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# A novel bHLH transcription factor *PebHLH35* from *Populus euphratica* confers drought tolerance through regulating stomatal development, photosynthesis and growth in *Arabidopsis*

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ABSTRACT

Plant basic helix-loop-helix (bHLH) transcription factors (TFs) are involved in a variety of physiological processes including the regulation of plant responses to various abiotic stresses. However, few drought-responsive bHLH family members in *Populus* have been reported. In this study, a novel bHLH gene (*PebHLH35*) was cloned from *Populus euphratica*. Expression analysis in *P. euphratica* revealed that *PebHLH35* was induced by drought and abscisic acid. Subcellular localization studies using a *PebHLH35*-GFP fusion showed that the protein was localized to the nucleus. Ectopic overexpression of *PebHLH35* in *Arabidopsis* resulted in a longer primary root, more leaves, and a greater leaf area under well-watered conditions compared with vector control plants. Notably, *PebHLH35* overexpression lines showed enhanced tolerance to water-deficit stress. This finding was supported by anatomical and physiological analyses, which revealed a reduced stomatal density, stomatal aperture, transpiration rate, and water loss, and a higher chlorophyll content and photosynthetic rate. Our results suggest that *PebHLH35* functions as a positive regulator of drought stress responses by regulating stomatal density and stomatal aperture, photosynthesis and growth.



## 1. Introduction

The sustainability of plant yields worldwide is seriously threatened by an array of abiotic stresses, including drought, salinity, and extreme temperatures, among which drought stress is the major environmental factor limiting plant growth, development, and productivity [1]. Plants have evolved sophisticated mechanisms, including morphological, physiological, and biochemical adaptations, to reduce the adverse effects of abiotic stress [2,3]. Transcription factors (TFs), in particular, play crucial roles in the response of plants to these environmental factors [4].

Basic helix-loop-helix (bHLH) genes constitute a large family of TFs found in eukaryotic organisms. A total of 167 genes in *Arabidopsis* and 162 genes in rice have been predicted to encode bHLHs [5,6]. Recent research have indicated that some plant bHLH TFs regulate plant responses to abiotic stress. *Poncirus trifoliata PtrbHLH* and apple *MdClbHLH1* are suggested to respond to cold stress [6,7]. The *Arabidopsis* gene *FIT1* interacts with *AtbHLH38* and *AtbHLH39* to regulate iron uptake gene expression for iron homeostasis [8]. *AtNIG1* and *bHLH92* have been suggested to be involved in plant salt stress signaling [4,9]. In response to drought stress, rice *OsbHLH148* confers drought tolerance by interacting with OsJAZ proteins, which function in jasmonate signaling [10]. *Arabidopsis bHLH122* is a positive regulator of drought tolerance, NaCl tolerance, and osmotic signaling [11].

*Populus euphratica* is widely used as a model species for conducting research on abiotic stress resistance in woody plants [12,13]. However, less information is available on the response of bHLH TFs in *P. euphratica*. In this work, *PebHLH35*, a drought-responsive bHLH family member in *P. euphratica*, was initially identified via high-throughput sequencing. The objective of this study was to characterize the functions of *PebHLH35*. Our results indicate a role for this TF in the adaptation of *Populus* to water-deficit stress.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Two-year-old seedlings were used in this study. Plants grown in a controlled experimental greenhouse for 2 months were exposed

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to drought and abscisic acid (ABA). Drought stress was imposed by withholding watering for 0, 5, 10, 15, and 20 days [13]. For ABA treatment, the leaves of young trees were sprayed with 200 μM ABA [14]. *Arabidopsis* seeds were sterilized and then sown on half-strength Murashige and Skoog medium. After stratification, the plate-grown seedlings were transferred to a tissue culture room. After germination, the *Arabidopsis* seedlings were transplanted to soil in a greenhouse (22 °C/16 h of light, 8500 lux, and 70% relative humidity).

### 2.2. PebHLH35 identification, sequence analysis, and gene expression analysis

Total RNA was extracted by the CTAB method from leaves of *P. euphratica* and cDNA synthesis was performed [15]. All primers are listed in Table S1 in the Supplementary material. A phylogenetic analysis of *PebHLH35* was performed using amino acid sequences from various species with PhyML and MEGA 5 by the maximum likelihood (ML) method. Quantitative real-time PCR (qPCR) was applied to evaluate the expression levels of *PebHLH35* under different treatments. qPCR and the statistical analyses were conducted as described by Chen et al. [16].

### 2.3. Plasmid construction, plant transformation, and subcellular localization

We constructed 35S-*PebHLH35*-GFP to overexpress *PebHLH35* in *Arabidopsis* (ecotype Col-0) and used 35S-GFP as a vector control (VC) [17]. A solution of 6 mg/ml mannose was used for transgenic selection, and three homozygous $T_3$ lines (*oxPebHLH35*#5, #9, and #22) were subjected to a detailed analysis. To examine the subcellular localization of *PebHLH35*, the 35s-*PebHLH35*-GFP fusion protein was observed using a confocal laser scanning microscope (DM16000 CS; Leica, Wetzlar, Germany).

### 2.4. Morphological characterization

Fifty seeds for one line in a batch were used to compare the germination rates of the overexpression and VC plants. The germination rates were recorded 7 days after sowing. The primary root length was measured after growing vertically for 7, 9, and 11 days. The leaf number in 4-week-old seedlings was recorded. The leaf area was computed using Photoshop CS4. Plant height was measured every 5 days during the bolting period.

### 2.5. Drought treatment

For the drought treatment experiments, *Arabidopsis* seeds were sown under 0, 100, 200, and 300 mM mannitol and the germination rates were recorded for each treatment level. Seedlings of the *oxPebHLH35* and VC plants were watered for 18 days after being transplanted to soil and then water was withheld for 15 days, followed by rewatering.

### 2.6. Physiological measurements

Ten leaves from *oxPebHLH35* and VC plants grown under normal conditions for 25 days were used to measure rapid water loss [15]. The photosynthetic rate and transpiration rate were measured using the Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). The maximum quantum yield of PSII (Fv/Fm) was measured using a Dual-PAM-100 measuring system (Walz Heinz GmbH, Effeltrich, Germany). The number of wilted leaves was counted after withholding water for 5, 10, and 15 days. Dead or withered, chlorotic, drooping, and yellowing leaves were considered to be wilted [18]. The number of plants that survived and continued to grow was recorded after rewatering for 7 days. The density and aperture of the stomata in fully expanded leaves of similar size and the growth period were recorded and photographed using a scanning electron microscope (Hitachi S-3400N; Chiyoda-ku, Tokyo, Japan) [19,20]. The chlorophyll content was measured as described by Shu et al. [21].

## 3. Results

### 3.1. Molecular identification of PebHLH35 from P. euphratica

According to our previous study of the drought-responsive transcriptome in *Populus*, we found striking expression differences in the bHLH TF family in response to drought stress [13]. Among identified TFs, *PebHLH35* (GenBank number: KJ363186), which demonstrated increased gene expression under water-deficit stress as measured by both high-throughput sequencing [13] and microarray analysis [22], was chosen for further characterization. *PebHLH35* is 744 bp in length and encodes 247 amino acid residues with a predicted molecular mass of 28.90 kDa and an isoelectric point of 5.74. Structural analyses of the *PebHLH35*-predicted protein using InterPro suggested that PebHLH35 has a typical MYC-type bHLH domain (IPR011598) and a coiled coil region. Phylogenetic analyses suggested that plant bHLH proteins are monophyletic and constitute 26 subfamilies [23]. Twelve well-characterized bHLH proteins that belong to bHLH subfamily III (a + c) are listed in Fig. 1A. Based on the results of our ML phylogenetic analysis, *PebHLH35* was classified into this subfamily. However, two other bHLH family members *OsbHLH148* and *AtbHLH122* which also confer drought tolerance belong to subfamilies IVd and IX, respectively. According to previous phylogenetic analysis, subfamilies IVd and III (a + c) were probably established in the common ancestors and were evolved later than IX [23].

To identify homologs of *PebHLH35*, a phylogenetic tree was constructed using the protein sequences of these genes (Fig. 1B). The closest homologs to *PebHLH35* were Potri.018G141700 (*Populus trichocarpa*), *AtbHLH27* (AT4G29930), and *AtbHLH35* (AT5G57150). To date, no function has been assigned to these proteins.

### 3.2. PebHLH35 expression is induced by drought and ABA

*PebHLH35* expression was measured in *P. euphratica* leaves subjected to drought and ABA exposure. Under drought treatment, the transcription of *PebHLH35* was not induced immediately, but the expression level increased between 5 through 20 days of withholding water (Fig. 1C). Under ABA treatment, *PebHLH35* expression was induced rapidly, peaked at 4 h, and then decreased after 6 h (Fig. 1D).

### 3.3. PebHLH35 is localized to the nucleus

To determine the subcellular localization of *PebHLH35*, a 35S-*PebHLH35*-GFP fusion protein was analyzed. Fluorescence from 35S-GFP was discovered in the cytoplasm and nucleus, whereas fluorescence from the 35S-*PebHLH35*-GFP fusion was detected only in the nucleus, demonstrating that *PebHLH35* is localized to the nucleus (Fig. 1E). This is consistent with its function as a TF.

### 3.4. Phenotype of PebHLH35-overexpressing lines under well-watered conditions

*OxPebHLH35* and VC plants were used to evaluate the performance of *PebHLH35* at different developmental stages under well-watered conditions. The *oxPebHLH35* plants germinated 1 day earlier than the VC plants (i.e., 4 vs. 5 days after sowing, respectively). Consequently, the primary roots of the *oxPebHLH35*



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