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Functional analysis of the *StERF79* gene in response to drought stress in potato (*Solanum tuberosum* L.)

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Abstract

Background The AP2/ERF (APETALA 2/ethylene-responsive element binding factors) is a class of superfamily of plant-specific transcription factor that play an important regulatory role in many physiological and biochemical processes in plants.

Results In this study, overexpression of the *StERF79* gene increased drought tolerance in potato plants, whereas *StERF79* RNA interference expression (RNAi) lines decreased drought tolerance in potato plants. In addition, the superoxide dismutase (SOD), peroxide dismutase (POD), and catalase (CAT) activities, as well as proline (Pro) content of *StERF79* transgenic lines, showed significantly higher results than those of the wild type (WT) potato plants under natural drought stress conditions, while the malondialdehyde (MDA) content was lower. The StERF79 transcription factor can respond to drought stress by interacting with a DRE *cis*-acting element in the promoter region of the downstream target gene (*StDHN-2*), and activating its expression, the result was validated by using yeast one hybrid (Y1H), Dual-Luciferase and β-glucuronidase (GUS) staining assays both in vivo and in vitro. The *StDHN-2* gene is a member of the dehydrin (DHN) subfamily of the potato plant late embryonic developmentally abundant (LEA) protein family. LEA, hydrophilic proteins found in plants, serve as cellular dewatering protectants to prevent desiccation during various stresses.

Conclusion The results could provide novel knowledge into the functional analysis of the *StERF79* gene in positive regulation of the *StDHN-2* gene to drought response and its possible mechanisms in potato plants.

Clinical trial number Not applicable.

Keywords Potato, Genetic transformation, Drought stress, *StERF79*, DRE element

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Introduction

There are many abiotic factors affecting the growth and development of plants throughout their life process, among which drought is the major one [1], that restricts crop growth, yield, and quality, has caused serious consequences for the global agricultural economy. Plants have evolved complex and diverse regulatory approaches to cope with the adverse effects of biotic or abiotic stresses for their survival [2], in which plant transcription factors (TFs) play a significant role in cells as a class of proteins that regulate the expression of genes, and they promote or repress the transcription of genes by binding to specific sequences on DNA [3, 4].

AP2/ERF (APETALA2/ethylene-responsive element binding factors) transcription factors are one of the largest families of TFs in plants, and play an important regulatory role in many biological processes such as plant growth and development, hormonal signaling, metabolites regulation, and response to adverse conditions [5]. They are characterized by the presence of the highly conserved AP2 DNA-binding domain, and classified into four major subfamilies based on domain architectures and specific amino acid residues. These subfamilies are DREB (Dehydration Responsive Element-Binding), ERF (Ethylene-Responsive-Element-Binding protein), AP2 (APETALA2), RAV (Related to ABI3/VP), and Soloists (a few unclassified factors) [6–8]. With the development of sequencing technology, genes of the AP2/ERF superfamily have been studied in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), maize (*Zea mays*) and carrot (*Dacus carota*) [8–11], and have also been found in *Solanaceae* plants family, especially some economically important plant crops, such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*) and pepper (*Capsicum annuum*) [12–14]. Researchers have identified 2195 *AP2/ERF* genes and updated the annotation of *AP2/ERF* genes in model plants and several *Solanaceae* plant family members as given in Table 1 [15].

Table 1 Numbers of *AP2/ERF* genes in eight *Solanaceae* plants family, *Arabidopsis thaliana* and *Oryza sativa*

Species	Previously annotated genes	Newly annotated genes	Total
Oryza sativa	163	14	177
Arabidopsis thaliana	141	1	142
Nicotiana benthamiana	323	32	355
Petunia axillaris	187	32	219
Capsicum annuum	181	67	248
Capsicum baccatum	173	74	247
Solanum tuberosum	212	42	254
Solanum melongena	153	23	176
Solanum pimpinellifolium	127	59	186
Solanum lycopersicum	167	24	191
Total	1827	368	2195

AP2/ERF transcription factors bind directly to the GCC box/dehydration response element (DRE)/C-repeat (CRT) *cis*-acting element in the promoter region of downstream target genes to regulate its expression [6]. Among them, the DRE element (TACCGACAT), which is the main binding site for AP2/ERF transcription factors in plant abiotic stress response, with a core conserved sequence of A/GCCGAC, was identified from the promoter region of the *Arabidopsis RD29A* gene [16]. It was found that the JERF1 transcription factor could specifically bind to the DRE *cis*-acting element in the promoter region of the ABA synthesis gene *NtSDR* in tobacco, which led to the up-regulation of the expression of the relevant genes and an increase in the ABA content in the plants, which in turn enhanced the plant's tolerance to abiotic stresses such as low temperatures [17]. In addition, overexpression of the *TSRF1* gene in tobacco also up-regulated the expression of the *NtSDR* gene, which led to an increase in ABA and proline content in transgenic tobacco, which improved the drought tolerance of transgenic tobacco [18]. The OsDERF1, as a negative regulator in osmotic regulation [19], can bind to the GCC box in the *OsERF3* promoter region, and *OsERF3* contains an ethylene-responsive element binding factor associated with amphiphilic repressor (EAR) motif that represses ethylene production during transcription, suppressing the drought tolerance of plants. The mutant EAR lines showed greater tolerance to drought compared to both wild-type and over-expression plants [20]. In maize, the ZmDREB1A protein activated the expression of *COR15A*, *KIN1*, *KIN2* and other downstream functional genes by binding to their DRE/CRT elements, causing the plants to exhibit higher drought and frost tolerance [21, 22]. Similarly, the TaERF3 transcription factor bound to the GCC box in the promoter region of seven abiotic stress-responsive genes and positively regulated drought and salt tolerance in wheat [23]. The AgDREB1 and AgDREB2 may act as transcriptional activators that regulate downstream genes by binding to the corresponding DRE/CRT elements, thereby enhancing stress tolerance in celery (*Apium graveolens* L.) [24]. The AP2/ERF transcription factors also can interact with various other transcription factors from different signaling pathways, forming a complex regulatory network. For example, in apples, the MdERF38 interacts with the positive regulatory factor MdMYB1 for anthocyanin biosynthesis, promoting binding to target genes and improving drought resistance [25]. In rice, the *OsERF109* gene regulates plant drought tolerance by interacting with the *OSACO2*, *OSACS6*, and *OsERF3*, the key genes in the ethylene biosynthesis pathway [26]. Abscisic acid (ABA) is a major mediator of drought [27], which prevents transpirational water loss by mediating stomatal conductance [28]. The molecular regulatory mechanisms of ABA-mediated

drought are categorized into ABA-dependent and ABA-independent pathways, including ABA receptors, transcription factors (AP2/ERF, MYB/MYC, WRKY, bZIP, etc.), and their corresponding *cis*-acting elements (DRE, MBS, W-box, ABRE, etc.), as well as downstream functional genes (*LEA*, *DREB2B*, *PP2C*, *NCED3*, etc.), which ultimately respond to drought stress by affecting the morphophysiology of the plants [29]. The late embryogenesis abundant (*LEA*) gene family is a class of plant gene family whose members are abundantly expressed in late embryonic stages and under adverse conditions such as drought [30]. The LEA protein family can be categorized into the following eight subgroups based on amino acid sequences and conserved structural domains: LEA1, LEA2, LEA3, LEA4, LEA5, LEA6, dehydrin (DHN), and seed maturation proteins (SMP). It has been found that classes of ASR proteins that are induced to be expressed by ABA are also LEA proteins, belonging to the LEA7 group [31–33]. In potato, 74 LEA proteins belonging to different subgroups have been identified [34].

The modern potato crop is considered to be a drought-sensitive crop, and under drought stress its yield and quality decrease dramatically [35]. With incessant global climate change, the adverse effect of drought on potatoes is also a challenging, improving agronomic measures, identifying drought-tolerant physiological and biochemical traits, and developing drought-tolerant genotypes of potatoes to increase its yield and quality [36]. Based on the potato ERF transcriptome database [37], our previous work identified and screened 15 drought-responsive *StERFs* genes, among which StERF79 showed the most significant expression differences under five treatments, including polyethylene glycol (PEG), ethylene (ET), salicylic acid (SA), methyl jasmonate (MeJA), and mannitol [38]. In this study, we have briefly investigated the role of the *StERF79* gene and its function in potato plants under drought-stress conditions. Firstly, we developed the overexpression and interference expression lines of the *StERF79* gene. Secondly, to elucidate its specific drought resistance pathway, the dehydrin gene *StDHN-2* was selected, and *cis*-acting element analysis was performed on approximately 2000 bp of the upstream promoter region of the gene, and the result showed that the presence of DRE elements, suggesting that it may be a downstream functional gene of *StERF79*. The results of this study may provide a theoretical basis for analyzing the mechanism of genetic drought tolerance in potato crop. These findings provide a fresh prospective for improving the potato drought resistance using molecular, physiological, and antioxidant processes by *StERF79* gene up-regulation of *StDHN-2* gene.

Materials and methods

Plant materials and growth conditions

Potato (*Solanum tuberosum* L.) cultivar 'Atlantic' plantlets were cut into stem segments in vitro with one or two leaves and were inoculated in Murashige and Skoog (MS) solid medium containing 3% sucrose and 0.7% agar, and placed in a light incubator at a temperature of 23 °C, 16 h of light and 8 h of darkness, and grown for about 21 d for subsequent experiments. To obtain micro tubers for potato genetic transformation experiments, we inoculated potato cultivar 'Atlantic' plantlets in MS solid medium containing 8% sucrose and 0.7% agar, firstly in a light incubator at a temperature of 23 °C, 16 h of light and 8 h of dark culture conditions for 28 d, and then transferred to the dark conditions again for 40–65 d to obtain the micro tubers [39]. The qRT-PCR analysis described below was used to investigate the relative mRNA expression of the *StERF79* gene in roots, stems, leaves, and tubers of potato plants. All experimental samples were harvested and frozen in liquid nitrogen and stored at -80 °C for further analysis. All experimental steps were carried out using three biological and three technical replicates.

Next, the Bentham's tobacco (*Nicotiana Benthamian* L.) seeds were cultivated in pots (10 × 10 cm) supplemented with nutrients soil and vermiculite with a ratio of 1:1, and the moisture content of the soil was maintained (70–75%). These pots were placed in an incubator with a 16 h light (2000 Lx)/8 h dark photoperiod at 23 °C for three to four weeks for growth.

Cloning of *StERF79* gene

Total RNA from plant tissues was extracted according to the instructions for the Tengen TRNzol Universal Total RNA Extraction Reagent (Tiangen, Beijing, China). The RNA was converted into cDNA by reverses transcription using the instructions of the Tengen FastKing One-Step De-Genomic cDNA First Strand Synthesis Premix Kit (Tiangen, Beijing, China) [40], and for the extraction of plant genomic DNA, the cetyltrimethylammonium bromide (CTAB) method was used [41].

The sequences of the *StERF79* gene (ID: Soltu.DM.03G031550.1) and its downstream gene *StDHN-2* (ID: Soltu.DM.02G024660.1) were retrieved from the potato database Spud DB (http://spuddb.uga.edu). The 2000 bp of the upstream promoter region of the *StDHN-2* gene was submitted to PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for *cis*-acting element analysis [42], and according to the result (Fig. S1), we selected 500 bp of the promoter region of *StDHN-2* gene for amplification. The coding sequence (CDS) of the *StERF79* gene was cloned using the cDNA of potato cultivar 'Atlantic' leaves as a template, the reaction system was as follows: OE-StERF79-F 1 μL, OE-StERF79-R 1 μL,

cDNA template 1 μL, 2×Easy Taq PCR Super Mix(+dye) 10 μL, ddH_2O 7 μL, the list of all primer sequences are shown in Table S1, then pre-denatured at 94 °C for 5 min, and performed 35 cycles in 94 °C for 30 s, 54.9 °C for 30 s, 72 °C for 67 s, finally 72 °C for 10 min and stored at 4 °C.

Real-time fluorescent quantitative PCR assay

The concentration of cDNA was measured with an ultra-micro UV spectrophotometer (Quawell Q5000), and the concentration was diluted to about 100 ng/μL. The qRT-PCR analysis was performed according to the Servicebio® 2×Universal Blue SYBR Green qPCR Master Mix kit instructions. The relative mRNA expression of the target gene was determined using a qRT-PCR instrument (Roche LightCycler®96, Switzerland), and the reaction system was 10 μL of 2×Universal Blue SYBR Green qPCR Master Mix, 0.4 μL of each forward and reverse primers, 1 μL of cDNA template (100 ng/μL), and 8.2 μL of nuclease-free water, the total reaction system was 20 μL. Furthermore, the reaction conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The StEF1α (GenBank No. AB061263.1) was used as the standard reference gene. Three biological replicates and three technical replicates were performed for each experiment, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [40]. For qRT-PCR analysis, the specific primers were designed using NCBI's primer-BLAST online analysis tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The list of all primer sequences are shown in Table S1. The melting curve analysis of the qRT-PCR experiments are shown in Fig. S2.

Genetic transformation of potato and identification of Transgenic plants

The coding sequence of the *StERF79* gene was amplified according to the designed specific primers (Table S1) and inserted into the linearized vector pCAMBIA1300-35 S-EGFP using homologous recombination, and the enzyme digestion sites were *Sac* I and *Kpn* I. The recombinant plasmid was named pCAMBIA1300-35 S-StERF79 (Fig. S3A). The interference expression vectors were constructed using amiRNA interference technology, and the interference precursor fragments were amplified using the precursor primers (I II III IV) designed on the WMD3 online tool (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (Table S1), and the interfering fragment was inserted into the pMD™ 18-T cloning vector using double digestion method, then the small fragment (490 bp) were recovered by double digestion of the recombinant plasmid, which was inserted into the expression vector pCAMBIA1300-35 S-EGFP using T4 ligase, the digestion sites were *Kpn* I and *Xba* I. Finally, the interference expression vector (pCAMBIA1300-35 S-amiRNA-StERF79) was constructed, depicted in Fig. S3B.

Agrobacterium suspensions containing recombinant plasmids were cultured in LB liquid medium (containing 50 μg/mL Rif, 50 μg/mL Kan) and activated to OD_{600} = 0.5, centrifuged, and re-suspended with an equal volume of 3% MS liquid medium, respectively, for further investigations. The micro tubers obtained as described in Sect. 2.1, were cut, peeled, and removed the bud eyes, cut into 2–3 mm thick chips, and dipped into the bacterial suspensions for 7 min, then, the residual bacterial suspension on the surface of the chips was wiped dry and placed into MS solid medium for 2 d of co-cultured. Afterward, chips were cultured on differentiation medium until the growth of callus. After differentiation, the seedlings were cut and inoculated in the rooting screening medium for screening and identification of transgenic plants (three screenings per line). DNA was extracted from the plants obtained from the preliminary screening root, and the transgenic plants were further identified by PCR amplification with the unique screening marker primers (HYG-F, HYG-R) on the carrier. Next, the transgenic plants could be detected with the target gene expression level, and the correct overexpression and interference expression lines were finally obtained.

Drought tolerance analysis of Transgenic plants

Transgenic lines and wild-type potato plants grown for 21 d in MS medium were planted in pots (10 × 10 cm) and supplemented with soil nutrients and vermiculite (1:1, v/v). The plantlets were cultivated in a greenhouse with a light intensity of 2000 Lx, a temperature of 23 ± 2 °C, and a photoperiod of 16 h of light and 8 h of darkness. The plantlets were watered every 7 d, and after four weeks of growth, the watering was stopped, and plants with uniform growth were selected for drought treatment. During this period, the water content of the soil was measured as well as the wilted state of the plants was observed and photographed. For the determination of leaf water loss rate, leaves of four-week-old plants were selected and kept under normal conditions (temperature of 22 °C and relative humidity of 20%) for 5 h to lose the water from leaves. The weights of the leaves were determined at the intervals of 1 h. The relative water content (RWC) of the leaves was determined after 14 d without watering, and the total fresh weight (FW) was calculated before and after the treatments. Then the leaves were placed in ddH_2O for 12 h, dried on the surface, and SFW was calculated, and the dry weight was calculated by keeping the leaves in an oven at 75 °C. The relative water content was measured by a formula as given, RWC (%) = [(FW)-(DW)/(SFW)-(DW)]×100% [43].

The MDA content was determined by thiobarbituric acid method (TBA), MDA content =

$[6.45\times(OD_{532}\text{-}OD_{600})\text{-}0.56\times OD_{450}\times V\times A]/W$ (V denotes the total volume of the reaction in ml; A denotes the dilution multiple of dilution; W denotes the protein content in mg/ml) [44]. Pro content was determined by acid ninhydrin method [45], Pro content = $(C\times V/V_a)/W$ (C denotes the concentration of proline found on the standard curve; V denotes the total volume of the extract in ml; V_a denotes the volume of the measuring liquid in ml; W denotes sample weight in g).

Furthermore, weighed 0.5 g of potato plant leaves, were ground in ice bath with phosphate buffer, centrifuged and the supernatant was taken as enzyme solution for use, superoxide dismutase (SOD) activity was determined by riboflavin-NBT reduction method [46], and the absorbance value of the reaction solution was measured at OD_{560}, SOD activity (U/g·FW) = $(A_{CK}\text{-}A_E)\times V/(W\times 0.5\times A_{CK})$ (A_{CK} denotes control absorbance value; A_E denotes the absorbance value of the experimental group; V denotes the volume of the measuring liquid; W denotes sample weight). Peroxidase (POD) activity was determined by the guaiacol colorimetric method [47], POD activity (ΔA_{470}/min·g·FW) = $\Delta A_{470}\times V/V_a/W$ (ΔA_{470} denotes the change in absorbance per minute; V denotes the volume of reactive liquid; V_a denotes the volume of enzyme liquid; W denotes sample weight). Catalase (CAT) activity was determined by H_2O_2 decomposition as described in Aebi [48], CAT activity (ΔA_{240}/min·g·FW) = $\Delta A240\times V/V_a/W$ (ΔA_{240} denotes the change in absorbance per minute; V denotes the volume of reactive liquid; V_a denotes the volume of enzyme liquid; W denotes sample weight). Microsoft Excel was used to enter and organize the data. SPSS software was used for significance analysis, and significance was analyzed by one-way analysis of variance (ANOVA). Data were graphed through GraphPad Prism 8. All the above experiments were performed in three biological replicates, respectively.

Yeast one-hybrid assays (Y1H)

The specific amplification primers were designed (Table S1), and 500 bp of the upstream promoter region of the *StDHN-2* gene was amplified and inserted into the pHIS2.1 vector using homologous recombination method, the recombinant plasmid was named bait vector pHIS2-prDHN-2 (Fig. S4A). Similarly, the CDS of the *StERF79* gene was amplified and inserted into the pGADT7 vector, the recombinant plasmid was named the reporter vector pGADT7-StERF79 (Fig. S4B). The bait vector pHIS2-prDHN-2 was first transfected into yeast Y187 receptor cells according to the PEG/LiAc-mediated method, with the p53-His2.1 acting as a positive control, the bacterial suspensions were coated on two-deficient-tryptophan-histidine (SD-Trp-His) medium containing different 3-AT concentrations (0 mM, 10 mM, 30 mM, 50 mM, 80 mM, 100 mM), and followed a 48–96 h incubation period at 30 °C, for the self-activation assay of the bait vectors. Then the bait vector pHIS2-prDHN-2 and the reporter vector pGADT7-StERF79 were co-transfected into Y187 yeast receptor cells, with the pGADT7-53 m + p53His acting as the positive control, pGADT7-53 m and pHis2.1 acting as the negative controls, respectively. Then coated on triple-deficient-tryptophan-histidine-leucine (SD-Trp-His-Leu) medium and incubated at 30 °C for 48–72 h to observe their growth.

Dual luciferase assay (LUC)

The specific primers were designed based on the sequence of the pGreenll-0800-Luc vector and 500 bp upstream of the promoter region of the *StDHN-2* gene, the pGreenll-0800-Luc was digested by *Kpn* I and *Bam*H I enzymes, the 500 bp was inserted into the pGreenll-0800-Luc vector, and the recombinant plasmid was named as p0800-LUC-prDHN-2 vector (Fig. S5A). Similarly, the CDS of *StERF79* was inserted into the pGreenII-62-SK vector, and the enzyme cleavage sites were *Xba* I and *Kpn* I, the recombinant plasmid was named as p62-SK-StERF79 vector (Fig. S5B). The *Agrobacterium* suspensions containing p62-SK-StERF79, p0800-LUC-prDHN-2, pGreen62-SK, and pGreen0800-LUC were cultured and activated in Luria-Bertani (LB) liquid medium (25 mL) containing 50 μg/mL rifampicin (Rif), 50 μg/mL kanamycin (Kan), 100 mM acetosyringone (ACE), and 100 mM 2-morpholinoethanesulfonic acid (MES), respectively, to an OD_{600} of 0.5–0.6. After centrifugation, the bacterial precipitates were re-suspended with equal volumes (25 mL) of 1% (sucrose concentration) MS liquid medium (containing 500 mM $MgCl_2$, 100 mM ACE, 100 mM MES), respectively, and injected into the backside of tobacco leaves after an ice bath for 2–3 h, the backside of tobacco leaf was divided into two infested areas, namely, control group: p0800-LUC-prDHN-2 + pGreen62-SK, and experimental group: p62-SK-StERF79 + p0800-LUC-prDHN-2. The infested area was marked and the tobacco plants were placed at temperature of 25 °C under dark conditions for 48–72 h. At the end of the culture, the injected tobacco leaves were cut and uniformly sprayed on the backside of the tobacco leaves using 1 $mmol\cdot L^{-1}$ fluoresceinase substrate reaction solution, D-luciferin, placed in a dark condition for 5–7 min. The expression of fluorescence signals was observed by the Plant View 100 system (Guangzhou Boluteng Bio-Technology Co. Ltd., China), and LUC values were determined using a dual fluorokinase reporter gene assay kit (Beyotime).

GUS staining assay

The 500 bp of the promoter region of *StDHN-2* gene was inserted into pBI121 vector (with GUS tag), and the

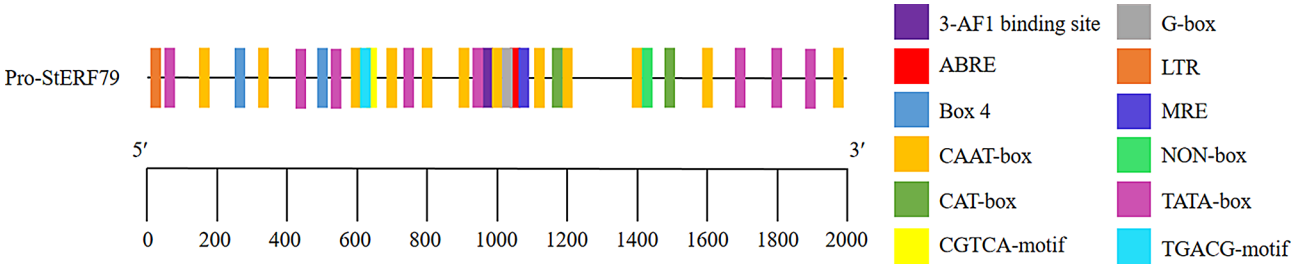

Fig. 1 *Cis*-acting element 2000 bp upstream of the promoter region of *StERF79* gene

Table 2 *Cis*-acting element analysis of *StERF79* gene

Name	Sequence	Number	Function
3-AF1 binding site	TAAGAGAGGAA	1	light responsive element
ABRE	ACGTG	3	abscisic acid responsive element
Box 4	ATTAAT	5	light responsive element
CAAT-box	CAAT	41	common element in promoter and enhancer
CAT-box	GCCACT	2	element related to meristem expression
CGTCA-motif	CGTCA	1	MeJA-responsive element
G-Box	CACGTG	3	light responsive element
LTR	CCGAAA	1	low-temperature responsive element
MRE	AACCTAA	1	MYB binding site involved in light responsiveness
NON-box	AGATCGACG	1	element related to meristem expression
TATA-box	TATA	42	core promoter element
TGACG-motif	TGACG	1	MeJA-responsive element

recombinant plasmid was named pBI121-GUS-prDHN-2 (Fig. S6A), the CDS of *StERF79* gene was inserted into the pBI121 (without GUS tag, *Bam* HI and *Sac* I were used to cut off the GUS tag) vector, and was named here as pBI121-StERF79 (Fig. S6B). *Agrobacterium* suspensions containing pBI121-StERF79, pBI121-GUS-prDHN-2 and pBI121 were activated respectively, according to the steps mentioned above in Sect. 2.7, re-suspended, and injected into the backside of tobacco leaves after an ice bath, the area of infestation was marked with a marker pen. The pBI121-StERF79 was used as a negative control, pBI121-StERF79 + pBI121, and pBI121 were used as positive controls, and the pBI121-StERF79 + pBI121-GUS-prDHN-2 as the experimental group. The injected tobacco was incubated at 25 °C under dark conditions for 48–72 h. The GUS staining solution was configured according to the instructions of the GUS Staining Kit of Beijing Coolaber Biological Company. The 70% ethanol solution was configured for leaf decolorization. Until the negative control (pBI121-StERF79) material was white and then it was possible to observe and take pictures to record the expression of the blue spots that were positive for GUS staining. In addition, GUS Reporter Gene Quantification Kit and Bradford Protein Concentration Measurement Kit (Coolaber) were used to measure GUS activity.

Results

Identification and cloning of *StERF79* gene

The full sequence of *StERF79* gene (Soltu.DM.03G031550) was retrieved from the potato database Spud. The sequence analysis showed that *StERF79* gene is a contiguous gene, because it has only one exon with a cDNA length of 1078 bp, and a total CDS coding sequence of 618 bp encoding 205 amino acids. The *StERF79* gene was extracted 2 kb genomic sequences upstream of its transcriptional start codon, and this sequence was then utilized to analyze for *cis*-regulatory elements using the online tool Plant CARE identified three elements that can respond to ABA and two other elements that respond to MeJA, as well as some light-responsive elements (Fig. 1) (Table 2). The cDNA of potato cultivar 'Atlantic' leaves was used as a template for cloning of the CDS of *StERF79* gene by PCR, and the PCR product was detected by 1% agarose gel electrophoresis to obtain the 618 bp of the targeted band (Fig. S7).

Tissue specific expression analysis of *StERF79* gene

The expression of *StERF79* gene in different tissues of potato cultivar 'Atlantic' was measured by qRT-PCR analysis. The results showed that the relative expression level of *StERF79* gene was the lowest in the stems, and while the levels in the leaves and tubers were higher than in other tissues, and was not significantly different from each other (Fig. 2).

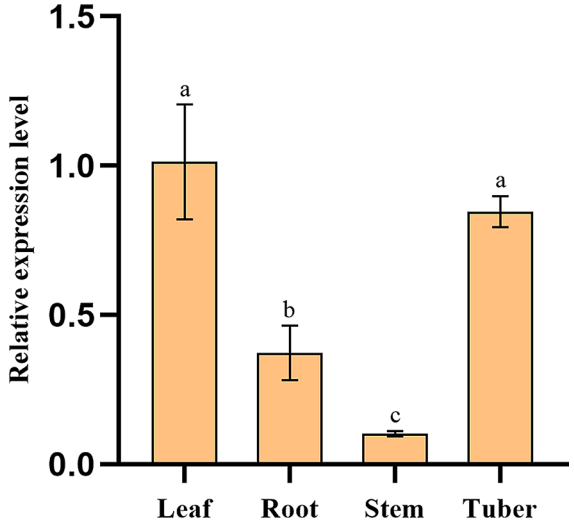

Fig. 2 The relative expression level of the *StERF79* gene in different tissues of potato. The x-axis represents different tissues of potato, and the y-axis represents the relative expression level of the *StERF79* gene. Error bars indicate the standard error of the means (SD) based on three technical replicates. Different lowercase letters represent significant difference ($p < 0.05$). Data = mean ± SD, $n = 3$

Genetic transformation of the *StERF79* gene in potato

Potato chips of the cultivar 'Atlantic' were infected with *Agrobacterium* suspensions containing the pCAMBIA1300-35 S-StERF79 and the pCAMBIA1300-35 S-amiRNA-StERF79, respectively. Next, these chips were co-cultured of 2 d (Fig. 3A, E) and again cultivated on differentiation medium to produce callus (Fig. 3B, F) and differentiated buds (Fig. 3C, G). Afterward, the differentiation buds were cut and inoculated in rooting screening medium containing cefatothin (Cef) and Hygromycin (HYG) for the screening of transgenic plants (Fig. 3D, H), and these plants that were screened for three times and rooted were initially identified as transgenic plants.

The DNA specificity of transgenic plants was detected by PCR. The wild-type (WT) plants were used as the negative control, while the empty vector pCAMBIA1300-35 S-EGFP was used as the positive control. The screening marker gene *HYG* on the vector was amplified by PCR and the targeted bands of expected sizes were attained by 1% agarose gel electrophoresis detection (Fig. 4A). These results indicated that the *StERF79* gene and its interfering fragments had been successfully transferred into potato plants. Furthermore, the relative expression level of *StERF79* gene was detected by qRT-PCR analysis. The results showed that the relative expression of *StERF79* gene in overexpression lines, such as OE-1, OE-2, and OE-3 was significantly higher compared to wild-type plants, which were 8.67-fold, 9.48-fold, and 4.17-fold, respectively (Fig. 4B). In contrast, the expression in the interference-expressing lines, such as RNAi-1,

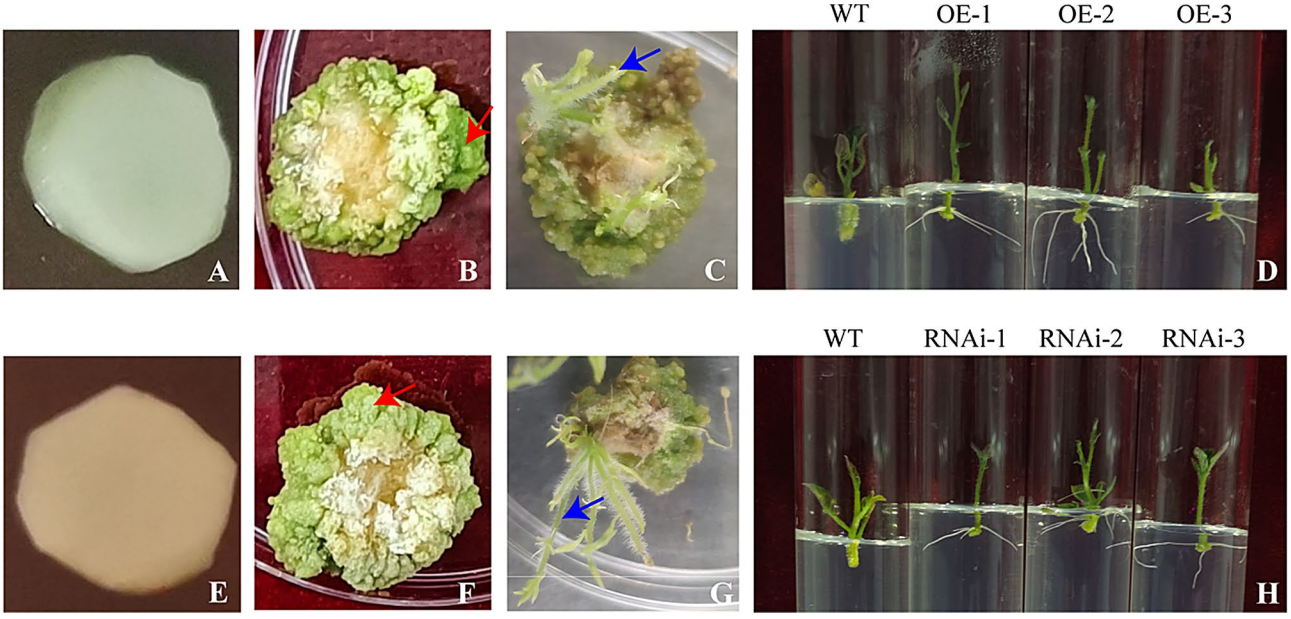

Fig. 3 Genetic transformation of *StERF79* gene in potato. (**A**): Potato chip co-culture with *Agrobacterium* suspensions containing the pCAMBIA1300-35 S-StERF79 plasmid. (**B**): Formation of callus of the pCAMBIA1300-35 S-StERF79 potato chip. (**C**): Differentiation of callus of the pCAMBIA1300-35 S-StERF79 potato chip. (**D**) Rooting screening of the pCAMBIA1300-35 S-StERF79 plants. (**E**): Potato chip co-culture with *Agrobacterium* suspensions containing the pCAMBIA1300-35 S-amiRNA-StERF79 plasmid. (**F**): Formation of callus of the pCAMBIA1300-35 S-amiRNA-StERF79 potato chip. (**G**): Differentiation of callus of the pCAMBIA1300-35 S-amiRNA-StERF79 potato chip. (**H**): Rooting screening of the pCAMBIA1300-35 S-amiRNA-StERF79 plants. Red arrows: Callus of the chip. Blue arrows: Buds differentiated from callus. WT: Wild-type plants of 'Atlantic'. OE-1 ~ OE-3: Plants overexpressing the *StERF79* gene. RNAi-1 ~ RNAi-3: Plants interfering expression of the *StERF79* gene. The same as below

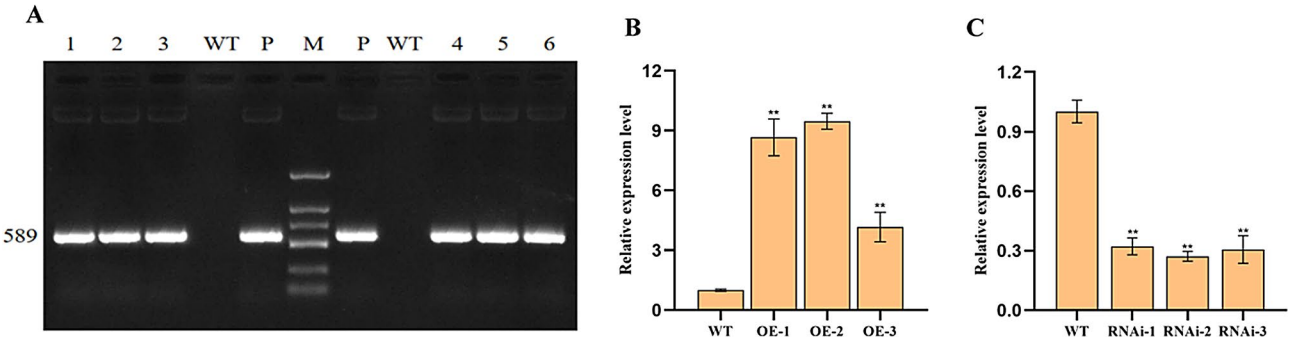

Fig. 4 Characterization of the *StERF79* gene transgenic plants. (**A**): DNA identification of transgenic plants. M: DL 2000 marker; P: Positive control plasmid; WT: Negative control; 1–3: Overexpression of transgenic lines. 4–6: Interference expression of transgenic lines. (**B**): Relative expression levels of the *StERF79* gene in wild-type and overexpression plants. (**C**): Relative expression levels of the *StERF79* gene in wild-type and interference-expressing plants. $p < 0.01$ (shown as '**'). Data = mean ± SD, $n = 3$

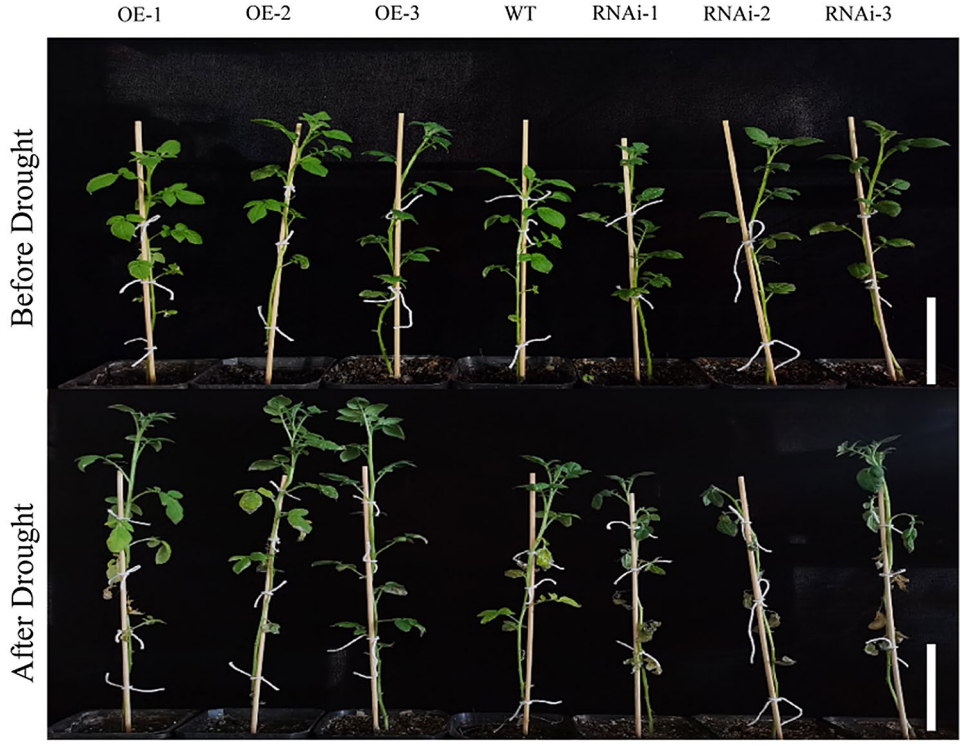

Fig. 5 Phenotypic characterization of transgenic plants before and after drought. WT: Wild-type plants of 'Atlantic'. OE-1 ~ OE-3: Plants overexpressing the *StERF79* gene. RNAi-1 ~ RNAi-3: Plants interfering expression of the *StERF79* gene. The scale bale represents 10 cm

RNAi-2, and RNAi-3 was significantly lower compared to wild-type plants, which were 0.32-fold, 0.27-fold, and 0.31-fold, respectively (Fig. 4C). The above statement concluded that *StERF79* overexpression (OE-1, OE-2, and OE-3) and interference expression (RNAi-1, RNAi-2, and RNAi-3) potato plants were constructed.

StERF79 gene activates the expression of *StDHN-2* and positively regulates drought stress in potatoes

Three lines of OE-n (overexpression line) and RNAi-n (interference expression line) were selected for drought resistance analysis of the *StERF79* gene. After two weeks of natural drought treatment, we found that the growth of OE-n plants was significantly stronger than RNAi-n plants after drought treatments, and the degree of leaf wilting was OE-n < WT < RNAi-n (Fig. 5).

After that, the water loss rate and relative water content (RWC) of the isolated potato leaves were measured, and the results showed that the water loss rate of the leaves was OE-n < WT < RNAi-n (Fig. 6A); RWC was OE-n > WT > RNAi-n (Fig. 6B).

To further analyze the drought resistance of the *StERF79* gene, the activities of key antioxidant enzymes were measured, such as SOD (Fig. 7A), POD (Fig. 7B),

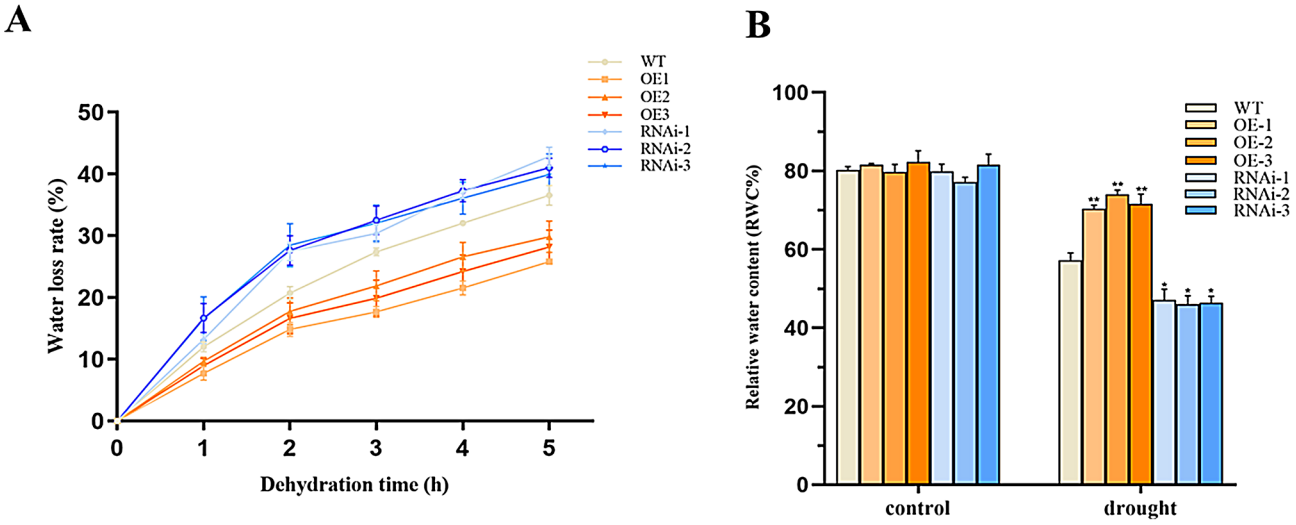

Fig. 6 Determination of water loss and relative water content of isolated leaves of transgenic plants. (**A**): leaf water loss rate. (**B**): Relative water content (RWC). $p > 0.05$ (shown as nothing), $0.01 < p < 0.05$ (shown as '*') and $p < 0.01$ (shown as '**'). Data = mean ± SD, $n = 3$

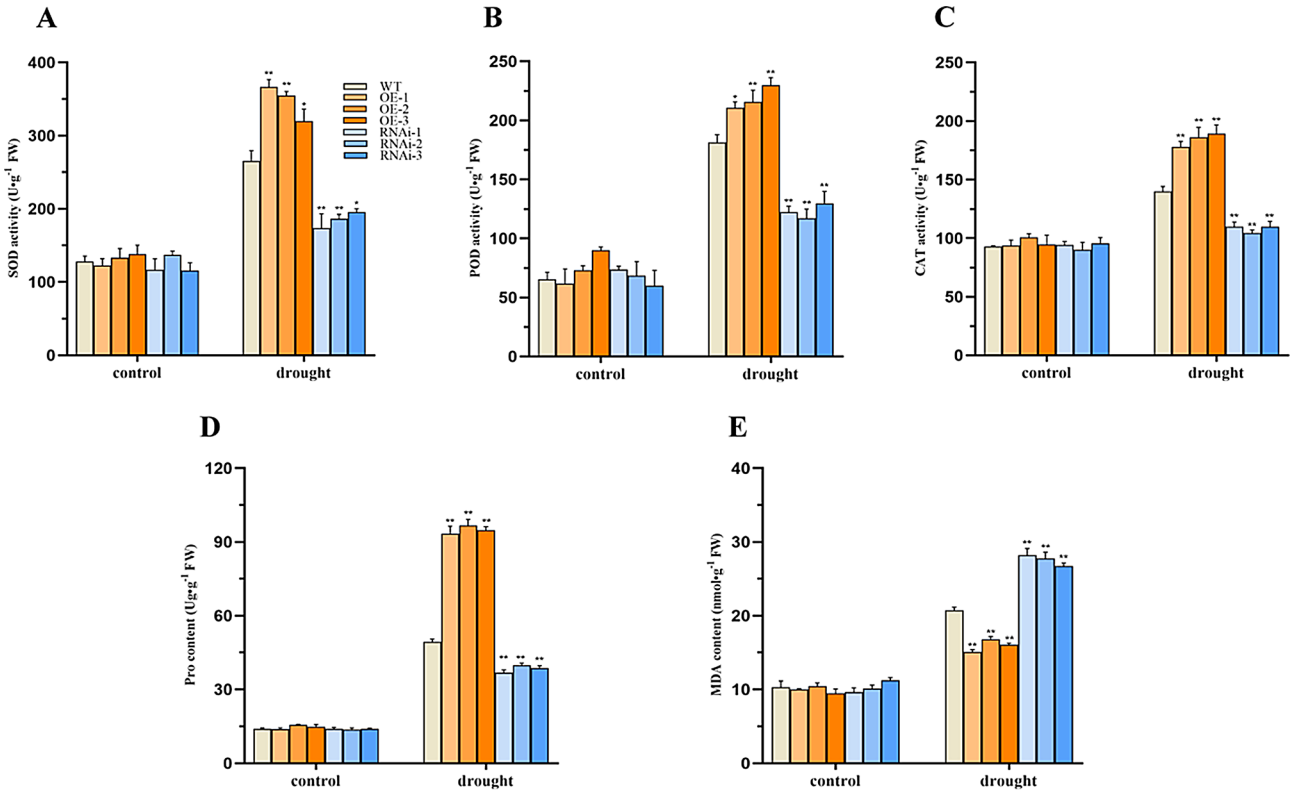

Fig. 7 Analysis of drought-related physiological indices in transgenic plants. (**A**): SOD activity. (**B**): POD activity. (**C**): CAT activity. (**D**): Pro content. (**E**): MDA content. $p > 0.05$ (shown as nothing), $0.01 < p < 0.05$ (shown as '*') and $p < 0.01$ (shown as '**'). Data = mean ± SD, $n = 3$

and CAT (Fig. 7C), and found that there was almost no difference in the activities of these three enzymes in WT, OE-n, and RNAi-n plants before drought stress treatment. After drought treatment, the enzyme activities of OE-n lines were significantly higher than those of WT plants, while in RNAi-n lines, they were significantly lower than those of WT plants. The results were as follows: the activities of SOD were 1.38, 1.34, and 1.20 times higher in the OE-n lines, respectively, compared to WT plants. Conversely, in the RNAi-n lines showed 0.65, 0.70, and 0.73 times higher results, respectively, than those of WT plants; POD activities were 1.16, 1.19, and 1.27 times higher in the OE-n lines, respectively, compared to WT plants. In contrast, in the RNAi-n

lines showed 0.67, 0.65, and 0.72 times higher results, respectively, than those of WT plants; CAT activities were 1.27, 1.33, and 1.35 times higher in the OE-n lines, respectively, compared to WT plants. Conversely, in the RNAi-n lines showed 0.78, 0.75, and 0.78 times higher results, respectively, than those of WT plants. Proline is a substance that will increase greatly in plants under drought stress, and the results found that its content was OE-n > WT > RNAi-n (Fig. 7D), Pro content was 1.89, 1.96, and 1.92 times higher in the OE-n lines than in the WT, respectively, while it was 0.74, 0.81, and 0.78 times higher in the RNAi-n lines than in the WT, respectively; MDA is the product of membrane lipid peroxidation, and its content can reflect the degree of stress damage to plants. After drought treatment, the MDA content was OE-n < WT < RNAi-n (Fig. 7E), and its content was 0.73, 0.81, and 0.77 times higher in the OE-n lines than in the WT, respectively, while it was 1.36, 1.34, and 1.29 times higher in the RNAi-n lines than in the WT, respectively.

To preliminarily analyze the regulatory relationship between the *StERF79* gene and downstream genes. The qRT-PCR analysis was used to test the relative expression levels of the *StERF79* gene and *StDHN-2* gene before and after drought treatment. It was found that the expression level of the *StDHN-2* gene before drought treatment was significantly higher in OE-n lines than WT-1, on the contrary, it was significantly lower in RNAi-n lines than WT-1 (Fig. 8A). Similarly, after drought stress treatment, the expression level of *StDHN-2* gene was OE-n > WT-2 > RNAi-n (Fig. 8B). The above results indicated that the StERF79 transcription factor activates the expression of the downstream functional gene *StDHN-2*, which has a positive regulatory effect on the drought resistance of potato plants.

StERF79 transcription factor interacts with DRE elements to activate expression of the downstream gene *StDHN-2*

Yeast one-hybrid assays

The binding interaction between the StERF79 transcription factor and the DRE element in the promoter region of the *StDHN-2* gene was verified by Yeast one-hybrid assays. Firstly, the self-activity of the bait vector was detected, single colonies of yeast receptor cell Y187 containing pHIS2-prDHN-2 were picked, and mixed in ddH_2O, and the bacterial suspensions was spot-coated on two-deficient-tryptophan-histidine (SD-Trp-His) medium containing different concentrations of 3-AT. The results showed that the 3-AT concentration of 50 mM inhibited the self-activity of the pHIS2-prDHN-2 vector, eliminating the investigational background for subsequent experiments (Fig. 9A). Then the bait vector pHIS2-prDHN-2 and reporter vector pGADT7-StERF79 were co-transfected into Y187 yeast receptor cells, and the yeast suspensions co-transfected with pGADT7-53 m and p53His was used as a positive control, the yeast suspensions co-transfected with pGADT7-53 m and pHis2.1 was used as a negative control, and which were coated on the triple-deficient-Tryptophan-Histidine -Leucine (SD-Trp-His-Leu + 50mM 3-AT) medium at 30 °C for 48–72 h. The results showed that the yeast could grow normally, which indicated that the *StERF79* gene could

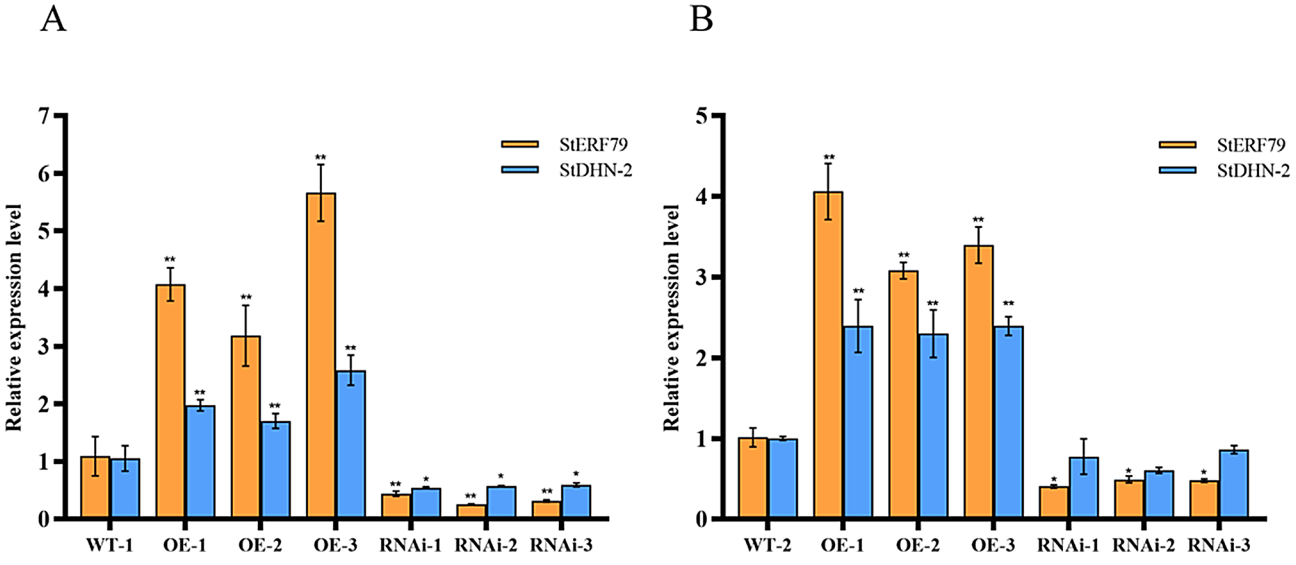

Fig. 8 Relative expression analysis of *StERF79* and *StDHN-2* genes before and after drought treatment. (**A**): Relative expression level before treatment. (**B**): Relative expression level after treatment. WT-1: Wild-type plants of 'Atlantic' before drought treatment. WT-2: Wild-type plants of 'Atlantic' after drought treatment. OE-1 ~ OE-3: Plants overexpressing the *StERF79* gene. RNAi-1 ~ RNAi-3: Plants interfering expression of the *StERF79* gene. '*' above bar indicate the significant difference of the changes between WT and transgenic plants with the level of $p > 0.05$ (shown as nothing), $0.01 < p < 0.05$ (shown as '*') and $p < 0.01$ (shown as '**'). Data = mean ± SD, $n = 3$

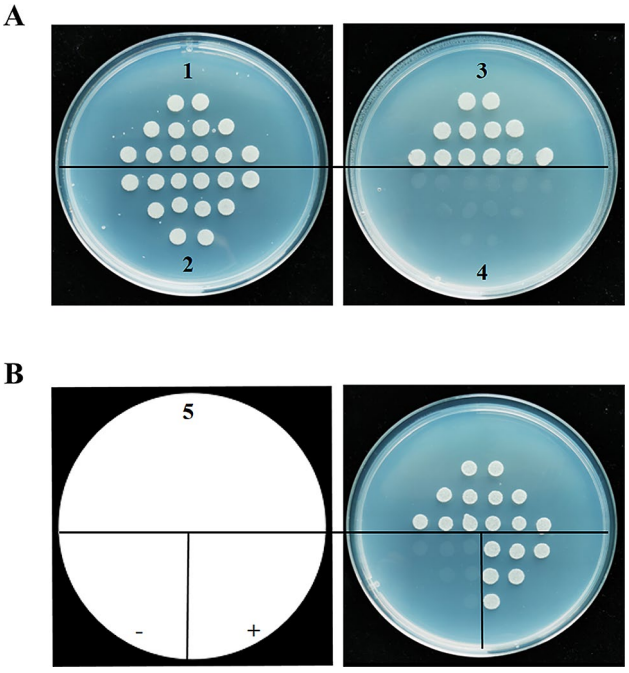

Fig. 9 Yeast one-hybrid assays for StERF79 and DRE elements. (**A**): Self-activating activity of the pHIS2-prDHN-2 vector. 1, 2 media: SD-Trp-His. 3, 4 media: SD-Trp-His + 50mM 3-AT. 1: p53-His2.1. 2: pHIS2-prDHN-2. 3: p53-His2.1. 4: pHIS2-prDHN-2. (**B**): Interaction of StERF79 to the DRE element in the promoter region of the *StDHN-2* gene. 5 media: SD-Trp-His-Leu + 50mM 3-AT; '+': positive control, pGADT7-53 m + p53His; '-': negative control, pGADT7-53 m + pHis2.1; 5: pHIS2-prDHN-2 + pGADT7-StERF79

interact with the downstream DRE element in the promoter region of the *StDHN-2* gene (Fig. 9B).

Dual luciferase and GUS staining assay

To further verify the reliability of the yeast one-hybrid assay, the dual luciferase and GUS staining experiments were performed. The *Agrobacterium* suspensions containing p62-SK-StERF79 and p0800-LUC-prDHN-2 vectors were mixed in equal volumes and injected into the backside of the tobacco leaves. The *Agrobacterium* suspension containing p0800-LUC-prDHN-2 + pGreen62-SK was used as the control group for the experiments, and stronger fluorescence signals were observed in the area of the experimental group (Fig. 10A-E). Meanwhile, the RLU values of firefly luciferase and sea kidney luciferase in the experimental group were also significantly higher than those in the control group (Fig. 10F). The above results demonstrated that the StERF79 activated the expression of the downstream gene *StDHN-2* by affecting DRE elements.

For GUS staining experiments, the expression of the blue spots of the experimental group on the leaves was observed to express a stronger blue color than the control groups (Fig. 11A). In addition, we extracted total proteins from tobacco leaves of the experimental and control groups, respectively, for the determination of the expression level of the *GUS* reporter gene, which was quantified according to the amount of fluorescent material produced by the total plant proteins per unit time, the results showed that the activity of GUS in the experimental group was higher than that in the control groups, and showed highly significant differences compared to positive control groups (Fig. 11B). In summary, it is exhibited that the transcription factor StERF79 up-regulated the *StDHN-2* gene by an interaction of DRE elements.

Discussion

In the AP2/ERF transcription factor family, although the ERF subfamily has often been reported to play a regulatory role in ethylene and jasmonic acid signaling pathways by activating the expression of specific genes in plant defense against pathogens, and enhancing plant disease resistance [49], ERF subfamily members also play an important role in abiotic stresses such as drought and low temperature [50]. The *OsERF71* gene positively regulates ABA signal transduction in rice, and overexpression of the *OsERF71* gene can promote the up-regulation of *ABI5*, *PP2C68*, *RAB16C*, and *RAB16D* genes in roots. In addition, *OsERF71* also directly regulates the transcription of the lignin biosynthesis genes, such as *CCR1*, *CCR10*, and *C4H*, and induces radial root growth, changes root architecture, and improves drought tolerance [51–52]. Conversely, AP2/ERF can also act as a negative regulator of ABA signaling. It has been shown that ERF18/ORA47 can initiate the transcriptional expression of the PP2C-like phosphatase gene *ABI2*, and at the same time, ABI1 regulates the upstream promoter region of *ORA47* gene, which in turn promotes the transcription of the *ORA47* gene, and this regulatory relationship builds up a regulatory loop between ABI1, ORA47 and ABI2, which effectively inhibits the ABA biosynthesis and reduces the drought tolerance of plants [53]. Ethylene, as a major phytohormone, also plays an important role in plant adversity response. For example, a study identified a cold-responsive transcription factor, VaERF092, from amur grape (*Vitis amurensis* L.), and heterologous expression of *VaERF092* in *Arabidopsis thaliana* enhanced cold tolerance, suggesting that VaERF092, as a key transcription factor in the signaling pathway downstream of ethylene, enhances the tolerance of grapes to low-temperature stress [54]. Likewise, in this study, we found that overexpression of the potato *StERF79* gene enhanced drought tolerance in plants. It has been shown that AP2/ERF transcription factors bind directly to DRE/CRT (dehydration responsive element/C-repeat) *cis*-acting elements on the promoters of downstream target genes with the core conserved sequence A/GCCGAC, and these downstream target genes are mostly responsive genes related to drought, cold and salt stress [55]. In *Arabidopsis thaliana*, the CBF1 transcription factor can bind

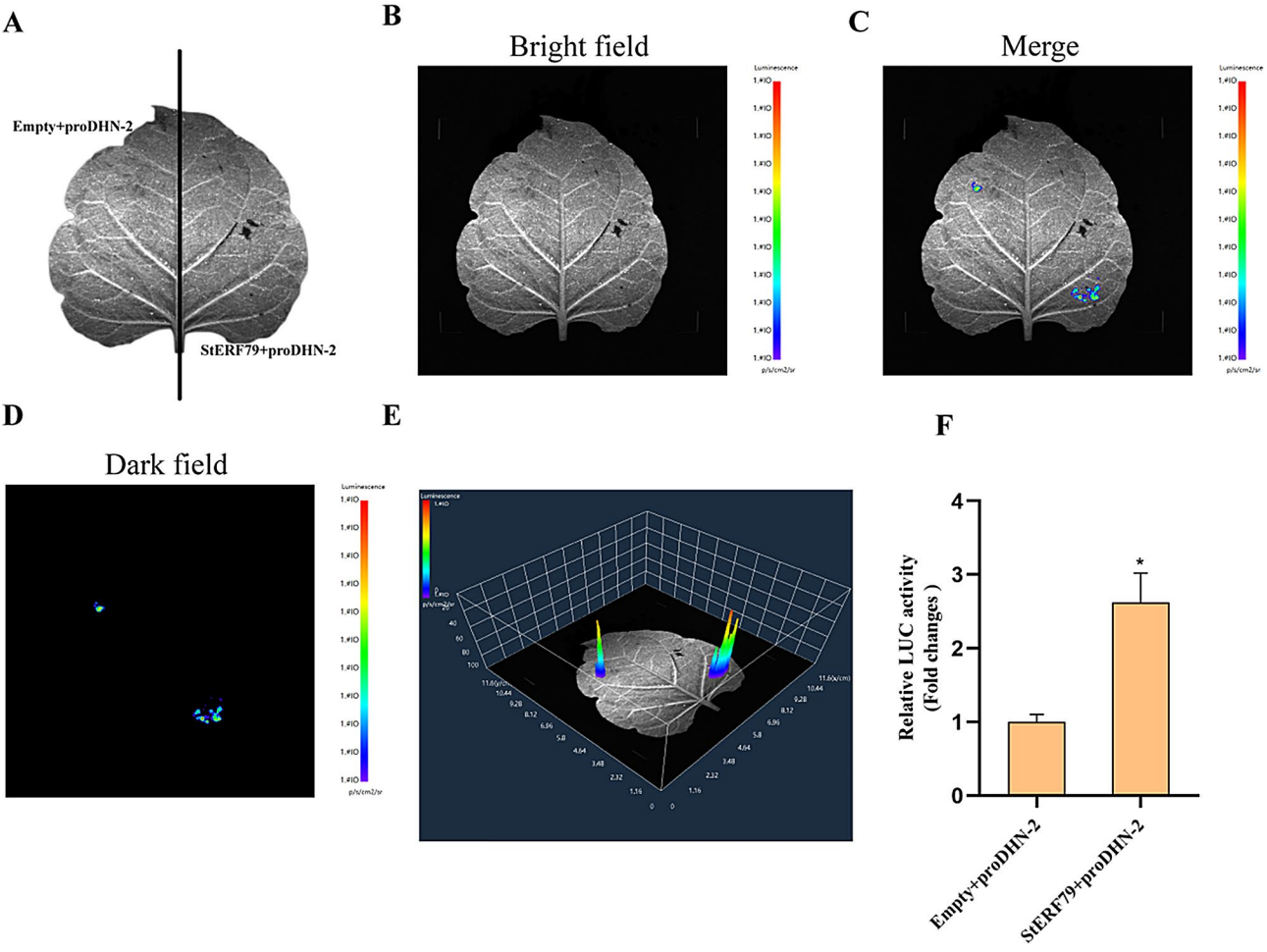

Fig. 10 Dual luciferase assay for StERF79 and DRE elements. (**A**): injection area. (**B**): fluorescent observation under bright field. (**C**): combination field. (**D**): fluorescent observation under dark field. (**E**): fluorescence intensity. (**F**): Relative LUC activity. Error bars indicate standard error of the means (SD) based on three technical replicates. '*' above bar indicate the significant difference of the changes between experimental and control group with the level of $0.01 < p < 0.05$ (shown as '*')

to the DRE/CRT motif in the COR15a promoter, resulting in freezing tolerance [56]; similarly, the ERF protein Tsi1 in tobacco binds specifically to GCC and DRE/CRT sequences, improving the salt tolerance of the plant [50]. Our Prior studies reported that the yeast two-hybrid technique was used to screen the interacting proteins of StERF79, and it was found that it would interact with 18 proteins, including Calmodulin (CaM), Ethylene-insensitive3 (EIN3) protein, and Late embryogenesis abundant (LEA), etc., the interaction between StERF79 and LEA protein in the nucleus was further verified by bimolecular fluorescence complementation technology (BiFC) [38]. According to our study, the *cis*-acting element analysis on the sequence of about 2000 bp upstream of the *StDHN-2* gene, and found that there were two DRE elements, while the sequence of GCCGA, was presented within 500 bp upstream (Fig. S1), and the StERF79 transcription factor can bind to the DRE elements in the promoter of the downstream gene *StDHN-2* and activiated its expression.

In a previous investigation, it was elaborated that the LEA proteins are involved in different plant development processes, stress responses and water deficiency responses. Among them, the DHN protein subfamily is a highly hydrophilic protein. Under drought stress conditions, plants produce large amounts of reactive oxygen species (ROS), and their accumulation leads to cellular lipid peroxidation and the production of large amounts of MDA. Our results are consistent with the above study, which showed that the transgenic potato lines overexpressing the *StERF79* gene under drought stress were also activated and enriched in the expression of the *StDHN-2* gene, and the content of MDA was significantly reduced in the OE-n lines, compared to the wild-type lines. Another report unveiled the citrus dehydrin gene *CuCOR19* on the anti-lipid peroxidation effect of cold-stressed transgenic tobacco [57]. Similarly, plants synthesize some key antioxidant enzymes such as SOD, POD, and CAT to scavenge reactive oxygen species that protect

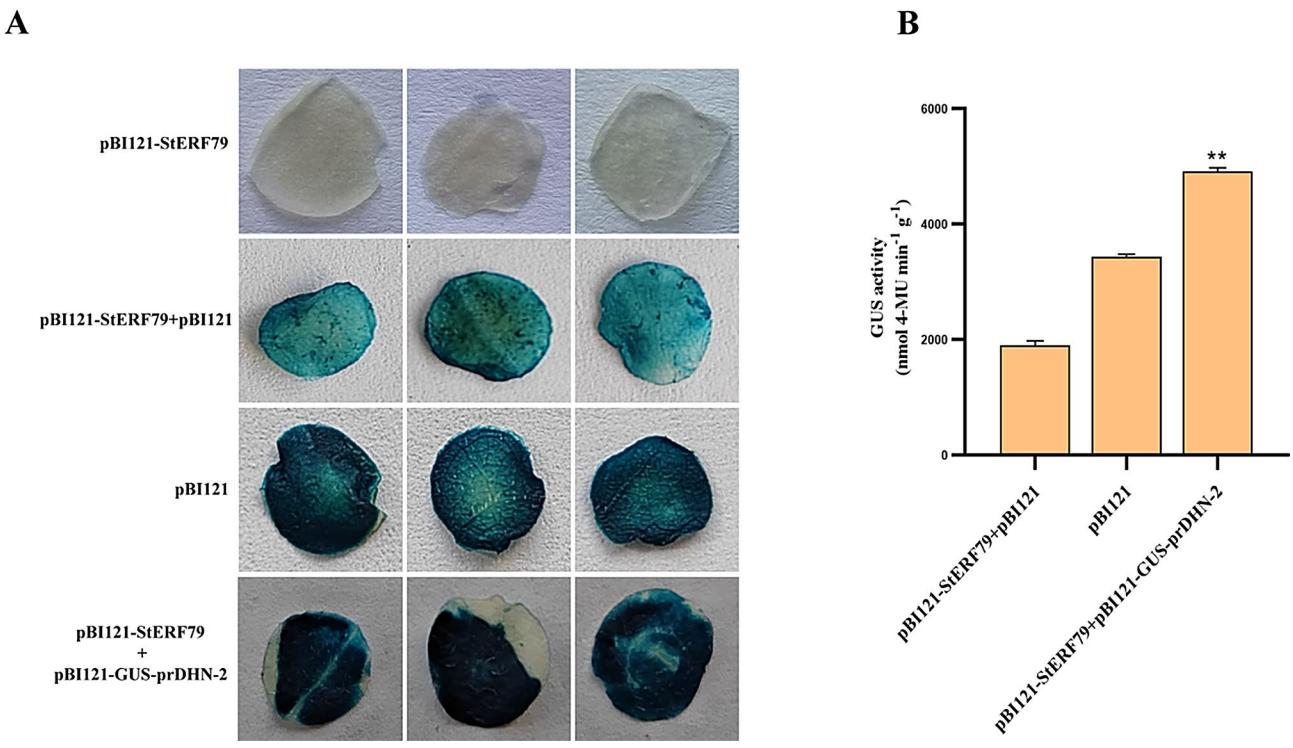

Fig. 11 GUS staining assay for StERF79 and DRE elements. (**A**): Expression of blue positive spots in tobacco leaves. (**B**): Determination of GUS activity. '**' above bar indicate the highly significant difference of the changes between experimental and control group with the level of $p > 0.05$ (shown as nothing), $p < 0.01$ (shown as '**'). pBI121-StERF79: negative control. pBI121-StERF79 + pBI121, pBI121: positive control. pBI121-StERF79 + pBI121-GUS-prDHN-2: experimental group

cells from oxidative damage, and in our results, the overexpression transgenic lines had the highest antioxidant enzyme activity, our results consistent with the fact that the *CaDHN5* gene scavenges free radicals increased antioxidant enzyme activity and thus positively regulated salt stress and osmotic stress in *Capsicum annuum* [58]. In addition, plants accumulate large amounts of proline when subjected to drought stress, and as an osmoregulatory substance, it can help to maintain intracellular water balance and slow down water loss, thereby protecting the cellular structure and function of plants. In our results, water loss rate and relative water content (RWC) were measured on isolated leaves of potato plants, and it was found that the overexpression transgenic lines had the strongest water retention capacity under drought stress. Proline content was correspondingly the highest in the overexpression transgenic lines, which is consistent with the previous study on the *CmLEA-S* gene that makes transgenic tobacco drought-resistant and salt-tolerant, for example, by enhancing the accumulation of osmoprotectants [59]. In summary, drought stress may activate an increase in abscisic acid content, which in turn stimulates the signals to the StERF79 transcription factor and activates the expression of the downstream gene *StDHN-2*, ultimately induce changes in plant morphophysiological indicators, such as scavenging of reactive oxygen species and accumulation of the osmotic substance proline, which improves the plant's tolerance to drought (Fig. 12). The StERF79 protein may also interact with StEIN3 to regulate the ethylene signaling pathway in response to harsh environmental conditions based on the yeast two-hybrid results [38].

Potato (*Solanum tuberosum* L.), as a major economic staple crop, is rich in carbohydrates, dietary fiber, protein, vitamins, and many other nutrients [60]. Since potato is a typical shallow-rooted crop, its yield and quality are highly susceptible to drought stress [35]. With the continuous emergence of global climate extremes, the adverse effects of drought on potatoes are a crucial problem to be solved. Improving agronomic measures, identifying drought-resistant cultivars, and improving the physiological and biochemical traits, can be used to improve potato production, yield, and quality. There are two ways to select drought-resistant varieties of potato, namely, selection by traditional breeding methods and the study of drought-resistant genes and their direct introduction into plants to improve plant drought resistance. The results of this study provide good genetic resources for potato molecular drought-resistant breeding. However, the positive regulation of drought tolerance in potato by the StERF79 transcription factor is still in the basic research stage, which is limited to in vitro

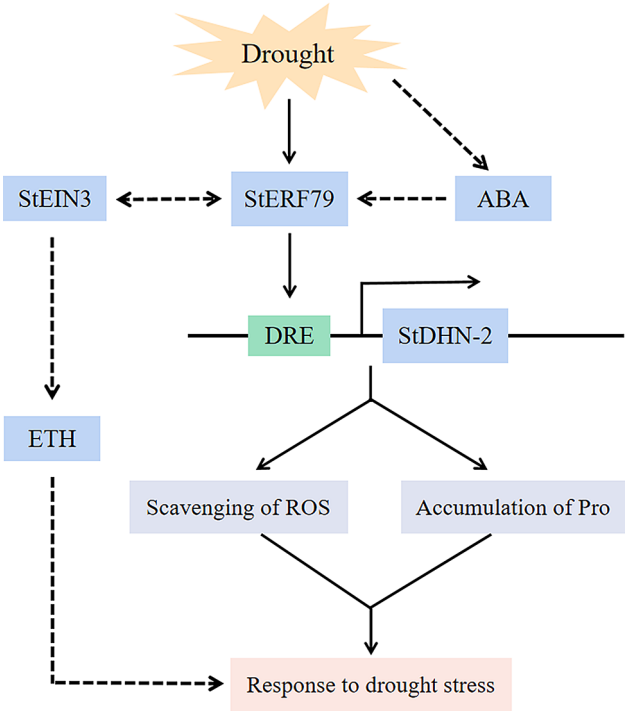

Fig. 12 Prediction pathway model of *StERF79* gene response to drought stress in potato. Drought causes an increase in abscisic acid content in potato, and activates the expression of the StERF79 transcription factor, which effects to the DRE element in the promoter region of the downstream response gene, *StDHN-2*, and activates the expression of *StDHN-2* in response to drought stress. The StERF79 protein may interact with StEIN3, which regulates the ethylene synthesis pathway in response to drought stress

and potted seedlings, and has not been applied to the actual production of potato in practice, compared with the growth conditions in the laboratory, the growth of potato plants in natural environments will face a greater challenge, and the factors affecting drought tolerance of potato will be more complex and diversified. In addition, due to the lack of a complete and systematic evaluation system for drought resistance in potato, the use of only a few simple physiological indicators as indicators of drought resistance lacks a certain degree of accuracy, therefore, the drought resistance mechanism of StERF79 transcription factor and its application in practical production can be further refined in future research.

Conclusions

In this study, the *StERF79* gene was cloned, and it was found to be expressed in potato roots, stems, leaves, and tubers using qRT-PCR analysis, and its relative mRNA expression was lowest in the stems, compared to leaves. Then, the transgenic (OE-1, OE-2, OE-3, RNAi-1, RNAi-2, RNAi-3) lines were constructed. Moreover, the StERF79–overexpressed potato plants showed an uptrend for various physiological indicators, such as SOD, POD, CAT, and proline, while MDA content was observed to be decreased under drought stress conditions, compared to wild-type potato plants. Finally, using yeast one hybrid, double luciferase, and GUS staining experiments, our study validated that the StERF79 transcription factor interacted with the DRE element in the promoter region of the *StDHN-2* gene and activated its expression, which improved drought tolerance in potato. In conclusion, the result provides a novel overview of the StERF79 transcription factor and its specific function in drought tolerance of potato plants.

Abbreviations

ABA	Abscisic acid
CAT	Catalase
Cef	Cefsulodine
DHN	Dehydrin
DRE	Dehydration response element
EGFP	Enhanced green fluorescent protein
ERF	Ethylene-responsive element binding factors
Hyg	Hygromycin
LEA protein	Late embryogenesis abundant protein
POD	Peroxidase
Pro	Proline
SOD	Superoxide dismutase
qRT–PCR	Quantitative real-time PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06417-w.

Supplementary Material 1: Table S1 Primer information used in this study. Figure S1 Schematic diagram of the location of DRE elements in the promoter region of *StDHN-2* gene. Figure S2 Melting curve of qRT-PCR analysis. Figure S3 Schematic diagram of pCAMBIA1300-35 S-StERF79 and pCAMBIA1300-35 S-amiRNA-StERF79 vectors construction. Fig S4 Schematic diagram of pHIS2-prDHN-2 and pGADT7-StERF79 vectors construction. Figure S5 Schematic diagram of p0800-LUC-prDHN-2 and p62-SK-StERF79 vectors construction. Figure S6 Schematic diagram of pBI121-GUS-prDHN-2 and pBI121-StERF79 vectors construction. Figure S7 Cloning of the *StERF79* gene. M: 2000 marker. 1, 2, 3, 4: electrophoretic bands of the target gene.

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Author contributions

Jingjing Wei: Conceptualization, Methodology, Software, Data curation, Validation, Writing – original draft. Ning Zhang: Conceptualization, Supervision, Writing – review & editing. Yurong Deng: Methodology, Software. Shengyan Liu: Methodology, Software. Liang Yang: Methodology, Software. Xiaofeng Wang: Methodology, Software. Ruiting Wen: Writing – review & editing. Huaijun Si: Writing – review & editing. All authors reviewed the manuscript.

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Data availability
All data supporting the findings of this study are available within the paper and its Supplementary Information. We do not have any research data outside the submitted manuscript file.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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