The Ethylene Response factor SmERF1 Improves Salinity Tolerance and Impacts Seed Size in Tobacco

Wenping Hua

Shaanxi Normal University College of Life Sciences

Chen Chen

Shaanxi Normal University

Weiwei Kong

Shaanxi Normal University

Zhezhi Wang (✉ zzwang@snnu.edu.cn)

Shaanxi Normal University <https://orcid.org/0000-0002-6111-1551>

Research article

Keywords: *Salvia miltiorrhiza*, SmERF1, ABA, plant resistance, seed size

Posted Date: December 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-132370/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Ethylene response factor (ERF) proteins play vital roles in plant resistance and plant development. However, little is known about the ERF transcription factors of *Salvia miltiorrhiza*, which is a famous Chinese herb with good resistance to stress.

Result Screened from our previous transcriptome data, *SmERF1*, an ERF transcript factor, was isolated from *S. miltiorrhiza*. *SmERF1* had a single AP domain and was classified in the ERF E3 subfamily. *SmERF1*-expressing tobacco plants showed slower growth, less biomass and a decrease in chlorophyll only at the seedling stage, and there was no significant difference in other growth stages. In addition, seeds of tobacco plants with *SmERF1* expression were smaller and lighter than those of wild plants, similar to some AP2 TFs. Under NaCl treatment, transgenic tobacco lines showed better tolerance to salinity, and the proline content, SOD and POD activities of transgenic lines were higher than those of wild-type plants, while MDA content was lower than that of wild-type plants. Furthermore, we determined the phytohormones related to plant resistance and showed that transgenic tobacco plants had higher ABA levels and lower GA levels than wild tobacco. The expression of *SmERF1* regulated the expression of key enzyme genes related to plant hormone biosynthesis, such as *NtSDR*, *NtGA20ox*, *NtACO* and *NtACS*.

Conclusions The *SmERF1*-expression in tobacco affect plant growth at seedling stage, and increase plant tolerance to salt. And the expression of *SmERF1* cause tobacco seeds smaller and lighter. Our study suggested that *SmERF1* enhanced tobacco tolerance to salt and impacted seed size through an ABA-dependent pathway.

Background

The ethylene-responsive factor (ERF) proteins are a large family of transcription factors known to play significant roles in various plant biological processes, such as plant growth, development, metabolism and biotic and abiotic stress responses[1–3]. ERF members contain one conserved AP2/ERF domain consisting of approximately 60 to 70 amino acids in the DNA-binding region, which can bind to CC-box containing DNA fragments and activate expression of the defense-related genes [4]. An increasing number of studies have shown that ERFs modulate multiple responses to abiotic and biotic stresses. The overexpression of *ERF1* in Arabidopsis can increase resistance to both *Botrytis cinerea* and *Pseudomonas syringaetomato* [5]. Transgenic plants overexpressing *OsDERF1* (a member of the ERF family from rice) exhibited reduced tolerance to drought stress in rice at the seedling stage, while knockdown of *OsDERF1* expression conferred enhanced tolerance at the seedling and tillering stages[6]. The tomato ERF TSRF1 (or TERF1) positively regulates pathogen resistance in tomato and tobacco [7]. AtORA59 and AtERF1 in Arabidopsis could activate the expression of the *PDF1.2* gene and further activate the expression of resistance genes in response to stress[8].

Evidence suggests that ERF transcription factors are involved in regulating plant hormone synthesis and integrating hormone signaling pathways. For example, the expression of the cotton ERF gene *GbERF*, the

tobacco ERF gene *NtCEF1* and the tomato ERF gene *JERF1/3* has been shown to be induced by abscisic acid (ABA)[9]. *JERF1* was inducible by ethylene (ET) and ABA[7]. The soybean ERF gene *GmERF135* participates in ABA and ET signaling pathways to regulate the expression levels of stress-related genes[10]. CrERF5 from *Catharanthus roseus* responds to both ethylene and jasmonate (JA) signals[11]. Moreover, some ERFs have been shown to regulate plant hormone biosynthesis. Rice TSRF1, peach PpERF3 and Arabidopsis ORA47 were shown to regulate ABA level[12–14]. Apple MdERF2 and AtERF11 can affect ET or gibberellic acid (GA) biosynthesis [15, 16]. ABA, GA and ET, are well known to play major roles in mediating plant defense responses against biotic and abiotic stresses[17].

Salvia miltiorrhiza Bunge is a well-known medicinal plant, the dry roots of which are widely used in the treatment of cardiovascular, cerebrovascular, hyperlipidemia, and acute ischemic stroke diseases. Due to its good curative effect on cardiovascular and cerebrovascular diseases, the demand for *S. miltiorrhiza* has increased. Over 10% of traditional Chinese patent medicines and simple preparations contain *S. miltiorrhiza*. The annual consumption of *S. miltiorrhiza* has exceeded 16 million kilograms in China[18]. With the increased demand for *S. miltiorrhiza*, wild resources are gradually being exhausted and the cultivation of new resources has become a research hotspot. The contents of active substances and plant active resistance are the main evaluation indexes of *S. miltiorrhiza* quality.

Based on the genome data of *S. miltiorrhiza*, 79 ERFs were identified[19]. SmERF6, SmERF1L1 and SmERF128 can positively regulate the biosynthesis of tanshinones [20–22], while SmERF115 can positively regulate the phenolic acid biosynthesis by affected the expression of key enzymes in the biosynthetic pathway of phenolic acids[23]. Additionally, SmERF115, SmERF1L1 and SmERF128 ERFs were responsive to methyl jasmonate (MeJA) or yeast extraction (YE) treatment, which are thought to be involved in active ingredient biosynthesis and plant resistance[24–27]. However, functions of most other ERFs in *S. miltiorrhiza* are still unknown. In this study, we isolated one novel ERF gene (named *SmERF1*) by screening our transcriptome library[28], which was also responsive to methyl jasmonate MeJA or YE treatment. Our study showed that the expression of *SmERF1* affected the expression of ABA and GA biosynthesis, affected seed size, and enhanced the tolerance of transgenic tobacco to drought, revealing the important roles of SmERF1 in improving crop agricultural characteristics.

Results

solation and characterization of SmERF1

The isolated full-length *SmERF1* (GenBank Accession No. KC405081.1) contained an ORF comprising 549 bp, encoding a polypeptide of 182 amino acids with a molecular weight of 19.6 kDa and a pI of 9.64. A single conserved AP2 DNA-binding domain with 63 amino acids was identified in SmERF1 by multiple sequence alignment (Fig. 1A). SmERF1 was predicted to be a nuclear-localized protein. SmERF1 showed high identities ($\geq 74\%$) with ERF proteins from other plants, such as tobacco (*NtERF5*, AAU81956), *Lithospermum erythrorhizon* (*LeERF-1*, GQ246186.1) and *Artemisia annua* (*AaERF1*, AEQ93554.1).

Phylogenetic analysis revealed that SmERF1 was classified in the B3 subfamily, together with NtERF5 and AaERF1 (Fig. 1B).

Expressing SmERF1 in tobacco resulted in smaller tobacco seed size

Young leaves from transgenic plants selected with Basta were used to extract DNA and RNA. After detecting the *CaMV35S* promoter sequence in transgenic plants, 15 transgenic tobacco lines were confirmed (Fig S1A). Through qPCR verification, four lines (OE-3, OE-4, OE-7 and OE-10) with high expression levels of SmERF1 were obtained and used for follow-up experiments (Fig S1B). There were some differences only at the seedling stage, and there was no significant difference in other growth stages between transgenic and wild plants (see results in 3.4).

Furthermore, we harvested T2 generation seeds from four tobacco transgenic lines of *SmERF1*. We found that the seed size of each tobacco transgenic line was smaller than that of the wild-type plants (WT) (Fig. 2A). According to the statistical analysis, the thousand kernel seed weights of OE-3, OE-4, OE-6, OE-7 and OE-10 were 59%, 63%, 55%, 56% and 58% of the WT, respectively (Fig. 2B).

Expression of SmERF1 in tobacco affected young tobacco seedling growth

During the germination stage, white buds appeared on the WT seeds of tobacco at 7 days after sowing, while the buds on tobacco transgenic seeds (T1) appeared later at 9 days. Two weeks after sowing, the growth rate of transgenic tobacco was significantly slower than that of wild plants (Fig. 3A). The biomass of transgenic tobacco seedlings was 48% ~ 0.53% (Fig. 3B). In addition, we observed that the leaves of transgenic tobacco seedlings were yellower than those of wild-type seedlings (Fig. 3A). The chlorophyll content of transgenic seedlings was 82%-84% of that of the WT (Fig. 3D); however, there was no significant difference in the germination rates between transgenic and WT seeds under normal conditions (Fig. 3C).

Expressing SmERF1 in tobacco enhances salt tolerance

To detect tolerance to salt, two-month-old plantlets of T2 generation transgenic and WT plants were treated with 400 mM NaCl. Leaves of all wild-type plants wilted and rolled up, and some old leaves turned yellow, while leaves of transgenic lines showed better status than that of WT, and only old leaves rolled up (Fig. 4A).

The physiological parameters, including SOD, POD, MDA content, proline content, and transgenic and WT tobacco plants, were compared under both normal and 400 mM NaCl conditions (Fig. 4B). (1) After NaCl treatment, the POD activity of transgenic tobacco plants was significantly higher than that of WT plants. Under normal conditions, the POD activities of transgenic tobacco plants were higher than those of WT plants, but there was no significant difference. (2) After NaCl treatment, the transgenic tobacco plants also showed markedly higher SOD activity than that of WT plants. However, under normal conditions, there were no significant differences. (3) The MDA content of the transgenic tobacco plants was significantly lower than that of WT under both conditions. (4) There was no significant difference in

proline content between transgenic tobacco plants and WT under normal conditions, while proline content was 1.35–1.48 times higher in transgenic tobacco plants than WT after NaCl treatment.

Expressing *SmERF1* in tobacco regulates ABA and GA biosynthesis

Absciscic acid (ABA) and gibberellins (GA) are a pair of classic phytohormones that antagonistically mediate several plant developmental processes, including seed maturation, seed dormancy and germination, primary root growth, and flowering time control (Shu et al., 2018). We found that the ABA contents of transgenic tobacco lines were 1.53–2.51 times higher than those of WT (Fig. 4-1c), and the GA contents of transgenic tobacco lines were 0.60–0.74 times lower than those of WT (Fig. 4-1d). The results from qRT-PCR showed that the expression of *NtSDR* (short-chain dehydrogenase/reductase, GenBank Accession No. AJ223177.1) was activated in *SmERF1*-overexpressing tobacco, which encodes a key enzyme of ABA biosynthesis. *NtGA20ox* (GenBank Accession No. JQ413251), a key enzyme-encoding gene of GA biosynthesis, was repressed in *SmERF1*-overexpressing tobacco. The study showed that the *SmERF1*-overexpressing seedlings had higher ABA content and lower GA content than WT plants (Fig. 4b), indicating that *SmERF1* can regulate the plant hormone response of transgenic tobacco seedlings to drought through the ABA pathway.

Moreover, we also tested the expression of *NtACS* (GenBank Accession No. NM_001326220.1) and *NtACO* (GenBank Accession No. NM_001325967), two key enzyme-encoding genes in plant hormone ethylene biosynthesis. Our qRT-PCR analysis showed that the transcription of both *NtACS* and *NtACO* increased in transgenic tobacco lines, which suggested acceleration of ET production.

Discussion

Over 10% of traditional Chinese patent medicines and simple preparations contain *S. miltiorrhiza*. The annual consumption of *S. miltiorrhiza* has exceeded 16 million kilograms in China [18]. More and more genes involved in *S. miltiorrhiza* resistance have been studied. For example, genes of three multigene universal stress proteins (SmUSPs) were cloned, and their expression enhanced *Escherichia coli* tolerance to salt and heat stress [29]. Overexpression of *SmSnRK2.6* (sucrose non-fermenting-1-related protein kinase 2) improved *S. miltiorrhiza* tolerance to abiotic stresses [30]. Overexpression of *SmLEA* (late embryogenesis abundant proteins) improves drought and salinity tolerance in *S. miltiorrhiza* [31]. Ectopic expression of tomato *prosystemin* (*LePS*), Arabidopsis *DREB1A/CBF3* and *AtDREB1A* was also used to improve *S. miltiorrhiza* resistance against biotic and abiotic stress separately [32–34].

ERF proteins play vital roles in a variety of stress responses in plants. Some ERF genes, such as *NtERF5*, *TaERF3* [35], *GmERF3* [36], *GmERF113* [37] and tomato *JERF1* [12], were thought to be candidates to improve crop-plant resistance because their overexpression enhances resistance to multiple diseases and improves tolerance to drought, salt, and freezing in transgenic plants [1]. To date, 79 ERFs have been identified from the *S. miltiorrhiza* genome [19] and the functions of four *SmERFs* in regulation of active ingredient biosynthesis have been investigated [20, 21, 23]. Few reports have described the resistance functions of *S. miltiorrhiza* ERFs in plants. In our study, the growth of transgenic tobacco lines with

SmERF1 expression was significantly better than that of wild-type plants, and the proline content, SOD and POD activities of transgenic lines were higher than those of wild-type plants, while the MDA content was lower than that of wild-type plants. These results implied that *SmERF1* expression could enhance plant salt tolerance under salt stress.

Some ERFs were shown to regulate hormone levels in plants. For instance, apple MdERF2 can negatively affect ethylene biosynthesis by suppressing MdACS1 transcription [15]. TSRF1 activates the expression of a putative rice abscisic acid (ABA) synthesis gene, *SDR*, resulting in enhanced ABA levels [12]. Peach PpERF3 regulates ABA biosynthesis by activating PpNCED2/3 transcription [13]. ORA47 of *Arabidopsis thaliana* can activate ABA biosynthesis genes (*NCED3* and *NCED9*) [14]. Overexpression of *AtERF11* resulted in elevated bioactive GA levels by upregulating the expression of *GA3ox1* and *GA20ox* genes [16]. ABA, GA and ET, are well known to play major roles in mediating plant defense responses against biotic and abiotic stresses [17]. Our qPCR results show that the expression levels of *NtSDR*, a key gene of ABA biosynthesis, was upregulated in *SmERF1*-expressing tobacco, while the expression of *NtGA20ox*, a key gene of GA biosynthesis was downregulated. In the *SmERF1*-expressing tobacco seedlings, the expression of *NtACS* and *NtACO*, two key enzyme-encoding genes in the ethylene biosynthesis, were upregulated compared with wild-type plants. It was also implied that the expression of *SmERF1* may also cause an increase of ethylene. Our further study also indicated that *SmERF1*-expressing tobacco seedlings had higher ABA content and lower GA content than wild tobacco. There was antagonism between ABA and GA [38], and ABA could also induce ET biosynthesis [39]. Therefore, we inferred that *SmERF1* can regulate the plant hormones of transgenic tobacco seedlings in response to drought through the ABA pathway. ABA is believed to be a general inhibitor of plant growth during the early development of seedlings [40]. It is indicated that the higher ABA content resulted in slower growth and decreased biomass in *SmERF1*-expressing tobacco seedlings.

Seed size is the most important agronomic traits in crop domestication [41], and it is desirable to increase seed yield because grains represent significant sources of food. Many transcription factors have been identified as regulators on controlling seed formation. AtMYB56, encoding an R2R3 MYB transcription factor, positively regulates *Arabidopsis* seed size by coordinately controlling the expansion of endothelium layer and proliferation [42]. ARF2 (AUXIN RESPONSE FACTOR 2) and NGAL2 can negative regulate seed size by suppressing cell proliferation [43, 44]. The AP2-type transcription factors *Aechmea fasciata* AfAP2-2, rice SMALL ORGAN SIZE1 (SMOS1) and *Larix kaempferi* LkAP2L2 also affect seed size [45–47]. Furthermore, SMOS1 may integrate auxin and BR signaling to control rice grain size [46]. In the current research, *SmERF1* only contains one AP2 DNA binding domain belonging to the ERF subfamily, differing from members of the AP2 subfamily containing two AP2 DNA binding domains. However, expressing *SmERF1* in tobacco also causes seeds to be smaller and lighter than wild-type tobacco, similar to AfAP2-2 [45].

Plant hormones also respond during seed development. ABA play a negative role in seed morphogenesis. ABA suppresses seed development by negatively regulating ABI5 (ABA response factor), while ABA-deficient mutants (*abi5*) can produce larger and heavier seeds for the increase of embryo cell number and

endosperm proliferation[48]. GAs also play important roles in the developing embryo and endosperm in seeds [49]. Loss-of-function mutation in *OsGA20ox2* (a key GA biosynthesis gene) resulted in lower GA levels, and delayed seed morphogenesis and maturation in rice[50]. Overexpression of *GASA4* (a member of gibberellic acid-stimulated Arabidopsis family), which is expressed in response to GAs, result in increase of seed size and total seed weight, while the *gasa4* mutant has smaller seeds[51]. Our study also indicated that *SmERF1*-expressing seedlings had higher ABA content and lower GA content than WT plants. We inferred that SmERF1 may regulate the plant hormones of transgenic tobacco seedlings, resulting in smaller seed sizes. Crosstalk exists between ABA and GA, and these two phytohormones antagonistically mediate plant developmental processes [52]. Decreasing the gibberellin/abscisic acid (GA/ABA) ratio can weak seed repression of soybean seed germination [53]. The delayed germination of seeds, slower growth and loss of mass of *SmERF1*-expressing tobacco seedlings all verified the change in GA/ABA ratio. Additionally, ET participates in integration with ABA and Gas [49]. Overexpression of ACC deaminase resulted in reduced levels of ET, GAs and IAA, and smaller seeds in *Brassica napus* [54]. In the *SmERF1*-expressing seedlings, the increase in ET may also contribute to sharp seed shape.

Conclusion

S. miltiorrhiza is a famous medicinal plant in Chian. We isolated one ERF transcription factor, SmERF1, from *S. miltiorrhiza*, and expressed SmERF1 in tobacco. The *SmERF1*-expression in tobacco affect plant growth at seedling stage, and increase plant tolerance to salt. And the expression of *SmERF1* cause tobacco seeds smaller and lighter. The ABA level were increased and GA levels were decreased in *SmERF1*-expression tobacco plant. Our study suggested that SmERF1 enhanced tobacco tolerance to salt and impacted seed size through an ABA-dependent pathway.

Methods

Clone and analysis of SmERF1

According to the unigene (unigene-25151) sequence from our transcriptome data[28], we isolated the cDNA sequences of *SmERF1* from *S. miltiorrhiza* with accession number: KC405081.1. Multiple sequence alignments were performed using ClustalW2 (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>), and the results were rendered using the Boxshade Server. The conserved domain of SmERF1 was searched using the Conserved Domain Search Service (<https://www.ncbi.nlm.nih.gov/guide/domains-structures/>). The subcellular location of SmERF1 protein was predicted with WoLF PSORT (<https://wolfpsort.hgc.jp/>). The phylogenetic tree was constructed with MEGA X software using a Maximum Likelihood (ML) method based on an JTT model, and 500 bootstrap test replicates were used during the construction with other parameters as default[55].

Plant Materials

Mature seeds of *S. miltiorrhiza* Bunge were surface sterilized as described previously (Yan and Wang, 2007) and cultured on MS basal media (Murashige and Skoog, 1962) for germination. One-month-old seedlings were used for RNA isolation.

Tobacco (*Nicotiana tabacum*) plants for agroinfiltration were grown in a growth chamber at 22 °C under 16 h of light for 6 weeks. One-month-old tobacco plants were used for transient transformation. Seeds (T2 generation) collected from *SmERF1*-expressing tobacco plants were surface-sterilized with 25% bleach and planted on Murashige–Skoog (MS) medium for germination, then transplanted into soil and maintained at 25 °C with a 16 h light/8 h dark cycle. Seedlings after transplant were used for treatments and various tests.

Extraction Of Rna, Cdna Synthesis

Genomic DNA was isolated from young leaves of 2-month-old *S. miltiorrhiza* seedlings or tobacco seedlings with the Biospin Plant Genomic DNA Extraction Kit (BioFlux, China). Total RNA was extracted from transgenic tobacco and wild type plants, using BIOZOL reagent (BIOER, Hangzhou) according to the manufacturer's instructions. The quality and concentrations of genomic DNA and RNAs were determined by 1.0% agarose gel electrophoresis and analysis on a spectrophotometer (SHIMADZU UV-2450, Japan). cDNA was synthesized with Revert Aid First Strand cDNA Synthesis Kit (Takara, Dalian).

Plant Transformation And Transgenic Plant Verification

We cloned the *SmERF1* sequence with the attB recombination sites and inserted into pDONOR221 *via* the Gateway BP clonase (Invitrogen Corporation) reaction to create entry clones. Then, the sequence was moved into the destination vector pEarleyGate100 using Gateway LR clonase (Invitrogen Corporation) to create the expression vector (pO-ERF). The new constructs of the pO-ERF vector were transformed into *Agrobacterium tumefaciens* strain *GV3101* *via* a freeze-thaw method[56]. Then, the transient transformation of tobacco leaves was accomplished according to Sparkes et al. [57]. The expression vector pO-ERF contained a Basta resistance selection marker; therefore, transformants were selected on 1/2 strength MS medium supplemented with IBA 0.5 mg/L and Basta 10 mg·mL⁻¹.

To determine whether the recombinant vector was inserted into the plant, we detected the *CaMV35S* promoter sequence in all transgenic tobacco lines (T0, T1 generation) by qPCR, primers were shown in Table 1.

Table 1
Primers used in this study

Genes (GenBank Accession)	Sequence
<i>SmERF1</i> (for clone and vector construction)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCTAGCTCACATAATGACGTTCTGA-3' (attB sites was underlined) 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC CGACGTTAATTACTGTAAGCCGACTTC-3'(attB site was underlined)
<i>SmERF1</i> (for qPCR)	5'-GCCTAGCTCACATAATGACGTTCTGA-3' 5'-CGACGTTAATTACTGTAAGCCGACTTC-3'
<i>CAMV-35</i>	5'-AATCTTCGTCAACATGGTGGAGC-3' 5'-GCTGTCGTCGCCGAGAATA-3'
<i>NtActin</i> (U60495.1)	5'- CGTTATGGTTGGAATGGGACAGAA-3' 5'- AAGAACAGGGTGCTCCTCGTGG-3'
<i>NtSDR</i> (AJ223177.1)	5'- GAAGAGGAGGTCGCAAAGGCA-3' 5'- CACTGATGGGTATTTTCGGATGAGA-3'
<i>NtGA2Oox</i> (JQ413251)	5'- GAAACCAGACCTCACTTTAGGGACA-3' 5'- TGAAAGAGCCATAAATGTATCGCCT-3'
<i>NtACS</i> (NM_001326220.1)	5'- TCGGGCTCGTTTCAACACAGA-3' 5'- GAACATCCCGTGTCTTTTCCCTA-3'
<i>NtACO</i> (NM_001325967)	5'- TCCAAGATGACAAAGTAAGCGGC-3' 5'- TGTTTGCTCTCCGCTGCCTC-3'

Transgenic and wild-type tobacco lines with good growth for approximately 6 weeks in tissue culture were transplanted to the artificial culture room after seedling refining. The culture temperature was 24 °C, the humidity was 60%, and the light/darkness was 14/10 h.

Expression analyses of SmERF1 in transgenic tobacco

After selective culture, the transgenic plants were transferred to MS culture. Approximately 6 weeks after subculture, young seedlings (T0 generation) were transplanted into humus soil. Seeds (T1 and T2 generation) were collected when they were mature. The sizes of T2 seeds were observed with a stereomicroscope. The thousand seed weights of tobacco transgenic lines were also evaluated.

Detection of ABA and GA levels in transgenic plants of tobacco (T2)

Whole plant samples were collected from each transgenic plant. The contents of ABA and GA were detected according to the manuals of ELISA kits from ShaanXi Maiyuan Biotechnology Co., Ltd.

Detection of chlorophyll content of transgenic plants of tobacco (T2)

Two-week seedlings after germination of T2 generation seeds were used to calculate biomass and detect the chlorophyll content. Chlorophyll (Chl) content was measured according to the method described by Frank et al. with slight modification[58]. Briefly, 200 mg of fresh seedlings was collected and ground with sterile mortar and pestle. The samples were transferred into 10 mL tubes, and then 10.0 mL of 80% acetone was added. After mixing well, the tubes were stratified at 4 °C for 24 h. The samples were centrifuged at 12000 g at 4 °C for 3 min, and then the OD value of the supernatant was assayed with a spectrophotometer (SHIMADZU UV-2450, Japan) at 665 nm and 649 nm wavelengths. Chlorophyll content (a & b) was calculated using the following formulas where A is the absorbance[59]: Chl (a) = $13.95 \times A_{665} - 6.88 \times A_{645}$; Chl (b) = $24.96 \times A_{649} - 7.32 \times A_{665}$.

Resistance Analyses Of Transgenic Tobacco

The seeds of the T1 generation of transgenic and wild-type tobacco were germinated on filter paper. When the seedlings grew to approximately 1 cm in size, they were transplanted into the sowing and seedling substrate, one in each pot, cultivated in the artificial culture room, and watered once every 3 days. Two months after transplantation into the soil, the tobacco seedlings were watered with 400 mmol/L NaCl solution every other day. After 7 days of treatment, the growth states of the tobacco lines were observed, and the aerial parts of these seedlings were quickly frozen with liquid nitrogen for the detection of physiological indexes as follows.

The activities of catalase (CAT, EC 1. 11. 1. 6), peroxidase (POD, EC 1. 11. 1. 7) and superoxide dismutase (SOD, EC 1. 15. 1. 1) were assayed using the CAT Assay Kit (A007–1, Nanjing Jiancheng, Nanjing, China), Plant POD Assay Kit (A084–3, Nanjing Jiancheng, Nanjing, China) and Total SOD Assay Kit (S0102, Nanjing Jiancheng, Nanjing, China), respectively, as described by Shi *et al.*[60, 61]. The MDA content was extracted using thiobarbituric acid reagent, and quantified by determining the absorbance of the supernatant at 450 nm, 532 nm and 600 nm as previously described[61].

Quantitative Real-time Pcr (qrt-pcr)

The quantitative reactions were performed on an IQ5 real-time PCR detection system (Bio-Rad), using SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa). PCR amplifications included the following conditions: 50 °C for 2 min and 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Gene-specific primers for qPCR are listed in Table 1. *Nicotiana tabacum* actin (Accession No. U60495.1) was used as an internal control. Their relative expression was calculated via the $2^{-\Delta\Delta Ct}$ method [62]. Each

sample was collected from five independent plants. Three independent biological replicates were used for each plant sample. Each data point represents the average of three experiments.

Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Turkey-Kramer test ($P < 0.05$) using SPSS software (version 19.0). Levels of statistical significance were marked with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Abbreviations

ABA: abscisic acid; ERF: ethylene response factor; GA: gibberellic acid; MDA: malondialdehyde; MeJA: methyl jasmonate; POD: peroxidase; SOD: superoxide dismutase; YE: yeast extraction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Chinese National Natural Science Foundation (Grant no. 31800259), the Natural Science Foundation of Shaanxi Province, China (Grant No. 2018JZ3001, 2019JQ-917), the Shaanxi Provincial Key R&D Project (Grant No. 2020SF-314), and the Fundamental Research Funds for the Central Universities (Grant No. GK202006003). The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

H WP, K WW, and W ZZ conceived and designed the experiments, H WP and C C wrote the manuscript. H WP, K WW carried out the experiments. C C and W ZZ helped to design the experiments and analyzed

data.

All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Yaya Huang, Xiaoya Zhu, and Donghao Wang for their help with the experimental operations.

References

1. Xu ZS, Chen M, Li LC, Ma YZ. Functions of the ERF transcription factor family in plants. *Botany*. 2008;86(9):969–77.
2. Jofuku KD, Den Boer B, Van Montagu M, Okamoto JK. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell*. 1994;6(9):1211–25.
3. Okamoto JK, Caster B, Villarroel R, Van Montagu M, Jofuku KD: The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. *Proceedings of the National Academy of Sciences* 1997, 94(13):7076–7081.
4. M OT HS. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell*. 1995;7(2):173–82.
5. Berrocal-Lobo M, Molina A, Solano R. Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *Plant J*. 2002;29(1):23–32.
6. Wan L, Zhang J, Zhang H, Zhang Z, Quan R, Zhou S, Huang R. Transcriptional activation of *OsDERF1* in OsERF3 and OsAP2-39 negatively modulates ethylene synthesis and drought tolerance in rice. *PLoS One*. 2011;6(9):e25216.
7. Wu L, Chen X, Ren H, Zhang Z, Zhang H, Wang J, Wang XC, Huang R. ERF protein JERF1 that transcriptionally modulates the expression of abscisic acid biosynthesis-related gene enhances the tolerance under salinity and cold in tobacco. *Planta*. 2007;226(4):815–25.
8. Zarei A, Körbes AP, Younessi P, Montiel G, Champion A, Memelink J. Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the *PDF1.2* promoter in Arabidopsis. *Plant molecular biology*. 2011;75(4–5):321–31.
9. Wang X, Liu S, Tian H, Wang S, Chen JG. The small ethylene response factor ERF96 is involved in the regulation of the abscisic acid response in Arabidopsis. *Frontiers in plant science*. 2015;6:1064.
10. Zhao MJ, Yin LJ, Ma J, Zheng JC, Wang YX, Lan JH, Fu JD, Chen M, Xu ZS, Ma YZ. The roles of GmERF135 in improving salt tolerance and decreasing ABA sensitivity in soybean. *Frontiers in plant science*. 2019;10:940.
11. Pan Q, Wang C, Xiong Z, Wang H, Fu X, Shen Q, Peng B, Ma Y, Sun X, Tang K. CrERF5, an AP2/ERF transcription factor, positively regulates the biosynthesis of bisindole alkaloids and their precursors in *Catharanthus roseus*. *Frontiers in Plant Science* 2019, 10(931).

12. Quan R, Hu S, Zhang Z, Zhang H, Zhang Z, Huang R. Overexpression of an ERF transcription factor *TSRF1* improves rice drought tolerance. *Plant Biotechnol J*. 2010;8(4):476–88.
13. Wang X, Zeng W, Ding Y, Wang Y, Niu L, Yao JL, Pan L, Lu Z, Cui G, Li G, et al. PpERF3 positively regulates ABA biosynthesis by activating *PpNCED2/3* transcription during fruit ripening in peach. *Horticulture research*. 2019;6:19.
14. Chen HY, Hsieh EJ, Cheng MC, Chen CY, Hwang SY, Lin TP. ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) regulates jasmonic acid and abscisic acid biosynthesis and signaling through binding to a novel cis-element. *New Phytol*. 2016;211(2):599–613.
15. Li T, Jiang Z, Zhang L, Tan D, Wei Y, Yuan H, Li T, Wang A. Apple (*Malus domestica*) MdERF2 negatively affects ethylene biosynthesis during fruit ripening by suppressing MdACS1 transcription. *The Plant journal: for cell molecular biology*. 2016;88(5):735–48.
16. Zhou X, Zhang ZL, Park J, Tyler L, Yusuke J, Qiu K, Nam EA, Lumba S, Desveaux D, McCourt P, et al. The ERF11 transcription factor promotes internode elongation by activating gibberellin biosynthesis and signaling. *Plant Physiol*. 2016;171(4):2760–70.
17. Verma V, Ravindran P, Kumar PP. Plant hormone-mediated regulation of stress responses. *BMC plant biology*. 2016;16:86.
18. Lu S: *Salvia miltiorrhiza*: an economically and academically important medicinal plant. In: *The Salvia miltiorrhiza Genome*. Springer; 2019: 1–15.
19. Ji AJ, Luo HM, Xu ZC, Zhang X, Zhu YJ, Liao BS, Yao H, Song JY, Chen SL. Genome-wide identification of the AP2/ERF gene family involved in active constituent biosynthesis in *Salvia miltiorrhiza*. *The Plant Genome* 2016, 9(2).
20. Huang Q, Sun M, Yuan T, Wang Y, Shi M, Lu S, Tang B, Pan J, Wang Y, Kai G. The AP2/ERF transcription factor SmERF1L1 regulates the biosynthesis of tanshinones and phenolic acids in *Salvia miltiorrhiza*. *Food Chem*. 2019;274:368–75.
21. Zhang Y, Ji A, Xu Z, Luo H, Song J. The AP2/ERF transcription factor SmERF128 positively regulates diterpenoid biosynthesis in *Salvia miltiorrhiza*. *Plant molecular biology*. 2019;100(1–2):83–93.
22. Bai Z, Li W, Jia Y, Yue Z, Jiao J, Huang W, Xia P, Liang Z. The ethylene response factor SmERF6 co-regulates the transcription of *SmCPS1* and *SmKSL1* and is involved in tanshinone biosynthesis in *Salvia miltiorrhiza* hairy roots. *Planta*. 2018;248(1):243–55.
23. Sun M, Shi M, Wang Y, Huang Q, Yuan T, Wang Q, Wang C, Zhou W, Kai G. The biosynthesis of phenolic acids is positively regulated by the JA-responsive transcription factor ERF115 in *Salvia miltiorrhiza*. *J Exp Bot*. 2018;70(1):243–54.
24. Simons L, Bultman TL, Sullivan TJ. Effects of methyl jasmonate and an endophytic fungus on plant resistance to insect herbivores. *J Chem Ecol*. 2008;34(12):1511.
25. Thaler JS, Stout MJ, Karban R, Duffey SS. Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecol Entomol*. 2001;26(3):312–24.
26. Zhou M, Memelink J. Jasmonate-responsive transcription factors regulating plant secondary metabolism. *Biotechnol Adv*. 2016;34(4):441–9.

27. Contreras A, Leroy B, Mariage PA, Wattiez R. Proteomic analysis reveals novel insights into tanshinones biosynthesis in *Salvia miltiorrhiza* hairy roots. *Sci Rep*. 2019;9(1):5768–8.
28. Hua WP, Zhang Y, Song J, Zhao LJ, Wang ZZ. *De novo* transcriptome sequencing in *Salvia miltiorrhiza* to identify genes involved in the biosynthesis of active ingredients. *Genomics*. 2011;98(4):272–9.
29. Wang XF, Su J, Yang N, Zhang H, Cao XY, Kang JF. Functional characterization of selected universal stress protein from *Salvia miltiorrhiza* (*SmUSP*) in *Escherichia coli*. *Genes (Basel)*. 2017;8(9):224.
30. Jia Y, Bai Z, Pei T, Ding K, Liang Z, Gong Y. The protein kinase *SmSnRK2.6* positively regulates phenolic acid biosynthesis in *Salvia miltiorrhiza* by interacting with *SmAREB1*. *Frontiers in plant science*. 2017;8:1384–4.
31. Wu Y, Liu C, Kuang J, Ge Q, Zhang Y, Wang Z. Overexpression of *SmLEA* enhances salt and drought tolerance in *Escherichia coli* and *Salvia miltiorrhiza*. *Protoplasma*. 2014;251(5):1191–9.
32. Chen C, Zhang Y, Qiakefu K, Zhang X, Han LM, Hua WP, Yan YP, Wang ZZ. Overexpression of tomato prosystemin (*LePS*) enhances pest resistance and the production of tanshinones in *Salvia miltiorrhiza* Bunge. *J Agric Food Chem*. 2016;64(41):7760–9.
33. Wei T, Deng K, Liu D, Gao Y, Liu Y, Yang M, Zhang L, Zheng X, Wang C, Song W, et al. Ectopic expression of DREB transcription factor, *AtDREB1A*, confers tolerance to drought in transgenic *Salvia miltiorrhiza*. *Plant Cell Physiol*. 2016;57(8):1593–609.
34. Wei T, Deng K, Wang H, Zhang L, Wang C, Song W, Zhang Y, Chen C. Comparative transcriptome analyses reveal potential mechanisms of enhanced drought tolerance in transgenic *Salvia miltiorrhiza* plants expressing *AtDREB1A* from Arabidopsis. *Int J Mol Sci* 2018, 19(3).
35. Rong W, Qi L, Wang A, Ye X, Du L, Liang H, Xin Z, Zhang Z. The ERF transcription factor *TaERF3* promotes tolerance to salt and drought stresses in wheat. *Plant Biotechnol J*. 2014;12(4):468–79.
36. Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J, Ma Y. Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *J Exp Bot*. 2009;60(13):3781–96.
37. Zhao Y, Chang X, Qi D, Dong L, Wang G, Fan S, Jiang L, Cheng Q, Chen X, Han D, et al. A novel soybean ERF transcription factor, *GmERF113*, increases resistance to *Phytophthora sojae* infection in soybean. *Frontiers in plant science*. 2017;8:299.
38. Liu X, Hou X. Antagonistic regulation of ABA and GA in metabolism and signaling pathways. *Front Plant Sci*. 2018;9:251.
39. Zhang M, Yuan B, Leng P. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *J Exp Bot*. 2009;60(6):1579–88.
40. Humplik JF, Bergougnoux V, Van Volkenburgh E. To stimulate or inhibit? That is the question for the function of abscisic acid. *Trends Plant Sci*. 2017;22(10):830–41.
41. Li N, Li Y. Maternal control of seed size in plants. *J Exp Bot*. 2015;66(4):1087–97.

42. Zhang Y, Liang W, Shi J, Xu J, Zhang D. *MYB56* encoding a R2R3 MYB transcription factor regulates seed size in *Arabidopsis thaliana*. *Journal of integrative plant biology*. 2013;55(11):1166–78.
43. Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ. The AUXIN RESPONSE FACTOR 2 gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. *Development*. 2006;133(2):251–61.
44. Zhang Y, Du L, Xu R, Cui R, Hao J, Sun C, Li Y. Transcription factors SOD7/NGAL2 and DPA4/NGAL3 act redundantly to regulate seed size by directly repressing *KLU* expression in *Arabidopsis thaliana*. *Plant Cell*. 2015;27(3):620–32.
45. Lei M, Li ZY, Wang JB, Fu YL, Xu L. Ectopic expression of the *Aechmea fasciata* APETALA2 gene *AfAP2-2* reduces seed size and delays flowering in *Arabidopsis*. *Plant physiology biochemistry: PPB*. 2019;139:642–50.
46. Li N, Xu R, Li Y. Molecular networks of seed size control in plants. *Annu Rev Plant Biol*. 2019;70:435–63.
47. Li A, Yu X, Cao B, Peng L, Gao Y, Feng T, Li H, Ren Z. LkAP2L2, an AP2/ERF transcription factor gene of *Larix kaempferi*, with pleiotropic roles in plant branch and seed development. *Russian journal of genetics*. 2017;53(12):1335–42.
48. Cheng ZJ, Zhao XY, Shao XX, Wang F, Zhou C, Liu YG, Zhang Y, Zhang XS. Absciscic acid regulates early seed development in *Arabidopsis* by ABI5-mediated transcription of SHORT HYPOCOTYL UNDER BLUE1. *Plant Cell*. 2014;26(3):1053–68.
49. Savadi S. Molecular regulation of seed development and strategies for engineering seed size in crop plants. *Plant Growth Regul*. 2018;84(3):401–22.
50. Ye H, Feng J, Zhang L, Zhang J, Mispan MS, Cao Z, Beighley DH, Yang J, Gu XY. Map-based cloning of seed dormancy1-2 identified a gibberellin synthesis gene regulating the development of endosperm-imposed dormancy in rice. *Plant Physiol*. 2015;169(3):2152–65.
51. Roxrud I, Lid SE, Fletcher JC, Schmidt ED, Opsahl-Sorteberg HG. GASA4, one of the 14-member *Arabidopsis* GASA family of small polypeptides, regulates flowering and seed development. *Plant Cell Physiol*. 2007;48(3):471–83.
52. Vishal B, Kumar PP. Regulation of seed germination and abiotic stresses by gibberellins and abscisic acid. *Front Plant Sci*. 2018;9:838.
53. Shuai H, Meng Y, Luo X, Chen F, Zhou W, Dai Y, Qi Y, Du J, Yang F, Liu J, et al. Exogenous auxin represses soybean seed germination through decreasing the gibberellin/abscisic acid (GA/ABA) ratio. *Sci Rep*. 2017;7(1):12620.
54. Walton LJ, Kurepin LV, Yeung EC, Shah S, Emery RJ, Reid DM, Pharis RP. Ethylene involvement in silique and seed development of canola, *Brassica napus* L. *Plant Physiol Biochem*. 2012;58:142–50.
55. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular biology evolution*. 2018;35(6):1547–9.
56. Chen H, Nelson RS, Sherwood JL. Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. *Biotechniques*. 1994;16(4):664–8,

670.

57. Sparkes IA, Runions J, Kearns A, Hawes C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature protocols*. 2006;1(4):2019–25.
58. Frank W, Ratnadewi D, Reski R. *Physcomitrella patens* is highly tolerant against drought, salt and osmotic stress. *Planta*. 2005;220(3):384–94.
59. Alam MN, Zhang L, Yang L, Islam MR, Liu Y, Luo H, Yang P, Wang Q, Chan Z. Transcriptomic profiling of tall fescue in response to heat stress and improved thermotolerance by melatonin and 24-epibrassinolide. *BMC Genom*. 2018;19(1):224.
60. Shi H, Jiang C, Ye T, Tan DX, Reiter RJ, Zhang H, Liu R, Chan Z. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers.] by exogenous melatonin. *J Exp Bot*. 2015;66(3):681–94.
61. Shi H, Wang X, Tan DX, Reiter RJ, Chan Z. Comparative physiological and proteomic analyses reveal the actions of melatonin in the reduction of oxidative stress in Bermuda grass (*Cynodon dactylon* (L). Pers.). *J Pineal Res*. 2015;59(1):120–31.
62. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3(7):RESEARCH0034.

Figures

Multisequence alignment and phylogenetic tree of ERF proteins from different plants. A: Multisequence alignment of ERF proteins by Clustalw2. B: Maximum Likelihood (ML) phylogenetic tree of B3 subgroup ERF protein sequences from different plant species. AtERF1, 2, 6, 13, 14, 15, ERF1, AT1G06160.1, AT5G43410.1, AT3G23220.1, AT3G23230.1, AT4G18450.1 (*Arabidopsis thaliana*), NtERF5 (*Nicotiana tabacum*), LeERF (*Lithospermum erythrorhizon*), AaERF1,2 (*Artemisia annua*).

Figure 2

Mature seeds and thousand kernel weights of SmERF1-expressing tobacco and WT. A Mature seed morphology of SmERF1-expressing tobacco and WT under a microscope. B Thousand kernel weights of SmERF1-expressing tobacco and WT.

Figure 3

Development of SmERF1 expression and WT tobacco seedlings. A The growth of SmERF1-expressing and WT tobacco seedlings after two weeks of germination. B The biomass of SmERF1-expressing and WT tobacco seedlings. C Germination rate of SmERF1-expressing and WT tobacco seedlings. D The chlorophyll contents of SmERF1-expressing and WT tobacco seedlings.

Figure 4

4 The resistance detection of SmERF1-expressing tobacco plants under NaCl treatment. A Morphology of SmERF1-expressing tobacco plants after 7 days of NaCl treatment. B The POD, SOD, MDA and proline content of SmERF1-expressing tobacco plants under NaCl treatment.

Figure 5

SmERF1 expression affects hormone biosynthesis in tobacco transgenic plants. A The contents of ABA and GA in SmERF1-expressing tobacco plants. B The expression levels of several key enzyme-encoding genes of ABA, GA and ET.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [S1new.jpg](#)
- [S2figERF1expression.tif](#)