RESEARCH PAPER

An ABA-responsive DRE-binding protein gene from Setaria italica, SiARDP, the target gene of SiAREB, plays a critical role under drought stress

Cong Li*, Jing Yue*, Xiaowei Wu, Cong Xu and Jingjuan Yu†

State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: yujj@cau.edu.cn

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Abstract

The DREB (dehydration-responsive element binding)-type transcription factors regulate the expression of stress-inducible genes by binding the DRE/CRT cis-elements in promoter regions. The upstream transcription factors that regulate the transcription of DREB transcription factors have not been clearly defined, although the function of DREB transcription factors in abiotic stress is known. In this study, an abscisic acid (ABA)-responsive DREB-binding protein gene (SiARDP) was cloned from foxtail millet (Setaria italica). The transcript level of SiARDP increased not only after drought, high salt, and low temperature stresses, but also after an ABA treatment in foxtail millet seedlings. Two ABA-responsive elements (ABRE1: ACGTGTC; ABRE2: ACGTGGC) exist in the promoter of SiARDP. Further analyses showed that two ABA-responsive element binding (AREB)-type transcription factors, SiAREB1 and SiAREB2, could physically bind to the ABRE core element in vitro and in vivo. The constitutive expression of SiARDP in Arabidopsis thaliana enhanced drought and salt tolerance during seed germination and seedling development, and overexpression of SiARDP in foxtail millet improved drought tolerance. The expression levels of target genes of SiARDP were upregulated in transgenic Arabidopsis and foxtail millet. These results reveal that SiARDP, one of the target genes of SiAREB, is involved in ABA-dependent signal pathways and plays a critical role in the abiotic stress response in plants.

Key words: Abscisic acid (ABA), abiotic stress, dehydration-responsive element (DRE), foxtail millet, SiARDP1, SiAREB, signal pathway, transcription factor.

Introduction

The growth of plants and productivity of crops are limited by environmental stresses, such as drought, high salinity, soils, and low temperatures. To respond and adapt to these stresses, a large number of specific genes are induced, such as molecular chaperones, osmotic adjustment proteins (Tamura et al., 2003), ion channels (Ward and Schroeder, 1994) and others (Ingram and Bartels, 1996; Thomashow, 1999). Most of these functional proteins are regulated by specific transcription factors (Zhu, 2002; Chinnusamy et al., 2004; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

Abscisic acid (ABA)-responsive element binding (AREB) transcription factors are members of the group A subfamily of the bZIP transcription factor family and play a key role in ABA-responsive abiotic stress (Jakoby et al., 2002; Correa et al., 2008). AREB proteins bind to ABA-responsive elements (ABREs), which are major cis-elements in the ABA-responsive gene promoter region (Giraudat et al., 1994; Busk and Pages, 1998). The AREB transcription factors respond mainly to drought and high salinity stresses and are involved in the regulation of gene expression in the ABA-dependent signal transduction pathway (Finkelstein et al., 2002; Fujita et al., 2005).

The APETALA2 (AP2)/ethylene-responsive factor (ERF) proteins constitute a transcription factor family, and there are
145 ERF/AP2 members in Arabidopsis thaliana (Sakuma et al., 2002). Dehydration-responsive element binding (DREB) transcription factors, a subfamily of the ERF/AP2 family, were first isolated using yeast one-hybrid screening of Arabidopsis cDNA (Stockinger et al., 1997; Liu et al., 1998). The proteins bind to an 8-bp conserved sequence (ACCGACA), named the dehydration responsive element (DRE), in the rd29A promoter (Yamaguchi-Shinozaki and Shinozaki, 1994). Since then many DREB-type transcription factors have been cloned and identified, and most have been confirmed to enhance abiotic stress tolerance in plants (Dubouzet et al., 2003; Qin et al., 2007; Agarwal et al., 2010). DREB transcription factors DREB1 and DREB2 are involved in different stress response pathways. The DREB1 genes were rapidly induced by cold stress and activated the expression of their target genes (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Overexpression of DREB1 enhanced transgenic Arabidopsis tolerance to cold, dehydration, and salt stress, and induced accumulation of osmoprotectants, such as proline and various sugars (Gilmour et al., 2000). Several DREB1-homologous genes, OsDREB1 and OsDREB2 from rice, HvCBF2 and HvCBF from barley (Hordeum vulgare), ZmDREB1A from maize (Zea mays), and PgDREB2A from sorghum, have been identified (Dubouzet et al., 2003; Xue, 2003; Qin et al., 2004; Skinner et al., 2005; Agarwal et al., 2010). The DREB2 genes were induced by osmotic stresses (Liu et al., 1998). However, overexpression of DREB2A did not increase stress tolerance in transgenic Arabidopsis. A domain analysis showed that DREB2A contains a negative regulatory domain. An active form of DREB2A (DREB2A-CA), in which the negative regulatory region was deleted, could improve drought tolerance and activate the expression of the target genes in transgenic Arabidopsis (Sakuma et al., 2006). Most DREB2 genes were induced by drought and high salinity. ZmDREB2A, TaDREB1, and PgDREB2 also respond to low temperature, and ZmDREB2A was also induced by high temperature (Shen et al., 2003; Qin et al., 2004; Egawa et al., 2006).

The ABA-independent and ABA-dependent signal transduction pathways are the main abiotic stress response pathways in plants (Yamaguchi-Shinozaki and Shinozaki, 2005). Most of the DREB transcription factors are reported to be involved in the ABA-independent pathway; however, a few are responsive to ABA signalling, such as DBF1, CBF4, and OsDREBIF (Kizis and Pagés, 2002; Haake et al., 2002; Wang et al., 2008). Currently, few reports on the mechanisms involved in the upstream regulation of ABA-induced DREB-type transcription factor genes have been published.

Foxtail millet (Setaria italica), an ancient crop in China, provides rich nutrient elements and has excellent drought tolerance (Barton et al., 2009). It grows in arid or marginal soils and is of great significance in developing countries (Bettinger et al., 2010). Being a close relative to major food crops, and bioenergy grasses, and having a small diploid genome (~510 Mb), it has become an ideal model species (Doust et al., 2009; Lata et al., 2012). Recently, the reference genome sequence and genome-wide association studies (GWAS) of diverse foxtail millet varieties have been generated (Jia et al., 2011; Bennetzen et al., 2012; Zhang et al., 2012). Because of its excellent drought tolerance and water-use efficiency, research on the mechanisms of drought tolerance of foxtail millet is very important. In the present study, an ABA-responsive DREB-like protein gene, SiARDP, was cloned from foxtail millet cDNA using a yeast one-hybrid screening assay. The transcription levels of SiARDP increased under exogenous ABA treatment, as well as under drought and high salt stress. We cloned and identified two ABA-responsive AREB transcription factor genes from foxtail millet. In vitro and in vivo assays showed that these two AREB transcription factors could bind to the ABRE elements in the promoter region of SiARDP. Meanwhile, we examined the functions of SiARDP in Arabidopsis and foxtail millet, and found SiARDP was an important regulator for abiotic stress responses during seed germination and seedling development. The results show that in foxtail millet SiARDP might be involved in different signalling pathways, and two AREB proteins could be involved in the regulation of SiARDP.

**Materials and methods**

**Plant materials and treatments**

Seeds of foxtail millet (Setaria italica, cv. Jigu 11) were germinated on moist gauze for 24 h at 28°C, and then grown in pots filled with nutrient soil and vermiculite mixed 1:1 (v/v) under a 16 h:8 h (light:dark cycle) at 28°C and 60% relative humidity for 2 weeks. Then, the soil and vermiculite attached to the seedling roots were washed away. The seedlings were fixed in plastic foam, transferred to 1/3 Hoagland solution and grown hydroponically at 26°C for 3 d. They were then subjected to various treatments. During culture, the Hoagland solution was changed every day, and an aeration system was used. For salt, dehydration, and ABA treatments, the seedling roots were immersed separately in 1/3 Hoagland solution containing 100 mM NaCl, 20% PEG-6000 and 10 μM ABA, respectively, and kept for the time indicated. For the cold treatment, 17-day-old seedlings grown in soil were maintained at 4°C for the time indicated. Meanwhile, seedlings cultured in 1/3 Hoagland solution without treatment for the corresponding times indicated were used as controls.

**Yeast one-hybrid assay**

All of the bait and mutant bait sequences were inserted into the pAbAi vector at the HindIII and XhoI sites to create the bait vectors. The bait vectors were transformed into yeast strain Y1HGold following the protocol of the Yeastmaker™ Yeast Transformation System 2 (Clontech, USA). The bait strains were screened on synthetic defined (SD) medium lacking uracil and containing different concentrations of Aureobasidin A (AbA).

The foxtail millet cDNA library was constructed following the protocol of the Matchmaker™ Gold Yeast One-Hybrid Library Screening System (Clontech, USA).

SiARDP, SiAREB1 and SiAREB2 were cloned into pGADT7-AD at the NdeI and XhoI sites as prey vectors. They were transformed into bait strains and grown on SD medium lacking leucine and containing 800 ng ml⁻¹ AbA for 3 d at 30°C.

**RNA extraction and DNA analysis**

Total RNA was isolated using TRIzol reagent (Invitrogen, USA). After digestion with RNase-free DNase I (Takara, Japan), ~2 μg of total RNA was used for reverse transcription by M-MLV Reverse Transcriptase (Promega, USA).
Reverse transcription polymerase chain reaction (RT-PCR) was performed using 2× Taq PCR StarMix with Loading Dye (GenStar, China). PCR conditions were 95°C for 3 min, followed by 24 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Quantitative RT-PCR (qRT-PCR) assays were performed with a LightCycler 480 II real-time PCR detection system (Roche, USA) using the UltraSYBR Mixture (CWBIO, China). The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 57°C or 60°C for 1 min. The ΔΔCt method was used to calculate the expression levels of relevant genes.

Plasmid construction for subcellular localization analysis
The full-length sequences of SiARDP, SiAREB1, and SiAREB2 without the stop codons were inserted into a modified pUC-RFP plasmid. The AT-hook motif nuclear-localized protein 22 gene, AHL22 (BR000358), without the stop codon, was cloned into a modified pUC-RFP plasmid as the positive control. The primers used are shown in Supplementary Table S2 at JXB online. The genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter. Each plasmid (20 μg) was transformed into millet protoplasts. After culturing for 16 to 20 h, fluorescence was observed under a confocal laser microscope (Leica HQ).

Protoplast isolation and transfection
Seeds of foxtail millet were germinated in pots filled with nutrient soil and vermiculite mixed 1:1 (v/v) under 12 h:12 h (light:dark) conditions at 26°C for 3 d, and then were moved to the dark for another 4–6 d. Tissues from the stems and leaves were used. A bundle of foxtail millet plants was cut into 0.5–1 mm wide strips. Protoplast isolation and transfection were carried out according to the procedure described by Zhang et al. (2011). Finally, the protoplasts were resuspended gently in 1 ml W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, and 2 mM MES at pH 5.7) and cultured in the dark at 26°C for 16 h.

Transcriptional activation in yeast assay
The open reading frames of SiARDP, SiAREB1, and SiAREB2 were inserted into the pBD-GAL4 plasmid (Stratagene, USA) at the EcoR1 and SalI sites under the control of the yeast alcohol dehydrogenase 1 (ADH1) promoter. The pBD-GAL4 plasmid was used as the negative control and the pGAL4 vector as the positive control. These plasmids were independently transformed into the yeast YRG-2 strain. These transformed yeast cells were grown on SD medium containing 0, 100, 150, and 175 mM NaCl for 8 d at 22°C, and then the fresh/dry weights of the WT and transgenic Arabidopsis were measured. Additionally, 35-day-old seedlings from WT and transgenic Arabidopsis grown on MS medium containing 0, 150, 200, and 250 mM NaCl for 4 d at 22°C were used in the dehydration treatment.

Stress treatments of transgenic Arabidopsis and foxtail millet
Approximately 70 seeds from the wild-type (WT) and each selected line of the T3 generation of transgenic Arabidopsis were used for the phenotypic analysis. For the high salinity treatment, seeds of WT and transgenic Arabidopsis were sown on the MS medium containing 0, 100, 150, and 175 mM NaCl for 8 d at 22°C, and then the fresh/dry weights of the WT and transgenic Arabidopsis were measured. Approximately, 35-day-old seedlings from WT and transgenic Arabidopsis were grown on MS medium containing 0, 150, 200, and 250 mM NaCl for 4 d at 22°C. For the dehydration treatment, ~70 seeds from WT and transgenic Arabidopsis were sown on MS medium containing 0, 200, and 300 mM mannitol for 8 d at 22°C, and then the fresh/dry weights of the WT and transgenic Arabidopsis were measured. To test drought tolerance, 30 seeds each from WT and transgenic Arabidopsis were grown on MS medium for 1 week and transferred to pots filled with soil and vermiculite (1:1, v/v) for an additional 2 weeks at 22°C and 60% relative humidity. Water was withheld for 14 d as the control condition. The survival rate was counted 5 d after rewatering. Approximately 48 seeds from WT and transgenic millet were germinated on moist gauze for 24 h at 28°C and then transferred into pots filled with soil and vermiculite (1:1, v/v) for an additional 2 weeks under conditions of 28°C day:25°C night. Two-week-old seedlings were not watered for 14 d, and then were rewatered and grown under normal conditions for 5 d. The survival rate was calculated.

DNA extraction
Total DNA of non-transgenic Arabidopsis and transgenic Arabidopsis were used to examine the expression levels of Rd29A, Rd29B, Rd17, and MT2A by qRT-PCR. The primers are shown in Supplementary Table S1 at JXB online.

Chromatin immunoprecipitation (ChIP) assay
Approximately one million foxtail millet protoplasts were transfected with 35S:SiAREB1-GFP or 35S:SiAREB2-GFP. Then, the
protoplasts were incubated at 26°C in the dark for 16 h. The harvested protoplasts were resuspended in a W5 solution containing 1% formaldehyde and crosslinked for 20 min. ChIP was performed using the EpiQuik Plant ChIP Kit (Epigentek, Germany) according to the instructions of the manufacturer. The anti-GFP antibody (Sigma, USA) was used. To calculate the enrichment, the C\text{T} values were normalized against the input C\text{T}, where ΔC\text{T} = C\text{T (sample)} − C\text{T (input)}.

The primer sequences are listed in Supplementary Table S2 at JXB online.

Results

Isolation and identification of SiARDP

To isolate cDNAs encoding DRE binding proteins from foxtail millet, the one-hybrid library screening system was used. A triplicate 16-bp DNA fragment (ATACTACCGACATGAG) between positions −156 and −171 in the rd29A promoter, which contains a DRE core sequence (ACCGAC) at its centre, was cloned into pAbAi as a bait plasmid. A triplicate 16-bp mutant DNA fragment in which the DRE core sequence ACCGAC was substituted with AAAAAA was used as a negative control. The bait plasmid and control were independently transformed into yeast. These transformed yeast cells could grow on media lacking leucine and containing 800 ng ml\textsuperscript{−1} AbA, but the transformed mutant yeast cells did not (Fig. 1A). The cloned cDNA showed high homology with the grass DREB-type transcription factors, such as PgDREB2A and ZmDREB2A (Supplementary Figure S1C at JXB online), indicating that the transcription factor belonged to the DREB2 family. It was named SiARDP.

To confirm the yeast one-hybrid results, an EMSA was performed. SiARDP was expressed as a Flag-tagged fusion protein in E.coli. Because DREB transcription factors bind to the DRE core element ACCGAC in the rd29A promoter (Liu et al., 1998), the sequence was used as a probe (Fig. 1B). The results are shown in Fig. 1B. The SiARDP fusion protein bound the probe, and the signal was gradually diminished by the addition of the unlabelled DNA probe but not by the addition of the mutant unlabelled DNA probe.

Additionally, the ability of SiARDP to activate transcription was examined in yeast. The full-length SiARDP sequence

![Fig. 1. DNA binding ability and transcriptional activation assay of SiARDP. (A) Yeast one-hybrid assay in which SiARDP binds to the DRE core element. The bait sequences are shown in Supplementary Table S2 at JXB online. (B) SiARDP bound to various elements. The DRE (highlighted) was labelled with these probe sequences. (C) Transcriptional activation assay of SiARDP. The plus symbol indicates that the yeast was transformed with the pGAL4 plasmid as the positive control. The minus symbol indicates that the yeast was transformed with the pBD-GAL4 plasmid as the negative control. This figure is available in colour at JXB online.](https://academic.oup.com/jxb/article-abstract/65/18/5415/548766 by guest on 26 July 2018)
was inserted into pBD-GAL4, and SiARDP was fused with the DNA sequence encoding the GAL4 DNA-binding domain. The fusion gene was driven by the yeast ADH1 promoter. The yeast cells carrying the pBD-SiARDP plasmid grew on medium lacking threonine and histidine. Compared with the GAL4 negative control, SiARDP strongly activated the histidine reporter gene and β-galactosidase activity (Fig. 1C). The result indicates that full-length SiARDP can activate transcription.

**SiARDP gene expression and protein localization**

The expression patterns of SiARDP under abiotic stresses were analysed using qRT-PCR. The seedlings were treated with different stresses for 0, 1, 3, 6, 12, and 24 h, and untreated seedlings at the corresponding time points were used as controls. The relevant expression ratios (the relevant expression level of SiARDP in the treatment/the relevant expression level of SiARDP in the untreated control at the same time point) of each time point were calculated. The results showed that the expression of SiARDP increased during drought, low temperature, and high salinity treatments. SiARDP expression was also induced by ABA treatment. The transcript level of SiARDP was obviously induced after 3 h of ABA treatment and reached its highest level at 12 h. The amount of SiARDP showed a significant decrease after the 24-h treatment (Fig. 2A). These results imply that SiARDP may be involved in different stress responses. The expression levels of SiARDP in different foxtail millet organs were also examined. The transcript levels of SiARDP were highest in leaf, and lower in root, stem, and inflorescence (Fig. 2B).

We examined the subcellular localization of SiARDP and SiARDP fused to GFP, as well as AHL22 fused to RFP as a positive control. The fusion genes driven by the CaMV 35S promoter were transiently expressed in foxtail millet protoplasts. The results indicate that SiARDP was located in the nucleus (Fig. 3A–D).

**Overexpression of SiARDP enhances abiotic stress tolerance of transgenic Arabidopsis**

To analyse the function of SiARDP, it was overexpressed under the control of the CaMV 35S promoter in Arabidopsis. At least 30 transgenic Arabidopsis plants were obtained using a vacuum infiltration method (Bechtold et al., 1998), and three independent homozygous T3 generation lines with relatively high expression levels of the transgene (Fig. 4A) were used for further investigations.

SiARDP was responsive to high salinity, low temperature, and dehydration stress; therefore, our study focused on the abiotic stress tolerance of transgenic Arabidopsis. To analyse the influence of salt and dehydration stress on seed germination and growth, the seeds of non-transgenic and transgenic Arabidopsis were germinated on MS medium containing different concentrations of NaCl or mannitol. Almost all of the seeds germinated on the medium, but the seedling growth of transgenic Arabidopsis overexpressing SiARDP and non-transgenic Arabidopsis was different under different abiotic stress conditions. No obvious difference was observed between the transgenic and non-transgenic plants under normal conditions. However, the SiARDP-overexpressing transgenic seedlings were more tolerant than the non-transgenic seedlings under 100, 150, and 175 mM NaCl or 200 and 300 mM mannitol stress conditions (Fig. 4B). The fresh/dry weight of seedlings showed that the influence of the abiotic stress on the transgenic seedlings was weaker than on the non-transgenic plants (Fig. 4C).

To further determine the effect of SiARDP overexpression on high salinity tolerance, 5-day-old plants growing on a normal medium were transferred to media containing different concentrations of NaCl and maintained for 5 d. Under normal conditions, no obvious differences were observed between WT and transgenic seedlings. The survival rates of the three SiARDP transgenic lines growing on a medium containing 250 mM NaCl were significantly higher than that of WT plants (Fig. 4D and E). Electrolyte leakage assays showed that the level of ion leakage in the transgenic plants was lower than that in WT plants, especially under high salinity stress (Fig. 4F).
obvious differences between WT and transgenic plants under the high salinity treatment (data not shown).

**SiARDP regulates stress-responsive gene expression**

The transgenic *Arabidopsis* expressing *SiARDP* showed a higher tolerance to abiotic stress compared with the non-transgenic *Arabidopsis*. To analyse the expression of stress responsive genes in the transgenic *Arabidopsis*, four stress-relevant genes were chosen. Without stress treatment, the expression of *rd29A* and *MT2A* increased to significantly higher levels in the transgenic *Arabidopsis*, especially in line 4, than in WT (Fig. 7A), and *Rd29B* and *Rd17* showed slightly higher transcript levels in *SiARDP*-transgenic *Arabidopsis* lines than in control lines. These results imply that *SiARDP* may regulate stress tolerance genes in transgenic *Arabidopsis*.

We chose 13 stress-relevant genes from foxtail millet to analyse the function of *SiARDP* and to study the relationship between stress tolerance and gene expression. The 13 genes were categorized according to putative gene functions. qRT-PCR analysis was used to examine changes in the expression levels of these genes. The expression of four LEA genes, *Si007326m*, *Si002813m*, *Si036287m*, and *Si023261m*, four genes encoding dehydrins, *Si003296m*, *Si000619m*, *Si029046m*, and *Si026926m*, and three other drought stress relevant genes, *Si000619m*, *Si018287m*, and *Si035445m*, increased in transgenic foxtail millet plants under normal conditions (Fig. 7B). Additionally, we found that the expression levels of two salt and low temperature tolerance-relevant genes, *Si023013m* and *Si038484m*, were not obviously altered in transgenic foxtail millet (Fig. 7B). There was at least one DRE core element in the 1000-bp promoter region of these 13 genes (Supplementary Table S1 at *JXB* online). These results indicate that *SiARDP* may activate the expression levels of the drought stress-relevant genes through direct binding to the DRE core element in their promoter regions.

**Identification of SiAREB1 and SiAREB2**

To further study the stress signal transduction pathway mediated by *SiARDP* in foxtail millet, we analysed the promoter of *SiARDP* and found two ABRE motifs (Supplementary Figure S2 at *JXB* online). The AREB/ABF subfamily binds to the ABRE core sequence, and *SiARDP* might be regulated by AREB-type transcription factors in foxtail millet. We found six putative AREB-type genes in foxtail millet. Among these genes, two multiple stress-inducible genes, named *SiAREB1* and *SiAREB2*, were chosen for further research. *SiAREB1* encoded a protein of 357 amino acids, and *SiAREB2* encoded a protein of 280 amino acids. Both proteins had a basic leucine zipper (bZIP) domain. *SiAREB1* harbours three N-terminal and one C-terminal conserved domains, and *SiAREB2* has two N-terminal and one C-terminal conserved domains.

The expression levels of *SiAREB1* and *SiAREB2* were induced by dehydration, high salinity, and ABA treatments, but they were not affected by cold stress (Supplementary Figure S3A at *JXB* online). Both *SiAREB1* and *SiAREB2*
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were highly expressed in leaf tissue (Supplementary Figure S3B at JXB online). A subcellular localization assay showed that both SiAREB1 and SiAREB2 fused to GFP were located in the nucleus of the foxtail millet protoplast (Fig. 3F–L). The abilities of SiAREB1 and SiAREB2 to activate transcription were examined using a yeast transcriptional activation system. Yeast cells separately transformed with pBD-SiAREB1 and pBD-SiAREB2 were grown on medium lacking tryptophan and histidine. SiAREB1 and SiAREB2 strongly activated β-galactosidase compared with the negative control (Supplementary Figure S4 at JXB online). These results suggest that SiAREB1 and SiAREB2 are AREB-type transcription factors associated with abiotic stress in foxtail millet.

SiAREB1 and SiAREB2 bind to the ABRE motifs in the promoter of SiARDP

To examine whether SiAREB1 and SiAREB2 are involved in the regulation of SiARDP, a yeast one-hybrid assay was performed. Two bait sequences were inserted into a bait
AREB proteins are very important, 2005 Egawa, a transient Supplementary Figure S3A). Fig. 8C−1 Fig. 8C−1 Fig. 8B; Johnson Fig. 8B 24x27 on 26 July 2018 by guest Downloaded from https://academic.oup.com/jxb/article-abstract/65/18/5415/548766 SiAREB1 were transformed separately into the bait strains or mutant SiAREB1 on media containing 800 bait strains grew on media lacking uracil, but did not grow core elements were also created. The bait strains and mutant containing AAAAAAA as a substitute for ABRE1 and ABRE2 SiARDP containing ABRE2 (ACGTGGC). Both sequences were in the plasmid. One has three 17-bp sequences containing ABER1 (ACGTGTC) and another has three 17-bp sequences containing ABRE2 (ACGTGGC). Both sequences were in the SiARDP promoter. Meanwhile, mutant bait constructs containing AAAAAAA as a substitute for ABRE1 and ABRE2 core elements were also created. The bait strains and mutant bait strains grew on media lacking uracil, but did not grow on media containing 800ng ml⁻¹ ABA (data not shown). SiAREB1 and SiAREB2 were independently inserted into the expression vector pGADT7-AD. Then, the two vectors were transformed separately into the bait strains or mutant bait strains. The bait yeast cells transformed with pGADT7-SiAREB1 or pGADT7-SiAREB2 grew on media lacking leucine and containing 800ng ml⁻¹ ABA, but the transformed mutant bait yeast cells did not (Fig. 8A).

To confirm the results of the yeast one-hybrid assay, an EMSA was performed. The DRE core element ACCGAC in the rd29A promoter was used as probe 3 (P3) and probe 4 (P4), respectively (Fig. 8B). The sequence that contained two ABRE elements in the promoter of SiARDP was used as probe 2 (P2). The sequence that contained ABRE2 and that contained ABRE1 in the SiARDP promoter were used as probe 3 (P3) and probe 4 (P4), respectively (Fig. 8B). Full-length SiAREB1 and SiAREB2 were expressed as glutathione S-transferase (GST) fusion proteins in E. coli. We examined whether SiAREB1 and SiAREB2 could bind to these probes. Both SiAREB1 and SiAREB2 had the ability to bind to P2 and P3 but not P4 (Fig. 8C). Both SiAREB1 and SiAREB2 proteins bound efficiently to P2, while the binding affinities of SiAREB1 and SiAREB2 for P3 were weaker. With the addition of the unlabelled core probe, the signal was reduced, but the addition of the mutant probe did not interfere with binding. These results indicate that SiAREB1 and SiAREB2 specifically bind to the ABRE2 element in the promoter of SiARDP.

Additionally, to analyse whether SiAREB1 and SiAREB2 could bind to the promoter of SiARDP in vivo, a transient ChIP-qPCR was performed on WT foxtail millet protoplasts. A sequence analysis showed that two ABRE elements, A1 and A2, existed in a 1-kb region upstream of the translation start site (Fig. 8D). qRT-PCR was used to detect the results of the ChIP analysis. As shown in Fig. 8E, the transient expression levels of the SiAREB1-GFP and SiAREB2-GFP fusion proteins indicated that they could bind to the SiARDP promoter. The SiAREB1 protein’s interaction with the SiARDP promoter was stronger than the interaction of the SiAREB2 protein. These results indicated that SiAREB1 and SiAREB2 directly regulate SiARDP in vivo, and that SiAREB1 may be the main regulatory factor.

Discussion

The regulatory networks of abiotic stress are complicated. ABA signalling plays an important role in plants that are under abiotic stress, such as drought conditions (Busk and Pagès, 1998; Rock, 2000; Yamaguchi-Shinozaki and Shinozaki, 2006). AREB proteins are very important transcription factors in the ABA-responsive signal pathway, and their activation of AREB transcription factors is necessary for ABA-dependent phosphorylation (Johnson et al., 2002; Kobayashi et al., 2005; Furihata et al., 2006). In previous research, the AREB and DREB transcription factors were reported to belong to the ABA-dependent and ABA-independent signal pathways, respectively. However, a few of the DREB-type transcription factors were found to be involved in the ABA-dependent pathway (Egawa et al., 2006). In the present study, the transcript level of SiARDP was upregulated not only by dehydration, high salinity, and low temperature treatments, but also by exogenous ABA. We found two ABRE core elements, ABRE1 and ABRE2, in the promoter of SiARDP. Two AREB proteins, named SiAREB1 and SiAREB2, were identified as AREB transcription factors and confirmed to bind to ABRE2 (Fig. 8C and E). The transcript levels of SiAREB1 and SiAREB2 increased, similarly to the level of SiARDP, under dehydration, salt, and ABA treatments, but not under the low temperature treatment (Supplementary Figure S3A at JXB online). In addition, three genes, SiARDP, SiAREB1, and SiAREB2, exhibited the same tissue-specific expression patterns in foxtail millet (Fig. 2B and Supplementary Figure S3B at JXB online). The co-expression of these three
genes in the same tissues at the same time indicated that SiAREB1 and SiAREB2 may be involved in the regulation of SiARDP. These results suggest that SiARDP is a member of two abiotic stress signal transduction pathways. One is the ABA-dependent signal pathway for drought and salt stress regulated by SiAREB1 and SiAREB2, and the other appears to be an ABA-independent pathway for low-temperature stress regulated by other transcription factors (Fig. 9).

ABRE is a major cis-acting element in the ABA-dependent signalling pathway. Most of the ABA-inducible genes contain ABRE elements in their promoters, and a single ABRE element is not enough for ABA-dependent transcription (Shen et al., 1996; Hobo et al. 1999). SiAREB1 and SiAREB2 specifically bind to P3, which contains an ABRE2 element, but not to P4, which contains ABRE1 (Fig. 8B and C). Analysis of the P3 sequence found that a G-box-like element exists in the sequence, while a similar element does not exist in the P4 sequence. The results of the yeast one-hybrid assay showed that SiAREB1 and SiAREB2 bind to the ABRE1 element. This is probably because the triplicate 17-bp sequence improves the affinity of SiAREB1 and SiAREB2 proteins to the ABRE1 element. Furthermore, the ChIP-qPCR assay showed that SiAREB1 and SiAREB2 bind to both A1 (containing the AREB1 element) and A2 (containing the ABRE2 element) in the promoter region of SiARDP (Fig. 8D and E) in vivo. There are ~72 bp between A1 and A2, and this short sequence could not be sheared completely by sonication. This may be the main reason why A1 and A2 were enriched in the ChIP-qPCR assay. The results imply that SiAREB1 and SiAREB2 can bind to the promoter of SiARDP and that the
peripheral sequences of the ABRE element are very important to AREB transcription factor binding.

The DREB proteins are very important transcription factors in abiotic stress signal transduction pathways in plants. The stability of the DREB2 proteins is very important to their function in Arabidopsis. The full-length DREB2A protein was unstable in the nucleus and was degraded by the ubiquitin-proteasome pathway (Qin et al., 2008), while the DREB2A-CA (constitutive active form) with a deleted negative regulatory domain showed stable expression in the nucleus and upregulated some stress-induced genes (Sakuma et al., 2006). Some of the DREB2 proteins that do not contain the negative regulatory domain, such as ZmDREB2A, OsDREB2B, and PeDREB2 (Qin et al., 2007; Chen et al., 2009; Matsukura et al., 2010), enhance drought resistance in transgenic plants. SiARDP, reported here, also did not contain the negative regulatory region and is stable in the nucleus. Overexpression of SiDREB2 improved both drought and high salinity stress tolerance in transgenic Arabidopsis. SiARDP, and the results were consistent with the expression patterns of SiARDP under dehydration and salt stress (Figs 2A, 4B and D, and 5A). However, the overexpression of SiARDP only enhanced drought tolerance in transgenic millet (Fig. 6B). Similarly, DREB2C responds to salt, mannitol, and cold, but overexpression of DREB2C only improves thermostolerance in Arabidopsis (Lim et al., 2007). The expression level of

![Graphs showing gene expression levels in transgenic Arabidopsis and transgenic foxtail millet.](https://academic.oup.com/jxb/article-abstract/65/18/5415/548766)
**ZmDREB2A** was increased by drought, salt, cold, and heat in maize, while overexpressing it enhanced drought tolerance and thermotolerance in transgenic *Arabidopsis*. These studies suggest that DREB2 proteins have different functions in different plants, and heterologous expression may be an important reason.

The DREB genes enhance stress tolerance by regulating their target genes. Overexpressing *SiARDP* induced the expression of drought and salt stress-relevant genes (*Rd17*, *MT2A*, *Rd29A*, and *Rd29B*) in transgenic *Arabidopsis* (Fig. 7A). In a previous study, *Rd17*, *Rd29A* and *Rd29B* were induced by drought and salt stress, and *MT2A* was induced by drought stress. In transgenic millet most of the induced genes were related to drought stress. Although the precise functions of these induced genes in the transgenic foxtail millet are still unknown, previous studies have implied that these proteins, especially LEA, play a role in protecting the cells from the irreversibly damaging effects of a water deficit (Ingram and Bartels, 1996; Thomashow, 1999; Zhu, 2001). Meanwhile, two salt and low temperature stress-relevant genes, *Si038484m* and *Si023013m*, were not induced. The target genes of *SiARDP* in transgenic *Arabidopsis* and transgenic millet are different, and there is a positive relationship between the stress induction of genes and stress tolerance. These results indicate that *SiARDP* is involved in the drought stress signalling pathway in foxtail millet.

Plant abiotic stress tolerance involves complex physiological and biochemical processes. In these processes, transcription factors are important to the plant's ability to adapt to stresses. In the present study, we focused on how *SiARDP* was involved in the ABA-responsive signalling pathway, as well...
Grass (Foxtail millet)  

- **Drought, High salinity**  
  - Signal Perception  
    - ABA-dependent pathway  
      - SiAREB  
        - ABRE  
          - DRE  
            - ABREs  
              - Cold  
                - ABA-independent pathway  
                  - SiARDP  
                    - Target genes

**Fig. 9.** Model of the SiARDP response to abiotic stress in foxtail millet. SiARDP is regulated by SiAREB transcription factors in the ABA-dependent pathway under drought and high salinity stress. Under cold stress, SiARDP may be involved in the ABA-independent pathway.

As the ability of SiARDP to increase abiotic stress tolerance in plants. Based on our study, we propose a regulatory role for SiAREB transcription factors in regulating the expression of SiARDP under drought and salt stress (Fig. 9). Our findings show that SiAREB1 and SiAREB2 bind to the ABRE elements in the promoter region of SiARDP and activate the expression of SiARDP, and that the target genes of SiARDP are then activated in response to drought and salt stress.

**Supplementary material**

Supplementary data can be found at JXB online.

- **Supplementary Table S1.** Stress-relevant genes in overexpressing foxtail millet SiARDP plants.
- **Supplementary Table S2.** Primers and bait sequences used in this study.
- **Supplementary Figure S1.** Comparison of amino acid sequences and phylogenetic analysis of the foxtail millet SiARDP with other DREB proteins.
- **Supplementary Figure S2.** ABREs identified in the SiARDP promoter.
- **Supplementary Figure S3.** Expression pattern assay of SiAREB1 and SiAREB2.
- **Supplementary Figure S4.** Transcriptional activation assay of SiAREB1 and SiAREB2.

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