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Over-expression of *JcDREB*, a putative AP2/EREBP domain-containing transcription factor gene in woody biodiesel plant *Jatropha curcas*, enhances salt and freezing tolerance in transgenic *Arabidopsis thaliana*

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ABSTRACT

Jatropha curcas L. is an all-purpose biodiesel plant and is widely distributed in tropical and subtropical climates. It can grow well on poor quality soil which is not qualified for crop cultivation. This is very important for relieving land, food and energy crises. However, tropical and subtropical distribution limits the production of *J. curcas* seed. So it is valuable to know the molecular mechanism of *J. curcas* response to adverse abiotic environmental factors, especially freezing stress, in order to change the plant's characteristics. Until now there are just a few reports about *J. curcas* molecular biology. In this paper, we cloned and characterized a DNA binding protein from this plant, designated as JcDREB. Sequence analysis and yeast one-hybrid assays show that JcDREB can effectively function as a transcription factor of DREB protein family belonging to A-6 subgroup member. Expression patterns of *JcDREB* showed that it was induced by cold, salt and drought stresses, not by ABA. Over-expression of *JcDREB* in transgenic Arabidopsis exhibited enhanced salt and freezing stresses. Understanding the molecular mechanisms of *J. curcas* responses to environmental stresses, for example, high salinity, drought and low temperature, is crucial for improving their stress tolerance and productivity. This work provides more information about A-6 subgroup members of DREB subfamily.

1. Introduction

Recently the demand for the biodiesel from vegetable oil has greatly increased because of energy and food crises. The world population is still growing fast over limited land and the consumption rate for gas, water and other resources continue to rise. Most people are reluctant to switch from using traditional energy source to renewable energy. Vegetable oil, such as soybean oil, rapeseed oil, castor oil, and corn oil, is a renewable energy. *Jatropha curcas* is a biodiesel plant, whose seed oil content is as high as 37%. Jatropha oil is a green fuel and renewable energy [1]. It is registered as a fuel and fuel additive with the World Environmental Protection Agency [2]. Like vegetable oil, Jatropha oil attracts more attention than other oils.

First of all, *J. curcas* is more productive (fuel/hectare) than other crops. Secondly, *J. curcas* is a drought-resistant perennial that grows well in wasteland and dry land whose poor soil quality is not suitable for either agricultural use or crop cultivation. Jatropha is an easy-to-grow perennial, producing seeds for 50 years. Depending on the soil quality and rainfall, oil can be extracted from the Jatropha nuts after 2–5 years. Also, *J. curcas* is a wonder and all-purpose plant for its world wide use as a source of folk medicine, lamp oil and soap, while providing control for soil erosion, reclaiming land, and live fence.

Even though *J. curcas* is native to Central America, it is now widely distributed in tropical and subtropical areas, such as China, India, and Africa. The distribution in tropical and subtropical limits cultivation range of *J. curcas*. There is a lot of wasteland in North of China. For example, Xinjiang province, whose vast territory occupies one sixth of China's land, has only 2.47% of arable land. The limiting factor for expanding *J. curcas* plants is the cold temperature in the winter, while genetic engineering is a powerful tool for improving *J. curcas* stress tolerance. Many reports show that increased tolerance had been achieved through over-expression of AP2/EREBP (ethylene responsive element binding protein) transcription factor (TF). The AP2/EREBP participates in plant hormone signal transduction as well as in plant's responses to biotic pathogens and abiotic environmental stresses [3]. Meanwhile many studies have revealed that AP2/EREBP transcription

Abbreviations: ABA, abscisic acid; CaMV, cauliflower mosaic virus; CBF, C-repeat binding factors; CRT, C-repeat; DREB, DRE-binding protein; DRE, dehydration-responsive element; ERF, ethylene-responsive factor.

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factor is also involved in flower development, floral organ identity, establishment of floral meristem identity, suppression of floral meristem indeterminacy, and development of the ovule and seed coat [4–7]. AP2 also plays an important role in determining seed size and weight, activation wax biosynthesis, accumulation of seed oil and protein as well as controlling of storage compound biosynthesis [8–11].

In *Arabidopsis thaliana*, the estimated 145 members of AP2/EREBP are divided into five groups: AP2, DREB (dehydration-responsive element-binding protein), RAV, ERF and 'others' [12,13]. DREB protein is a *trans*-acting factor that can bind to the DRE/CRT (C-repeat) sequence which contains an A/GCCGAC motif to activate the gene expression in the stress-signaling pathway in plants. The DREB subfamily is further classified into six small groups (A1–A6) based on the similarities in their binding domains. Except DEAR1 and GhDBP1, which are transcription repressor, most of DREB family members function as a transcription activator [14,15]. This indicates that DREB transcription factors play important and complex roles in the stress-related regulation network in plants. Until now, several DREBs have been isolated in a wide variety of plants, from the herb Arabidopsis [16] to wood *Populus trichocarpa* [17], from the monocot rice [18] to dicot cabbage [19], from non-vascular plant moss [20] to vascular plant soybean [21], from crop oat [22] to commercial ornamental flower chrysanthemum, etc. [23]. Yet, there has been no study, so far, on JcDREB (DREB from *J. curcas*) despite extensive research on its oil extraction, seed composition, proteomics under cold stress, ERF protein, detoxification of seed cake and antitumor activities of curcin from the seed [24–30]. In this paper we focus on one of the DREB family members and its function in stress condition. The results show that *JcDREB* has similar roles as those DREB genes in Arabidopsis.

2. Materials and methods

2.1. Plant materials and chemical treatments

Seeds of *J. curcas* were collected from Panzhihua, Sichuan Province, China. The process of sterilization is as following: 70% ethanol for 5 min, 0.1% $HgCl_2$ for 10 min. Following rinsing four times with sterile distilled water, cotyledons were obtained and placed in 100 mL flask containing 40 mL sterile MS medium and 0.8% (w/v) agar (pH 5.8). Three days later, the rooted cotyledons were transferred into pots with 1:1 (v/v) vermiculite: peat medium and incubated at 28 °C with a 16 h light/8 h dark for one month. Leaves from one-month old plants (with 2–3 true leaves) were used for Northern blot. All stress treatments (salt, dehydration, and abscisic acid (ABA) treatments) were performed as follows: dipped root of seedlings into a beaker containing 200 mL solution of 300 mM NaCl, 20% PEG, and 100 μM ABA. The control roots were dipped into water. For chilling treatment, seedlings were put into a 4 °C growth chamber and the controls at 28 °C with light conditions as mentioned above. After treatments, leaves were harvested and frozen immediately in liquid nitrogen for further analyses.

2.2. DNA and RNA gel blot analyses

CTAB method was used for DNA extraction. Genomic DNA (about 20 μg) was digested completely with 20 U of restrictive enzymes *Bam*HI, *Xba*I, *Eco*RI, and *Hind*III (all of these enzymes together or separately) overnight, fractionated on a 1.0% agarose gel, and then blotted onto the Hybond-N^+ nylon membrane. The membrane filter was hybridized at 68 °C with a full-length DIG-labeled *JcDREB* cDNA fragment (DIG Random Primer Kit from Roche Diagnostics). Hybridization, washing and immuno-staining were performed according to manufacturer's instructions.

Total RNA was extracted from *J. curcas* young leaves as described by Invitrogen Trizol kit. About 30 μg of total RNA was fractionated on a 1.0% formaldehyde-agarose gel and then transferred onto Hybond-N^+ nylon membrane in 20 × SSC. The following steps were the same as above.

2.3. In vivo DRE-binding activities of the JcDREB proteins

The entire coding regions of *JcDREB* and the Arabidopsis *AtDREB2* (as a positive control) were fused into the BamHI–XhoI sites of the activation domain of YepGAP vector (pAD). Transformation method is from Clontech's protocol. All constructs were transformed into the host yeast strain YM4271, which carries two reporter genes *His3* and *LacZ* under the control of *rd29A* promoter containing the DRE sequence (T**ACCGAC**AT) or mutated DRE (mDRE) sequence (TA**TTTT**CAT) according to Liu et al. [16]. Analysis of the transformed yeast cells were performed on SD medium without His plus 10 mM 3-amonotrizole, a competitive inhibitor of the HIS3 gene product, to test the expression of the HIS3 gene. According to Clontech's protocol, we analyzed β-galactosidase activity of the transformed yeast colonies by the colony lift filter assay as follows: colonies on the plate were transferred to 3 mm filter paper, and submerged in liquid nitrogen for 10 s. After the filter was thawed at room temperature, the filter was placed on top of another filter presoaked in Z buffer/X-gal solution (ingredients per 100 mL: X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 0.134 g; β-mercaptoethanol, 0.27 mL; $Na_2HPO_4{\cdot}7H_2O$, 1.61 g; $NaH_2PO_4{\cdot}H_2O$, 0.55 g; KCl, 0.075 g; $MgSO_4{\cdot}7H_2O$, 0.0246 g). Incubate the filters at 30 °C (or room temperature) and check periodically for the appearance of blue colonies.

2.4. Trans-activation assay by the yeast one-hybrid system

The constructs pLexA-*JcDREB* (whole coding region of *JcDREB*) and pLexA-GAL4 (positive control) were transformed into EGY48 strain containing p8op-*LacZ* element, following Clontech protocol. Selection media for the transformants were SD medium without Leu, Ura and His. After three days of growing at 30 °C on selection media, transformants were analyzed to determine the transcription activation of JcDREB protein according to the expression of the *LacZ* gene on the colony lift filter using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

2.5. Transgenic Arabidopsis with JcDREB over-expression

The coding region of *JcDREB* was fused into plant over-expression vector p3301-121 under the control of the CaMV35S promoter. The construct was then transformed into Arabidopsis by Agrobacterium-mediated floral dip method [38]. Wild type Arabidopsis was named CK, while the transgenic *JcDREB* Arabidopsis was represented as D1, D2, etc. indicating different transgenic lines.

2.6. Analysis of stress tolerance in transgenic JcDREB plants

Four-week old wild type and transgenic seedlings were transferred to MS medium containing 200 mM NaCl, and allowed to grow under 16 h white light/8 h dark at 23 °C for one week. The results were observed and photos were taken. Leaves were cut from wild type and transgenic *JcDREB* plants and floated in MS liquid medium with 300 mM NaCl for 48 h under light at 23 °C.

Plants were grown in 8-cm pots of 1:1 (v/v) mixture of peat/vermiculite under light at 23 °C for four weeks. Four-week old plants were exposed to freezing stress at −8 °C for 20 h and then returned to 23 °C for 14 days.

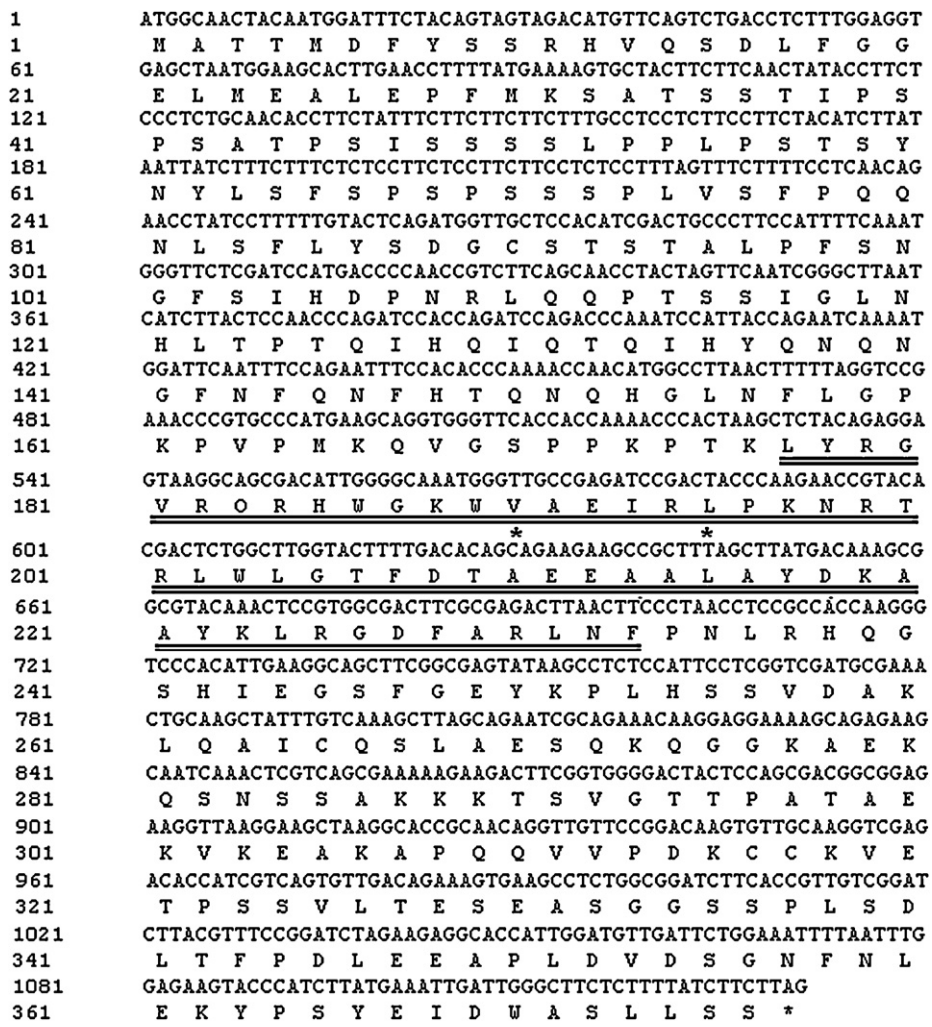

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *JcDREB*. The double black line represents AP2/EREBP domain. The asterisks mark the two residues "V" and "L" at 14th and 19th amino acid in AP2/EREBP domain. Numbers on the left indicate positions of the nucleotide and the amino acid.

3. Results

3.1. Isolation and sequence analysis of JcDREB cDNA

To isolate the genes encoding DRE-binding protein from *J. curcas*, we used low temperature (4 °C) to treat *J. curcas* when four true leaves came out. The full-length of *JcDREB* clone (Fig. 1) was 1319 bp containing 5′ and 3′ flanking regions of 68 bp and 120 bp, respectively. *JcDREB* encoded a polypeptide of 376 amino acids with a predicted molecular mass of 41.1 kDa and a p*I* of 7.85. Analysis of the deduced amino-acid sequence revealed that this protein had an AP2/EREBP DNA-binding domain of 58 amino acids that is the largest family in plant DNA binding proteins (Fig. 1). Furthermore, the AP2/EREBP domain (Fig. 1) had conserved Vla (V) and Leu (L) residues at the 14th and 19th positions, respectively. The comparison of its genomic DNA with cDNA sequence confirmed that *JcDREB* genomic DNA length is the same as that of its cDNA (data not shown). Thus, it was concluded that there should be no intron in the genomic DNA of *JcDREB*.

On the basis of the sequence alignment (Fig. 2B), JcDREB showed a very high similarity with other AP2/EREBP proteins in the AP2 domain from tomato LeDREB3 (98.28%), cotton GhDP2 (98.28%), Arabidopsis RAP2.4 (98.28%), soybean GmDREBb (94.83%) and maize ZmDBF1 (91.38%). However JcDREB was not highly homologous to the whole protein sequence of following proteins: LeDREB3, 48.59%; RAP2.4, 42.57%; GhDP2, 41.37%; ZmDBF1, 40.15% and GmDREBb, 38.51%. This result showed that JcDREB might function as a transcription factor in plants as a novel member of AP2/EREBP proteins.

Following Sakuma's et al. [12] classification of 145 AP2/ERF transcription factors in Arabidopsis, we performed a phylogenetic analysis of the DREB between JcDREB and other DREB proteins. Fig. 2A indicated that JcDREB, together with cotton GhDBP2, Arabidopsis RAP2.4, tomato LeDREB3, soybean GmDREBb and maize ZmDBF1, was classified into the A-6 group. Although there were 9 A-6 group members of the DREB subfamily in Arabidopsis and 11 in *P. trichocarpa* [12,17], there was not enough information about their DNA-binding properties, expression patterns or gene functions. Here, we provided more information about the role of A-6 group members.

3.2. DNA gel blot analysis of the JcDREB gene

The copy number of *JcDREB* in genome was estimated by southern blotting analysis (Fig. 3A). Genomic DNA isolated from young leaves was digested with *Bam*HI, *Xba*I, *Eco*RI and *Hind*III. Two major bands were detected in *Xba*I and *Hind*III digestions and one band was observed in *Eco*RI and *Bam*HI digestions (Fig. 3A). *JcDREB* was clearly a single-copy gene in the genome.

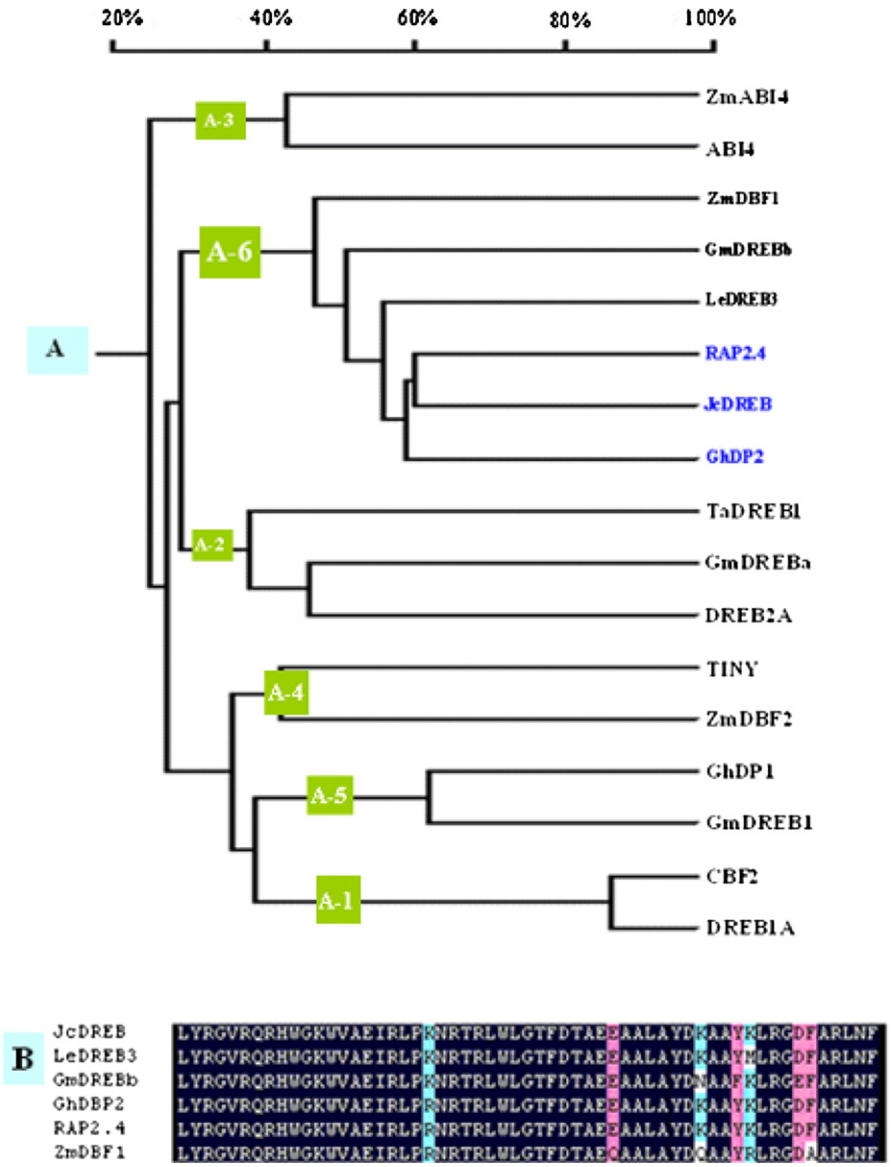

Fig. 2. Phylogenetic analysis of JcDREB with other AP2/EREBP transcription factors. (A) The phylogenetic tree of the AP2/EREBP domain was constructed by Clustal W and the scale indicates branch lengths. A-1 to A-6 indicate groups proposed by Sakuma et al. [12]. A-6 group member shows dark blue. The accession number of each protein is as follows: cotton GhDP1 (AAO43165) and GhDP2 (AAT39542); maize ZmDBF2 (AAM80485), Zm DBF1 (AAM80486) and ZmABI4 (AAM95247); Arabidopsis DREB1A (BAA33791), DREB2A (BAA33794), CBF2 (AAD15976), TINY (NP_197953), ABI4 (ABE65896) and RAP2.4 (NP_177931); rice OsDREB1C (BAA90812); soybean GmDREB1 (AAP47161), GmDREBb (AAQ57226) and GmDREBa (AAT12423); wheat TaDREB1 (AAL01124); tomato LeDREB3 (AAO13360). (B) Multiple alignment of the AP2 domain of DRE-binding protein JcDREB with the same A-6 subgroup proteins from cotton, Arabidopsis, soybean and maize. Boxes in black indicate identical amino acids. The accession numbers of each protein see (A).

3.3. Expression of JcDREB mRNA in response to various abiotic stresses

JcDREB mRNA profile in response to various abiotic stresses was analyzed by Northern-blotting assay. Four-week-old *J. curcas* seedlings were subjected to four separate abiotic stresses: low temperature (4 °C), 300 mM NaCl, 20% PEG6000, and 100 μM abscisic acid (ABA), and treated for serial time periods and transcript levels were examined at each time point. As shown in Fig. 3B, under cold treatment, *JcDREB* expression increased within 30 min, expressed strongly in 2 h and 6 h, and then slightly decreased in 24 h. This was consistent with the observation that *J. curcas* grew well in low temperatures and even could withstand a light frost [2]. For treatment with 300 mM NaCl and PEG6000, the expression pattern was almost the same. Increased expression started at 0.5 h and continued to increase for 24 h. Salt and drought expression pattern is a good explanation as to why *J. curcas* can grow on poor and dry land. However, the expression of *JcDREB* gene did not change significantly under ABA treatment. The results suggested that *JcDREB* responds to salt, drought and cold stresses via an ABA-independent pathway at transcriptional level.

3.4. DRE-binding and trans-activation activity of JcDREB in yeast

To analyze the function of JcDREB protein *in vivo*, the entire coding region of *JcDREB* was fused into yeast expression vector pYepGAP, and the recombinant plasmid was transformed into yeast cells carrying wild type DRE or mDRE motif (Fig. 4A). In DRE yeast strains, transformants harboring pAD-JcDREB or pAD-AtDREB2 (positive control) could grow on SD media minus His

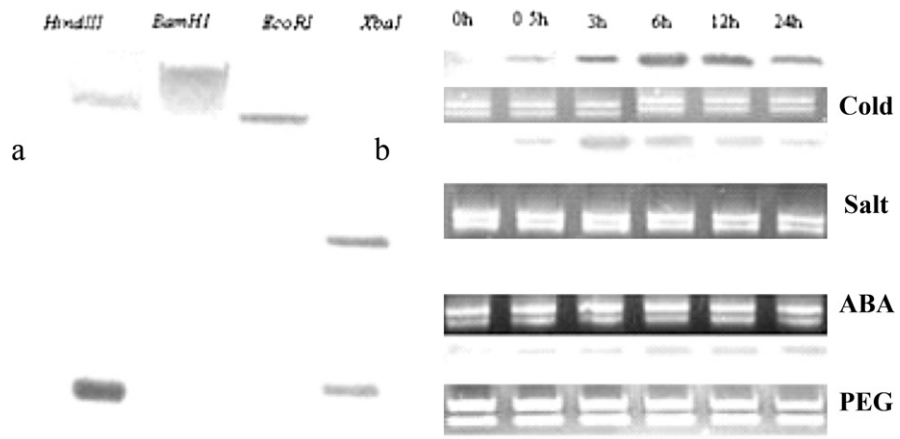

Fig. 3. DNA and RNA blot analysis of *JcDREB* cDNA in *J. curcas*. DNA blot analysis of *JcEREB* gene. Genomic DNA of the leaves of *J. curcas* was digested with *Bam*HI, *Xba*I, *Eco*RI, and *Hind*III (*JcDREB* gene containing *Xba*I and *Hind*III restriction sites), respectively. The full-length *JcDREB* cDNA was labeled with a digoxygenin as a probe. Expression patterns of the JcDREB gene in response to various treatments. Northern analysis was performed with total RNA (30 μg) isolated from the leaves of *J. curcas* seedling exposed to low temperature (4 °C), 300 mM NaCl, 100 μM abscisic acid (ABA) and 20% PEG6000, respectively marked as a, b, c, d. The entire coding region of *JcDREB* was labeled with a digoxygenin as a probe. To ensure equal loading of RNA samples, ribosomal 28s and 18sRNA in the gel was stained with ethidium bromide. Filters were washed under high stringency condition.

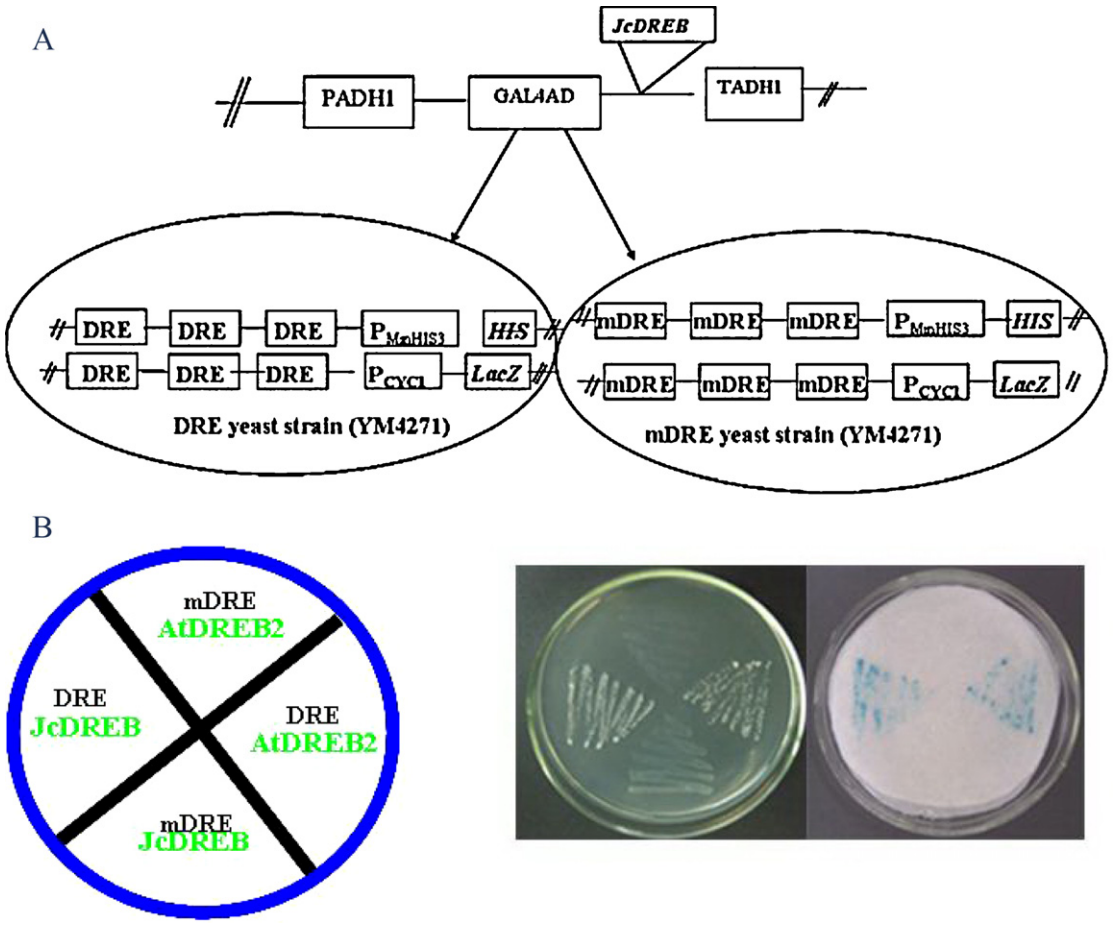

Fig. 4. JcDREB transactivated the dual reporter genes in the yeast one-hybrid system. (A) Construction of pAD-JcDREB plasmids. The encoding region of *JcDREB* was fused to the activation domain of GAL4, and then the plasmids were transformed into yeast cells harboring DRE-controlled or mDRE-controlled reporter genes, respectively. P_{ADH1} is the promoter of the *ADH1* gene; T_{ADH1} is the terminator of the *ADH1* gene. (B) The *middle plate* indicates the transformed yeast cells grow on SD medium lacking His in the presence of 10 mM 3-aminotriazole at 30 °C and the *right plate* for β-galactosidase activity. The expression of *AtDREB2* was used as control. The *left figure* shows the position of each transformed yeast cell, the *around circle part* demonstrate the DRE motifs and the mDRE motifs, and the *inside circle part* indicates yeast cells harboring AtDREB2 proteins and JcDREB proteins.

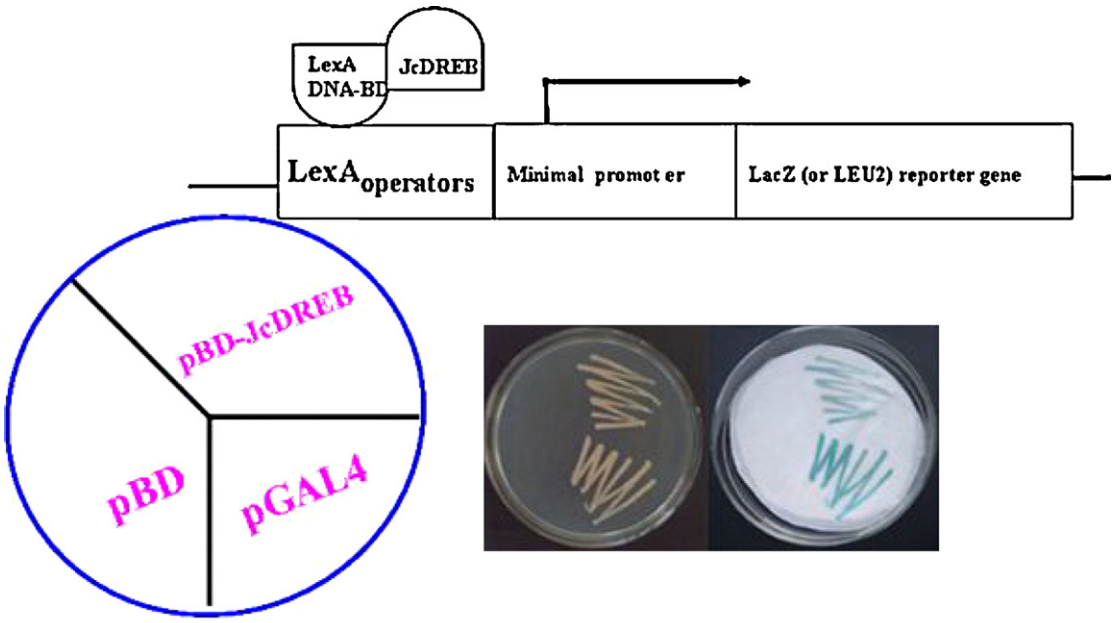

Fig. 5. Transcription activation of JcDREB protein in yeast. (A) The encoding region of JcDREB were fused to the LexA DNA binding domain (LexA) in the yeast expression vector and transformed into yeast strain EGY48 (*p8op-LacZ*). (B) Heterogenous expression of the *JcDREB* gene in yeast strain. The *left panel* shows the position of each transformed yeast cell; the *middle panel* indicates the growth of yeast cells on SD medium lacking Leu, Ura, His and the *right panels* for the assay of *β*-galactosidase activity.

in the presence of 10 mM 3-AT, whereas the transformants of mutant DRE yeast carrying the same plasmids could not (Fig. 4B). Together with the results of β-galactosidase activity assays, it indicated that *JcDREB* could specifically bind the DRE element and activate the transcription of the downstream *His3* and *LacZ* in yeast cells.

To investigate the transcription activation activity of JcDREB, we fused the coding regions of *JcDREB* with the LexA DNA-binding domain of vector pLexA (Fig. 5A) and transformed the recombinant plasmid pLexA-BD-JcDREB into yeast EGY48 strain. This strain has autonomously replicating reporter plasmid p8op-*lacZ*. Yeast cells with plasmids pLexA-BD-JcDREB or pLexA-BD-GAL4 (as a positive control) could grow well on SD media lacking Leu, Ura and His and showed blue colour by colony-lift filter assay of β-galactosidase (Fig. 5B), while cells harboring plasmid pLexA-BD (as a negative control) could not. It suggested that JcDREB protein functioned as a transcription activator in yeast.

3.5. Constitutive expression of JcDREB in transgenic Arabidopsis enhances salt and freezing tolerance

Several reports have shown that ectopic expression DREB family genes in plants could enhance the tolerance to abiotic stresses, such as dehydration, drought, freezing, salinity, and heat [16,35], as well as biotic stress such as virus infection [18]. To determine whether *JcDREB* was involved in the stress pathway, transgenic Arabidopsis was developed with constitutively ectopic expression *JcDREB* controlled by CaMV35S promoter. Compared to wild type, we investigated the tolerance to salt and freezing stresses in 35S::JcDREB plants (Fig. 6). For salt tolerance, leaves of wild type were bleached either in 300 mM NaCl solution or MS medium containing 200 mM NaCl, while the leaves of transgenic *JcDREB* plants kept some green (Fig. 6a). As observed, under the freezing condition, all wild-type plants died, but the 35S::JcDREB plants were tolerant to freezing stress (31.6% survived; Fig. 6b). These results indicated that *JcDREB* conferred freezing and salt tolerances to transgenic plants.

4. Discussion

AP2/EREBP transcription factor is the largest family in plant and most of them have important regulatory functions in environment or biotic stress response and plant development [3–11]. In this study, we isolated and identified *JcDREB* from woody oil plant *J. curcas*. Studying the molecular mechanisms of *JcDREB* responses to environmental stresses help us to improve plants' tolerance. DREB transcription factors could be classified as DREB1 and DREB2 group, based on the similarity to AP2 domain of DREB-type proteins in Arabidopsis [39,40]. We found JcDREB contains a conserved AP2 domain. Analysis of the deduced amino-acid sequence of JcDREB revealed that it had an AP2/EREBP DNA-binding domain which had conserved Vla (V) and Leu (L) residues at the 14th and 19th positions, respectively.

In *J.curcas*, JcDREB can specifically bind to the DRE element T**ACCGAC**AT and further activate transcription of downstream stress responsive genes as shown in our one-hybrid assay, suggesting that JcDREB belongs to the DREB family.

JcDREB expression level was changed in response to salt, drought and cold stresses, which was somewhat similar to *LeDREB3*, a member of group A-6. *LeDREB3* gene expression was significantly induced by NaCl, drought, cold and H_2O_2, but not by exogenous ABA treatment [31]. According to current research, it seemed that certain A-6 sub-group members were induced by NaCl and cold, while other A-6 members were induced by more stress signals such as high salinity, drought, low temperature and biotic stresses [31–33]. However, it was complex for the ABA dependent or independent pathways. For example, A-6 group members *GhDP2* and *ZmDBF1* were strongly induced by ABA treatment, while *JcDREB, LeDREB3* and *RAP2.4* were non-responsive to ABA treatment [31–34]. Moreover, *RAP2.4* also mediates light and ethylene signaling pathways [34].

According to this report and previous studies, we knew that many DREB genes were involved in stress tolerance and plant development, such as A-1 group member *DREB1*, A-2 group member *DREB2*, A-3 group member *ABI4*, A-4 member *ZmDBF2*, A-5 member *GhDBP1*, and A-6 member *RAP2.4* [16,32–34,36]. However,

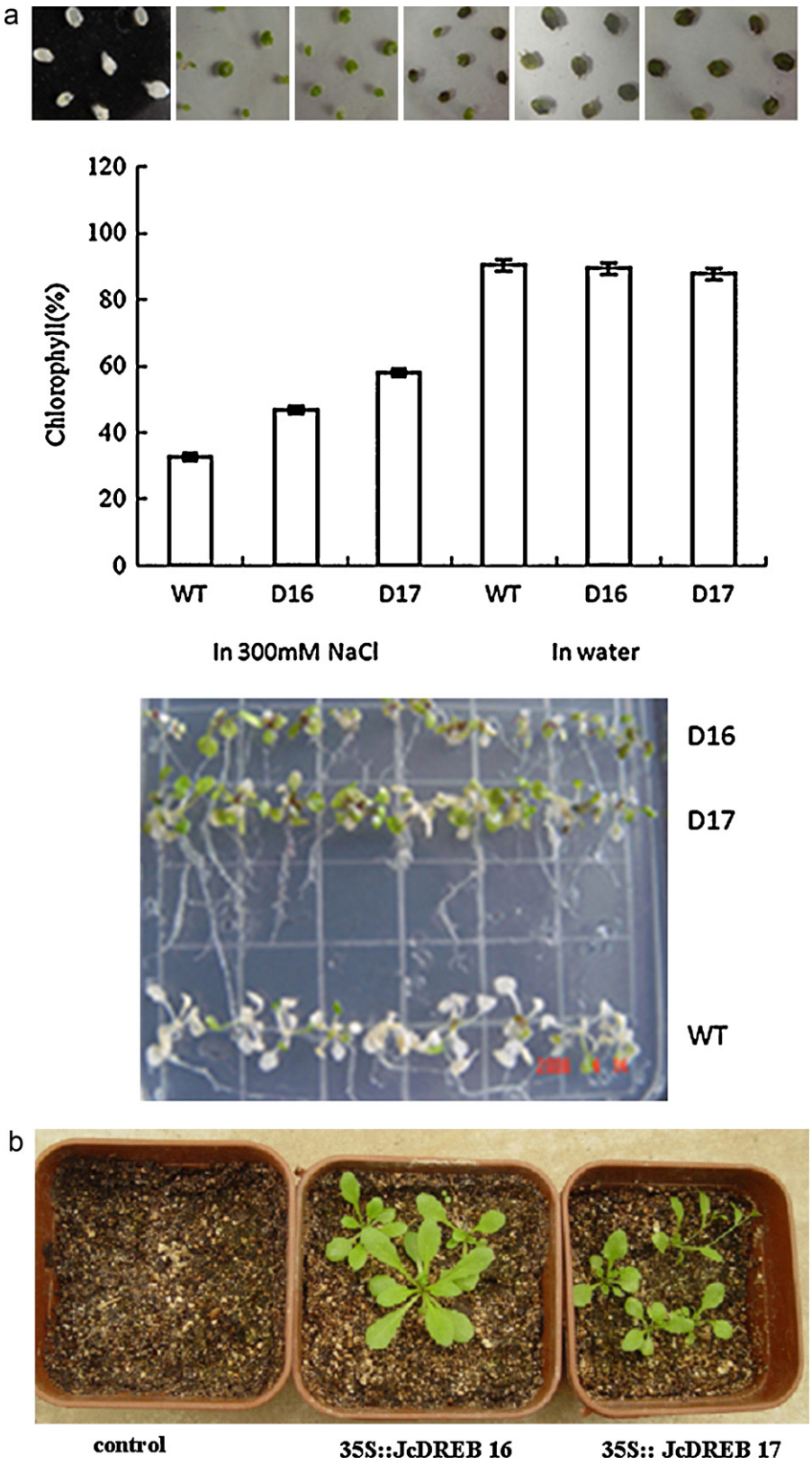

Fig. 6. Transgenic Arabidopsis plants (*35S::JcDREB*) under freezing and salt stresses. (A-a) For salt stress, 4-week-old plants were gently pulled out of the normal MS medium and transferred into MS medium containing 200 mM NaCl. (b) Cut leaves from 4-week-old transgenic and wild-type plants, then floated them in 300 mM NaCl MS medium for 48 h under light at 23 °C. (B) For freezing stress, 4-week-old plants stayed at −8 °C for 20 h and sent back to 23 °C for 2 weeks.

there might exist different mechanisms to mediate stress tolerance. For example, Arabidopsis *DREB1* and *DREB2* are induced by cold or dehydration through activation of the expression of *rd29A*, but induction of *rd29A* expression by exogenous ABA treatment was not inhibited, suggesting that their function was independent of ABA-pathway [16]. Similarly, RAP2.4 and GhDBP1. RAP2.4-overexpressing plants had higher drought and salinity tolerance and could mediate light and ethylene signaling independent on

ABA pathway [34]. *abi4* was A-3 group member mutant, which had decreased sensitivity to ABA inhibition of germination and altered seed-specific gene expression, showing the role for ABI4 in regulating seed responses to ABA and/or seed-specific signals. Maybe this function was because *abi4* mutant altered several genes' expressions, such as *Em6* and other late embryogenesis genes, sugar modulated gene *ApL3*, and photosynthesis-related gene plastocyanin [37]. However, tranformation with different DREBs may lead to various abiotic tolerances in transgenic plants. For example, over-expression of Arabidopsis CBF1/DREB1B gene could improve the drought tolerance in transgenic tomato [41]. Also, over-expressing ZmDBF2 enhanced tolerance to salt and drought stresses in transgenic Arabidopsis due to activate transcription regulation in ABA-dependent pathway [32]. Over-expression of rice gene OsDREB1F could increase drought, salt and low temperature tolerance in rice and Arabidopsis [42]. Chen et al. (2008) found that over-expression of OsDREB gene leaded to enhanced drought tolerance in rice [43]. These results suggest that, although all DREBs share high sequence homology in AP2 domain and are induced by stresses, it is necessary to study the specific expression profile and role in stress tolerance of each gene. This is different from a previous report which suggested that some DREB genes may function in a conserved way, though the structure of DREB family proteins are well conserved in plants [43]. So if we want to know more functions of A-6 group members of DREB family, we need study more members. Therefore, a lot of work is still needed for the characterization of more A-6 group genes to obtain a better comprehensive overall picture of DREB regulation.

To sum up, the tolerance to salt and freezing stresses in JcDREB over expressing plants was significantly improved compared to wild type (Fig. 6), thus over-expression of JcDREB protein in Arabidopsis can improve the tolerance to salt and freezing stresses, and this strategy might be adopted to develope new *J. curcas* species with high tolerance to abiotic stresses.

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