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RESEARCH ARTICLE

Molecular cloning and characterization of two novel *DREB* genes encoding dehydration-responsive element binding proteins in halophyte *Suaeda salsa*

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Abstract The dehydration-responsive element-binding (DREB) proteins play an important role in regulating expression of stress-inducible genes under abiotic stresses. In this study, two genes encoding putative DREB proteins, named *SsDREBa* and *SsDREBb*, were cloned from halophyte *Suaeda salsa* L. using RACE method. The deduced SsDREBa and SsDREBb proteins contain a typical AP2/ERF domain. Multiple sequence alignments and phylogenetic analysis revealed that the two *SsDREB* genes of *S. salsa* were highly similar in AP2/ERF domains at the nucleotide and amino acid levels and belong to the A-6 subgroup of the DREB transcription factor subfamily. A subcellular localization assay showed that both SsDREBs localized to the nucleus. Yeast one-hybrid experiments testified that both proteins were able to specifically bind to the DRE sequence and activate the expression of the downstream *HIS* reporter gene in yeast. Quantitative real-time PCR analysis demonstrated that under normal conditions, the expression level of *SsDREBa* was the most high in the roots and no *SsDREBa* mRNAs were detected in the stems; *SsDREBb* expressed at relatively higher levels in the leaves than in the roots and stems. The expression of *SsDREa* and *SsDREBb* genes in *S. salsa* roots and leaves was remarkably induced by high-salt and dehydration treatments, but not by cold and ABA, and exhibited stronger induction in roots and leaves, respectively. These results indicate that the *SsDREBa* and *SsDREBb* are novel stress-responsive transcription factors, which are involved in the drought and high-salt stress responses through ABA-independent pathways and could be used for production of stress-tolerant transgenic crops.

Keywords *Suaeda salsa* L. · DREB transcription factors · Abiotic stresses · Subcellular localization · Yeast one-hybrid · Quantitative real-time PCR

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Introduction

Various abiotic stresses such as drought, salinity and extreme temperature heavily affect plant growth and crop yields (Mahajan and Tuteja 2005). In order to survive under harsh environments, plants have evolved complex molecular mechanisms to sense and adapt to the environmental changes. Transcription factors (TFs), which bind to specific cis-acting elements in the promoters of downstream genes, play central roles in regulating the expression of stress- responsive genes and consequently enhance stress tolerance of plants (Yamaguchi-Shinozaki and Shinozaki, 2006; Agarwal and Jha 2010; Huang et al. 2012).

To date, various types of TFs involved in plant abiotic responses have been isolated from higher plants and most of them are grouped into several large families, such as b-ZIP, WRKY, MYB, MYC, NAC and AP2/ERF (Umezawa et al. 2006; Agarwal et al. 2006). According to the sequence similarity and number of AP2/ERF domains, the AP2/ERF TFs, which constitute a large superfamily, are

further divided into five subfamilies including AP2, RAV, ERF, DREB and others (Sakuma et al. 2002; Nakano et al. 2006). The dehydration responsive element binding TFs (DREBs), which specifically bind to DRE elements existed in the promoters of stress-responsive genes, are activated by drought, salt and cold stress in many plant species (Magnani et al. 2004; Agarwal et al. 2006; Chen et al. 2007; Gao et al. 2009). Since the first *DREB* gene *CBF1* was isolated from *Arabidopsis thaliana* (Stockinger et al. 1997), many *DREB* genes have been cloned and characterized in different plants, such as wheat (Xu et al. 2008), barley (Choi et al. 2002; Xu et al. 2009), rice (Tian et al. 2005; Zhang et al. 2013), soybean (Chen et al. 2007; Marcolino-Gomes et al. 2013), cotton (Huang and Liu 2006; Huang et al. 2008), *Populus euphratic*a (Chen et al. 2009a), *Caragana korshinskii* (Wang et al. 2010), *Medicago falcate* (Niu et al. 2010), chicory (Liang et al. 2014), and *Broussonetia papyrifera* (Sun et al. 2014).

DREBs contain a conserved DNA binding AP2/ERF domain, which consists of a three-stranded β-sheet and one α-helix running almost parallel to the β-sheet (Magnani et al. 2004; Lata et al. 2011). In the β-sheet of AP2/ERF domains, two conserved functional amino acids (valine and glutamic acid) at the 14th and 19th residues, respectively, are thought to be crucial sites for consensus recognition and binding of DREBs to the DRE core sequence (Liu et al. 1999; Sakuma et al. 2002). However, recent studies have indicated that the 19th glutamic acid (Glu) residue was found not to be conserved in some plant species and be replaced by valine and leucine (Dubouzet et al. 2003; Agarwal et al. 2006; Wang et al. 2011; Zhou et al. 2012). The core DRE element contains a TACCGACAT sequence, which is essential for specific interaction with DREBs in response to cold, drought, and/or high-salt treatments (Yamaguchi-Shinozaki and Shinozaki 2006, 2009).

DREB genes form a large multigene family and can be divided into six small subgroups (subgroups A1–A6), with A-1 and A-2 constituting the two largest groups (Sakuma et al. 2002). The A-1 subgroup is a major regulator of cold stress responses (Agarwal et al. 2006). For example, about 12–20 % of cold-induced genes in *Arabidopsis thaliana* can be activated by *AtCBF1-3* (Van Buskirk and Thomashow 2006). But recently, it was reported that some *DREB1*s are also responsive to other external stimulations due to the crosstalk between low temperature and other abiotic stresses (Wang et al. 2011; Yang et al. 2011; Jiang et al. 2011). Overexpression of *DREB1A* from *Arabidopsis* in rice plants resulted in improved tolerance to drought and salinity (Oh et al. 2005). Most of the *DREB2* (A-2) genes are regulated by salt, water stress or heat shock, but not by cold (Dubouzet et al. 2003; Liu et al. 1998; Nakashima et al. 2000). However, recent reports have shown that some A-2 group genes are also induced by ABA or cold (Xu et al. 2008; Chen et al. 2009a; Matsukura et al. 2010), indicating crosstalk between different groups. The overexpression of *SbDREB2A* improved salt and drought tolerance in transgenic tobacco plants and increased the level of expression of downstream stress-related genes under stress conditions (Gupta et al. 2014). Other *DREB* subgroup genes have also been reported to be stress responsive and/or to impart stress tolerance in transgenic plants. Such as *TINY2* (A-4), *PpDBP1* (A-5), *ZmDBF1* (A-6) and *CkDBF* (A-6) were identified as stress-response regulation genes (Wei et al. 2005; Kizis and Pages 2002; Liu et al. 2007; Wang et al. 2010). Overexpression of *HARDY*, an A-4 subgroup AP2/ERF gene from *Arabidopsis*, enhances salt and drought tolerance in transgenic *Trifolium alexandrinum* L. (Abogadallah et al. 2011). Overexpression of *PpDBF1* induces expression of stress-related genes and improves tolerance to high salinity in transgenic tobacco (Liu et al. 2007). Overexpression of *CkDBF* in transgenic tobacco plants resulted in higher tolerance to osmotic and high salinity stresses and induction of a downstream stress-responsive gene under normal conditions (Wang et al. 2010). However, up to now, most reports about DREBs focused on A-1 and A-2 groups, limited investigation was carried out in other groups. Due to multiple and complicated roles of members of the *DREB* family in plant acclimation to stress environment, a functional analysis of each transcription factor belonging to this family should be carried out. To date, many *DREB* genes have been isolated and characterized in a wide variety of glycophytes (Xu et al. 2011). However, only a few studies regarding *DREB* TFs have been carried out on halophytes like *AsDREB* from *Atriplex halimus* (Khedr et al. 2011), *SsDREB2A* from *Salicornia brachiata* (Gupta et al. 2010), *PpDBF1*from *Physcomitrella patens* (Liu et al. 2007) and *AhDREB1* from *Atriplex hortensis* (Shen et al. 2003a).

The Chenopodiaceae *Suaeda salsa* L., a C3 euhalophyte, is one of the most important halophytes in China for both industrial application and scientific research (Wang et al. 2001; Zhao et al. 2002). *S. salsa* is a native halophyte in the Bohai coast and can grow in the intertidal zone where soil salt reaches up to 3 %. In contrast to some other halophytic plants, *S. salsa* does not have salt glands or salt bladders on its leaves. Treatment of *S. salsa* with 200 mM NaCl significantly increased its growth and net photosynthetic rate (Pang et al. 2005). However, the salt-stress-tolerance signal-regulating mechanisms in this species are poorly understood. The reports about isolating and studying the genes in response to salt stress in *S. salsa* are limited (Guo et al. 2006; Pang et al. 2011; Li et al. 2011) and none *DREB* homologues have been identified from *S. salsa* up to now. In the present work, the isolation and characterization of two *DREB*-like genes from *S. salsa* were reported. Their

subcellular localizations, DRE-binding and transcriptional activation activities and expression patterns under abiotic stresses were investigated. The results of this study will enable better understanding the regulatory mechanisms of abiotic stress response in *S. salsa.*

Materials and methods

Plant materials and stress treatments

Seeds of *S. salsa* were grown in plastic pots containing sand and watered daily with Hoagland nutrient solution according to Pang et al. (2005). The seeds germinated under a light intensity of 400–420 μmol m^{-2} s^{-1} during a 16-h light/8-h dark cycle at 20/25 °C for 2 weeks. For the stress treatments, six-week-old seedlings after germination were carefully removed from soil to avoid injury and hydroponically grown in Hoagland nutrient solution containing 400 mmol/L (2.3 %) NaCl (salinity stress), 20 % PEG (dehydration stress) or 100 μmol/L ABA, respectively, for 0, 0.5, 2, 4, 8, 12 and 24 h. For the cold-stress treatment, potted plants were transferred to a growth chamber at 4 °C for 0, 0.5, 2, 4, 8, 12 and 24 h. In order to get reliable results, the *S. salsa* seedlings of consistent growth were subjected to each series of treatments and the un-treated *S. salsa* seedlings were used as control. Excised leaf and root samples of plants subjected to different treatments were harvested and immediately frozen in liquid nitrogen and stored at −80 °C for extraction of DNA or total RNA and qRT-PCR assay. All experiments were repeated in biological triplicate.

Gene cloning and sequence analysis

Genomic DNA was isolated from seedling with Plant genomic DNA Extraction Kit (Takara, Dalian, China). Based on the alignment of amino acid sequence encoded by the known *DREB/CBF* genes from various plants available in the GenBank database, a pair of degenerate primers was designed to amplify the conserved AP2/ERF domain of *DREB* genes in *S. salsa*. The forward primer DREB-S was 5′-TGGGGG(T)AAG(A)TGGGTC(T)GCC(A/T)GAA(G)ATC(T)-CG-3′ and the reverse primer DREB-AS was 5′-ACG(A/T)GAG(A/T)GAG(A)TGG(A/T/C)AG-A(T)GGC(T)TTG(A)TA-3′, corresponding to the amino acid sequences WGKWVAEI and YKPLHSSV, respectively. Then, a 180-bp fragment was amplified by PCR with the genomic DNA as template and DREB-S and DREB-AS as primers and sequenced. After sequence analysis, two *DREB/CBF* DNA fragments from *S. salsa* were obtained and named *SsDREBa* and *SsDREBb*, respectivly. The sequence information of the two DNA fragments was then used to design the primers for obtaining the full-length sequences of the two genes.

Total RNA was isolated from leaves of *S. salsa* treated with 400 mM NaCl for 6 h with a Plant RNA Kit (Promega, USA) and the contaminant DNA has been removed with DNase I in the RNA extraction process. The isolation of the full length cDNA sequences was carried out using the RNA ligase-mediated rapid amplification of 5′ and 3′ ends (RLM-RACE) method, according to the GeneRacer Kit (Invitrogen, USA). The cDNA pools for 5′- and 3′-RACE (Rapid Amplification of cDNA Ends) were generated from 2.5 μg of total RNA by Superscript II Reverse Transcriptase using the ligated mRNA as template and the GeneRacer Oligo (dT) 3′ AP as primer according to the manufacturer's protocol (Supplementary Table S1). For *SsDREBa* 5′-RACE, two gene-specific nested primers SsDa5′ GSP1 and SsDa5′ GSP2 were designed based on the known partial sequence. The first PCR was performed on the cDNA pool using SbDa5 'GSP1 and adapter-specific primer 5′ AAP (Supplementary Table S1). The amplification products of the first PCR was diluted ten times as the template and SbD5′ GSP2 and adapter-specific primer 5′ AUAP were used as the primers for the second PCR (Supplementary Table S1). For *SsDREBa* 3′-RACE, The first round PCR was done using gene-specific primers SsDa3′ GSP1 and the adapter-specific primer 3′AUAP by PCR (Supplementary Table S1). The second PCR amplification was performed on the products of the first PCR using SbDa3′GSP2 and 3′AUAP (Supplementary Table S1). Two gene-specific nested primers for *SsDREBb* 5′-RACE were SsDb5′ GSP1 and SsDb5′ GSP2 and those for *SsDREBb* 3′-RACE were SsDb3′ GSP1 and SsDb3′ GSP2 (Supplementary Table S1). Amplification was all done in the following conditions: 1 cycle of 95 °C for 3 min, 35 cycles of 94 °C for 1 min, 58 °C for 45 s, and 72 °C for 1 min 10 s, followed by a 10 min final extension at 72 °C.

The 5′- and 3′-RACE fragments of each gene were cloned into separate pGEM-T Easy plasmid vectors (Promega, USA) and sequenced. The full-length cDNA sequences were obtained by combining the 5′ and 3′ end sequences with an overlap fragment using DNAMAN software. Full-length cDNAs of *SsDREBa* and *SsDREBb* were individually amplified using primers SsDa-S, SsDa-AS and SsDb-S, SsDb-AS (Supplementary Table S1). The amplified sequences were separately subcloned into a pGEM-T Easy plasmid vector. The full-length nucleotide sequences of the two *S. salsa DREB*s were sequenced and submitted to GenBank. Sequence analyses were performed using the program BLASTX (National Centre for Biotechnology Information, USA). The ORF of *SsDREB* genes and the properties of protein encoded by them were predicted by DNAStar software. The subcellular localization was predicted using ESLPred (http://www.imtech.res.in/raghava/eslpred/). Multiple sequence alignment

was performed employing Clustalx and GeneDoc softwares. A phylogenetic neighbour-joining analysis was conducted on deduced amino acid sequences using the program MEGA 5.1.

Subcellular localization of SsDREBa and SsDREBb proteins

*SsDREB*s were combined with green fluorescent protein (GFP) to yield fusion proteins. The entire coding sequences of *SsDREBa* and *SsDREBb* were individually amplified by PCR with primers SsDa-S, SsDa-AS and SsDb-S, SsDb-AS, and then subcloned into pXDG-vector (Chen et al. 2009b) to obtain expression vectors pXDG-GFP-SsDREBa and pXDG-GFP-SsDREBb. Particle bombardment was performed to introduce the two fusion constructs into onion epidermal cells as previously described (Chen et al. 2002) and the plasmid pXDG-vector containing the *GFP* open reading frame (ORF) alone under the control of 35S promoter was used as the negative control. The bombarded epidermal peels of onion were cultured on Murashige–Skoog (MS) medium at 25 °C for 16–24 h in the dark and GFP fluorescence in the onion epidermal cells was observed under a laser confocal scanning microscope (LSM 510; Zeiss, Germany) at a wavelength of 488 nm.

DRE-binding and transactivation assay of the SsDREBa and SsDREBb proteins in yeast

The yeast one-hybrid experiment was performed to examine DNA binding and transcriptional activation activities of *SsDREB*s. Three tandem repeats of the core sequence of the DRE (5′-TACCGACAT-3′) and its mutant sequence (mABRE: 5′-TATTTTCAT-3′) were cloned into the *Sac* I/*Spe* I restriction sites of the yeast pHIS2.1 cloning reporter vector upstream to the *HIS3* minimal promoter according to the protocol described by Clontech (Clontech, Mountain View, CA, USA). The entire coding regions of *SsDREB*s were separately cloned into the *Xho* I and *Sma* I sites of the YepGAP expression vector containing no GAL4 activation domain (AD) (Liu et al. 1998). The recombinant YepGAP expression vector containing *SsDREBa* or *SsDREBb* cDNAs and the pHIS2.1 vector containing three tandem repeats of the DRE or mDRE were co-transformed into the yeast strain Y187. The growth status of the transformed yeast cells was compared on SD/-His/-Ura/-Trp/+ 10 mM 3-AT plates to test the expression of the *HIS* reporter gene. Empty YepGAP was used as a negative control.

Gene expression analysis by real-time quantitative PCR

Total RNA was extracted from harvested leaves and roots using total RNA extraction kit (Promega, USA) and the contaminant DNA has been removed with DNase I in the RNA extraction process. Using PrimeScript RT reagent Kit (Takara, Dalian, China), cDNAs were produced on 1 μg of each RNA sample according to the manufacturer's instructions. The gene specific primers for qPCR were designed using Oligo 7.0 program (MBI Inc, Cascade, CO) (Supplementary Table S2), which excluded the highly conserved AP2 domain and had high efficiency and specificity, and were detected by agarose gel electrophoresis and a Roche 2.0 Real-Time PCR Detection System. Before proceeding with the actual experiments, a series of template and primer dilutions were tested to determine the optimal template and primer concentration for maximum amplification of the target during the experiments. In all of the experiments, appropriate negative controls containing no template RNA were subjected to the same procedure to exclude or detect any possible contamination. Each sample was amplified by qRT-PCR using a Roche 2.0 Real-Time PCR Detection System with the SYBR Green Supermix (Takara, Dalian, China). Each 20-μl reaction contained 10 μl SYBR Premix Ex Taq, 0.4 μl each of 10 μM primers, 0.4 μl ROX Reference DyeII (50×), 2.0 μl cDNA template, and 6.8 μl dd H_2O. The amplification program was as the following: 30 s denaturation at 95 °C, then 45 cycles of 5 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. *Actin* gene of *S. salsa* (GenBank accession no. FJ587488) was used as the internal control to normalize the amount of cDNA in the qPCR reaction. All PCRs were replicated at least three times. The resulted PCR products by all the primers were subjected to sequence to confirm the specificity. The mRNA fold difference was relative to that of untreated samples used as calibrator. The relative expression level of genes was calculated using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen 2001). The results of gene expression level are presented as a mean value of the three assay replicates. Fold changes were used for quantitative analysis.

Results and discussion

Isolation and sequence analysis of the *SsDREBa* and *SsDREBb* genes

To obtain partial sequence of the conserved AP2 domain region of the *S. salsa DREB* genes, the amino acid sequences encoded by *DREB* genes from *Atriplex hortensis* (*AhDREB*, GenBank accession no. AF274033), *Atriplex halimus* (*As-DREB*, GenBank accession no. JF451138) and *Atriplex canescens* (*AcDREB*, GenBank accession no. JN632583) were aligned using the DNAMAN 6.0 software. Based on the amino acid sequences WGKWVAEI and YKPLHSSV from conserved AP2 domain region which showed a high degree

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1      CGAATTATCTACCCTACCCTTTTCTTCAACGTTTCAAATCAATTATCGACACCTTTTCTTTTATAAATCATAATT
76     TCATATTTCAAAACACATATATGGCAGCTGCAATTGATAGATATAATAATCCCAACAACAATCCTCTTGTTTTAG
                                 M  A  A  A  I  D  R  Y  N  N  P  N  N  N  P  L  V  L
151    ATCCTCTTAGTGCGGAGCAAAACAGAGCATTCGATCCAACAAACCCTTGTTTATTCTACTCTTCAAACAACAATA
       D  P  L  S  A  E  Q  N  R  A  F  D  P  T  N  P  C  L  F  Y  S  S  N  N  N
226    ATTTTTACATGTTTGATCAAATGGGTATTGCTCAATCTCCTTCAATTGGAATCCTTGATCTTATTCCATCCCACC
       N  F  Y  M  F  D  Q  M  G  I  A  Q  S  P  S  I  G  I  L  D  L  I  P  S  H
301    AGCAGCTTCTTCCTTCATCAATGTCTCCCACTCTAAATTCAAAAACAAACGAACACTTTCTTCTTTCTCCTAAAC
       Q  Q  L  L  P  S  S  M  S  P  T  L  N  S  K  T  N  E  H  F  L  L  S  P  K
376    CTACACTCATGAAACACATCAAGCCTACAAAACTATACAGAGGAGTAAGACAGAGACATTGGGGAAAATGGGTAG
       P  T  L  M  K  H  I  K  P  T  K  L  Y  R  G  V  R  Q  R  H  W  G  K  W  V
451    CTGAGATCAGATTGCCTAAGAATCGAACTCGTCTTTGGCTCGGAACATTCCAAACTGCTGAAGAAGCTGCTTTGG
       A  E  I  R  L  P  K  N  R  T  R  L  W  L  G  T  F  Q  T  A  E  E  A  A  L
526    CTTATGATAAAGCGGCTTACAAGCTTCGAGGAGAATTCGCTCGTTTAAATTTTCCACATCTTCAACATCATGGAC
       A  Y  D  K  A  A  Y  K  L  R  G  E  F  A  R  L  N  F  P  H  L  Q  H  H  G
601    CTCATGTAACAACTCAATTTGGGCTATATAAACCTCTTCATTCTTCGGTGAATGCTAAACTTGATGCAATTTGTC
       P  H  V  T  T  Q  F  G  L  Y  K  P  L  H  S  S  V  N  A  K  L  D  A  I  C
676    AAAGTTTGGACATTTGCTTAGACCAGGGGAAAACAGACTACCCCTGTTTTCCCTCTGTTTCAACTTCTTCTAATC
       Q  S  L  D  I  C  L  D  Q  G  K  T  D  Y  P  C  F  P  S  V  S  T  S  S  N
751    CTATTATGGATTCAGCTTCATCTAGTGCTGATGTTTTGGTTCCTAAACCCGAGGTTTCGGATGAGTCGTCGTCGT
       P  I  M  D  S  A  S  S  S  A  D  V  L  V  P  K  P  E  V  S  D  E  S  S  S
826    CGGGTGCTACATGTTCACCGGAGTCCGGTGTAACATTATTGAATTTCACTGAATCTTCATCTTCCTGGAATGAAT
       S  G  A  T  C  S  P  E  S  G  V  T  L  L  N  F  T  E  S  S  S  S  W  N  E
901    CTAATGGTTACTCCTTGGAAACGATTCCTTCCCTTGAAATTGACTGGGATTCTATTTAGATTTTGTGCTTCATTT
       S  N  G  Y  S  L  E  T  I  P  S  L  E  I  D  W  D  S  I  *
976    GATCTGGAGTTTGCCTTAGTTCTATAAATTGGTGTTTTTGAAAATTTGCAGGGGTGAGGATGTATATTAGAATGA
1051   CGGAATGAATATGGGGAGTTGTTGGAAATATTATTACAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1 The full-length sequence of *SsDREBa* cDNA and deduced amino-acid. The AP2/ERF domain is *underlined*. A basic region that might function as a nuclear localization signal is shown *within box*. Two Serine-rich regions are indicated with *dashed underlines*, respectively. This sequence has been submitted to the GenBank database with accession number KM365207

of similarity among the DREBs of the various Chenopodiaceae plants, two degenerate primers (DREB-S and DREB-AS) were designed, and then an about 180 bp fragment was amplified from *S. salsa* genome DNA. Unexpectedly, we got two kinds of *DREB* sequence fragments, named *SsDREBa* and *SsDREBb*. The full-length cDNA sequences of the *SsDREBa* and *SsDREBb* genes (GenBank accession no. KM365207 and GenBank accession no. KM365208) were ultimately isolated from *S. salsa* total RNA by RACE-PCR. The isolated full-length cDNA of *SsDREBa* has 1,115 bp consisting of an ORF of 861 bp, a 5′-UTR of 95 bp and 3′-UTR of 156 bp. *SsDREBa* encodes a protein of 287 amino acid residues with a predicted molecular mass of 31.9 kDa and a calculated pI of 6.24 (Fig. 1). The *SsDREBb* cDNA is 1,475 bp in length and contains an ORF of 1,095 bp, encoding a protein of 364 amino acids with a predicted molecular mass of 39.5 kDa and a calculated pI of 6.09 (Fig. 2). The protein database searches revealed that each of two proteins contained one typical AP2/ERF conserved domain of 60 amino acids (Fig. 3). Either of AP2/ERF conserved domain was predicted to fold into a secondary structure consisted of three β-sheets and one α-helix using Anthepro software (Fig. 3). This structure may play a key role in recognizing and binding to DRE/ERF cis-acting element located in stress-responsive gene promoter (Shen et al. 2003b; Qin et al. 2004). Previous studies showed that the conserved valine residues (14th) and the glutamic acid residue (19th) in the AP2/ERF domain were responsible for determination of DNA binding specificity (Sakuma et al. 2002; Agarwal et al. 2006). Valine residues at the 14th position is absolutely conserved in many plants and determines the protein's specific binding activity, whereas the

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1     TTAGATTAAGA AAAAATGGC AGCTACAAC AATGGATAT GTTGTTTA GTAGTAATAA TAGTACCGA TCCATTTGGT
                         M  A  A  T  T  M  D  M  L  F  S  S  N  N  S  T  D  P  F  G
76    AATGAGCTCATG GAAGCTCTTT CACCTTTC ATCAAAACC AATTCATCT TCATCTTCT TCATCACCT TCTTCCACC
      N  E  L  M  E  A  L  S  P  F  I  K  T  N  S  S  S  S  S  S  S  P  S  S  T
151   ATTTC TAATAACAC TCCATCTTT TTCTTCTTT CTCTCTCCC TCAATCTTC CACCTCAT TTTCTCTCTC CTCATAC
      I  S  N  N  T  P  S  F  S  S  F  S  L  P  Q  S  S  T  S  F  S  L  S  S  Y
226   TCTCA AAATCCATC TTCTTCTTT CTACCAGGA TGGTTGCTC CTACACTGA GCAGGCCC CCTTTTTTGG GCTTACC
      S  Q  N  P  S  S  S  F  Y  Q  D  G  C  S  Y  T  E  Q  A  P  F  F  G  L  T
301   CATTC TGACCCATC TACTACACT TGGGCTTAC CACCCACTT CAACCAACC CCAATTCA CCTACCCTCA GGCCCAG
      H  S  D  P  S  T  T  L  G  L  T  T  H  F  N  Q  P  Q  F  T  Y  P  Q  A  Q
376   GCCCA GGCCCAACT ACCCATGTA CTCATACCC ACCACCTCT CCCTTCCTA CCAGTCCA CCAACATGAA CACCTTT
      A  Q  A  Q  L  P  M  Y  S  Y  P  P  P  L  P  S  Y  Q  S  T  N  M  N  T  F
451   CTCTC CCCAAAGCC CGTCCCCAT GAAGACCAC CGGCCCACC CCAAAAGCC CACGAAGC TTTACAGGGG TGTCCGC
      L  S [P  K  P  V  P  M  K  T  T  G  P  P  Q  K  P  T  K] L  Y  R  G  V  R
526   CAACG TCACTGGGG AAAATGGGT CGCTGAGAT CCGACTACC CAAGAACCG AACCCGGT TATGGCTTGG TACCTTC
      Q  R  H  W  G  K  W  V  A  E  I  R  L  P  K  N  R  T  R  L  W  L  G  T  F
601   GATAC AGCTGAAGA AGCTGCTTT GGCTTATGA CAAAGCAGC TTACAAGCT AAGAGGTG ACTTCGCCAG GCTCAAC
      D  T  A  E  E  A  A  L  A  Y  D  K  A  A  Y  K  L  R  G  D  F  A  R  L  N
676   TTCCC TAACCTTCG TCATGAAGG GTCCCACAT CGGGGGAGA ATTCGGGGA GTACAAAC CCCTTCACTC CTCAGTC
      F  P  N  L  R  H  E  G  S  H  I  G  G  E  F  G  E  Y  K  P  L  H  S  S  V
751   AACGC TAAACTCGA AGCTATTTG TGAGAGCTT AGCCAAACA GGGGAATGA AACCACCA AACAGGGGAA ATCAAAG
      N  A  K  L  E  A  I  C  E  S  L  A  K  Q  G  N  E  T  T  K  Q  G  K  S  K
826   TCAAA GAAAGTAGC TGCTTCGTC ATCAACAAC AAGTTCATC ATCTTCGTC AACTGTGA CATCATCAGC CGTGGTG
      S  K  K  V  A  A  S  S  S  T  T  S  S  S  S  S  S  T  V  T  S  S  A  V  V
901   CAGCA ACAGCAACA GCAGGAGCT GCTGCACCA CCCGGCAGA GGTGAAAGC CGAAAGCG CATCCGACAG CGAGGTG
      Q  Q  Q  Q  Q  Q  E  L  L  H  H  P  A  E  V  K  A  E  S  A  S  D  S  E  V
      *  *  *  *  *  *
976   GGATC CGGTGGATC ATCCCCGCT GTCGGACTT GACATTCGG AGACGGTGA AGAGTTAG GAGCGGAGCA ATACTTG
      G  S  G  G  S  S  P  L  S  D  L  T  F  G  D  G  E  E  L  G  A  E  Q  Y  L
1051  TTGGAG TCATGTCCAT CTCATGA GATAGATTGG GATGCTAT CTTATCATCA TCATCTTA ATGTTACAT GTTAGAG
      L  E  S  C  P  S  H  E  I  D  W  D  A  I  L  S  S  S  S  *
1126  GGAGTC TAGTTTGGTC ATTATGT TATTATAAAA TTAAAGTA AAAGTGAAGG TTGTGTTG TAAATTTTT TAGGTAG
1201  TGTTTT AAGGGAAAGG AGTAGAA ACTCTACAAT GAAGTTTT TGGAAATTGT AGGGGCTC CAAAATTTA TGTAAAC
1276  CCCATG GATGAAGCAA GTGCTTT TTTGATCTTG CCTTTGAT CCTTGATGGG TAGTCTTT TTTTTTTTT TTAATCT
1351  AATGTC TTCATTATGT TGATAGT AGAGGGTTCT CATCATCT AGGATTGTAA TTTTTATT TTTGTCATC ATTAGTC
1426  AAGAAG TAATATTATA ATTATTA TTAGTAGTAA AAAAAAAA AAAAAAAAA
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Fig. 2 The full-length sequence of *SsDREBb* cDNA and deduced amino-acid. The AP2/ERF domain is *underlined*. A basic region that might function as a nuclear localization signal is shown *within box*. Two Serine-rich regions and a Glutamine-rich region are indicated with *dashed* and *asterisked underlines*, respectively. This sequence has been submitted to the GenBank database with accession number KM365208

amino acid residues present in the 19th position may vary slightly (Cao et al. 2001). In this work, SsDREB proteins contain valine residues at the 14th position and leucine residues (not glutamic acid residues) at the 19th position in the AP2/ERF domains. The replacement has been also found in the other known DREB A-6 group proteins (Huang et al. 2008; Wang et al. 2010; Khedr et al. 2011). A valine residue replacement at the 19th position in the AP2/ERF domains is also found in DREB1-type factors from monocotyledons rice, wheat, and barley (Dubouzet et al. 2003; Qin et al. 2004). Therefore, it can be speculated that the 14th residue is more important and more conserved than the 19th residue in the regulation of the DRE-binding activity of DREB. Liu et al. (2006) reported that Ala37 which resides in the α-helix of the AP2 domain was also essential for binding with DRE and the GCC box.

Sequence analysis revealed that *SsDREBa* and *SsDREB*b shared 41.7 and 31.6 % identity at the coding nucleotide and amino acid levels. Whereas, the AP2 domains of the two *SsDREB*s were highly similar and shared 75.5 and 90.6 % identity at the nucleotide and amino acid levels, respectively. A basic region in SsDREB N-terminal region might function as a nuclear localization signal, suggesting that SsDREBs may localize to the nucleus (Figs. 1, 2).

Based on the classification of 145 AP2/ERF TFs in *Arabidopsis* (Sakuma et al. 2002), a phylogenetic analysis of the DREBs between SsDREBs and other DREB proteins was carried out. As shown in Fig. 4, SsDREBs, *Arabidopsis* RAP2.4, maize ZmDREB1, ZmDREB2, and ZmDBF1, as well as nine other DREB proteins, were classified into the A-6 group. Both DREBs from *S. salsa* had a relatively close evolutionary relationship with those from *Salicornia bigelovii* and *Atriplex halimus*, which, like *S. salsa*, belong to the Chenopodiaceae family. The SsDREBa has the highest similarity (65 %) to SbDREB from *Salicornia bigelovii*. The SsDREBb is grouped together with AsDREB from *Atriplex halimus* (79 % similarity). It seems reasonable to assume that members of the same group possess not only structural similarity and conserved function but also close relative relationship. Comparison of SsDREBs with other known DREB proteins revealed that SsDREBs was not highly homologous to

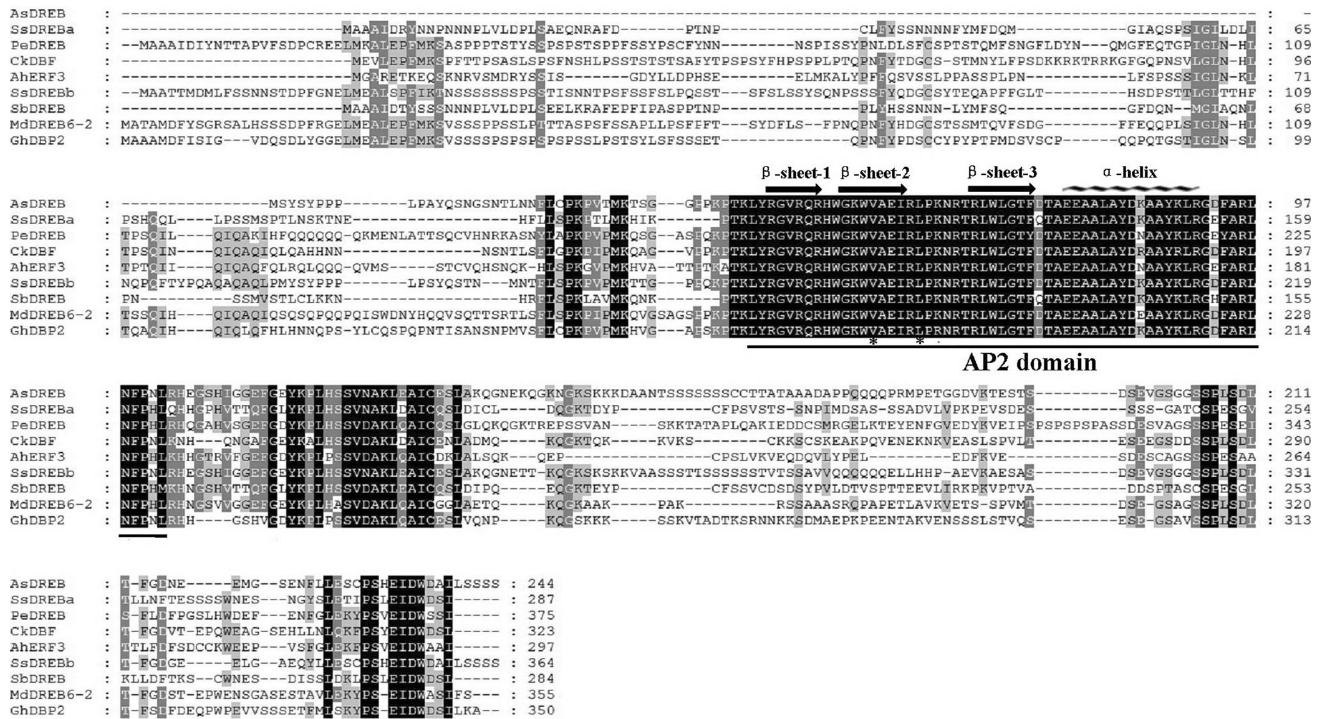


Fig. 3 Comparison of the deduced amino acid sequences of *S. salsa* SsDREBs with homologs from 7 other plants: AsDREB (JF451138); SsDREBa (KM365207); SsDREBb (KM365208); SbDREB (JF894301); CkDBF (GU573848); PeDREB (EF597499); AhERF3 (JN613348); MdDREB6-2 (JQ669823); GhDBP2 (AY619718). The conserved AP2/ERF domain is *underlined* and *asterisks* indicate the conserved valine and glutamic acid residues at positions 14 and 19 inside the motif. Three β-sheets and one amphipatic α-helix are marked over the corresponding sequences

other DREB proteins. However, they shared very high identity with other proteins in AP2/ERF domain (Fig. 3). Genomic PCR products amplified by primers designed from the 5′ and 3′ untranslated region revealed that no introns were present in either *SsDREB* gene, which is consistent with the known *DREB* genes from other plants (Niu et al. 2010; Wang et al. 2010).

Subcellular localization of SsDREBa and SsDREBb proteins

TFs were commonly expected to localize to the nucleus. Previous studies showed, NLSs (nuclear localization signals), which are required for nuclear localization, were frequently identified in the N-terminus of DREB TFs (Sakuma et al. 2002; Agarwal et al. 2006). In this present study, basic amino acid-rich regions KTNEHFLLSPKPTLMKHIKPTK and PKPVPMKTTGPPQKPTK were identified at the N-terminals of SsDREBa and SsDREBb, respectively (Figs. 1, 2). To investigate their subcellular localization, the *SsDREBa* and *SsDREBb* cDNA sequences were fused to the C-terminus of the *GFP* reporter gene respectively and empty *GFP* plasmid was used as a negative control. Then the three constructs were transferred into onion epidermal cells, respectively, through particle bombardment. As shown in Fig. 5, GFP fluorescence from hGFP::SsDREBa and hGFP::SsDREBb fusion proteins was found exclusively in the nucleus, whereas the control GFP protein was uniformly distributed throughout the whole cell. Similar results were previously reported for *BpDREB2* (Sun et al. 2014) and *CkDBF* (Wang et al. 2010). These results suggest that SsDREBs are nuclear proteins, possibly serving as transcription factors.

DRE/CRT-binding and transactivation activity of the SsDREBa and SsDREBb proteins in yeast

The DNA-binding and transactivation activities of the two SsDREB proteins were investigated using a yeast one-hybrid system. The full-length cDNAs of both *SsDREB*s were individually inserted into the yeast expression vector YepGAP with lacking transactivation domain and transformed into two Y187 yeast strains that carried the *HIS3* reporter genes under the control of the DRE or mutated DRE motif, respectively. Empty vector YepGAP was used as a negative control. Growth of the yeast strains on selective media was then analyzed. As shown in Fig. 6, yeast cells harboring the wild-type DRE motifs and either of the *SsDREB*s grew well on SD media lacking histidine in the presence of 10 mM 3-AT, whereas the yeast cells

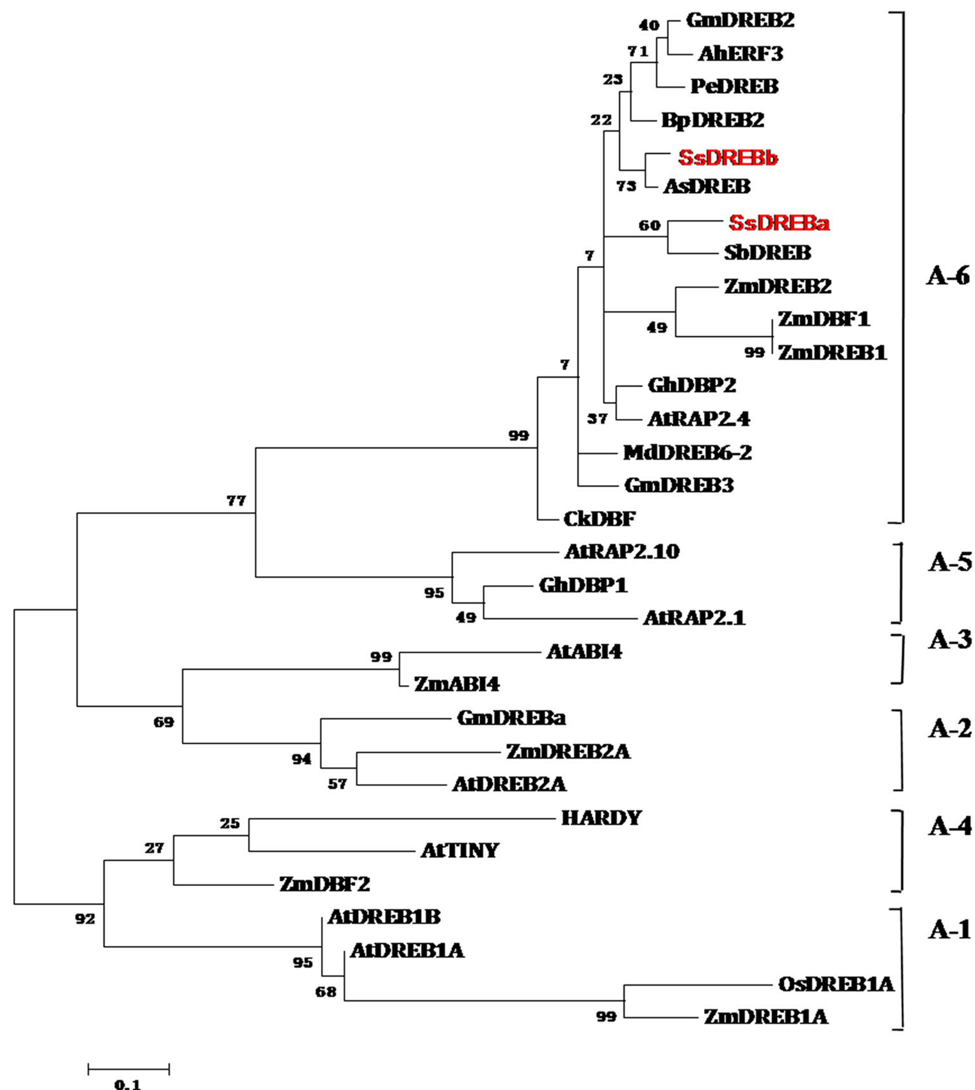


Fig. 4 Phylogenetic relationship of DREB transcription factors based on amino acid sequences comparison of the full length proteins. Multiple sequence alignment and phylogenetic tree were conducted with Clustalx and MEGA5.1. The *scale* indicates branch lengths. A-1 to A-6 indicate subgroups proposed by Sakuma et al. (2002). SsDREBa and SsDREBb are shown in *red*. DREB protein sequences were retrieved from GenBank. The accession number of the above proteins are as follows: SsDREBa (KM365207); SsDREBb (KM365208); AhERF3 (JN613348); AsDREB (JF451138); AtABI4 (AF085279); AtDREB1A (NM_118680); AtDREB1B (NM_118681); AtDREB2A (NP_196160); AtRAP2.1 (AY086838); At RAP2.10 (NM_119854); At RAP2.4 (NP_177931); AtTINY (AC005405); BpDREB2 (DQ211836); CkDBF (GU573848); GhDBP1 (AY174160); GhDBP2 (AY619718); GmDREB2 (AY296651); GmDREB3 (DQ208969); GmDREBa (AY542886); HARDY (AT2g36450); MdDREB6-2 (JQ669823); OsDREB1A (AAN02486); PeDREB (EF597499); SbDREB (JF894301); ZmABI4 (AY125490); ZmDBF1 (AAM80486); ZmDBF2 (AAM80485); ZmDREB1 (EU963205); ZmDREB2 (FJ805750); ZmDREB1A (AF450481); ZmDREB2A (BAE96012)

transformed with mutant DRE (mDRE) motifs and the *SsDREB*s could not grow in the same medium (Fig. 6). These results further suggested that both *SsDREB* genes encode transcriptional activators that can bind specifically to the DRE elements and activate transcription of downstream stress-responsive genes.

The yeast one-hybrid assay showed that SsDREBa and SsDREBb possess DNA-binding and transcriptional activation abilities. Amino-acid sequence analysis showed that two Serine-rich segments are present in the C-terminal region of SsDREBa (Fig. 1). SsDREBb also contains two Serine-rich segments located in the N- and C-terminal region, respectively (Fig. 2). Sakuma et al. (2006) found that deletion of a region containing a Ser/Thr-rich motif led to a constitutively active form of *AtDREB2A* which was reported not to activate downstream genes under normal growth conditions. Xu et al. (2008) reported that overexpression of *TaAIDFa*, which encodes a DREB protein lacking a Ser/Thr-rich region (a putative phosphorylation site) relative to TaAIDFb/c, activated transcription of

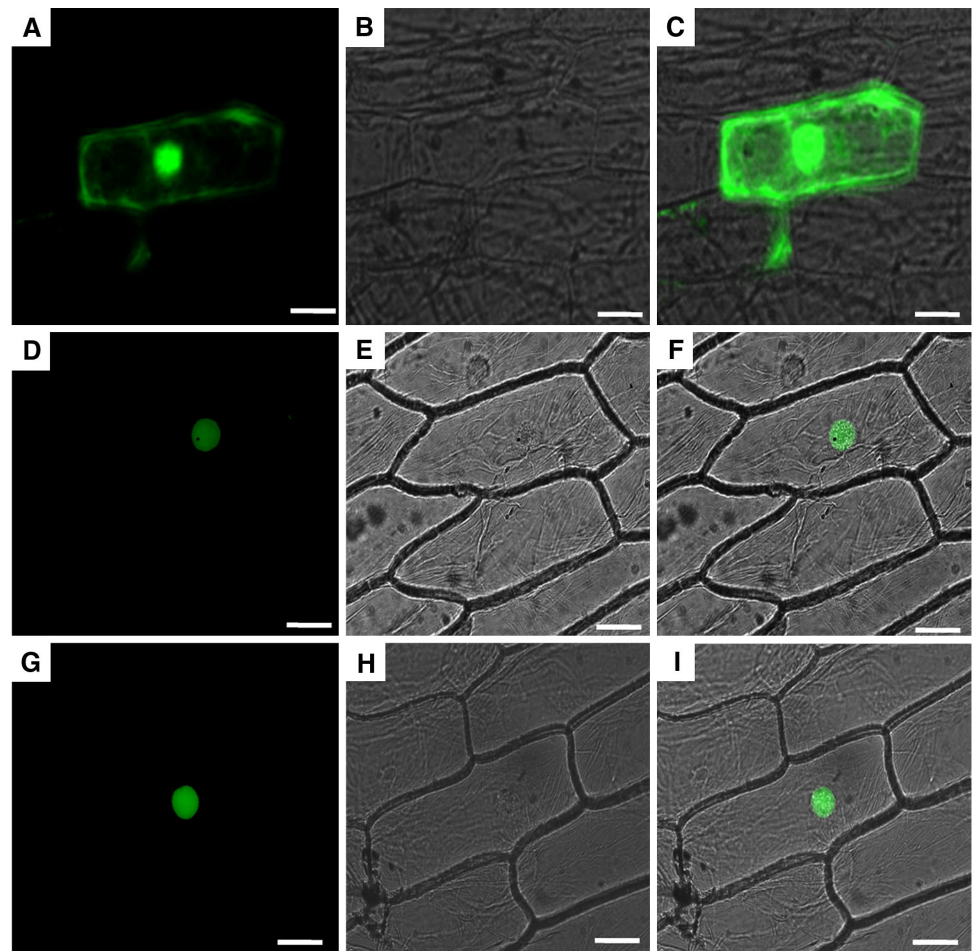


Fig. 5 Subcellular localization of SsDREB proteins in onion epidermal cells. Onion epidermal cells were transiently transformed with SsDREB-GFP. The GFP control plasmid (**a–c**), the fusion construct for SsDREBa-GFP (**d–f**) and SsDREBb-GFP (**g–i**) were introduced onion epidermal cells by particle bombardment method. The subcellular localization of SsDREB-GFP fusion proteins and GFP were tracked by fluorescence confocal microscopy 24 h after bombardment. The photographs were taken in *dark field* for green fluorescence (**a**, **d**, **g**), in *bright light* to illustrate the morphology of the cells (**b**, **e**, **h**) and in combination (**c**, **f**, **i**). The *scale bar* represents 50 μm

RD29A, *COR15A*, and *ERD10* in transgenic *Arabidopsis* plants. Here, we propose that the Serine-rich segments in the C-terminal regions of the two SsDREB proteins might function as phosphorylation sites that regulate transcription activity of SsDREBs. The Serine-rich segment in the N-terminal region of SsDREBb may be responsible for controlling its DRE-binding ability by phosphorylation. Sequence analysis of SsDREBb identified a Glutamine-rich region next to the serine-rich segment in the C-terminal (Fig. 2). In general, Glutamine-rich regions located in the C-terminal region can act as essential structural motifs required for transcriptional activation activity (Bruhn et al. 1992; Dai et al. 2003).

Expression of the two *SsDREB* genes in response to various abiotic stresses by quantitative real-time PCR

The expression patterns of the *SsDREBa* and *SsDREBb* genes under cold, salt and drought stress conditions were investigated using quantitative real-time PCR. Firstly, we analyzed the tissue-specific expression of *SsDREB*s in *S. salsa*. The expression levels of *SsDREBa* and *SsDREBb* in roots, stems and leaves of *S. salsa* under normal conditions were monitored. The expression level of *SsDREBa* was the most high in the roots of *S. salsa*, which was almost 6-fold higher than that in the leaves. No *SsDREBa* mRNAs were detected in the stems. *SsDREBb* were expressed at relatively higher levels in the leaves than in the roots and stems (Fig. 7a).

Next, the expression patterns of *SsDREBa* and *SsDREBb* genes in roots and leaves under salt, dehydration and cold stresses were investigated. The expression patterns of *SsDREBa* and *SsDREBb* differed under various conditions. Under 400 mM NaCl treatment, the expression of *SsDREBa* was enhanced in both leaves and roots, but with different patterns. In leaves, the expression level of *SsDREBa* was increased slightly under salt stress at 4 h and then diminished. In roots, the expression of *SsDREBa* was strongly induced by NaCl and the mRNA abundance accumulated gradually from 0.5 h to 12 h, with the highest abundance of about 14-fold increase at 12 h, and then dropped rapidly at 24 h (Figs. 7b). The transcript level of *SsDREBb* was obviously increased in both the leaves and roots under salt stress, with the highest abundance at 4 h in the leaves and 2 h in the roots (Figs. 7b). The greatest increase was about 16-fold in leaves and 5-fold in roots

respectively and the relative transcript levels of *SsDREBb* were higher in leaves than in roots on average (Figs. 7b). We used 20 % PEG6000 to mimic dehydration stress to monitor the expression patterns of *SsDREB*s in *S. salsa* leaves and roots. *SsDREBa* expression was slightly up-regulated in leaves at 8 h and then decreased under dehydration stress (Figs. 7c). Dehydration stress (PEG) treatment sharply up-regulated the expression of *SsDREBa* in roots with a peak level at 8 h and diminished slightly after that (Figs. 7c). The expression of *SsDREBb* was enhanced distinctly in both PEG treated leaves and roots. Expression of *SsDREBb* in leaves was induced after 0.5 h of dehydration, peaked at 8 h with the abundance about 8.5-fold, then decreased to untreated level at 24 h (Fig. 7c). Similarly, expression of *SsDREBb* in roots was also induced by dehydration treatment and peaked at 4 h with the abundance about 2.6-fold (Fig. 7c). Under cold stress (4 °C) treatment, the expression level of *SsDREBa* was down-regulated in leaves during the 24-h test period, but there was no obvious expression change in root (Fig. 7d). *SsDREBb* gene remained constitutively expressed in *S. salsa* leaves and roots during cold treatment (Fig. 7d).

DREBs show variation in some conserved motifs and biological functions in divergent species, and are dichotomized as DREB1- and DREB2-types (Dubouzet et al. 2003). Expression of DREB1-type genes was specifically responsive to low-temperature stress in *Arabidopsis* and rice (Liu et al. 1998; Dubouzet et al. 2003). In contrast, DREB2-type genes were induced by dehydration and high-salt stresses (Liu et al. 1998; Dubouzet et al. 2003). Our results revealed that the expression of each *SsDREB* gene was significantly induced by salt and dehydration, not by cold, which is similar to that of DREB2-types. *ZmDBF1* and *BpDREB2*, A-6 group members from maize and paper mulberry, respectively, were reported to be responsive to salt and dehydration, not to cold (Kizis and Pages 2002; Sun et al. 2014). However, some other members of DREB A-6 subgroup which contain *RAP2.4* from *Arabidopsis* (Rae et al. 2011), *CkDBF* from *Caragana korshinskii* (Wang et al. 2010), *AhERF5* from peanut (Chen et al. 2012), *GhDBP2* from cotton (Huang et al. 2008) and

Fig. 7 Quantitative real-time RT-PCR analysis of *SsDREBa* and *SsDREBb* expression levels. **a** The expression levels of *SsDREBa* and *SsDREBb* in the roots, stems and leaves of unstressed plants. **b**, **c**, **d** and **e** The relative expression of *SsDREBa* and *SsDREBb* in response to salinity stress (400 mM NaCl), dehydration stress (20 % PEG), low temperature (4 °C), and 100 μM ABA, respectively, for the indicated times. Total RNA was extracted from the leaves and roots of six-week-old seedlings of *S. salsa* in the same position after 0, 0.5, 2, 4, 8, 12 and 24 h of exposure to various stresses. The *actin* mRNA was used as a control to normalize samples. Experiments were repeated three times. *Error bars* represent the standard errors of three biological replicates of a single treatment

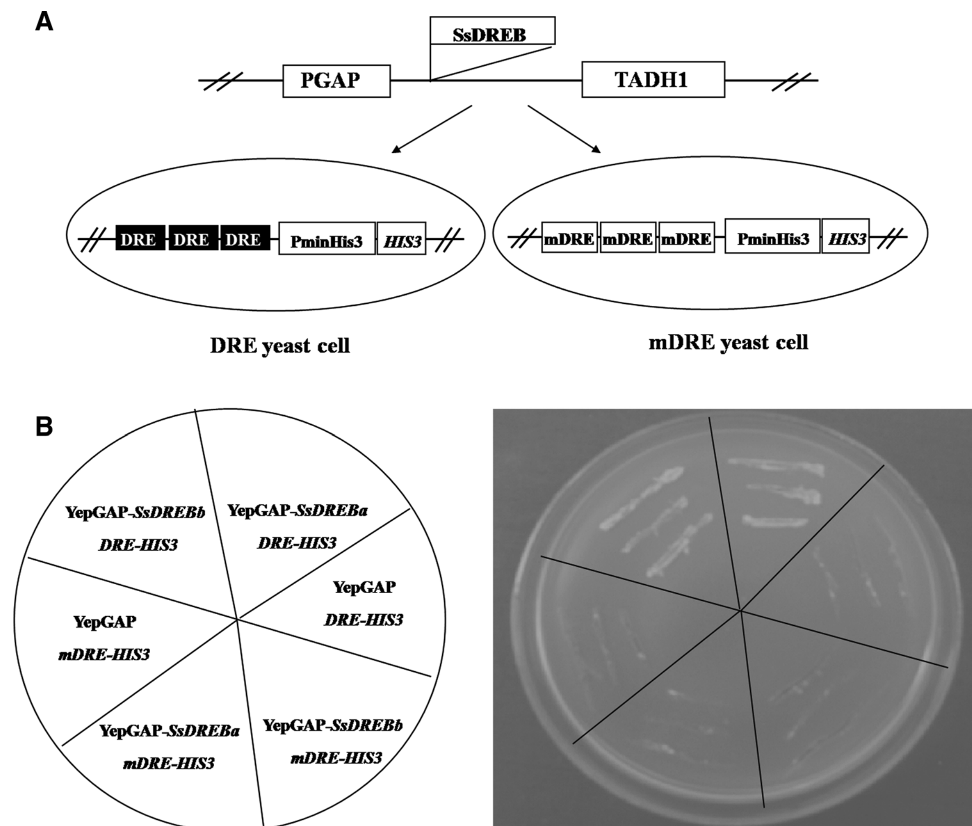
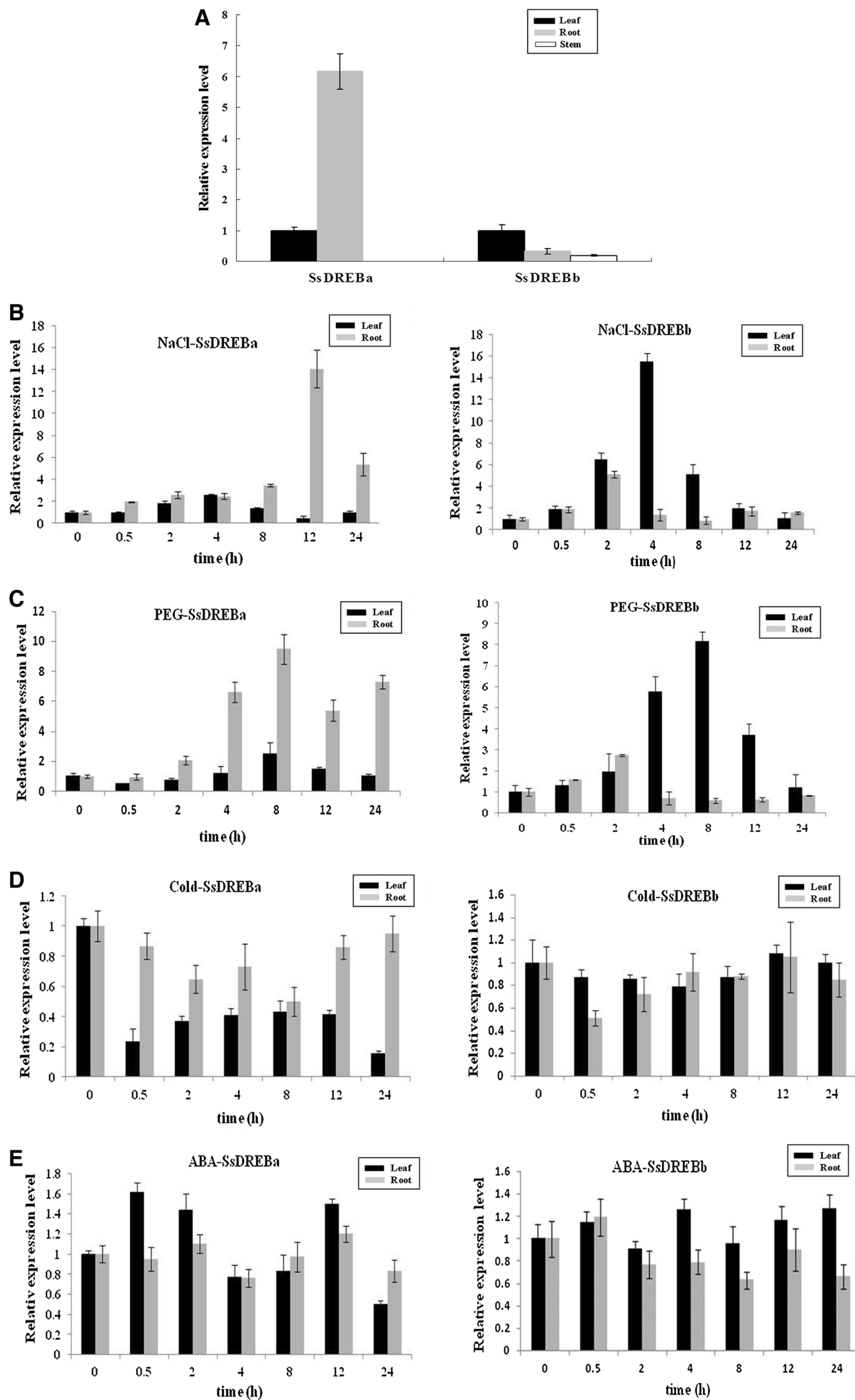


Fig. 6 Analysis of DNA-binding and transactivation activities of SsDREBa and SsDREBb proteins in yeast one-hybrid system. **a** Map of the YepGAP-SsDREB vectors. The plasmid expressed the SsDREBa or SsDREBb protein in the yeast and activated the expression of reporter gene *HIS3*. **b** The *left* sketch illustrates the location of different transformed yeast strains on plate. *Right plate* shows the growth state of transformed yeast strains on SD/-His/-Ura/-Trp/+ 10 mM 3-AT at 30 °C for 3 days. Empty YepGAP was used as a negative control


A
Relative expression level
Leaf
Root
Stem
SsDREBa
SsDREBb
B
NaCl-SsDREBa
NaCl-SsDREBb
Relative expression level
time (h)
C
PEG-SsDREBa
PEG-SsDREBb
D
Cold-SsDREBa
Cold-SsDREBb
E
ABA-SsDREBa
ABA-SsDREBb
Relativ expression level

GmDREB2 from soybean (Li et al. 2005) were induced by high salinity, dehydration and cold. The strong induction of *SsDREB*s by salt and drought environmental stimuli indicates that the genes may be involved in the cross-talk between salt and drought stresses.

The *SsDREB*a gene was up-regulated in roots and leaves by salinity and dehydration treatments, but it was more responsive in roots than in leaves (Fig. 7b, c). AhERF5 were also reported to be inducible by salt at relatively higher levels in the roots than in the leaves. However, it had a contrary response to drought stress with up-regulation in leaves and inhibition in roots (Chen et al. 2012). The expression level of *SsDREBb* exhibited more significant changes in leaves than in roots after exposure to salt and dehydration treatments (Fig. 7b, c). Similarly, the relative expression level of the *CkDBF* was higher in the leaves than in the roots under salt stress (Wang et al. 2010). The stronger induction of *SsDREBa* in roots and *SsDREBb* in leaves by dehydration and salt treatments implies that the two *SsDREB* paralogous may play distinct roles in resistance regulation to adverse environment in *S. salsa* leaves and roots. The conclusion is also supported by the result that relative transcript level of *SsDREBa* was higher in the roots and that of *SsDREBb* was higher in the leaves. The *SsDREB*s were induced by dehydration and salt, but displayed a stronger induction under high salinity. In contrast to some other halophytic plants, *S. salsa* does not have salt bladders or salt glands on its leaves. Therefore, the high induction of *SsDREB*s under salt stress may partly contribute to alleviate the salt-induced osmotic and ionic stresses on plant cells.

The phytohormone abscisic acid (ABA) plays an important role in regulation of gene expression in response to different environmental stresses (Verslues and Zhu 2005; Pospísilová et al. 2009). To explore the possible ABA regulatory pathway underlying the response of *SsDREB*s to abiotic stresses, the effect of exogenous application of the phytohormone ABA on the expression of *SsDREB*s was assessed. As shown in Fig. 7e, *SsDREBa* expression was almost unaltered by ABA treatment. The transcript levels of *SsDREBb* were largely unchanged in leaves, but decreased slightly in roots.

The induction of stress-responsive gene are controlled through two pathways, the ABA-dependent and ABA-independent regulatory systems (Yamaguchi-Shinozaki and Shinozaki 2006). Many studies showed that ABA, whether endogenous or exogenous, is involved in gene regulatory pathways in response to drought, high salt and cold stresses and plays a crucial role in inducing the expression of some stress-responsive genes and facilitating adaptation to different abiotic stresses (Verslues and Zhu 2005; Hirayama and Shinozaki 2007). Our research results indicated that *SsDREB* genes were not responsive to ABA treatment, which suggests that *SsDREB* genes are involved in the drought and high-salt stress responses through ABA-independent pathways. *GmDREB2* and *BpDREB2* were also reported not to be responsive to ABA treatment (Li et al. 2005; Sun et al. 2014). However, some other members of DREB A-6 subgroup including *ZmDBF1*, *GhDBP2* and *CkDBF* were induced by exogenous ABA, implying that they may act as a cross-point between ABA-independent and ABA-dependent stress signaling pathways (Kizis and Pages 2002; Huang et al. 2008; Wang et al. 2010). Therefore, it could be proposed that the A-6 DREB proteins from various plants may be responsive to abiotic stress through ABA-independent or ABA-dependent pathways and that crosstalk between the two regulatory pathways exists.

In summary, we have cloned two *SsDREB*s from *S. salsa* and confirmed that the proteins encoded by *SsDREB*s belonged to A-6 subgroup members of the DREB subfamily based on sequence comparison and phylogenetic analysis. A subcellular localization assay revealed that both SsDREBs localized to the nucleus. Yeast one-hybrid experiments verified that SsDREBs possessed DRE element-binding specificity and transcriptional activation activity. Expression of *SsDREB*s was shown to be upregulated by high-salt or dehydration treatment, but no significant change was observed under ABA or low-temperature condition. Further studies are needed to explore the roles of these genes in abiotic resistance.

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Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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