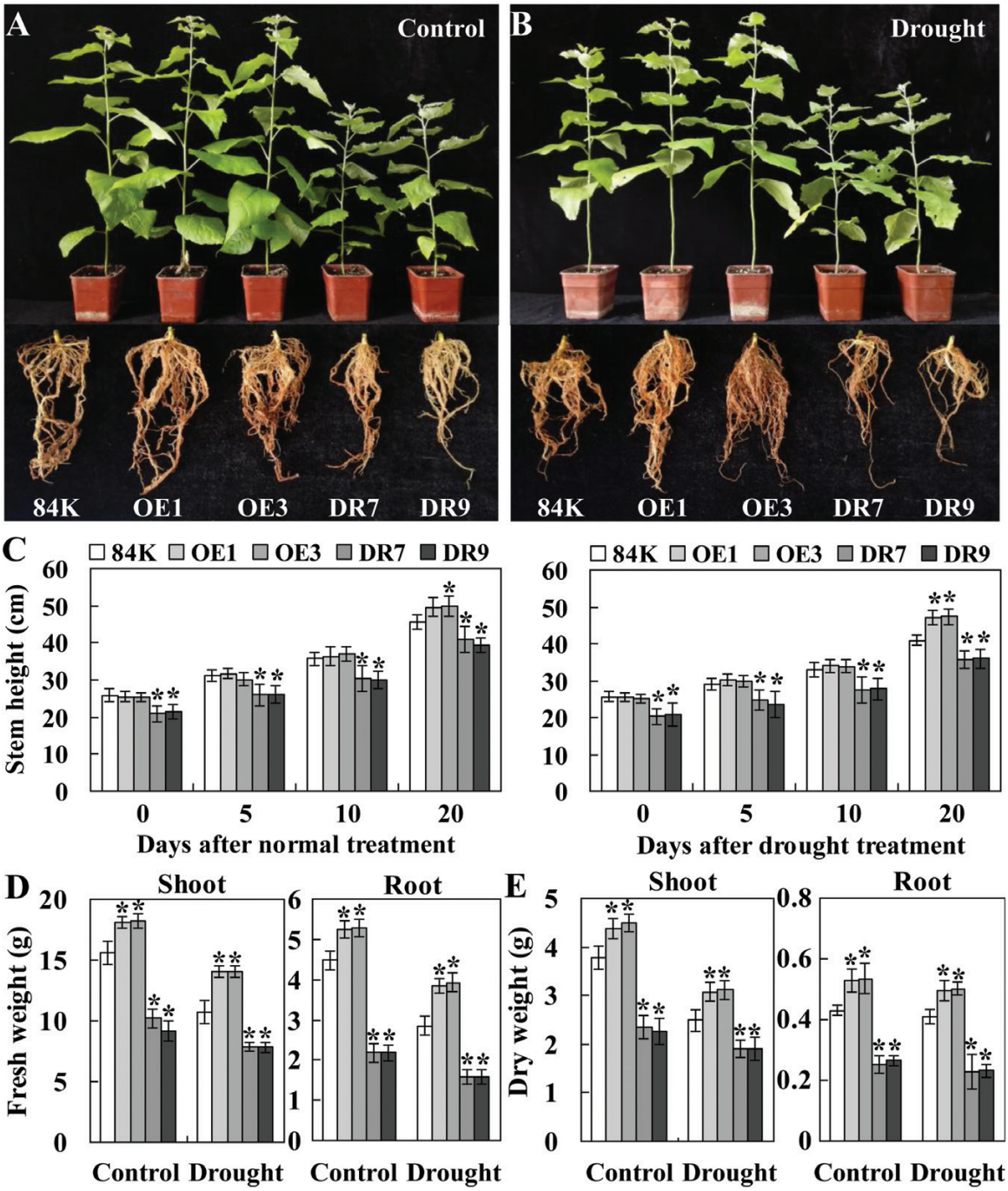

Fig. 3. Biomass and drought tolerance of *PagWOX11/12a* transgenic plants. (A, B) Phenotypes of 84K and transgenic (OE and DR) plants. Four-week-old poplar plants were cultivated in soil for 20 d and subsequently grown under normal conditions (A) or subjected to drought stress for 20 d (B). (C) Stem height of 84K and transgenic plants under normal and drought conditions. (D, E) Shoot and root biomass of 84K and transgenic plants under normal and drought conditions. The error bars represent ±SD from multiple biological replicates, and the asterisks indicate significant differences between 84K plants and transgenic plants (*P<0.05).

that these constructs had been successfully transformed into yeast Y2HGold cells. On SD/−Trp medium supplemented with X-α-Gal, the cells harboring the full-length CDS of *PagWOX11/12a* or its C-terminal domain appeared blue because of the transactivation of the α-galactosidase gene, whereas cells containing pGBKT7 or the N-terminal domain did not. These results suggest that the full-length PagWOX11/12a sequence and its C-terminal polypeptide have transcriptional activation ability.

PagERF35 binds to the DRE of the PagWOX11/12a *promoter to regulate its expression*

The DRE present within the *PagWOX11/12a* promoter was shown to be involved in responsiveness to drought stress (Yamaguchi-Shinozaki and Shinozaki, 1994). To further investigate the responsiveness of *PagWOX11/12a* to drought stress, a Y1H assay was performed to screen an 84K poplar cDNA library and identify potential transcription factors that regulate

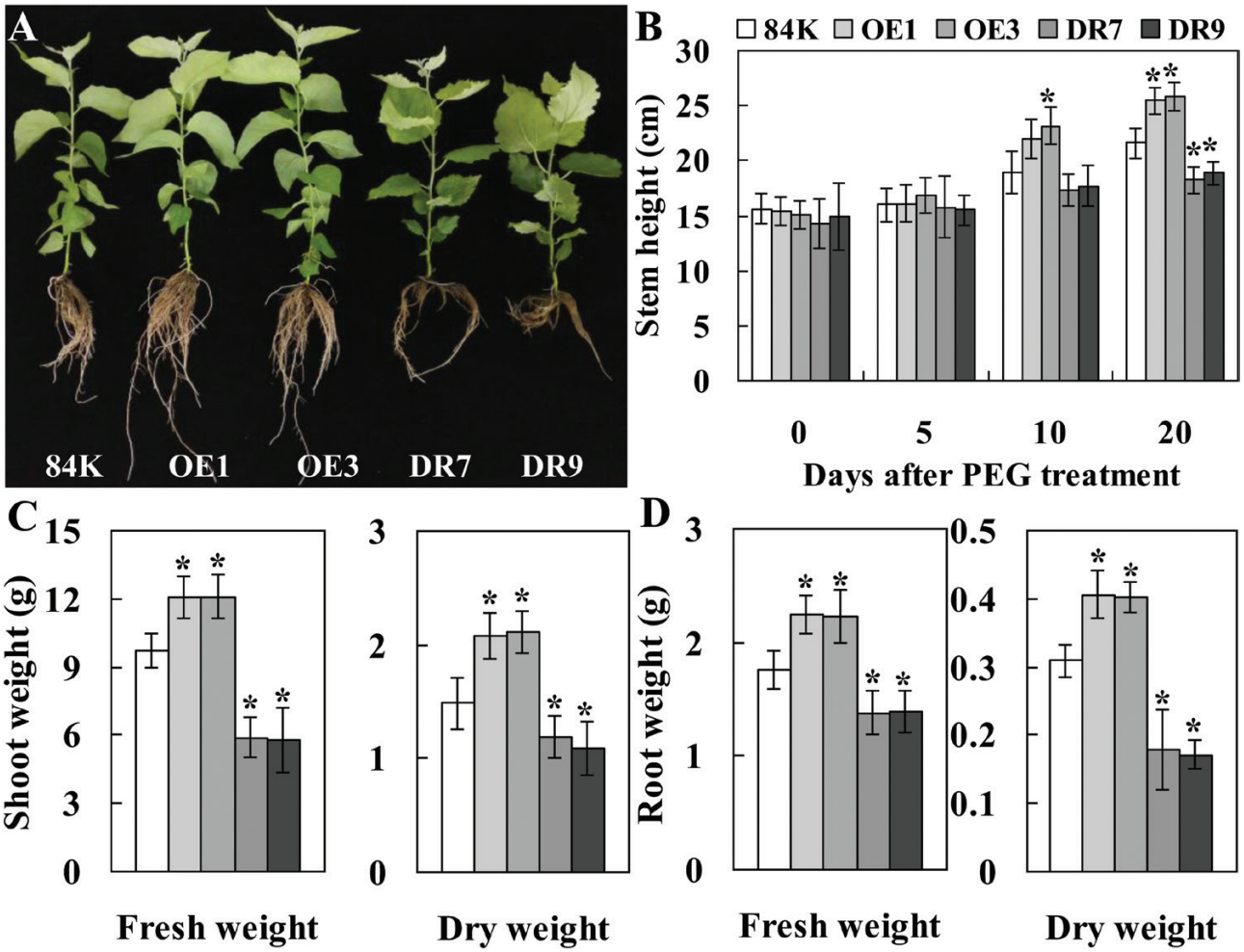

Fig. 4. *PagWOX11/12a* enhances drought tolerance and biomass growth under drought conditions. (A) Phenotypes of 84K and transgenic (OE and DR) plants under PEG-simulated drought treatment. Four-week-old poplar plants were hydroponically cultivated in Hoagland's nutrient solution for 10 d and subsequently subjected to drought stress with 10% PEG6000 for 20 d. (B) Stem height of 84K and transgenic plants under drought conditions. The plant heights were measured periodically after the beginning of the treatment. (C, D) Shoot and root biomass of 84K and transgenic plants under drought conditions. Drought treatment was performed as indicated in (A). The error bars represent ±SD from multiple biological replicates, and the asterisks indicate significant differences between 84K plants and transgenic plants (*P<0.05).

PagWOX11/12a. After putative positive clones were restreaked on high-stringency medium supplemented with 15 mM 3-AT, the clone harboring an ERF transcription factor grew better than did the others (Fig. 6A). Phylogenetic analysis indicated that the ERF transcription factor was most similar to members of the PtERF-B4 group (including PtERF35/RAP2.4, PtERF70, PtERF71, and PtERF76) from *P. trichocarpa* (Zhuang *et al.*, 2008) and of the AtERF-VII family (including AtERF71/HRE2, AtERF72/RAP2.3, and ATERF73/HRE1) from Arabidopsis (Yao *et al.*, 2017) (Supplementary Figs S4, S5). The ERF transcription factor was highly homologous to PtERF35 (Potri.005G195000.1) and thus was named PagERF35. To confirm the specificity of the interaction between PagERF35 and the DRE, we mutated the DRE (mDRE) and replaced the DRE in the promoter fragment. The mini-promoters with 3× DRE or 3× mDRE and the promoters with DRE or mDRE were used in a Y1H assay, and the results showed that PagERF35 specifically bound to the DRE motif and that its binding ability was completely lost when the core DRE motif was mutated to mDRE (Fig. 6A).

To further confirm that the PagERF35 protein binds to the DRE sequence in the *PagWOX11/12a* promoter and regulates *PagWOX11/12a* transcription, using Arabidopsis protoplasts and ChIP assays, we performed transient assays in which transgenic 84K plants expressed *PagERF35-GFP*. Constructs expressing *PagERF35* and the firefly luciferase gene driven by the DRE or *PagWOX11/12a* promoter as a reporter were co-transformed into Arabidopsis protoplasts. The relative ratios of firefly luciferase activity driven by the DRE and *PagWOX11/12a* promoter co-transformed with the PagERF35 effector were ~3- and 2.5-fold those of the mock vector without PagERF35. However, when the DRE motif within the promoter region of *PagWOX11/12a* was mutated to the mDRE, the relative luciferase activity decreased to the level of the control (Fig. 6B). To test the transformation efficiency, we used the 35S::*GUS* gene as a positive control and found that it was highly expressed in the roots of transgenic plants (Supplementary Fig. S6A). In addition, we randomly selected four transiently transformed poplar plants and found that *PagERF35-GFP* gene expression was

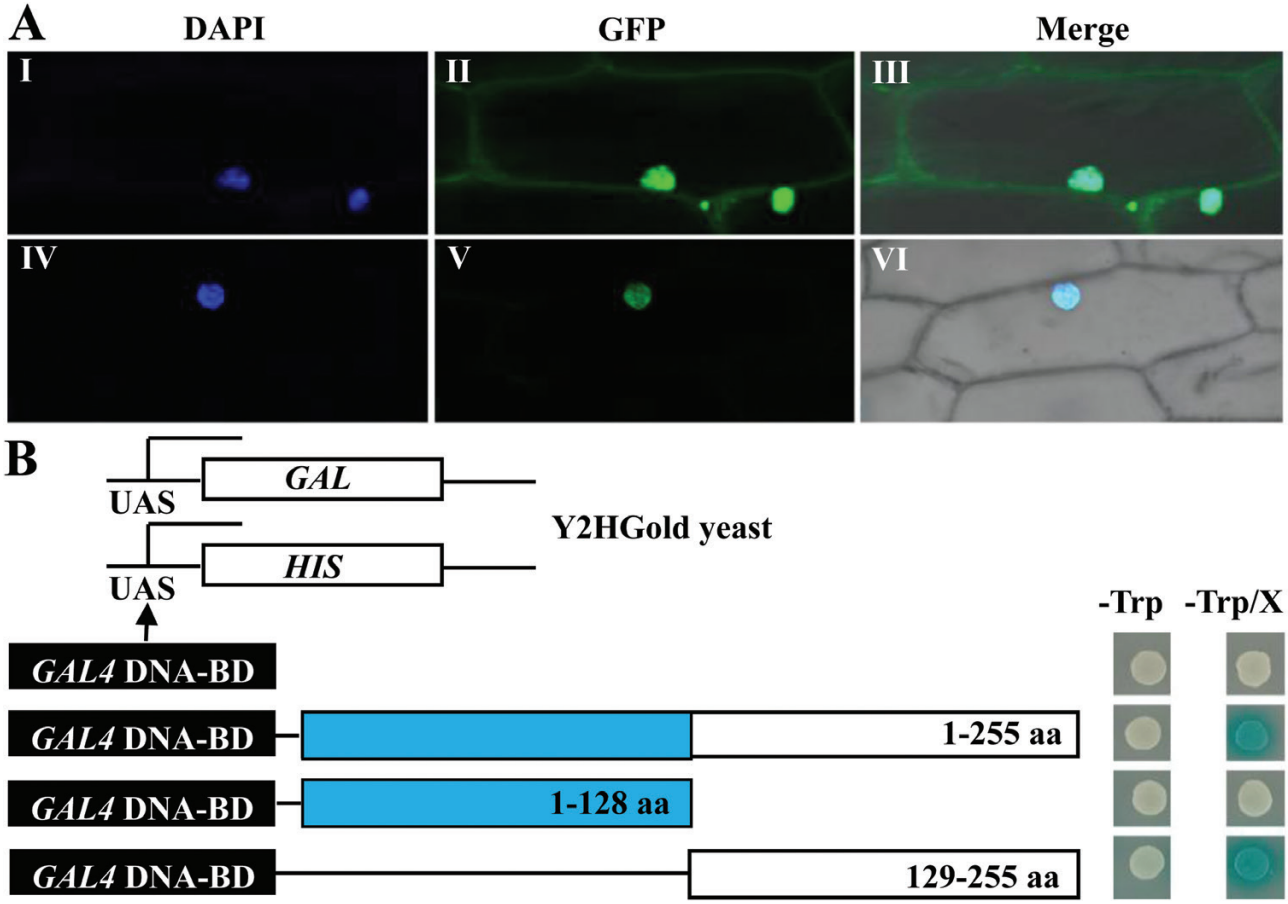

Fig. 5. Subcellular localization and transactivation activity of PagWOX11/12a protein. (A) The control *GFP* (I–III) and *PagWOX11/12a-GFP* (IV–VI) constructs were expressed in onion epidermal cells, showing the nuclear localization of PagWOX11/12a. (B) Schematic diagram of the *PagWOX11/12a* cDNA fragments encoding different portions of PagWOX11/12a fused to the GAL4 DNA-binding domain of the pGBKT7 vector. The fusion plasmids and pGBKT7 empty vectors (negative control) were individually transformed into the yeast strain Y2HGold, after which the transformed yeast cells were grown on SD/−Trp medium or SD/−Trp medium with X-α-Gal for 2–3 d and then used for β-galactosidase assays.

readily detected in all four (Supplementary Fig. S6B). These results indicate that the transient transformation method is highly efficient. Therefore, the roots of the transgenic plants could be used for the ChIP assay. After isolation of cross-linked chromatin, the immunoprecipitated DNA was analyzed by PCR. The promoter region of *PagWOX11/12a* containing the DRE was detected when the chromatin was prepared before immunoprecipitation (Input) and when PagERF35::GFP was precipitated (ChIP), whereas the promoter fragment was rarely detected when the chromatin was immunoprecipitated without the anti-GFP antibody (Mock). In addition, qPCR revealed that, compared with that in the mock, the promoter fragment of *PagWOX11/12a* in the ChIP was significantly enriched >3-fold (Fig. 6C). These results confirmed that PagERF35 could activate the expression of *PagWOX11/12a* by specifically binding to the DRE in its promoter *in vivo*.

PagERF35 *and* PagWOX11/12a *show similar expression patterns under abiotic stress*

The expression of *PagERF35* and *PagWOX11/12a* in the roots of 84K plants was determined by qRT–PCR (Fig. 6D). We found that the expression of *PagERF35* and *PagWOX11/12a* not only was induced by drought stress but also had a similar expression pattern in response to drought stress. These results further support that PagERF35 regulates the expression of *PagWOX11/12a* under drought stress conditions and indicate that they may be involved in a common regulatory module.

Discussion

The WOX family is a plant-specific homeobox transcription factor family that controls many key developmental processes by promoting cell division and/or preventing premature cell differentiation (Van der Graaff *et al.*, 2009; Yadav *et al.*, 2010). Although the determination of meristems by WOXs is well documented (Nakata *et al.*, 2012; Ohmori *et al.*, 2013) and some *WOX* genes have been identified to be developmental regulators that mediate root formation and growth (Cho *et al.*, 2013; B. Liu *et al.*, 2014; Hu and Xu, 2016), their roles in modulating plant development under abiotic stress remain elusive. Here, we show that PagWOX11/12a is involved in drought tolerance by regulating the root system in poplar. In addition, *PagWOX11/12a* can be activated directly by PagERF35, a stress-related transcription factor, to promote root elongation and biomass growth, thus enhancing drought tolerance.

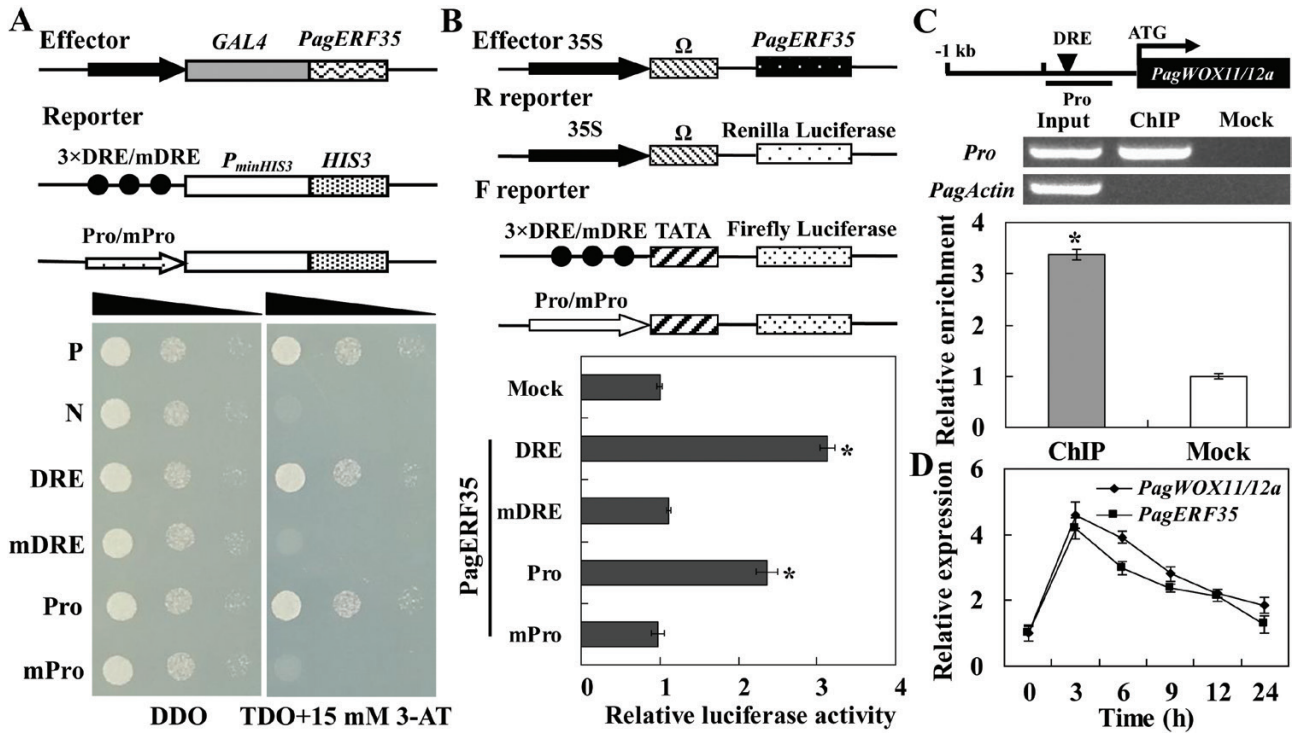

Fig. 6. Identification of the upstream regulator of *PagWOX11/12a*. (A) Y1H analysis. A diagram of the reporter and effector vectors is shown. The reporter constructs and the effector plasmids were co-transformed into yeast Y187 cells, and the positive transformants were determined by spotting serial dilutions of yeast onto SD/–His/–Leu/–Trp medium supplemented with 3-AT. P, positive control (p53HIS2+pGAD-53); N, negative control (p53HIS2+pGAD-PagERF35). (B) Transient expression assay. A schematic of the reporter and effector constructs used for the transient expression assays is shown. Co-expression of *PagERF35* and DRE or mDRE, *PagWOX11/12a* promoter fragments containing the DRE motif (Pro) or the corresponding mutant (mPro) in Arabidopsis protoplasts. All luciferase activities were expressed relative to the mock (value set at 1.0). Mock: F empty reporter, R reporter, and effector. The error bars represent ±SD from multiple biological replicates, and the asterisks indicate significant differences with respect to the control (*P<0.05). (C) ChIP-PCR analysis. Simplified gene structure indicating the locations of the amplified promoter region containing the DRE. The abundance of the *PagWOX11/12a* promoter sequence was obtained for chromatin preparation before immunoprecipitation (Input), and was immunoprecipitated with (ChIP) and without anti-GFP antibodies (Mock). *PagActin* was used as a negative control. Three independent biological replicates of the ChIP assay were performed. (D) Expression patterns of *PagWOX11/12a* and *PagERF35* in the roots of 4-week-old 84K plants subjected to drought treatment, as shown in Fig. 1A.

Previous studies have shown that overexpression of *WOX11* genes such as *OsWOX11*, *PtoWOX11*, *PeWOX11a*, and *PeWOX11b* in transgenic plants resulted in increased numbers of adventitious roots and ectopic roots (Zhao *et al.*, 2009; B. Liu *et al.*, 2014; Xu *et al.*, 2015). In the present study, we found that transgenic poplar plants overexpressing *PagWOX11/12a* exhibited significantly enhanced MR formation and elongation and total LR length. In contrast, *PagWOX11/12a* repression with SRDX resulted in markedly delayed LR development (Fig. 2). These observations suggest that *PagWOX11/12a* plays an important role not only in adventitious root induction but also in MR and LR development, leading to high root biomass.

Roots play key roles in water and mineral uptake, and in the detection of drought stress conditions. Thus, roots release signals to induce resistance and/or alter their architecture for optimal growth and development under these conditions (Atkinson and Urwin, 2012; Lee *et al.*, 2016). Recent studies have uncovered several key root morphological traits, including root length, root density, and root diameter, which are important for plants in terms of drought resistance (Hund *et al.*, 2009; Henry *et al.*, 2011; Comas *et al.*, 2013). In addition, vigorous root growth and a robust root system were shown to be closely associated with high plant water potential under drought conditions, and overexpression of *OsERF48*, *HARDY*, or *PtabZIP1L* promoted root growth and biomass, leading to enhanced drought resistance (Karaba *et al.*, 2007; Dash *et al.*, 2017; Jung *et al.*, 2017). Interestingly, Cheng *et al.* (2014) reported that the expression of *OsWOX11*, *OsWOX12A*, and *OsWOX12B* in rice was induced by salt, cold, and drought stress conditions. Transgenic rice plants overexpressing *OsWOX11* exhibited improved drought resistance by controlling root hair development in rice (Cheng *et al.*, 2016), but the mechanism by which *WOX11/12a* regulates abiotic stress tolerance is not clear. In addition, rice plants overexpressing another *WOX* member, *OsWOX13*, driven by a water deficit-specific promoter (*Rab21*), performed better than non-transgenic plants under water deficit conditions (Minh-Thu *et al.*, 2018). Some cotton *WOX* genes, including *GhWOX10*, *GhWOX13a*, and *GhWOX13b*, are induced by PEG-simulated drought stress (Yang *et al.*, 2017). However, the functions of these genes under drought stress were not further characterized in cotton. We also found that *PagWOX11/12a* was strongly induced by drought stress in roots (Fig. 1). Therefore, we propose that the root system promotion by *PagWOX11/12a* might be associated with drought stress. To test this hypothesis, we generated *PagWOX11/12a* transgenic poplar plants (OE and DR)

and found that the root length and total biomass of OE plants were greater than those of 84K and DR plants under drought conditions (Figs 2–4). These results provide strong evidence that *PagWOX11/12a* promotes root elongation and biomass growth under drought stress, leading to increased drought tolerance.

Studies have shown that several transcription factors, such as AP2/ERF, NAC, and MYB, are associated with root development and stress tolerance (Redillas *et al.*, 2012; Zhao *et al.*, 2015; Jung *et al.*, 2017). Plants overexpressing *OsNAC5*, *OsNAC10*, *AtMYB96*, or *PtabZIP1L* were highly resistant to drought stress (Jeong *et al.*, 2010, 2013; Seo *et al.*, 2011; Dash *et al.*, 2017). Notably, the root system modulated by overexpressing *OsERF48*, *OsERF71*, or *HYR/OsERF137* was shown to mediate drought tolerance in rice (Ambavaram *et al.*, 2014; Lee *et al.*, 2016; Jung *et al.*, 2017). However, the molecular mechanism governing the modulation of root development by these regulators under abiotic stress remains unclear. In this work, we identified an AP2/ERF member, PagERF35, and found that it could specifically bind to the DRE *cis*-acting element within the promoter of *PagWOX11/12a* and regulate its expression (Fig. 6A, B). In addition, *PagERF35* and *PagWOX11/12a* are both induced by drought conditions in the roots, and their expression patterns are similar (Fig. 6C). Interestingly, sequence alignment and phylogenetic analysis indicated that PagERF35 was most similar to published ERF proteins of Arabidopsis, AtERF71/HRE2 and AtERF73/HRE1 (Supplementary Fig. S5), which are responsive to hypoxia through ethylene-dependent and/or -independent pathways (Licausi *et al.*, 2010; Yang *et al.*, 2011) and activate the expression of downstream genes by binding to the GCC box or DRE/CRT as transcriptional activators, increasing the root length in different ways (Seok *et al.*, 2014; Lee *et al.*, 2015). Therefore, we propose that PagERF35 directly regulates the expression of *PagWOX11/12a* and initiates the drought response by modulating root development in poplar. These phenomena could explain how *PagWOX11/12a* responds to drought stress and which regulatory mechanism ERFs are used in modulating root systems under drought stress.

In conclusion, our study reveals that PagWOX11/12a can promote root elongation and biomass growth, especially under drought conditions, thus mediating the role of PagERF35 in response to drought stress. This work could be useful in the molecular breeding of poplar clones that are highly tolerant to abiotic stress.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Sequence alignment and phylogenetic analysis of PagWOX11/12a and other plant WOX11 proteins.

Fig. S2. *Cis*-acting elements and activity analyses of the *PagWOX11/12a* promoter via GUS staining in poplar.

Fig. S3. Expression analysis of *PagWOX11/12a* transgenic poplar plants by qRT–PCR.

Fig. S4. Phylogenetic tree of PagERF35 and ERF proteins from *P. trichocarpa*.

Fig. S5. Phylogenetic tree of PagERF35 and ERF proteins from *A. thaliana*.

Fig. S6. GUS staining and semi-quantitative RT–PCR to check the transient transformation efficiency.

Table S1. Primer sequences used in this study.

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