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Isolation and functional characterization of the *JcERF* gene, a putative AP2/EREBP domain-containing transcription factor, in the woody oil plant *Jatropha curcas*

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Abstract A cDNA clone, named *JcERF*, was isolated from *Jatropha curcas* seedlings (a woody oil plant). It was classified as an ERF subfamily member based on multiple sequence alignment and phylogenetic characterization. The deduced amino acid sequences of the *JcERF* clone showed no significant sequence similarity with other known ERF proteins except for the conserved AP2/EREBP DNA-binding domain. Expression of the *JcERF* gene was rapidly induced upon salinity, drought, ethylene and mechanical wounding treatments. No significant changes in the *JcERF* expression were observed under ABA stress. Gel retardation assay revealed that the JcERF protein could bind specifically to the GCC box as well as to the C/DRE motif. Also it can be inferred from the gel-shift that there is a possibility that the near sequence of the GCC box has an important effect on the DNA-binding activity. In yeast, the JcERF protein specifically bound to the DRE sequence and activated the transcription of two reporter genes *His3* and *LacZ* driven by the DRE sequence. When fused to the LexA DNA-binding domain, the full-length *JcERF* functioned effectively as a trans-activator in the yeast one-hybrid assay. Overexpression of *JcERF* cDNA in transgenic *Arabidopsis* enhanced the salt and freezing tolerance. Meanwhile the seed germination of *JcERF* transgenic plants was not affected by various concentrations ABA in MS medium. Taken together, the results showed that JcERF functioned as a novel transcription factor and it exhibited a mechanism of plant response to environmental factors like the other AP2/EREBP regulons that also exist in tropical woody plants.

Keywords AP2/ERF-domain · DRE element · GCC box · *Jatropha curcas* · Salt and freezing tolerance

Abbreviations

ABA	Abscisic acid
CBF	C-repeat binding factor
CRT	C-repeat
DREB	DRE-binding protein
DRE	Dehydration-responsive element
ERF	Ethylene-responsive factor
CaMV	Cauliflower mosaic virus
ET	Ethylene
MeJA	Methyl jasmonate
SA	Salicylic acid

Introduction

Ethylene-responsive element binding-factors (ERF) are a large subfamily of AP2/EREBP-type transcription factors that are present in the plant kingdom (Riechmann et al. 2000). ERF proteins from the second largest transcription factor family in plants have been characterized from various plant species. In *Arabidopsis* and rice, 122 and 139 ERF proteins have been

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annotated, respectively (Nakano et al. 2006). ERF1-ERF4 was isolated in tobacco, and specifically binds to the GCC box (Ohme-Takagi and Shinshi 1995). In 2004, Huang's lab characterized four transcriptional activators from tomato by yeast one-hybrid assay: stress-responsive factor TSRF1, ethylene-responsive factor TERF1 and jasmonate-responsive factors JERF1 and JERF3 (Huang et al. 2004; Zhang et al. 2004a, 2004b; Wang et al. 2004). The ERF proteins have a highly conserved novel plant-specific DNA binding domain consisting of 58–59 amino acids and have been found related to the AP2 domain. Based on the ERF proteins' binding *cis*-acting element, they are divided into at least two major classes. One class binds to the GCC box, a *cis*-acting element in the analysis of promoters of several basic pathogenesis-related (PR) gene promoters (Ohme-Takagi and Shinshi 1995). The GCC box (GCCGCC) is a core sequence from the 11-bp consensus sequence TAAGAGCCGCC first isolated in tobacco (Ohme-Takagi and Shinshi 1995). The other class of ERF proteins interacts with the DRE/CRT element usually present in the promoters of cold and dehydration-inducible genes, including *rd29A*/*cor78*, *cor15a*, *cor47* and *cor6.6/kin2* (Thomashow 1998).

ERFs are involved not only in plant hormone signal transduction, but also in plants' responses to biotic pathogens, and environmental stresses (Nakano et al. 2006). Multiple ERF genes are induced by disease-related stimuli, such as phytohormone ethylene, jasmonic acid and salicylic acid or pathogen infection (Lee et al. 2004; Menke et al. 1999; Zhang et al. 2004a; Wang et al. 2004). Also many ERF genes are regulated by wounding and abiotic stresses, especially salt stress (Lee et al. 2004; Yi et al. 2004; Nishiuchi et al. 2004).

The roles of ERF proteins in plants have been studied extensively in many previous reports. Park et al. (2001) isolated and characterized an AP2/EREBP type transcription factor, designated as Tsi1, which could bind specifically to both the GCC and the DRE/CRT sequences. Overexpression of *Tsi1* in transgenic tobacco improved tolerance to salt and pathogens. ORCA3 is a member of the AP2/ERF-domain transcription factor family in periwinkle. Overexpression of *Orca3* in transgenic periwinkle cell-suspension cultures induced several primary and secondary metabolic genes that are involved in terpenoid indole alkaloid (TIA) biosynthesis and production (van der Fits and Memelink 2001).

Although most ERF proteins are transcription activators, a small subfamily of ERF proteins can act as transcriptional repressors in plant responses to various hormones or environmental cues. The *Arabidopsis* genome contains eight ERF repressors, namely AtERF3, AtERF4, and AtERF7-12 (Yang et al. 2005). Song et al. (2005) revealed that AtERF7 played an important role in ABA responses and might be part of a transcriptional repressor complex regulated by protein kinase PKS3. Transient expression assays with tobacco protoplasts have demonstrated that ERF3 repressed transcription via the NsERF3 promoter activated by NtWRKYs (Nishiuchi et al. 2004).

Jatropha curcas L., belonging to the family Euphorbiaceae, is a drought-resistant oil plant or small tree which is widely distributed in tropical and subtropical areas, especially in Central and South America, Africa, India and Southeast Asia (Schmook et al. 1997). Different parts of *J. curcas* have traditionally been used for various purposes and have considerable potential. The plant can be used to prevent and/or control erosion and reclaim land. It can be grown as a live fence or used for animal feed, and it also has medicinal value (Openshaw 2000). Most importantly, the seeds of *J. curcas* contain viscous oil that can be used for soap-making in the cosmetics industry and as a diesel/kerosene substitute or extender (Openshaw 2000). Recently, research on this plant has focused on oil extraction, the composition of the seed, detoxification of seed cake and antitumor activities of curcin from the seed (Shah et al. 2005; Akintayo 2004; Aregheore et al. 2003; Lin et al. 2003). However, the understanding of the molecular aspects of this plant's response to adverse abiotic environmental factors (e.g., cold, drought and salt stress) is relatively rudimentary. In order to characterize a putative regulatory molecular response to salt stress, we isolated a full-length *JcERF* gene from *J. curcas* treated with high salt. Also we investigated its expression pattern, and DNA-binding and transcription activation ability. Moreover, the functions of transgenic *JcERF Arabidopsis* under several types of stresses were evaluated.

Materials and methods

Plant materials and chemical treatments, plant hormones and wounding

Mature seeds of *J. curcas* were collected from Panzhihua, Sichuan Province, China. The seeds were surface-sterilized in 70% ethanol for 5 min and then in 0.1% $HgCl_2$ for 10 min. The seeds were rinsed four times with sterile distilled water, and then the cotyledons were taken out from the seeds and placed in 100-ml flasks containing 40 ml MS medium (Murashige and Skoog 1962) and 0.6% (w/v) agar, pH 5.8. The medium was autoclaved at 121°C (1.1 kg/cm^2) for 20 min. Three days

later, the rooted cotyledons were transferred into pots with 1:1(v/v) vermiculite with peat medium and incubated at 28°C with a 16 h light/8 h dark photoperiod for 4 weeks. Four-week-old light-grown intact plants (with 2–3 leaves) were used for Northern blot analysis. The stress treatments were performed as follows: for salt, dehydration, and ABA treatments, we dipped the roots of seedlings into a beaker containing solutions of 100-ml 300 mM NaCl, 20%PEG, 100 μM ABA, respectively. The controls were put into water. For mechanical wounding, mature leaves were cut six times per leaf with a razor blade in line with the main vein. Ethylene treatment was performed with 2-ml 40% ethephon, and 1 g $NaHCO_3$ dissolved in 200-ml H_2O (Zhang et al. 2004b; Ethylene gas is liberated by ethephon in such conditions). The control and treated *J. curcas* plants were placed in a sealed Plexiglas chamber before leaves were harvested and frozen in liquid nitrogen for further analysis.

RNA gel blot analysis

Total RNA was extracted from *J. curcas* samples as described by Tang et al. (2005a). About 20 μg of total RNA was fractionated in a 1.0% agarose gel containing formaldehyde and then transferred onto Hybond-N^+ nylon membrane in 20 × SSC. The membrane filter was hybridized at 65°C with a full-length *JcERF* cDNA fragment, which was DIG-labeled using the DIG Random Primer Kit (Roche Diagnostics, Indianapolis, IN, USA). The hybridization, washing and immunostaining were performed according to the manufacturer's instructions (Roche Diagnostics). RNA hybridizations using two independent sets of RNA samples were performed, with identical results. The results from one experiment are presented.

DRE-binding activity of JcERF protein in yeast

To analyze the DRE-binding activity of JcERF, the entire encoding regions of *JcERF* and the *Arabidopsis AtDREB2* (as a positive control) were fused into the *Bam*HI-*Xho*I sites of the activation domain of the YepGAP vector (pAD). According to Clontech's protocol, the recombinant plasmids pAD-JcERF and pAD-AtDREB2 were transformed into the yeast strain YM4271 carrying two reporter genes *His3* and *LacZ* under the control of the *rd29A* promoter containing the DRE sequence (T**ACCGAC**AT) or mutated DRE (mDRE) sequence (T**ATTTTC**AT) according to Liu et al. (1998) respectively. The transformed yeast cells were analyzed on SD medium minus His plus 10 mM 3-amonotrizole (a competitive inhibitor of the *His3* gene product) to test the expression of the *His3* gene. The β-galactosidase activity of the transformed yeast cells was examined by colony lift filter assay following Clontech's instructions.

Transcriptional activity investigation by using yeast one-hybrid assay

The entire coding region of *JcERF* and GAL4 (as a positive control) were cloned into pLexA and transformed into EGY48 containing p8op-LacZ as described by the manufacturer (Clontech). The transformants were selected by growth on SD medium minus Leu, Ura and His at 30°C for 3 days. Subsequently in order to test the expression of the LacZ gene, a colony lift filter assay was performed using *o*-nitrophenyl-ß-D-galactopyranoside (ONPG) as a substrate to determine the transcription activation of the JcERF protein.

Binding assay in vitro

The full-length encoding region of JcERF was fused in a frame into the *Bam*HI-*Xho*I sites of the pGEX-4T vector and expressed in *Escherichia coli* BL21 cells. A pGEX-JcERF fusion protein was purified using affinity chromatography. DNA probes containing the wild type and mutated forms of the GCC box and C/DRE motif were end-labeled with ^{32}P-dATP for gel retardation assays. The oligonucleotides used in the analysis were as follows: DRE1: GATATACTGCCGACATGAGTTC; mDRE1: GATATACTATTTTCATGAGTTC; DRE2: GGGGTCGACCGGGCCACCGACGCACCGGCT CGAG; mDRE2: GGGGTCGACCGGGCCAGAATT GCACCGGCTCGAG; ERF: AGTGCCAAAAGCCG CCACACCCCT; mERF: AGTGCCAAAATCCACT ACACCCCT. ERE: CGCAGACATAGCCGCC ATTT; mERE: CGCAGACATATCCTCCATTT; ABRE: TGGAAGCTTCGCGCCACGTGGGCA; mABRE: TGGAAGCTTCGCGCCTGCATGGCA. The core sequence or mutated core sequence is underlined. Gel mobility shift assays were conducted as described previously (Urao et al. 1993).

Plant transformation

Two plant expression vectors, p3301-121-JcERF and p3301-121, which include the *bar* gene that was selected for its phosphinothricin (PPT) resistance, were transformed into *Arabidopsis thaliana* with the Agrobacterium-mediated floral dip method (Clough and Bent 1998). The T1 seeds were harvested, dried at

25°C, and plated on half-strength sterile MS medium with 10 mg/l PPT selection pressure, subsequently surviving T1 seedlings were transferred to soil to set T2 seeds. T2 seeds and seedlings were used for the following analysis.

Analysis of salt, freezing tolerance and seed germination in transgenic plants

35S::JcERF transgenic plants were grown in 8-cm pots filled with a mixture of peat/vermiculite (1:1, v/v) under light at 23°C for 4 weeks. Four-week-old plants were exposed to freezing stress. Freezing stress was conducted by exposure of plants to a temperature of –8°C for 20 h, after which the temperature was returned to 23°C for 14 days. Leaves were cut from controls and transgenic *JcERF* plants (4 weeks old) and floated in MS liquid medium with 300 mM NaCl for 48 h or 72 h under light at 23°C. According to Aono et al. (1993), we measured chlorophyll content and the results are shown as percentages relative to the control. We repeated experiments on salt and freezing stress tolerance three times, respectively. Surface sterilized T2 seeds from controls and transgenic plants were laid on MS media containing various concentrations of ABA and germinated at 23°C under a 16 h light/8 h dark photoperiod.

Results

Isolation and characterization of JcERF cDNA

The full-length *JcERF* clone (GenBank Accession No. DQ109673) is 1,051 bp containing an open reading frame of 774 bp and encoding a putative protein of 258 amino acids with a predicted molecular mass of 28.09 kDa and a p*I* of 9.39. Analysis of the deduced amino acid sequence revealed that this protein had a typical AP2/EREBP DNA-binding domain exhibiting 67.2–96.5% sequence identity with the AP2-EREBP domains of other members of the family. Moreover, JcERF has conserved Ala (A) and Asp (D) residues at the 14th and 19th positions in the AP2/EREBP domain (Fig. 1A), which indicates that JcERF may be a member of the ERF subfamily among the AP2/EREBP proteins (Feng et al. 2005; Sakuma et al. 2002).

Sakuma et al. (2002) divided ERF subfamily proteins in *Arabidopsis* into six subgroups (B-1 through B-6) on the basis of the similarity of the amino acid sequence of the DNA-binding domain. The JcERF protein identified as belonging to the B-3 subgroup (Fig. 1B). On the basis of the sequence alignment (Fig. 1B), JcERF has limited sequence identity with AtERF5, RAP2.2, NtCEF1 and JERF3 among the ERF subfamily in the database, exhibiting only 12.94–18.09% homology in the open reading fragment at the amino acid level, which suggests a functional difference between JcERF and other previously identified proteins and appears to be a new member of AP2/EREBP gene family.

Expression patterns of the *JcERF* gene under various treatments

To investigate whether the expression of *JcERF* is regulated by multiple factors, its mRNA accumulation profile was determined under various abiotic stresses by Northern blot assay. Four-week-old intact *J. curcas* seedlings were treated in a solution containing various chemical reagents for various time periods, and transcript levels were monitored at each time point. As indicated in Fig. 2, *JcERF* expression began to increase within 0.5 h and reached a peak at 2 h, 10 h, 6 h, 10 h, and subsequently decreased under wound, drought (PEG), ethylene and high-salt treatment, respectively. The transcript level of the *JcERF* gene also continued high until 24 h in 300 mM NaCl treatment. Meanwhile, the transcript of *JcERF* was nearly undetectable at 0 h in the above treatments, which demonstrated that the *JcERF* gene is inducible. Like *OsDREB2A* (Dubouzet et al. 2003), *JcERF* expression is faintly detectable in plants treated with 100 μM ABA. Taken together, the results suggested that *JcERF* is not only involved in the high salt, drought or wound stress signal pathway but is related to ethylene-mediated signal transduction at the transcriptional level.

DRE-binding activity of JcERF proteins

To examine whether JcERF protein can specifically bind the DRE element, the entire coding region of *JcERF* was fused in-frame with the GAL4 activation domain in a pAD vector, and the recombinant plasmid pAD-JcERF was transformed into yeast cells carrying the dual reporter genes *His3* and *LacZ* under the control of the DRE or mDRE motif, respectively. As shown in Fig. 3A, yeast cells harboring pAD-JcERF or pAD-AtDREB2 (positive control) with DRE-His3 could grow on SD media minus His in the presence of 10 mM 3-AT, whereas pAD-JcERF or pAD-AtDREB2 (negative control) plus mDRE-His3 could not grow on the same media. In β-galactosidase activity assays, yeast cells that could grow on SD media minus His with 10 mM 3-AT became blue by the colony lift filter assay. These results demonstrated that JcERF could specifically bind the DRE element but not the mDRE element.

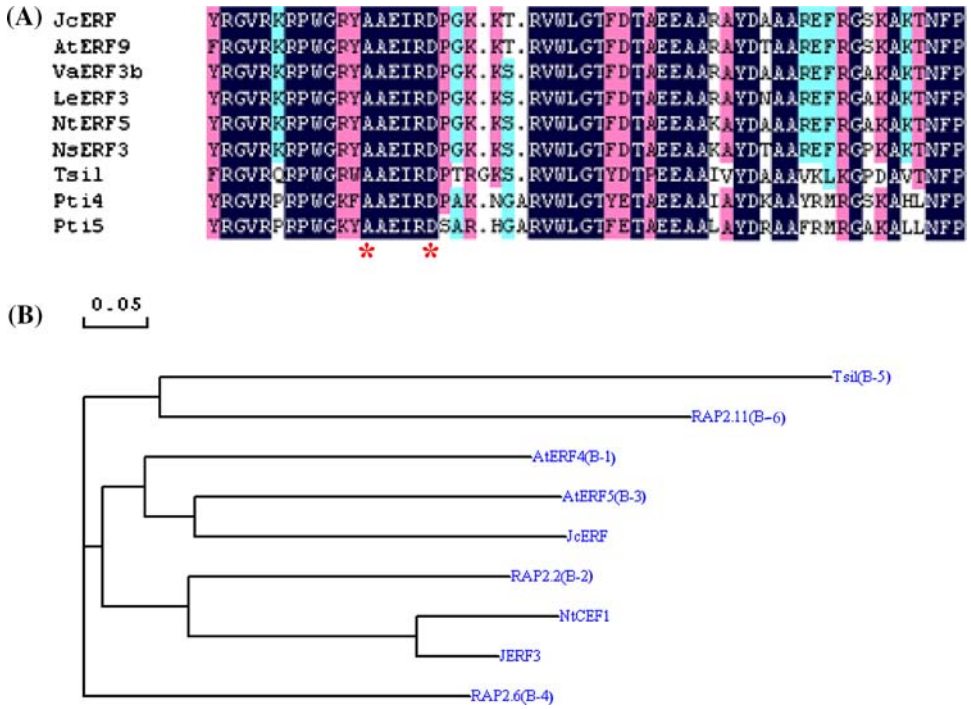

Fig. 1 Amino acid sequence alignment and dendrogram of JcERF proteins. **(A)** Comparison of deduced amino acids of JcERF with other EREBP/AP2 proteins in the ERF domain: Tsil and NtERF5 from tobacco (*Nicotiana tabacum*), NsERF3 from *N. sylvestris*; AtERF9 from *Arabidopsis*; LeERF3, Pti4 and Pti5 from tomato (*Lycopersicon esculentum*); VaERF3b from *Vitis aestivalis*. Black boxes represent 100% similarity. The 14th Ala (**A**) and 19th Asp (**D**) residues in the AP2/EREBP domain, which are conserved in the ERF subfamily, are marked with the bold asterisks in red. **(B)** Phylogenetic tree of JcERF protein with other AP2/EREBP proteins

Transcriptional activation ability of JcERF

Previous results have shown that acidic C-terminal of the ERF proteins might function as a transcriptional activator domain (Liu et al. 1998). The ability of JcERF to regulate transcriptional activation was tested using the yeast one-hybrid assay. The encoding region of *JcERF* was fused into the DNA-binding domain of the pLexA-BD vector, and the recombinant plasmid pLexA-BD-JcERF was transformed into yeast strain EGY48 with two reporter genes *Leu2* and *LacZ* under the control of LexA operators. The yeast cells with vector pLexA-BD (as a negative control) could not grow on SD media minus Leu, Ura and His because of the absence of the LexA activation domain in pLexA-BD vector (Fig. 3C). However, the cells harboring pLexA-BD-JcERF or pLexA-BD-GAL4 (as a positive control) could grow well on the same media and became blue correspondingly in β-galactosidase activity assays (Fig. 3C). These results indicated that JcERF was functional as a transcriptional activator in yeast.

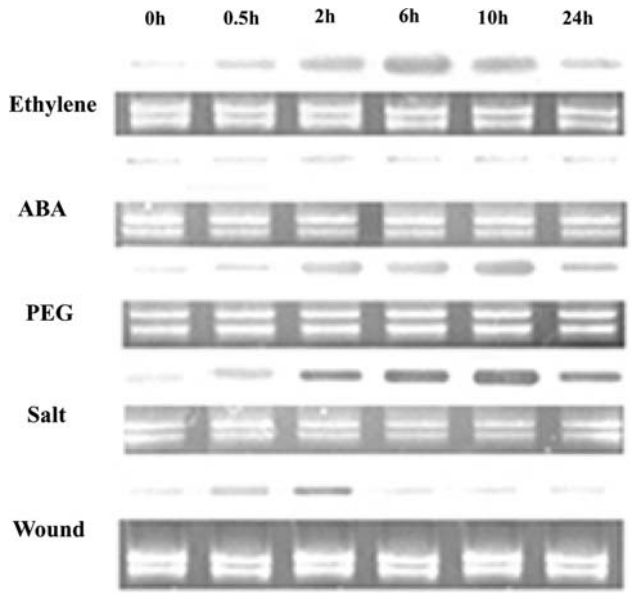

Fig. 2 Expression of *JcERF* in response to ethylene, cold, PEG, high-salt and wound stresses. Each lane was loaded with 20 μg of total RNA from four-week-old *J. curcas* seedlings that had been treated with ethylene, cold (4°C), 20% PEG, 300 mM NaCl and wound. The amount of ethidium bromide-stained rRNA was shown to verify equal loading of RNA in each lane. The RNA gel blots were hybridized with the encoding region of *JcERF* as probe

JcERF protein specifically binds to both the GCC box and DRE element in vitro

Accumulated evidence indicates that some proteins in the ERF subfamily have dual binding activity (the GCC box and the C/DRE sequence) (Lee et al. 2005; Park et al. 2001; Hao et al. 2002; Zhang et al. 2004a). To test whether JcERF binds to the *cis*-acting elements GCC box and C/DRE in vitro, gel retardation assays were performed with the encoding region of recombinant JcERF protein. As shown in Fig. 3B, we could observe a slowly migrating band with strong intensity, which

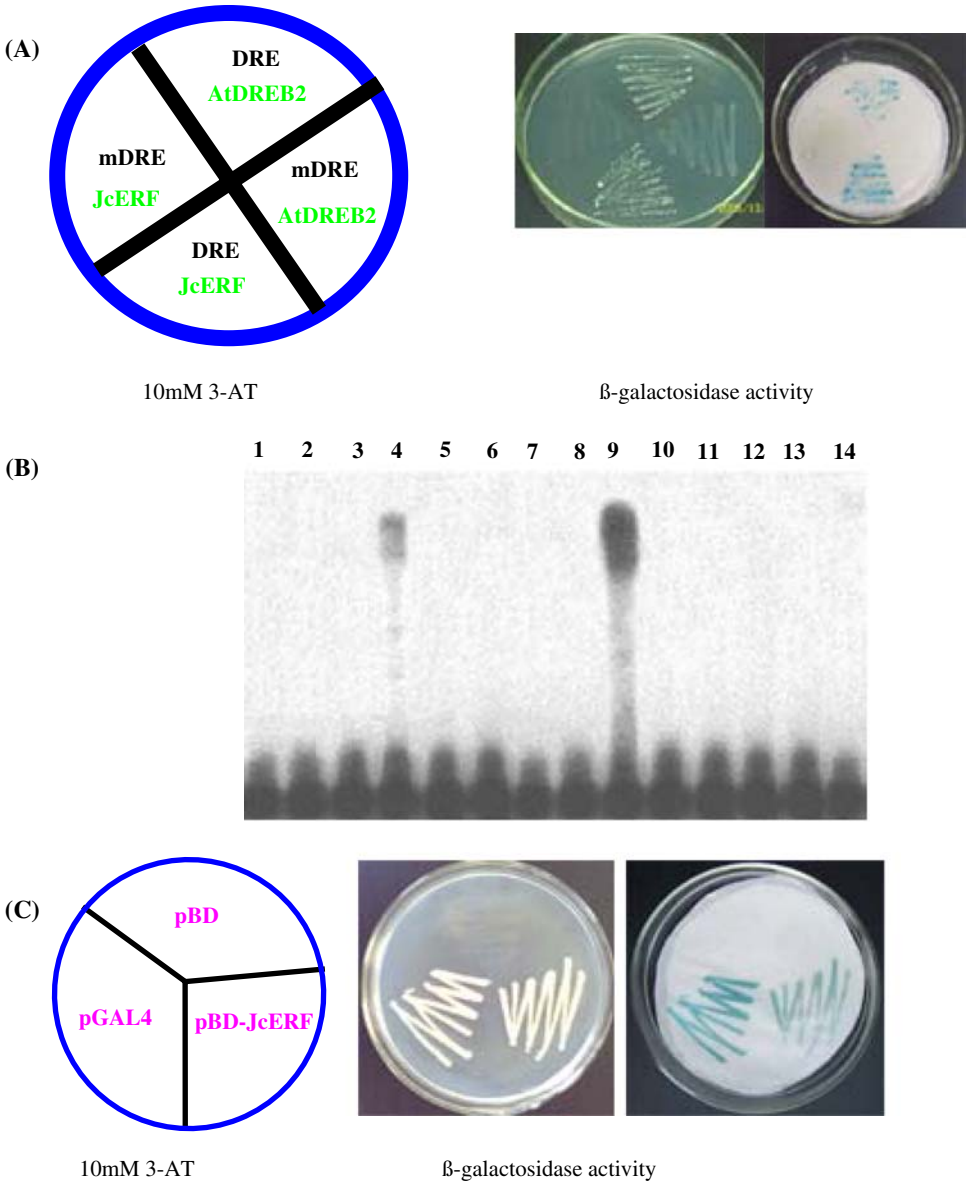

Fig. 3 Characterizations of JcERF Protein (**A**) Transcriptional binding activity of JcERF. The *left panel* shows the position of each transformed yeast cell. The *middle panel* indicates the transformed yeast cells with growth on SD medium minus His in the presence of 10 mM 3-aminotriazole at 30°C and the *right panel* for ß -galactosidase activity. The expression of *AtDREB2* was used as a control. (**B**) Gel retardation assay shows the JcERF protein binding to the DRE2 and ERE elements. lane 1, JcERF protein with DRE1 probes; lane 2, JcERF protein with the mutated DRE1 (mDRE1) probes; lane 3, the free radiolabeled DRE2 probes without JcERF protein; lane 4, the DRE2 probes with JcERF protein; lane 5, the DRE2 probes with JcERF protein and 500-fold molar excess of unlabeled cold DRE2 sequences as competitors; lane 6, the mutated DRE2 (mDRE2) probes with JcERF protein; lane 7, the free radiolabeled ERE probe without JcERF protein; lane 8, the ERE probes with JcERF protein and 500-fold molar excess of cold unlabeled ERE sequences as competitors; lane 9, the ERE probes with JcERF protein; lane 10, the mutated ERE (mERE) probes with JcERF protein; lane 11, the ERF probes with JcERF protein; lane 12, the mutated ERF (mERF) probes with JcERF protein; lane 13, the ABRE probes with JcERF protein; lane 14, the mutated ABRE (mABRE) probes with JcERF protein. (**C**) Transcriptional activation of LexA DNA binding domain fusion protein with full-length JcERF. The *left panel* shows the position of each transformed yeast cell; the *middle panels* show the growth of yeast cells on selective medium, *right panels* for the assay of reporter activity

indicated DNA-protein complexes were formed between JcERF and the GCC box, when we used ERE as a probe (lane 9). However, there was no band when mERE (mutated GCC box) was used as probe. At the same time, there was no migrating band with ERF (GCC box) or mutated ERF probe. The difference between ERE and ERF is the nucleotide sequence outside the GCC box. The results demonstrate that the JcERF

protein specifically binds to the GCC box, and the near nucleotide sequences of the GCC box have an important effect on the binding activity of JcERF protein. With the C/DRE and mutated C/DRE (mC/DRE) sequences as probes, JcERF was specifically bound to the DRE2 motif but not to the DRE1 or the mutated DRE1 and 2 sequences (lanes 1–6). Also lane 13–14 showed that JcERF could not bind the ABRE motif. Taken together, the results in Fig. 3B suggest that JcERF binds to both the GCC box and the C/DRE motif in vitro.

Constitutive expression of *JcERF* in transgenic *Arabidopsis* enhances salt and freezing tolerance

To investigate the role of *JcERF* in plants, we generated transgenic *JcERF Arabidopsis* plants in which *JcERF* is driven by the CaMV35S promoter. The tolerance to freezing and high-salt stresses of the *35S::JcERF* plants was compared with controls (Fig. 4). We incubated the leaves of transgenic plants and controls in 300 mM NaCl solution in order to determine if the transgenic *Arabidopsis* plants had improved salt tolerance. The leaves of the controls were totally bleached in solution, while most of the transgenic *JcERF* plants remained green (Fig. 4 A). The chlorophyll content of the controls and transgenic *JcERF* plants differed significantly after 48 h salt treatment (Fig. 4A). For the freezing tolerance test, four-week-old plants were incubated at –8°C for 20 h, and then returned to 23°C for 14 days. The controls did not survive under these conditions, but the *35S::JcERF* plants were highly tolerant to freezing stress (70.4% or 38/54 survived; Fig. 4B). There was no difference in seed germination between transgenic and controls on plates containing various concentrations of ABA (Fig. 4C for 200 nM ABA; data not shown for other concentrations ABA). These results indicate that the *35S::JcERF* plants had higher tolerance to freezing and high-salt stresses.

Discussion

The AP2/EREBP proteins in plants are the largest transcription factor family (Riechmann et al. 2000), which indicates that the proteins might play an important role in plant development and responses to ethylene, disease and other adverse environmental stresses. In the present paper, a *JcERF* gene (ERF-like transcription factor) was isolated from the oil plant *J. curcas* seedlings under high-salt stress (data not shown). Sequence alignment showed that it had little similarity to other known ERF proteins outside of the highly conserved ERF DNA-binding domain (Fig. 1). This result demonstrates that JcERF is a novel member of the ERF proteins family, and is a transcription activator that may play a distinct role in plant responses to environmental stresses.

Several reports have demonstrated that the 14th valine (V) and the 19th glutamic acid (E) may be decisive for DNA-binding specificity of DREB proteins in the AP2 domain (Sakuma et al. 2002; Hao et al. 2002). Particularly, the 14th valine (V) was a key to determining the specific binding activity between this protein and the DRE sequence. Li et al. (2005) concluded that all three DREB-like proteins from soybean could bind to the DRE element regardless of glutamic (E) or leucine acid (L) at the 19th residue. At the 19th position, OsDREB1A protein containing valine (V) but not glutamic (E) acid, could bind to GCCGAC more preferentially than to ACCGAC, while OsDREB2A protein containing valine and glutamic acid at the 14th and 19th positions respectively bound to GCCGAC and ACCGAC at the same efficiency (Dubouzet et al. 2003). However, recent research of several ERF proteins showed that the 14th and 19th amino acid in the ERF domain might be not significant for DNA-binding as was previously reported. Fig. 1 shows that there are many ERF proteins containing alanine (A) and aspartic (D) in the 14th and 19th amino acids, such as JERF3, AtERF9, NtCEF1, Tsil and RAP2.2, and most of the ERF proteins, such as Tsil and JERF3, can bind to both the C/DRE element and the GCC box (Park et al. 2001; Wang et al. 2004). Our results are in agreement with studies of these ERF proteins. The gel retardation assay of JcERF in this study indicated that JcERF containing an alanine acid (A) at the 14th position and aspartic acid (D) not glutamic acid (E) bind to ACCGAC (Fig. 3B; lane 4) but not to GCCGAC (lane 1). The core sequence ACCGAC, named DRE2, is from the rab17 promoter that regulates DRE-binding proteins through the drought-responsive element (Kizis and Pages 2002). Meanwhile the core sequence of the DRE1 element is GCCGAC, commonly referred to as C-repeat/DRE, which is found in the promoters of the *Arabidopsis rd29A*/*cor15A* genes. DRE/CRT is involved in cold, drought and osmotic stress (Liu et al. 1998). It can be inferred from the differences between DRE1 and DRE2 that JcERF protein might be involved in drought or osmotic-responsive pathway. The reason that JcERF protein binds DRE2 might be that the region is an AEIR amino acid sequence, not a conserved C/SEV/LR amino acid sequence (DREB1 group A-1) between the 14th and 19th amino acid. Sakuma et al. (2002) thought that this region played an

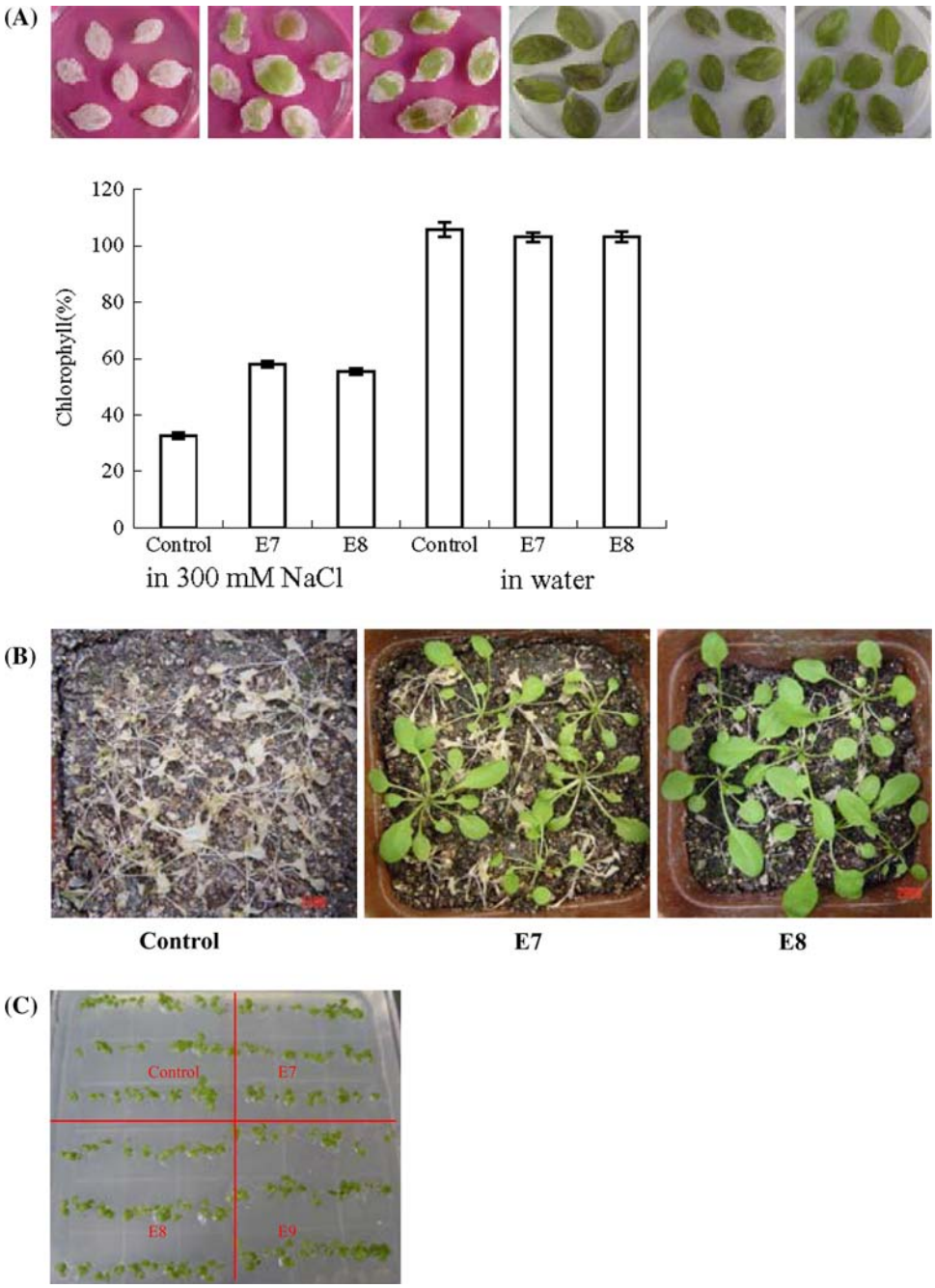

Fig. 4 Tolerance of the *35S::JcERF* transgenic plants exposed to freezing and high-salt stress. (**A**) For high-salt treatment, leaves from 4-week-old T2 transgenic plants and controls were cut and floated in MS medium containing 300 mM NaCl for 48 h or 72 h under light at 23°C. Phenotype differences were observed and photos were taken at 72 h and chlorophyll contents were measured at 48 h for leaves in 300 mM NaCl. Data are shown as percentage relative to controls from three independent experiments +SD. (**B**) For freezing treatment, 4-week-old T2 transgenic plants were exposed to a temperature of –8°C for 20 h and returned to 23°C for 14 days. (**C**) Effect of ABA on seed germination. Phenotype was observed 21 days after germinated seeds were plated on MS plate supplemented with 200 nM ABA

important role in the DNA binding sites between DREB1 and DREB2. This would explain the different functions of DREB1 and DREB2 (DREB1 is involved in cold while DREB2 is involved in salt stress). Meanwhile, we observed that JcERF protein could only bind to ERE probe (Fig. 3B; lane 9), but not to ERF probe (Fig. 3B; lane 11) in the gel-shift assay, although both sequences (ERF and ERE) contained GCC box. This result may indicate the possibility that the near sequence of the GCC box has an important effect on the DNA-binding activity.

Different members of the ERF family have been shown to respond differently to plant hormones, pathogens or environmental stresses. Zhang et al. (2004a) reported that the *JERF1* gene was regulated by ethylene, MeJA, ABA and salt stresses. The transcripts of *CaPF1* were observed not only under ethylene, MeJA and pathogens, but also under cold stress (Yi et al. 2004). Moreover, the At4g34410 gene, which belongs to the B-3 subgroup, was markedly induced by high salt and drought stress (Feng et al. 2005). In the present study, we observed that the transcript levels of

the *JcERF* gene could be enhanced by ethylene, drought (PEG), wound and salt stresses. Onate-Sanchez and Singh (2002) thought that transcription factors have different responses to signal transduction pathways as a result of the differences in DNA binding site preference, post-translational control and/or specific interactions with other proteins. For example, the *JcERF* gene was induced by mechanical wounding stress in a rapid and transient way due to the proximal W boxes in the promoter of the *JcERF* gene. Nishiuchi et al. (2004) also reported that the proximal W boxes in the promoter of the *ERF3* gene may function as *cis*-acting elements for wound-inducible immediate early expression. Taken together, the results that indicate JcERF is induced by abiotic stress implied that there is a possibility that a cross-talk signal pathway exists in *J. curcas*.

Many reports have indicated that many overexpressed ERF subfamily genes induce the transactivation of some GCC and C/DRE-motif-containing genes to trigger a subset of stress responses, and enhance resistance to biotic and abiotic stress, such as pathogens, salt, drought and freezing stresses (Fujimoto et al. 2000; Memelink et al. 2001; Park et al. 2001; Tournier et al. 2003; Lee et al. 2004). GCC box-containing *PR* genes and the DRE/CRT-containing *LT145* (*erd10*) gene were constitutively expressed in transgenic tobacco plants, which caused ectopic expression of *CaERFLP1* in transgenic tobacco plants to improve the tolerance against bacterial pathogens and salt stress (Lee et al. 2004). Overexpression of *CaPF1* in *Arabidopsis* resulted in constitutive overexpression of stress-related genes such as *PR* and *COR* genes (*cor47*, *cor6.6*, *rd29A*, *GST* and *PDF1.2*) and pathogen and freezing tolerance under normal growth conditions (Yi et al. 2004). Tang et al. (2005b) reported that the *CaPF1* gene in *Pinus virginiana* Mill. confers tolerance to heavy metals, to heat, and to bacterial pathogens, as by the survival rate of transgenic plants and the decrease in the number of pathogen cells. He measured the activity of three antioxidant enzymes: ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD), and found that the level of these three enzyme activities was higher in transgenic *Virginia pine* plants with *CaPF1* gene overexpressed, compared to the controls. Then he inferred that higher enzyme activity might protect cells from the oxidative damage caused by stresses. Based on the dual DNA-binding activity of the JcERF protein to both the GCC and CRT/DRE boxes, we conclude that JcERF play a role in plants responses to disease, salt, drought and cold stresses. Overexpression of *JcERF* in transgenic *Arabidopsis* under the control of CaMV35S promoter shows that *JcERF* can enhance the transgenic plants' salt and freezing tolerance. In conclusion, the data suggest that *JcERF* plays multiple roles in the regulation of GCC- and DRE/CRT mediated gene expression.

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