The Transcription Factor HcERF4 Confers Salt and Drought Tolerance in Kenaf (*Hibiscus Cannabinus* L.)

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Research Article

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Abstract

Ethylene response factors (ERF) are members of the APETALA2/ERF transcription factor family, and they play an important role in plant growth, development, and response to various environmental stresses. In the present study, an ERF transcription factor *HcERF4* was isolated and characterized from kenaf. The protein encoded by the *HcERF4* has 233 amino acid residues with a theoretical isoelectric point of 8.89 and a predicted molecular weight of 25.53 kDa. *HcERF4* had an over than 86.97% identity to *HsERF4*(XP_039019980.1), and shared a closest phylogenetic relationship with *Hibiscus syriacus*. Subcellular localization analysis shows that *HcERF4* is located in the nucleus. Transactivation assays in yeast demonstrated that *HcERF4* functions as a transcriptional activator. The expression of *HcERF4* was enriched in leaf and root, and can be induced by salt or drought treatments in kenaf. The VIGS-silenced *HcERF4* plant showed significantly reduced plant height, stem diameter, fresh weight, and relative water content (RWC) compared with wild type plants under salt or drought stress condition; In addition, the contents of MDA, O_2^-, H_2O_2, and free proline is significantly increased, and the activities of SOD and CAT are significantly reduced. The DAB/NBT staining results showed that the H_2O_2 and O_2^- contents in *HcERF4*-silenced plants were consistent with the determination. Based on these results, it is proposed that *HcERF4* plays an important role in regulating salt and drought stress in kenaf.

Key Message

HcERF4 transcription factor modulates drought and salinity stress in kenaf (*Hibiscus cannabinus* L.).

Introduction

Soil salinization and drought can affect seed germination, crop growth and yield, which severely limits sustainable agricultural production(Zhang et al. 2021a; Zhang et al. 2021b). According to reports, soil salinity directly affects 20% of the world's agricultural land, and the global loss caused by drought is about 30%. In fact, it is predicted that by 2050, drought and salinization will cause severe salinization of more than 50% of arable land(Chen et al. 2021c; Kirungu et al. 2019). The high salt environment will destroy the ion balance in plant cells, promote the accumulation of excess sodium and chloride ions, cause severe osmotic stress, and affect the water absorption capacity of plant cells(Ketehouli et al. 2019). The negative effects of salt stress may cause abnormalities in all physiological and metabolic processes of plants, there by seriously threatening plant growth and ultimately leading to plant death. Recently, lots of research have reported salt and drought stress, and some plants with strong resistance to salt and drought have been screened out. For example, *Jerusalem artichoke*(Zou et al. 2020); *Sophora alopecuroides*(Yan et al. 2020); poplar(Li et al. 2021a; Sow et al. 2021; Wei et al. 2020); *Apocyni Veneti Folium*(Chen et al. 2019) and Tartary buckwheat(Huang et al. 2021; Li et al. 2020) are reported to be plants with strong stress resistance. At the same time, kenaf(Kashif et al. 2020a) is also.

Kenaf (*Hibiscus cannabinus* L.) is an annual dicotyledonous plant in the *Malvaceae* family. It is one of the most important natural fiber producing species after cotton and jute(Giwa Ibrahim et al. 2019). It is widely distributed in Asia and Africa and mainly grows in temperate to tropical regions(Duan et al. 2020). Kenaf has a high yield of biomass and is widely used, including papermaking, ropes, textiles, animal feed, compounding agents, construction and filtration materials, and can also be used for board making and as a source of biomass energy (Li et al. 2021b; Tang et al. 2019). Based on the characteristics of strong resistance, such as saline(Wei et al. 2019), drought(An et al. 2020b) and heavy metal(Chen et al. 2021b), it is also considered as a good candidate for bioremediation. To uncover the molecular mechanism involved in salt tolerance in kenaf, our previous proteomic study has revealed several key regulatory pathways and identified a lot of proteins associated with salt-stress tolerance (Kashif et al. 2020b). In addition, a lot of transcription factors have been characterized to be-associated with drought stress response by transcriptome analysis(An et al. 2020b). However, the salt and drought resistance mechanism of kenaf still needs further study.

Transcription factors, also known as trans-acting factors, are a type of DNA binding protein that specifically binds cis-acting elements(Do et al. 2020). The interaction between transcription factors and cis-acting elements or other proteins can be transcription activation or inhibition. To date, numerous transcription factors belonging to different transcription factor families, such as MYB, NAC, AP2/ERF, bZIP and WRKY, have been identified and implicated in the regulation of stress responses(Singh et al. 2002). Ethylene transcription factors (ERFs) are a large family of transcription factors unique to plants with conserved AP2/ERF domains(Faraji et al. 2020). They play a transcriptional regulatory role by combining ethylene response sequence motifs such as GCC-box or DRE/CRT motifs. Many previous studies have found that ERF TFs play a role in regulating plant growth and development, plant organ development, cell division, differentiation, flower development and fruit maturation(Chen et al. 2021a; Feng et al. 2020a; Zhang et al. 2020). In addition, ERFs have also been reported in abiotic stress regulation, including drought, salt, extreme temperature, and so on(Gao et al. 2020; Han et al. 2020; Kavas et al. 2020a). The AP2/ERF family has been identified in *Arabidopsis*(Xing et al. 2019), rice(Chen et al. 2021a; Jisha et al. 2015; Neogy et al. 2019), wheat (Rong et al. 2014; Zhang et al. 2020) and other plant species. However, AP2/ERFs are a large family of transcriptional factor, and the role may different in different plant species. Among members of ERF proteins identified to date, *Brassica rapa BrERF4* improved tolerance to salt and drought stresses in *Arabidopsis*(Seo et al. 2010); ERF4 and MYB52 transcription factors play antagonistic roles in regulating homogalacturonan de-methyl esterification in *Arabidopsis* seed coat mucilage(Ding et al. 2021); ERF4 affects fruit firmness through TPL4 by reducing ethylene production(Hu et al. 2020); *Arabidopsis* ERF4 is a transcriptional repressor capable of modulating ethylene and abscisic acid responses(Yang et al. 2005); overexpression of *GmERF4* enhances salt and drought tolerance in transgenic tobacco plants(Zhang et al. 2010). In our previous transcriptome analysis, we found that the expression level of *HcERF4* significantly decreased under cadmium stress(Chen et al. 2020), but its role in response to salt and drought stress is remains unclear.

Virus-induced gene silencing VIGS is a loss-of-function technology for the study of plant gene function (Kant and Dasgupta 2017), which aims to interfere with the transcription of candidate genes and to study

the biological functions of these genes in organisms. It is established on the basis of RNA-mediated post-transcriptional gene silencing (Gunupuru et al. 2019) (PTGS), which acts as an antiviral defense system in plants. A partial fragment (200-400bp) of the candidate gene is inserted into the modified viral vector to produce a recombinant vector. Inoculation of infected plants with *Agrobacterium* carrying recombinant virus binary vector can produce virus-associated small interfering RNA (siRNA) with a length of 21-24 nucleotides(Schachtsiek et al. 2019). siRNA can mediate the degradation of targeted endogenous gene transcripts, leading to the silencing of candidate gene expression. Therefore, gene functions can be effectively identified by inducing changes in plant phenotype or physiological indicators (Xiao et al. 2020). The potential of VIGS has been confirmed in some plant families, such as *Solanaceae*(Tomar et al. 2021a; Tomar et al. 2021b), *Cruciferae*(Reyes et al. 2017) and *Gramineae*(Tavakol 2018). VIGS has become a versatile genomics tool for knocking out the transcription level of plant genes, especially genes related to plant disease resistance, stress resistance(Wang et al. 2021), growth and development(Feng et al. 2020b), and metabolic regulation. Other studies have also shown the advantages of VIGS, such as easy operation, wide application range, high efficiency and independence of genetic transformation.

Materials And Methods

Plant materials and growth conditions

Kenaf cultivar of SF192 were used in this study. About 300 healthy and plump seeds were initially soaked in distilled water for 1 hour, then surface sterilized with 3%H_2O_2 for 10 minutes, and finally wash them with distilled water three times. Put the sterilized seeds neatly into a plastic nursery pot (27 cm×18 cm×9 cm) lined with double-layer paper towels, and place the kenaf seeds in a light incubator to grow, and incubate at 27°C for 14 hours (280μmol). m^{-2} s^{-1}), incubate in the dark at 25°C for 10 hours, with a relative humidity of 60-65%. After about 4 days, the seedlings that grew evenly were selected and transferred to a seedling tray (35 holes) containing 0.5×Hoagland solution for hydroponic cultivation. Seedlings are planted at intervals of one hole, with 21 seedlings per trays, and the seedlings are fixed with sponge strips. Keep the kenaf seedlings in a greenhouse at 28°C, 16h light /8h dark, and 60% relative humidity.

Cloning and bioinformatics analysis of HcERF4

According to transcriptome data(Chen et al. 2021b) of kenaf, the *HcERF4* gene sequence was obtained to design the primer *HcERF4*-F/R (Table 1), and the RNA of healthy growing kenaf leaves was extracted by the TRIzon method. cDNA was synthesized using HiScript®Ⅱ 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd), and used as template to amplify the full length of the gene. The reaction program is 95℃ 5min, 95℃ 30s, 60℃ 30s, 72℃ 30s, 35 cycles, 72℃ 5min, 12℃ storage. The amplified target fragments were recovered from gel, and ligated with 1ul pEASY-Blunt(TransGen Biotech Co., Ltd) to transform into *E. coli* DH5a (TransGen Biotech Co., Ltd), screened with LB (Luria-Bertani□medium plates containing kanamycin, and sent positive clones to Tsingke Biotechnology Co., Ltd. for sequencing, and verifying its sequence, the vector was named Blunt-*HcERF4*.

The DNAMAN8.0 and Jalview software was used to predict the amino acid sequence of the gene. Conserved domain analysis and homology comparison analysis uses the Blast function of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the ProtParam tool in ExPASy (https://web.expasy.org/protparam/) to predict the molecular weight and isoelectric point of the encoded protein amino acid sequence Basic physical and chemical properties. The three-dimensional structures and transmembrane regions or orientation prediction of *HcERF4* were predicted by the Phyre2 server(http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). The phylogenetic tree was constructed using MEGA7.0 software. Use the online software Plant-mPLoc to predict subcellular location of *HcERF4* (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/).

Subcellular localization analysis of HcERF4

The Open reading frame (ORF) of *HcERF4* cDNA sequence was amplified by specific primers designed by Primer 5 (GFP-*HcERF4*-F/R (EGFP vector construct) in Table 1) without a stop codon through the RT-PCR method. Then, the *HcERF4* was inserted into pBI121-EGFP with *BamH* Ⅰ enzyme (Takara Bio Co., Ltd). The recombinant and empty vector were introduced into *Agrobacterium* GV3101 separately. The *Agrobacterium* is cultured in LB liquid medium, the bacteria are collected, and resuspended in a 10mM $MgCl_2$ (containing 120μM AS) suspension to adjust the OD600 to about 0.6. 1mL of *Agrobacterium* solution was injected the epidermis of tobacco leaves (approximately one-month-old) suing a syringe. The transformed seedlings were marked and cultured in the dark for 2 days, and then sampled the *Agrobacterium*-injected leaves for subcellular localization analysis using a laser scanning confocal microscope (Nikon C2-ER, Japan).

Transactivation assay of HcERF4 in yeast

The coding sequence of *HcERF4* was sub-cloned into the GAL4-binding domain of the pGBKT7 vector to produce the plasmid of pGBKT7-*HcERF4*. Afterwards, the pGBKT7-*HcERF4* recombinant was introduced into the yeast strain Y2H and plated on SD/-Trp medium. Empty pGBKT7 vector was used as a negative control. After PCR detection, 3ul of the positive transformants were spotted on SD/-Trp/-His/-Leu and SD/-Trp/-His/-Leu/ X-α-gal plates to observe yeast growth at 30℃ for 3 days.

Expression analysis of HcERF4 in kenaf plant

Refer to 2.1 for the disinfection method of kenaf seeds. After the seeds germinated, the 4-day-old seedlings were transplanted into the seedling tray containing 1/4 Hoagland nutrient solution for hydroponic culture, with 35 plants in each tray. Two weeks later, the seedlings were exposed to drought and salt stress. Drought and salt stress were applied by adding PEG 6000 (polyethylene glycol, 10% w/v, 20% w/v) and NaCl (100 mM, 200 mM) to the nutrient solution, respectively. All treatments involved three biological replicates. Leaf samples were harvested after stress treatment for 0, 1, 3, 6, 12, 24 and 48 h, and unstressed seedlings were used as control. Afterward, the collected plant materials were directly frozen in liquid nitrogen and stored at - 80 ℃ for total RNA extraction and qRT-PCR analysis.

qRT-PCR was used to detect the expression of *HcERF4* under stress conditions. See Table S1 for primers for *HcERF4* and *Actin* expression analysis. Total RNA was extracted from seedlings leaves under different stress conditions (control, 100 mM NaCl, 200 mM NaCl, 10% PEG and 20% PEG) suing TRIzol reagent according to the manufacturer's instructions (Vazyme Biotech Co., Ltd). The RNAs were then reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd) kit according to the suggested protocol. For qRT-PCR analysis, a total of 20μl reaction system containing 1μl cDNA template, each of 0.4μl 10uM forward primer and reverse primer, 10μl ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech Co., Ltd), and 8.2μl ddH_2O, was used with a Bio-Rad CFX96 (Bio-Rad Laboratories). The PCR conditions are set as follows: 95°C 30sec; 95°C 10sec, 60°C 30sec, 40 cycles; 95°C 15sec, 60°C 60sec, 95°C 15sec. The $2^{-\triangle\triangle Ct}$ method was used to determine the expression abundance of *HcERF4*, and *Actin* was used as an internal reference gene to calculate the relative change of *HcERF4* expression in the qRT-PCR experiment. Each group performed 3 biological replicates, and each reaction performed 3 technical replicates.

VIGS-induced gene silencing vector construction and transformation

The online software SGN VIGS Tool (https://vigs.solgenomics.net/) was used to predict the gene silencing region, and primer premier5.0 was used to design gene-specific primers name of TRV-*HcERF4*-F/R (Table 1), *HcERF4* fragment (227bp) was amplified from kenaf leaf cDNA. The resulting product was cloned into pTRV2 by seamless cloning *BamH* I digestion, and the recombinant vector pTRV2-*HcERF4* was produced. The pTRV1(Helper plasmid), pTRV2(Empty plasmid) and pTRV2-*HcERF4* Recombinant vectors were introduced into *Agrobacterium* strain GV3101, and cultured in YEB medium containing kanamycin, rifampicin and streptomycin at 180 rpm for 12 h until the OD600 reached 1.5. After shaking for 12 hours, centrifuge at 8000 rpm for 10 minutes, and re-suspend in a resuspension solution (pH 5.6) containing 10 mM 3-(N-morpholino) ethane sulfonic acid, 10 mM magnesium chloride, and 200 mM AS (acetosyringone). The OD600 of the resuspension is about 0.8-1.0. The resuspension of pTRV1 was mixed with pTRV2 and pTRV2:*HcERF4* at a ratio of 1:1, and allowed to stand at room temperature for 3 hours in the dark or incubated at 28°C at 220 rpm for 1 hour before injection. Before the appearance of the first true leaf, approximately 1 ml of *Agrobacterium*-containing osmotic medium was infiltrated into the kenaf cotyledons. Seedlings infiltrated with pTRV1 and pTRV2 were used as negative controls. Each treatment has three seeding trays, and each seedling tray was infiltrated with 21 seedlings. After two weeks, new leaves grown after injection were randomly selected and detected with *HcERF4* specific primers (Table 1) to determine the silenced seedlings.

Salt and drought treatments for VIGS silenced-plants

After VIGS silencing for 15 days, the wild type plants, pTRV2 plants and pTRV2-*HcERF4* silenced plants were treated with 1/4 Hoagland solution containing 150mM NaCl or 15%PEG, respectively, and the treatment solution was changed every two days. After 7day of stress treatment, the phenotype of each treated plant was photographed and recorded. The plant height, stem diameter, fresh weight and relative leaf water content (RWC) were measured respectively.

Physiological indexes detection

The leaves were collected after 7 days under salt and drought stress. Use the hydroxylamine oxidation method(Li et al. 2021b) to measure the MDA content, O_2^- and H_2O_2 in VIGS silent and wild-type plants. In addition, the activities of SOD, POD, and CAT were measured according to the protocol described by Chen et al (Chen et al. 2020). The proline content was measured according to the spectrophotometric method(Jimenez-Bremont et al. 2006). All these experiments are three biological repetitions.

Histochemical detection of O_2^- and H_2O_2

Superoxide radical (O_2^-) detection was performed using the nitro blue tetrazolium (NBT)(Liu and Friesen 2012) in situ staining method, and histochemical detection of H_2O_2 was carried out by means of the 3,3'-diaminobenzidine (DAB) staining method(Daudi and O'Brien 2012). Seedlings after VIGS silencing were treated with 150mM NaCl or 15%PEG for one week. Leaves were soaked in freshly prepared NBT (2mg/ml NBT in 50mM sodium phosphate buffer, pH 7.5) or DAB (1mg/ml DAB, pH 3.8) solution, and store overnight at room temperature. Drain the staining solution from the test tube, soak the seedlings in absolute ethanol, and heat them in a boiling water bath for 10 minutes to decolorize, and photograph the stained seedlings against a contrasting background for proper recording.

Statistical analysis

All of the above experiments were repeated at least three times independently, and the data shown are the mean ± SD. In this research, statistical analyses were performed using the statistical tools (Student's t-test) of Excel 2010 software (Microsoft Corp., Albuquerque, NM, USA). The significance level was defined as * ($P < 0.05$), ** ($P < 0.01$) and ***($p<0.001$).

Results

Cloning and bioinformatics analysis of HcERF4

The ERF transcriptional factor, *HcERF4*, was cloned from kenaf according to the transcriptome data (Chen et al. 2020). The open reading framer (ORF) is 702bp in length, encoding 233 amino acids, containing a typical AP2 motif from 25 to 57. (Fig. 1A). The molecular weight of the protein is 25.53 KDa and the theoretical pI value is 8.89. The average hydrophilic coefficient (GRAVY) of *HcERF4* is -0.640, indicating it is a hydrophilic protein. The instability coefficient of this protein is 65.71, which means it is an unstable protein. To understand the phylogenetic relationships between *HcERF4* and its homologous proteins, multiple sequence alignment was carried out using the Jalview program, the result showed that they shared high sequence similarity, especially the AP2/ERF domain (Fig. 1B). The three-dimensional structure predicated by Phyre2 showed that *HcERF4* has one trans-membrane *α*-helices (Fig. 1C). Phylogenetic analysis based on amino acid sequence of *HcERF4* and homologs from other species revealed that the *HcERF4* gene has the closest homology relationship with two ERF4 proteins in *Hibiscus syriacus* and *Gossypium hirsutum* which belong to the same family of *Malvaceae* (Fig. 1D).

HcERF4 is a nuclear-localized protein

Firstly, the online software WoLF PSORT was used to predict subcellular localization, and the results showed that *HcERF4* is localized in the nucleus. In order to further determine whether *HcERF4*, as an ERF-type transcription factor, is localized in the nucleus, we conducted transient expression experiments in tobacco leaf cells using an *HcERF4* (green fluorescent protein) fusion vector. Compared with the epidermal cells transformed with an empty GFP vector alone, the GFP fluorescence signal was only observed in the nucleus with the *HcERF4*-GFP fusion construct (Fig. 2). These results determined that *HcERF4* is a nucleus localized protein.

HcERF4 acts as a transcriptional activator in kenaf

The yeast test was used to study the transcriptional activity of the *HcERF4* transcription factor (Fig. 3). The yeast transformants containing either pGBKT7 (negative control) or pGBKT7-*HcERF4* grew normally on SD/Trp (single dropout supplements) medium. However, only the yeast transformed with pGBKT7-*HcERF4* grew normally on SD/-Trp-His-Leu (triple dropout supplements) medium. When added with X-α-gal on the medium, the yeast transformed with pGBKT7-*HcERF4* showed blue, the yeast transformed with pGBKT7 could not. These results indicate that the *HcERF4* transcription factor has transcriptional activation activity.

Expression profile analysis of HcERF4 response to stress

qRT-PCR was employed to analyze the *HcERF4* expression profile response to stress. Firstly, the *HcERF4* expression level in different tissues was investigated, and the result indicated that *HcERF4* was expressed extensively in all tested tissues including leaves, roots and stems under normal condition. *HcERF4* has the highest expression level in leaves, followed by in roots, and the lowest in stems (Fig. 4A).

To further reveal its detailed expression model to salt or drought stress, kenaf leaves samples from different stress condition and different time were selected for qRT-PCR analysis. Under salt stress conditions, the expression of *HcERF4* in kenaf leaves increased with the increase of salt concentration (Fig. 4B). With the duration of high salt concentration, there is a trend of first increasing and then decreasing. The expression level of *HcERF4* under 100mM NaCl treatment reached the highest at 3h, which was 4.77 folds that of the control, and under 200mM NaCl stress, the expression level reached the highest at 1h, which was 17.05 folds that of the control, and the expression level gradually recovered after 12h. Under drought stress, with the increase of PEG concentration, the gene expression level showed a trend of first increasing and then decreasing (Fig. 4C). With the increase of stress time, under 10% PEG stress, the expression level reached its peak at 1 hour, which was 19.55 folds that of the control, and then increased with time. It showed a gradual downward trend, and the expression level showed an upward trend in 24h. Under 20%PEG treatment, the expression level reached the highest at 1h, and was 6.43 folds that of the control, and then gradually decreased with the increase of time. Our results show that the expression pattern of *HcERF4* is different under different stress treatments. Under the same stress

treatment, the expression patterns of *HcERF4* at different concentrations are also different. These results indicate that *HcERF4* may be involved in salt and drought signal response pathways.

VIGS (Virus-induced gene silencing) analysis of HcERF4

To reveal the role of *HcERF4* under salt and drought stress in kenaf, VIGS experiment analysis were performed. The pTRV2-*HcERF4* recombinant vectors were constructed and the positive plasmid was transferred into *Agrobacterium* GV3101, and the primers of *HcERF4* fragment were used for PCR verification. A fragment of about 250 bp was obtained, indicating that the expression vector has been successfully transformed into *Agrobacterium* GV3101, and were injected into kenaf seedlings with the syringe infiltration method. After 14 days, infected leaves (sampled from the third true leaf) were selected for qRT-PCR analysis. As a represent example shown in Fig. 5, the result showed that the expression of *HcERF4* in two of the five VIGS seedlings (2# and 5#) was significantly lower than that of the control, indicating that the *HcERF4* was successfully silenced in these two kenaf seedlings (Fig. 5). The authentic silenced plant seedlings were used in subsequent phenotype and physiological analyses.

HcERF4 VIGS silenced kenaf reduced the tolerance to salt stress

In order to study the role of *HcERF4* in response to salt stress, the *HcERF4* silenced kenaf seedlings were subjected to stress treatments under 150mM NaCl stress for 7 days. As shown in Fig. 6, there was no significant difference in appearance of CK and pTRV2, while *HcERF4* silenced plants showed significantly lower plant height, smaller leaf area, and less developed root system compare with CK and pTRV2 (Fig. 6A). The agronomic characteristics of VIGS plants including plant height, stem diameter, fresh weight and relative water content (RWC) were measured under salt stress condition. As shown in Fig. 6, the plant height (Fig. 6B), stem diameter (Fig. 6C), fresh weight (Fig. 6D) and RWC (Fig. 6E) of *HcERF4* silenced plants were significantly lower than those of CK and pTRV2 plants. The results showed that *HcERF4* VIGS silenced inhibited the growth of kenaf seedlings and reduced its salt tolerance.

The physiological indexes were analyzed and the results showed that the contents of MDA, O_2^- and H_2O_2 in pTRV2-*HcERF4* plants were significantly increased by 64.3%, 43.9% and 21.6% under salt stress, while the contents of MDA, O_2^- and H_2O_2 in pTRV2 plants have no major changes (Fig. 7A, B, C). Silenced plants under salt stress The CAT and SOD were significantly reduced by 65.6% and 91.3% (Fig. 7D, F), and POD activity was significantly increased by 69.8% (Fig. 7E). Salt stress also significantly increased the proline content in silent plants (Fig. 7G). These results indicate that *HcERF4* silent plants are sensitive to salt stress.

Silencing of HcERF4 in kenaf reduced the tolerance to drought stress

In order to study the role on the drought tolerance, *HcERF4* VIGS silenced seedlings were treated with 15% PEG6000 for 7 days. By observing the phenotypes, it was found that there was no significant difference between CK and pTRV2 plants. However, *HcERF4* silenced plants were significantly lower than CK and

pTRV2 plants, and the leaves are curled and wilted shapes, the root system is under developed (Fig. 8A). This phenomenon indicates that the silence of VIGS reduces the drought tolerance of kenaf.

As shown in Fig. 8, after the drought stress treatment, the agronomic indicators of the treated plants were determined. Among them, CK and pTRV2 had no significant differences in plant height (Fig. 8B), stem diameter (Fig. 8C), fresh weight (Fig. 8D) and relative water content (Fig. 8E). However, after the VIGS silencing, the indicators decreased with exception of stem diameter, indicating that the *HcERF4* may positively regulates the drought resistance in kenaf.

To study the effect of *HcERF4* on drought tolerance, the content of MDA content and antioxidant activity were determined. The content of MDA can reflect the degree of damage to cell membranes caused by reactive oxygen species. Under drought stress, the MDA content of silenced plants was significantly reduced, but the MDA content of pTRV2 plants did not change significantly (Fig. 9A). After drought stress, the O_2^- and H_2O_2 content of silent plants increased by 1.28 and 1.31 folds, respectively, compared with the control (Fig. 9B, C), and there was no significant change in pTRV2 plants. Further study the oxidative stress under drought stress by analyzing the activity of antioxidant enzymes. The results showed that drought stress had a significant effect on the antioxidant enzyme activity of silenced plants. SOD is the first line of defense against ROS-mediated oxidative stress. After drought stress, the SOD activity of silent plants was significantly reduced, about 5.98 folds lower than that of the control, and the difference of pTRV2 under 15% PEG stress was not significant (Fig. 9D). Under drought stress, POD activity decreased significantly, about 1.48 folds lower than the control (Fig. 9E). CAT activity and POD activity showed similar trends. Compared with the control, the CAT activity of silent plants under drought stress was significantly reduced by 3.08 folds (Fig. 9F). However, the chlorophyll and proline contents of silent plants under drought stress were not significantly different from those of control plants (Fig. 9G). These results indicated that the decline of POD and SOD activity after *HcERF4* silencing led to the increase in ROS levels, thereby aggravating membrane damage and reducing the drought tolerance in kenaf.

Histochemical detection of O_2^- and H_2O_2

Additionally, in order to analyze the accumulation of ROS in the *HcERF4*-silenced and control plants after salt stress, NBT and DAB staining were used to detect O_2^- and H_2O_2 levels. As shown in Fig. 10, after 7 days of salt stress or drought stress, the NBT and DAB stained area of silent plants was significantly larger than that of wild-type plants. In addition, the $O_2^-$and H_2O_2 content in the silent plants was significantly higher than that of wild-type plants (Fig. 7B-C and Fig. 9B-C). These results indicated that *HcERF4* silenced plants aggravated its susceptibility to salt and drought stress.

Discussion

Adverse environmental conditions such as salinity, drought, and cold severely affect the growth and development of plants, posing a global threat to the continuous growth and yield of most crops. In order

to survive and maintain the integrity of the entire plant, plants have evolved certain adaptive mechanisms to deal with these adversities, have a certain ability to perceive stress signals, and quickly adjust their biological responses(Zhao et al. 2021). Plants regulate the expression of stress-related genes, and regulation may involve many biochemical and physiological processes. Transcription factors interact with cis-acting elements in the promoter regions of various stress-related genes and have been found to be important components for regulating gene expression. APETALA2/Ethylene Responsive Factor (AP2/ERF) TF family is one of the largest TF families in the plant kingdom. ERFs play important roles throughout the plant life cycle and contribute to the ability of plants to withstand various abiotic and biotic stresses. To date, a great deal of stress-related AP2/ERF transcription factors have been functionally characterized in different species. For example, the ectopic expression of *Phaseolus vulgaris PvERF35* promotes salt tolerance in tobacco(Kavas et al. 2020b); *MdERF38* promotes drought stress-induced anthocyanin biosynthesis in apple(An et al. 2020a); *OsERF101* regulates drought stress response in reproductive tissues(Jin et al. 2018); *GsERF71* positively regulates alkaline stress tolerance in *Arabidopsis*(Yu et al. 2017).

In a previous study, overexpression of a *BrERF4* gene from Brassica rape was reported. Overexpression of *BrERF4* increased the tolerance of Arabidopsis to salt and drought stress, and significantly affected the growth and development of transgenic plants (Seo et al. 2010). Here, we found that the kenaf *HcERF4* gene can be induced under salt and drought stress. To determine whether the *HcERF4* gene is related to salt and drought stress, we silenced *HcERF4* in kenaf plants. Under stress conditions, there was no phenotypic difference between control plants and empty plants. The morphology of *HcERF4* silent plants was significantly affected by salt and drought stress (Fig. 7A, Fig. 9A), indicating that silencing *HcERF4* may reduce the salt tolerance of kenaf plants. According to reports, most ERF genes play an important role in plant morphology, growth and development (Feng et al. 2020a). For example, *TaERF8* associated with plant height and yield in wheat. The analysis of related agronomic traits of the *TaERF8-2B* overexpression transgenic line showed that *TaERF8-2B* has a regulatory effect on plant configuration and yield-related traits (Zhang et al. 2020). Overexpression of a Brassica rape *BrERF4* gene increased the tolerance of *Arabidopsis* to salt and drought stress, and significantly affected the growth and development of transgenic plants (Seo et al. 2010).

Although these functions of ERF genes have been studied to a certain extent in other plants, so far, the role of ERF genes in kenaf, especially the functions under abiotic stress is completely unknown. In the present study, a kenaf ERF gene, *HcERF4*, was functionally characterized. Sequence alignment and phylogenetic analysis showed that *HcERF4* contained a typical AP2 conserved domain located in the middle region (amino acids 25-57). *HcERF4* had an over than 86.97% identity to *HsERF4* (XP_039019980.1), and with a closest phylogenetic relationship to *ERF4* of *Hibiscus syriacus* and *Gossypium hirsutum* which belong to the same family of *Malvaceae*. Subcellular localization results showed that the *HcERF4* induced fluorescence in nuclei only, indicating it is a nuclei localized protein. The ERFs can act as transcription activators such as *AtERF1*, *AtERF2* and *AtERF5* of *Arabidopsis*; however, some of the ERFs can also act as repressors, take *AtERF1*, *AtERF2* and *AtERF5* for an example, they play negative roles in gene expression by being repressors (Fujimoto et al. 2000). The present study showed

HcERF4 has transcriptional activation activity in yeast indicating it can serve as a nuclei-localized transcription activator in regulatory pathways.

Virus-induced gene silencing VIGS is a powerful technology for plant gene function analysis, especially for plants that without a mature regeneration system. VIGS technique possess the characteristics of convenient and fast, and most importantly, it can analyze the gene function in its original plant genetic background. In present research, the expression of *HcERF4* was highly induced by salt or drought, and showed a concentration and time dependent manners, indicating that this gene might be involved in abiotic stress responses. To verify that the role of *HcERF4* in kenaf in response to abiotic stress, the *HcERF4* VIGS knocked down kenaf seedlings were treated with high salt and PEG imitated drought stress. The morphology of *HcERF4* silenced plants was significantly affected by salt and drought stress (Fig. 7A, Fig. 9A), in addition, the plant height, biomass, and relative water content (RWC) were significantly lower than those of WT plants, indicating that silencing of *HcERF4* reduced the salt and drought tolerance of kenaf.

According to previous reports, most ERF genes play an important role in plant morphology, growth and development (Feng et al. 2020a). For example, *TaERF8* is associated with plant height and yield in wheat. The *TaERF8-2B* overexpression transgenic line showed that it has a regulatory effect on plant configuration and yield-related traits (Zhang et al. 2020). *PsAP2* VIGS silenced poppy showed significantly reduced tolerance to abiotic and biotic stress, while overexpression *PsAP2* in tobacco enhanced its abiotic and biotic stress tolerance(Mishra et al. 2015). Overexpression of *MbERF11* from *Malus baccata* enhanced the tolerance to cold and salt stress of transgenic *Arabidopsis* (Han et al. 2020).

The activity of proline, malondialdehyde (MDA) and antioxidant enzymes can be used to indicate the degree of damage caused by stress (Jin et al. 2018). The higher the MDA content, the higher the degree of membrane peroxidation of plant cells and the more severe the damage to the cell membrane (Sun et al. 2014). Salt stress increased the proline content of silent kenaf plants. Plant antioxidant enzyme system plays an important role in resisting external environmental stress. They can inhibit the accumulation of free radicals, thereby reducing the occurrence and lethal effects of oxidative damage. The silencing of *HcERF4* reduces the tolerance of kenaf plants to salt and drought stress, and leads to a decrease in the activities of SOD and CAT, and an increase in the content of MDA, O_2^- and H_2O_2. Knock down of *HcERF4* may reduce its salt tolerance and drought tolerance by changing these physiological indicators under adversity.

Conclusions

The present study characterized an ethylene transcription factors (ERFs), *HcERF4*, from kenaf. The expression profile of *HcERF4* has been characterized in different tissues, and could be induced by both salt and drought stress conditions. In addition, sequence alignment and phylogenetic analysis showed that *HcERF4* contained a typical AP2 conserved domain, located in nuclei with transcriptional activation

activity. Virus-induced gene silencing (VIGS) knocked down plants of *HcERF4* showed more sensitive to salt or drought stress, indicating that *HcERF4* plays an important role in the resistance of kenaf to abiotic stress. This study is of great significance for understanding the regulation mechanism of *HcERF4* in kenaf salt and drought stress, and also provides useful information for improving the salt and drought tolerance of kenaf.

Declarations

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Author contributions JY: Data curation; Methodology, Formal analysis, Roles/Writing - original draft. **MT:** Investigation, Formal analysis. **HZ; CC:** Investigation, Data curation, Formal analysis.**DL; SC; YH; ZH:** Software, Methodology, Formal analysis**. QW; XW; JP:** Formal analysis, Validation**. PC:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Project administration.

Data availability The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest The authors declare that they have no known competing financial interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Primers used in this study

Name of Primer	Sequence of Primer(5'-3')	Purpose
HcERF4-F	ATGGCACCGAGGGAGAAAAC	Cloning
HcERF4-R	GGCGATTTCCGGAAGAGG	Cloning
TRV-HcERF4-F	agaaggcctccatggggatccGCCAGAGCCTACGACGCC	VIGS
TRV-HcERF4-R	cgtgagctcggtaccggatccCCGGTAACCGCCTCACCT	VIGS
Q-HcERF4-F	AACCATAGCCCTAGCCAGAGTAGC	qPCR
Q-HcERF4-R	CCGCCTCACCTTGTACGAGATTG	qPCR
ACTIN-F	GTGAGGATATTCAACCCCTTGTCT	qPCR
ACTIN-R	CATCTTTCTGTCCCATACCAACC	qPCR
GFP-HcERF4-F	*GCTCTAGA* ATGGCACCGAGGGAGAAAAC	Subcellular localization
GFP-HcERF4-R	*CGGGATCC* GGCGATTTCCGGAAGAGG	Subcellular localization
pGBKT7-HcERF4-F	aggccgaattcccggggatccATGGCACCGAGGGAGAAAA	Transactivation assay
pGBKT7-HcERF4-R	ccgctgcaggtcgacggatccGGCGATTTCCGGAAGAGG	Transactivation assay

Figures

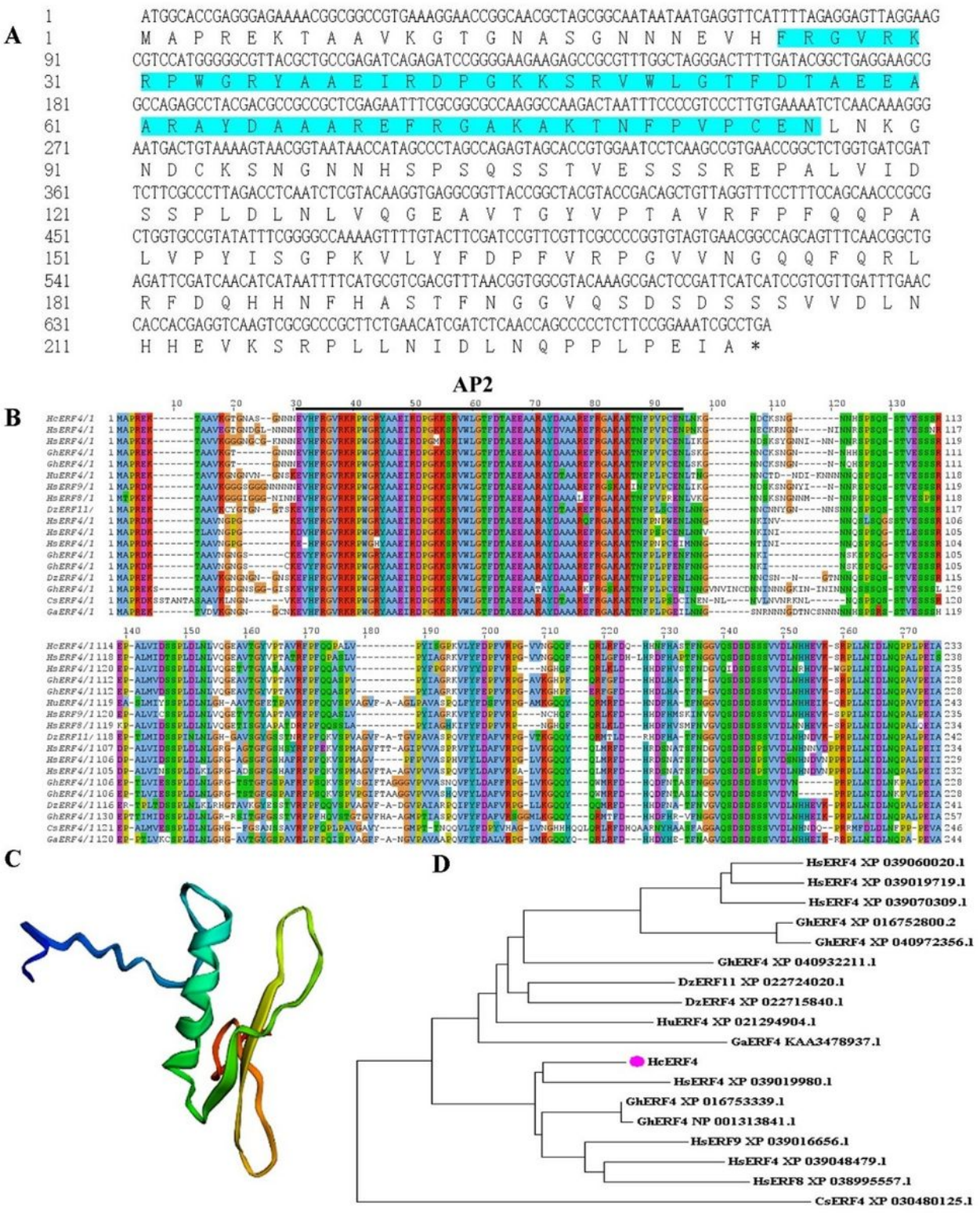

Figure 1

The coding sequence of HcERF4 and its deduced amino acids; Comparisons of HcERF4 and its homologous sequences; transmembrane domain prediction and phylogenetic analyses of the HcERF4 protein. (A) Nucleotide and deduced amino acid sequences of HcERF4 cDNA. Numbers at left indicate nucleotide (upper) and amino acid (lower) positions. The asterisk indicates the stop codon. The AP2/ERF domain is shown as a blue box. (B) The sequence alignments generated by Jalview. (C) Predicted 3D

structure of HcERF4 generated using the Phyre2 server. (D) A phylogenetic tree of HcERF4 and its homologous sequences constructed by using the neighbor-joining method. The Pink circle symbol showed the position of HcERF4.

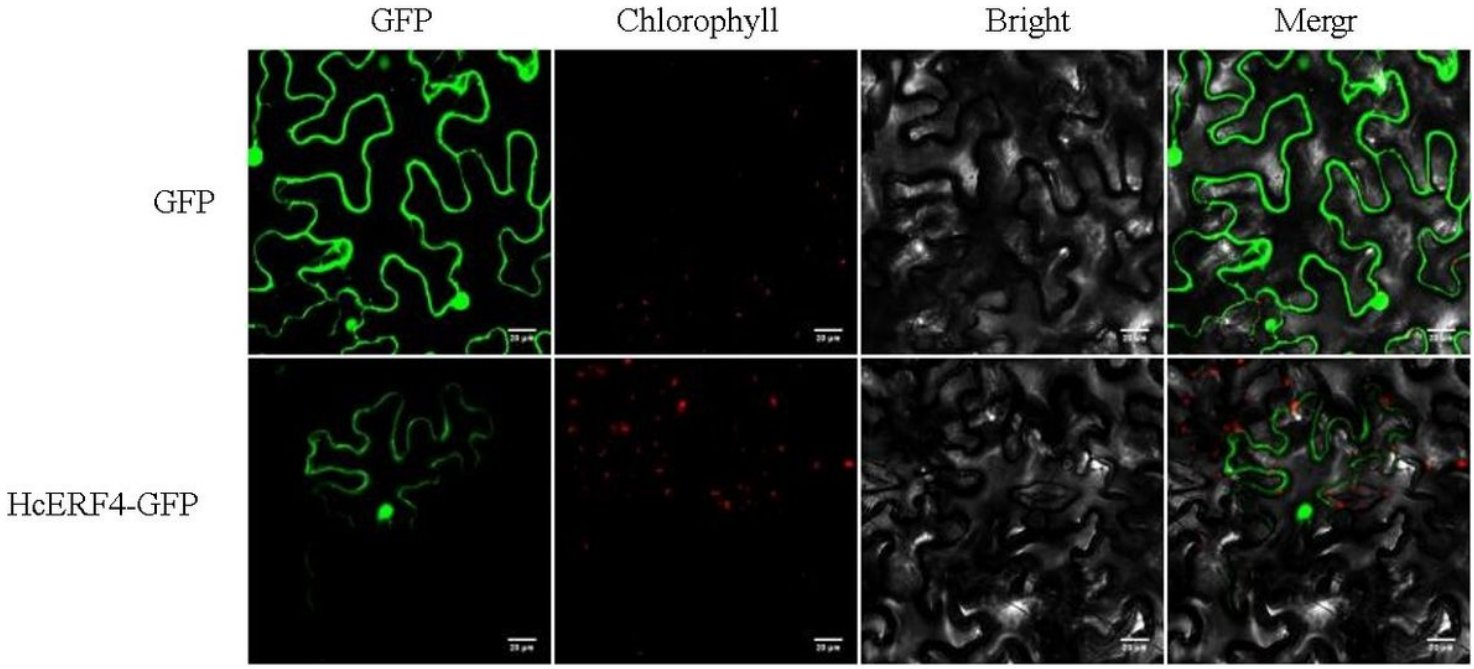

Figure 2

Subcellular localization of HcERF4 protein in tobacco leaf epidermal cells. Images of cells expressing GFP (control group, upper column) or HcERF4: GFP (lower column) fusion protein were displayed and examined under fluorescence channel (first column), chloroplast fluorescence channel (second column), bright field (third column) and superposition of bright field and fluorescence illumination (last column).

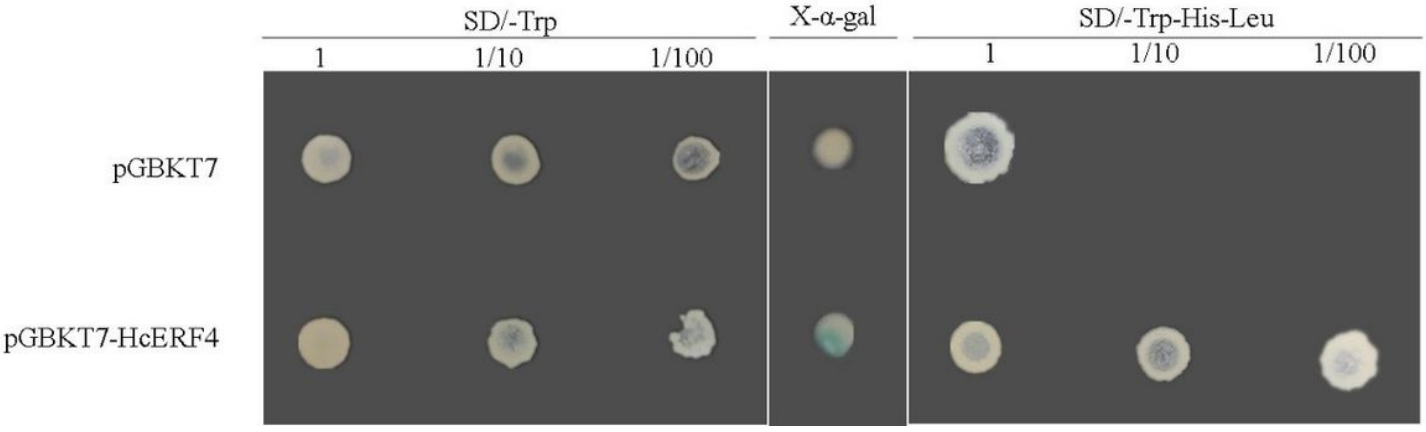

Figure 3

Transcription activity analysis of HcERF4. The transformants with pGBKT7 (up) and pGBKT7-HcERF4 (down) grow normally on SD-Trp medium, and only the transformants pGBKT7- HcERF4 (down) can grow normally on SD-Trp-His-Leu medium.

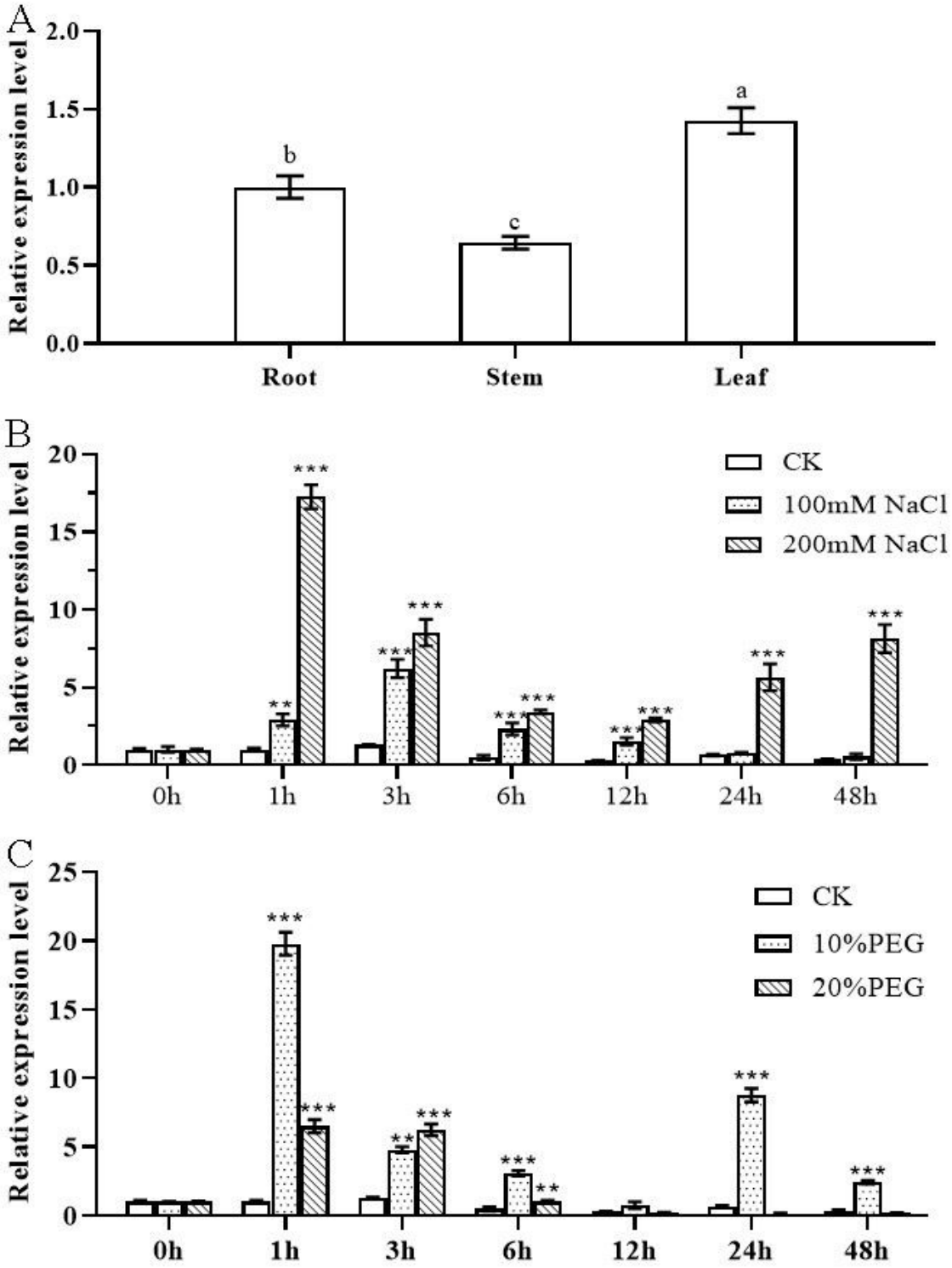

Figure 4

Time-course expression level of HcERF4 in kenaf SF192 using qRT-PCR method. A: Expression patterns of HcERF4 in root, stem and leaf in normal condition. B, C: Expression patterns of HcERF4 in control, salt (100mM NaCl, 200mM NaCl) and drought (10% PEG, 20% PEG) in leaf at the following time point: 0h, 1h, 3h, 6h, 12h, 24h, and 48h. The reference genes HcACTIN were used as controls in this study. The error bar

display is based on three repeated SDs. *Represents the significance level at P<0.05; **represents the significance level at P=0.01; *** represents the significance level at P<0.001.

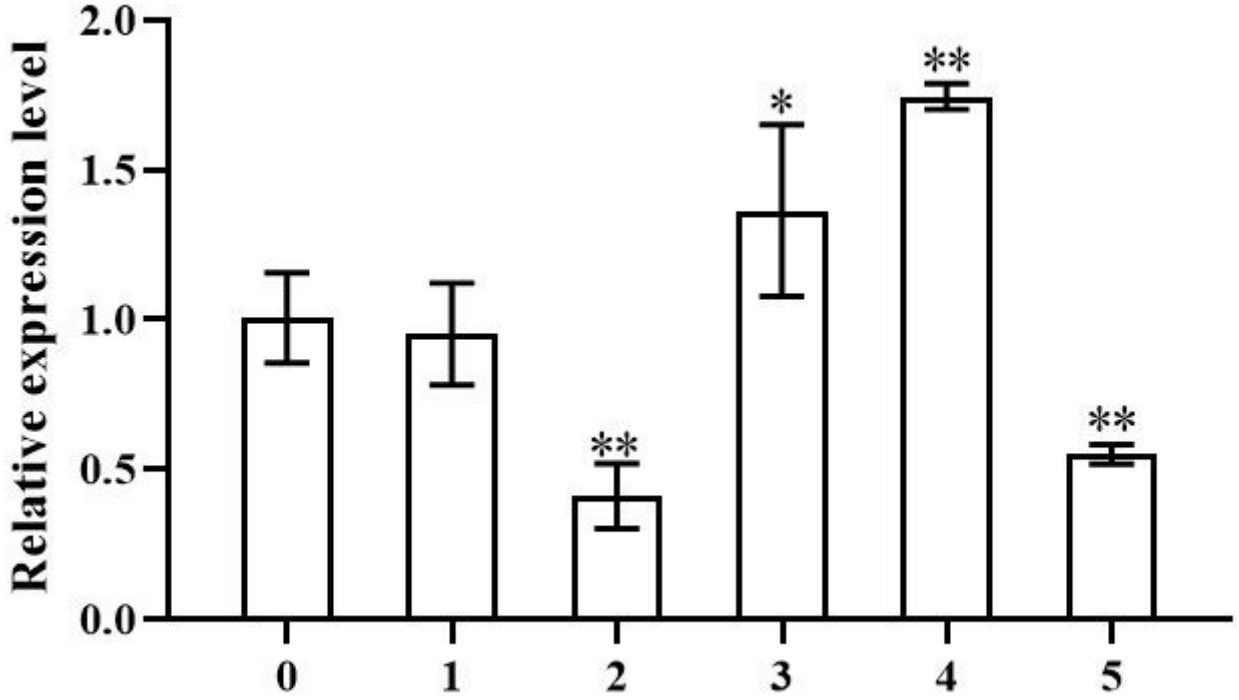

Figure 5

qRT-PCR analysis of transgenic plants. The 0 represents control plant of empty vector pTRV2, 1-5 represents gene silencing plants of HcERF4. Values are means ± SD (n=3). The asterisk in the table indicates p-value < 0.05 level difference is significant.

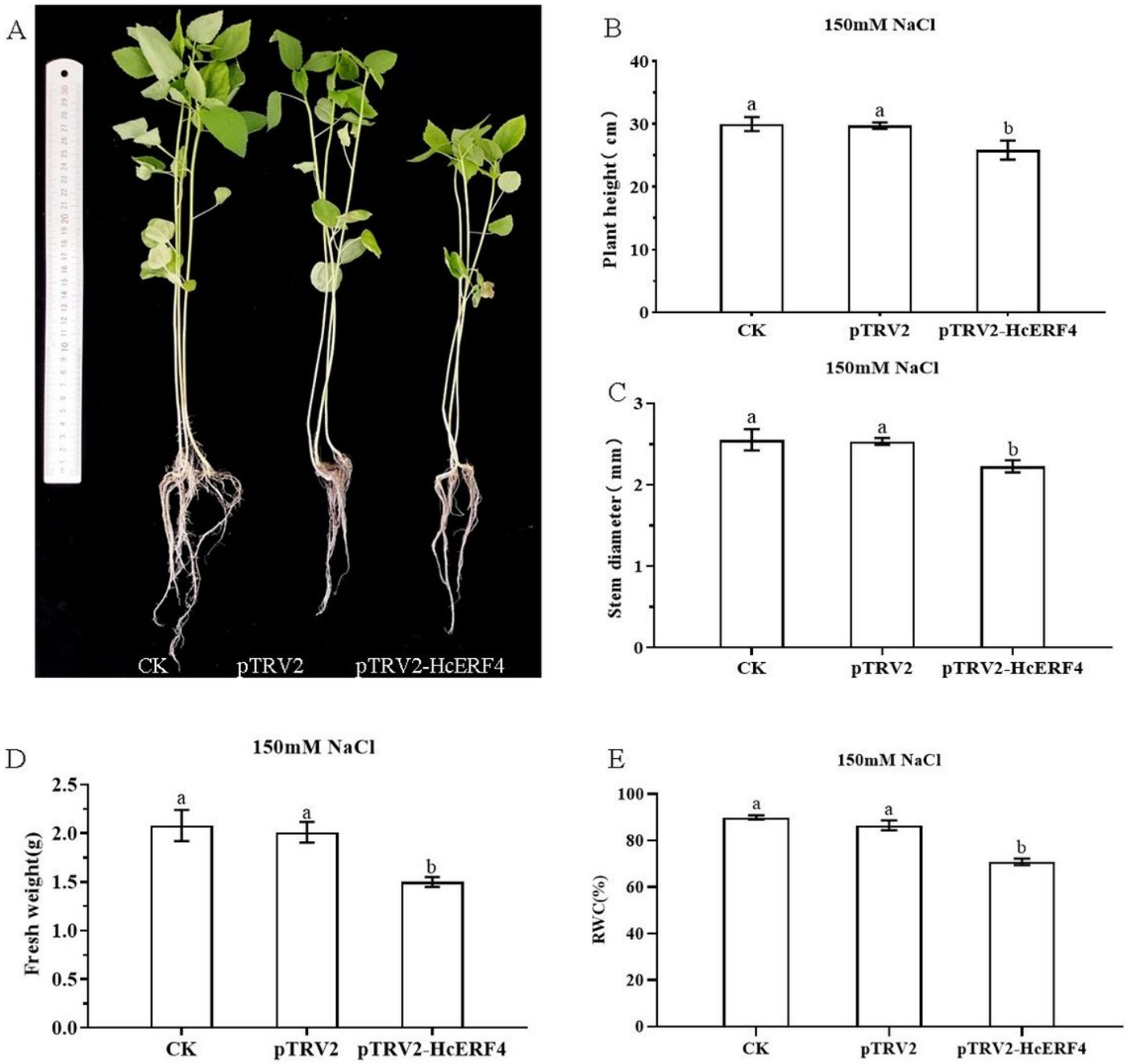

Figure 6

Morphology difference and determination of botanical characters of VIGS plants under salt stress. A: Plant phenotype. B: Plant Height; C: Stem diameter; D: Leaf area, E: RWC. Values are means ± SD (n= 3). Different letters indicate significant differences of Duncan's test with a $p<0.05$.

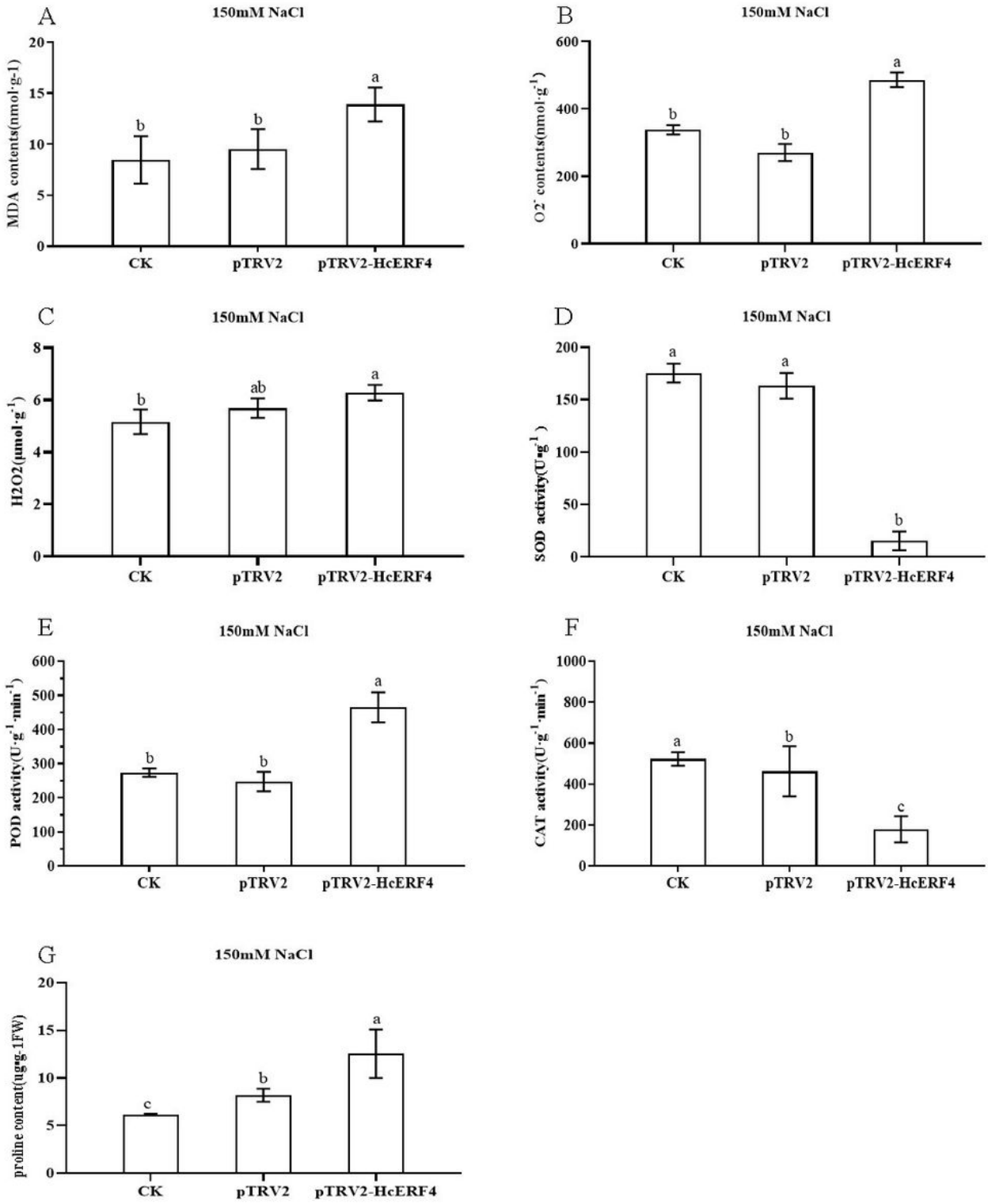

Figure 7

Determination results of physiological VIGS plants under salt stress. A: MDA contents; B: O2-¬¬contents; C: H2O2 contents; D: SOD activity; E: POD activity; F: CAT activity; G: Proline content. The value of each bar represents the mean ± SD. Bars with different lowercase letters indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests.

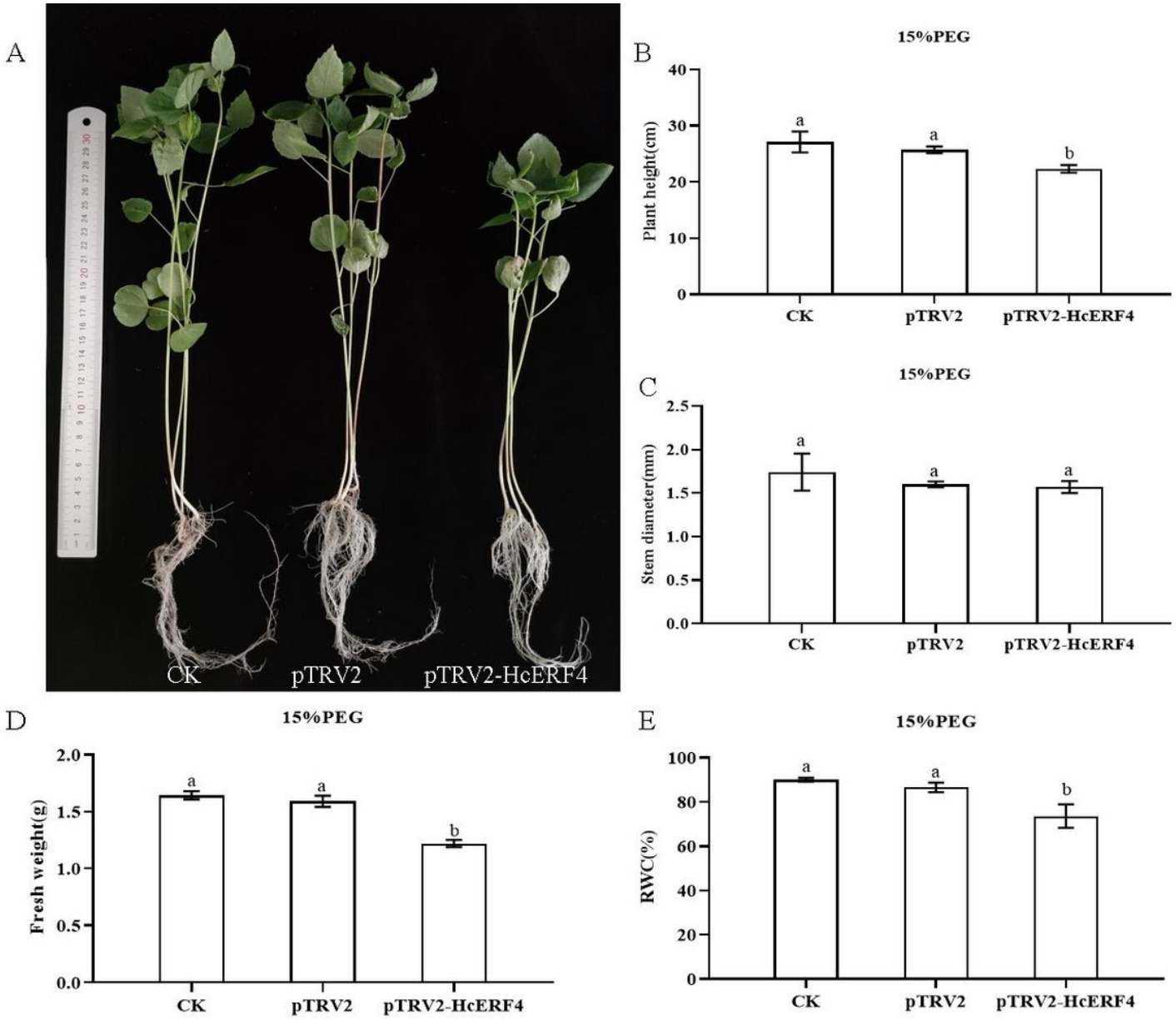

Figure 8

Morphology difference and determination of botanical characters of VIGS plants under drought stress. A: Plant phenotype. B: Agronomic indicators. B: Plant Height; C: Stem diameter; D: Leaf area, E: RWC. Values are means ± SD (n =3). Bars indicate SD. Different letters indicate significant differences of Duncan's test with a $p < 0.05$.

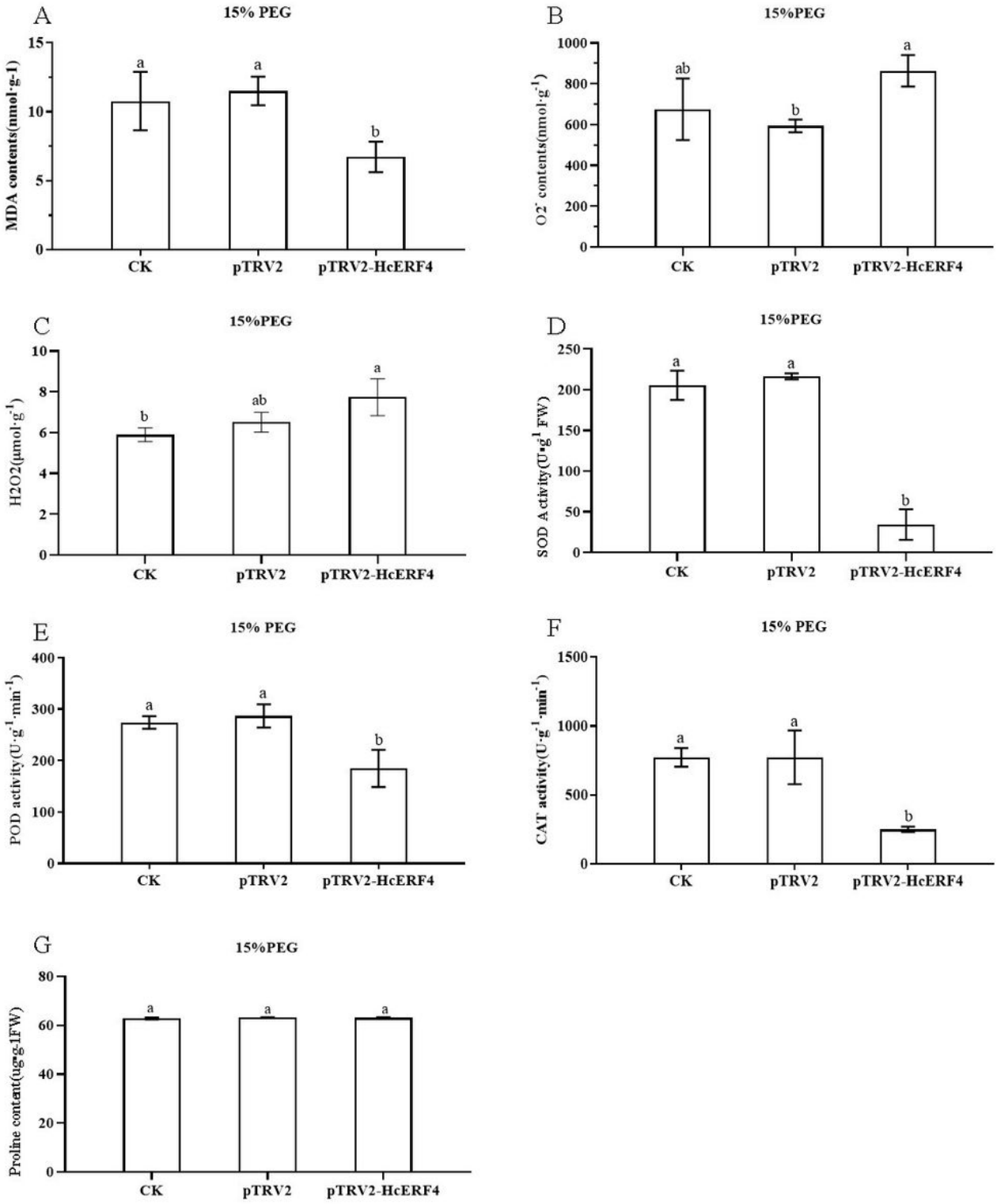

Figure 9

Determination results of physiological VIGS plants under drought stress. A: MDA contents; B: O2-¬- contents; C: H2O2 contents; D: SOD activity; E: POD activity; F: CAT activity; G: Proline content. The values are means ± SD (n =3). Different lowercase letters in the table indicate p - value < 0.05 level difference is significant.

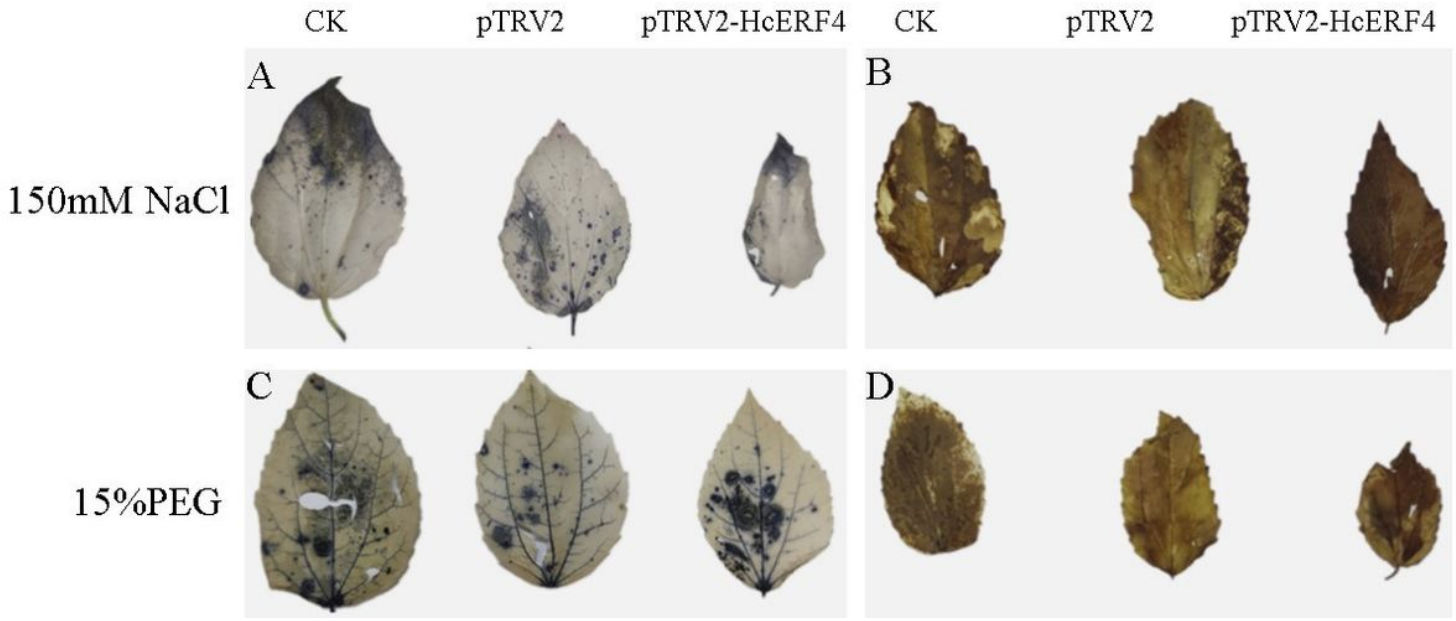

Figure 10

NBT and DAB staining of silenced plants. A: NBT staining to detect O2- content after 150mM NaCl stress; B: DAB staining to detect H2O2 content after 150mM NaCl stress; C: 15% PEG to detect O2- content after NBT staining; D: 15% PEG stress Post DAB staining to detect H2O2 content