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ORIGINAL ARTICLE

Secretory peptide *PdEPF2* enhances drought tolerance by modulating stomatal density and regulates ABA response in transgenic *Arabidopsis thaliana*

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Abstract Water deficit limits the growth and productivity of plants worldwide. Improved water use efficiency (WUE) and drought tolerance are important adaptations to address these limitations. In this study, an epidermal patterning factor (EPF), *PdEPF2*, from a fast-growing poplar clone NE-19 (*Populus nigra* × (*Populus deltoids* × *Populus nigra*)) was isolated. Quantitative reverse transcription polymerase chain reaction showed that transcription of this gene was induced by drought and abscisic acid (ABA). To study the biological functions of *PdEPF2*, transgenic *Arabidopsis* plants harboring (35S:*PdEPF2*) in which *PdEPF2* was constitutively expressed were generated. Compared with the wild type and *epf2-3* mutant, the transgenic plants ectopically expressing *PdEPF2* showed favorable osmotic parameters, such as seed germination rate, primary root length, proline and chlorophyll content, Fv/Fm, photosynthetic rate, and instantaneous leaf WUE, under drought stress. In addition, the transgenic *Arabidopsis* plants displayed enhanced drought tolerance as a result of decreased stomatal density, which would limit transpiration and reduce water loss. Compared with the wild-type, plants that overexpressed *PdEPF2* had decreased sensitivity to exogenous ABA during germination and seedling development, whereas the *epf2-3* mutant showed increased sensitivity to ABA. Furthermore, *PdEPF2* positively regulated expression of two ABA signaling-related genes, *ABI1* and *ABI2*. These findings indicate that *PdEPF2* may enhance drought tolerance by regulating stomatal density and the response to the ABA signaling pathway.

Keywords Abscisic acid · Drought · *PdEPF2* · Stomatal density · Water use efficiency

Introduction

Plants have developed adaptations to cope with adverse environmental conditions, such as drought, high salinity, and cold, as these abiotic stresses can limit growth and development (Zhu et al. 2007; Nakashima et al. 2009; Xing et al. 2011). Moreover, drought is a primary environmental constraint on plant growth and crop production, especially in arid and semi-arid regions. Water use efficiency (WUE) (defined as yield per unit of water) represents the relationship between biomass production and water consumption. Specifically, leaf WUE is the ratio of photosynthesis to transpiration and is also referred to as transpiration efficiency (Karaba et al. 2007). Under water-limited conditions, high WUE is necessary to maintain high biomass production (Han et al. 2013) and thus high WUE is imperative to improve and stabilize crop productivity under drought conditions (Bhatnagar-Mathur et al. 2007).

Increased WUE is achieved when plants enhance photosynthetic assimilation and reduce transpirational water loss. Stomata are major regulators of carbon dioxide uptake and water loss in plants (Hetherington and Woodward 2003). In addition, stomatal conductance regulates gas exchange through stomatal movements (opening and closing) and density (Chaerle et al. 2005; Yoo et al. 2009).

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Alteration of the stomatal aperture in response to drought is a short-term means through which plants cope with fluctuating water availability, although this strategy affects photosynthesis and WUE (Chaves et al. 2003; Kim et al. 2010). To adapt to long-term water deficits, plants adjust stomatal density during development. This modification of stomatal density varies between species and with drought severity (Quarrie and Jones 1977; Xu and Zhou 2008; Silva et al. 2009) and is closely associated with WUE and stomatal development (Yoo et al. 2010).

Stomatal development has been well studied in *Arabidopsis*. Each developmental transition is regulated by three basic helix–loop–helix (bHLH) transcription factors, namely SPEECHLESS (MacAlister et al. 2007), MUTE (Pillitteri et al. 2007) and FAMA (Ohashi-Ito and Bergmann 2006). In addition, different epidermal patterning factor (EPF) family members play different roles in stomatal development. For example, EPF2 modulates protodermal cell differentiation into meristemoid mother cells (Hara et al. 2009). EPFf peptides interact with three leucine-rich repeat receptor-like kinases (ERECTA, ERECTA-LIKE1, and ERECTA-LIKE2) and a leucine-rich repeat receptor-like protein called TOO MANY MOUTHS (TMM). These receptors contain extracellular leucine-rich repeat domains that often promote protein–protein interactions (Shpak et al. 2005; Bhave et al. 2008). Biochemical studies using biosensor chips have shown that ER-EPF2 was the predominant ligand-receptor pairs (Lee et al. 2012). In addition, stomatal development is regulated by environmental factors such as light, CO_2, temperature, humidity, drought, and abscisic acid (ABA) (Royer 2001; Casson et al. 2009; Casson and Hetherington 2010; Tricker et al. 2012; Chater et al. 2014).

Stomatal development, including the density and size of stomata, is the primary defense mechanism of plants. Regulation of stomatal development may lead to increased WUE and drought tolerance (Boccalandro et al. 2009; Wang et al. 2014). Most previous research has focused on herbaceous plants, such as *Arabidopsis thaliana*, and few studies have examined mechanisms in woody plants. Poplars (*Populus* spp.) are among the fastest-growing trees in temperate climates, and their high productivity strongly depends on water availability (Tschaplinski and Blake 1989; Zsuffa et al. 1996; Monclus et al. 2006). In addition, poplar is generally easy to regenerate in vitro and is susceptible to *Agrobacterium*-mediated transformation (Han et al. 2000; Confalonieri et al. 2003).

We predicted 15 EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) members in *Populus trichocarpa* (data unpublished). In a previous study, we isolated *PdEPF1* from poplar and demonstrated that *PdEPF1* improved WUE and conferred drought tolerance in *Populus* (Wang et al. 2015). In the present study, an additional member of the EPF family, *PdEPF2*, was isolated for further analysis. Morphological and physiological experiments revealed that overexpression of *PdEPF2* reduced stomatal density and water loss, thus increasing drought tolerance. Moreover, our data showed that *PdEPF2* is likely to be involved in response to the ABA signaling pathway.

Materials and methods

Plant materials and growth conditions

Cuttings of the poplar genotype NE-19 (*Populus nigra* × (*Populus deltoids* × *Populus nigra*)) with 15-cm-long stems were planted in April, 2013, in an open field at the experimental nursery of Beijing Forestry University, Beijing, China for genetic analysis. For expression analysis, uniformly developed plants (95–110 cm high, with 25–35 leaves and 15–35 cm root length) were subjected to drought and ABA treatments. For drought treatment, water was withheld from plants for 0, 3, 6, 9, or 12 days. Treatment with ABA treatment was performed by spraying the leaves once with a 250 μM ABA solution, following which young leaves were collected at 0, 3, 6, 9, or 12 h after the treatment. For both treatments, young leaves (the first one to three leaves from the shoot apex) were harvested, frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA isolation.

Arabidopsis mutant and transgenic lines used were in the Columbia-0 (Col-0) background. *A. thaliana* ecotype Col-0 was selected as the wild-type (WT) control. Seeds of Col-0 and the mutant *epf2-3* (SALK_047918) were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH, USA). The mutant homozygous for T-DNA insertion within *AtEPF2* (At1g34245) was verified by polymerase chain reaction (PCR). Transgenic plants harboring and expressing 35S:*PdEPF2* and *epf2-3*/*PdEPF2* were generated using the agrobacterium tumefaciens-mediated floral dip method (Zhang et al. 2006). 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 analyses. All 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 (1/2MS; Murashige and Skoog 1962) plates supplemented with 3 % (w/v) sucrose and 0.6 % (w/v) agar. The seeds were stratified for 2 days at 4 °C before transference 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. Ten days after germination, seedlings were transplanted

and grown at a density of four plants per 7 × 7 × 6.5 cm pot containing a mixture of soil and vermiculite (2:1) under a 16/8 light/dark photoperiod at 22 °C, 70 % relative humidity, and 150 μmol m^{-2} s^{-1} irradiation.

Poplar gene cloning and plasmid construction

Total RNA isolation was performed using the CTAB reagent method (Chang et al. 1993). First-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase and an oligo (dT) primer (Promega, Madison, WI, USA) in accordance with manufacturer's instructions. The full-length cDNA of *PdEPF2* was retrieved by homologous cloning-using the gene-specific primers FW 5′-ATGAAGTTCTTAGTTGGAGCCC-3′ and RV 5′-TCATGCTGAAGGCACATGG-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 *PdEPF2* 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:*PdEPF2*.

Phylogenetic tree construction

To understand the relationship between *PdEPF2* and other *EPFL* family members from *Arabidopsis*, a phylogenetic analysis of *PdEPF2* was performed using full-length amino acid sequences from *Arabidopsis* and *Populus trichocarpa* using phylogeny (http://www.phylogeny.fr/). The sequences used were retrieved from PopGenIE (http://popgenie.org/) and TAIR (https://www.arabidopsis.org/).

RT-PCR and qRT-PCR analysis

Total RNA from each sample was extracted by the CTAB method, and 1 μg RNA was used for reverse transcription. Subsequently, 100 ng cDNA was used as the template for reverse-transcription (RT)-PCR amplification. The PCR products were electrophoresed in a 2 % agarose gel stained with ethidium bromide. Quantitative (R) RT-PCRs were performed in 96-well plates containing 100 ng template (1 μL), 0.6 μL (10 μM) forward primer, 0.6 μL (10 μM) reverse primer, 10 μL SYBR® Green Master Mix, 2 μL ROX™ Reference Dye and 5.8 μL RNase-free ddH2O, comprising a total volume of 20 μL. The reaction was amplified for 40 cycles at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 32 s. Transcript levels of all candidate genes was determined using the $2^{-\Delta\Delta CT}$ method and relative transcript levels were calculated and normalized as described previously (Willems et al. 2008). The reactions were performed in biological triplicates using RNA samples extracted from three independent plant materials and all experiments were repeated three times. *UBQ* was used as an internal control to quantify the relative transcript level of *PdEPF2* in each sample (Wang et al. 2014). Gene-specific primers were designed using the Primer6 (PRIMER-E, Ivybridge, UK) software listed in Online Resource 1.

Southern blot analysis

Southern blot analysis was performed to demonstrate transgene integration and gene copy number. Following digestion with XbaI overnight, DNA samples (30 μg) were separated by electrophoresis on a 1 % agarose gel and then transferred to a nylon membrane. DNA probes specific for *PdEPF2* coding sequence were labelled with digoxigenin (DIG). Southern blot hybridization was performed according to the protocol of the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany). After hybridization, the DNA filter was washed sequentially as follows: twice with 2× SSC and 0.1 % SDS for 5 min at room temperature and twice with 0.5× SSC and 0.1 % SDS for 15 min each at 65 °C.

ABA and drought tolerance assays

To determine the germination efficiency of transgenic seeds under stress conditions, seeds were germinated and seedlings grow on 1/2MS agar plates (supplemented with 300 mM mannitol, and 0.6 μM ABA) (Luo et al. 2013). Germination was scored daily, and seedlings were photographed after 10 days. For the root length assay, 5-day-old seedlings grown on 1/2MS agar plates were transferred to vertically oriented 1/2MS agar plates supplemented with or without 300 mM mannitol for 12 days before root length was measured and photographed. The solute potential of 300 mM mannitol was −0.74 MPa. Seventy seeds from each line were used to determine germination rates and six seeds from each line were used to compare root length. All experiments were repeated three times.

To test drought tolerance at subsequent developmental stages, the seedlings transplanted into the soil and watered for 3 weeks before water was withheld. After 2 weeks of water deficit, the pots were re-watered for 1 week. The control comprised plants that were well-watered continuously. Soil was collected at three stages (well-watered, drought, and re-watered), weighed immediately (fresh weigh), then oven-dried to a constant weight for 16 h at 105 °C and weighed for determination of soil water content (dry weigh). The percentage soil water contents was calculated as (fresh weight − dry weight)/fresh weight × 100.

To investigate ABA-regulated gene expression, total RNA was extracted from 2-week-old seedlings floated in 100 μM ABA solution for 6 h under continuous white light

(150 µmol m^{-2} s^{-1}). The synthetic ABA used was a racemic mixture.

Physiological measurements

The following physiological measurements were conducted on seedlings after water was withheld for 10 days. Photosynthetic and transpiration rates were measured using the Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). The leaves were held at an ambient CO_2 concentration of 400 µmol mol^{-1}, a photosynthetic photon flux density of 600 µmol m^{-2} s^{-1}, and a chamber temperature of 24 °C. The maximum quantum yield of PSII (Fv/Fm) was measured using a Dual-PAM-100 measuring system (Walz Heinz GmbH, Effeltrich, Germany). Six fully expand leaves were detached from different plants of each line, then for each line 1.70 cm^2 leaf discs were obtained with a 6-mm- diameter single-hole punch. Leaf chlorophyll was extracted with 80 % (v/v) acetone (Shu et al. 2010). A UV/visible spectrophotometer (YHB-061; GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used to measure the absorbance at 663 nm for chlorophyll a and 645 nm for chlorophyll b. Chlorophyll a concentration was calculated using the equation $C_a = 12.72D_{663} - 2.59D_{645}$, where D is absorbance at 663 or 645 nm. Chlorophyll b was calculated using the equation $C_b = 22.88D_{645} - 4.67D_{663}$. Total chlorophyll concentration was equal to $C_a + C_b$. The concentration of proline was estimated using the acid-ninhydrin method by measuring the absorbance at 520 nm. After water was withheld for 14 days, six plants from each line (ox*PdEPF2#1*, *#2*, *#3*, WT, *epf2-3*, and *epf2-3/PdEPF2*) were sampled and oven-dried for 72 h at 70 °C to measure total biomass.

Water loss measurements

Rosette leaves detached from transgenic plants, WT, *epf2-3*, and *epf2-3/PdEPF2* grown under non-stress conditions for 3 weeks were used to measure the rate of water loss. Leaves weighing approximately 0.5 g were harvested, weighted and used immediately for experiments. The leaves were placed on a laboratory bench and weighed every 30 min (Ma et al. 2010). The percentage loss of fresh weight was calculated on the basis of the initial weight of the leaves. The experiments were replicated three times.

Stomatal density and stomatal aperture

The stomatal density and aperture of fully expanded leaves were recorded and photographed using a scanning electron microscope (Hitachi S-3400 N; Chiyoda-ku, Tokyo, Japan). Leaves of rosette stage plants were sampled from the ox*EPF2* lines, WT and the *epf2-3* mutant. The stomatal aperture assays were supplemented with 10 µM ABA for 2.5 h. The samples were fixed as described by Cao et al. (2007). The samples were first fixed in 25 % glutaraldehyde for 24 h. The leaves were then dehydrated in 30, 50, 70, 85, and 95 % ethanol (15 min each) and then twice in 100 % ethanol (15 min each). The dehydrated samples were then treated with isoamyl acetate: ethanol (1:1) and 100 % isoamyl acetate (15 min each).

Statistical analysis

For qPT-PCR, RNA samples were extracted from three independent plant materials and all experiments were repeated three times. Six root lengths were measured per strain, and three independent experiments were repeated. For physiological measurements, six leaves were measured per plant, and three individual plants were included in each group. For stomatal density, three leaves were measured per plant, and three individual plants were included in each group. All statistical analyses and plots were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Statistical comparisons used one-way analysis of variance (ANOVA) in SPSS. Different letters indicate significant differences at $\alpha = 0.05$ (one-way ANOVA). Data were presented as the mean ± standard error (SE).

Results

Identification and molecular characterization of *PdEPF2*

The *PdEPF2* gene (GenBank accession number KR611990), comprising a complete open reading frame of 351 bp and encoding 116 amino acid residues with a predicted molecular mass of 12.88 kDa and an isoelectric point of 8.63 (http://web.expasy.org/compute_pi/), was cloned from the high WUE poplar genotype NE-19 (*Populus nigra* × (*Populus deltoids* × *Populus nigra*)) (Hao et al. 2011; Xing et al. 2011).

To identify homologous proteins of PdEPF2, a phylogenetic tree was constructed between the poplar and *Arabidopsis* EPF family members through their amino acid sequence alignment. PdEPF2 was clustered with AtEPF2 (Fig. 1a). The multiple protein sequence alignment revealed that PdEPF2 contained eight conserved cysteine residues at the C-terminal end (Fig. 1b), consistent with AtEPF1, AtEPF2, and AtEPFL7 (Hara et al. 2009; Shimada et al. 2011).

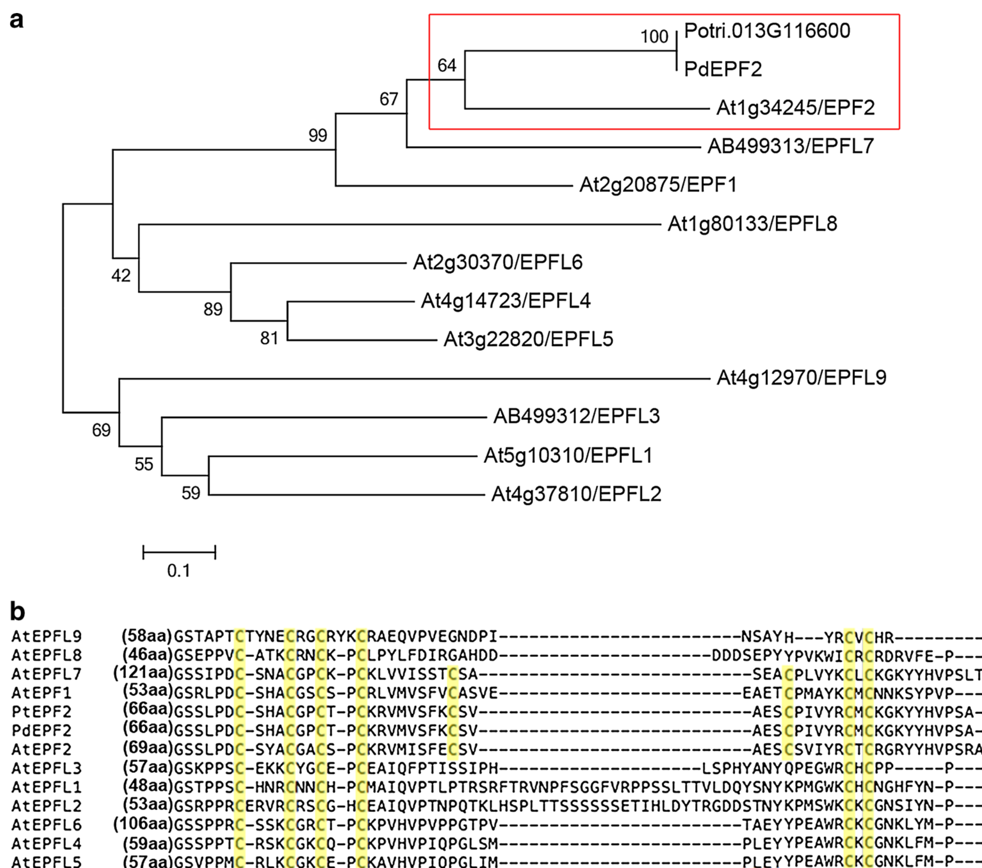

Fig. 1 *PdEPF2* gene of *Populus nigra* × (*Populus deltoides* × *Populus nigra*). **a** Phylogenetic relationships among poplar and *Arabidopsis* EPF family members. The phylogenetic tree was constructed with the neighbor-joining method using MEGA 6. **b** Multiple sequence alignment of the C-terminal region of *PdEPF2* and homologous EPFs from *Arabidopsis*. The number of preceding amino acid residues is indicated in *parentheses*. Conserved cysteine residues are marked in *yellow*. (Color figure online)

Expression profile of *PdEPF2*

Transcription of *PdEPF2* was tissue-specific. Transcripts were detected in roots, stems, young leaves, mature leaves, and senescent leaves of NE-19 under non-stress growth conditions. The results exhibited that *PdEPF2* 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, *PdEPF2* transcription initially increased but subsequently decreased during days 3–12 of drought treatment (Fig. 2b). ABA is an important secondary signaling molecule, and its exogenous application can cause effects similar to those of osmotic stress and may modulate some drought-responsive genes (Zhu 2002). To investigate the involvement of *PdEPF2* in responses to ABA stress, the transcript level of *PdEPF2* under 250 μM ABA was quantified by qRT-PCR. Transcripts of *PdEPF2* accumulated rapidly to about 5.2-fold 3 h after ABA application before slightly declining (Fig. 2c). These results indicate that the expression of *PdEPF2* was induced by both drought and ABA treatments.

Analysis of transgenic *Arabidopsis* overexpressing *PdEPF2*

To elucidate the in vivo functions of *PdEPF2*, the 35S:*PdEPF2* construct was transformed into WT *Arabidopsis* and the *epf2-3* mutant to generate overexpression and complementation lines, respectively. We raised 11 independent transgenic lines, of which three were selected on the basis of qRT-PCR results. As expected, *PdEPF2* transcripts were undetectable in the WT plants. Three overexpression lines (lines 1, 3, and 5) showed higher transcript levels than the other overexpression lines (Online Resource 2), and were designated ox*PdEPF2#1*, *#2*, and *#3*. One complementary line was selected for further analysis (Online Resource 3). DNA hybridization showed that *PdEPF2* was integrated into the genomes of the transgenic *Arabidopsis* as one (*oxPdEPF2#1, #2, #3* and

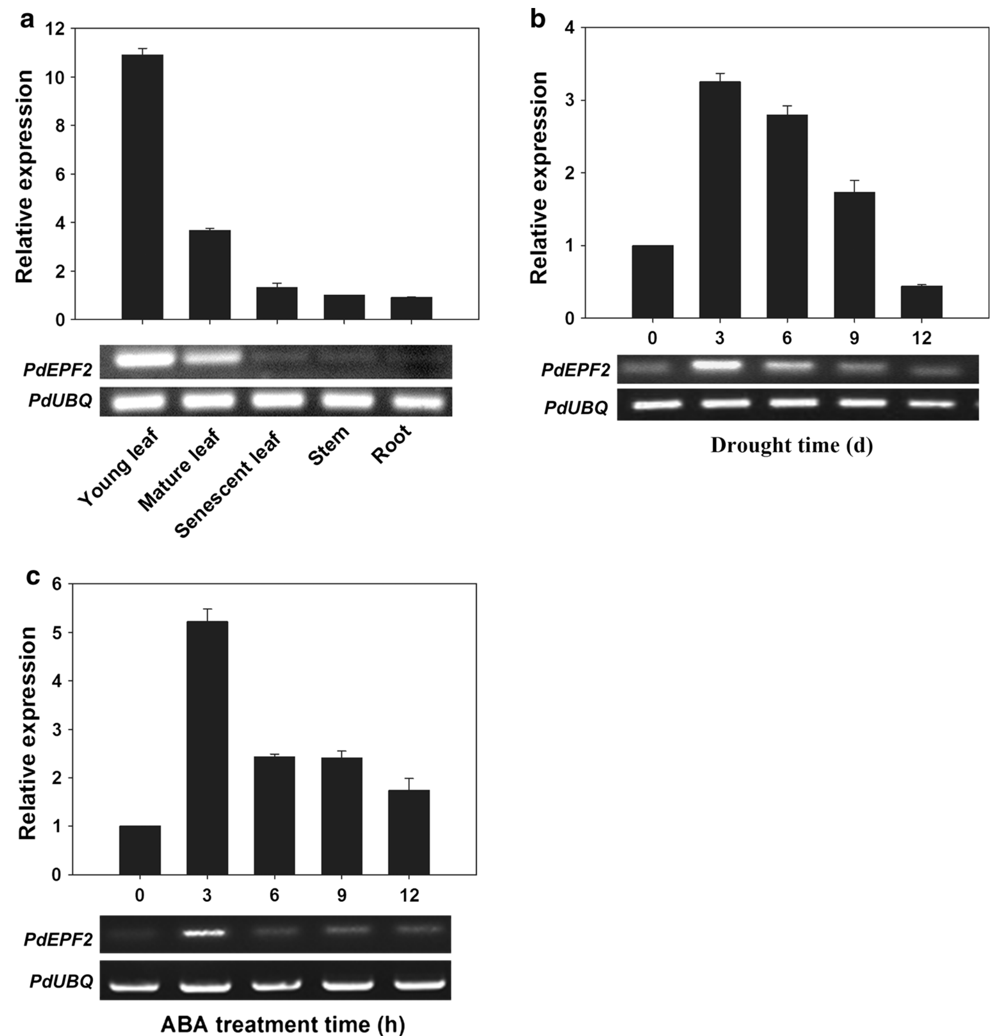

Fig. 2 Expression patterns of *PdEPF2*. **a** Tissue-specific expression pattern of *PdEPF2* in poplar. "Young leaf", the first one to three leaves from the shoot apex; "mature leaf", a fully expanded leaf; "senescent leaf", basal most two to three leaves above the root system. The level of the *PdEPF2* transcript in the stem was set at 1. **b**, **c** Expression of *PdEPF2* in response to drought and ABA in poplar. Data represent mean ± SE ($n = 3$)

epf2-3/PdEPF2), whereas no hybridization was observed in wild type (Online Resource 4).

Phenotypes of ox*PdEPF2* plants under drought conditions

Mannitol was used to simulate drought stress. Seeds of transgenic plants, WT, *epf2-3*, and the complementary line *epf2-3/PdEPF2* were sown on 1/2MS agar medium supplemented with or without 300 mM mannitol. In the presence of mannitol, seedlings from the ox*PdEPF2* plants were greener (Fig. 3a) and showed more rapid germination than WT and *epf2-3* seeds (Fig. 3b).The transgenic plants showed more green cotyledon than the WT and the mutant *epf2-3* (Fig. 3c). When 5-day-old seedlings grown on 1/2MS medium were transferred to vertical agar plates containing 1/2MS medium supplemented with 300 mM mannitol, the primary root was 1.34 times as long in ox*PdEPF2* plants as in WT plants; however, when compared with the *epf2-3* mutant, the difference was significant (2.17 times). The mutant complementation restored the phenotype to that of the WT. Furthermore, compared with seedlings grown on standard 1/2MS medium, the primary root length of ox*PdEPF2*, WT, and *epf2-3* seedlings subjected to osmotic stress decreased by approximately 50, 63.05, and 76.65 %, respectively (Fig. 3d).

The capacity of ox*PdEPF2* plants to respond to severe drought stress was investigated further. Watering of 3-week-old transgenic plants, WT, *epf2-3*, and the mutant complementation line *epf2-3/PdEPF2* was withheld for 2 weeks, after which the plants were re-watered for 1 week. The soil water contents in the three phases of the experiment were 58.65 ± 1.99 g/g % (well-watered), 8.66 ± 1.83 g/g % (drought), and 46.64 ± 1.97 g/g % (re-watered), respectively. During water deprivation, plants of the WT and *epf2-3* mutant wilted more severely than the transgenic plants, and the mutant complementation line showed a similar response to that of the WT (Fig. 3e). These results indicated that the transgenic plants showed enhanced drought tolerance.

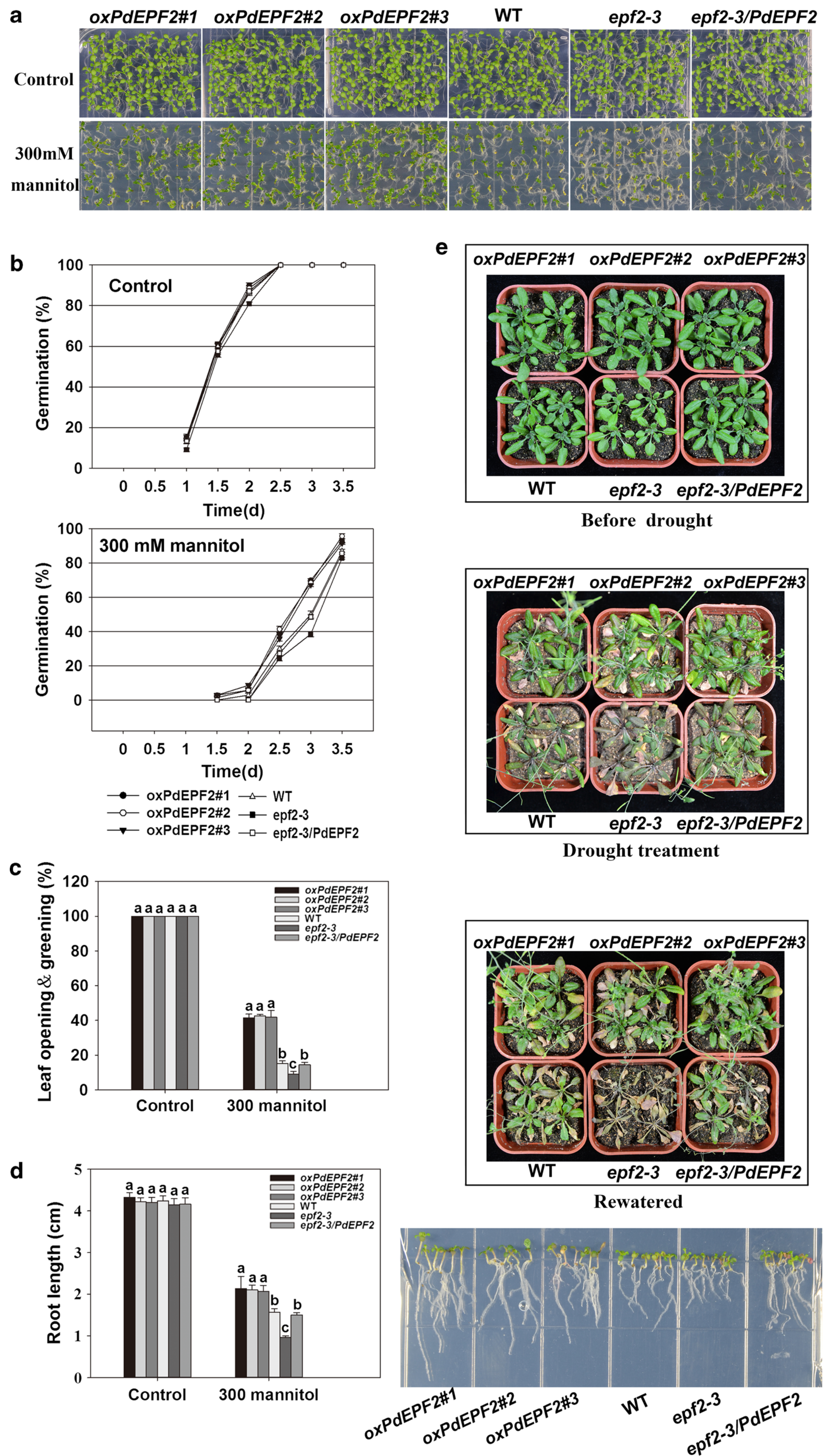

Fig. 3 Overexpression of *PdEPF2* in *Arabidopsis* under drought stress. **a–c** Phenotype, germination time, and cotyledon greening at the seed germination stage of seedlings grown on 1/2MS medium supplemented with or without 300 mM mannitol. A seeds was considered to have germinated when the radicle had completely penetrated the seed coat. Photographs were taken 10 days after stratification. Data shown represent the means (±SE) of three independent experiments (70 seeds for each test). **d** Root length of ox*PdEPF2* s, WT, *epf2-3*, and *epf2-3/PdEPF2* seedlings grown on 1/2MS medium supplemented with or without 300 mannitol. Photographs were taken after 12 days of growth on the media. Data are mean ± SE ($n = 6 \times 3$) of three independent experiments. *Different letters* indicate a significant difference at $\alpha = 0.05$ (one-way ANOVA). **e** Morphological differences in drought experiments. The seedlings were grown in soil for 3 weeks under well-watered conditions; thereafter, water was withheld for 2 weeks, then plants were re-watered for 1 week

Physiological analysis of ox*PdEPF2* plants under drought stress

To investigate the physiological basis of the transgenic plants' drought tolerance, we monitored changes in proline and chlorophyll content and F_v/F_m after water was withheld for 10 days. Although there were no differences under well-watered conditions, proline content in the transgenic lines was significantly higher than that of the WT (31 %) and *epf2-3* plants (54 %) under drought stress (Fig. 4a). Likewise, at all tested drought levels, chlorophyll content in the transgenic plants was significantly higher than that of WT and *epf2-3* (1.35 and 2.12 times, respectively) (Fig. 4b). F_v/F_m was significantly higher in the ox*PdEPF2* plants under drought stress. When water was withheld for 10 days, F_v/F_m of the ox*PdEPF2*, WT, and *epf2-3* plants decreased from 0.83 to 0.79, 0.82 to 0.70, and 0.82 to 0.63, respectively (Fig. 4c). During water deprivation, growth of

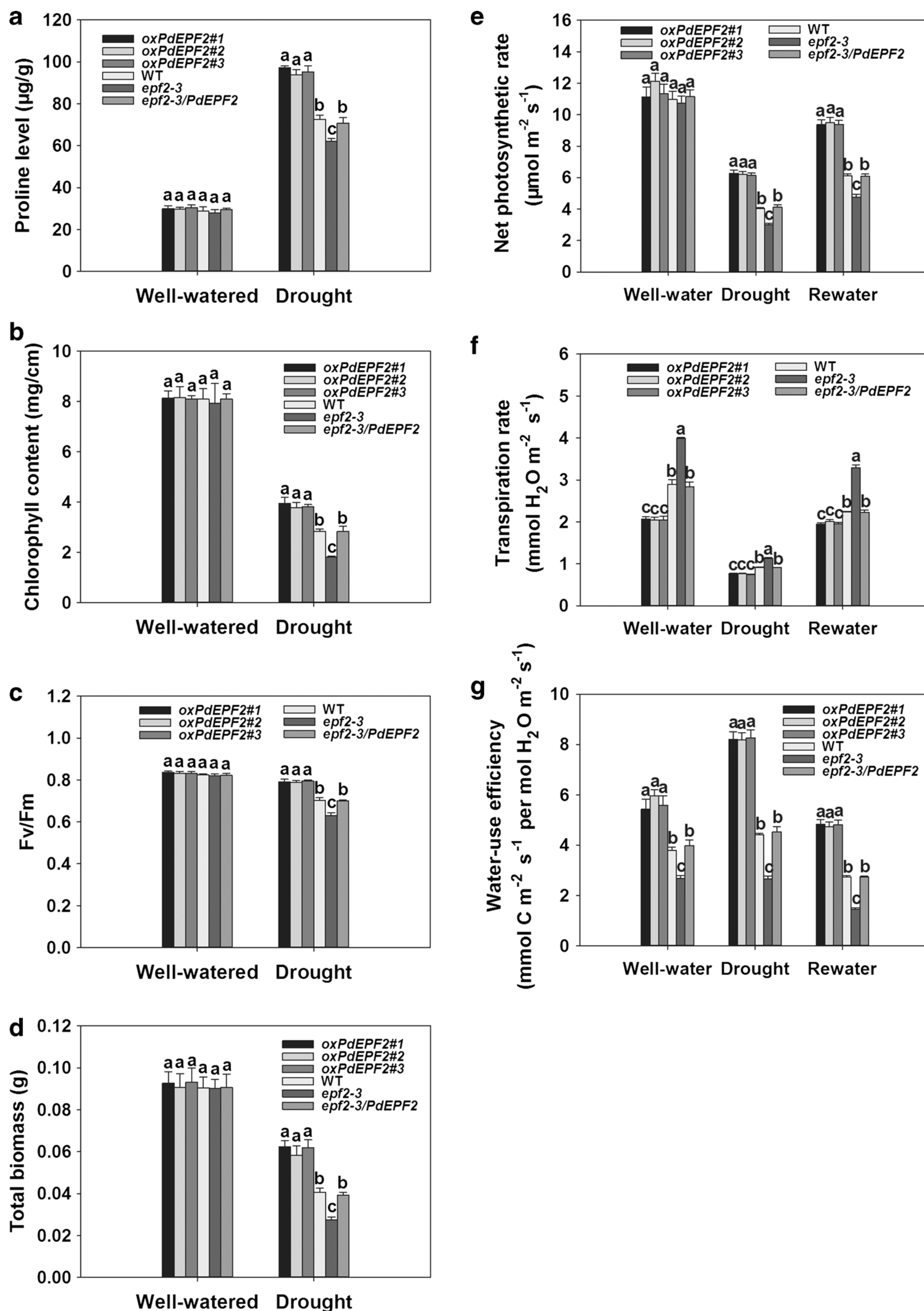

Fig. 4 Physiological analysis of ox*PdEPF2* lines under drought stress. **a–c** Changes in proline content, chlorophyll content, and F_v/F_m of transgenic plants, WT, *epf2-3* mutant, and *epf2-3/PdEPF2* complementation line after water was withheld for 10 days. **d** Total plant biomass over the 14-day experimental period. Data are mean ± SE ($n = 6$). **e–g** Net photosynthetic rate, transpiration rate, and instantaneous WUE of leaves at three different stages. Data represent mean ± SE ($n = 6$). *Different letters* indicate a significant difference at $\alpha = 0.05$ (one-way ANOVA)

the ox*PdEPF2* lines continued, resulting in higher biomass, despite all lines suffering wilting, whereas no difference in biomass was observed among the lines under well-watered conditions (Fig. 4d). In addition, the ox*PdEPF2* plants maintained a significantly higher photosynthetic rate and lower transpiration rate than those of the WT and *epf2-3* plants under drought stress (Fig. 4e, f). Based on the higher photosynthetic capability and lower transpiration level, the ox*PdEPF2* plants showed increased leaf WUE compared with the WT and *epf2-3* mutant (Fig. 4g). These observations indicated that a positive relationship existed between *PdEPF2* expression and drought stress tolerance.

Overexpression of *PdEPF2* decreases stomatal density

Stomatal density is an important index of tolerance to drought stress in plants, as it affects water loss. Compared with the WT and *epf2-3* plants, the transgenic plants showed a decrease in water loss (Fig. 5a) consistent with their superior drought tolerance. Given the close relationship between water loss and stomatal density, we recorded the number of stomata per unit leaf area (Fig. 5b). The number of stomata per mm^2 in the ox*PdEPF2* plants was <50 % that of the *epf2-3* mutant plants and approximately 30 % less than that of WT plants. The *epf2-3* mutant showed a significantly higher stomatal density than the WT, whereas the *epf2-3/PdEPF2* complementation line showed a decreased stomatal density, as observed for the WT (Fig. 5c). These results indicated that overexpression of *PdEPF2* may reduce water loss by influencing stomatal density.

Overexpression of the *PdEPF2* gene decreases ABA sensitivity in seeding stage

Substantial evidence suggests that ABA plays a crucial role in responses to abiotic stresses, including drought and salinity (Shinozaki and Yamaguchi-Shinozaki 2007; Jin et al. 2011). Hence, the ABA-response phenotypes of the ox*PdEPF2* plants were examined further. To observe germination and early seedling development, seeds were incubated on 1/2MS medium supplemented with or without 0.6 μM ABA. In the absence of ABA, no significant difference was observed among the different lines. In the presence of exogenous ABA, the ox*PdEPF2* plants showed more green cotyledon (Fig. 6a) and seeds showed faster germination compared with the WT and mutant *epf2-3* plants (Fig. 6b). These results indicated that ox*PdEPF2* plants showed reduced sensitivity to ABA. To investigate

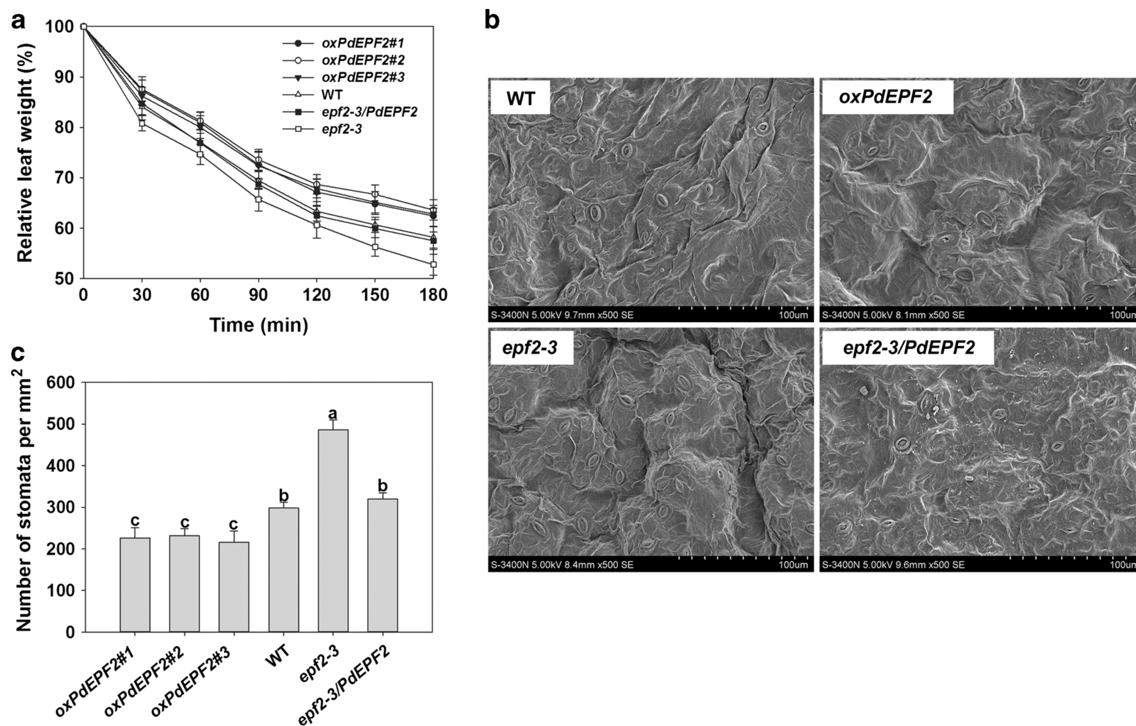

Fig. 5 Effect of *PdEPF2* on stomatal density and leaf water loss. **a** Water loss from detached leaves; the water loss of 0.5 g detached leaves per plant was measured at the indicated time points in triplicate. Three measurements were averaged at each time point. Data are mean ± SE. **b** Scanning electron micrograph of the abaxial leaf epidermis. *Scale bars* 100 μm. **c** Abaxial stomatal densities in WT, transgenic plants, *epf2-3* mutant, and the *epf2-3/PdEPF2* complementation line. *Different letters* indicate a significant difference at $\alpha = 0.05$ (one-way ANOVA)

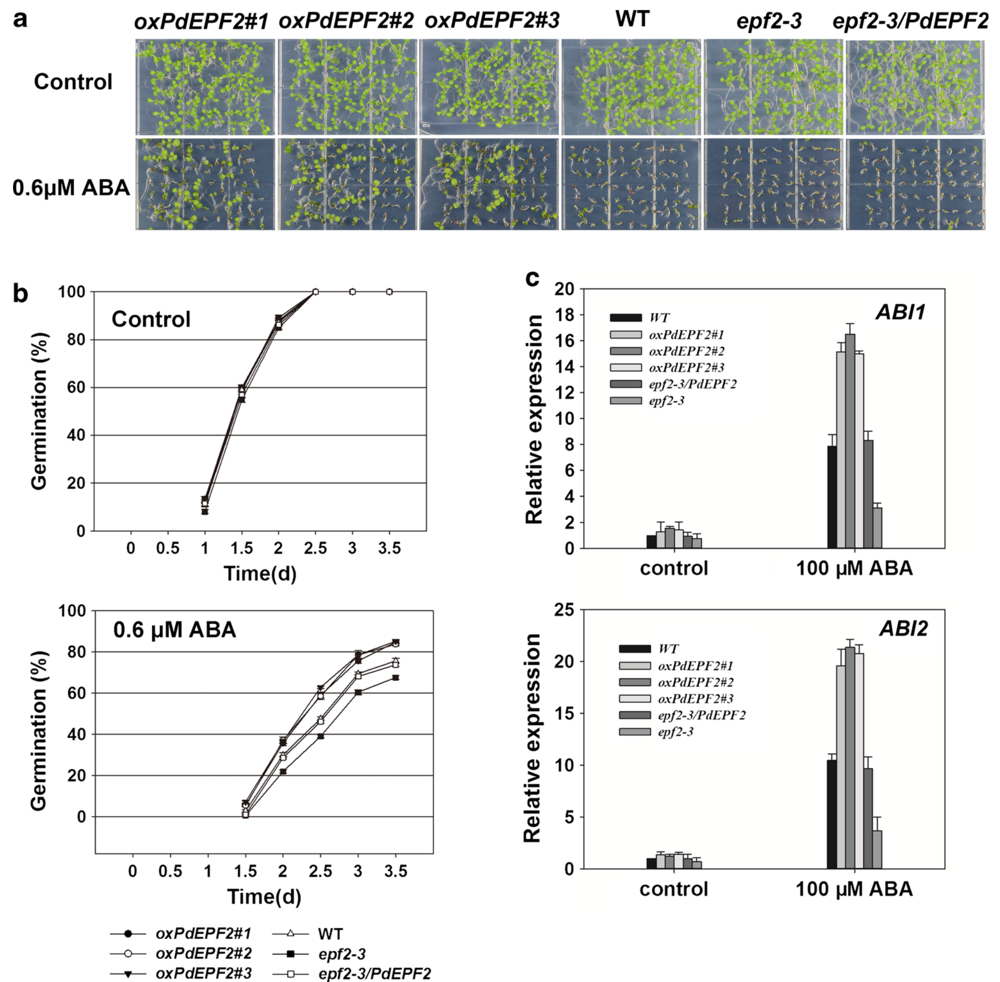

Fig. 6 Overexpression of *PdEPF2* in *Arabidopsis* decreased sensitivity to ABA. **a**, **b** Phenotype and time course of seed germination on 1/2MS medium supplemented with or without 0.6 µM ABA. Data are mean ± SE from three independent experiments (70 seedlings per experiment). **c** Expression of *ABI1* and *ABI2* in transgenic plants. Two-week-old seedlings were grown for 6 h on 1/2MS agar medium supplemented with or without 100 µM ABA. Quantitative RT-PCR was used to analyze ABA-responsive gene expression. The *Arabidopsis actin* gene was used as the internal control. Data are mean ± SE of three independent measurements

molecular changes in response to ABA stress, we evaluated the transcription of two genes, *ABI1* and *ABI2*, which are both associated with type 2C protein serine/threonine phosphatases and act as key regulators in the response to ABA (Merlot et al. 2001). The overexpression lines showed higher transcript levels of *ABI1* and *ABI2* compared with those of WT plants after ABA treatment. The mutant *epf2-3* showed lower transcript levels than those of WT plants, whereas the complementation line (*epf2-3/PdEPF2*) showed similar transcript levels to those of WT plants (Fig. 6c). These results indicated that *PdEPF2* may be involved in response to the ABA signaling pathway.

It is well known that ABA induces stomatal closure. We therefore compared stomatal apertures in the different lines under ABA treatment. The aperture length:width ratio was used as a measure of stomatal closure (Ren et al. 2010). As shown in Online Resource 5, all lines showed a reduced stomatal aperture in response to ABA treatment. However, no obvious difference in the length:width ratio of stomata was observed in the different lines.

Discussion

Drought is one of the most prevalent environmental stressors to plants. Therefore, identifying genes that confer drought tolerance, and thus enable plants to better survive under water deficit, is important for crop production and plant breeding. The present study identified novel roles for *PdEPF2* in the response to drought stress, and transcription analyses indicated that *PdEPF2* was differentially expressed and induced in response to drought and ABA (Fig. 2b, c). Furthermore, *PdEPF2* transgenic plants differed phenotypically from WT and *epf2-3* mutant plants under drought stress. First, transgenic plants showed superior germination capacity and increased primary root length under osmotic stress conditions (Fig. 3a–d). Second, drought-induced proline content was significantly higher in transgenic plants than that of WT and *epf2-3* mutant plants (Fig. 4a). Proline is positively associated with abiotic stress tolerance (Liu and Zhu 1997; Xiang et al. 2007). Third, although net CO_2 assimilation under well-watered

conditions did not vary and photosynthetic rates decreased in all plants under water deprivation, the reduction in photosynthetic rate was least severe in transgenic plants (Fig. 4e), which was consistent with the observed changes in chlorophyll content and F_v/F_m (Fig. 4b, c). The transgenic lines maintained higher WUE (Fig. 4g) through reduction in the transpiration rate (Fig. 4f).

Transpiration and CO_2 uptake occur primarily through stomatal pores, and conductance depends on stomatal density and pore aperture size (Hetherington and Woodward 2003; Nilson and Assmann 2007). In addition, reduced transpirational water loss is a key determinant of drought tolerance (Xiong et al. 2002). We observed that overexpression of *PdEPF2* decreased the rate of water loss (Fig. 5a) and stomatal density (Fig. 5b, c), which resulted in enhanced tolerance of water deficiency. This likely occurred because the reduced stomatal density resulted in decreased transpiration during drought conditions, although we cannot rule out that other changes caused increased efficiency in water uptake in the ox*PdEPF2* plants. Interestingly, the decreased stomatal density did not affect photosynthesis capacity, although gas exchange might be limited by the reduced number of stomata.

Generally, in C_3 plants, photosynthetic rates become saturated as stomatal conductance increases because of limitations unrelated to stomata, such as the regeneration of ribulose 1,5-bisphosphate (Zeiger and Field 1982). Consequently, lower stomatal density does not necessarily affect carbon assimilation or biomass accumulation and therefore ox*PdEPF2* plants showed continued growth and development, resulting in higher biomass compared with the other lines under drought conditions (Fig. 4d). This finding is supported by previous work that showed that overexpression of both *HARDY* and *HDG11*, and mutations of *GTL1* and *GPA1*, reduced stomatal density and improved drought tolerance while not affecting carbon assimilation or biomass accumulation (Karaba et al. 2007; Yu et al. 2008; Nilson et al. 2010; Yoo et al. 2010). However, transpiration rate increases linearly over a certain range of stomatal conductance and therefore decrease in stomatal density could reduce transpiration rate, thereby increasing WUE without affecting carbon assimilation (Yoo et al. 2009).

Quantitative RT-PCR confirmed that exogenous ABA application induced *PdEPF2* expression (Fig. 2c), indicating that *PdEPF2* may respond to the ABA signaling pathway. *HDG11*, a transcription factor involved in the interplay between ABA biosynthesis, ABA signaling and stomatal development (Chater et al. 2014), has been shown to negatively regulate stomatal development via transactivation of the *ERECTA* promoter (Yu et al. 2008). Biochemical studies using a biosensor chip have shown that EPF2–ERECTA, as the ligand–receptor pair, regulates initiation of stomatal development (Lee et al. 2012). These studies indicate that the EPF2–ERECTA–MPK signal transduction pathways involved in stomatal development may share signaling pathways involved in ABA response. In addition, appraisal of ABA sensitivity in the current study showed that *epf2-3* was more sensitive to ABA before germination, whereas ox*PdEPF2* was less sensitive than WT plants to ABA (Fig. 6a, b). Analysis of two ABA-regulated genes suggested that the reduced sensitivity of *PdEPF2* to ABA may be due to activation of *ABI1* and *ABI2* (Fig. 6c). Previous studies have shown that *ABI1* and *ABI2* are important for ABA signal transduction and act by negatively regulating ABA responses (Merlot et al. 2001). The significant up-regulation of *ABI1* and *ABI2* is consistent with the decreased ABA sensitivity of ox*PdEPF2* plants during germination and seedling growth. Some evidence indicates that *ABI1* and *ABI2* negatively affect a plant's stress tolerance (Jung et al. 2008; Seo et al. 2010). However, this negative relationship is not consistently supported. For example, transgenic *Arabidopsis* plants overexpressing *GsWRKY20* showed reduced ABA sensitivity but were more tolerant of drought stress, possibly through up-regulation of *ABI1* and *ABI2* (Luo et al. 2013). In addition, overexpression of *OZF2* in *Arabidopsis* improved salt stress tolerance and reduced ABA sensitivity, and the level of *ABI2* transcripts was increased (Huang et al. 2012). Furthermore, transgenic plants overexpressing *ZmPP2C* showed reduced expression of *ABI1* and *ABI2* but were less sensitive to ABA and displayed decreased salt and drought tolerance (Liu et al. 2009). Consequently, stress tolerance depends on the expression of stress response genes as well as plant physiology and drought tolerance and thus is the result of their integrated effects. In the present study no obvious difference in stomatal aperture was observed in the different lines after treatment with ABA (Online Resource 5). ABA promotes stomatal closure mainly through import of Ca^{2+} and export of K^+, Cl^-, and malate in guard cells (Chen et al. 2010; Kim et al. 2010). These results demonstrate that the molecular mechanisms of *PdEPF2* response to ABA are complex. Furthermore, different mechanisms might be involved at different stages of growth or due to other unknown factors.

In conclusion, in this study we identified and isolated *PdEPF2* and showed that its expression in *Arabidopsis* plants improved drought tolerance. Our findings suggest that the gene may be useful in the breeding of drought-tolerant plants and in improving crop production under drought conditions. Further experiments are required to generate additional transgenic poplars and to elucidate the regulatory mechanisms of drought tolerance in woody plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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