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An ABRE Promoter Sequence is Involved in Osmotic Stress-Responsive Expression of the *DREB2A* Gene, Which Encodes a Transcription Factor Regulating Drought-Inducible Genes in Arabidopsis

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In plants, osmotic stress-responsive transcriptional regulation depends mainly on two major classes of *cis*-acting elements found in the promoter regions of stress-inducible genes: ABA-responsive elements (ABREs) and dehydration-responsive elements (DREs). ABRE has been shown to perceive ABA-mediated osmotic stress signals, whereas DRE is known to be involved in an ABA-independent pathway. Previously, we reported that the transcription factor DRE-BINDING PROTEIN 2A (DREB2A) regulates DRE-mediated transcription of target genes under osmotic stress conditions in Arabidopsis (*Arabidopsis thaliana*). However, the transcriptional regulation of *DREB2A* itself remains largely uncharacterized. To elucidate the transcriptional mechanism associated with the *DREB2A* gene under osmotic stress conditions, we generated a series of truncated and base-substituted variants of the *DREB2A* promoter and evaluated their transcriptional activities individually. We found that both ABRE and coupling element 3 (CE3)-like sequences located approximately −100 bp from the transcriptional initiation site are necessary for the dehydration-responsive expression of *DREB2A*. Coupling our transient expression analyses with yeast one-hybrid and chromatin immunoprecipitation (ChIP) assays indicated that the ABRE-BINDING PROTEIN 1 (AREB1), AREB2 and ABRE-BINDING FACTOR 3 (ABF3) bZIP transcription factors can bind to and activate the *DREB2A* promoter in an ABRE-dependent manner. Exogenous ABA application induced only a modest accumulation of the *DREB2A* transcript when compared with the osmotic stress treatment. However, the osmotic stress-induced *DREB2A* expression was found to be markedly impaired in several ABA-deficient and ABA-insensitive mutants. These results suggest that in addition to an ABA-independent pathway, the ABA-dependent pathway plays a positive role in the osmotic stress-responsive expression of *DREB2A*.

Keywords: ABA • *Arabidopsis thaliana* • Osmotic stress • Promoter analysis • Transcription factor.

Abbreviations: ABF, ABA-responsive element-binding factor; ABRE, ABA-responsive element; AREB, ABA-responsive element-binding protein; 3-AT, 3-amino-1,2,4-triazole; bHLH, basic helix–loop–helix; bZIP, basic leucine zipper; CE3, coupling element 3; ChIP, chromatin immunoprecipitation; DRE, dehydration-responsive element; DREB, dehydration-responsive element-binding protein; GUS, β-glucuronidase; HA, hemagglutinin; qRT-PCR, quantitative real-time PCR

Introduction

In contrast to animals, plants cannot avoid unfavorable circumstances by adjusting their location. Instead, they have developed sophisticated sensing and response mechanisms related to environmental stresses such as drought, high salinity and extreme temperatures. Numerous researchers have reported a number of genes that provide the ability to tolerate and overcome the adverse effects of these stresses and have revealed complicated transcriptional regulatory networks

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associated with these genes that involve specific *cis*-acting elements in their promoters and interacting transcription factors (Zhu 2002, Bartels and Sunkar 2005, Nakashima et al. 2009b, Medina et al. 2011).

Many osmotic stress-responsive genes in plants are also responsive to the phytohormone ABA, indicating that ABA functions as a signal mediator for osmotic stress in plants (Cutler et al. 2010, Raghavendra et al. 2010, Fujita et al. 2011). The ABA-responsive element (ABRE; PyACGTG/TC) is a well-studied *cis*-element involved in ABA-induced gene expression (Hattori et al. 2002, Fujita et al. 2011). Multiple ABREs or combinations of ABREs with coupling elements are required for ABA-responsive transcription to occur (Shen et al. 1996, Hobo et al. 1999, Narusaka et al. 2003, Zhang et al. 2005, Gómez-Porras et al. 2007). The ABRE-BINDING PROTEIN/FACTOR (AREB/ABF) family belongs to group A of the basic leucine zipper (bZIP) transcription factor family and is composed of nine members in Arabidopsis (*Arabidopsis thaliana*) (Choi et al. 2000, Uno et al. 2000, Corrêa et al. 2008). Gain- and loss-of-function studies on the *AREB/ABF* genes have revealed positive and negative effects of these genes, respectively, on the osmotic stress tolerance of plants (Kang et al. 2002, Kim et al. 2004, Yoshida et al. 2010).

Dehydration-responsive element/C-repeat (DRE; A/GCCG AC) is another example of the *cis*-elements that govern osmotic stress-responsive transcription (Agarwal et al. 2006, Nakashima et al. 2009b). DRE was first identified as an essential *cis*-element in the *RESPONSIVE TO DESSICATION 29A* (*RD29A*) promoter for ABA-independent expression in response to dehydration and cold stresses (Yamaguchi-Shinozaki and Shinozaki 1994). The ABA independence of DRE-mediated transcription has been supported by several reports. A truncated *RD29A* promoter containing DREs without an ABRE was found to exhibit a loss of induction activity in response to exogenous ABA application but to retain induction activity under osmotic stress treatments (Yamaguchi-Shinozaki and Shinozaki 1994, Narusaka et al. 2003). Moreover, the osmotic stress-responsive transcription of *RD29A* was observed to be maintained in ABA-deficient mutant plants (Yamaguchi-Shinozaki and Shinozaki 1993).

DRE-BINDING PROTEIN 2A (DREB2A) was identified in a yeast one-hybrid screen using the *RD29A* promoter as bait (Liu et al. 1998). DREB2A harbors an ethylene-responsive element-binding factor/APETALA2-type (ERF/AP2-type) DNA-binding domain and functions as a transcriptional activator by interacting with a DRE *cis*-element in the promoter region of downstream genes (Liu et al. 1998, Nakashima et al. 2000, Sakuma et al. 2002). Overexpression of full-length DREB2A does not result in the activation of downstream gene expression. However, overexpression of a constitutively active form of DREB2A that lacks the negative regulatory domain confers enhanced tolerance to dehydration, high salinity and high temperature in plants (Sakuma et al. 2006a, Sakuma et al. 2006b). Conversely, T-DNA insertional mutants of *DREB2A* exhibit a loss of tolerance to high temperature (Sakuma et al. 2006b). Subsequent genome-wide transcriptomic analyses revealed that DREB2A contributes to the acquisition of environmental stress tolerance in plants. Microarray data from a *DREB2A* gain-of-function mutation suggest that DREB2A regulates a number of genes that are involved in osmotic and/or heat stress tolerance and that a number of these genes contain more than one DRE sequence in their promoter region (Sakuma et al. 2006a, Sakuma et al. 2006b, Maruyama et al. 2009). Recent studies suggest that DREB2A might collaborate with other transcription factors, including members of the AREB/ABF family, in downstream gene regulation through direct interaction (Lee et al. 2010, Elfving et al. 2011).

DREB2A expression is also stress inducible. When Arabidopsis plants are exposed to abiotic stress conditions, such as heat and high osmolarity, the *DREB2A* transcript level increases rapidly and reaches levels up to 250-fold higher than the basal level. In contrast, transcriptional induction of *DREB2A* in response to exogenous ABA is weak, which supports our categorization of *DREB2A* transcription under osmotic stress conditions as ABA-independent. However, a weak but significant induction of *DREB2A* expression in response to ABA application can be detected (Liu et al. 1998, Nakashima et al. 2000, Sakuma et al. 2002).

From many studies performed in recent years, we are currently beginning to understand the roles of DREB2A and its orthologs in plants. However, we still have little information regarding how plants regulate the expression of *DREB2A* in response to unfavorable circumstances. In this study, we report that the *DREB2A* promoter contains ABRE and coupling element 3 (CE3)-like sequences that are necessary for promoter activity under osmotic stress conditions, such as dehydration and high salinity. Based on establishing the significant but partial effect of ABA signaling cascades on *DREB2A* expression, we suggest a model in which *DREB2A* expression requires both ABA-dependent and ABA-independent signaling cascades.

Results

Truncation analysis of the *DREB2A* promoter

To define the *DREB2A* promoter region involved in the response to osmotic stress, a number of truncated promoter fragments were isolated from Arabidopsis genomic DNA and fused to the *GUS* (β-glucuronidase) reporter gene in the plant expression vector pGK-GUS (Qin et al. 2008). Transgenic Arabidopsis plants harboring each promoter–*GUS* construct were generated and were exposed to controlled dehydration conditions for 5 h. The transcript levels of endogenous *DREB2A* and *GUS* were measured by quantitative real-time PCR (qRT-PCR) (**Fig. 1**). The transcriptional response of *GUS* was abolished between the N8 and N9 constructs, or between the C5 and C6 constructs (**Fig. 1**). These data indicated that the region (designated Region I) between −147 and −54 bp from

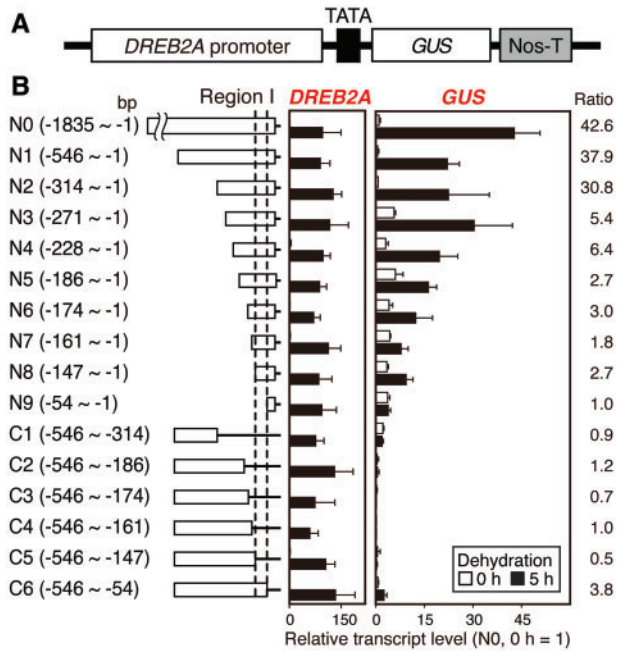

Fig. 1 Truncation analysis of the *DREB2A* promoter region. (A) Schematic map of the promoter–*GUS* construct used in this study. (B) The left panel shows the relative locations and lengths of the promoter fragments that were fused with the *GUS* reporter gene and introduced into Arabidopsis plants. The middle and right panels show the relative transcript levels of endogenous *DREB2A* and *GUS*, respectively, before (open bar) or after (filled bar) the dehydration treatment. The values represent averages from more than three independent transgenic lines for each construct, and the error bars indicate the SD. The ratios indicate the fold change increases of the *GUS* transcript levels after the dehydration treatment compared with values obtained before the treatment for each line.

the transcription initiation site was responsible for the dehydration-responsive expression of *DREB2A* (**Fig. 1**).

ABRE is necessary for *DREB2A* expression under osmotic stress

For further characterization of the *cis*-elements that influence *DREB2A* promoter activity, we screened the Region I sequence using two web-based plant *cis*-element databases: PlantCARE (Lescot et al. 2002) and PLACE/Signal Scan (Higo et al. 1999). Based on these screenings, ABRE and CE3 (GACGCGTGTC)-like sequences were identified as putative *cis*-elements in Region I (**Fig. 2A**). Although no clear-cut prediction was available, two additional GC-rich boxes were identified and designated Box1 and Box2 (**Fig. 2A, B**). In addition, we obtained genomic sequences upstream of the *DREB2A* orthologs in other plant species from the Phytozome database (http://www.phytozome.net/) and compared them with the *DREB2A* promoter sequence. We found that the CE3–ABRE cassette is conserved in the promoter region of *DREB2A* orthologs in many other eudicot plants, although CE3 was found to be replaced by

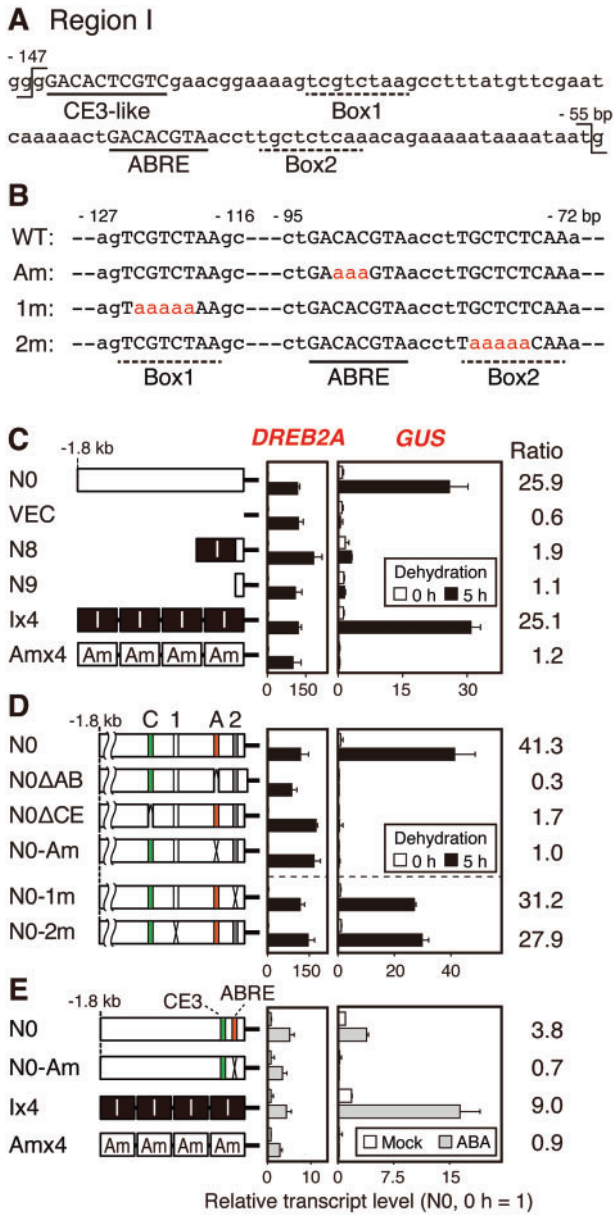

Fig. 2 Analysis of the ABRE and CE3-like sequences in Region I. (A) Sequence of Region I of the *DREB2A* promoter. Predicted *cis*-elements (solid line) and GC-rich boxes (dotted line) are underlined and indicated. (B) Nucleotide substitutions introduced into the ABRE, Box1 and Box2 sequences. Each candidate *cis*-element is underlined and in upper case. Substituted nucleotides are in red. (C–E) qRT-PCR analysis of various promoter–*GUS* constructs. The left panel presents the schematic structure of the promoter in each reporter construct. The middle and right panels show the relative transcript levels of endogenous *DREB2A* and *GUS*, respectively, before (open bar) and after (filled bar) dehydration (C, D) or ABA treatment (E). The error bars indicate the SD. The ratios indicate the fold change increase of *GUS* transcript levels following dehydration or ABA treatment. Abbreviations: C, CE3-like; A, ABRE; 1, Box1; and 2, Box2.

ABRE in plants outside the *Arabidopsis* genus (**Supplementary Fig. S1**).

Because the transcriptional activity of a single copy of the Region I sequence was not strong enough to define, a new promoter–*GUS* construct was prepared containing four unidirectional tandem repeats of Region I (I × 4). In transgenic Arabidopsis plants, I × 4 showed dehydration stress-induced accumulation of *GUS* mRNA as N0 did (**Fig. 2C**). Next, we introduced base substitutions into each of the putative ABRE sequences in the I × 4 construct and designated this new construct Am × 4 (**Fig. 2B, C**). This ABRE-mutated construct resulted in the loss of induction of transcriptional activity in response to dehydration (**Fig. 2C**). These results raised the possibility that the ABRE sequence might be one of the core elements in the *DREB2A* promoter involved in the response to dehydration stress. To test this hypothesis, we examined the promoter activity of the N0 derivatives, including base substitutions in ABRE, Box1 or Box2 (N0-Am, N0-1m or N0-2m, respectively) and truncations of the ABRE or CE3-like (N0ΔAB or N0ΔCE, respectively) sequence in transgenic plants under dehydration conditions (**Fig. 2B, D**). All of the ABRE- or CE3-like-mutated promoters were unable to respond to dehydration treatment, whereas mutations in Box1 (N0-1m) and Box2 (N0-2m) had only minor effects on the promoter activity (**Fig. 2B, D**). Because ABRE was originally identified as a *cis*-element involved in ABA signaling, we suspected that the ABRE sequence in the *DREB2A* promoter might receive an ABA signal. Two-week-old plants containing one of several different promoter–*GUS* constructs were treated with ABA for 5 h, and their transcriptional activities were measured individually by qRT-PCR (**Fig. 2E**). We were able to detect the responses of the wild-type constructs (N0 and I × 4: 3.8- and 9.0-fold increases, respectively) to ABA, but the fold changes were smaller than the increases observed in response to dehydration stress (**Fig. 2C**: 25.9- and 25.1-fold increases, respectively). The ABRE-mutated constructs (N0-Am and Am × 4) did not respond to ABA (**Fig. 2E**). These data suggest that the ABRE sequence in Region I is responsible for the dehydration stress-induced activity of the *DREB2A* promoter and is also involved in the weak induction of *DREB2A* by ABA.

AREB/ABFs bind to the ABRE sequence of the *DREB2A* promoter

ABRE belongs to the larger G-box *cis*-element group, which is generally considered to be a target of the bZIP or basic helix–loop–helix (bHLH) transcription factor families (Choi et al. 2000, Uno et al. 2000, Sibéril et al. 2001). Therefore, we hypothesized that members of these transcription factor families might influence ABRE-dependent transcriptional regulation of the *DREB2A* promoter. Because the ABRE sequence in the *DREB2A* promoter is distinct from the standard target sequence of bHLH (CANNTG; Sibéril et al. 2001), we attempted to identify candidate transcription factors in the bZIP family that bind to the ABRE sequence. Using the methods described by Satoh et al. (2004), we screened a pool comprised of representative members of each bZIP family subgroup using the I × 4 and N0 constructs (**Supplementary Fig. S2**). An active form of AREB1 (AREB1ΔQT; Fujita et al. 2005), which represents subgroup A, was associated with the most prominent transcription activity of the *DREB2A* promoter. The bZIP family proteins that belong to subgroups B, C, E and I were also associated with transcription activity, although this activity was weak (**Supplementary Fig. S2**). The AREB/ABF family members are important for ABRE-mediated gene expression (Choi et al. 2000, Uno et al. 2000, Kang et al. 2002, Kim et al. 2004, Corrêa et al. 2008, Yoshida et al. 2010, Fujita et al. 2011). Therefore, we selected the AREB/ABF family as one of the candidate families for transcription factors that interact with ABRE in the *DREB2A* promoter.

Among the nine members of the AREB/ABF family, AREB1, AREB2 and ABF3 were selected because they have been reported to regulate environmental stress responses and ABA signaling coordinately in the vegetative stage (Fujita et al. 2005, Yoshida et al. 2010). To test the abilities of the three AREB/ABF members to bind to the *DREB2A* promoter, we used a yeast one-hybrid (Y1H) system (**Fig. 3A, B**). Either a 42 bp fragment of the *DREB2A* promoter proximal to the ABRE sequence (WT) or a mutated ABRE sequence (MT) was introduced into the yeast genome (**Fig. 3A**). The three AREB/ABF members were expressed individually in the yeast cells as fusion proteins with the GAL4 activation domain, and their binding affinities for the promoter were evaluated. On synthetic dextrose media (SD media) containing 3-amino-1,2,4-triazole (3-AT), the yeast lines expressing each tested AREB/ABF member showed more rapid growth than did the void vector control (VEC) in an ABRE-dependent manner (**Fig. 3B**). These results suggested that the AREB/ABF members can bind to the ABRE sequence in yeast. AREB1 is activated by phosphorylation, and two constitutively active forms (AREB1ΔQT, internal deletion; AREB1M8, phosphorylation mimic) and one constitutively inactive form (AREB1M5, phosphorylation negative) of this protein have been reported (Fujita et al. 2005, Furihata et al. 2006). We adapted these derivatives of AREB1 for use in our Y1H system and found no significant differences among the binding signals of these mutant forms (**Fig. 3B**).

To confirm the interaction of AREB/ABFs with the ABRE sequence in the *DREB2A* promoter in planta, chromatin immunoprecipitation (ChIP) was performed using Arabidopsis plants expressing hemagglutinin (HA)-tagged AREB1 driven by the 35S promoter (**Fig. 3C–E**). Because the relationship between stress signal acceptance and the DNA-binding affinity of the AREB/ABF proteins is unknown, we treated the plants with ABA or dehydration before ChIP. In both mock- and stress-treated plant lines, positive binding signals were detected by PCR (**Fig. 3D**) and quantitative PCR (**Fig. 3E**). Taken together with the results from the Y1H system, these findings suggest that the three AREB/ABF members could interact directly with the ABRE sequence of the *DREB2A* promoter. In addition, the

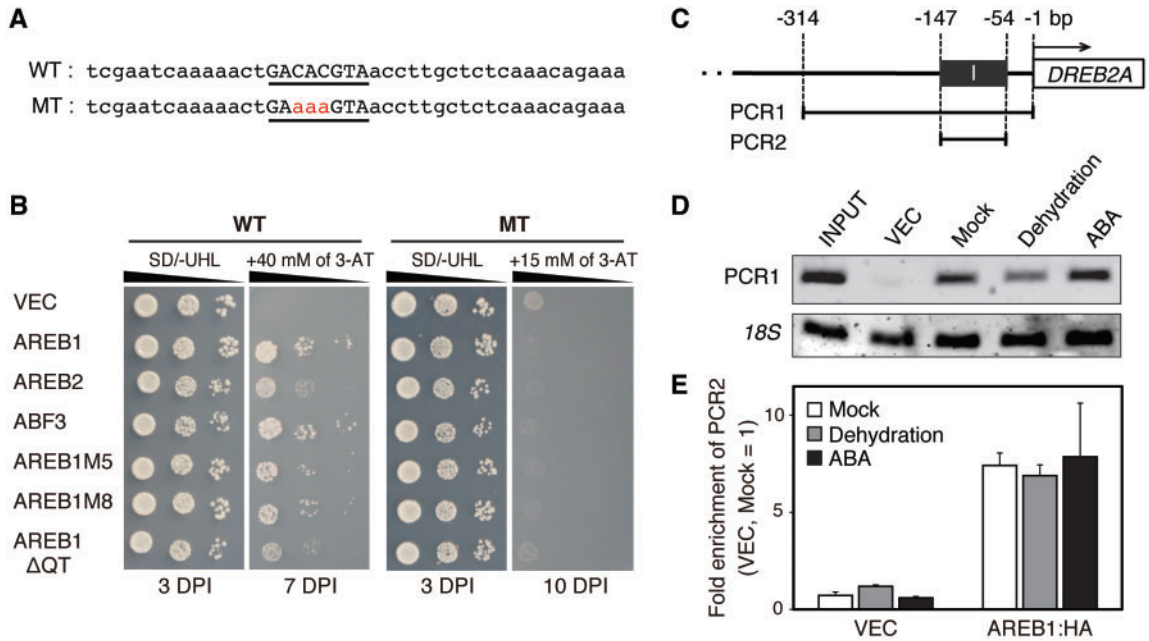

Fig. 3 Interaction of the AREB/ABF transcription factors with the *DREB2A* promoter. (A) The 42 bp sequence proximal to the ABRE sequence used as bait for yeast one-hybrid analysis. WT includes the intact promoter sequence, and MT includes the ABRE-inactivated sequence. The ABRE sequences are underlined and in upper case. (B) Analysis of the ability of the AREB/ABFs to bind to the *DREB2A* promoter. Growth of yeast cells on SD/−UHL media or media supplemented with 3-AT (40 or 15 mM). VEC indicates the empty vector control. DPI refers to day post-inoculation. (C) Schematic map of the amplified promoter regions (PCR1 and 2) in the chromatin immunoprecipitation assays. In the filled block, I refers to Region I. (D) Enrichment of a *DREB2A* promoter region (PCR1) visualized in an ethidium bromide-stained agarose gel. As a loading control for the template, *18S* rDNA (*18S*) is used. Lanes are identified as follows: input control (INPUT), void vector control (VEC), mock treatment (Mock), 2 h of dehydration treatment (Dehydration) and 5 h of ABA treatment (ABA). (E) Fold enrichment of another *DREB2A* promoter region (PCR2) analyzed by quantitative PCR. The values indicate the average amounts of the fragment normalized to the amount of *18S* rDNA. The error bars indicate the SD.

activation of AREB1 by an ABA or stress signal seems to be distinct from its binding affinity for the target sequence.

AREB/ABF transcription factors can activate the *DREB2A* promoter

We evaluated the transactivity of the three AREB/ABF members with respect to the *DREB2A* promoter through a transient expression assay using Arabidopsis mesophyll protoplasts (**Fig. 4**). In addition to the three AREB/ABF members, we used AREB1ΔQT, which exhibits constitutive transactivity in the absence of ABA (Fujita et al. 2005). An RD29Bpro construct, including five tandem repeats of doubled ABREs containing a 77 bp fragment from the *RD29B* promoter region, was used as a positive control reporter. This construct has been reported to show a steady response to ABA-dependent AREB/ABF activation in a transient expression assay system (**Fig. 4A**) (Fujita et al. 2005, Furihata et al. 2006, Yoshida et al. 2010). In comparison with the RD29Bpro reporter construct, all of the tested AREB/ABF members showed reduced but still significant transactivity related to the N0 construct in an ABA-dependent manner (**Fig. 4B**). Similar to the loss-of-function analysis in the Y1H system, the ABRE-mutated MT construct showed no activity associated with any of the AREB/ABF members (**Figs. 3B**, **4B**). These results suggest that the investigated AREB/ABFs might be among the transcription factors that interact with the ABRE sequence in the *DREB2A* promoter and activate its expression.

The stress-responsive activity of the *DREB2A* promoter is impaired in ABA-insensitive and ABA-deficient mutants

Studies on two triple null mutants [null for *AREB1*, *AREB2* and *ABF3* (*areb1 areb2 abf3*; *areb* triple) or *SnRK2D*, *SnRK2E* and *SnRK2I* (*srk2d srk2e srk2i*; *srk2* triple)] have shown AREB/ABFs and SnRK2s to be key factors influencing gene expression as part of a signaling cascade in response to ABA and osmotic stress signals (Fujii and Zhu 2009, Fujita et al. 2009, Nakashima et al. 2009a, Yoshida et al. 2010). Microarray and qRT-PCR data from these reports revealed transcriptional alleviation of *DREB2A* under high salinity and dehydration stress conditions in these two triple mutants (**Supplementary Table S1**) (Fujita et al. 2009, Yoshida et al. 2010). Based on these data, we performed dehydration treatments using representative ABA mutant lines (**Fig. 5A**). ABA INSENSITIVE 1 and 2 (ABI1 and ABI2), which encode type 2C protein phosphatases, have negative regulatory roles in ABA signaling, and *abi1-1C* and *abi2-1C*

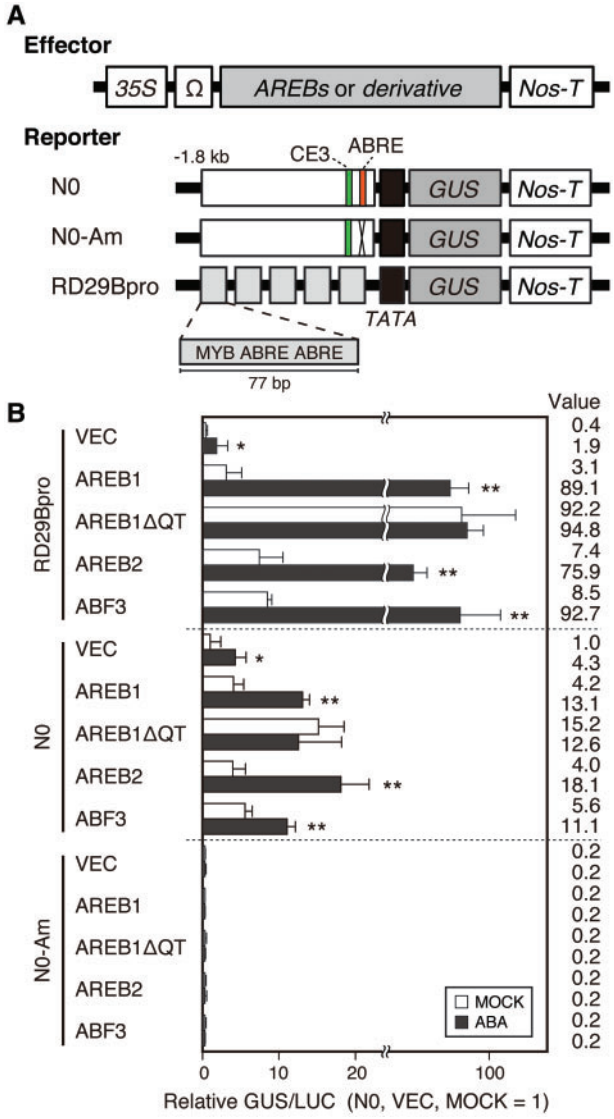

Fig. 4 Transactivation of *DREB2A* promoter fragments by AREB/ABF transcription factors. (A) Schematic view of the constructs used for transient expression analysis. (B) Transactivation of *DREB2A* promoter fragments by AREB/ABFs with or without ABA. Values represent the mean activity ratios of β-glucuronidase (GUS) and luciferase (LUC). The error bars indicate the SD. VEC indicates the empty effector plasmid. Asterisks indicate significant differences that were observed due to ABA treatment (Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$).

plants mutant for these genes exhibit ABA insensitivity (Leung et al. 1997, Raghavendra et al. 2010, Umezawa et al. 2010). The *ABA-DEFICIENT 2* (*ABA2*) and *9-cis-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) genes encode key enzymes in the ABA biosynthetic pathway, and *aba2-2* and *nced3-2* mutant plants exhibit ABA deficiency (Cheng et al. 2002, Nambara and Marion-Poll 2005). Three-week-old Arabidopsis plants of these mutant lines and the wild type were subjected to dehydration, and their *DREB2A* transcript levels were measured by qRT-PCR before treatment and after 5 h of treatment (**Fig. 5A**). The transcript levels of *RESPONSIVE TO DESSICATION 20* (*RD20*) and *RESPONSIVE TO ABA 18* (*RAB18*) were also monitored as representative ABA-inducible genes and not under regulation by DREB2A (Sakuma et al. 2006a, Fujii and Zhu 2009, Fujita et al. 2009, Yoshida et al. 2010). We observed significantly impaired stress-inducible expression of *DREB2A* in both *areb* triple and *srk2* triple mutant plants, in agreement with previous microarray data (**Fig. 5A**; **Supplementary Table S1**) (Fujita et al. 2009, Yoshida et al. 2010). The other mutant lines showed similar impaired induction of *DREB2A* transcript levels (**Fig. 5A**).

We observed a reduced transcriptional response of *DREB2A* to dehydration stress in these mutant lines. Next, we analyzed whether these mutations in the SnRK2–AREB signaling cascade affected the *DREB2A* promoter activity. The N0 construct was introduced into the *areb* triple mutant plants, and the transcript level of the *GUS* gene was measured before and after dehydration treatment (**Fig. 5B**). We detected significantly impaired dehydration induction of *GUS* expression in the mutant plants compared with wild-type plants harboring the N0 construct (**Fig. 5B**). These results suggest that *DREB2A* expression in response to dehydration stress is influenced by ABA signaling cascades. However, the remaining dehydration stress-responsive expression was detected at significant levels in all of the mutants analyzed, which suggests that ABA-independent signaling influences the *DREB2A* expression under dehydration stress conditions.

Discussion

In this study, we performed a truncation assay for the *DREB2A* promoter to restrict the promoter region that affects transcriptional regulation under dehydration stress (**Fig. 1**). We discovered that ABRE and CE3-like sequences, which are known to be *cis*-elements involved in ABA-induced gene expression, are important for the dehydration and high-salinity stress-responsive promoter activity of *DREB2A* (**Fig. 2**; **Supplementary Fig. S3**). This discovery was unexpected because *DREB2A* gene expression has been reported to be weakly induced by exogenously applied ABA and strongly induced by dehydration and high salinity stresses (Liu et al. 1998, Nakashima et al. 2000, Sakuma et al. 2002). The comparison of the promoter sequence of Arabidopsis *DREB2A* with its orthologs in other sequenced eudicot species revealed a conservation of the CE3/ABRE–ABRE cassette with a similar positional relationship (**Supplementary Fig. S1**). This raises the possibility of a crucial role for the CE3/ABRE–ABRE cassette in the *DREB2A* promoter activity. Multiple ABRE motifs were found in the promoter of a *DREB2A* ortholog in the lycophyte, *Selaginella moellendorffii*, which might suggest an ancient origin for this ABRE regulation. In addition, an HSE sequence for the heat shock-responsive expression of *DREB2A* (Yoshida et al. 2011) is also conserved in these eudicot species in the vicinity of the CE3/ABRE–ABRE cassette (**Supplementary Fig. S1**).

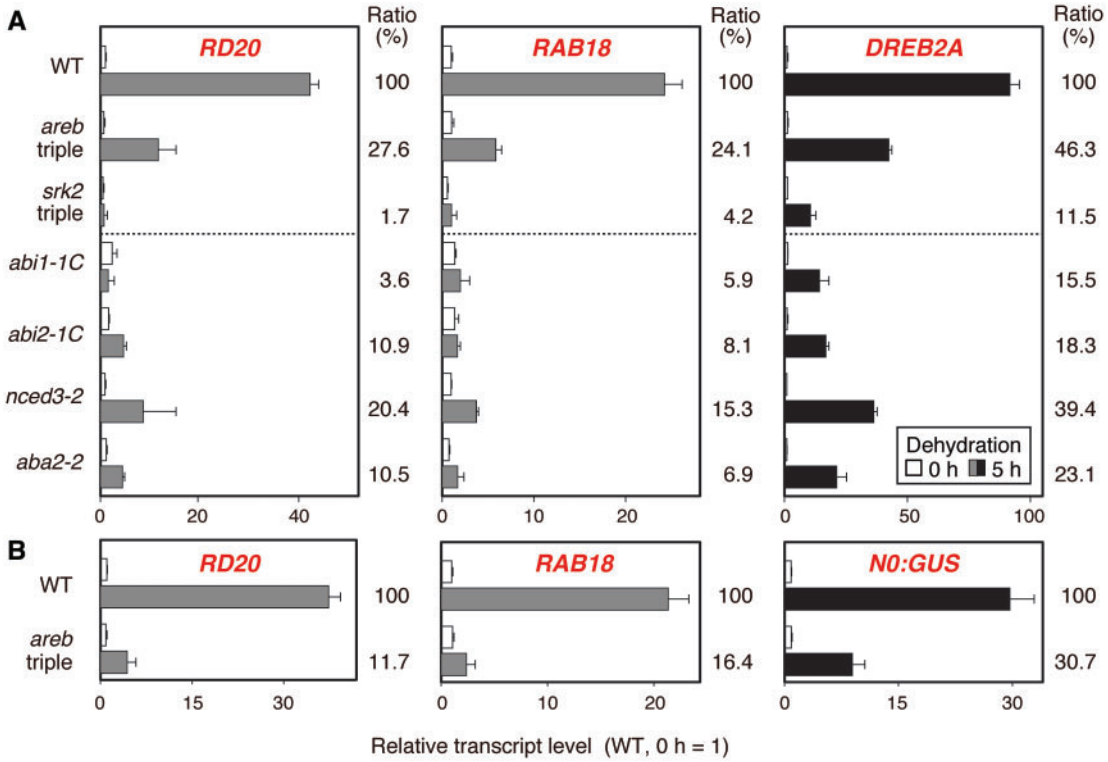

Fig. 5 *DREB2A* promoter activity in various ABA-insensitive and ABA-deficient mutants. (A) *DREB2A*, *RD20* and *RAB18* transcript levels in ABA-related mutants before and after dehydration. WT indicates the wild-type strain. The values shown represent the averages of more than three replicated samples, and the error bars indicate the SD. (B) *GUS*, *RD20* and *RAB18* transcript levels in transgenic WT or the *areb* triple plants harboring the *DREB2A* promoter–*GUS* fusion gene (*N0:GUS*) before and after dehydration. The values shown represent the averages of more than three independent transformants, and the error bars indicate the SD.

This promoter element positioning may facilitate the proper expression of *DREB2A* and its orthologs under various stress conditions.

We examined the relationship between *DREB2A* expression and an ABA signaling cascade. Subsequent analyses revealed that the *DREB2A* promoter can be a direct target of AREB1, AREB2 and ABF3. Our in vitro and in vivo data indicate that the three AREB/ABF members could recognize the ABRE sequence in the *DREB2A* promoter and activate it in response to osmotic stress conditions under which activation levels were reduced (**Figs. 3**, **4**). In addition, loss-of-function experiments demonstrated positive roles for ABA and ABRE in the transcriptional regulation of *DREB2A* (**Fig. 5**). All of the analyzed ABA-deficient and ABA-insensitive mutant lines presented significantly impaired transcriptional responses of *DREB2A* to osmotic stress treatments, and the *DREB2A* promoter–*GUS* construct was associated with a similar phenomenon (**Fig. 5**; **Supplementary Fig. S3**). Collectively, our results established the contribution of ABA signaling cascades to *DREB2A* transcriptional regulation through the *DREB2A* promoter region under osmotic stress conditions.

Although an influence of ABA signaling cascades on *DREB2A* expression was revealed in this study, we hypothesize that the *DREB2A* expression that occurs in response to osmotic stress is not equivalent to that in response to ABA. The transcriptional response of *DREB2A* to exogenously applied ABA is weaker than the response to osmotic stress (**Fig. 2**; Liu et al. 1998, Nakashima et al. 2000, Sakuma et al. 2002), and >20% of *DREB2A* expression was retained in ABA-deficient mutants, whereas the expressional responses of *RD20* and *RAB18* were almost abolished in these mutant lines (**Fig. 5A**; **Supplementary Fig. S3**). Therefore, we hypothesized that the *DREB2A* promoter requires other stress signals to stimulate expression, which is expected to be independent of ABA signaling.

Several clues to explain this ABA-independent pathway can be obtained from different resources. In our previous study, only one-fifth of genes downstream of the three SnRK2s were found to be regulated by the three AREB/ABFs addressed here (Fujita et al. 2009). In the case of *DREB2A*, the *srk2* triple mutation is >2-fold more effective in response to the osmotic stress-responsive transcription than the *areb* triple mutations (**Fig. 5A**; **Supplementary Table S1**; Fujita et al. 2009, Yoshida et al. 2010). These data support the conclusion that factors other than AREB/ABFs can influence *DREB2A* expression under the control of the SnRK2s. Considering the reported possibility that SRK2E, which is one of the three SnRK2 members, is capable of receiving ABA-independent osmotic stress

signals (Yoshida et al. 2006), it might be expected that *DREB2A* expression is regulated by an ABA-independent pathway under the control of the SnRK2 members. Although downstream factors for such a pathway have not yet been identified, this possibility may explain the greater effect of the *srk2* triple mutation on *DREB2A* expression compared with the ABA-deficient mutations (**Fig. 5A**). Several reports have suggested a considerable number of candidates as unknown signal mediators of *DREB2A* expression in response to osmotic stresses, such as WRKY, MYB or other bZIP family members (Liao et al. 2008, Zhou et al. 2008, Shang et al. 2010, Xie et al. 2010).

The *DREB2A* promoter sequences also provide us with several lines of evidence. The ABRE sequence contains an ACGT core, which is considered to be a target for plant bZIP proteins (Sibéril et al. 2001, Fujita et al. 2011). Additionally, an ACGT core can be found in other *cis*-elements of different transcription factor family members in plants, such as NAC and MYC (Boter et al. 2004, Takasaki et al. 2011). The CE3-like sequence, as a putative coupling element for the ABRE in the *DREB2A* promoter, should be studied further. CE3 was originally identified as a coupling element involved in achieving ABA induction in the barley *HVA1* promoter (Shen et al. 1996). A genome-wide in silico analysis found that only a few Arabidopsis ABRE *cis*-elements are coupled with CE3 compared with the rice sequences (Gómez-Porras et al. 2007). These data support the hypothesis that the osmotic stress-responsive expression of *DREB2A* is regulated in a different manner to that of other ABA-responsive genes in Arabidopsis.

As a stress-responsive transcription factor, DREB2A governs many stress-responsive genes that may lead plants to tolerate unfavorable circumstances as well as potentially having adverse effects on plant growth and reproduction (Sakuma et al. 2006a, Sakuma et al. 2006b, Maruyama et al. 2009). Therefore, a complex series of steps is expected to be involved in the regulation of *DREB2A* expression in plants. In this study, we demonstrated that ABA signaling cascades play important roles in *DREB2A* expression in response to osmotic stress. The ABRE and CE3-like sequences in the *DREB2A* promoter might function as *cis*-elements related to *DREB2A* expression, and the ABRE sequence could physically interact with AREB1, AREB2 and ABF3 (**Fig. 6**). Furthermore, a lack of ABA signaling resulted in a significant decrease of the *DREB2A* promoter activity under osmotic stress conditions, and the presence of ABA-independent signaling cascades was revealed through the limited effect of an ABA signal on *DREB2A* expression. In addition to the recently revealed post-translational regulation of DREB2A activity in response to osmotic stress (Qin et al. 2008), multiple signaling cascades contribute to the osmotic stress-responsive expression of *DREB2A* via both ABA-dependent and ABA-independent pathways (**Fig. 6**). The existence of such multiple and complex pathways for regulating *DREB2A* expression could allow plants to respond rapidly and precisely to osmotic stress.

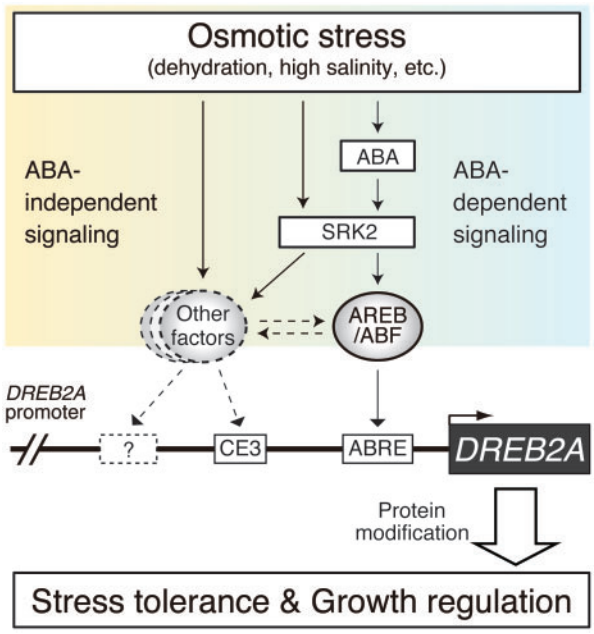

Fig. 6 Schematic model of *DREB2A* transcriptional regulation under osmotic stress. The ABRE and CE3-like sequences in the *DREB2A* promoter region might recognize bifurcated osmotic stress signals via both ABA-independent and -dependent pathways. The solid lines refer to the signal cascades revealed in this study or reported previously. The dotted lines refer to unidentified but circumstantially expected signal cascades.

Materials and Methods

Plant material and growth conditions

The plants used in this study were *A. thaliana* (L.) Heynh. ecotype Columbia. Seeds were sterilized and stratified at 4°C for 2 d in the dark, then sown and grown on growth medium (GM) agar plates in a growth chamber under 16 h of fluorescent light ($40 \pm 10\ \mu mol\ m^{-2}\ s^{-1}$) at 22°C and 70% relative humidity (Fujita et al. 2005).

Arabidopsis transformation was achieved using the floral-dip method (Clough 2005). Harvested T_1 candidate seeds were selected on GM agar plates containing $50\ mg\ l^{-1}$ kanamycin sulfate or $10\ mg\ l^{-1}$ hygromycin with $25\ mg\ l^{-1}$ cefotaxime. To obtain homozygous lines, T_2 and T_3 generations were subjected to the same antibiotic resistance selection protocol. The verified T_3 lines were used for further analyses.

Isolation of *DREB2A* promoter fragments and promoter–GUS construction

All members of a truncation series of the *DREB2A* promoter were amplified from Arabidopsis genomic DNA. Nucleotide substitutions in the promoter construct were generated by mismatched amplification with each specific primer set (**Supplementary Table S2**). Each amplified promoter fragment was inserted into the *Bam*HI site of pGK-GUS (Qin et al. 2008)

or pGH-GUS (see **Supplementary data**). The sequence of each construct was verified by comparison with The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) database. Verified constructs were individually introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by a freeze–thaw method and were subsequently used for Arabidopsis transformation.

Stress and ABA treatment

Three-week-old Arabidopsis plants (principal growth stage 1.08–1.10; Boyes et al. 2001) grown on GM agar plates were subjected to dehydration treatments. For each treatment, 3–5 plants were placed on a piece of Parafilm™ at the same distance (1.0–1.5 cm) and held under 60–65% relative humidity. Following treatment, samples were collected and stored at −80°C. As negative controls, non-treated samples were stored directly at −80°C.

Two-week-old Arabidopsis plants (principal growth stages 1.02–1.03) grown on GM agar plates were used for high salinity or ABA treatments. For each treatment, 5–6 plants were soaked in water containing 50 μM ABA or 250 mM NaCl. Following the treatments, samples were placed on a piece of paper to remove surface water before storage at −80°C. In mock treatments, the same volume of solvent in which the ABA stock solution had been prepared was used. Stored samples were used for RNA extraction and qRT-PCR.

RNA extraction and qRT-PCR

Total RNA was isolated from each plant sample using RNAiso plus™ (TAKARA BIO INC.; http://www.takara-bio.co.jp/) according to the manufacturer's instructions. Isolated RNA was employed for cDNA synthesis using Superscript III™ reverse transcriptase (Invitrogen™; http://www.invitrogen.com/). qRT-PCR was performed using a 7500 real-time PCR system and 7500 system SDS software, version 1.4.0.25 (Applied Biosystems™; http://www.appliedbiosystems.com/). SYBR® Premix Ex Taq™ (TAKARA BIO INC.) was used for amplification. Arabidopsis *18S* rRNA was used as a quantitative control for the amount of template. The sequences of the primer sets are shown in **Supplementary Table S2**.

Computational analysis

The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al. 2002) was used for scanning the *cis*-elements in the *DREB2A* promoter sequence. The significance of candidate *cis*-elements was evaluated through scanning further with the other plant *cis*-element database PLACE (http://www.dna.affrc.go.jp/PLACE/; Higo et al. 1999).

Yeast one-hybrid (Y1H) assay

We used a modified MATCHMAKER™ One-Hybrid System (Clontech Laboratories; http://www.clontech.com/) to perform the Y1H assay. Specifically, pHisi-1 and pLacZi vectors, both harboring four tandem repeats of the *DREB2A* promoter fragment (WT, MT; **Fig. 3A**), were integrated into the genome of yeast strain YM4271 followed by selection on SD media agar plates without uracil or histidine (SD/−UH).

To verify promoter–protein interactions in yeast, putative target proteins and their derivatives were individually introduced into the pGADT7 vector (Clontech Laboratories) using *Bam*HI and *Xho*I restriction enzyme sites. Following sequence verification, each construct was transformed into yeast lines harboring each repeated *DREB2A* promoter region using a *Fast*™ Yeast Transformation kit (Geno Technology Inc.; http://www.gbiosciences.com/). Transformed colonies were spotted on SD agar media that did not contain uracil, histidine or leucine (SD/−UHL) but did contain 0, 15 or 40 mM 3-AT in a concentration gradient (OD_{600}). After several days of incubation at 30°C, the interaction affinity was evaluated based on the growth status of each spot.

Chromatin immunoprecipitation (ChIP)

Samples of 1–1.3 g were collected from whole 3-week-old Arabidopsis plants (principal growth stage 1.08–1.10) harboring the doubled HA (2 × HA)-tagged AREB1 protein-expressing construct (see **Supplementary data**) and subjected to ABA or dehydration treatments, as described above. ChIP was performed using an EpiQuik™ Plant ChIP Kit (Epigentek; http://www.epigentek.com/) according to the provided user guide. To immunoprecipitate the HA-tagged proteins, an anti-HA antibody (catalog No. H3663, Sigma-Aldrich; http://www.sigmaaldrich.com/) was used. The primer sets employed to amplify the *DREB2A* promoter regions and *18S* rDNA are shown in **Supplementary Table S2**.

Transient expression assay with Arabidopsis mesophyll protoplasts

Transient transformation using Arabidopsis mesophyll protoplasts was achieved as described by Yoshida et al. (2010). The effector constructs were adapted from our previously reported research (Fujita et al. 2005, Furihata et al. 2006, Yoshida et al. 2010).

Accession numbers

Sequence data from this article can be found in the TAIR database under the following accession numbers: ABA2 (AT1G52340), ABF3 (AT4G34000), ABI1 (AT4G26080), ABI2 (AT5G57050), AREB1/ABF2 (AT1G45249), AREB2/ABF4 (AT3G19290), DREB2A (AT5G05410), NCED3 (AT3G14440), RAB18 (AT5G66400), RD20 (AT2G33380), RD29A (AT5G52310), RD29B (AT5G52300), and SRK2D (AT3G50500), SRK2E (AT4G33950), SRK2I (AT5G66880).

Supplementary data

Supplementary data are available at PCP online.

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