Biochemical and Biophysical Research Communications 450 (2014) 453–458

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Yan Dong [a,b], Congpeng Wang [a], Xiao Han [a], Sha Tang [a], Sha Liu [a], Xinli Xia [a,*], Weilun Yin [a,*]

[a] College of Biological Sciences and Technology, National Engineering Laboratory for Tree Breeding, Beijing Forestry University, Beijing 100083, China
[b] Liaoning Forestry Vocational-Technical College, Shenyang 110101, China

ARTICLE INFO

Article history:
Received 25 May 2014
Available online 6 June 2014

Keywords:
Populus euphratica
bHLH
Transcription factor
Drought
Stomata

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, 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 functions 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

* Corresponding authors. Fax: +86 10 62336400.
E-mail addresses: xiaxl@bjfu.edu.cn (X. Xia), yinwl@bjfu.edu.cn (W. Yin).

http://dx.doi.org/10.1016/j.bbrc.2014.05.139
0006-291X/

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 were 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

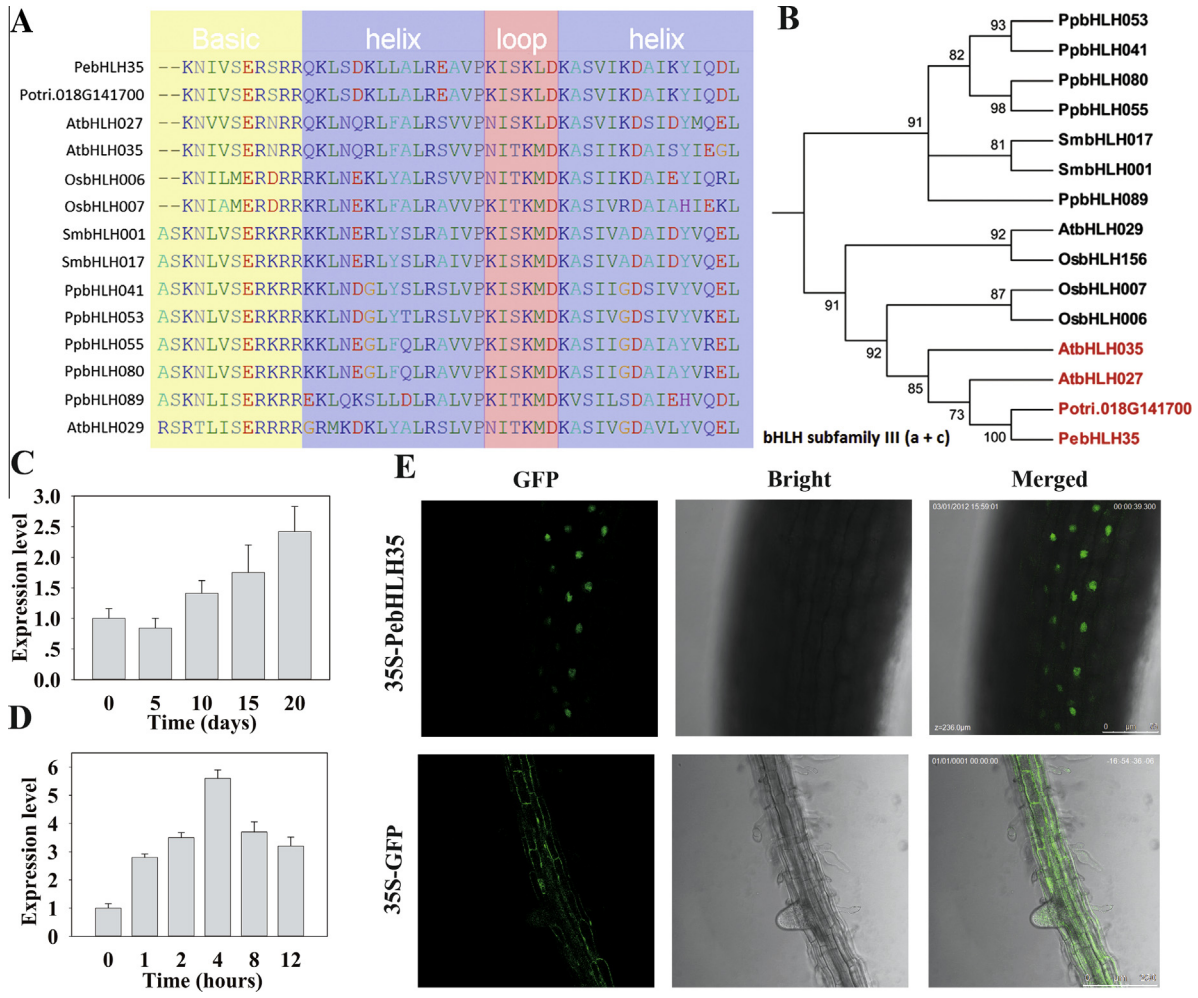

Fig. 1. Structure of the bHLH domain and conserved features of the PebHLH35 protein sequence. (A) Alignment of the bHLH domain of representative plant proteins. A representative of 13 genes from bHLH subfamily III (a + c) is shown. The shaded boxes indicate the position of the DNA-binding basic region (yellow), the two a-helixes (blue), and the variable loop region (red). (B) The phylogenetic relationships between PebHLH35 and its homologs in other representative plant species in bHLH subfamily III (a + c). Expression of *PebHLH35* in response to drought (C) and ABA (D) in *P. euphratica*. *PeActin* was used as an internal control. The data are the mean ± SE (n = 3 experiments). (E) The subcellular localization of *PebHLH35* as revealed using GFP fusion proteins. Confocal images of GFP fluorescence transformed with 35S-GFP or 35S-*PebHLH35*-GFP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1 day earlier than the VC plants (i.e., 4 vs. 5 days after sowing, respectively). Consequently, the primary roots of the *oxPebHLH35* plants were consistently longer (1.30- to 1.51-fold) than those of the VC plants at each time point (Supplemental Fig. S1A and C). The leaf number and leaf area of 4-week-old *oxPebHLH35* plants were greater than those of VC plants under the same well-watered conditions. Compared to the VC plants, the average leaf number was 1.37- to 1.47-fold greater and the average leaf area was 1.80- to 2.17-fold greater in the *oxPebHLH35* plants (Supplemental Fig. S1B, D and F). Additionally, the *oxPebHLH35* plants blossomed at 23–24 days after germination, whereas the VC plants blossomed 1–2 days later. At 26 days after germination, the inflorescence length of the *oxPebHLH35* plants ranged from 1.07 to 1.27 cm, while that of the VC plants only averaged 0.13 cm. At 42 days after germination, the height of the *oxPebHLH35* plants reached about 32.44 cm, which was 1.18- to 1.25-fold higher than the VC plants (Supplemental Fig. S1E and G).

3.5. Phenotype of oxPebHLH35 plants under drought conditions

To study the effect of osmotic stress on seed germination, different concentrations of mannitol were used. There was no obvious difference in germination rate, but, similar to the results obtained for well-watered plants, the germination time of the VC plants was 1 to 2 days later than that of the *oxPebHLH35* plants at 0 and 100 mM mannitol. In 200 mM mannitol, the germination rate of the *oxPebHLH35* plants was 48%, whereas that of VC plants was only 10%. Notably, the germination of the VC plants was seriously inhibited in 300 mM mannitol; at this concentration, 11–29% of the *oxPebHLH35* plants germinated (Fig. 2D).

The measurement of water loss from detached leaves showed that the *oxPebHLH35* plants lost water more slowly than the VC plants during dehydration for 3 h (Fig. 2C). When water was withheld for 5 days, no obvious differences were found between the VC and *oxPebHLH35* plants. After 10 days without water, the leaf-wilting rate of the VC plants was approximately 24%, whereas the *oxPebHLH35* plants showed less wilting. After 15 days without water, 91% of the VC plants had withered leaves, whereas 47–59% of the transgenic plants had no withered leaves (Fig. 2A and B). After being rewatered for 7 days, the *oxPebHLH35* plants showed a high survival rate (45–60%); in comparison, none of the VC plants survived (Fig. 2E).

3.6. PebHLH35 overexpression regulates stomatal development and photosynthesis

PebHLH35 overexpression regulated the stomatal density and aperture in the transgenic plants. The number of stomata per

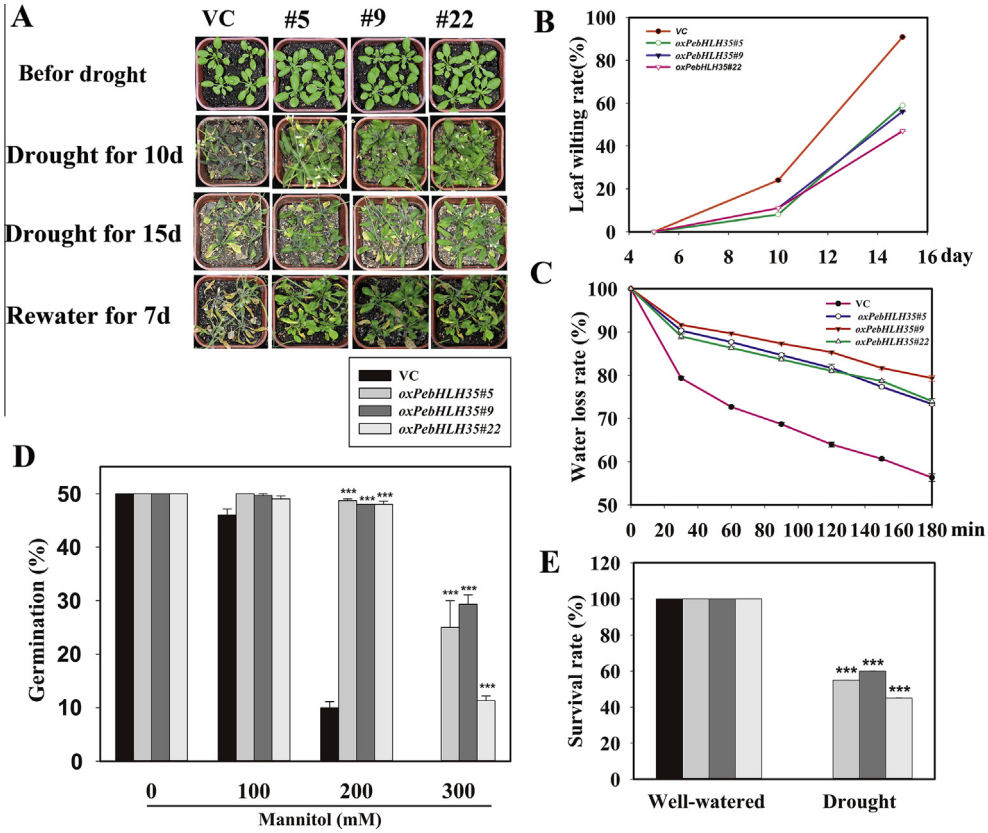

Fig. 2. Phenotypic comparison of *oxPebHLH35s* and VC plants under drought stress. (A) Phonotype of the *oxPebHLH35s* and VC plants under drought stress. (B) Comparison of the leaf wilting rate between *oxPebHLH35s* and VC plants after water was withheld for 5, 10 and 15 d. Values are means percentage of the number of wilted leaves to total number of leaves from three replicated experiments (30 seedlings per experiment). (C) Water loss from the detached leaves of *oxPebHLH35s* and VC plants. Values are means percentage of initial fresh weight from ten leaves from five replicated experiments. (D) Comparison of osmotic effects on the germination rate of *oxPebHLH35* and VC plant seeds. $n = 5$ independent replicates (50 seeds per experiment). (E) Survival rates of *oxPebHLH35s* and VC plants after water was withheld for 15 days, and then rewatered for 7 days. $n = 3$ independent replicates (50–60 seedlings per experiment). Each bar in (D) and (E) represents the mean ± SE. (***) denotes a significant difference ($P < 0.001$) according to the ANOVA results.

mm^2 in the *oxPebHLH35* plants was lower 69–117 (or 22.40–37.98%, respectively) than that in the VC plants (Fig. 3A and B). Under normal conditions, the stomatal aperture index of the VC plants was 0.51, while that of *oxPebHLH35* plants was 0.46–0.48. The stomatal aperture index of the VC plants was not obviously changed under well-watered and water-deficit conditions, but that of the *oxPebHLH35* plants was progressively reduced with increased drought stress. The stomatal aperture indices of *oxPebHLH35* plants for which water was withheld for 11 days decreased to 0.39–0.40, which was about 23% lower than that of the VC plants (Fig. 3D and E).

An analysis of photosynthetic performance showed that the *oxPebHLH35* plants maintained a significantly higher photosynthetic rate and lower transpiration rate than the VC plants. The photosynthetic rate of the *oxPebHLH35* plants was higher (25.23–41.8%) than that of the VC plants under well-watered conditions. Although the photosynthetic rates were reduced with an increasing duration of drought stress for all plants, the photosynthetic rate of the *oxPebHLH35* plants was consistently higher (55.16–70.60%) than that of the VC plants (Fig. 3F). The transpiration rate of the *oxPebHLH35* plants was lower (20.49–24.91%) than that of the VC plants under well-watered conditions, and after withholding water for 11 days, the transpiration rate of the *oxPebHLH35* plants was reduced by 0.79- to 1.31-fold (Fig. 3G).

Furthermore, the chlorophyll contents were evaluated under well-watered conditions. The chlorophyll content of the *oxPebHLH35* plants was higher by 13.7–24% compared to that of the VC plants. When water was withheld for 11 days, the chlorophyll content of the *oxPebHLH35* plants was higher by 65.90–87.80% than that of the VC plants (Fig. 3C).

Although the Fv/Fm values were similar between the VC and *oxPebHLH35* plants under normal conditions, they were significantly higher in the *oxPebHLH35* plants under drought stress. The Fv/Fm values of the *oxPebHLH35* plants for which water was withheld for 11 days decreased from 0.83 to 0.80, while the Fv/Fm rate of the VC plants decreased from 0.83 to 0.69 (Fig. 3H).

3.7. Expression analysis of stress-responsive genes regulated by PebHLH35

To uncover the downstream genes of *PebHLH35*, six drought-related genes (*ERD4*, *ATDR4*, *MDAR*, *ALDH3H1*, *PLC1*, and *AtMYC2*) and eight stomatal development or regulation-related genes (*FAMA*, *MUTE*, *SPCH*, *SCRM*, *SCRM2*, *PP2CA*, *TPK1*, and *CYP707A3*) were analyzed by qPCR in *oxPebHLH35* plants and VC plants under well-watered and water-deficit conditions. The results showed that, under well-watered conditions, *FAMA* and *PLC1* were up-regulated by >2.00-fold in *oxPebHLH35* plants and that the expression levels in *oxPebHLH35* plants were significantly higher than those in the VC plants. We found that *FAMA* and *PLC1* were strongly induced under drought stress (Fig. 4A and B).

4. Discussion

PebHLH35, which belongs to bHLH subfamily III (Fig. 1B), was firstly isolated from *P. euphratica*, and induced rapidly under

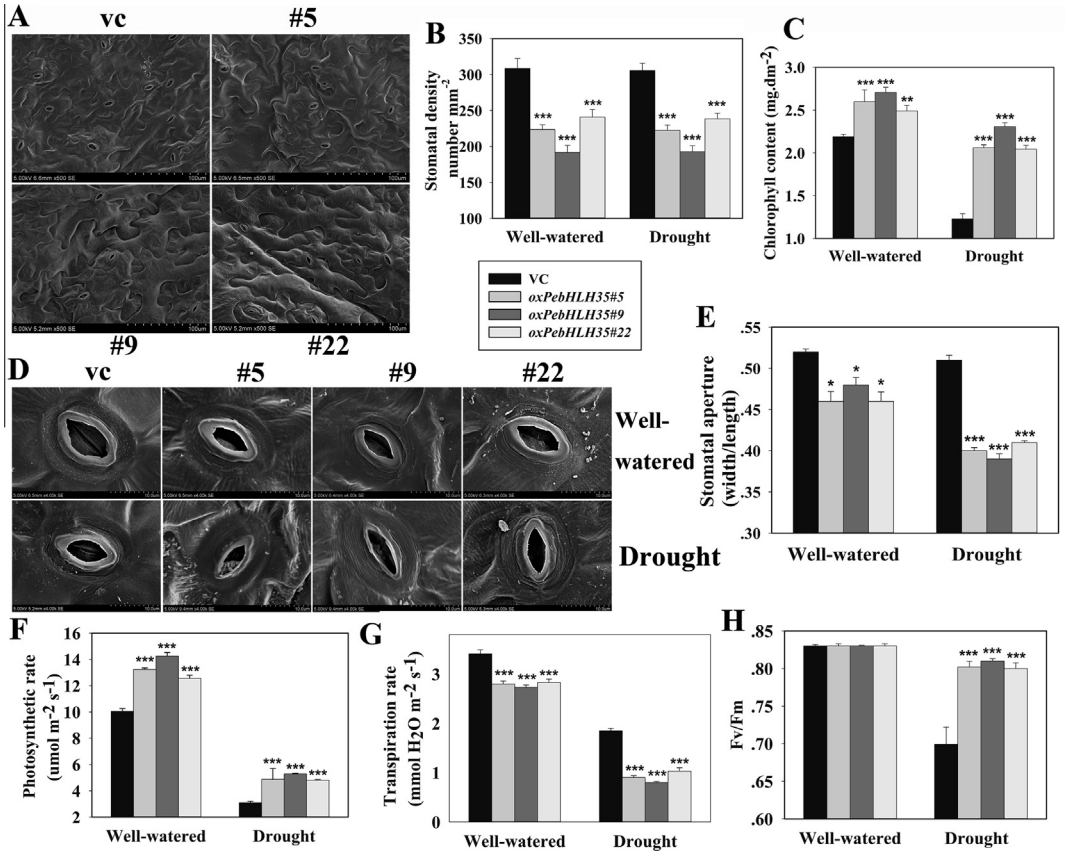

Fig. 3. *PebHLH35* overexpression increases drought resistance in *Arabidopsis*. (A) Representative scanning electron micrograph of the abaxial leaf epidermis. The scale bars represent 100 μm. (B) The abaxial stomatal densities in the *oxPebHLH35s* and VC plants. (C) Changes in the chlorophyll content in the *oxPebHLH35s* and VC plants. (D) Representative images of stomatal aperture in the *oxPebHLH35s* and VC plants. The scale bars represent 10 μm. (E) Changes in stomatal aperture of the *oxPebHLH35s* and VC plants. The values are mean ratios of width to length. (F) Changes in the photosynthetic rate of the *oxPebHLH35s* and VC plants. (G) Changes in the transpiration rate of the *oxPebHLH35s* and VC plants. (H) Changes in the chlorophyll fluorescence of the *oxPebHLH35* and VC plants. Water was withheld from the plants for 11 days. Each test represents the average of three replicates. Nine technical replicates were included in each replicates. Each bar represents the mean ± SE ($n = 27$). *, **, and *** denote a significant difference ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) according to the ANOVA results.

drought stress (Fig. 1C), which is consistent with recent high-throughput sequencing and microarray results [13,22]. In this study, we generated *oxPebHLH35 Arabidopsis* plants, and our results suggest that *PebHLH35* is involved in earlier seeding establishment and vegetative growth (Supplemental Fig. S1). Recent studies have indicated that root growth is closely correlated with drought tolerance. Longer roots and a larger root surface area can improve plant tolerance to drought stress [24]. In our study, there was no obvious difference in the lateral roots of the *oxPebHLH35* and VC plants, but there were significant differences in primary root growth (Supplemental Fig. S1A and C). The longer primary root of *oxPebHLH35* increased the surface area of the root, and provided greater capacity for the absorption of ions and water from soil.

Stomata is the primary source for water loss, and stomatal closure results in reduced water loss; this is an adaptive response to maintain a high water potential in plants under water-deficit stress [25]. Stomatal development regulated by transcription factors is an important process to response to drought stress [26]. The report suggests that transgenic *Arabidopsis* overexpressing *bHLH122* enhance drought tolerance by decreasing stomatal aperture [11]. The *Arabidopsis GTL1* transcription factor regulates drought tolerance by reducing stomatal density and transpiration [27]. *PebHLH35* overexpression reduced stomatal density, stomatal

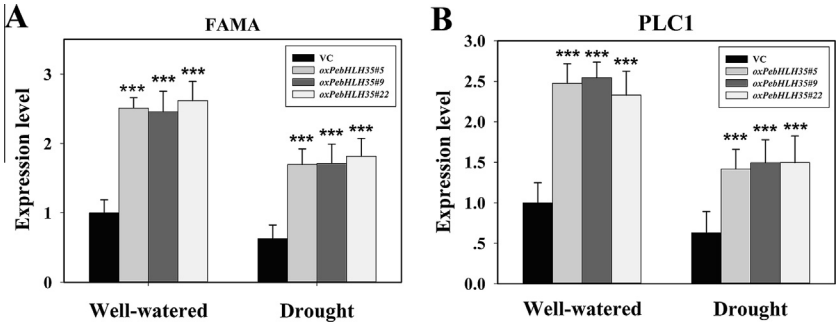

Fig. 4. Quantitative PCR analysis of two marker genes in the *oxPebHLH35s* and VC plants. *AtActin* (At5g09810) was used as an internal control. Each bar represents the mean ± SE ($n = 15$). ***denotes a significant difference ($P < 0.001$) according to the ANOVA results.

aperture, and transpiration rate significantly (Fig. 3B, E, and G). We found that *PebHLH35* regulates not only stomatal density but also stomatal aperture, and the reduction in stomatal density and stomatal aperture conferred by *PebHLH35* overexpression contributed to the deceased transpiration rate and, hence, reduced water loss.

Photosynthesis is an essential process for maintaining plant growth and development. Recent reports have demonstrated that stomatal closure is one of the first responses to drought stress and that it leads to a decline in photosynthetic rate [28]. In our experiments, the photosynthetic rate decreased in both *oxPebHLH35* plants and VC plants under limited water, and the photosynthetic rate in transgenic plants decreased more slowly than that in VC plants. Recent reports have shown that drought-tolerance improvement can be accompanied by the maintenance of a high photosynthetic rate [14], which was seen in our results. Stomata are points of entry for both CO_2 and water, and this presents plants with a functional dilemma [29]. Stomatal closure affects the absorption of CO_2, thereby reducing the photosynthetic capacity and carbon assimilation of plants. In our study, the stomatal density and aperture of the *oxPebHLH35* plants were lower than those of the VC plants, but the photosynthetic rate of the *oxPebHLH35* plants was higher than that of the VC plants. These results indicate that a moderate decrease in stomatal density and aperture in the *oxPebHLH35* plants did not diminish the supply of CO_2 for carbon assimilation, but it did reduce the loss of water. This was consistent with the previous study that decreased stomata conductance within a certain range could cause lower transpiration but equivalent net photosynthesis rate [27].

Several studies have suggested the measurement of chlorophyll fluorescence parameters (e.g., Fv/Fm) as a rapid and accurate approach for detecting and quantifying drought stress tolerance in plants [30]. Higher Fv/Fm of overexpression of *OsbHLH148* indicated *OsbHLH148* increases transgenic rice tolerance to drought stress [10]. Our data show that the Fv/Fm was similar in both *oxPebHLH35* and VC plants under well-watered conditions. The slight reduction in Fv/Fm in the *oxPebHLH35* plants under severe drought conditions compared to the corresponding case in the VC plants indicates that the components of the photosynthetic apparatus may have been damaged in the VC plants, and that the *oxPebHLH35* plants were more efficient in protecting their photosynthetic apparatus under drought stress conditions.

In conclusion, a range of physiological and biochemical responses was induced in the *oxPebHLH35* plants by drought stress. Our results demonstrate that the heterologous expression of *PebHLH35* in *Arabidopsis* confers drought tolerance by reducing stomatal density, stomatal aperture, transpiration rate, water loss, and by improving chlorophyll content and photosynthetic rate.

Acknowledgments

The research was supported by the Hi-Tech Research and Development Program of China (2013AA102701), the National Natural Science Foundation of China (31270656), the 111 Project (B13007), and the Scientific Research and Graduate Training Joint Programs from BMEC (Stress Resistance of Poplar).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.139.

References

[1] H.-B. Shao, L.-Y. Chu, C.A. Jaleel, P. Manivannan, R. Panneerselvam, M.-A. Shao, Understanding water deficit stress-induced changes in the basic metabolism of higher plants-biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe, Crit. Rev. Biotechnol. 29 (2009) 131–151.
[2] L. Xiong, K.S. Schumaker, J.-K. Zhu, Cell signaling during cold, drought, and salt stress, Plant Cell Online 14 (2002) S165–S183.
[3] K. Shinozaki, K. Yamaguchi-Shinozaki, Gene networks involved in drought stress response and tolerance, J. Exp. Bot. 58 (2007) 221–227.
[4] Y. Jiang, B. Yang, M.K. Deyholos, Functional characterization of the *Arabidopsis* bHLH92 transcription factor in abiotic stress, Mol. Genet. Genomics 282 (2009) 503–516.
[5] G. Toledo-Ortiz, E. Huq, P.H. Quail, The *Arabidopsis* basic/helix-loop-helix transcription factor family, Plant Cell Online 15 (2003) 1749–1770.
[6] X.-S. Huang, W. Wang, Q. Zhang, J.-H. Liu, A basic helix-loop-helix transcription factor, PtrbHLH, of *Poncirus trifoliata* confers cold tolerance and modulates peroxidase-mediated scavenging of hydrogen peroxide, Plant Physiol. 162 (2013) 1178–1194.
[7] X.-M. Feng, Q. Zhao, L.-L. Zhao, Y. Qiao, X.-B. Xie, H.-F. Li, Y.-X. Yao, C.-X. You, Y.-J. Hao, The cold-induced basic helix-loop-helix transcription factor gene MdClbHLH1 encodes an ICE-like protein in apple, BMC Plant Biol. 12 (2012) 22.
[8] Y. Yuan, H. Wu, N. Wang, J. Li, W. Zhao, J. Du, D. Wang, H.-Q. Ling, FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis*, Cell Res. 18 (2008) 385–397.
[9] J. Kim, H.-Y. Kim, Functional analysis of a calcium-binding transcription factor involved in plant salt stress signaling, FEBS Lett. 580 (2006) 5251–5256.
[10] J.S. Seo, J. Joo, M.J. Kim, Y.K. Kim, B.H. Nahm, S.I. Song, J.J. Cheong, J.S. Lee, J.K. Kim, Y.D. Choi, OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice, Plant J. 65 (2011) 907–921.
[11] W. Liu, H. Tai, S. Li, W. Gao, M. Zhao, C. Xie, W.X. Li, BHLH122 is important for drought and osmotic stress resistance in *Arabidopsis* and in the repression of ABA catabolism, New Phytol. 2 (2013) 1–7.
[12] E.A. Ottow, A. Polle, M. Brosche, J. Kangasjärvi, P. Dibrov, C. Zörb, T. Teichmann, Molecular characterization of PeNhaD1: the first member of the NhaD Na+/H+ antiporter family of plant origin, Plant Mol. Biol. 58 (2005) 75–88.
[13] S. Tang, H. Liang, D. Yan, Y. Zhao, X. Han, J.E. Carlson, X. Xia, W. Yin, *Populus euphratica*: the transcriptomic response to drought stress, Plant Mol. Biol. 83 (2013) 539–557.
[14] X. Han, S. Tang, Y. An, D.-C. Zheng, X.-L. Xia, W.-L. Yin, Overexpression of the poplar NF-YB7 transcription factor confers drought tolerance and improves water-use efficiency in *Arabidopsis*, J. Exp. Bot. 64 (2013) 4589–4601.
[15] H.-S. Ma, D. Liang, P. Shuai, X.-L. Xia, W.-L. Yin, The salt-and drought-inducible poplar GRAS protein SCL7 confers salt and drought tolerance in *Arabidopsis thaliana*, J. Exp. Bot. 61 (2010) 4011–4019.
[16] J. Chen, X. Xia, W. Yin, Expression profiling and functional characterization of a *DREB2*-type gene from *Populus euphratica*, Biochem. Biophys. Res. Commun. 378 (2009) 483–487.
[17] X. Zhang, R. Henriques, S.-S. Lin, Q.-W. Niu, N.-H. Chua, Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method, Nat. Protoc. 1 (2006) 641–646.
[18] D. Li, S. Song, X. Xia, W. Yin, Two CBL genes from *Populus euphratica* confer multiple stress tolerance in transgenic triploid white poplar, Plant Cell, Tissue Organ Cult. 109 (2012) 477–489.
[19] Z.-M. Pei, K. Kuchitsu, J.M. Ward, M. Schwarz, J.I. Schroeder, Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and abi1 and abi2 mutants, Plant Cell Online 9 (1997) 409–423.
[20] W.-H. Cao, J. Liu, X.-J. He, R.-L. Mu, H.-L. Zhou, S.-Y. Chen, J.-S. Zhang, Modulation of ethylene responses affects plant salt-stress responses, Plant Physiol. 143 (2007) 707–719.
[21] Z. Shu, X. Zhang, C.G. ChenJ, D. Xu, The simplification of chlorophyll content measurement, Plant Physiol. Commun. 46 (2010) 399–402.
[22] D.-H. Yan, T. Fenning, S. Tang, X. Xia, W. Yin, Genome-wide transcriptional response of *Populus euphratica* to long-term drought stress, Plant Sci. 195 (2012) 24–35.
[23] N. Pires, L. Dolan, Origin and diversification of basic-helix-loop-helix proteins in plants, Mol. Biol. Evol. 27 (2010) 862–874.
[24] L.P. Manavalan, S.K. Guttikonda, L.-S.P. Tran, H.T. Nguyen, Physiological and molecular approaches to improve drought resistance in soybean, Plant Cell Physiol. 50 (2009) 1260–1276.
[25] Y. Ohashi, N. Nakayama, H. Saneoka, K. Fujita, Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants, Biol. Plant 50 (2006) 138–141.
[26] G. Castilhos, F. Lazzarotto, L. Spagnolo-Fonini, M.H. Bodanese-Zanettini, M. Margis-Pinheiro, Possible roles of basic helix-loop-helix transcription factors in adaptation to drought, Plant Sci. 223 (2014) 1–7.
[27] C.Y. Yoo, H.E. Pence, J.B. Jin, K. Miura, M.J. Gosney, P.M. Hasegawa, M.V. Mickelbart, The *Arabidopsis* GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1, Plant Cell Online 22 (2010) 4128–4141.
[28] S.A. Anjum, X.-Y. Xie, L. Wang, M.F. Saleem, C. Man, W. Lei, Morphological, physiological and biochemical responses of plants to drought stress, Afr. J. Agric. Res. 6 (2011) 2026–2032.
[29] L. Taiz, E. Zeiger, Plant Physiology, Sinauer Associates, Sunderland, MA, USA, 2002.
[30] G.C. Percival, C.N. Sheriffs, Identification of drought-tolerant woody perennials using chlorophyll fluorescence, J. Arboric. 28 (2002) 215–223.