Cloning and characterization of the dehydration-responsive element-binding protein 2A gene in *Eruca vesicaria* subsp *sativa*

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ABSTRACT. *Eruca vesicaria* subsp *sativa* is one of the most tolerant Cruciferae species to drought, and dehydration-responsive element-binding protein 2A (DREB2A) is involved in responses to salinity, heat, and particularly drought. In this study, a gene encoding EvDREB2A was cloned and characterized in *E. vesicaria* subsp *sativa*. The full-length *EvDREB2A* cDNA sequence contained a 388-bp 5'-untranslated region (UTR), a 348-bp 3'-UTR, and a 1002-bp open reading frame that encoded 334 amino acid residues. The theoretical isoelectric point of the EvDREB2A protein was 4.80 and the molecular weight was 37.64 kDa.

The genomic sequence of *EvDREB2A* contained no introns. Analysis using SMART indicated that EvDREB2A contains a conserved AP2 domain, similar to other plant DREBs. Phylogenetic analysis revealed that EvDREB2A and DREB2As from *Brassica rapa*, *Eutrema salsugineum*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, and *Arachis hypogaea* formed a small subgroup, which clustered with DREB2Bs from *A. lyrata*, *A. thaliana*, *Camelina sativa*, and *B. rapa* to form a larger subgroup. EvDREB2A is most closely related to *B. rapa* DREB2A, followed by DREB2As from *E. salsugineum*, *A. thaliana*, *A. hypogaea*, and *A. lyrata*. A quantitative real-time polymerase chain reaction indicated that *EvDREB2A* expression was highest in the leaves, followed by the roots and hypocotyls, and was lowest in the flower buds. *EvDREB2A* could be used to improve drought tolerance in crops.

Key words: *Eruca vesicaria* subsp *sativa*; Gene cloning; Dehydration-responsive element-binding protein 2A

INTRODUCTION

Dehydration-responsive element-binding (DREB) proteins are transcription factors of the APETALA2/ethylene-responsive element-binding factor (AP2/ERF) family, which recognizes the dehydration-responsive element (DRE) core sequence motif (A/GCCGAC) in the promoters of stress-inducible genes (Yamaguchi-Shinozaki and Shinozaki, 1994). The DREB family is one of the most promising regulons in genetic engineering for the improvement of abiotic stress tolerance in plants (Zhao et al., 2012). Among the DREB gene family, the A-2 subgroup, including *DREB2A*, is specifically involved in responses to salinity, heat, drought, and cold (Liu et al., 1998; Nakashima et al., 2000; Dubouzet et al., 2003; Sakuma et al., 2006; Qin et al., 2007). Signal transduction involves upregulation of *DREB2A* and the activation of various genes involved in stress tolerance in different plant species (Lata and Prasad, 2011; Mizoi et al., 2012; Morimoto et al., 2013).

Eruca vesicaria subsp *sativa* is one of the most tolerant Cruciferae species to drought stress (Sun and Zhang, 1999; Prakash and Bhat, 2007). Recently, we found *Eruca* lines that were highly tolerant to polyethylene glycol (PEG)-simulated drought stress (Huang et al., 2015). In this study, we cloned and characterized *DREB2A* from *E. vesicaria* subsp *sativa*. *EvDREB2A* expression profiles in different tissues of the plant were also examined.

MATERIAL AND METHODS

Plant and tissue collection

E. vesicaria subsp *sativa* PI 251498, which is highly tolerant to PEG-simulated drought stress (Huang et al., 2015), was used for cloning *DREB2A*. Seeds were germinated on filter paper immersed in liquid Murashige and Skoog medium without sugar or organic

components. Seven days after seed inoculation, the roots and hypocotyls of at least three plants were harvested separately and frozen immediately in liquid nitrogen, and then stored at -80°C. Young leaves and flower buds were collected from the field and frozen immediately in liquid nitrogen and stored at -80°C.

Cloning full-length *EvDREB2A* cDNA and genomic DNA

Total RNA was isolated from the different tissues using TRIzol Total RNA Extraction Reagent (TaKaRa, Dalian, China). First-strand cDNA was synthesized using 1 mg total RNA and 1 μL ReverTra Ace® (100 U; Toyobo, Osaka, Japan) according to the manufacturer protocol. DREB2A amino acid sequences from various higher plant species (*Brassica rapa*, XP_009125600.1; *Arabidopsis lyrata*, XP_002871157.1; *A. lyrata*, EFH47416.1; *Arabidopsis thaliana*, AED90870.1; *Arachis hypogaea*, ABC60025.1; *Eutrema salsugineum*, AAS58438.1; *Salicornia brachiate*, ADE35085.1; *Zea mays*, NP_001105876.2; and *Arundo donax*, JAF93766.1) were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and highly conserved regions were retrieved. A pair of degenerated primers (DREB2A core-FP and DREB2A core-RP; Table 1) was designed to amplify a 278-bp fragment of the *DREB2A* coding region using cDNA from leaves and roots as templates. Two gene-specific primers (5'-RACE-DREB2A and 3'-RACE-DREB2A; Table 1) were designed for 5'- and 3'-rapid amplification of cDNA ends (RACE) to clone full-length *EvDREB2A* cDNA using a SMART™ RACE Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA). Primers based on sequences of the 5'- and 3'-RACE fragments (DREB2A-FP and DREB2A-RP; Table 1) were used to amplify full-length *EvDREB2A* from cDNA and genomic DNA. All of the polymerase chain reaction (PCR) products were cloned into a pMD-18T vector (TaKaRa) and sequenced. At least five independent clones were sequenced for each PCR product to ensure accuracy.

Table 1. Polymerase chain reaction (PCR) primer sequences used in the study.

PCR system	Primer name	Primer sequence (5'-3')	Amplicon size (bp)
Core fragment PCR	DREB2A-FP	TGGGAAGGAGATGGCAGTTTATGATCA	278
	DREB2A-RP	TACCCCAAATCCTCTGCCTAACTCC	
5'-RACE	5'-RACE-DREB	CCCCAAATCCTCTGCCTAACTCCCCTGAAA	476
3'-RACE	3'-RACE-DREB	CCCAAGAAGCGGAAAGTACCAGCGAAAGGA	1215
cDNA PCR	DREB cDNA FP	TAACCCAAAAGGAGAAATATCACTAG	1738
	DREB cDNA RP	ACAAAAGAAATTCGGGTCACAAGGTCAA	
Genomic DNA PCR	DREB FP	TAACCCAAAAGGAGAAATATCACTAG	1738
	DREB RP	ACAAAAGAAATTCGGGTCACAAGGTCAA	
Quantitative real-time PCR	DREB-qRT-FP	CCCAAGAAGCGGAAAGTACCAGCGAAAG	278
	DREB-qRT-RP	CGCCCTGAAGTCCCAACCGTACA	
	actin qRT-FP	CGCCGCTTAACCCTAAGGCTAACAG	322
	actin qRT-RP	TTCTCTTTAATGTCACGGACGATTT	

RACE = rapid amplification of cDNA ends.

Sequence analysis

ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to perform open reading frame (ORF) analysis and deduce the amino acid sequence. The organization

of *EvDREB2A* genomic DNA was determined using the Splign software (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). The theoretical isoelectric point (pI) and molecular weight (Mw) were computed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Nucleotides and the derived amino acid sequences of *EvDREB2A* were BLAST-searched against *DREB2A* genes from different plant species on the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST). A motif scan of the deduced EvDREB2A protein was conducted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de), and the secondary structure was predicted using the SOPMA software (http://npsa-pbil.ibcp.fr/). A phylogenetic tree was constructed using the DNAMAN software (Lynnon Biosoft) based on the amino acid sequences.

Expression analysis by quantitative real-time PCR

Total RNA from leaves, roots, hypocotyls, and flower buds was extracted for quantitative real-time PCR. The primers DREB2A qRT-FP and DREB2A qRT-RP (Table 1) were used to detect *EvDREB2A* expression, and the constitutively expressed actin gene was used as an internal control to normalize *EvDREB2A* transcript levels. The quantitative real-time PCRs were performed in triplicate according to the manufacturer (Hangzhou Bioer Technology Co., Ltd., China) instructions using the comparative Ct method: the PCR amplification was performed in a reaction mix with a final 15-μL volume, comprising 1.2 μL cDNA, 0.2 μL each gene-specific primer, 7.5 μL 19 PCR mix, and 5.9 μL purified water. The results are reported as means ± SD.

RESULTS

Cloning and sequence analysis of *EvDREB2A*

By using degenerated primers based on the highly conserved sequence of *DREB2A* from different plant species, a 278-bp conserved fragment was amplified from *E. vesicaria* subsp *sativa* root, hypocotyl, leaf, and flower bud mRNA. By aligning and splicing sequences of the conserved region and the 3'- and 5'-RACE PCR products, a 1738-bp full-length *DREB2A* cDNA was predicted. A 1738-bp sequence was amplified from cDNA and genomic DNA using primers based on the 3'- and 5'-RACE sequences. Analysis by ORF Finder indicated that the *EvDREB2A* cDNA sequence contained a 388-bp 5'-untranslated region (UTR), a 1002-bp ORF region that coded 334 amino acids, and a 348-bp 3'-UTR (Figure 1). The genomic *EvDREB2A* sequence did not contain introns.

Analysis of the deduced amino acid sequence

The pI of the EvDREB2A protein was 4.80 and the Mw was 37.64 kDa. A comparison of the amino acid sequence homologies and biochemical properties of DREB2As from the different plant species is presented in Table 2.

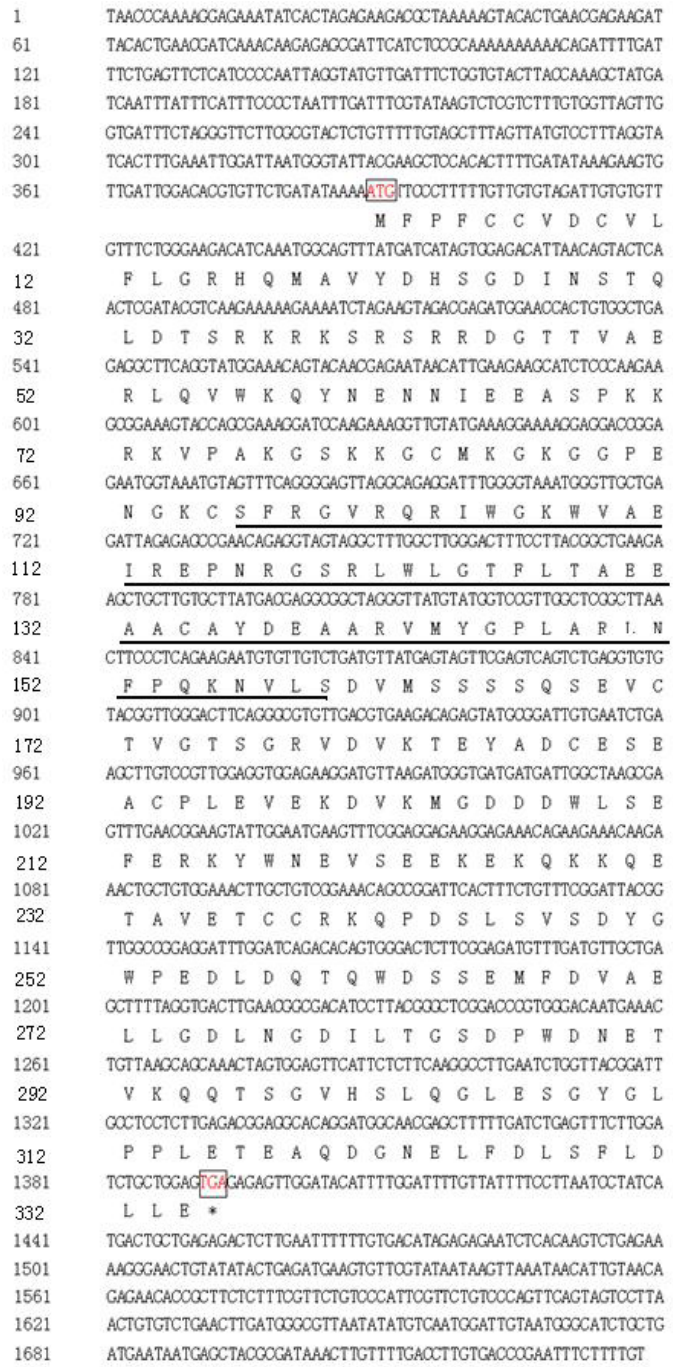

Figure 1. Full-length cDNA sequence of *Eruca vesicaria* subsp *sativa DREB2A* and the deduced amino acid sequence. The initiator and stop codons are shown in boxes. Sequences before the initiator and after the stop codons are the 5'- and 3'-UTR, respectively. The AP2 domain is underlined.

Table 2. Comparison of amino acid sequences and biochemical properties of DREBs from different plant species.

DREB	GenBank accession No.	Number of amino acids	Similarity (%)	Identity (%)	Molecular weight (Dalton)	Isoelectric point
Eruca vesicaria sativa DREB2A		334	-	-	37,646.62	4.8
Brassica rapa DREB2A-like	XP_009125600.1	305	75	70.2	34,440.86	4.75
Eutrema salsugineum DREB2A	AA558438.1	344	68.0	64.1	38,170.38	5.02
Arabidopsis lyrata DREB2A	XP_002871157.1	338	67	62.3	38,372.53	4.98
Arabidopsis thaliana DREB2A	AED90870.1	335	63.9	58.6	37,699.84	5.17
Arachis hypogaea DREB2A-like	ABC60025.1	338	63.1	57.5	38,010.16	5.17
Arabidopsis lyrata DREB2B	XP_002882709.1	324	53.7	47.9	36,447.69	5.42
Arabidopsis thaliana DREB2B	AEE74994.1	330	53	47	37,114.78	5.02
Camelina sativa DREB2B-like	XP_010489429.1	343	51.3	46.1	39,268.04	4.73
Brassica rapa DREB2B	XP_009135143.1	384	49.2	42.4	43,050.41	4.48
Nicotiana tomentosiformis DREB2C	XP_009612863.1	369	44.1	35.1	40,850.64	4.99
Triticum aestivum DREB3B	AAX13278.1	341	42	31	37,239.93	4.88
Triticum aestivum DREB4B	AAX13283.1	346	41.5	31.7	37,764.6	4.9
Arabidopsis thaliana DREB2C	NP_565929.1	341	41.1	30.4	37,826.97	4.68
Brassica rapa DREB2C	XP_009141887.1	274	40.9	31	30,056.2	4.83
Triticum urartu DREB2B	EMS45041.1	361	40.5	31.4	39,441.38	5.04
Salicornia brachiate DREB2A	ADE35085.1	353	40.1	34.1	39,398.46	4.87
Populus hopeiensis DREB2A	ADA59486.1	283	39.8	33.2	31,062.75	5.65
Arundo donax DREB2A	JAF93766.1	343	39.7	28.5	37,503.41	4.78
Sorghum bicolor DREB	ACA79910.1	262	39.5	30.8	28,588.88	5.43
Camelina sativa DREB2C	XP_010517453.1	342	38.4	28.8	38,262.39	4.86
Triticum aestivum DREB3A	AAX13277.1	389	37.9	28.1	42,285.34	5.02
Triticum aestivum DREB4A	AAX13282.1	394	37.4	28.8	42,829.05	5.03
Zea mays DREB2A	NP_001292873.1	367	36.7	28.9	39,574.67	5.02
Leymus chinensis DREB2C	AEC53580.1	375	36.4	27.4	40,567.86	4.99
Ricinus communis DREB2C	XP_002520794.1	386	35.8	30.9	42,743.03	4.83
Arabidopsis thaliana DREB2E	NP_181368.1	244	33.3	27.5	27,367.84	8.42
Arabidopsis thaliana DREB2G	AED92565.1	307	32.5	25	34,231.99	6.34
Arabidopsis thaliana DREB2F	AEE79677.1	277	31.5	23.8	31,569.97	5.33
Arabidopsis thaliana DREB2D	NP_177681.2	206	29.9	23.1	22,578.47	6.13
Arabidopsis thaliana DREB1D	AED96156.1	224	28	21.1	24,954.45	5.54
Arabidopsis thaliana DREB2H	AEC09817.1	157	27.1	22.1	17,790.42	10.02
Arabidopsis thaliana DREB1C	AEE85064.1	216	25.4	16.2	24,262.47	5
Arabidopsis thaliana DREB1A	AEE85065.1	216	24.9	17.1	24,234.44	5.08
Arabidopsis thaliana DREB1B	AEE85066.1	213	24.3	16.2	23,827.9	4.99

The BLASTp analysis revealed that the deduced EvDREB2A amino acid sequence shared the highest identity (70.2%) with *B. rapa* DREB2A (GenBank accession No. XP_009125600.1), followed by *A. lyrata* DREB2A (XP_002871157.1, 62.3% identity), *A. thaliana* DREB2A (58.6% identity), and *A. hypogaea* DREB2A-like (ABC60025.1, 57.5% identity). The putative secondary structure of the deduced amino acid sequence is presented in Figure 2.

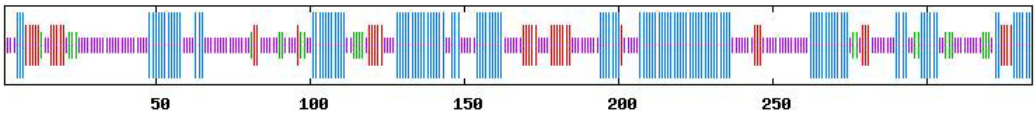

Figure 2. Secondary structure of EvDREB2A as predicted by SOPMA software. The longest lines, second longest lines, third longest lines, and shortest lines indicate alpha helices, extended strands, beta turns, and random coils, respectively.

The SMART analysis revealed that EvDREB2A contained a conserved AP2 domain (96-159aa; Figure 1). Multiple alignments of the EvDREB2A protein with other Cruciferae DREB2As are presented in Figure 3. EvDREB2A is highly similar to other Cruciferae DREB2As, and the amino sequences are identical in regions such as 74-90 and 95-129 aa (Figure 3).

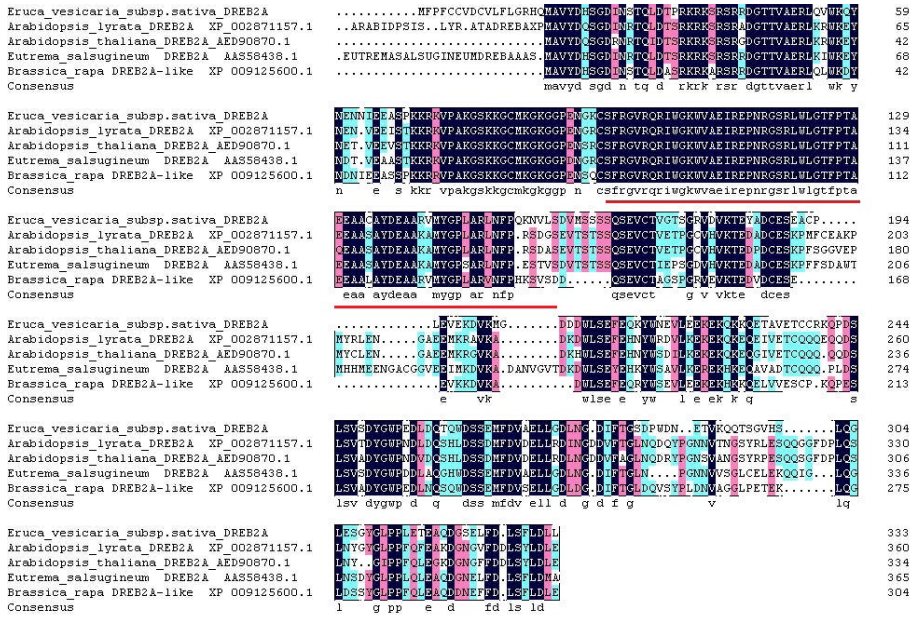

Figure 3. Multiple alignments of the N-termini of Cruciferae DREB2A proteins. The AP2 domain is underlined.

Sequence alignments and phylogenetic analyses

As shown in Figure 4, monocot DREBs clustered into one major group (M), while dicot DREBs clustered into three major groups (D1, D2, and D3). In group D1, EvDREB2A and DREB2As from *B. rapa*, *E. salsugineum*, *A. thaliana*, *A. lyrata*, and *A. hypogaea* formed a small subgroup, which clustered with DREB2Bs from *A. lyrata*, *A. thaliana*, *Camelina*

sativa, and *B. rapa* to form a larger subgroup. EvDREB2A was most closely related to *B. rapa* DREB2A, followed by DREB2As from *E. salsugineum*, *A. thaliana*, *A. hypogaea*, and *A. lyrata*.

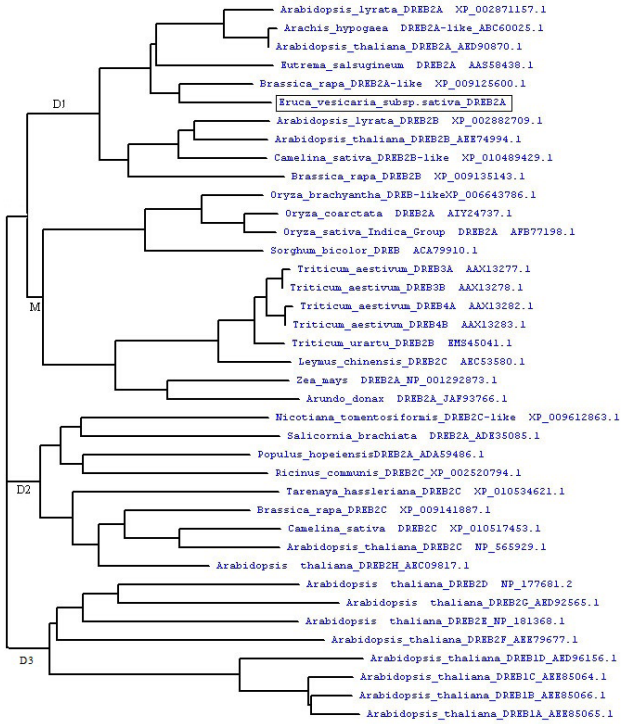

Figure 4. Phylogenetic relationships among EvDREB2A and other plant DREB proteins.

Tissue-specific *EvDREB2A* expression

The qRT-PCR revealed that *EvDREB2A* expression was highest in the leaves, followed by the roots and hypocotyls, and was lowest in the flower buds (Figure 5).

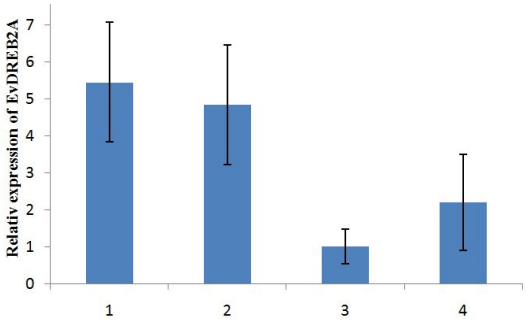

Figure 5. Relative *EvDREB2A* mRNA expression in different tissues. *EvDREB2A* expression was normalized to the amount of actin mRNA. Data are reported as means ± SD. Column 1 = leaf; column 2 = root; column 3 = flower bud; column 4 = hypocotyl.

DISCUSSION

In this study, we cloned the full-length *DREB2A* cDNA and genomic DNA sequences of *E. vesicaria* subsp *sativa*. The full-length *EvDREB2A* cDNA sequence contained a 388-bp 5'-UTR, a 348-bp 3'-UTR, and a 1002-bp ORF that encoded 334 amino acid residues. The genomic *EvDREB2A* sequence did not contain introns, which is similar to *DREB2A*s in *A. thaliana* (Liu et al., 1998) and *Salicornia brachiata* (Gupta et al., 2010), but different to *DREB2A*s in rice (Dubouzet et al., 2003) and *Pennisetum glaucum* (Agarwal et al., 2007), which contain one intron in the genomic *DREB2A* sequence. The SMART analysis revealed that EvDREB2A contains a conserved AP2 domain, which is similar to that found in other plant DREBs (Sakuma et al., 2002). The phylogenetic analysis revealed that EvDREB2A and DREB2As from *B. rapa*, *E. salsugineum*, *A. thaliana*, *A. lyrata*, and *A. hypogaea* formed a small subgroup, which clustered with DREB2Bs from *A. lyrata*, *A. thaliana*, *C. sativa*, and *B. rapa* to form a larger subgroup. EvDREB2A was most closely related to *B. rapa* DREB2A, followed by DREB2As from *E. salsugineum*, *A. thaliana*, *A. hypogaea*, and *A. lyrata*.

The results of previous studies have indicated that *DREB2A* expression is strongly induced by dehydration, heat, and salt, and weakly induced by exogenous abscisic acid treatment (Liu et al., 1998; Nakashima et al., 2000; Sakuma et al., 2002; Gupta et al., 2010; Sadhukhan et al., 2014) and in some cases by cold (Wei et al., 2015; Chen et al., 2016). In *Eucalyptus grandis*, *DREB2A* is expressed in the roots, shoots, and leaves, and expression in the leaf is highest under unstressed conditions (Wei et al., 2015). In the present study, we found that *EvDREB2A* expression was highest in the leaves, followed by the roots and hypocotyls, and was lowest in the flower buds. *EvDREB2A* could be used to improve drought tolerance in crops.

Conflicts of interest

The authors declare no conflict of interest.

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