



Overexpression of *MdEPF2* improves water use efficiency and reduces oxidative stress in tomato

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ABSTRACT

Increasing drought frequency and diminishing groundwater resources are critical environmental constraints that seriously reduce global plant production. However, these limitations might be partially addressed by improving crop drought tolerance and water-use efficiency (WUE). We isolated an epidermal patterning factor (EPF), *MdEPF2*, from apple (*Malus domestica*). Transcript levels of that gene were higher after plants were treated with abscisic acid and drought. To investigate its function in drought tolerance, we ectopically expressed *MdEPF2* in *Solanum lycopersicum* cultivar 'Micro-Tom'. When compared with the wild type (WT), stomatal density was significantly lower in the leaves from the transgenics, which enabled those plants to avoid dehydration. Under drought stress, transgenic plants had higher values for relative leaf water content, chlorophyll, photosynthetic rates, and WUE than did WT. The former also had lower accumulations of reactive oxygen species and malonyldialdehyde but greater activities of antioxidant enzymes, thereby leading to less oxidative damage. Our results suggested that *MdEPF2* is a functional ortholog of EPF2 in *Arabidopsis* and can be potentially used in apple breeding to improve WUE and drought tolerance in this economically important fruit crop.

1. Introduction

Changes in the global climate due to continuously rising levels of atmospheric carbon dioxide will exacerbate water deficits in large parts of the world (Zandalinas et al., 2017). Many crops are cultivated in areas where climatic conditions are not always ideal and rainfall is below optimal levels. Periods of drought limit plant growth and survival because water uptake from the soil via the roots is insufficient to meet transpirational requirements (Blum, 1996). Water deficits decrease leaf cell turgor, restrict cell expansion and the size of the photosynthetic source, and postpone development, all of which constrain the production and accumulation of biomass (Chaves et al., 2003). Improving water-use efficiency (WUE) i.e., the ratio of biomass production to water consumption, by reducing transpirational losses and conserving soil water status is an important priority on marginal lands

that face increasing drought and diminishing groundwater resources (Franks et al., 2015).

Stomata are microscopic two-celled pores on the leaf surface. Their opening and closing modulates plant-atmosphere gas exchange. Plants control their CO₂ uptake and transpiration mainly by regulating stomatal conductance, which is determined by stomatal movement and density (Chaerle et al., 2005). In the short term, leaves adjust the stomatal aperture to respond rapidly to environmental factors such as blue light, Ca²⁺, CO₂, abscisic acid, and changes in water status (Shimazaki et al., 2007; Chater et al., 2014). Altering the number of stomata, however, is a long-term response. Stomatal density can be modified in young leaves through sensing of environmental factors by mature leaves (Lake et al., 2001). Changes in stomatal density vary according to species and the severity of the drought. In response to a water deficit, stomatal densities decrease in *Triticum aestivum*, *Cucurbita maxima*

Abbreviations: WUE, water-use efficiency; EPF, epidermal patterning factor; EPFL, EPF-LIKE; WT, wild type; MMC, meristemoid mother cell; M, meristemoid; GMC, guard mother cell; GC, guard cell; bHLH, basic helix-loop-helix; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; ABA, abscisic acid; RWC, relative water content; BLAST, basic local alignment search tool; NCBI, National Center for Biotechnology Information; PLACE, a database of plant *cis*-acting regulatory DNA elements; PlantCARE, plant *cis*-acting regulatory elements enhancers and repressors; CTAB, hexadecyl trimethyl ammonium bromide; CaMV, cauliflower mosaic virus; OE, overexpression; Pn, rates of photosynthesis; gs, stomatal conductance; FW, fresh weight; TW, turgid weight; DW, dry weight; MDA, malonyldialdehyde; NBT, nitro blue tetrazolium; DAB, diaminobenzidine

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cotyledons, and *Spondias tuberosa* trees (Quarrie and Jones, 1977; Sakurai et al., 1986; Silva et al., 2009). For leaves of rice (*Oryza sativa*) and *Leymus chinensis*, the densities increase under moderate drought but decline under more severe drought (Xu and Zhou, 2008).

The process of stomatal development has been well studied in the model dicot plant *Arabidopsis thaliana* (hereafter, *Arabidopsis*). Stomatal lineage consists of meristemoid mother cells (MMC), meristemoids (M), guard mother cells (GMC), and guard cells (GC). Each developmental transition is orderly regulated by three basic helix-loop-helix (bHLH) transcription factors, namely SPEECHLESS for MMC to M, MUTE for M to GMC, and FAMA for GMC to GC (Lau and Bergmann, 2012). EPIDERMAL PATTERNING FACTOR (EPF) and EPF-LIKE (EPFL) encode a cysteine-rich secreted peptide family of 11 members (EPF1, EPF2, and EPFL1–9) that participate in several developmental processes in *Arabidopsis* (Hara et al., 2007, 2009; Lee et al., 2012). EPF2 is expressed in and secreted by MMCs and early meristemoids, where it inhibits surrounding cells from entering the stomatal lineage (Hara et al., 2009; Hunt and Gray, 2009). Loss-of-function mutants of EPF2 induce the production of excess meristemoid-like cells (Hara et al., 2009; Hunt and Gray, 2009), whereas overexpression of that gene blocks stomatal formation (Hara et al., 2009; Hunt and Gray, 2009). Clustered stomata form in the loss-of-function mutants of EPF1, a phenotype associated with defects in orienting spacing divisions (Hara et al., 2007; Lee et al., 2012). In contrast, EPF1 overexpression leads to few or no stomata, all of which is consistent with the belief that this gene regulates stomatal patterning. Stomagen/EPFL9 is expressed in the mesophyll tissue of young leaves (Sugano et al., 2009; Kondo et al., 2010). In contrast to EPF1 and EPF2, its ectopic overexpression promotes the formation of clustered stomata (Sugano et al., 2009; Kondo et al., 2010). Furthermore, silencing of EPFL9 via RNA interference leads to a reduction in stomatal density for those lines (Sugano et al., 2009).

Reactive oxygen species (ROS) are important regulatory molecules for vital cellular processes. Under normal growing conditions, they are produced at low levels in the chloroplasts, e.g., $\sim 240 \mu\text{M s}^{-1}$ for O₂ and 0.5 μM for H₂O₂ (Polle, 2001). Restrictions to the supply of CO₂ in water-stressed plants is attributed to a decrease in stomatal conductance (Breusegem et al., 2001), which then disturbs the photosynthetic electron transport chain, redirecting excess energy toward ROS generation. Those molecules can aggravate the effects of drought stress on plant cells because they influence membrane integrity and permeability, as well as levels of chlorophyll, proteins, and nucleic acids (Gill and Tuteja, 2010). To mitigate those negative effects, plants utilize a complicated antioxidant defense system to detoxify ROS, including low-molecular-mass non-enzymatic antioxidants (e.g., ascorbic acid, glutathione, and carotenoids); the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD); and enzymes involved in the Halliwell-Asada cycle, i.e., the ascorbate-glutathione cycle (Foyer and Halliwell, 1976). The activities of antioxidant enzymes are increased in order to detoxify excessive ROS produced under drought, salinity, ozone, or temperature stress.

Apple (*Malus domestica*) is one of the most economically important fruit crops grown in temperate regions, but its high productivity strongly depends upon water availability (Zohary and Hopf, 2000). In some dry or semi-dry areas, such as northern China, high WUE is a determining factor of success in apple breeding research (Wang et al., 2018). Most previous studies of EPFs have focused on herbaceous plants, such as *Arabidopsis*, barley (*Hordeum vulgare*), and rice (Hughes et al., 2017; Caine et al., 2018). The EPFs also appear to have vital roles in regulating stomatal development in woody plant species. Here, we identified a putative EPF homolog, MdEPF2 (MDP0000154038), from apple. Its ectopic overexpression in tomato (*Solanum lycopersicum*) led to decreased stomatal density. We then investigated how that reduction in density might affect gas exchange, WUE, and tolerance to drought and oxidative stress.

2. Materials and methods

2.1. Plant materials and treatments

Leaves, branches, bark, flowers, and fruits were collected from healthy ‘Royal Gala’ apple trees growing at the Horticultural Experimental Station of Northwest A & F University, Yangling (34°20'N, 108°24'E), China. All of the tissues were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extractions. Several cultivars of apple were planted at the Horticultural Experimental Station of Northwest A & F University, Yangling (34°20'N, 108°24'E), China. Buds of *Malus domestica* Borkh. ‘Honeycrisp’, and ‘Qinguan’ were grafted onto the apomixic rootstock of *M. hupehensis* Rehd., and placed in plastic pots (38 cm high, 23 cm diam) filled with a local 5:1 (v : v) loess soil : sand. All cultivars were placed in a greenhouse under ambient light, at 20–35 °C and a relative humidity of 50% to 75%. To avoid edge effects, the pots were rotated weekly. Uniform trees of each cultivar were used for two treatments over a 60-d period: (1) well-watered, irrigated daily to maintain 65% to 75% field capacity; or (2) moderate drought, irrigated daily to maintain 45% to 55% field capacity. Young leaves were sampled every 15 d by detaching and quickly frozen in liquid nitrogen and stored at -80°C . Each treatment had four biological replicates.

Seeds of *Malus hupehensis* Rehd. were stratified at 0 to 4 °C for 50 d, then planted in black plastic pots (12 cm × 12 cm) filled with soil and sand and placed in a greenhouse under ambient light conditions. Culturing in our hydroponics system followed the methods described by Bai et al. (2008). To examine the role of abscisic acid (ABA) on stomatal behavior and gene expression in response to drought, we sprayed the leaves of selected plants with a 10 μM ABA solution. Young leaves were detached from different plants at designated time points and immediately frozen in liquid nitrogen.

Seeds of tomato cv. ‘Micro-Tom’ from WT and MdEPF2-overexpressing plants (see below) were sown in 250-cm³ plastic pots in a growth chamber under controlled conditions of 50% relative humidity, 28 °C, and a long-day (14-h day/10-h night) photoperiod (photon flux density of 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The seedlings were watered regularly and supplied with half-strength Hoagland’s nutrient solution (pH 6.0) twice a week for 40 d to maintain healthy growth.

Various watering regimes were assigned to the WT and transgenic tomato plants (six-week old): well-watered, with soil relative water content (RWC) maintained at 65%–75%; or drought, with RWC kept at 35%–45%. Soil RWC was calculated as (fresh weight – dry weight) / (saturated water weight – dry weight). The pots were weighed and supplemented for water losses daily. After 50 d of treatment, we measured photosynthetic rates; leaf RWC; leaf water loss; concentrations of chlorophyll, malondialdehyde, and H₂O₂; electrolyte leakage; and activities by SOD, CAT, and POD.

2.2. Sequence retrieval, construction of phylogenetic tree, sequence alignment, and analysis of promoter sequences

The following databases were used for retrieving and comparing the putative EPFLs from apple, tomato and *Arabidopsis*: the Genome Database for Rosaceae (GDR; https://www.rosaceae.org/species/malus/malus_x_domestica); The *Arabidopsis* Information Resource (TAIR) database (<http://www.Arabidopsis.org>); the tomato (*Solanum lycopersicum*) genome database (<http://solgenomics.net/>) and the National Center for Biotechnology Information (NCBI) database. All *Arabidopsis* EPFL sequences were taken as queries for performing a TBLASTN (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov>) against the apple genomic database, and a BLASTP against NCBI and tomato database, with an E value threshold of e^{-12} for apple, and e^{-13} for tomato proteins. We searched for EPF2 homologous protein sequences in other species by using BLASTP in the NCBI database. All the sequences we obtained were submitted to Pfam (<http://pfam>).

sanger.ac.uk/) and SMART (<http://smart.embl-heidelberg.de/>) to verify the presence of the EPF domain, and were manually inspected and revised according to their homologues and information available in the *Malus* EST database.

To elucidate putative orthologs of EPFLs in apple or tomato, EPFL sequences from *Arabidopsis*, apple and tomato were aligned using MEGA5 software (NJ method, bootstrap 1000 replications), with default parameters. To analyze the phylogenetic relationships among *MdEPF2* and *EPF2*s from other plants, we constructed a minimum-evolution (ME) phylogenetic tree via MEGA 5 software (<http://www.megasoftware.net/>), based on amino acid sequences.

The molecular weight and isoelectric point for *MdEPF2* were calculated by DNASTar software (Madison, WI, USA). Signal peptide cleavage site was predicted by PSORT (<http://psort.hgc.jp/form.html>). Multiple sequence alignments for the *EPF1-2-L7* clade of *MdEPF2* and the sequences from *Arabidopsis* were performed by DNAMAN software (Lynnon Biosoft Inc., San Ramon, CA), with default parameters.

Regulatory elements in the promoter region were analyzed with the online program PLACE (a database of plant *cis*-acting regulatory DNA elements; <http://www.dna.affrc.go.jp/PLACE/>) and PlantCARE (plant *cis*-acting regulatory elements, enhancers and repressors; <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

2.3. RNA extraction and qRT-PCR

Total RNA was extracted by the CTAB method (Chang et al., 1993). First-strand cDNA synthesis was performed with 1 µg of total RNA, using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. For real-time quantitative RT-PCR (qRT-PCR) analysis, we used *MdMDH* serving as the internal control. Procedures for qRT-PCR were conducted with SYBR® Premix Ex Taq™ II (Takara, Dalian, China), and the relative expression level of *MdEPF2* was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All reactions involved three biological replicates based on RNAs extracted from three different samples. For each sample, more than three seedlings were used.

2.4. cDNA cloning and plasmid construction

Total RNA was extracted from 'Qinguan' apple leaves by a CTAB method (Chang et al., 1993). First-strand cDNA synthesis was performed with a RevertAid First Strand cDNA Synthesis Kit (Thermo-Scientific) according to the manufacturer's instructions. The full-length cDNA sequences of *MdEPF2* and *sGFP* were amplified with gene specific primers. The amplified *MdEPF2* was inserted into a pCambia2300 vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Afterward, *sGFP* was inserted to generate the fusion construct 35S:*MdEPF2-sGFP*. Sequencing confirmed that the plasmid had been transformed into *Agrobacterium* EHA105 by the heat-shock method (Hood et al., 1984). The primer sequences for qRT-PCR analysis and cDNA cloning were listed in supplemental table S1.

2.5. Genetic transformation of tomato

'Micro-Tom' tomato was transformed by *Agrobacterium*-mediated procedures (Guo et al., 2012). Antibiotic (kanamycin)-resistant seedlings were PCR-confirmed to select positive transgenic lines. Because homozygous overexpression (OE) lines OE-2, OE-7, and OE-8 did not segregate until the T3 generation, we chose them for further experiments.

2.6. Gas exchange parameters

Gas exchange parameters were measured with a portable photosynthesis system (CIRAS-3; PP Systems, Amesbury, MA, USA), using the third expanded leaf from the shoot apex. All photosynthetic

measurements were applied at a photon flux density of 500 µmol m⁻² s⁻¹, cuvette flow of 300 mL min⁻¹, temperature of 24 ± 2 °C, and an ambient CO₂ concentration of 400 ± 5 ppm. Rates of photosynthesis (Pn), transpiration, and stomatal conductance (gs) were obtained from five plants for each genotype.

2.7. Stomatal characteristics

Stomatal density and sizes were examined under a scanning electron microscope (JSM-6360LV; JEOL Ltd., Tokyo, Japan), using abaxial epidermis of five-week old leaves collected from five plants each of the *MdEPF2*-OE lines and the WT. All samples were fixed as described by Cao et al. (2007).

2.8. Water loss, leaf RWC, and WUE

Leaves were detached from WT and transgenic plants grown under non-stress (well-watered) conditions and weighed immediately before being placed on a laboratory bench and re-weighed every 30 min. Water losses were calculated based on changes from those initial weights. Other leaves were removed and immediately weighed to obtain fresh weight (FW) values. They were placed for 24 h in bottles filled with distilled water, then blotted to remove excess water and re-weighed to obtain the turgid weight (TW). Afterward, they were dried to a constant weight at 65 °C and re-weighed to obtain the dry weight (DW). The leaf RWC was calculated as (FW – DW) / (TW – DW). Water-use efficiency was calculated as the plant dry weight / water consumption. Biomass production was determined after drying whole plants to a constant weight at 65 °C.

2.9. Photosynthetic pigments

Photosynthetic pigments were measured following extraction with 80% acetone (Porra et al., 1989), and absorbance was determined with a UV/visible spectrophotometer at 663 nm and 646 nm. The chlorophyll concentration was calculated as $C = 17.32 D_{646} + 7.18 D_{663}$.

2.10. MDA, H₂O₂, and O₂⁻

The concentration of MDA in leaf extracts was assessed by the thiobarbituric acid reaction method (Hodges et al., 1999) while the level of H₂O₂ was measured as described by Patterson et al. (1984). Accumulations of the superoxide ion O₂⁻ and H₂O₂ were assayed by histochemical staining methods that used nitro blue tetrazolium (NBT) and diaminobenzidine (DAB), respectively (Wang et al., 2015; Rangani et al., 2016).

2.11. Activities of antioxidant enzymes

Leaf samples (0.1 g) were ground in a chilled mortar with 1% (w/v) polyvinylpyrrolidone, then homogenized with 1.2 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA-Na₂ and 0.3% Triton X-100. Each homogenate was centrifuged at 13,000 g for 10 min at 4 °C. The supernatant was used for the following assays. The activity of SOD (EC 1.15.1.1) was determined by monitoring the inhibition of the photochemical reduction of NBT according to the methods of Giannopolitis and Ries (1977), while that of CAT (EC 1.11.1.6) was defined as the decrease in absorbance at 240 nm due to consumption of H₂O₂ (extinction coefficient of 39.4 mM⁻¹ cm⁻¹) (Aebi 1984). Peroxidase (EC 1.11.1.7) was assayed based on absorbance at 470 nm (extinction coefficient 25.2 mM⁻¹ cm⁻¹) caused by the oxidation of guaiacol (Rao et al., 1996).

2.12. Statistical analysis

Data for tissue-specific expression and ABA treatments were

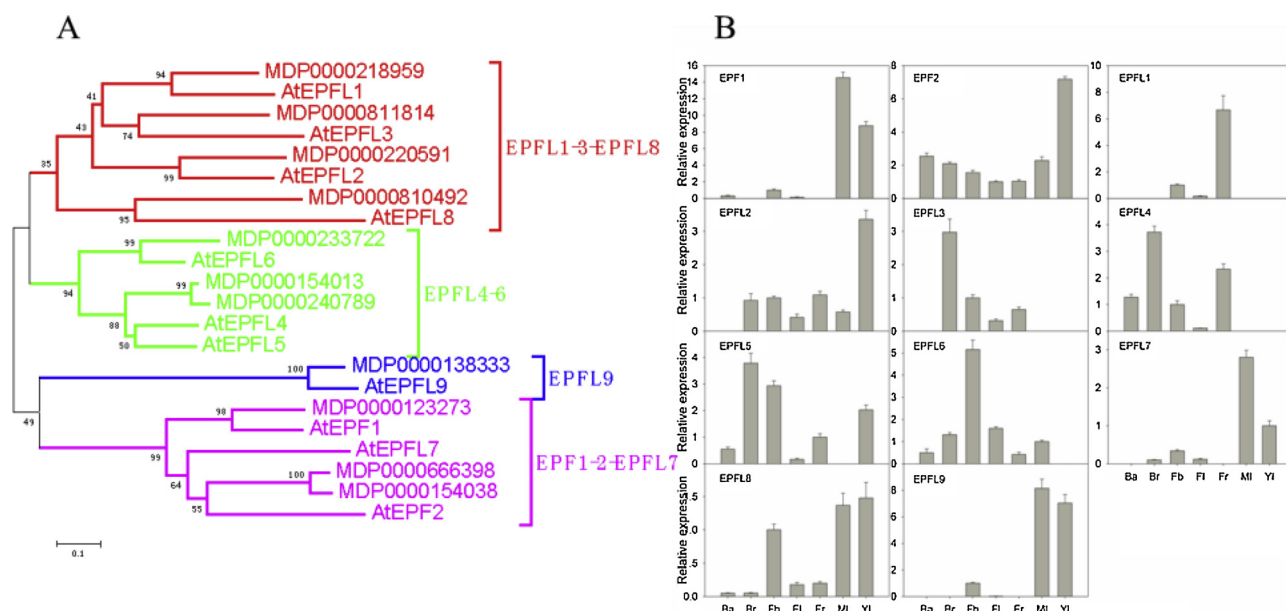


Fig. 1. Phylogenetic and expression analysis of *MdEPFL* family. (A) Phylogenetic relationship between EPFL family members in apple and *Arabidopsis*. (B) Expression pattern of *MdEPFLs* in different apple tissues. Ba, bark; Br, branches; Fb, flower buds; Fl, flowers; Fr, fruits; MI, mature leaves; YI, young leaves.

analyzed using one-way ANOVA, followed by Tukey's multiple range tests. Other data were examined by Student's *t*-tests and the SPSS 20 statistical package (SPSS, Chicago, IL, USA). All values were presented as the means \pm standard deviation (SD) of three to five replicate samples. A *P*-value of < 0.05 indicated a significant difference between means within the well-watered group or drought group.

3. Results

3.1. Identification and phylogenetic relations of the EPFL genes in apple

To identify members of the EPFL family in apple, all previously reported EPFL proteins in *Arabidopsis* were used as a TBLASTN query to search the apple genome (version 1.0). In all, 11 proteins with one conserved EPF domain were identified, determined with the on-line SMART and Pfam tools. To evaluate the evolutionary relationship between EPFL proteins in apple and *Arabidopsis*, a phylogenetic tree was constructed based on the full length EPFL protein sequences from these two species. As shown in Fig. 1A, the phylogenetic tree divided the EPFL proteins into four clades, and each EPFL protein in *Arabidopsis* had only one corresponding member in apple. Then, the EPFL family proteins were named based on their homologues in *Arabidopsis*.

To determine the tissue-specific expression pattern of *MdEPFLs*, we performed qRT-PCR with total RNA obtained from the branches, flowers, flower buds, fruits, barks, and leaves of 'Gala' apple (Fig. 1B).

As shown in Fig. 1B, these *MdEPFLs* exhibited different expression levels in different apple tissues. *MdEPFL2* and *MdEPFL8* were expressed in all the tissues examined, whereas *MdEPFL1* and *MdEPFL9* were only expressed in a few tissues. *MdEPFL2*, *MdEPFL2* and *MdEPFL8* were most highly expressed in young leaves, while several EPFL genes were not expressed in leaves (MI or YI); (Fig. 1B). The highest expression level of *MdEPFL2* in young leaves (Fig. 1B) was in accord with the function of EPF2 proteins in regulating stomatal patterns (Hara et al., 2009; Hunt and Gray, 2009), and implied that *MdEPFL2* might involve in stomatal development.

3.2. Expression profile of *MdEPFLs* during long-term drought treatment

To evaluate whether the *MdEPFL* genes are response to drought, changes of their expression levels were investigated in leaves of 'Honeycrisp' and 'Qinguan' apple during long-term drought treatment. For these two apple cultivars, 'Honeycrisp' is a drought sensitive cultivar and 'Qinguan' is a drought tolerant cultivar. *MdEPFL1*, *MdEPFL2* and *MdEPFL2* were induced by drought obviously. Moreover, *MdEPFL2* was induced by drought significantly in both cultivars, whereas *MdEPFL1* and *MdEPFL2* exhibited different expression patterns between different cultivars, with more significant induction in the drought sensitive cultivar 'Honeycrisp' (Fig. 2). *MdEPFL2* is the only gene that showed highest expression in young leaves and exhibited different expression patterns in different cultivars, so it was chose for further study.

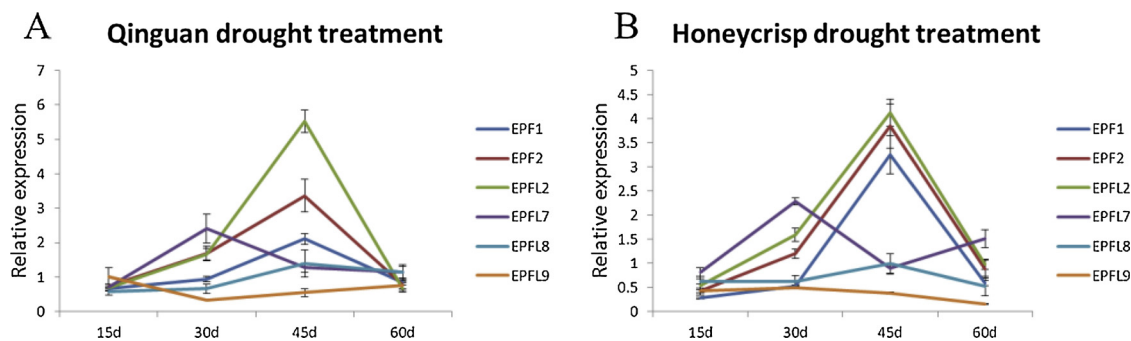


Fig. 2. Expression profile of *MdEPFLs* in apple leaves during long-term drought treatment. (A) Expression levels of *MdEPFLs* in 'Qinguan' leaves during drought. (B) Expression levels of *MdEPFLs* in 'Honeycrisp' leaves during drought.

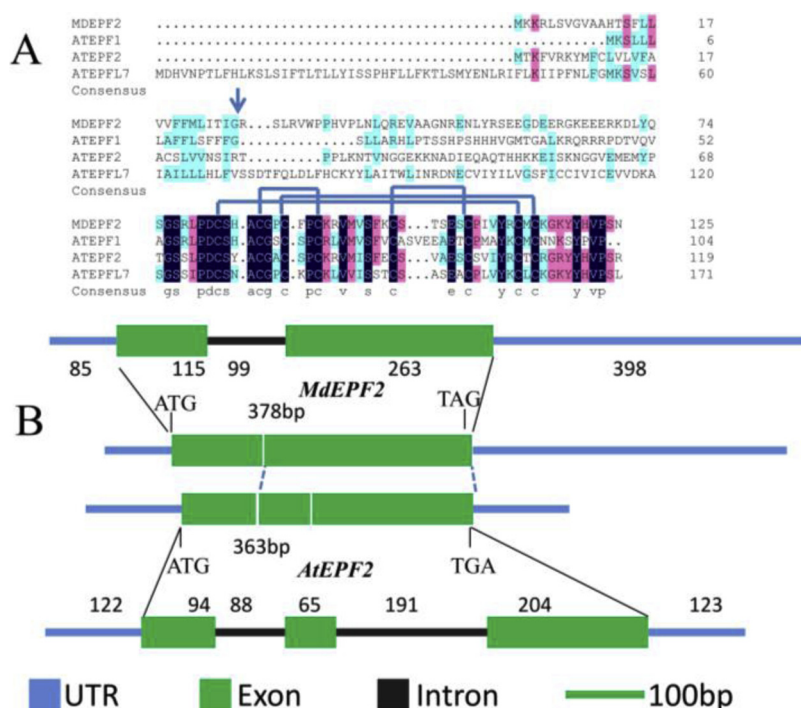


Fig. 3. Multiple sequence alignment and gene structure. (A) Alignment of MdEPF2 protein sequence with AtEPF1, AtEPF2, and AtEPFL7 proteins. Black boxes, identical residues; blue lines, pairs of cysteine residues forming disulfide bonds predicted for *Arabidopsis thaliana* EPFL genes. Arrow indicates signal peptide cleavage site predicted by PSORT (<http://psort.hgc.jp/form.html>). (B) Comparison of genomic sequences and cDNA sequence between MdEPF2 and AtEPF2. Numbers indicate sizes of untranslated region, (UTR), introns, and exons (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Identification and molecular characterization of MdEPF2

Based on previous studies on EPFL genes, we chose *MdEPF2* for further gene cloning and functional identification. Gene-specific primers, designed according to the mRNA sequence of MDP0000154038, were used to clone the full-length sequence of *MdEPF2* from ‘Qinguan’ apple. From this we obtained an mRNA sequence that contained a complete open reading frame of 378 bp. *MdEPF2* encodes a protein of 125 amino acids and has a molecular weight of 14.25 kDa and an isoelectric point of 8.65. Multiple sequence alignment showed that MdEPF2 shares 36.5% amino acid similarity with AtEPF2 and has eight cysteine residues that could putatively form four pairs of disulfides (Fig. 3A) (Ohki et al., 2011; Takata et al., 2013). It also has a putative N-terminal signal peptide of 27 amino acid residues and is expected to be secreted. Phylogenetic analysis and multiple sequence alignment indicated that MdEPF2 is most closely related to AtEPF2, based on those two proteins being clustered within the same clade (Fig. 1A) and the highest similarity in sequence alignment (Fig. 3A).

Although the lengths of the genomic and coding sequences were similar between *MdEPF2* and *AtEPF2*, a comparison of their cDNA and genomic sequences suggested that they have different gene structures, i.e., *AtEPF2* has three exons while *MdEPF2* has only two (Fig. 3B). Results from a further comparison suggested that the second exon of *MdEPF2* was possibly formed by the merger of the second and third exons of *AtEPF2* during the process of species evolution.

3.4. Phylogenetic analysis, promoter regulatory elements prediction and expression analysis under ABA treatment of MdEPF2

The EPF2 proteins are extensively distributed among land plant species (Takata et al., 2013). Our phylogenetic tree based on EPF2 protein sequences from apple and 43 other plant species (minimum-evolution method) revealed an evolutionary relationship from lower to higher plant species, with bryophytes located at the root of that tree (Fig. 4A). We also noted that proteins in the same families were clustered together. For example, MdEPF2 was clustered in the same clade with other EPF2s from members of Rosaceae, and was most closely related to *Prunus avium* and *P. persica*. Furthermore, MdEPF2 was most distantly related to the EPF2 proteins from monocotyledonous plants, as

illustrated by an obvious boundary between the EPF2 proteins from dicot and monocot species.

To investigate potential regulatory *cis*-acting elements responsive to water stress, we conducted a predictive analysis of the *MdEPF2* promoter and found that it contains four putative *cis*-elements: LTRECORE (an ABA-responsive element), an MBS (a MYB binding site involved in drought-inducibility), and two MYCs (cis-acting elements involved in drought-responsiveness) (Fig. S1). Abscisic acid has important roles in responses to various stresses, including drought, salt, and cold (Danquah et al., 2014). Exogenous application of ABA has impacts similar to those of osmotic stress, and may mediate some drought-responsive genes (Zhu, 2002). To investigate the expression pattern of *MdEPF2* in response to such treatment, we sprayed apple leaves with a 10 μ M ABA solution and used qRT-PCR protocols to quantify the transcription levels of that gene at different time points. As shown in Fig. 4B, expression increased by approximately three-fold at 2 h after treatment before sharply decreasing to the initial level. This was evidence of a quick response to the hormone. Combined with reports of the phenotype of *AtEPF2*-overexpressing *Arabidopsis* plants, including reduced stomatal density and increased WUE and dry weights (Doheny-Adams et al., 2012; Franks et al., 2015), we deduced that *MdEPF2* is critical to the regulation of drought tolerance in apple, perhaps by modulating stomatal density.

3.5. Tomato plants over-expressing MdEPF2 show reduced stomatal density and decreased water loss

Expression of *AtEPF2* helps determine stomatal formation (Hara et al., 2009; Hunt and Gray, 2009). To confirm whether *MdEPF2* functions in stomatal regulation, we transferred its overexpression vector 35S::MdEPF2-GFP into ‘Micro-Tom’ tomato to generate OE plants. After kanamycin-screening and verification via PCR for the presence of the transgene, we obtained 12 transformants and selected three lines with the highest levels of *MdEPF2* expression (Fig. S3). According to phylogenetic analysis (Fig. S2), there was only one member (SIEPF2, XP 004237867) corresponding to AtEPF2 in tomato. And *MdEPF2* overexpression did not interfere with the expression level of *SIEPF2* in transgenic tomato lines (Fig. S3).

Under normal growing conditions, the stomata density on the

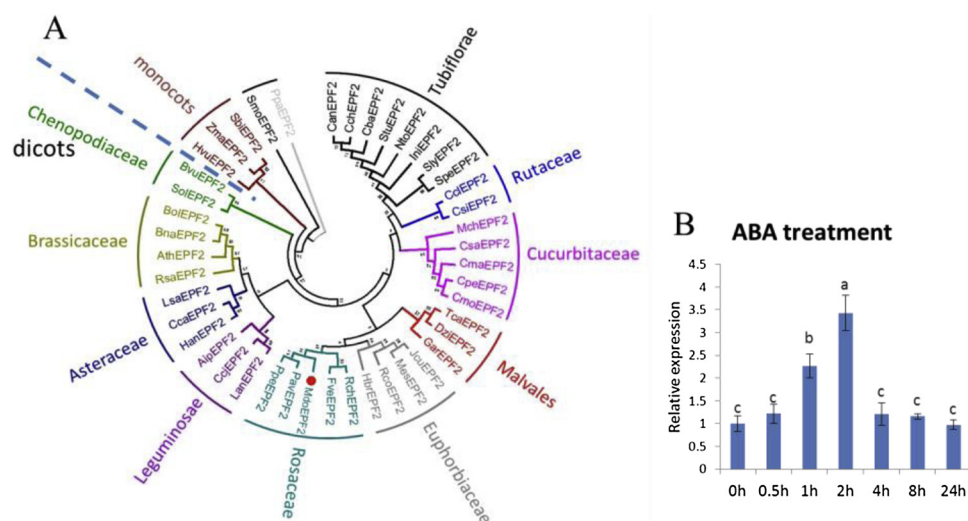


Fig. 4. Phylogenetic relationships among plant EPF2 proteins and *MdEPF2* expression in response to ABA treatment. (A) Phylogenetic analysis among plant EPF2 proteins. *MdEPF2* is marked by red dot. (B) Expression of *MdEPF2* in response to ABA treatment. Data are means \pm SD ($n = 3$). Different letters indicate significant differences over time, at $P < 0.05$. Can, *Capsicum annuum* (XP_016571569.1); Cch, *Capsicum chinense* (PHU20260.1); Cba, *Capsicum baccatum* (PHT50606.1); Stu, *Solanum tuberosum* (XP_006354046.1); Nto, *Nicotiana tomentosiformis* (XP_009615066.1); Ini, *Ipomoea nil* (XP_019154717.1); Sly, *Solanum lycopersicum* (XP_004237867.2); Spe, *Solanum pennellii* (XP_015071781.1); Ccl, *Citrus clementina* (XP_006434651.2); Csi, *Citrus sinensis* (XP_006473231.1); Mch, *Momordica charantia* (XP_022134337.1); Csa, *Cucumis sativus* (XP_004140931.1); Cma, *Cucurbita maxima* (XP_022992672.1); Cpe, *Cucurbita*

pepo subsp. *pepo* (XP_023550443.1); Cmo, *Cucurbita moschata* (XP_022939743.1); Tca, *Theobroma cacao* (XP_007020231.2); Dzi, *Durio zibethinus* (XP_022772392.1); Gar, *Gossypium arboreum* (XP_017605813.1); Jcu, *Jatropha curcas* (XP_012080631.1); Mes, *Manihot esculenta* (XP_021600136.1); Rco, *Ricinus communis* (XP_015583904.1); Hbr, *Hevea brasiliensis* (XP_021661140.1); Rch, *Rosa chinensis* (XP_024162463.1); Fve, *Fragaria vesca* subsp. *vesca* (XP_004292498.1); Mdo, *Malus domestica* (MDP0000154038); Pav, *Prunus avium* (XP_021799981.1); Ppe, *Prunus persica* (XP_020426255.1); Lan, *Lupinus angustifolius* (XP_019433845.1); Ccj, *Cajanus cajan* (XP_020208934.1); Aip, *Arachis ipaensis* (XP_016169582.1); Han, *Helianthus annuus* (XP_021984781.1); Cca, *Cynara cardunculus* var. *scolymus* (XP_024977368.1); Lsa, *Lactuca sativa* (XP_023770897.1); Rsa, *Raphanus sativus* (XP_018485341.1); Ath, *Arabidopsis thaliana* (at1g34245); Bna, *Brassica napus* (XP_013671225.1); Bol, *Brassica oleracea* var. *oleracea* (XP_013590663.1); Sol, *Spinacia oleracea* (XP_021847287.1); Bvu, *Beta vulgaris* subsp. *vulgaris* (XP_010692602.1); Hvu, *Hordeum vulgare* (ARS22227.1); Zma, *Zea mays* (AQK44000.1); Sbi, *Sorghum bicolor* (XP_002446602.2); Smo, *Selaginella moellendorffii* (XP_024515692.1); Ppa, *Physcomitrella patens* (XP_024378168.1) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

abaxial leaf surface was significantly lower for OE plants than for the WT, with the number per unit area ranging from 23% (Line OE8) to 35% (OE2), both values being smaller than those calculated for the untransformed WT plants (Fig. 5A, C). Stomatal size was also somewhat altered due to overexpression, with stomata from the transgenics being 18% (OE2) to 39% (OE7) longer than those from the WT, while their widths did not differ significantly among genotypes (Fig. 5B, D). Stomatal density is highly related to the degree of drought tolerance because it has a significant impact on water loss from the leaves (Doheny-Adams et al., 2012). Therefore, we tested rates of loss and found that, after 3 h of dehydration, the reduction in leaf weights was less from the three transgenic lines than from the WT plants, all evidence that water loss was slowed in response to *MdEPF2* overexpression (Fig. 5E). All of these data suggested that drought tolerance was enhanced in the transgenic plants due to such overexpression, which decreased those water losses by reducing their stomatal density.

3.6. *MdEPF2*-overexpressing plants show greater drought tolerance under long-term water deficits

When comparing between well-watered and water-deficit (35%–45% soil RWC) conditions, we noted that, after 50 d of treatment, all three transgenic lines performed better than the WT. Stressed transgenic plants were obviously taller (Fig. 6A) and had produced much more biomass than their WT counterparts (Fig. 6A, B). The OE plants also exhibited higher WUE under both normal and drought conditions than the WT, with those differences being statistically more significant under the latter scenario (Fig. 6C). All of these results suggested that overexpression of *MdEPF2* improved plant drought tolerance.

Reducing stomatal density can improve leaf RWC (Yoo et al., 2010), which then leads to enhanced drought tolerance. Our assessment of that parameter under both normal and drought conditions indicated that the leaf RWC values were higher in the transgenic plants than in the WT (Fig. 6D), which enabled the former to grow better in the stressed environment because they maintained higher leaf RWC due to

overexpression of *MdEPF2*.

3.7. *MdEPF2* overexpression improves photosynthetic rates in drought-stressed transgenic plants

Stomatal density can affect plant transpiration and leaf gas exchange, thereby influencing photosynthesis and, ultimately, biomass production. Our data revealed that, under stress conditions, Pn values were significantly higher in the transgenic lines than in the WT (Fig. 7A). However, no such contrasts were noted among genotypes under well-watered conditions. This more rapid rate of photosynthesis was associated with a greater accumulation of biomass in OE tissues under drought treatment (Fig. 6A, B). Values for gs and transpiration were also significantly reduced in the OE plants than in the WT under both well-watered and stress conditions (Fig. 7B, C). Therefore, this decrease in water losses from transgenic plants (Fig. 4E) might have been due to their lower rates of transpiration through the stomata, which likely enhanced their capacity to maintain higher WUE and leaf RWC (Fig. 6C, D). The significant decline in transpiration was not accompanied by a decrease in Pn (Fig. 6A, B), suggesting that reduced gs, which led to less transpiration and water loss, did not result in a concomitant decrease in biomass accumulation (Fig. 6B). Consequently, we could directly attribute the improved WUE by *MdEPF2*-OE plants to lower transpiration and a higher photosynthetic rate.

Chlorophyll is an important component of thylakoid pigment-protein complexes. Functioning of that complex is affected if the level of chlorophyll decreases, which then reduces the absorption of light energy by the chloroplasts. Because we had found that the OE plants had higher net photosynthetic rates (Fig. 7A), we compared chlorophyll concentrations among genotypes. Under normal conditions, no differences occurred. However, drought treatment caused a decline in concentrations in leaves from all plants, although levels were much higher in the transgenics (Fig. 7D). This was in accord with our finding of higher Pn values in the OE plants.

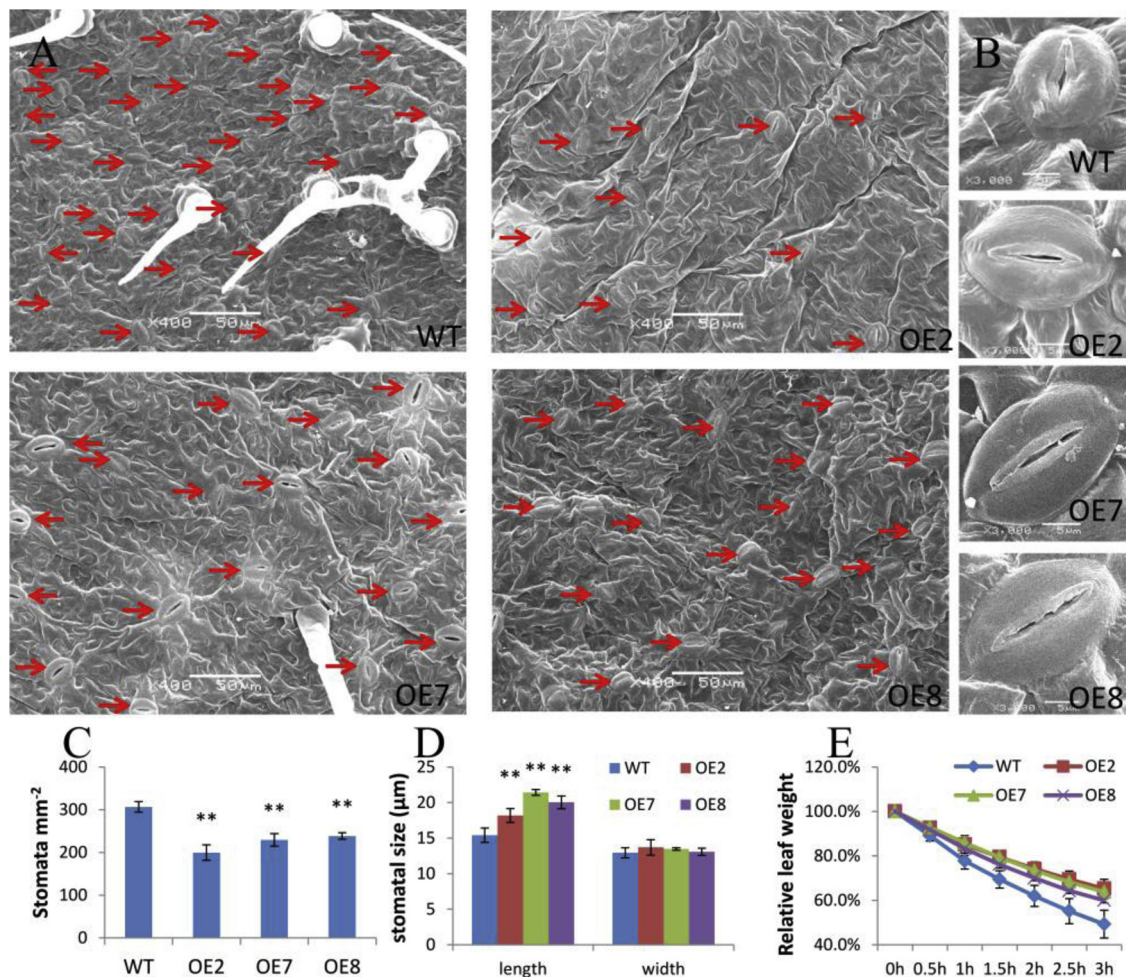


Fig. 5. Effect of *MdEPF2* overexpression on stomatal density, stomatal size, and leaf water loss. (A–B) Scanning electron micrographs of abaxial leaf epidermis from WT and *MdEPF2*-overexpressing plants. Scale bars represent 50 μm (A) and 5 μm (B). (C, D) Comparisons of stomatal density and stomatal size in leaf abaxial epidermis from WT and OE plants. (E) Water loss from detached leaves measured at indicated time points. Data are means \pm SD ($n = 5$). **, difference from WT is significant at $P < 0.01$.

3.8. *MdEPF2*-OE plants sustain less damage under long-term water deficits

Electrolyte leakage and MDA concentrations are typically used to assess the extent of drought-related damage to crop plants (Wang et al., 2012). Because our *MdEPF2* transgenic plants showed greater drought tolerance than the WT, we monitored fluctuations in leaf electrolyte leakage and MDA levels and found no visible differences in either parameter for any genotype grown under well-watered conditions. However, after the onset of long-term drought, values for both leakage and MDA concentrations were increased in all plants, albeit to a lesser degree in the transgenics. For example, electrolyte leakage rose by approximately 40% for the OE plants versus 46% for the WT (Fig. 8A) while MDA concentrations were approximately $9 \text{ nmol}^{-1} \text{ g}^{-1}$ and $11 \text{ nmol}^{-1} \text{ g}^{-1}$ for the transgenic lines and the WT, respectively (Fig. 8B).

Drought-induced accumulations of highly toxic ROS can lead to oxidative stress, which then damages various cell components. Histochemical treatment with NBT (for O_2^-) and DAB (for H_2O_2) revealed extensive staining in the leaves of all stressed genotypes, which indicated that ROS were greatly accumulated in response to drought treatment. However, those accumulations were much lower in the OE plants than in the WT (Fig. 8C). Values representing H_2O_2 levels also supported this conclusion, with concentrations being 15.0% to 18.3% lower in leaves from the transgenics than from the WT after the long-term water deficit (Fig. 8D).

The activities of antioxidant enzymes (e.g., SOD, CAT, and POD) involved in scavenging O_2^- and H_2O_2 change significantly under drought conditions (Jaleel et al., 2009). Here, we found no obvious differences in activities by any of those three enzymes among our transgenic and WT plants under well-watered conditions, but did note significant increases after drought treatment was applied. Moreover, those activities were approximately 21.4%–23.0% (SOD), 12.1%–12.7% (CAT), and 37.4%–41.3% (POD) higher in the OE plants than in the WT (Fig. 8E–G). All of these results suggested that plants over-expressing *MdEPF2* sustained less damage and were more drought tolerant under stress conditions.

4. Discussion

With the continued rise in levels of atmospheric greenhouse gases, drought has become a more critical factor affecting agricultural production worldwide (Zandalinas et al., 2017). Predictions of reduced water availability and an increasing frequency of extreme drought events that lead to substantial crop losses will present particular challenges for farmers (Korres et al., 2017). However, the ability to over-express *EPF* genes in various species, such as *Arabidopsis*, rice, barley, and *Populus*, could greatly increase their WUE and drought tolerance (Franks et al., 2015; Liu et al., 2016; Wang et al., 2016; Hughes et al., 2017; Caine et al., 2018). For this study, we cloned the *EPF* family gene *MdEPF2* from apple and conducted a preliminary investigation of its

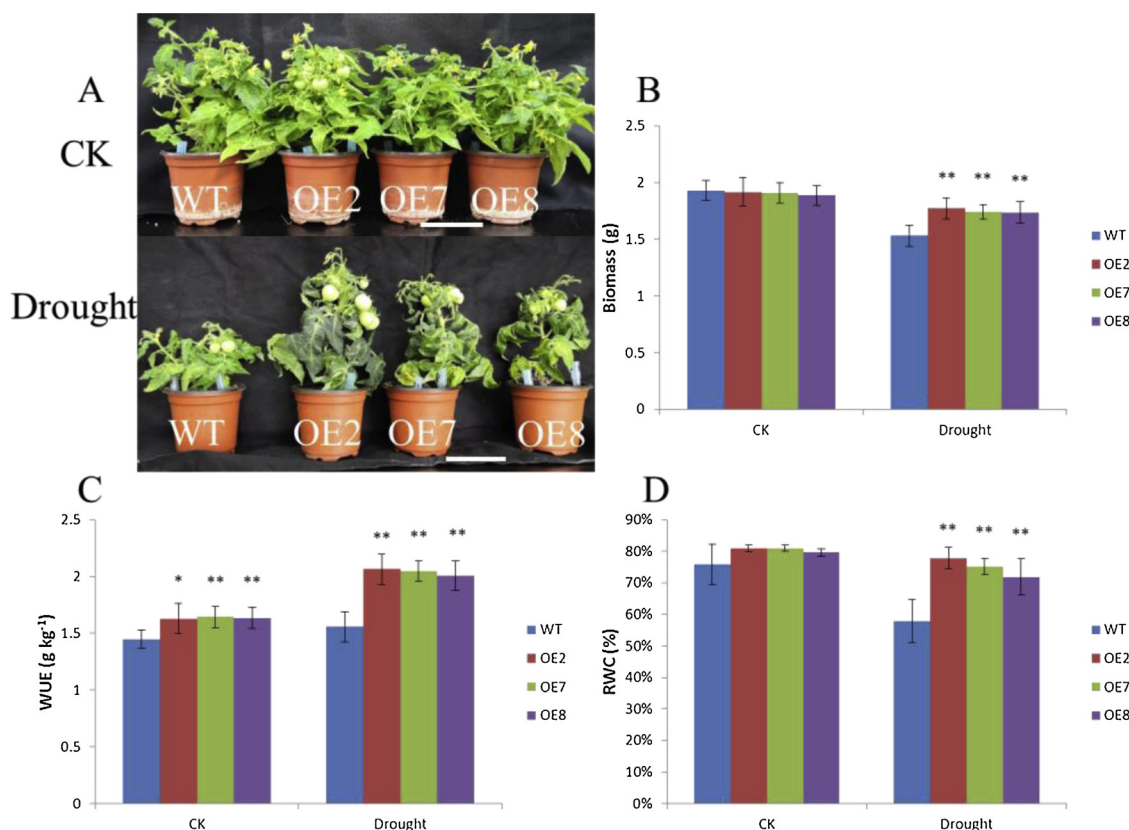


Fig. 6. Overexpression of *MdePPF2* in tomato confers drought tolerance during long-term drought. (A) Morphological differences. Bar = 5 cm. (B) Biomass production from OE lines and WT. (C) WUE in OE lines relative to control plants. Data are means \pm SD ($n = 5$). (D) Leaf relative water content from OE lines and WT. CK, well-watered control. *, **, differences among genotypes are significant at $P < 0.05$ and $P < 0.01$, respectively.

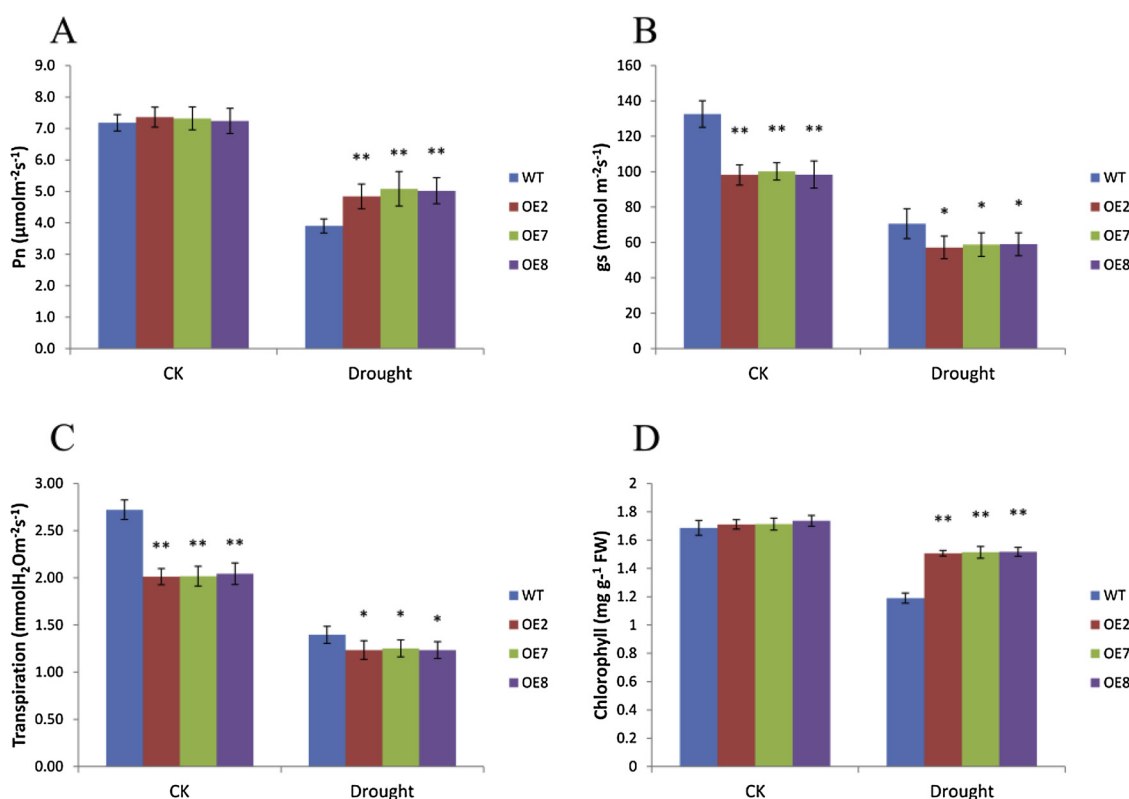


Fig. 7. Effect of changes in tomato stomatal density on net photosynthetic rate (A), stomatal conductance (B), transpiration (C), and chlorophyll concentrations (D). CK, well-watered control. Data are means \pm SD ($n = 5$). *, **, differences among genotypes are significant at $P < 0.05$ and $P < 0.01$, respectively.

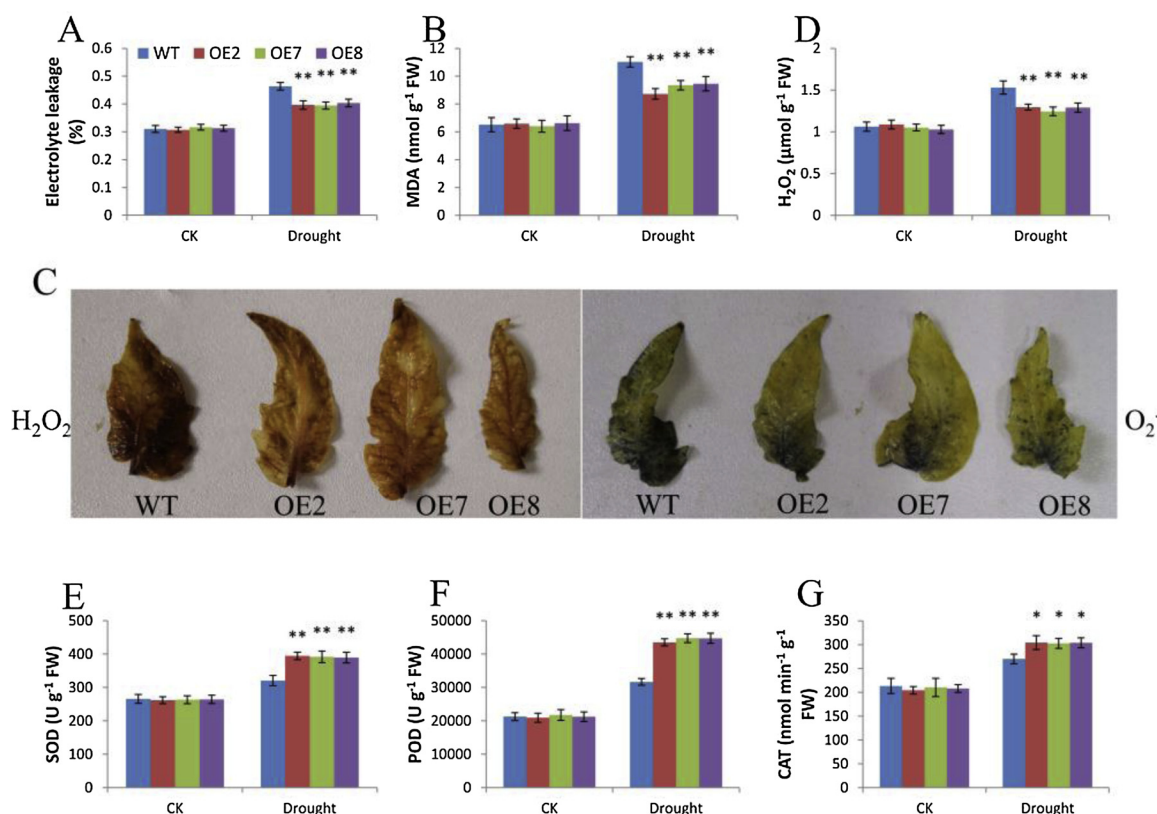


Fig. 8. Effects of *MdePF2* overexpression in tomato on degree of plant damage under long-term drought. (A) Electrolyte leakage. (B) MDA concentrations. (C) Results from staining to detect O₂⁻ and H₂O₂ in leaves from stressed WT and transgenic plants. (D) H₂O₂ concentrations. (E–G) Antioxidative enzyme activities in leaves from WT and transgenic plants under well-watered and drought conditions: SOD (E), POD (F), and CAT (G). CK, well-watered control. Data are means \pm SD ($n = 5$). *, **, differences among genotypes are significant at $P < 0.05$ and $P < 0.01$, respectively.

functions in controlling stomatal development and regulating tolerance to drought.

The *EPFL* genes are conserved in land plants and are classified, according to their mature peptide regions, into four clades: *EPFL1-2-EPFL7*, *EPFL1-3*, *EPFL4-6-EPFL8*, and *EPFL9* (Takata et al., 2013). Within the *EPF* structure, four pairs of disulfide bonds are structurally required for their activity (Ohki et al., 2011). Homologous genes from different species generally exhibit similar gene structure. For example, both of *MdUVR8* and *AtUVR8* contain 12 exons with similar gene structure (Zhao et al., 2016); and each exon of *MdFKBP12* is the same length as corresponding exons of *FKBPs* in other species (Dong et al., 2018). *PtEPF1* contains two exons and one intron similar to the gene structure of *MdePF2*, meanwhile, *PtEPF2* consists of three exons and two introns which shared similar gene structure with *AtEPF2*. Interestingly, comparative analysis of the cDNA sequences between *MdePF2* and *AtEPF2* suggested that, although they are of similar length, they exhibit different patterns of exon–intron composition (Fig. 3B). This exon/intron diversification of gene family members, including gain/loss, exonization/pseudoexonization, and insertion/deletion, has played an important role in the evolution of multiple gene families (Xu et al., 2012). Because the length of the second exon in *MdePF2* is similar to the addition of the second and third exons in *AtEPF2*, we suggest that a gain/loss of an intron within the second exon may have occurred during their evolution.

The expression levels of these apple *MdePF2* genes among the seven tissues were various, suggesting that they may play different roles in different tissues. In *Arabidopsis*, *EPF2* is expressed in the aerial organs of the plant (Hara et al., 2009). For *Populus* sp., expression of *EPF2* is higher in young leaves and mature leaves than in senescent leaves, and lowest in the roots (Liu et al., 2016). In this study, *MdePF2* was most highly expressed in young leaves (Fig. 1B), which is consistent with the

recognized role of *EPF2* in regulating stomatal development (Hara et al., 2009; Hunt and Gray, 2009; Liu et al., 2016). Expression of this gene was also significantly induced by ABA application (Fig. 4B), which was similar to the expression pattern of *PdePF2* in poplar (Liu et al., 2016). This phytohormone is a key regulator of abiotic stress responses. Under short-term drought, plants adjust their stomatal apertures, as influenced by ABA, by regulating the transmembrane transport of some ions (Chen et al., 2010; Kim et al., 2010). However, under a prolonged water deficit, plants produce leaves with altered stomatal density in order to reduce their maximum stomatal conductance (Franks et al., 2009; Doherty-Adams et al., 2012). *Arabidopsis* overexpressing *PdePF2* significantly up-regulated *ABI1* and *ABI2*, and decreased ABA sensitivity of overexpression plants during seed germination and seedling growth (Liu et al., 2016). This indicates that, besides to regulating stomatal development, *EPF2* gene also plays important roles in regulating the long-term growth and development of plants in response to ABA. Therefore, we might infer from these combined results that ABA regulates stomatal development and density by controlling *MdePF2* expression under long-term drought conditions.

Stomata on leaf surfaces are microscopic valves that optimize gas exchange. Transpiration and CO₂ uptake occur mainly through the stomatal apertures (Hetherington and Woodward, 2003; Nilsson and Assmann, 2007). We determined that overexpression of *MdePF2* in tomato led to a decrease in stomatal density but an increase in stomatal size (Fig. 5A, B). This result was in accordance with others. For example, leaves from *AtEPF2*-overexpressing *Arabidopsis* plants show significantly lower stomatal densities but larger stomata while *epf1epf2* mutants have higher stomatal density and smaller stomata when compared with the ‘Col-0’ control (Franks et al., 2009; Franks et al., 2015). Except for stomatal size, the decline in stomatal density in our transgenic leaves led to lower stomatal conductance (Fig. 7B) and a decline

in the transpiration rate (Fig. 7C). Therefore, the drop in transpirational water loss was likely due to the reduction in stomatal density, a key determinant of enhanced drought tolerance (Xiong et al., 2002). Studies also showed that water deficit decreased the stomatal density on leaf abaxial surface in tomato (Sam et al., 2000). However, our previous studies indicated that long-term drought treatment increased stomatal density in newly develop leaves in apple (Liang et al., 2018a, 2018b). The smaller leaves after drought treatment led us to speculate that the increase of stomatal density might due to the reduction of leaf size and cell size. Besides, species specificity in drought response may also be a factor.

Although photosynthetic rates under well-watered conditions did not vary but were decreased in all genotypes under water stress, that reduction was less severe in the transgenics (Fig. 7A). Because stomatal conductance was lower in the OE plants under both drought and well-watered conditions, we speculate that their photosynthetic rates became saturated due to limitations unrelated to stomatal factors, such as chlorophyll concentrations (Fig. 7D) and the regeneration of ribulose 1,5-bisphosphate, rather than because of changes in stomatal conductance (Farquhar and Sharkey, 1982). Similar result was found in the Arabidopsis mutant *sdd-1*. The increased stomatal density in this mutant had no significant influence on the leaf photosynthesis under constant light conditions (Schlüter et al., 2003). In addition, biomass accumulations were almost identical between stressed transgenic plants and well-watered WT plants (Fig. 6B), which suggested that a moderate decrease in stomatal density might have significantly reduced transpiration in the OE lines without a concomitant effect on CO₂ assimilation, enabling them to maintain higher WUE under the water deficit. This discovery is also in accord with previous reports that overexpression of *HARDY* and *HDG11*, as well as mutations of *GTL1* and *GPA1*, lead to decreased stomatal density and enhanced drought tolerance while having no effect on carbon assimilation (Karaba et al., 2007; Yu et al., 2008; Nilsson and Assmann, 2010; Yoo et al., 2010).

The decrease in chlorophyll content might be general symptom of oxidative stress under drought conditions, which could be attributed to chlorophyll degradation and photo-oxidation (Mafakheri et al., 2010). The lower capacity of light harvesting owing to decline in chlorophyll content ultimately caused the increase of ROS (Mafakheri et al., 2010). It is known that the homeostasis of ROS may act as secondary messengers in the stress-resonse signal transduction pathway, triggering defensive/adaptive responses. When ROS levels reach a certain threshold, they can trigger oxidative damage, leading to retarded growth and eventual cell death (Hou et al., 2009). In this study, the WT plants showed less biomass (Fig. 6) and more severe oxidative stress injury (Fig. 8) compared with transgenic plants after long-term drought treatment, suggesting that ROS could be another reason for biomass decline. To resist such damage, plants utilize various enzymatic and non-enzymatic systems to detoxify and scavenge excess ROS (Gill and Tuteja, 2010). Antioxidant enzymes such as SOD, CAT, and POD provide protection against cellular damage from oxidative stress. They also have important roles in conferring drought tolerance (Wang et al., 2011). Overexpression of *OsLG3* increases the activities of SOD and POD and helps improve drought tolerance (Xiong et al., 2018) while overexpression of *MdATG18a* in apple elevates CAT and POD activities and drought tolerance (Sun et al., 2018). We also found here that *MdEPF2*-OE plants showed higher SOD, CAT, and POD activities under drought stress (Fig. 8). This demonstrated that those transgenics had greater capacity for ROS-scavenging and were more tolerant of drought conditions. Previous studies indicates that *SIMAPK3* overexpressing tomato showed high transcript abundance and activities of antioxidant enzymes, such as SOD, POD and CAT (Muhammad et al., 2019; Long et al., 2014). MAPK3 was activated (phosphorylated) by EPF2 through MAPK cascade (Lee et al., 2015), suggesting that the enhanced antioxidant enzymes' activity in *MdEPF2* transgenic plants may also be related to the MAPK cascade. Further studies are needed to explore this mechanism.

Drought limits plant growth, production and survival rate in dry or semi-dry areas, and complex systems enable plants to respond to water deficit (Bray, 1997; chaves et al., 2003). In this study, we proved that *MdEPF2* overexpression could enhance drought tolerance of transgenic tomato plants, based on its function in stomatal density regulation and reduction of oxidative damage. Our results suggested that, with the application of CRISPR-CAS9 gene editing technology in apple (Malnoy et al., 2016; Nishitani et al., 2016) and further improvement (Osakabe et al., 2018), this gene might be potentially used in apple breeding to improve productivity and drought tolerance of this important economic fruit crop. This study is ectopic and simple, so further studies with apple transgenic materials are needed to clarify the molecular and physiological mechanisms of *MdEPF2* in response to drought.

Author contributions

The study was conceived by FM. QJ and KM collected the public datasets for apple and other species in this research, and contributed to bioinformatics analysis and manuscript preparation. QJ, QW, and KZ participated in qRT-PCR experiment. QJ and JY participated in phenotype and data analysis. FM, QJ, and KM participated in planning of experiments and revising the manuscript.

Declaration of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2019.03.009>.

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