

A novel Ap2/ERF transcription factor from *Stipa purpurea* leads to enhanced drought tolerance in *Arabidopsis thaliana*

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Abstract

Key message SpERF1 acts as a positive regulator, contributing to drought stress tolerance in *A. thaliana* through activating DRE/CRT elements in the promoters of abiotic stress-responsive genes.

Abstract *Stipa purpurea* is an endemic perennial grass species in alpine arid and semi-arid meadows on the Qinghai-Xizang Plateau, which is highly tolerant against drought and cold. ERF transcription factors are known to regulate gene expression under abiotic and biotic treatments. Herein, we isolated a full-length ERF gene CDS

from *S. purpurea* named *SpERF1*, which was induced by drought, cold, and jasmonic acid stresses. Subcellular localization revealed that SpERF1 is a nuclear protein, consistent with its roles as a transcription factor. Overexpression of *SpERF1* enhanced drought tolerance of transgenic *Arabidopsis thaliana* via the activation of DRE/CRT elements in the promoters of abiotic stress-responsive genes. Furthermore, increased accumulation of proline indicated that SpERF1 might be involved in proline synthesis in the transgenic lines, allowing them to have a better buffering capacity and membrane protection under drought stress. This study indicated that *SpERF1* might be an attractive target in the genetic engineering for improving stress tolerance in other crops. Moreover, SpERF1 protein function analysis increased our understanding of *S. purpurea*'s ability to adapt to the adverse conditions of the Qinghai-Xizang Plateau.

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A novel ERF transcription factor from *Stipa purpurea*.

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Introduction

Stipa purpurea (Poaceae) is distributed along a moisture gradient in alpine arid and semi-arid meadows on the Qinghai-Xizang Plateau (Yue et al. 2011). It plays critical roles in conserving water and soil, as a windbreak, and/or in sand fixation, and is an important natural forage resource for grassland animals. It is also an endemic constructive species, because of its excellent tolerance to harsh environments, including drought and cold. Our previous studies showed that the responses of *S. purpurea* to drought were complex, and higher expression levels of certain genes related to plant defense responses enhanced its acclimation

to drought (Yang et al. 2015a). Drought is a major environmental factor that has a crucial impact on the productivity and yield of crops and forage grass. Studies showed that extreme drought conditions have entered the northwest of the region over the past 300 years, and regional drought has been accelerating on the Qinghai-Xizang Plateau due to global climate change and human activities (Gou et al. 2007; Huang et al. 2011). Thus, it is important to identify the important regulatory genes associated with drought stress and to understand the mechanisms underlying these gene functions.

Plants have evolved with certain adaptation mechanisms, such that drought stress can be perceived sensitively and their physiology can be regulated rapidly. Such perception and regulation might involve many biochemical and physiological processes by modulating the expression of related genes. Transcription factors are essential for the regulation of gene expression by interacting with cis-acting elements in the promoter regions of various stress-related genes. Four major families of transcription factors have been identified in plants, including APETALA2/EREBP, bZIP, WRKY, and MYB, which mainly participate in controlling the plant stress response (Lindemose et al. 2013). AP2/ERF (APETALA2/Ethylene Responsive Factor) is a large group of plant-specific transcription factors, and 145 family members have been identified in *A. thaliana* and 167 in rice (Nakano et al. 2006). The subfamily of ethylene response factor (ERF) transcription factors induces resistance responses via various mechanisms that increase the plant's tolerance to multiple stresses. For example, overexpression of *TERF1* (from tomato) increased tolerance to drought and high salt in transgenic rice by regulating the expression of stress-responsive genes, including *Lip5*, *Wcor413-l*, *OsPrx*, *OsABA2*, *OsCDPK7*, *OsCDPK13*, and *OsCDPK19* (Gao et al. 2008). Similar studies also revealed that overexpression of tomato *JERF1* in rice could improve drought tolerance by activating the expression of two-key ABA biosynthesis enzyme genes in the ABA-dependent pathways (Zhang, et al. 2010).

RF proteins specifically interact with the GCC box (GCCGCC), which was first identified in *A. thaliana* and tobacco (Ohme-Takagi and Shinshi 1995; Solano et al. 1998). Earlier investigations indicated that the ERF proteins are mainly involved in the response to biotic stresses, such as pathogenesis, by recognizing the GCC box, which is present in promoters of pathogenesis-related (PR) genes. Expressions of the ERF genes are upregulated by JA/methyl jasmonate (MeJA), ethylene (ET), salicylic acid (SA), and pathogen infection, indicating that other ERFs are probably also important in plants overcoming disease through the ET, JA, and SA signaling pathways. Recent studies have shown that some ERF proteins also bind the

DRE/CRT (drought-responsive/C-repeat) element (G/ACCGAC) to participate in abiotic stress by regulating specific defense- and stress-related genes (Cheng et al. 2013; Huang et al. 2004). Under normal growth conditions, *A. thaliana* ERF1 protein binds to GCC box or DRE/CRT motifs, but binding with DRE/CRT is enhanced in some genes, such as *P5CSI*, under specific stress conditions (Cheng et al. 2013). In addition, some other ERFs that displayed improved tolerance to drought stress in transgenic rice and wheat plants, such as *TSRF1* (Quan et al. 2010) and *TaERF3* (Rong et al. 2014), also bound specifically to the DRE/CRT cis-element and activated the transcription of genes contained DRE/CRT boxes in the transgenic plants.

In this study, we reported the isolation and characterization of a novel ERF transcription factors from *S. purpurea*, designated *SpERF1*, which is responsive to drought stress. To investigate the function of *SpERF1*, we constructed 35S:SpERF1-GFP *A. thaliana* plants and confirmed the plants tolerance to drought stress. We also investigated the putative molecular mechanisms underlying the observed functions. Our results suggested that *SpERF1* increased the drought resistance of plants by regulation of stress-responsive genes. These results provide a better understanding of the adaptation of *S. purpurea* to harsh environments at the molecular level.

Materials and methods

Plant material and abiotic stress

Seeds of *S. purpurea* from the Qinghai-Xizang Plateau (35°14'37"N, 98°5'19"E) were germinated in the dark at 23 °C. The seedlings were transferred separately to soil pots and 1/4 Hoagland's nutrient solution (pH 5.5) under controlled conditions (28 °C day/25 °C night cycle, relative humidity of 75–80 %, 200-mmol photons m⁻² s⁻¹ light intensity). After 2 weeks of germination, drought stress was executed by withholding water for 10 days, and then the plants were re-watered for 5 days for recovery. In addition, PEG 6000 was added to 1/4 Hoagland's nutrient solution to a final concentration of 20 % (w/v) when the third leaf was fully expanded. For salt, MeJA, UV-B, ET (treatment with 1-aminocyclopropane-1-carboxylic acid; ACC), ABA, and cold treatment, seedlings were exposed to ultraviolet-B radiation (10 kJ m⁻² day⁻¹), cold (4 °C), NaCl stress (250 mM), ACC (10 μM) ABA (200 μM), and treated with 100-μM MeJA. Plants were harvested after treatment for 1, 3, and 6 h, with 0 h as the control, and then, all samples were kept at -80 °C.

The *A. thaliana* (ecotype: Col-0) plants were grown on 1/2 solid Murashige and Skoog (MS) medium for about

10 days. And then, all plants were transferred to soil in a growth chamber (photoperiod of 16-h light/8 h darkness) at 21 °C.

ERF gene fragment cloning

Total RNA was isolated from plant leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated by reverse transcribing 3 µg of DNA-free total RNA using Superscript III reverse transcriptase (Invitrogen) in a 20-µL reaction volume. Upstream primer E1 and downstream primer E2 were designed using the assembled ERF gene sequence (NCBI SRA: SRR825213). To obtain the full-length ERF cDNA from a *S. purpurea* sample, rapid amplification of cDNA ends (RACE) was performed using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, USA) according to the manufacturer's instructions. The full-length sequence of ERF was obtained using upstream primer ERF-F (5' ATGTTGCTGAACCTACGTCGG 3') and downstream primer ERF-R (5' CTAGCTGACCAGCTGCTCCAC 3').

Analysis of quantitative real-time PCR (qRT-PCR)

qRT-PCRs of all the investigated genes were performed as previously described (Bennett et al. 2015). All primers were designed by the Primer Express 2.0 software under default parameters. All the PCRs were performed under following conditions: 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 15 s. *Actin8* (GenBank: NM_103814) and *S. purpurea actin 1* (GenBank: KM216249) were used to normalize the *A. thaliana* and *S. purpurea* samples, respectively. The primer sequences are shown in Table A 1 in Supplementary material.

Sequence analysis of ERF gene

ERF protein sequences were aligned using the program Clustal W in BioEdit 7.0, followed by manual comparisons and refinement. Phylogenetic reconstruction of ERF sequences was performed using MEGA 5.1 based on the neighbor-joining method and bootstrap analysis of 1000 replications.

Generation and screening of transgenic *A. thaliana* plants

The open reading frame (without the stop codon) of *SpERF1* was inserted into vector pBIN, which contains a GFP gene under the control of the *Cauliflower mosaic virus* (*CaMV*) 35S promoter. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, which was then used for floral dip

transformation of *A. thaliana*. T0 seeds were selected on 1/2 MS medium-containing 50 mg L⁻¹ kanamycin, and the overexpressing *35S:ERF-GFP* transgenic lines (T1) were verified according to Sun et al. (2012). To detect the sub-cellular localization, root tissues were stained with 10-mg/mL propidium iodide for 5 min and washed once in water. Fluorescence of *35S:ERF-GFP* (Green fluorescence) and red fluorescence of transgenic plants were observed under a confocal microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Propidium iodide was excited using wavelengths of 600–640 nm, and GFP was observed with the 488-nm laser light.

Analysis of transgenic *A. thaliana* plants under drought conditions

For drought resistance analysis, WT and T3 generation seeds were surface sterilized, and then the seeds were sown separately on 1/2 MS medium added with 8 and 12 % polyethylene glycol (PEG) 6000 in the dark at 4 °C for 3 days. Samples were transferred to growth chambers. After 14 days, the lengths of the main root were measured. In addition, WT and transgenic plants were grown on 1/2 MS medium for 10 days and transferred to soil under a normal watering regime for 3 weeks. Water was withheld completely for 20 days, and then the plants were re-watered for 7 days for recovery. The relative water content (RWC) of leaves was calculated as follows: RWC (%) = [(FW – DW)/(TM – DW)] × 100 (Yang et al. 2015b). The leaves were weighed immediately after collection to calculate the leaves fresh weight (FW). To obtain the turgid mass (TM), leaf samples were floated in distilled water for 8 h, and then weighed. Dry weight (DW) was determined after drying samples at 80 °C in oven for 48 h. Three replicates per treatment were collected and analyzed.

Electrophoretic mobility shift assay (EMSA) analysis of SpERF1

The *SpERF1* was first cloned into pET32a, and then pET32a-*SpERF1* was transformed into the *Escherichia coli* strain RossetaTM. The His-*SpERF1* protein was induced with 0.5-mM isopropyl β-D-1-thiogalactopyranoside. Cells were lysed according to the instructions of the MagneHis Protein Purification System (Promega, WI, USA). After centrifugation, the supernatant was purified with a Ni-NTA spin column (Promega). Oligonucleotide probes were synthesized, annealed, and labeled by end-labeling a double-stranded oligonucleotide containing one ERF-binding site with a biotin label at the 3' end. The DRE/CRT and mutant DRE/CRT sequences used for EMSA are listed in Table A 2 in Supplementary material. The binding reactions were performed in a total volume of 20 µL containing

1 µg of purified protein, 100 fmol of probe, and binding buffer (20-mM HEPES–KOH, pH 7.5, 0.1-mM EDTA, 10-ng herring sperm DNA, 50-mM KCl, 0.1 % BSA, and 10 % glycerol). The chemiluminescence of biotin-labeled DNA was tested using a Light Shift Chemiluminescent EMSA Kit (Pierce, Rockford, Illinois, USA).

Proline content and electrolyte leakage measurements

The proline content was measured as previously described, with minor modifications (Demiral and Türkan 2005). In brief, the leaf samples were weighted and extracted in 10 mL of 3 % sulfosalicylic acid, after which the homogenate was centrifuged at 12,000g for 10 min. 2 mL of the extract was incubated with 2 mL of glacial acetic acid and 2 mL of acidic-ninhydrin (2.5 % w/v, ninhydrin, 40 % 6 M phosphoric acid, 60 % glacial acetic acid) at 100 °C for 40 min. The reaction mixture was then extracted with 5 mL of toluene for 1 min. L-Pro was used as the reference standard and the absorbance was measured at 520 nm. Three replicates per treatment were performed ($n = 10$).

To determine the membrane stability, the rate of electrolyte leakage (EL) was measured as previously described, with minor modifications (Wang et al. 2013). Briefly, samples were washed twice to remove surface-adhered electrolytes, and then added into 20 mL of distilled water and incubated at 25 °C for 30 min. The electrical conductivity of the solution (L_0) was measured using a conductivity meter (DDS-11A; Shanghai Leici Instrument Inc., Shanghai, China). Samples were then autoclaved at 120 °C for 20 min, and finally cooled down to room temperature. The last electrical conductivity was recorded as L_1 . The EL was calculated as follows: $EL = (L_0/L_1) \times 100$.

Results

Cloning and structural analysis of *SpERF1* gene

The full-length ERF gene sequence was cloned from *S. purpurea* using RACE technique, and was named *SpERF1* (Fig. 1a; GenBank accession: KU553347). Sequence analysis using the ExPASyCompute pI/Mw tool (http://web.expasy.org/compute_pi/) showed that the *SpERF1* cDNA was 756 bp, encoding a polypeptide of 251 amino acids with a calculated isoelectric point of 4.74 and a molecular mass of 26.20 kDa (Fig. 1). Further analysis using the simple modular architecture research tool (SMART) software (<http://smart.embl-heidelberg.de/>) indicated that the *SpERF1* protein contained an AP2 domain sequence (76 aa; 91–166), which is also referred to as the AP2-EREBP domain (Bowman and Smyth 1999).

SpERF1 was compared with two ERF proteins other species. The deduced amino acid sequence of *SpERF1* showed 70.49 % identity with *TiERF1* from *Thinopyrum intermedium* (GI:148009084), 67.41 % identity with *TaERF3* from *Triticum aestivum* (GI: 148009102). At the same time, we found that the ERF DNA-binding domain of *SpERF1* contained the RAYD element, but lacked the YGR element domain (Fig. 1b). In addition, a phylogenetic tree, which was reconstructed, including *SpERF1* and 147 ERF proteins, identified in the *A. thaliana* genome, suggested that *SpERF1* shared the closest genetic relationships with *AtERF1* (At4g17500) (Fig. 2 and Table A 3 in Supplementary material).

Expression pattern of *SpERF1* under stresses

To examine the response of *SpERF1* to various abiotic stresses, the expression of *SpERF1* was analyzed by qRT-PCR. The transcript level of *SpERF1* increased markedly from 0 to 1 h in response to PEG and MeJA treatment, but there were no significant differences from 1 to 6 h (Fig. 3). The highest expression levels of *SpERF1* were observed after drought treatment for 5 days, and the levels decreased at 10 and 5 days of re-watering, although the expression level remained higher than that of the untreated (Fig. 3). Meanwhile, compared with the control, expression of *SpERF1* was more than 11-fold higher after 6 h of ACC stress and more than fivefold higher after ABA stress, respectively (Fig. 3). In addition, the expression level of *SpERF1* decreased under salt and UV-B stresses. Under cold conditions, the expression level of *SpERF1* was significantly upregulated at 3 h of treatment, but downregulated at 6 h (Fig. 3). These results indicated that *SpERF1* might be involved in osmotic stresses, cold, and JA signaling.

Generation of transgenic *A. thaliana* plants and the subcellular localization of *SpERF1*

Transgenic plants were confirmed by detecting the *npt II* gene using specific PCR primers. The expression level of *SpERF1* in the T3 lines was determined using RT-PCR, which indicated that *SpERF1* was expressed differentially in the five homozygous lines (Fig. 4a). Two transgenic lines with the highest or lowest expression level (lines 5 and 2, respectively; designated as *SpERF1-5* and *SpERF1-2*, respectively) were selected for further analysis. The subcellular localization of a protein usually provides a clue to its function. As shown in Fig. 4b, the *SpERF1*-GFP fusion protein was specifically localized in the nuclei. These results suggested that *SpERF1* was a nuclear protein, which was consistent with its role as a putative transcriptional factor.

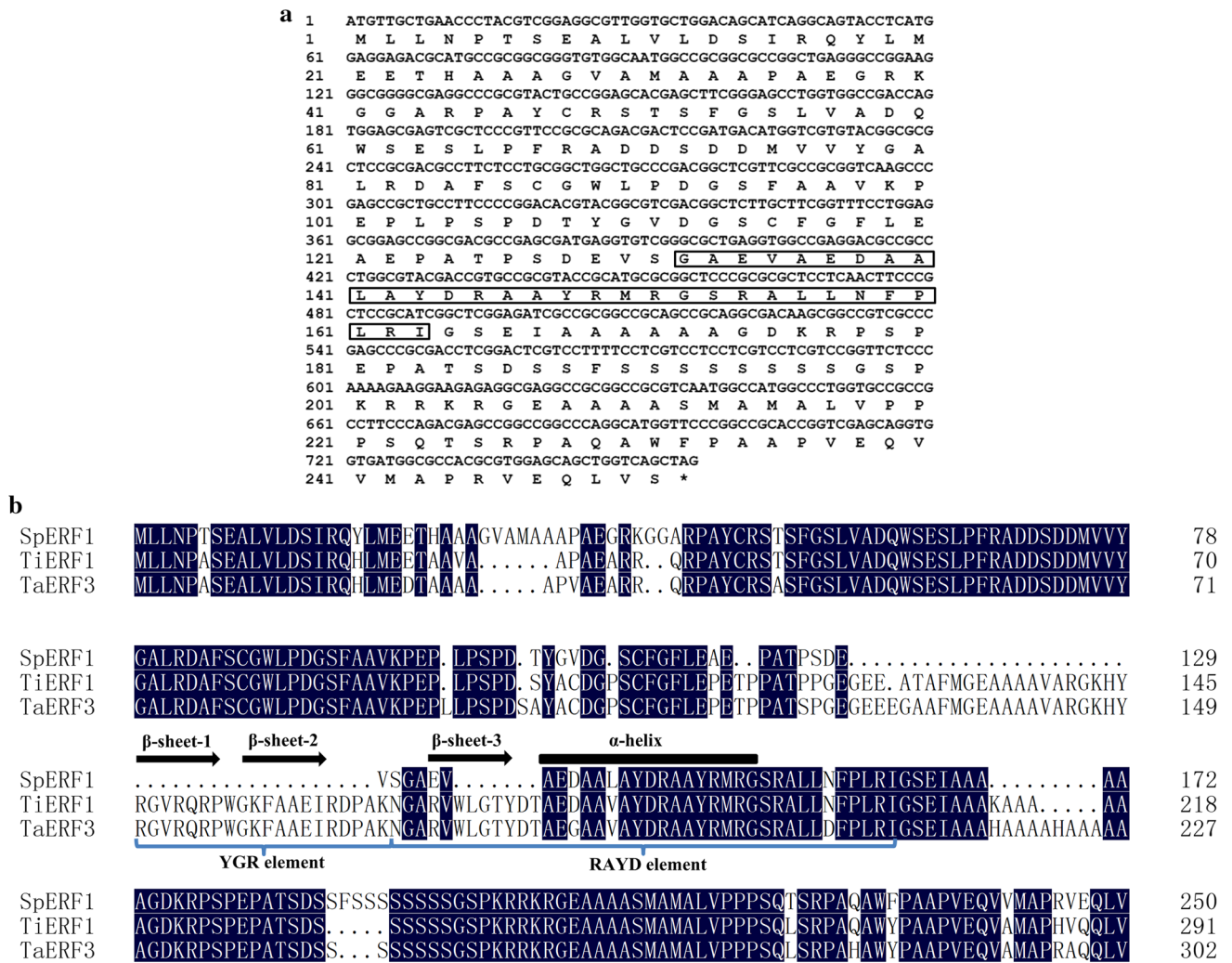


Fig. 1 Sequence analysis of the *SpERF1*. **a** Nucleotide and deduced protein sequence of *SpERF1*. The ERF domain is shown in boxes. **b** Multiple sequence alignment of *SpERF1* with its homologous sequences, including *Thinopyrum intermedium* TiERF1 (ABQ52686)

and *Triticum aestivum* (ABQ52687). Blue represents 100 % similarity. The amphipathic α -helix and three β -sheets are indicated over the corresponding sequences, and the conserved RAYD and YRG elements are shown by sky-blue brackets (color figure online)

Improved drought treatments tolerance in transgenic plants

To determine the biological function of *SpERF1* gene in stress tolerance, the response of transgenic *A. thaliana* overexpressing *SpERF1* to drought stress was analyzed. As shown in Fig. 5a, under normal growth conditions, the two transgenic lines showed no significant difference in primary root length compared with the WT. However, following treatment with 8 and 12 % PEG treatment, the primary roots of transgenic *A. thaliana* plants were much longer than those of the WT plants at 14 days (Fig. 5b–d). To further determine *SpERF1*'s function, the 3-week-old plants withheld from water for 20 days. Overexpression of *SpERF1* in transgenic plants increased drought tolerance compared with WT (Fig. 6a). In particular, the survival rates of transgenic plants were higher than those of WT

(Fig. 6a, b). When plants were re-watered, the transgenic plants recovered faster and showed a better survival than WT plants (Fig. 6a, b). Drought stress causes water loss in plants; after a 20-day drought period, the water content of the leaves of the control plants was significantly lower than the leaves of the transgenic plants (Fig. 6). These results suggested that overexpression of *SpERF1* rendered plants more resistant to drought.

EMSA of the SpERF1 fusion protein

Previous studies demonstrated that ERF proteins might prevent developmental changes by interacting with GCC box or DRE/CRT element and be involved in the activation of multiple stress signal pathways (Cheng et al. 2013; Hao et al. 1998). To examine whether SpERF1 directly interacts with the promoter-containing DRE/CRT element, we

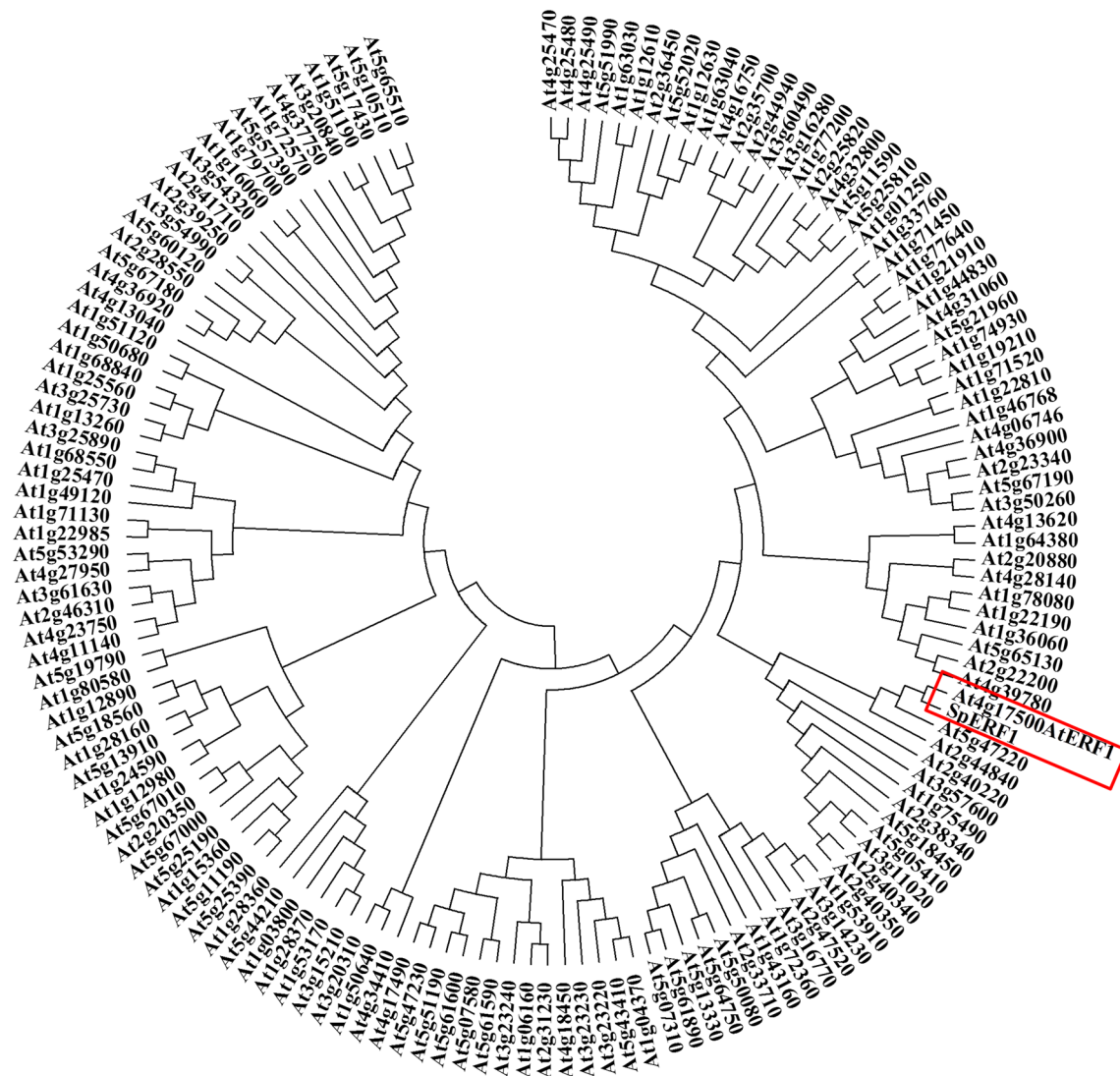


Fig. 2 Phylogenetic relationships between the *SpERF1* protein and the 147 ERF proteins from *A. thaliana*. The phylogenetic tree was reconstructed based on a complete amino acid sequence alignment of

ERF proteins using the NJ method (1000 replicates). *SpERF1* and *AtERF1* are shown in boxes. Information of 147 ERF proteins from *A. thaliana* are listed in Supplementary material

performed EMSA using a double-stranded oligonucleotide one ERF-binding site as probes. We established that the His-SpERF1 fusion protein could bind to the promoters of several genes, such as *HSP101*, *LEA4-5*, *RD29A*, *P5Cs*, *HSP70*, and *COR47*, all of which contain a DRE/CRT element (Fig. 7). Specific primers were designed to mutate the DRE/CRT element, whereas non-specific primers were used as the negative control. Using EMSA, a high-affinity DNA–protein complex was observed when DRE/CRT elements were incubated with His-SpERF1 fusion proteins. Excess unlabeled DRE/CRT element was able to compete for the DNA-binding activity of SpERF1. The mutated DRE/CRT element failed to interact with SpERF1 (Fig. 7). These results indicated that the SpERF1 protein was able to recognize and bind to the promoter of *HSP101*, *LEA4-5*,

RD29A, *P5Cs*, *HSP70*, and *COR47* via their DRE/CRT elements. We speculated that the SpERF1 proteins executed their functions via this recognition sequence to regulate downstream genes to enhance transgenic plants tolerance to drought stress.

SpERF1 interacts with DRE/CRT elements of promoters of genes upregulated in response to drought stress

To further confirm whether *SpERF1* regulates the promoters of *HSP101*, *LEA4-5*, *RD29A*, *P5Cs*, *HSP70*, and *COR47*, the expression of these genes was analyzed by qRT-PCR in *SpERF1*-overexpressing *A. thaliana* and WT plants. As shown in Fig. 8, the expression levels of all

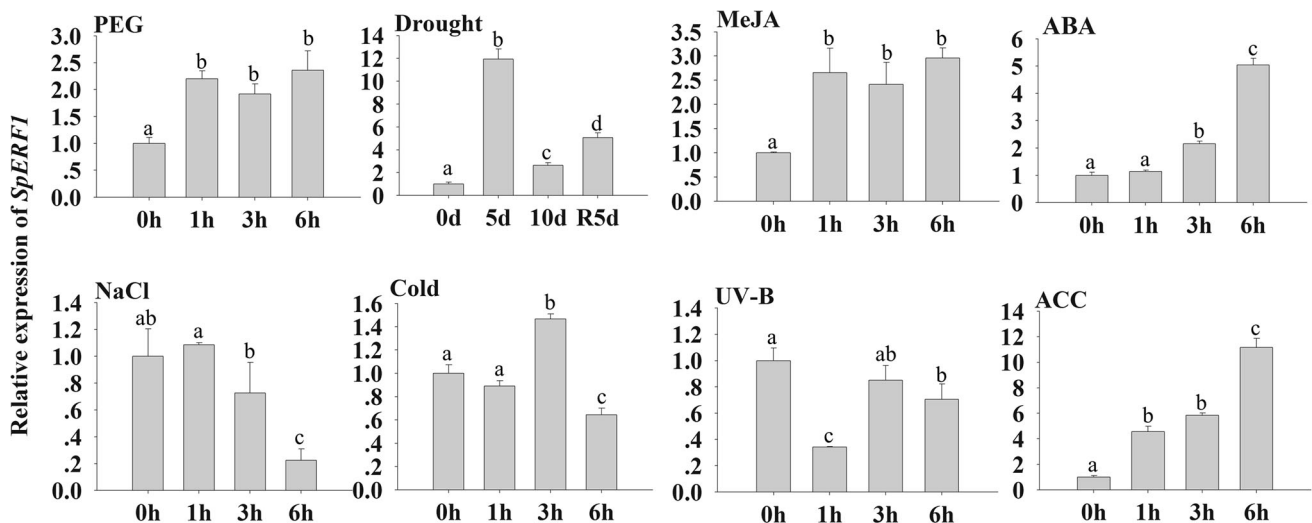


Fig. 3 Analysis of *SpERF1* expression in *S. purpurea* exposed to difference stresses. Treatments and quantitative real-time RT-PCR analysis were performed as described in “Materials and methods”. Expression values were calculated using $2^{-\Delta\Delta CT}$ method. Data are

mean values \pm SE obtained from three replicates and shown in Table A 4 in Supplementary material. Different letters within a column indicate a significant difference ($P < 0.05$; Tukey’s test)

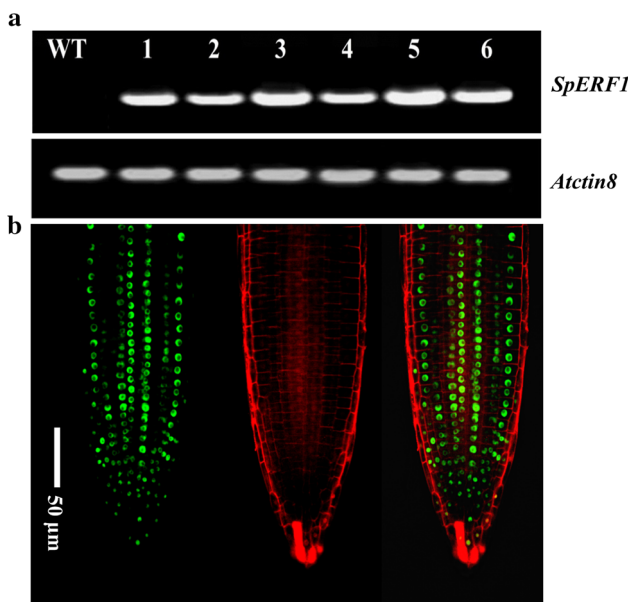


Fig. 4 Subcellular localization of the *35S:SpERF1-GFP* fusion protein. **a** Analyses of *SpERF1* expression in T3 lines and WT using reverse transcription-PCR (RT-PCR). WT wild-type plants, 1–5 transgenic *A. thaliana* plants, 6 positive control. **b** Confocal laser scanning microscopy images of GFP (green) and PI fluorescence (red) in the transgenic *A. thaliana* root. Bars 50 μ m (color figure online)

these genes in the transgenic lines were obviously higher than those of the WT plants. In particular, the expression levels of *HSP101* were more than 100-fold higher compared with their levels in the WT. After PEG treatment, these genes were also increased in WT and two transgenic lines, but significant differences were observed in the

magnitude of the changes between *SpERF1*-overexpressing *A. thaliana* and the WT. The magnitude of the increased expression levels of *LEA4-5* and *P5CS1* was higher than in the WT (Fig. 8). The present findings, combined with the EMSA results, implied that the SpERF1 transcription factor can upregulate expressions of these genes by recognizing DRE/CRT element in their promoters.

Changes in proline accumulation and electrolyte leakage (EL)

To evaluate the role of *SpERF1* in plant response to drought stress, the proline contents and EL were measured. Similar proline concentrations were found in the leaves of well-watered transgenic and WT plants (Fig. 9). When they were exposed to dehydration treatment, the proline contents of both types of plants increased significantly with exposure time. However, the proline content of the transgenic plants was significantly higher compared with the WT. In addition, under normal conditions, there were no significant differences between the EL of the transgenic and WT plants. Drought stress caused an increase in EL of the transgenic and WT plants; however, the transgenic lines displayed a lower EL than WT at each time point (Fig. 9).

Discussion

The AP2/ERF family, one of the large transcription factors families, is involved in plant responses to the biotic and abiotic stresses. ERFs are defined by the presence of an AP2/ERF domain, which comprises 60–70 highly

Fig. 5 Effects of drought stress on transgenic and WT seedlings. **a** Phenotype of *A. thaliana* under normal conditions. Phenotype of WT and *SpERF1*-overexpressing *A. thaliana* roots grown on MS medium-containing 8 % PEG (**b**) and 12 % PEG (**c**). The photograph was taken 14 days after germination (DAG). **d** The root length was measured after 8 % PEG or 12 % PEG treatment for 14 DAG. Data mean values \pm SE obtained from three replicates (* $P < 0.05$, ** $P < 0.01$; Student's *t* test)

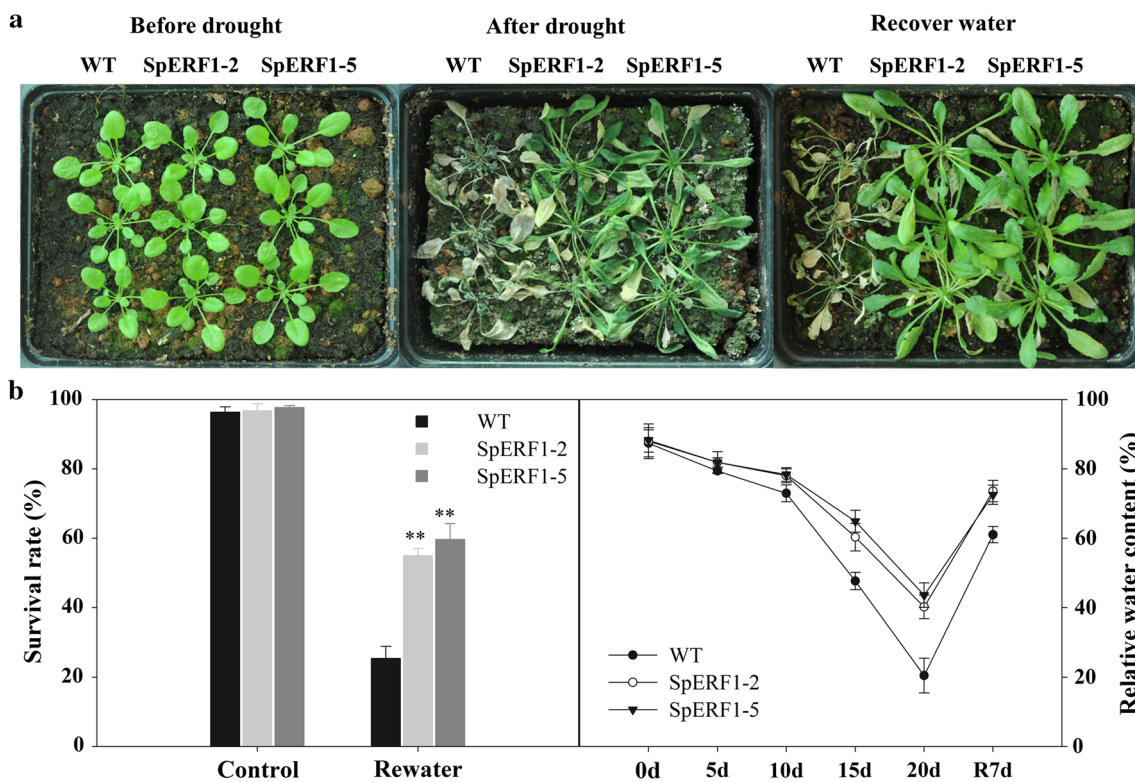
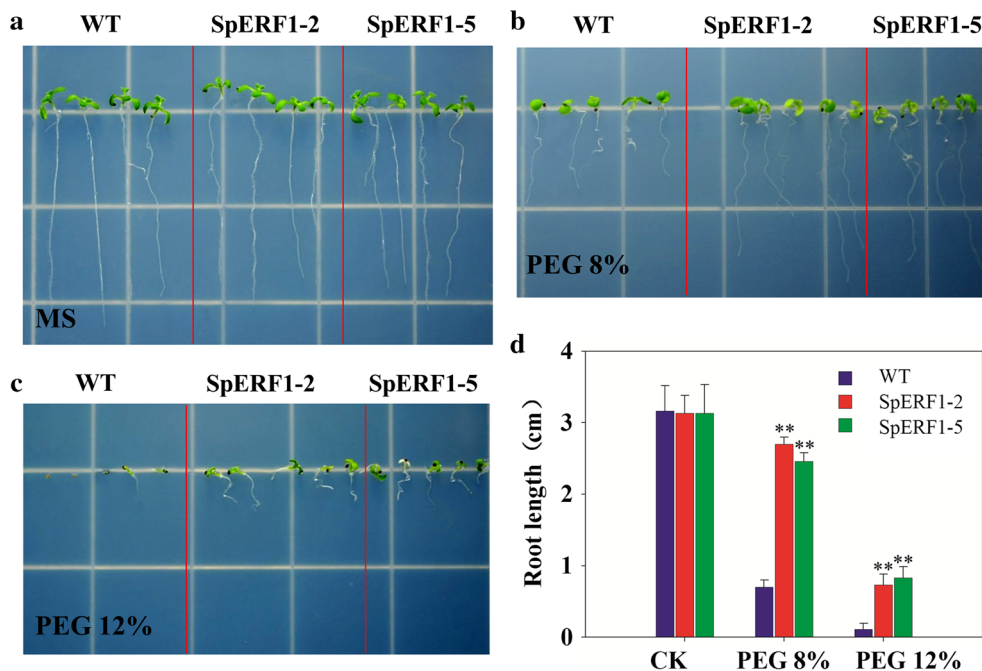


Fig. 6 Phenotypes of transgenic and wild-type plants under natural growth conditions and drought treatment. **a** Leaf phenotypes of wild-type and transgenic *A. thaliana* following exposure to drought stress.

Effects of drought on survival rate (**b**) and RWC of leaves (**c**) in WT and transgenic seedlings. Data are mean values \pm SE obtained from three replicates (* $P < 0.05$, ** $P < 0.01$; Student's *t* test)

conserved amino acids (Weigel 1995). In *A. thaliana*, the AP2/ERF family was classified into three subfamilies based on AP2/ERF domains, including the RAV subfamily,

the ERF subfamily, and the AP2 subfamily (Nakano et al. 2006; Sakuma et al. 2002). The RAV family proteins contain a single AP2/ERF domain and B3 domain; the ERF

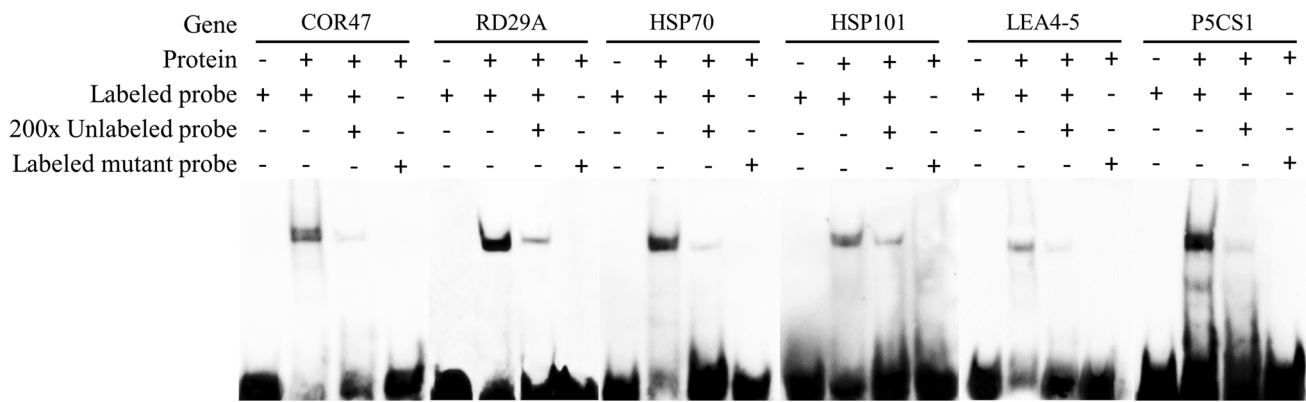
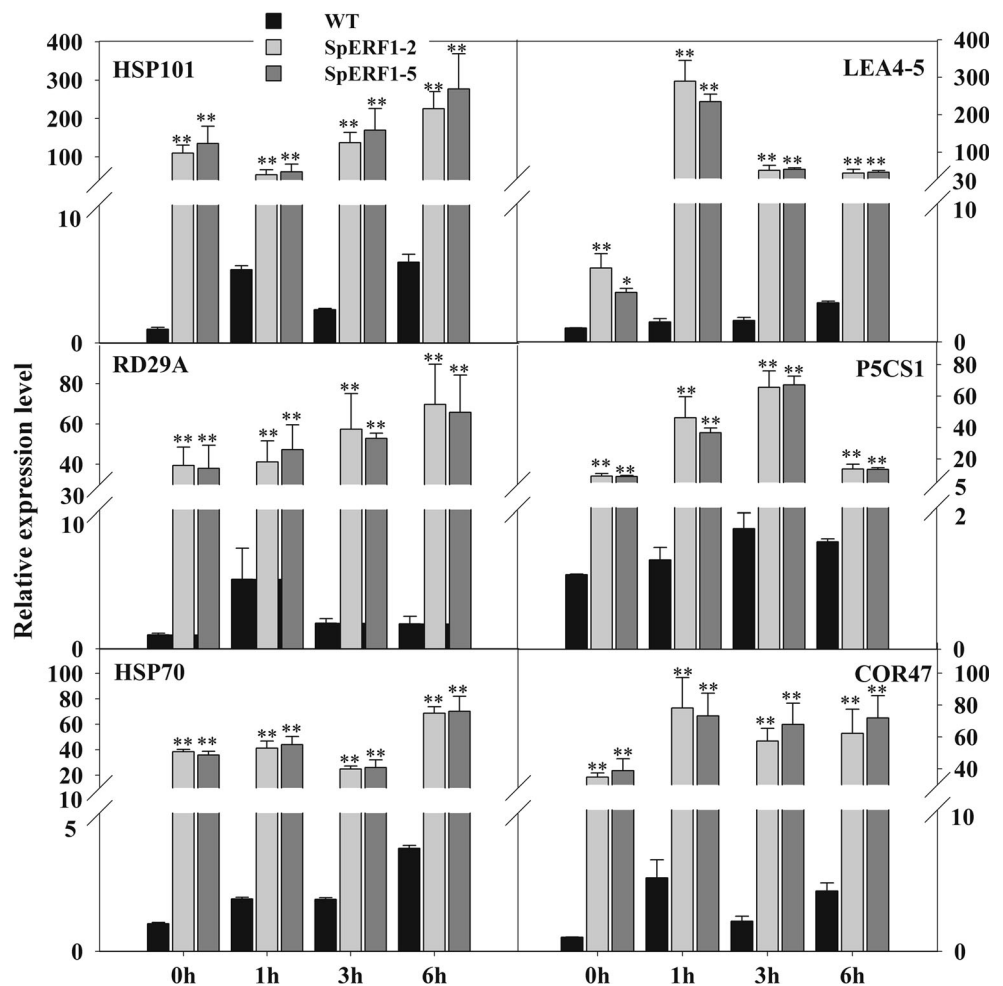


Fig. 7 *SpERF1* specifically binds to DRE elements of gene promoters in vitro. Electrophoretic mobility shift assay (EMSA) results showing full-length *SpERF1* binding to a DRE/CRT sequence. DRE/CRT and mDRE/CRT indicate the native and mutant DRE/CRT of the gene promoters from *A. thaliana*, respectively. Protein, His-

SpERF1 protein. Sequences of genes of labeled, as the probe and the labeled mutant probe are listed in Table A 2 in Supplementary material. Magnification $\times 200$, the unlabeled probe, was the wild-type gene sequence

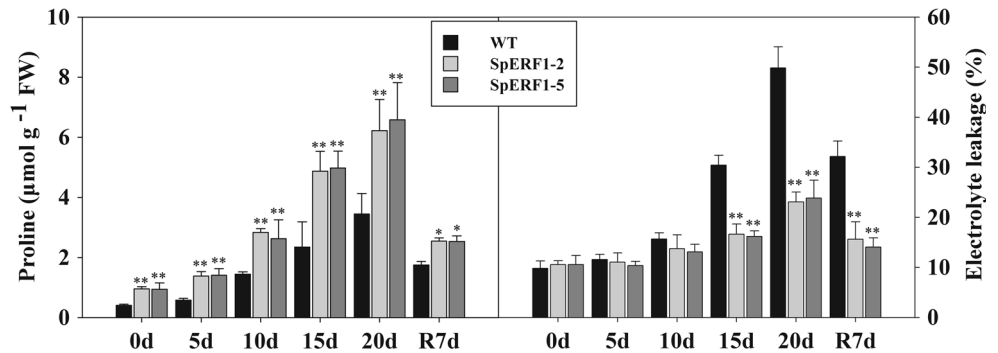
Fig. 8 Expression of stress-responsive marker genes with DRE elements in wild-type and transgenic *A. thaliana* under drought treatment. Expression values were calculated using the $2^{-\Delta\Delta CT}$ method and *AtActin1* as the endogenous control. Data are mean values \pm SE obtained from three replicates (* $P < 0.05$, ** $P < 0.01$; Student's *t* test)



family proteins contain a single AP2/ERF domain; and the AP2 family proteins contain double AP2/ERF domains (Nakano et al. 2006; Sakuma et al. 2002). In this study, we

cloned *SpERF1*, the first Ap2/ERF transcription factor from *S. purpurea*, which included an AP2/ERF domain (Fig. 1a). The AP2/ERF domain of *SpERF1* contains the

Fig. 9 Overexpression of *SpERF1* in *A. thaliana* increases the proline level and decreases water loss of whole plants. Data are mean values \pm SE obtained from three replicates (* $P < 0.05$, ** $P < 0.01$; Student's *t* test)



RAYD element, but lacks the YGR element domain (Fig. 1b). Furthermore, Nakano et al. reported that *A. thaliana* ERF subfamilies could be divided into 12 subgroups: groups I–X, VI–L, and Xb–L (Nakano et al. 2006). The results were different from the classification of Sakuma et al., in which the ERF subfamilies were classified into six groups: B-1 to B-6 (Sakuma et al. 2002). A phylogenetic tree, including the SpERF1 protein and the 147 *A. thaliana* Ap2/ERF proteins, was created, which showed that SpERF1 was clustered with *AtERF1* (At4g17500). According to these observations, SpERF1 belonged to group IX in the ERF subfamily of Ap2/ERF in *S. purpurea*.

In plants, ERF proteins play important roles in regulating abiotic and biotic stress responses. Increasing evidence shows that ERFs can be induced in response to various stresses, including drought, salt, extreme temperature, abscisic acid, and pathogen attack (Rong et al. 2014; Zhang et al. 2007, 2009). Here, we also found that the expression of *SpERF1* was about 2.5-fold higher after 1 h of PEG stress and 12-fold higher after 5 days of drought stress, respectively, than the control values (Fig. 3). The expression of SpERF1 increased markedly from 0 to 6 h in response to ABA and ACC treatments. Meanwhile, *SpERF1* was induced by cold and JA treatment (Fig. 3). Previous studies have revealed that some of ERF proteins that act as mediators of ethylene-related responses participate in the abiotic stresses response in several plants, and could be used to enhance the tolerance of transgenic plants to stresses (Fujimoto et al. 2000; Mizoi et al. 2012). For instance, osmotic stress could enhance *ERF1*, *ERF2*, *ERF5*, *ERF6*, and *ERF11* expression and accumulation of ET production in *A. thaliana*. ERF proteins were also involved in gene expression regulation in an ethylene-independent manner in soybean, rice, poplar, and tomato; however, their detailed functions are unknown (Sharma et al. 2010; Sharoni et al. 2011; Zhang et al. 2008; Zhuang et al. 2008). *SpERF1* transcripts were induced in response to MeJA, cold, ABA, ACC, and drought treatments, which implied that *SpERF1*

could be involved in an active response to drought, cold, JA, ABA, and ET signaling.

Unfortunately, there are no available mutants of *SpERF1*, and a transformation system of *S. purpurea* has not been established. Some investigations indicated that ectopic overexpression of plant genes in *A. thaliana* had provided important information for the function verification of foreign genes from non-model plants (Kishor et al. 1995; Ayarpadikannan et al. 2014). They were, however, limited for the specific purposes to reveal the real function of the foreign genes, especially for the genes network regulation, because of the diverse genomes of different species (Weising et al. 1988; Iyer et al. 2000). In addition, limitation of functional genomics analysis based on transgene overexpression also includes stable transformation in offspring, non-seed-specific high transgene expression, and phenotypic abnormalities, respectively (Budar et al. 1986; Weising et al. 1988; Iyer et al. 2000). Whether the foreign genes can be stably inherited in offspring is one of the key problems in gene function research and application. The foreign genes usually showed instability in early generations, but the genes could be stabilized through self-pollination generation by generation (Weising et al. 1988). Therefore, the *SpERF1* CDS driven by 35S CaMV promoter was introduced into WT *A. thaliana*, which a typical self-fertilize species T3 transgenic lines were used for further analysis. The signal of SpERF1-GFP fusion protein was observed exclusively in the nucleus of the transgenic *A. thaliana*, which is consistent with the concept that *SpERF1* is a transcription factor (Fig. 4), like other ERFs (Ayarpadikannan et al. 2014; Makhloufi et al. 2014). Overexpression of ERFs from various plants could improve the tolerance of transgenic plants to salt, drought, and freezing (Ayarpadikannan et al. 2014; Rong et al. 2014; Xu et al. 2007). *TaERF3* from wheat was transformed into wheat and its overexpression improved tolerance to drought and salt stresses (Rong et al. 2014). In this study, under normal condition, the overexpression of *SpERF1* did not disturb normal growth and development of transgenic lines in the T3 generation. Under drought stress, overexpression of *SpERF1* in *A. thaliana* significantly

improved drought tolerance. Longer primary root length was detected in the transgenic lines compared with the WT under dehydration (Fig. 5). By contrast, the transgenic lines displayed higher RWC and survival rate than the WT after drought treatment (Fig. 6). These results implied that transgenic plants might be better at conserving water under stress than the WT, and that *SpERF1*, acting as a positive regulator, played a vital role in enhancing plant tolerance to drought stresses.

ERF transcription factors can directly interact with GCC box elements in the promoters of downstream defense- and stress-related genes to regulate multiple stress responses (Oñate-Sánchez et al. 2007; Zhang et al. 2012). Although most of the studies on the binding of ERFs have focused on the GCC box element, recent studies have reported that the ERF1 protein from *A. thaliana* and wild radish (*Raphanus sativus*) could also bind to DRE/CRT elements (Ayarpadikannan et al. 2014; Cheng et al. 2013). Moreover, the ERF1 protein mostly binds to the DRE/CRT elements in the promoter regions of many abiotic stress-inducible genes. Meanwhile, the binding affinity of ERF1 to the DRE/CRT elements in the promoters of abiotic stress-inducible genes was much stronger compared with binding to the GCC box in the promoters of JA-responsive genes (Cheng et al. 2013). In this study, EMSAs showed that *SpERF1* could bind directly to the DRE/CRT elements in six stress-related genes (*HSP101*, *LEA4-5*, *P5CS1*, *RD29A*, *HSP70*, and *COR47*; Fig. 7). To further examine this possibility, the expression patterns of the six stress-related genes were analyzed using qRT-PCR in *SpERF1*-overexpressing lines and the WT. In the *SpERF1*-overexpressing plants, these genes were activated before the stress treatment, and produced higher levels of mRNA than those in the control plants under drought stress (Fig. 8). These results suggested that *SpERF1* could interact with DRE/CRT elements in promoters of stress-related genes and activated the expression of these genes.

The expressions of some HSP genes are induced not only by heat stress, but also by drought stress (Wang et al. 2004). In this study, two HSPs genes, *HSP70* and *HSP101*, showed higher expression levels in the *SpERF1*-overexpressing lines than in the WT. Under drought conditions, the expression levels of *HSP70* from wheat were upregulated by more than tenfold, and by almost eightfold in *A. thaliana*, as compared with controls (Grigорова et al. 2011; Sung and Guy 2003). *HSP101* is also involved in the response to different stresses by protecting membrane integrity and maintaining protein activity/synthesis (Gullì et al. 2007; Hong et al. 2003). *LEA4-5* and *COR47* belong to the LEA protein family, and their gene expressions were induced by drought, salt, and cold (Olvera-Carrillo et al. 2010; Welin et al. 1994). Overexpression of *AtLEA4-5* resulted in a higher RWC, biomass accumulation, and bud maintenance upon water deficit and recovery from severe dehydration compared with WT *A.*

thaliana (Olvera-Carrill, et al. 2010). *P5CS1* and *RD29A* are stress-response marker genes, and were induced in response to drought stress, especially during early drought treatment. Cheng et al. showed that overexpression of an ERF gene resulted in enhanced drought stress resistance by regulating *P5CS1* expression in *A. thaliana* (Cheng et al. 2013). Taken together, these findings suggested that the six stress-related genes with DRE/CRT elements that markedly increased in the *SpERF1*-overexpressing lines relative to WT might function together to enhance resistance to drought stress. In our previous study, the expression of *P5CS1* gene was upregulated in the leaves of *S. purpurea* under drought conditions (Yang et al. 2015a). However, whether it was regulated by the *SpERF1* transcription factor via binding DRE/CRT elements is still unknown due to the lack of the promoter sequence of the *P5CS1* gene from *S. purpurea*.

In this work, the *P5CS* expression in the transgenic plants was higher than that of the WT before and after drought treatment (Fig. 8). Previous research showed that proline synthesis was correlated with *P5CS* transcript levels under drought and salt stress (Kishor et al. 1995; Silva-Ortega et al. 2008). Proline, which is one of the most powerful osmoprotectants, plays a protective role in the response of plants to biotic stress or abiotic stress, and higher levels of proline were accumulated in the transgenic plants compared with the WT before and after drought stress (Fig. 9). This suggested that more proline was produced in the transgenic lines, allowing them to have a better buffering capacity under drought stress. Moreover, proline, as an extremely efficient ROS scavenger, can also stabilize proteins and membranes by reducing free radical levels and provide a source of carbon, nitrogen, and energy during cell rehydration (Matysik et al. 2002; Szabados and Saviouré 2010). The EL measurement indicates the stability of cell membrane and often is used as an indicator of drought tolerance. An increase in EL has been attributed to the oxidative damage to lipids that disrupts the membrane's structural integrity during rehydration (Epron and Dreyer 1992; Kasuga et al. 2004; Premachandra et al. 1990). Under drought conditions, sensitive varieties showed significantly higher EL than the tolerant ones in durum wheat (*Triticum durum Desf.*) as the dehydration time increased (Sayar et al. 2008). In this study, we also observed that EL increased in response to drought treatment in the transgenic and WT plants (Fig. 9); however, the EL was significantly higher in the WT compared with the transgenic plants after exposure to drought stress for 15 days.

Conclusions

We identified an ERF gene (*SpERF1*) from *S. purpurea* leaves. qRT-PCR analyses showed that its transcripts accumulated under drought, cold, JA, ET, and ABA

treatments, but were not induced by salt and UV-B. SpERF1-GFP proteins were located in the nucleus, consistent with its putative function as a transcription factor. SpERF1 acted as a positive regulator, enhancing stress tolerance in *A. thaliana* through the activation of DRE/CRT elements in the promoters of abiotic stress-responsive genes. These results provided a theoretical and experimental basis for the use of the gene in genetic engineering to elevate stress tolerance in other crops.

Author contribution statement XDS and YPY conceived and designed the experiments; YQY and CD performed the experiments; XL analyzed the data; MQ and JCD contributed reagents/materials/analysis tools; and YQY wrote the paper.

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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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