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ORIGINAL RESEARCH PAPER

Ectopic expression of secretory peptide PdEPF3 in *Arabidopsis* confers drought tolerance with reduced stomatal density

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Abstract
Drought is one of the most prevalent environmental stresses that affect plant growth and development. Improvement in drought tolerance is associated with reduced stomatal density and higher water use efficiency (WUE). In this study, an epidermal patterning factor (EPF), PdEPF3, from a fast-growing poplar clone, NE-19 [*Populus nigra* × (*Populus deltoides* × *Populus nigra*)], was characterized. Quantitative reverse transcription polymerase chain reaction showed that the transcription of *PdEPF3* was induced by drought. We further found that the transgenic *Arabidopsis* overexpressing *PdEPF3* had an earlier seedling germination and longer primary roots under osmotic stress treatments, compared with the WT and mutant *epf1-1*. In addition, ectopic overexpression of *PdEPF3* in *Arabidopsis* plants was able to enhance drought tolerance. This observation was associated with the reduced stomatal density of transgenic plants, which would limit transpiration and reduce water loss, consequently improving the WUE of plants. Interestingly, the reduction of stomatal density in transgenic plants overexpressing *PdEPF3* did not affect their photosynthetic capacity. These results indicate that *PdEPF3* could be used in transgenic breeding to enhance plant drought tolerance.

Keywords
drought; *PdEPF3*; stomatal density; water use efficiency (WUE)

Introduction

At present, 65% of the world's agroforestry production is affected by drought, with developing countries accounting for more than 80% of this proportion [1]. For the sustainable development of agriculture and environment, it is important to identify key genes that enhance drought tolerance without reducing yield or biomass [2]. With the development of molecular biology and genetics, more researchers are focusing on the use of technology to improve the water use efficiency (WUE) of plants. Water use efficiency is defined as the ratio of carbon assimilation to transpiration, or the ratio of biomass production to water consumption. Compared with other engineering techniques for water saving, breeding new varieties with a high WUE and drought resistance has several advantages, such as: low investment and sustainable efficiency [3].

As a part of the adaptation towards environmental stresses, plants have evolved delicate regulatory mechanisms to sense and respond to these stresses [4]. The most obvious and important management strategies for plants to cope with drought stress are minimizing water loss through closure of stomata and reduction in stomatal density [2]. Stomata, which are surrounded by two guard cells, are the primary regulators of

carbon dioxide uptake and water loss in plants [5]. As most of the water absorbed by a plant is lost to the air through transpiration, the regulation of stomatal behavior (movement and development) is an important factor affecting WUE and drought resistance [6]. The balance between carbon gain through photosynthesis and water loss through transpiration affects WUE. Under drought stress, plants tend to close their stomata to conserve water and improve WUE [7].Water use efficiency is an important basis for the functional identification of novel drought gene resources. Several studies have demonstrated the possibility of improving drought tolerance and WUE by reducing the stomatal density on leaves. By using genetic manipulation or mutation to reduce stomatal density, improvements in WUE have been achieved across several model species, including *Arabidopsis* [8–11], poplar [12], wheat [13], barley [14], and tobacco [2].

Over the past decade, a good understanding of the signaling pathways that control the formation of stomata has been developed. A central feature in stomatal development is the "one-cell spacing" rule, in which two stomata are separated by at least one intervening nonstomatal epidermal cell [15]. This spacing is presumed to provide the necessary position for gas exchange and ion exchange [5]. During the process of plant growth and development, the stomata or their precursors ensure "one-cell spacing" pattern by sending a cell-to-cell signal to the undifferentiated neighbor cells, which, in turn, modulates their cell division [16]. Cell-to-cell signaling is essential for many processes in plant growth and development. Secreted peptides are important cell-to-cell signaling components that coordinate and specify cellular functions in plants. Extracellular and cross-tissue communication via known peptide ligands, such as EPF1, EPF2, and STOMAGEN, received by TMM or ERECTA family receptors, could potentially provide the spatial information to position stomata away from the underlying cells [17–19].

We had previously reported the isolation of *PdEPF1* and *PdEPF2* from poplar, and demonstrated their role in improving the WUE and conferring drought tolerance in the plant [12,20]. In this manuscript, an additional member of the EPF family, *PdEPF3*, was isolated, and its functions were analyzed. We demonstrate that the ectopic overexpression of *PdEPF3* reduced the stomatal density and water loss mediated by, thus increasing drought tolerance.

Material and methods

Plants material and growth conditions

Arabidopsis mutant and transgenic lines were used in the Columbia-0 (Col-0) background. *Arabidopsis thaliana* ecotype Col-0 was used as the wild-type (WT) control. Seeds of Col-0 and the T-DNA insertion mutant *epf1-1* (SALK_137549) were obtained from the ABRC. All the seeds were surface sterilized for 1 min in 75% ethanol followed by 10 min in 1% NaClO and five washes in sterilized distilled water. Seeds were sown on half-strength Murashige and Skoog (1/2 MS) [21] plates supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar. The seeds were stratified for 2 days at 4°C before transfer to a growth room maintained at 22°C under a 16 h / 8 h (white light/dark) photoperiod, 80% relative humidity, and 150 μmol m^{-2} s^{-1} irradiation. Seven days after the germination, the seedlings were transplanted and grown at a density of four plants per pot (7 × 7 × 6.5 cm) containing a mixture of rich soil and vermiculite (2:1, v/v), under a 16/8 light/dark photoperiod at 22°C, 70% relative humidity, and 150 μmol m^{-2} s^{-1} irradiation.

PdEPF3 gene cloning, transformation, and expression analysis

Total RNA isolation was performed using the CTAB reagent method [22]. First-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase and an oligo (dT) primer (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The full-length cDNA of *PdEPF3* was retrieved by homologous cloning, using gene-specific primers FW 5′-ATGAGAAAAACCAAAGCCATGAAG-3′ and

RV 5′-TCATGGGACAGGGTAAGACTT-3′. The PCR products were cloned into the pMD18-T cloning vector (Promega, Madison, WI, USA) and were subsequently subjected to sequencing analysis. The amplified *PdEPF3* open reading frame was inserted into the XbaI and BamHI sites in the pBI121 vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter, to generate the fusion construct *35S:PdEPF3*.

The constructs were introduced into *Agrobacterium tumefaciens* (strain GV3101) and transferred into *Arabidopsis* plants (wild type and mutant) using the floral dip method [23]. The transformed lines were screened on hygromycin-supplemented media (containing 50 mg/L hygromycin) to generate independent transgenic lines. The homozygous T3 lines were used for further analysis.

For subcellular localization of *PdEPF3* in leaf epidermal cells, PdEPF3-GFP fusion proteins were observed using a confocal laser scanning microscope (DMI6000 CS; Leica, Wetzlar, Germany).

RT-PCR and qRT-PCR analysis

Total RNA from each sample was extracted by the CTAB method. Quantitative real time-PCR (qPCR) was performed as described by previously [24]. SYBR GREEN Master Mix (TianGen Bio Inc., Beijing, China) was used to monitor the kinetics of PCR product formation during qPCR. UBQ was used as an internal control to quantify the relative transcript level of *PdEPF3*. Gene-specific primers were designed using the Primer6 (PrimerE, Ivybridge, UK), and the primer sequences are shown in Tab. S1.

Morphological characterization

To compare the morphological differences among ox*PdEPF3*, WT, *epf1-1*, and *epf1-1*/ox*PdEPF3* plants under well-watered condition, rosette leaves were removed from 22-day-old seedlings, and the leaf area was computed using PHOTOSHOP CS5. The plant height was measured at 25 and 40 days after germination.

Characterization of germination rate and root length

For osmotic treatment experiments, *Arabidopsis* seeds were sown on 1/2 MS agar plates supplemented with 200 mM mannitol and 25 mM PEG6000 [25]. Three independent batches of seeds were used to compare the germination rate among ox*PdEPF3*, WT, *epf1-1*, and *epf1-1*/ox*PdEPF3* plants. For the root length assay, 5-day-old seedlings grown on 1/2 MS agar plates were transferred to vertically oriented 1/2 MS agar plates supplemented with 200 mM mannitol and 25 mM PEG6000 and grown for 10 days before the root length was measured. Six seeds from each line were used to compare the root lengths. All experiments were repeated three times.

Drought stress and water loss measurements

For testing the phenotype under drought stress, seedlings grown on 1/2 MS medium for 7 days were transferred to a mixed soil and grown for 3 weeks with sufficient watering. Each was planted with four seedlings planted. The plants were then subjected to drought stress treatment by withholding irrigation for 12 days. The plants were rewatered for 1 week after significant differences in wilting were observed. All the pots were subjected to under identical conditions within a growth chamber. The photographs were taken after withholding water or rewatering for the indicated times. To evaluate the water loss rate of the plants, rosette leaves of transgenic plants, WT, *epf1-1*, and *epf1-1*/ox*PdEPF3* growing under normal conditions for 3 weeks were detached. The leaves were harvested from the same stage of different plants (about 0.5 g) and weighed immediately on a piece of weighing paper. They were then placed on a laboratory bench and weighed every 30 min, with three replicates. The percentage of fresh weight lost was calculated based on the initial weight of the leaves.

Measurement of leaf stomatal density

To measure the stomatal density, leaves of the same age and from the same elative position were sampled from the overexpression transgenic plants, WT, *epf1-1*, and *epf1-1*/ox*PdEPF3* grown under identical conditions. The fixed sample method was used as described previously [20]. The fixed samples were dried and then set up on an aluminum plate for coating with gold. These samples were observed by scanning electron microscopy (Hitachi S-3400 N; Chiyoda-ku, Tokyo, Japan). For statistical analysis of stomatal density, three leaves were sampled for each plant, and three individual plants were included in each group. Different letters indicate significant differences at $\alpha = 0.05$ (one-way ANOVA). Data are presented as mean ±*SE*.

Leaf gas-exchange measurements

The net photosynthetic (A) and transpiration rates (E) were measured using the Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). Data were recorded during 9 a.m. – 11 a.m. At the time of data recording, the ambient CO_2 concentration was 400 μmol mol^{-1}, the photosynthetic photon flux density was 600 μmol m^{-2} s^{-1}, and the chamber temperature was 24°C. Water use efficiency (WUE) was calculated from instantaneous values of A and E.

Results

Molecular characterization of PdEPF3

We identified a putative poplar peptide which was homologous to PdEPF1 [12], and designated this peptide as PdEPF3 (GenBank accession number MF461306). A comparison showed that PdEPF3 shared a high sequence similarity and clustered with PdEPF1, and similar to PdEPF1 and AtEPF1, it also contains eight conserved cysteine residues at the C-terminal (Fig. 1A). The *PdEPF3* cDNA is 387 bp in length and encodes 128 amino acid residues with a predicted molecular mass of 14.25 kDa and an isoelectric point of 9.53 (http://web.expasy.org/compute_pi/). The subcellular localization results showed that the localization of PdEPF3-GFP could be in cytosol, plasma membrane, or cell wall. An empty vector with GFP served as the positive control (Fig. 1B).

Expression profile of *PdEPF3*

The transcription of *PdEPF3* was tissue-specific. Transcripts were detected in roots, stems, young leaves, mature leaves, and senescent leaves of NE-19 under nonstress growth conditions. The results showed that *PdEPF3* was expressed more highly in young leaves and mature leaves than in senescent leaves, but was the lowest in root (Fig. 2A). Under drought stress, *PdEPF3* transcription initially increased but subsequently decreased during days 3–12 of drought treatment (Fig. 2B).

Analysis of transgenic *Arabidopsis* overexpressing *PdEPF3*

To elucidate the in vivo functions of *PdEPF3*, the *35S:PdEPF3* construct was transformed into WT *Arabidopsis* and the *epf1-1* mutant, to generate overexpression and complementation lines, respectively. We raised nine independent transgenic lines, of which three were selected, based on the qRT-PCR results. As expected, *PdEPF3* transcripts were undetectable in the WT plants. Three of the overexpression lines (Lines 2, 4, and 5) showed higher transcript levels than the other overexpression lines (Fig. S1), and were designated as ox*PdEPF3#1*, *#2*, and *#3*.

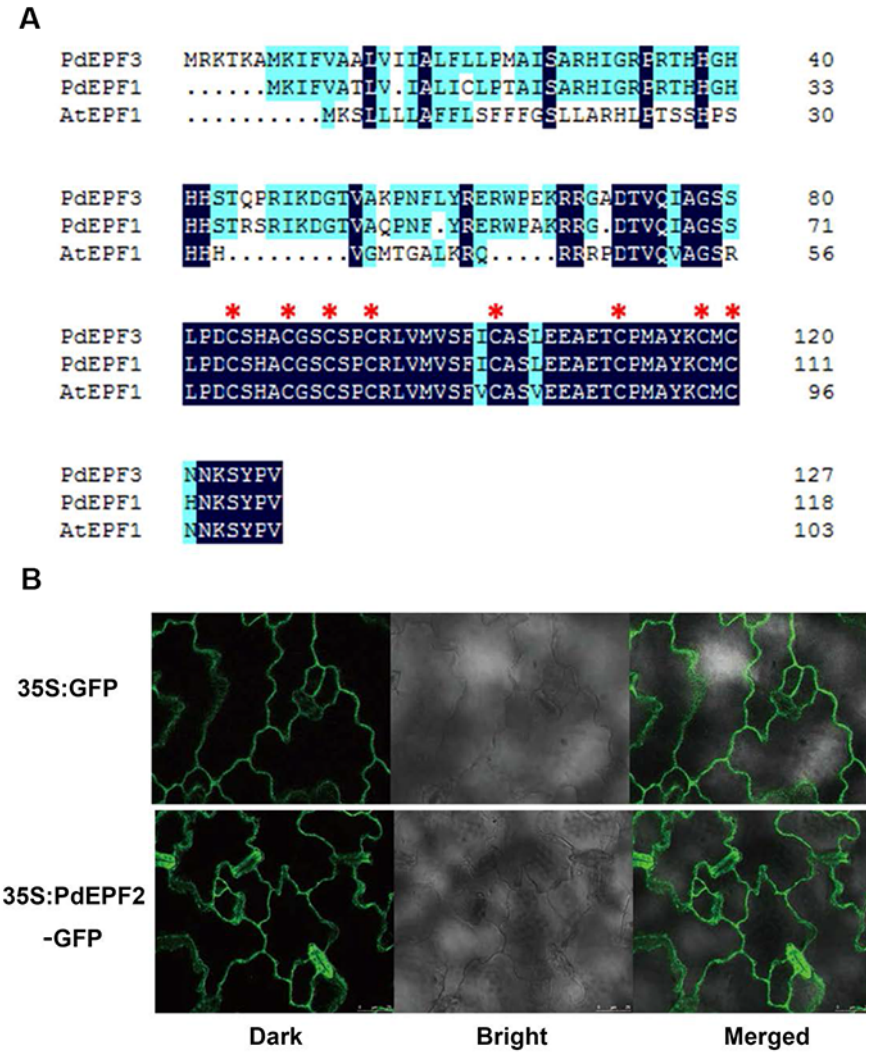

Fig. 1 The identification of poplar *PdEPF3* gene. (**A**) Alignment of the putative PdEPF3 mature signaling peptide with the signaling peptides PdEPF1 and AtEPF1. Conserved Cys residues are marked with red asterisks. Amino acid sequences for the mature peptide region were aligned using DNAMAN. (**B**) Subcellular localization of 35S:PdEPF3-GFP and control 35S:GFP expression in epidermal leaf cells. The photographs were taken in dark field, bright field, and merged field.

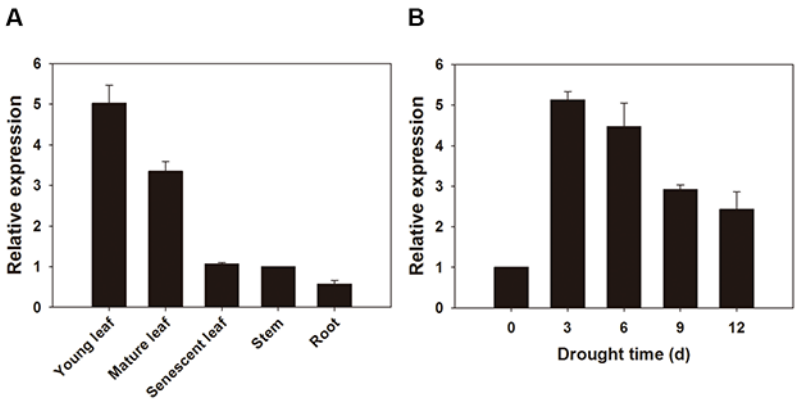

Fig. 2 Expression patterns of *PdEPF3*. (**A**) Tissue-specific expression pattern of *PdEPF3* in poplar. Young leaf – the first 1–3 leaves from the shoot apex; mature leaf – a fully expanded leaf; senescent leaf – lower most two to three leaves above the root system. The level of the *PdEPF3* transcript in the stem was set as 1. (**B**) Expression of *PdEPF3* in response to drought in poplar. Data represent means ±*SE* ($n = 3$).

Morphological analysis of ox*PdEPF3* lines under well-watered condition

At 22 days after germination, the ox*PdEPF3* lines had larger leaves than WT and *epf1-1*. The rosette leaf area of the ox*PdEPF3* lines was 13.7–21.4% and 44.16–53.93% larger than that of WT and *epf1-1*, respectively (Fig. 3A). As shown in Fig. 3B, after 25 days after germination, the inflorescence length of ox*PdEPF3* lines was longer than that of WT and *epf1-1*. Forty days after germination, the inflorescence length of all plants reached 400 mm. This result indicates that the growth rates of ox*PdEPF3* lines, WT, and *epf1-1* were different, but they eventually became consistent in morphology.

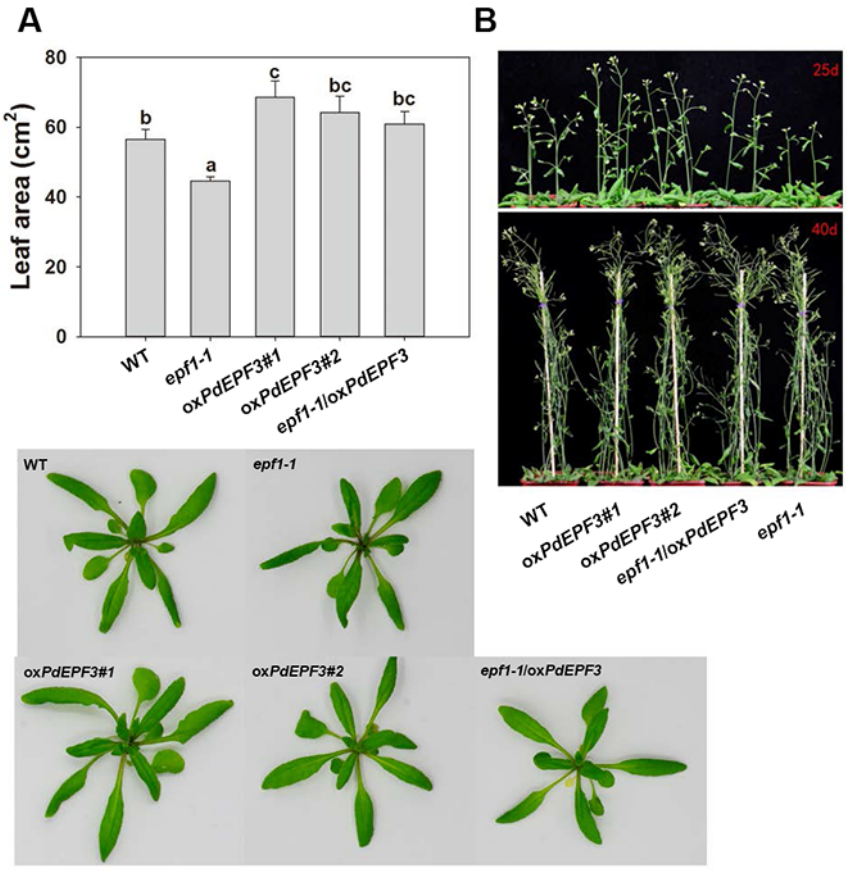

Fig. 3 Morphological analysis under well-watered condition. (**A**) Difference in rosette leaf area of 22-day-old seedlings. (**B**) Morphology of 25-day-old and 40-day-old seedlings grown under well-watered condition. Data represent means ±*SE* ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

Overexpression of *PdEPF3* enhanced the tolerance of *Arabidopsis* to drought stress

To study the drought-tolerance mechanism, various experimental setups were developed. The seeds of transgenic lines, WT, *epf1-1*, and the complemented line *epf1-1*/ox*PdEPF3* were sown on 1/2 MS culture with 200 mM mannitol and 25 mM PEG6000, for osmotic stress. After 7 days, in the presence of 200 mM mannitol, the ox*PdEPF3* lines had more vigorous germination (74.7–81.9%) than WT (63.3%) and the mutant (42.2%) (Fig. 4A). In the presence of 25 mM PEG6000, ox*PdEPF3* lines had more vigorous germination (83.4–84.8%) than WT (38.1%) and the mutant (28.7%) (Fig. 4B). However, in comparison, all the seeds of transgenic lines, WT, *epf1-1*, and *epf1-1*/ox*PdEPF3* had sprouted after 7 days under normal conditions (data not shown). In addition, when 5-day-old seedlings grown on 1/2 MS medium were transferred to vertical agar plates containing 1/2 MS medium supplemented with 200 mM mannitol and 25 mM PEG6000, the primary root lengths of the 10-day-old seedlings were also different. In the presence of 200 mM mannitol, ox*PdEPF3* lines had considerably longer (1.6- and 1.8-times, respectively)

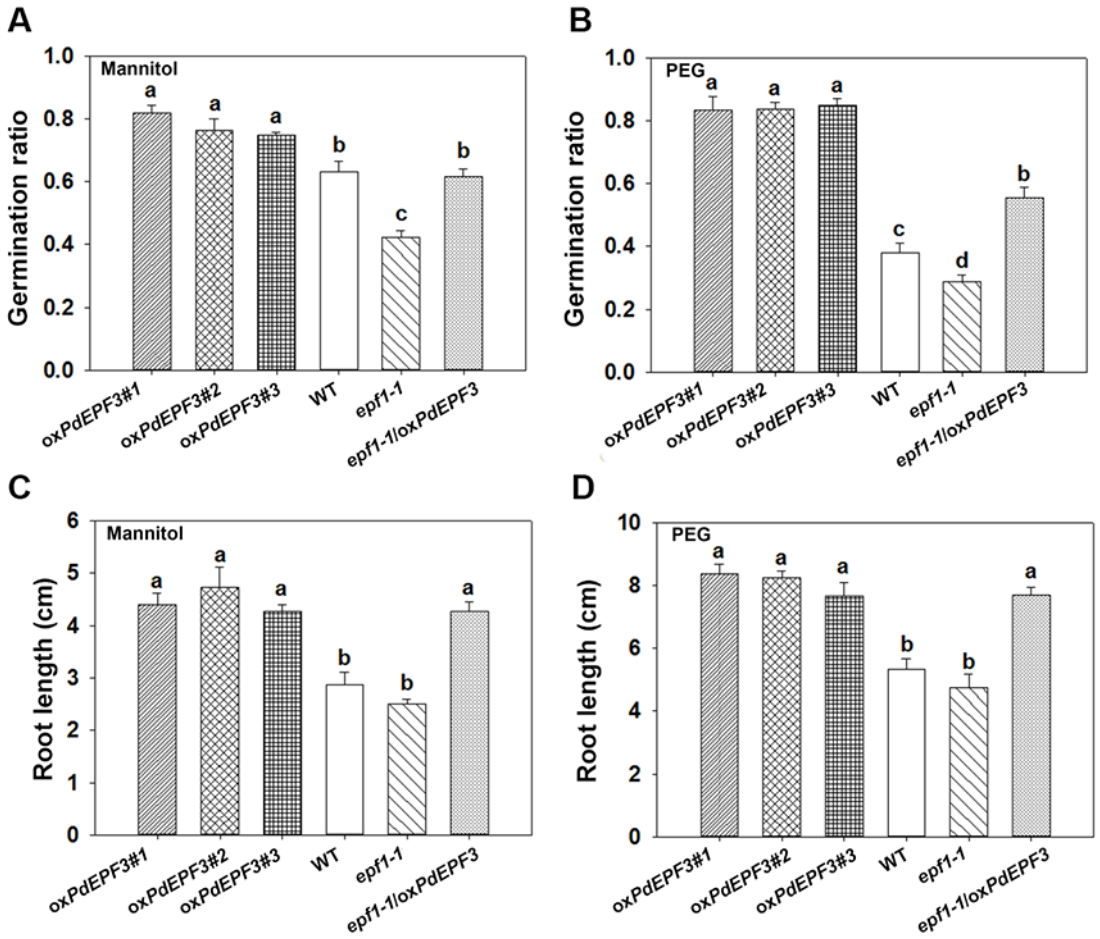

Fig. 4 Differences in the germination ratio and root length in *Arabidopsis*. (**A**) Germination rate under 1/2 MS + 200 mM mannitol treatment. (**B**) Germination rate under 1/2 MS +25 mM PEG6000 treatment. (**C**) Root length of 1/2 MS + 200 mM mannitol treatment. (**D**) Root length of 1/2 MS + 25 mM PEG6000 treatment. Data are means ±*SE* ($n = 6$). Different letters indicate significant difference ($p < 0.05$).

primary roots than that of WT and the mutant, *epf1-1* (Fig. 4C). In the presence of 25 mM PEG6000, ox*PdEPF3* lines had longer (1.5- and 1.7-times, respectively) primary roots than that of WT and the mutant *epf1-1* (Fig. 4D).

The assays of water loss from detached leaves showed that the *epf1-1* plants lost water much more quickly than the WT plants, and that the ox*PdEPF3* plants lost water much more slowly than both WT and *epf1-1* plants (Fig. 5A). The fresh weight of transgenic plants was reduced to 73.6% for Line 1, 68.5% for Line 2, 66.9% for Line 3, 59.9% in wild-type plants, and 45.6% in the *epf1-1* mutant.

The capacity of ox*PdEPF3* lines to respond to drought stress was investigated next. As shown in Fig. 5B, the ox*PdEPF3* plants did not show any difference in their phenotype compared with the WT and mutant plants under the control conditions, but they did show much stronger tolerance to drought stress than WT and the mutant plants under the drought stress. These results confirmed that *PdEPF3* overexpression can greatly improve the drought resistance of transgenic *Arabidopsis*.

Ectopic overexpression of *PdEPF3* decreases stomatal density and improves WUE in *Arabidopsis*

To clarify the physiological mechanism responsible for the increased drought tolerance of *PdEPF3*-overexpression lines, the stomatal density on the abaxial leaf epidermis of 3-week-old rosette leaves was studied. The stomatal density in the ox*PdEPF3* plants was 20–24% less than that of the WT plants, and almost 50% less than that of the *epf1-1* mutant plants (Fig. 6A). The reduced stomatal density apparently contributes to the

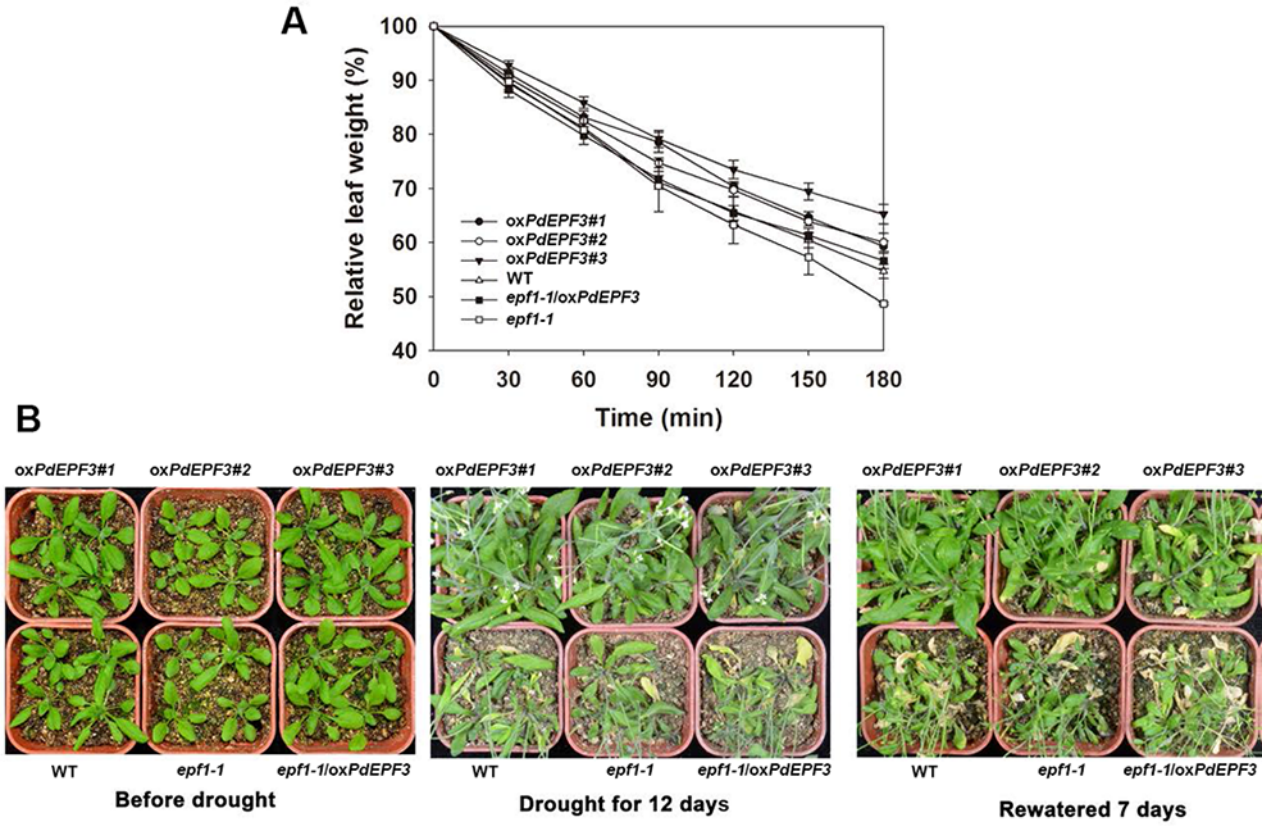

Fig. 5 Overexpression of *PdEPF3* confers drought tolerance in *Arabidopsis*. (**A**) Water loss from detached leaves; water loss from a 0.5-g sample of detached leaves per plant was measured at the indicated time points. Three measurements were averaged for each time point. Data are means ±*SE*. (**B**) Morphological differences in drought experiments. The seedlings were grown in soil for 3 weeks under well-watered conditions; thereafter, water was withheld for 12 days, and then the plants were rewatered for 1 week.

reduced rate of water loss in transgenic plants. The *epf1-1* mutant also showed a defect in the one-cell-spacing rule of stomatal pattern, marked by the red arrow in Fig. 6B. This overexpression phenotype of *PdEPF3* indicated that this protein is capable of negatively affecting stomatal patterning. Reduced stomatal density is known to affect water and CO_2 exchange [26]. Therefore, we measured the photosynthetic and transpiration rates of all lines and found that the ox*PdEPF3* plants maintained a significantly higher photosynthetic rate and lower transpiration rate than the WT and *epf1-1* plants under drought stress (Fig. 7A,B). Consequently, the WUE is higher for the transgenic plants (Fig. 7D). These findings indicated that the lower stomatal density was one of the reasons for the higher drought tolerance of *35S:PdEPF3 Arabidopsis* compared with the WT and *epf1-1* mutant.

Discussion

Water is essential for plant growth because it plays an important role in many cellular functions. Biomass production and accumulation are negatively affected by water deficit because such conditions reduce the leaf cell turgor, restrict cell expansion, and postpone development [22]. Researchers have found that most of the water loss from leaves occurs through the stomata, which is known as stomatal transpiration, and reduced transpiration is an important aspect of plant drought resistance [27]. Regulation of stomatal behavior has been an active area of research on drought stress. Stomatal density may be a target trait for plant engineering to improve the photosynthetic capacity, particularly in future high-CO_2 atmospheres [9,26,28,29].

In this present study, *PdEPF3* was cloned from poplar. According to the analysis, PdEPF3 shared high amino acid sequence similarity with and clustered with PdEPF1, which improves WUE and confers drought tolerance in transgenic poplar by decreasing

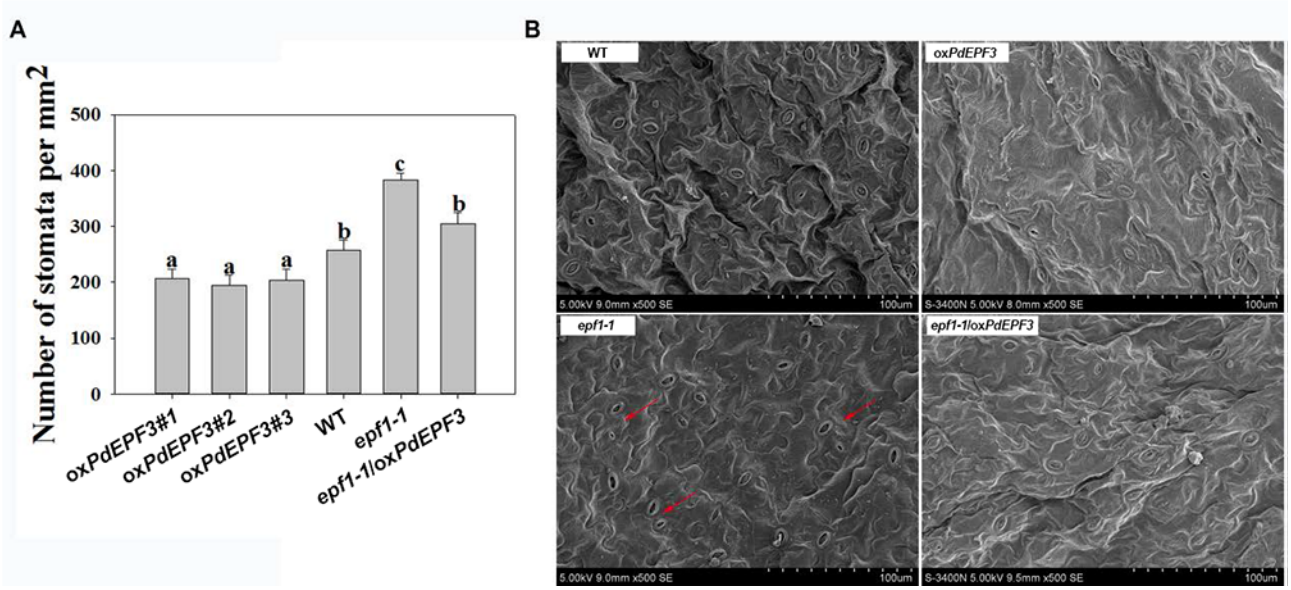

Fig. 6 Effect of *PdEPF3* on stomatal density. (**A**) Abaxial stomatal densities in WT, transgenic plants, *epf1-1* mutant, and the *epf1-1*/ox*PdEPF3* complementation line. Different letters indicate significant difference ($p < 0.05$). (**B**) Scanning electron micrograph of the abaxial leaf epidermis. Scale bars represent 100 μm.

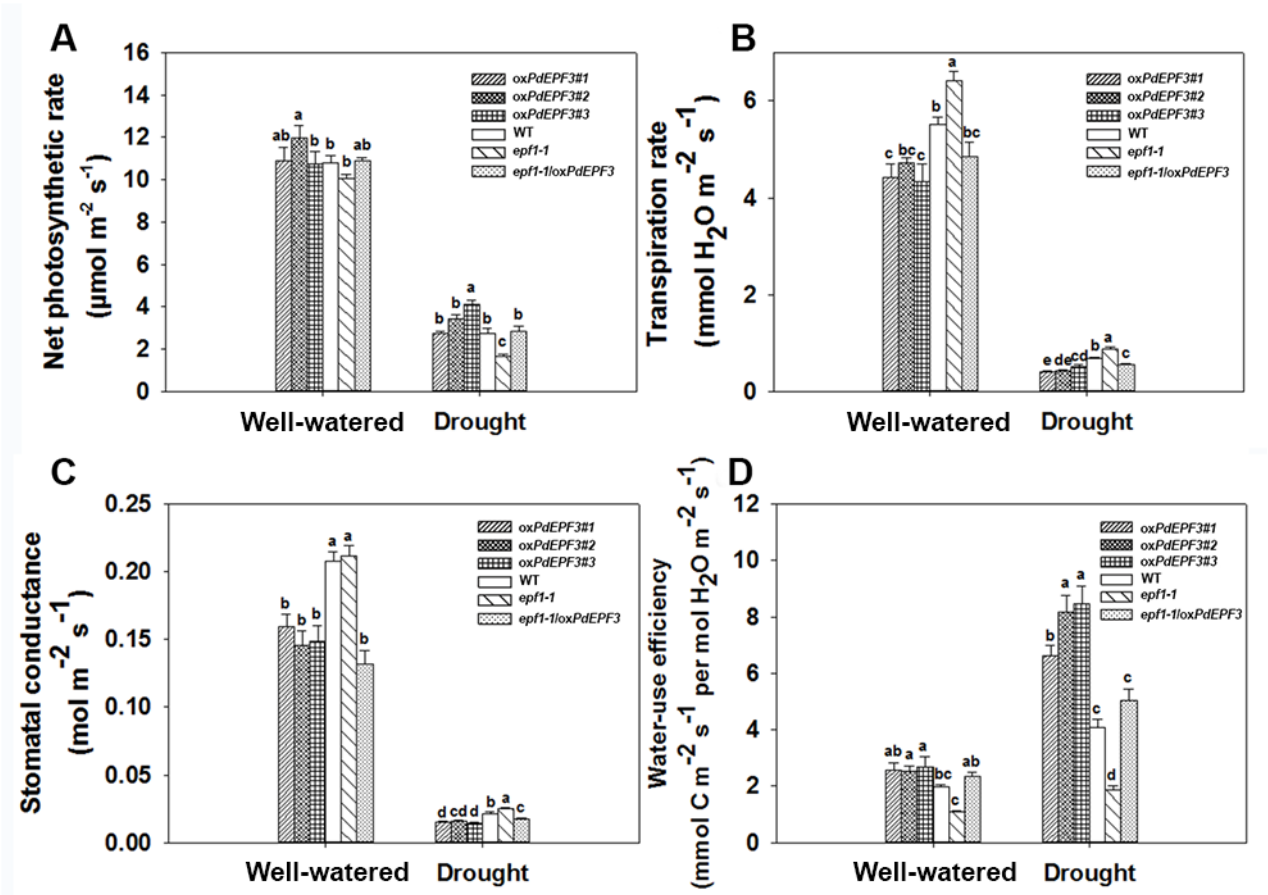

Fig. 7 Physiological analysis of ox*PdEPF3* lines under drought stress. Net photosynthetic rate (**A**) transpiration rate (**B**), stomatal conductions (**C**), and instantaneous WUE (**D**) of leaves under well-watered and drought conditions. Data represent means ±*SE* ($n = 9$).

the stomatal density [12]. PdEPF3 also contains eight conserved cysteine residues at the C-terminal, similar to PdEPF1 and AtEPF1 (Fig. 1A).

Under abiotic stress conditions, the expression of many genes is induced, and their products have important roles in stress responses and tolerance. In the current study, expression analysis showed that *PdEPF3* was concomitantly induced in response to water deficit in the NE-19 seedlings (Fig. 2B). Overexpression of *PdEPF3* in *Arabidopsis* exhibited earlier seedling germination time and longer primary roots under the osmotic stress conditions (Fig. 4). Roots are the organs that absorb water and nutrients from soil. Therefore, the root growth state directly effects the growth and development of the whole plant [30]. In addition, *PdEPF3* overexpression in transgenic plants also maintained a higher leaf water potential than that of wild type and *epf1-1* mutant under water-deficit conditions (Fig. 5). Stomata are the main portals of gas exchange between the plant above-ground organs and the atmosphere and have a strong effect on the characteristics associated with photosynthesis and transpiration [31]. *AtEPF1* is a well-characterized negative regulator of stomatal development in *Arabidopsis thaliana* [32]. Through ectopic overexpression of *PdEPF3*, we created *Arabidopsis* transformants with reduced stomatal density: the ox*PdEPF3* plants had 20–24% lower stomatal density than the WT plants, and almost 50% lower density than the *epf1-1* mutant (Fig. 6A). Decreased stomatal density was a primary factor underlying the enhanced drought resistance in ox*PdEPF3 Arabidopsis*. This decrease in stomatal density and conductance most likely allows plants to fine tune their responses to the environment and exercise a better control of CO_2 assimilation and water utilization [33,34]. Consistent with this, transgenic *Arabidopsis* overexpressing *PdEPF3* showed significantly higher WUE under drought conditions (Fig. 7D). Theoretically, stomatal density is negatively correlated with both photosynthesis and transpiration, whereas, our results indicated that the ox*PdEPF3* plants maintained a higher photosynthetic rate and lower transpiration rate than those of the WT and *epf1-1* plants under drought stress (Fig. 7A,B). Moreover, Franks et al. [10] reported that, in *Arabidopsis*, a reduction in stomatal conductance via reduced stomatal density in *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*)-overexpressing plants increased both instantaneous and long-term WUE, without significantly altering the photosynthetic capacity [10]. In rice, it has been reported that an *Arabidopsis* home domain-leucine zipper transcription factor, Enhanced Drought Tolerance / HOMEODOMAIN GLABROUS11 (EDT1/HDG11), was able to confer drought tolerance and increase the grain yield in transgenic plants. Furthermore, the improved drought tolerance was associated with a more extensive root system, reduced stomatal density, and higher water use efficiency [35]. The *Arabidopsis GT-2 LIKE 1* loss-of-function mutations (*gtl1*) result in increased water deficit tolerance and higher integrated WUE by reducing the daytime transpiration without a reduction in biomass accumulation [9]. Therefore, the above-reported traits exemplify the adaptive mechanisms in plants that help them survive under drought stress without loss of productivity or yield.

In this study, we identified and isolated *PdEPF3*, and showed that its expression in *Arabidopsis* plants improved drought tolerance. Our findings suggest that this gene may be useful in the breeding of drought-tolerant plants, and in protecting and optimizing the plant yields under future drier environments. Further experiments are needed to generate additional transgenic poplars, and to elucidate the regulatory mechanisms of drought tolerance in woody plants.

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Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3627/0:

Tab. S1 Gene-specific primers used for qRT-PCR and RT-PCR.

Fig. S1 qRT-PCR was used for the analysis of *PdEPF3* transcription.

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