

VrDREB2A, a DREB-binding transcription factor from *Vigna radiata*, increased drought and high-salt tolerance in transgenic *Arabidopsis thaliana*

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Abstract Mung bean (*Vigna radiata* L.) is commonly grown in Asia as an important nutritional dry grain legume, as it can survive better in arid conditions than other crops. Abiotic stresses, such as drought and high-salt contents, negatively impact its growth and production. The dehydration-responsive element-binding protein 2 (DREB2) transcription factors play a significant role in the response to these stress stimuli via transcriptional regulation of downstream genes containing the cis-element dehydration-responsive element (DRE). However, the molecular mechanisms involved in the drought tolerance of this species remain elusive, with very few reported candidate genes. No DREB2 ortholog has been reported for mung bean, and the function of mung bean DREB2 is not clear. In this study, a novel VrDREB2A gene with conserved AP2 domains and transactivation ability was isolated from mung bean. A modified VrDREB2A protein lacking the putative negative regulatory domain encoded by nucleotides 394–543 was shown to be localized in the nucleus. Expression of the VrDREB2A gene was induced by drought, high salt concentrations and abscisic acid treatment. Furthermore,

comparing with the wild type *Arabidopsis*, the overexpression of VrDREB2A activated the expression of downstream genes in transgenic *Arabidopsis*, resulting in enhanced tolerance to drought and high-salt stresses and no growth retardation. The results from this study indicate that VrDREB2A functions as an important transcriptional activator and may help increase the abiotic stress tolerance of the mung bean plant.

Keywords *Vigna radiata* · VrDREB2A · Transcription factor · Salt tolerance · Drought tolerance

Introduction

Drought and high-salt concentrations are two common abiotic stresses that are severe threats to plant growth and food production around the world (Nakabayashi and Saito 2015). Many analyses have shown that transcriptional regulatory pathways are important to abiotic stress responses (Atkinson and Urwin 2012). Several transcription factors have been characterized with respect to their roles in the signaling network and transcriptional regulation, especially in abiotic stress responses (Yoshida et al. 2014). The DREB (dehydration-responsive element binding) transcription factor family, with the core sequence A/GCCGAC, was originally isolated in *Arabidopsis* (Liu et al. 1998) and shown to bind to DRE (dehydration-responsive element) core cis-acting sequences in the promoters of stress-responsive genes and regulate their expression via an abscisic acid (ABA)-independent pathway under conditions of drought, high-salt and cold stress in plants (Sakuma et al. 2002, 2006a). DREB transcription factors belong to the APETLA2/ethylene responsive element binding protein (AP2/EREBP) family, which is characterized by a conserved APETLA2/

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ethylene-responsive element binding factor (AP2/ERF) domain and is induced under abiotic stress conditions (Kizis et al. 2001; Mizoi et al. 2012).

Many DREB factors are induced by abiotic stresses, including drought (Liu et al. 1998; Nakashima et al. 2000; Qin et al. 2007b; Sakuma et al. 2006a), low temperatures (Gutha and Reddy 2008; Li et al. 2005; Qin et al. 2004, 2007b), high salt (Chen et al. 2009; Cong et al. 2008; Dubouzet et al. 2003; Huang et al. 2008; Nakashima et al. 2000; Wang et al. 2008) and extreme heat (Schramm et al. 2008). Many DREB genes have been identified, isolated and characterized in different plant species under various abiotic stresses, such as *Arabidopsis* (Sakuma et al. 2002), rice (*Oryza sativa* L.) (Cui et al. 2011), maize (*Zea mays* L.) (Qin et al. 2007a), soybean (*Glycine max* L.) (Chen et al. 2007; Mizoi et al. 2013) and cowpea (*Vigna unguiculata* L.) (Sadhukhan et al. 2014).

The roles of the DREB2A gene from *Arabidopsis* in abiotic stress tolerance are well characterized (Sakuma et al. 2006a). However, similar studies are very limited in legume plants, especially in mung bean, and the molecular mechanisms controlling plant tolerance to drought and salt remain largely unknown (Chung et al. 2013). In cowpea, *VuDREB2A* was induced by drought and salt stresses, and heterologous expression of *VuDREB2A* resulted in significant drought stress tolerance in *Arabidopsis* (Sadhukhan et al. 2014). Two *GmDREB2A* homologs (*GmDREB2A;1* and *GmDREB2A;2*) have been identified in soybean, and their peptide sequences were very similar to each other, although the induction of *GmDREB2A;2* was stronger than that of *GmDREB2A;1*. The two *GmDREB2A* homologs exhibited strong inducibility under abiotic condition. Overexpression of *GmDREB2A;2* caused growth defects in the germination and seedling stages of the transgenic *Arabidopsis* (Mizoi et al. 2013). Moreover, DREB2A genes from different species, such as chrysanthemum (*Dendranthema vestitum*) (Liu et al. 2008), common wheat (Egawa et al. 2006), and *Pennisetum glaucum* (Agarwal et al. 2007), that result in physiological variations have been overexpressed in *Arabidopsis thaliana* and improved stress tolerance under drought, salt and freezing stresses in transgenic plants.

Mung bean (*Vigna radiata* L.) is an economically important grain legume widely grown in Asian countries for its protein-rich grains (Ahmad et al. 2011; Mishra et al. 2014). Drought and salt stresses are recognized as major constraints in the production and growth of mung beans (Hossain and Fujita 2010). Mung bean is moderately drought tolerant. Therefore, this distinctive characteristic makes it a valuable tropical legume for studying the molecular tolerance mechanisms of various abiotic stresses, including salinity and drought (Kim et al. 2004). However, no studies on the AP2/EREBP transcription factor gene in mung bean have been reported.

In the current study, we isolated and characterized the *VrDREB2A* gene in mung bean and demonstrated that the resulting protein is localized in the nucleus and exhibits transactivation ability. The expression of *VrDREB2A* was up-regulated under drought, high salt and ABA treatments. Furthermore, the overexpression of *VrDREB2A* enhanced the drought and high-salt tolerance of the transgenic *Arabidopsis* plants and did not cause growth retardation due to the activation of downstream genes.

Materials and methods

Plant growth

Mung bean (*Vigna radiata* L.) cultivar, VC2917, a high drought resistance accession used in this study was selected from the National Genebank of China, which is located at the Institute of Crop Science (ICS), in the Chinese Academy of Agricultural Sciences (CAAS), Beijing. The seeds were firstly germinated on moistened filter paper. When the roots had grown to approximately 1 cm, the seeds were transplanted into a soil/vermiculite (1:1, v/v) mix and grown in a greenhouse maintained at 22 °C under long days (16 h light, 8 h dark) with 50 % humidity. The 1-week old plants after germination were used for expression pattern analyses of *VrDREB2A* under different stress conditions.

For drought treatment, the seedlings were transferred to dry Whatman 3 MM paper for 1, 2 and 3 h. For salt treatment, the seedlings were watered with 1 M NaCl for 1, 6 and 24 h. For cold treatment, the seedlings were incubated at 4 °C for 1, 6 and 24 h. For ABA treatment, the leaves of seedlings were sprayed 200 μM ABA solution [0.05 % Tween-20 (v/v)] at 1, 6 and 24 h. After treatment, the whole seedlings were harvested, frozen in liquid nitrogen immediately, and stored at −80 °C for further analysis. Each treatment contained three biological repeats.

Isolation of *VrDREB2A* via degenerate PCR and RACE

RNA was isolated from 1-week old mung bean plants treated with 1 M NaCl for 3 h using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was amplified using reverse transcription PCR (RT-PCR) with MLV Reverse Transcriptase (Promega, Tokyo, Japan). A partial cDNA was obtained using PCR with degenerate primers designed from conserved regions of reported DREB2 sequences (see Table S1 in Supporting Information) and subcloned into pMD18-T simple (TaKaRa, Dalian, China). Then, the 3' and 5' regions were obtained using the RACE (rapid amplification of cDNA ends) PCR (Invitrogen, Carlsbad, CA, USA).

DNA/protein sequence and phylogenetic analyses

Nucleotide and protein sequences of DREB2A orthologs were retrieved from the NCBI Entrez database (<http://www.ncbi.nlm.nih.gov>). Multiple amino acid sequence alignment was performed using CLUSTALX 1.83 (Thompson et al. 1997). A phylogenetic tree was constructed using MEGA 6.06 (Tamura et al. 2007). The distances between the branches were calculated via the neighbor-joining method with 1,000 bootstrap replicates (Saitou and Nei 1987).

Localization of the VrDREB2A-GFP fusion protein

The full length of *VrDREB2A* gene was amplified with two primers: 5'- CTCGAGCATGGGTGCTTATGATCAA-GTTTCTG -3' (*XhoI* site underlined) and 5'- GGTACC-CTTCCTTGCTTGCTACCATTTC -3' (*KpnI* site underlined). The CDS of a modified *VrDREB2A* (designated *VrDREB2A-TR*) gene was removed; the putative negative regulatory domain encoded by nucleotides 394–543 through megaprimer PCR (Ke and Madison 1997). In this study, the pE3025-*GFP* vector was derived from the pSATS-*RFP*-NI vector, which was replaced the red fluorescent protein (*RFP*) gene with the green fluorescent protein (*GFP*) gene under the control of the CaMV dual 35S promoter, as well as a TEV enhancer (Li et al. 2011). The two PCR products were cloned into the pE3025-*GFP* vector to generate the pE3025-*VrDREB2A-GFP* and pE3025-*VrDREB2A-TR-GFP* constructs. They were confirmed by sequencing and used for transient transformation of onion epidermal cells via a gene gun (Bio-Rad, California, USA). GFP fluorescence was observed under a confocal fluorescence microscopy system (Nikon, Tokyo, Japan). The pE3025-*GFP* empty vector was used as a control.

Transactivation analysis in yeast

The entire coding sequence of *VrDREB2A* was amplified using two primers: 5'- GAATTCATGGGTGCTTATGATCAAGTTTCTGTG -3' (*EcoRI* site underlined) and 5'- GTCGACTCACTTCCTTGCTTGCTAGCATTTCCTTTG -3' (*Sall* site underlined). The PCR product was sub-cloned into the DNA-binding domain vector pBD GAL4 with a DNA-binding domain which could activate transcription of the dual report genes *His3* and *LacZ* both controlled by the GAL4 upstream activation sequence to construct plasmid pBD-*VrDREB2A*. The recombinant plasmid was then transferred into the yeast strain YRG-2 that had the reporter genes *His3* and *LacZ*. The yeast strain YRG-2 cannot grow on the SD plates without histidine, and cannot induce *LacZ* (β-galactosidase) activity. The transformed yeast culture containing pBD-*VrDREB2A*, pBD GAL4 or pGAL4 vector was dropped onto SD plates lacking tryptophan or

lacking both tryptophan and histidine. The plates were incubated at 30 °C for 3 d, and the resulting clones were used in a β-Gal assay to examine the transactivation ability of *VrDREB2A*. The pBD GAL4 empty vector (CK−) was used as the negative control, and pGAL4 vector (CK+) was used as the positive control.

Generation of VrDREB2A-overexpressing Arabidopsis

The *VrDREB2A* cDNA was cloned into the pMD18-T simple vector using the primers 5'- CCATGGGTGCTTATGATCAAGTTTCTGTG -3' (*NcoI* site underlined) and 5'- ACTAGTTTCCTTGCTTGCTAGCATTTCCTTTG -3' (*SpeI* site underlined) (TaKaRa, Dalian, China). An *NcoI*-*SpeI* fragment containing *VrDREB2A* cDNA was inserted into the pCAMBIA1302 vector containing the 35S CaMV promoter and a hygromycin (kanamycin) resistance marker. The plasmid was introduced into *Agrobacterium* EHA105 via heat shock. The pCAMBIA1302-*VrDREB2A* vector was transformed into *Arabidopsis* plants using the floral dip method (Clough and Bent 1998; Liu et al. 1998). Seeds of transformed *Arabidopsis* were selected on Murashige and Skoog (MS) medium containing 80 mg/ml hygromycin. Genomic PCR and qRT-PCR confirmed the successful transfer of 35S:*VrDREB2A*. Three independent lines of the T₃ generation were randomly chosen for further analysis.

Salt treatment

To determine the sensitivity of the seed germination process to NaCl, seeds from wild-type and transgenic plants were placed on MS agar plates or MS agar plates saturated with 210, 220 mM NaCl. The seeds were incubated at 4 °C for 72 h, and then transferred to a growth chamber with constant light (approximately 100 μmol m⁻² s⁻¹) at 22 °C for germination. After 14 d, the number of seed germination was recorded. Seeds were considered to have germinated when radicles were 1 mm long. Germination rate statistics are presented as the number of seeds germinated VS the number of seeds tested.

To assess salt tolerance, 5-day-old seedlings were carefully transferred to MS media containing different concentrations of NaCl (0 or 150 mM) and incubated for 7 d. The position of the root tips was marked at the time of transfer, and the root growth was measured from this mark after 7 d. Three independent biological replicates were conducted for salt experiments.

Drought treatment

To assess drought tolerance, one-week-old seedlings were transferred to pots filled with a soil/vermiculite (1:1, v/v) mix for another 2 weeks in a greenhouse under

conditions of continuous illumination of approximately $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 50 % relative humidity, a temperature of 22 °C, and long days (16 h light, 8 h dark), with regular watering every 4 d before water was withheld. After 16 d without water, all pots were watered simultaneously and plant recovery and survival rate were measured after 4 d. Survival statistics are presented as the number of plants survived.

The relative electrolyte leakage (EL) was determined as described (Hao et al. 2013). Fully expanded leaves (about 100 mg) of WT and transgenic lines were incubated in 15 ml ddH₂O. The initial level of EL (C_i) was measured using a conductance meter (Thermo Scientific, Beverly, USA) after shaken for 24 h at room temperature. Leaf tissue was killed in an autoclave at 121 °C for 30 min. The conductance of the incubation solution (C_{max}) was measured after 24 h incubation on a shaker. Relative EL was calculated as $\text{EL} = (\text{C}_i/\text{C}_{\text{max}}) \times 100$. Values are mean \pm SD of twelve independent plants.

qRT-PCR analysis

RNA samples were isolated using Invitrogen reagents (Invitrogen, USA), and reverse transcription was performed on 2 μg of RNA using M-MLV Reverse Transcriptase (Promega, Tokyo, Japan). qRT-PCR was performed using a CFX-96 Real-Time System (Bio-Rad, Hercules, California, USA) with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The specificity of the primer pairs (see Table S2 in Supporting Information) was confirmed by sequencing the PCR amplicons. *VrActin* (GenBank: AF143208.1) or *AtActin* (GenBank: At5G09810) was used as an internal control to normalize the amount of cDNA in the samples.

Statistical analysis

The above described experiments were performed in three independent biological repetitions. The results are presented as the mean \pm SD. Differences between groups were examined for statistical significance using Student's *t* test (**P* < 0.05, ***P* < 0.01) was regarded as statistically significant.

Results

Isolation and phylogenetic analysis of the *VrDREB2A* in mung bean

Firstly, a cDNA fragment (225 bp) was obtained using PCR with degenerate primers designed against the highly conserved regions of DREB2-type proteins. The full-length mRNA sequence was further isolated from total mung bean

RNA using the 5' and 3' RACE procedures. The full-length mRNA was 1158 bp and had a complete open reading frame of 386 amino acids. Further analysis of the deduced amino acid sequence revealed that this protein contained conserved consensus amino acid residues in the unique AP/EREBP domain (the 14th valine and 19th glutamic acid at 79–142 amino acids) and was highly homologous to previously isolated DREB2 proteins (Fig. 1a). In addition, this protein included a conserved nuclear localization signal. Therefore, it was assumed that the encoding gene was mung bean *DREB2A*.

To investigate the relationship between *VrDREB2A* and other DREB2A proteins, the deduced *VrDREB2A* amino acid sequence was compared with twelve other DREB2A proteins from *Arabidopsis thaliana* and other legume plants. Among these proteins, the reported *VrDREB2A* is closely related to cowpea *VuDREB2A* (GenBank: JN629045.3), with a 92 % match at the amino acid level, and has less similarity with the other DREB2A proteins from soybean (GmDREB2As, GenBank: AFU35562.1; GenBank: AFU35563.1) and the DREB proteins from *Arabidopsis* (AtDREB1A (GenBank: BAA33434.1) and AtDREB2A (GenBank: BAA36705.1)) (Fig. 1b). However, the characteristics and function of *VrDREB2A* remained unclear.

Localization and transactivation of the *VrDREB2A* protein

The nucleotides 394–543 of the *VrDREB2A* gene encode a putative negative regulatory domain, so we used a modified *VrDREB2A* construct (designated *VrDREB2A-TR*) to determine its subcellular localization. The full length of *VrDREB2A* and *VrDREB2A-TR* were fused in frame to the 5' terminus of the GFP reporter gene under the control of the cauliflower mosaic virus dual 35S promoter (CaMV 35S) and a tobacco etch virus (TEV) enhancer. Recombinant constructs encoding the *VrDREB2A-GFP*, *VrDREB2A-TR-GFP* fusion gene and *GFP* alone were introduced into onion epidermal cells. The *VrDREB2A-TR-GFP* fusion protein accumulated primarily in the nucleus (Fig. 2g–i), whereas GFP was present throughout the entire cell (Fig. 2a–c). Interestingly, the *VrDREB2A-GFP* fusion protein did not accumulate in the whole cell (Fig. 2d–f). Thus, *VrDREB2A-TR* was a nuclear-localized protein.

The transactivation ability of *VrDREB2A* was analyzed using a yeast assay. The pBD-*GAL4* had a DNA-binding domain which was controlled by the *GAL4* upstream activation sequence and could activate transcription of the dual report genes *His3* and *LacZ*. Yeast cells containing the fusion plasmid harboring *VrDREB2A* gene grew well on SD medium lacking histidine and could be stained

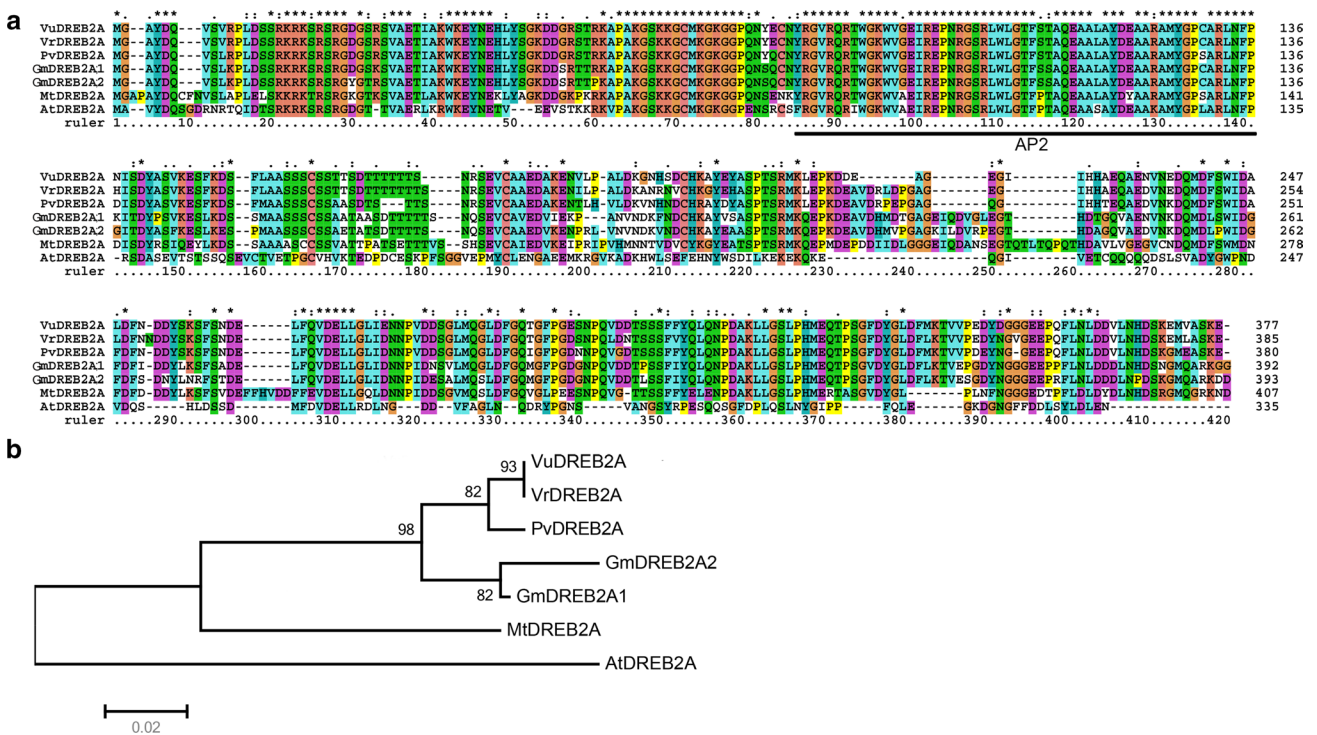


Fig. 1 Multiple sequence alignment and phylogenetic relationship of DREB2A homologs. **a** The conserved DREB2A DNA-binding domain is indicated as the underlined segment. Stars and squares indicate the DREB2A signature sequences. **b** The phylogenetic relationship of DREB2A homologs constructed using MEGA 6.06 software.

The bootstrapping value (out of 550 samples) for each node, obtained using the same software. The species designations are as follows: Mt, *Medicago truncatula*; At, *Arabidopsis thaliana*; Gm, *Glycine max*; Vu, *Vigna unguiculata*; Vr, *Vigna radiata*; Pv, *Phaseolus vulgaris*

blue using X-gal solution as well as yeast cells containing pGAL4 vector. On the contrary, yeast cells containing pBD GAL4 empty vector did not grow on SD medium lacking histidine, and could not stain blue (Fig. 2j). These results indicated that VrDREB2A showed transactivation activity. Taken together, these results reveal that VrDREB2A was consistent with its predicted function as a transcription factor.

Expression characteristics of the VrDREB2A in mung bean

qRT-PCR analysis showed that the transcription of the VrDREB2A gene was responsive to drought, high salt, and cold stresses as well as ABA treatment. Under drought conditions, VrDREB2A mRNA began to increase within 1 h and continued to increase after 3 h (Fig. 3a). Under high salt conditions, the expression pattern of VrDREB2A was similar to that during the drought treatment (Fig. 3b). Under cold conditions, the transcription of VrDREB2A was low and reduced its minimum level at 6 h (Fig. 3c). Interestingly, the expression of VrDREB2A was also induced by ABA treatment after 1 h and reached to its maximum at 24 h, suggesting that the gene was responsive to ABA

and involved in ABA-dependent signal pathways (Fig. 3d). Taken together, these results indicate that VrDREB2A was induced by drought, salt, and cold stresses as well as ABA stimulation, indicating that it might play an important role in the response to abiotic stresses and ABA treatment.

Overexpression of VrDREB2A improved the high salinity tolerance in transgenic Arabidopsis

The notable induction of VrDREB2A expression by multiple stresses indicated that this gene might be involved in stress resistance. Expression of VrDREB2A in transgenic Arabidopsis was detected via qRT-PCR (Fig. 4a). We randomly selected three independent T₃ VrDREB2A overexpression lines (L2, L5 and L8) for drought and salinity resistance testing. The overexpression of VrDREB2A in these three lines did not cause significant growth retardation compared with the wild type line.

To further evaluate the function of VrDREB2A in plants, we characterized the salinity tolerance phenotypes. In the germination stage, seed germination of the wild-type and transgenic plants did not differ under normal conditions (Fig. 4d). However, at a NaCl concentration of 210 or 220 mM, the seed of transgenic lines displayed significantly

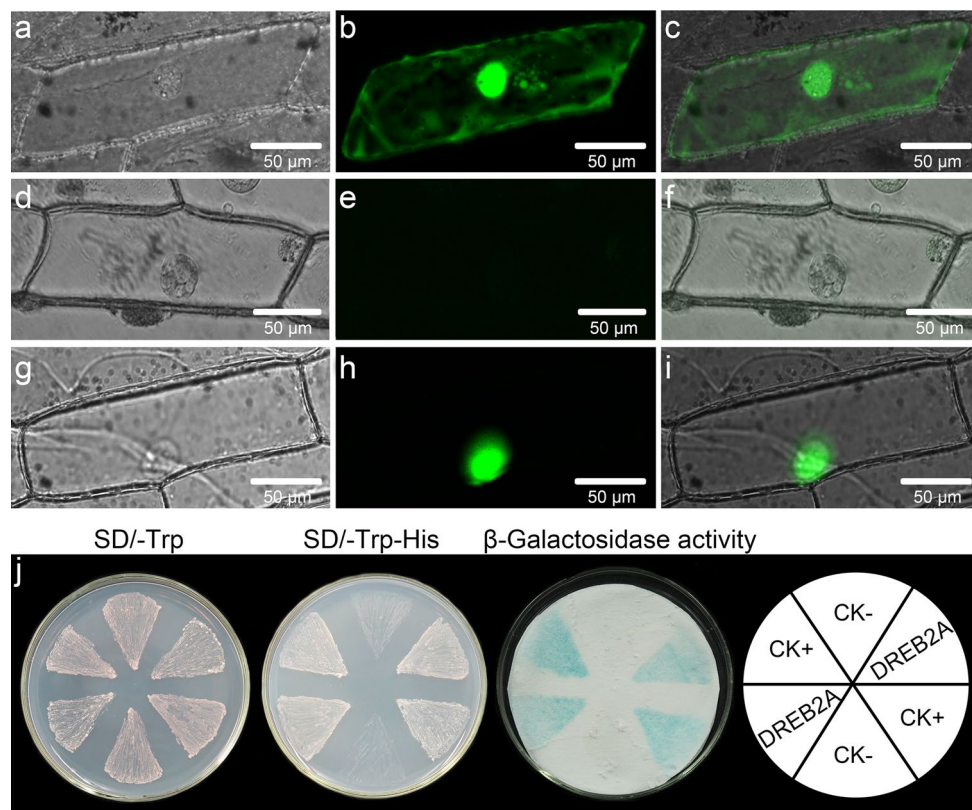


Fig. 2 Subcellular localization and transcriptional activation analysis of VrDREB2A. **a–c** Subcellular localization of pE3025-GFP. **d–f** Subcellular localization of pE3025-VrDREB2A-GFP. **g–i** Subcellular localization of pE3025-VrDREB2A-TR-GFP. VrDREB2A-GFP, VrDREB2A-TR-GFP and GFP alone were bombarded into the onion epidermal cells using DNA-coated gold particles, and GFP expression was visualized after 20 h at room temperature in the dark. Cells

expressing GFP alone were used as a control. **a, d, g** Bright field images; **b, e, h** GFP fluorescence (green); and **c, f, i** merged images. **j** All of the transformants containing pBD-VrDREB2A, pBD GAL4 or pGAL4 vector grew on SD/-Trp medium, SD/-Trp-His medium, or dyed b-galactosidase. The pBD GAL4 empty vector (CK-) was used as the negative control, and pGAL4 vector (CK+) was used as the positive control. Bars = 50 μ m

better germination rate than the wild type, and as the concentration of NaCl increased, the seed germination fulfilled down. At 220 mM NaCl seed germination of wild type was seriously inhibited (Fig. 4b). In the presence of 210 mM NaCl, 33.3 % of the wild-type seeds germinated, whereas the germination rates of the 35S:VrDREB2A L2, L5 and L8 transgenic lines were 70, 81.1 and 56.7 % (** $P < 0.01$, t test), respectively (Fig. 4e).

Similarly, when grown on MS plates containing 150 mM NaCl for 7 d, the primary root length of the wild-type plants was 1.2 cm, whereas the primary root lengths of the 35S:VrDREB2A L2, L5 and L8 transgenic lines were 1.7, 1.6 and 1.5 (* $p < 0.05$, t test), respectively (Fig. 4c, g). In conclusion, the transgenic plants exhibited longer primary roots than the WT plants, whereas no significant differences were observed under normal conditions (Fig. 4f, g). The results indicated that overexpression of VrDREB2A in *Arabidopsis* increased salt tolerance during germination and in the seedling stage.

Overexpression of VrDREB2A improved the drought tolerance of transgenic *Arabidopsis*

It was interesting that the expression of VrDREB2A was induced by drought stress; we further evaluated the function of VrDREB2A in plants response to drought stress. When without water for 16 d, 19.44 % of the wild-type plants survived, whereas the survival rates of the 35S:VrDREB2A L2, L5 and L8 transgenic plants were 49.31, 56.94 and 43.75 % (** $P < 0.01$, t test), respectively (Fig. 5b). Overall, the T₃ transgenic *Arabidopsis* plants overexpressing VrDREB2A showed enhanced drought tolerance, whereas the wild-type plants wilted compared to the L2, L5 and L8 transgenic lines after drought treatment (Fig. 5a). Meanwhile, the relative electrolyte leakage of the 35S:VrDREB2A transgenic lines were lower than that of the wild-type line (Fig. 5c). These results indicated that overexpression of VrDREB2A could increase drought tolerance in *Arabidopsis* plants.

Fig. 3 Expression patterns of *VrDREB2A* in response to different abiotic stress inmung bean by qRT-PCR analyze. 1-week-old seedlings were treated with the following treatments: **a** for drought treatment, seedlings were transferred to dry Whatman 3 MM paper for 1, 2, or 3 h; **b** seedlings were subjected to 1 M NaCl for 1, 6, or 24 h; **c** seedlings were placed in a growth chamber at 4 °C for 1, 6, or 24 h; **d** the leaves were sprayed with 200 μl ABA solution containing 0.05 % Tween20 (v/v) for 1, 6, or 24 h. After treatment, the whole seedlings were harvested, frozen in liquid nitrogen immediately, and stored at –80 °C. *VrActin* was used as a reference gene to measure the relative quantification. Data are presented as mean ± SD (n = 3) and error bars represent SD

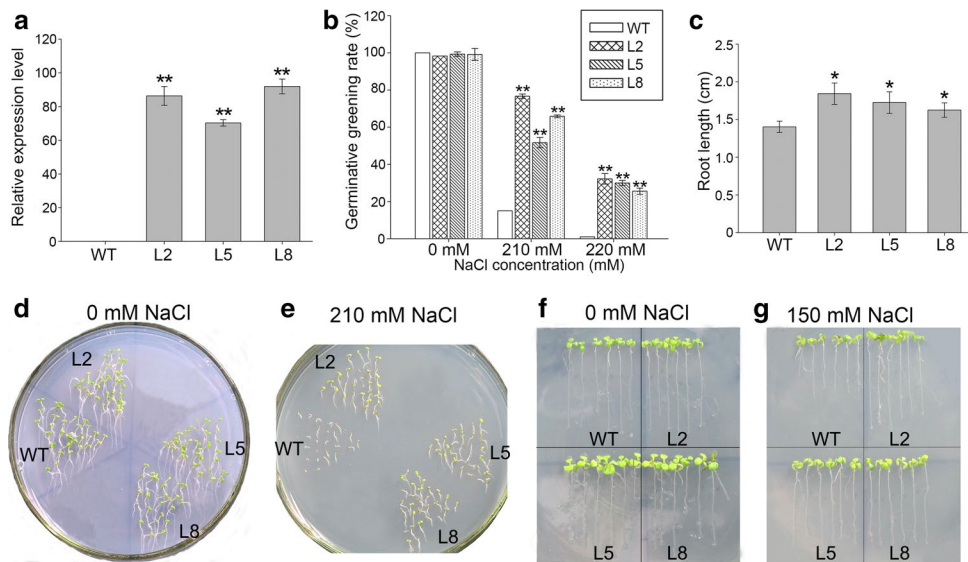
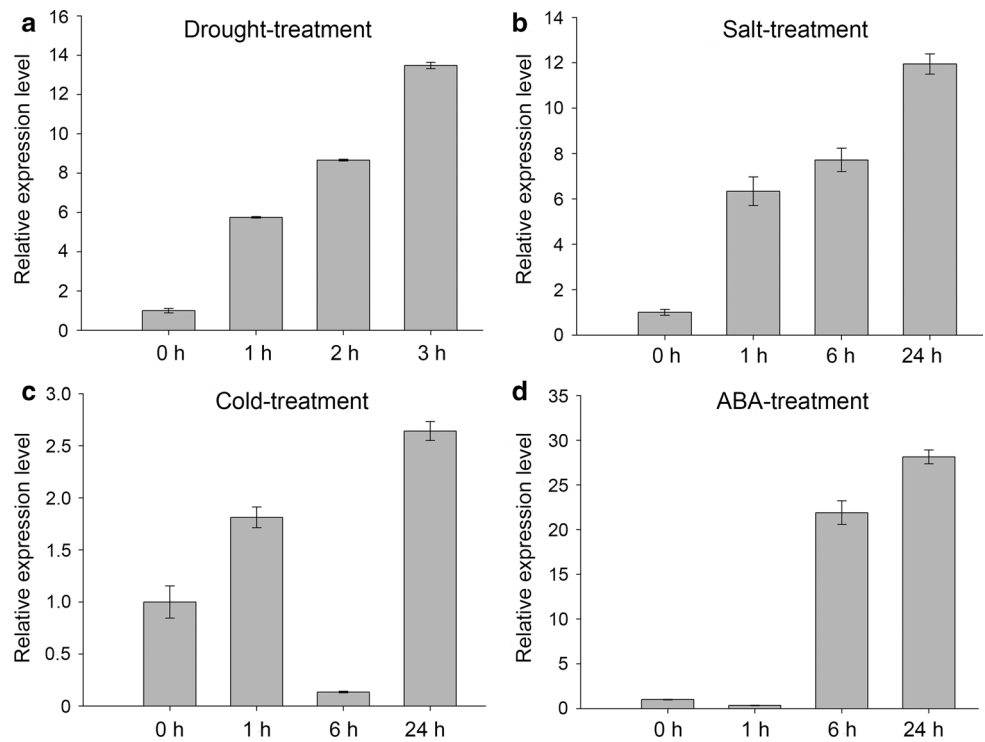


Fig. 4 The overexpression of *VrDREB2A* increased the salt tolerance in *Arabidopsis*. **a** *VrDREB2A* expression in transgenic lines (L2, L5 and L8) and wild type (WT). The values were normalized against *Atactin* expression. Data represent the mean ± SD of three technical replicates. **b** Germination rate of wild type and *VrDREB2A* transgenic lines (L2, L5 and L8) with 0, 210 or 220 mM NaCl for 14 d. Mean germination and SD were calculated from the results of three replicated experiments each using more than 60 seeds. **c** Primary root elongation of wild type and *VrDREB2A* transgenic lines (L2,

L5 and L8) seedlings in the presence of 0 mM or 150 mM NaCl for 7 d. All experiments included three replicates. *Error bars* represent the mean ± SD using 30 seeds. **d, e** Representative images of germination of wild type and *VrDREB2A* transgenic lines (L2, L5 and L8) with 0 mM or 210 mM NaCl for 14 d. **f, g** Representative images of root growth of wild type and *VrDREB2A* transgenic lines (L2, L5 and L8) with 0 mM or 150 mM NaCl for 7 d. *Asterisks* indicate significant difference from the wild type, Student’s *t* test, **P* < 0.05, ***P* < 0.01)

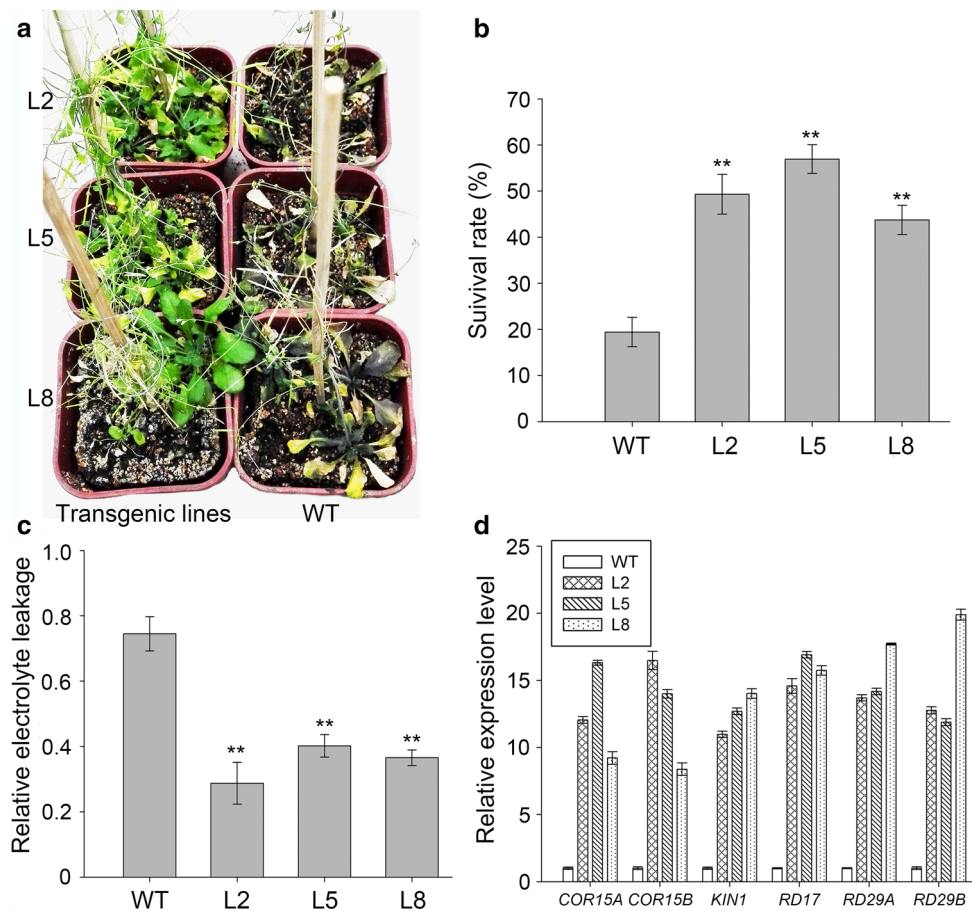


Fig. 5 The overexpression of *VrDREB2A* increased the drought tolerance of *Arabidopsis*. **a** Representative images of drought tolerance of 3-week-old *Arabidopsis* plants from which water was withheld for 16 d. **b** Survival rate for wild type, L2, L5 and L8, respectively. Survival statistics are presented as the number of plants survived/number of plants tested. The experiment included three replicates. Error bars represent the mean \pm SD and asterisks indicate a significant difference from the wild type ($n = 48$, Student's *t* test, * $P < 0.05$, ** $P < 0.01$). **c** The relative electrolyte leakage of wild type and transgenic plants (L2, L5 and L8) under normal condition was determined as described. **d** Expression analysis of *AtCOR15A*,

AtCOR15B, *AtKIN1*, *AtRD29A*, *AtRD29B* and *AtRD17*, downstream genes of *VrDREB2A* in *Arabidopsis*. Abiotic stress-responsive genes in *VrDREB2A* transgenic and wild-type plants were analyzed by qRT-PCR. Total RNA was extracted from 3-week-old seedlings grown under normal conditions. The graphs indicate the induction levels of *AtCOR15A* (At2g42540), *AtCOR15B* (At2g42530), *AtKIN1* (At5g15960), *AtRD29A* (At5g52310), *AtRD29B* (At5g52300) and *AtRD17* (At1g20440) in the transgenic lines L2, L5 and L8 compared with those of wild-type plants (WT). *Actin* was amplified as control. Data represent means and SE of three replications. Primers used are listed in Additional file (Supplementary Table S2)

Overexpression of the *VrDREB2A* in *Arabidopsis* activated the expression of downstream genes containing the DRE element

To understand the effects of *VrDREB2A* and the relationship between gene expression and stress tolerance in *Arabidopsis*, we analyzed the gene expression alterations of six genes containing DRE elements in their promoter regions (*AtCOR15A*, *AtCOR15B*, *AtKIN1*, *AtRD17*, *AtRD29A* and *AtRD29B*), all of which are downstream genes of *DREBs* in *Arabidopsis* (Li et al. 2011). In the transgenic plants, qRT-PCR analysis was performed using samples from WT and *VrDREB2A* transgenic plants. Expression levels of all six genes were enhanced in the *VrDREB2A* transgenic plants

under normal growth conditions (Fig. 5d). These results indicated that *VrDREB2A* up-regulated the expression of downstream genes related to the drought and salt stress responses.

Discussion

Studying the regulation of stress-inducible genes may improve our understanding of the mechanisms by which plants maintain growth and thrive under abiotic stress conditions (Dolferus 2014). Identifying the transcription factors that mediate responses to abiotic stress is an important prerequisite for the use of stress-inducible genes in crop

improvement. The DREB transcription factor is one of the most promising candidate genes conferring abiotic tolerance in several crops (Kizis et al. 2001; Roorkiwal et al. 2014). However, there have been no reports of mung bean *DREB* genes or the physiological processes regulated by these DREBs under abiotic stress conditions.

In this study, we successfully isolated *VrDREB2A* from mung bean, which is a drought-tolerant species (Kim et al. 2004), and demonstrated that it encoded a typical AP2/ERF domain. In addition, the phylogenetic analysis justified the classification of *VrDREB2A* in the DREB2 class of transcription factors, as its sequence was highly homologous to the cowpea protein *VuDREB2A*. Furthermore, *VrDREB2A* was a nuclear-localized protein and activated the transcription of dual reporter genes in yeast, which is characteristic of DREB2A from *Arabidopsis* and soybean (Mizoi et al. 2013). These data demonstrated that *VrDREB2A* was a new member of the DREB2 subclass and belonged to the AP2/EREBP family.

DREB2As play various roles in plants and participate in different pathways and crosstalk among pathways during the response to abiotic stresses. qRT-PCR analysis showed that the expression of the *VrDREB2A* gene was strongly induced by drought and salinity stresses. The rapid induction patterns of *VrDREB2A* in response to drought and high salt conditions were also characteristic of DREB2A proteins from *Arabidopsis* and other plant species. In particular, qRT-PCR data show that *VrDREB2A* is rapidly and strongly induced upon exposure to ABA treatment. This result indicated that *VrDREB2A* expression belongs to the ABA-dependent pathway in mung bean, which was completely different from the DREB2As isolated from other plants. This result confirmed that *VrDREB2A* is an abiotic stress response gene and may contribute to plant stress tolerance.

Many studies have indicated that transgenic methods are effective at improving the stress tolerance of crops. Earlier studies suggested that the overexpression of DREB2A proteins in plants increased abiotic stress, resulting in the induction of target stress-inducible genes in *Arabidopsis* (Kim et al. 2011; Sakuma et al. 2006b). Overexpression of *VuDREB2A* (Sadhukhan et al. 2014) and *GmDREB2A:2* (Mizoi et al. 2013) in *Arabidopsis* resulted in enhanced tolerance to drought stress. Homologous expression of *OsDREB2A* in rice improved survival rates under drought and salt stress conditions compared with wild type plants (Cui et al. 2011). Our results indicated that overexpression of *VrDREB2A* improved tolerance to drought and salt stress. In addition, several unique genes containing DRE cis-elements were up-regulated exclusively in the *DREB2A* overexpressing transgenic plants. These results indicated that DREB2A was also an important transcription factor regulating the expression of other stress responsive genes

via DRE cis-elements and that this protein might play a crucial role in mediating tolerance to multiple stresses.

Despite technological advances in the transformation of mung bean plants, the generation of transgenic mung bean plants is still difficult and time consuming (Somers et al. 2003). In our study, overexpression of *VrDREB2A* in *Arabidopsis* resulted in higher tolerance to drought and salt stress.

In conclusion, the *VrDREB2A* protein exhibited high transactivation activity and localized in the nucleus. Finally, the heterologous expression of *VrDREB2A* resulted in enhanced expression of *DREB2A* target, stress-inducible genes and improved the salt and drought stress tolerance of transgenic *Arabidopsis*. Therefore, our efforts in isolating mung bean *VrDREB2A* could provide a useful tool to increase plants tolerance against the increasing trend of droughts and/or salt restriction associated with global warming.

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