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Molecular cloning and expression analysis of a new stress-related *AREB* gene from *Arachis hypogaea*

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Abstract

An *AREB* gene, designated as *AhAREB1*, was cloned from peanut (*Arachis hypogaea* L.). The gene contains a 1 338-bp open reading frame that encodes a putative protein of 445 amino acids. The corresponding genomic DNA containing four exons and three introns was isolated and analyzed. An upstream 1 060-bp DNA promoter fragment of the *AhAREB1* gene was also amplified from peanut genomic DNA. Multiple sequence alignment of the deduced amino acids of AREB showed that the AhAREB1 protein shares high sequence homology with GmAREB1, SlAREB, and ABF2. Quantitative real-time PCR analysis showed that *AhAREB1* was induced by polyethylene glycol, NaCl, gibberellic acid, abscisic acid, and salicylic acid. The cloning and characterization of the *AhAREB1* gene will be useful for further studies establishing the biological role of *AhAREB1* in plants.

Additional key words: ABA, *AhAREB1*, GA_3, peanut, PEG, SA, water stress.

Introduction

Abiotic stresses such as drought, high salinity, and low temperature can severely affect plant growth and production. Plants activate the appropriate signal transduction pathway and induce a series of stress responsive gene expressions to initiate protective stress responses following stress signals (Hussain *et al.* 2011, Liu *et al.* 2011). The abscisic acid (ABA) dependent pathway *via* ABA response element (ABRE) is one of the main drought stress response pathways (Seki *et al.* 2007). ABA response element binding protein/ABRE binding transcription factor (AREB/ABF) belong to subfamily A of the basic leucine zipper (bZIP) transcription factor family. AREBs contain four conserved domains: three N-terminal (C1, C2, and C3) and one C-terminal (C4) (Furihata *et al.* 2006). The C-terminal (C4) domain contains a highly conserved bZIP structure which binds to DNA and other proteins and is activated by phosphorylation (Furihata *et al.* 2006). AREB/ABF transcription factors are plant specific and are involved in plant responses to hormones and stresses (Bensmihen *et al.* 2002, Finkelstein *et al.* 2002). Moreover, studies have shown that genetic manipulation of ABF/AREB family members can modify plant stress responses (Umezawa *et al.* 2006, Hossain *et al.* 2010, Hsieh *et al.* 2010, Huang *et al.* 2010) indicating an important role of these family members in stress responses.

Peanut (*Arachis hypogaea* L.) is an important crop often affected by drought, resulting in a serious drop in production (Wan and Li 2006). One of the effective ways to reduce the negative impact of drought on peanut production is through genetic engineering to increase drought-resistance. We have previously cloned a

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Abbreviations: ABA - abscisic acid; ABF - ABRE binding factor; ABRE - ABA response element; AREB - ABA-response element binding protein; bZIP - basic leucine zipper; CaMK - calmodulin-dependent protein kinase; CK - casein kinase; GA_3 - gibberellic acid; NCED - 9-*cis*-epoxycarotenoid dioxygenase; ORF - open reading frame; PEG - polyethylene glycol; PKC - protein kinase C; SA - salicylic acid; W-BOX - WRKY binding site; WRKY - superfamily of plant transcription factors.
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dehydration-inducible gene from peanut, designated as *AhNCED1* which encodes a putative 9-*cis*-epoxy-carotenoid dioxygenase (NCED; Wan and Li 2005). The expression of *AhNCED1* gene in mutant *Arabidopsis* increased ABA content and drought resistance (Wan and Li 2006) suggesting a role in drought stress responses. The subsequent cloning of the AhNCED1 promoter in peanut and the identification of two novel ABRE elements opened up the possibility for isolating the *AREB* gene which may lead to more practical approaches for genetic modification of drought tolerance in crop species (Liang *et al.* 2009).

Here we report the molecular cloning and expression analysis of a full-length cDNA for the *AREB* gene in peanut designated as *AhAREB1*. Quantitative real-time PCR reveals a significantly induced expression when treated with polyethylene glycol (PEG), NaCl, gibberellic acid (GA_3), ABA, and salicylic acid (SA). The *AhAREB1* organ specificity expression is also determined.

Materials and methods

Peanut (*Arachis hypogaea* L. cv. ShanYou 523) seeds were sown in mixture of *Vermiculite*, *Perlite* and soil (1:1:2) and grown in plastic pots in a growth chamber with a 16-h photoperiod, irradiance of 200 μmol m^{-2} s^{-1}, day/night temperature of 26/22 °C, and air humidity of 60 % (for details see Wan and Li 2005).

To amplify a specific *AhAREB1* fragment from peanut, degenerate primers (DP1, 5'-GATTT(C/T)GGG TCCATGAACATGGATGA-3'; DP2, 5'-T(T/C)TGT CTCCTCTC(A/C)AC(C/A)ACTTTCTC-3') were designed based on the conserved regions of the corresponding genes from *Arabidopsis* and soybean. RNA was obtained from peanut leaves that had been dehydrated for 2 h. The first-strand cDNA was synthesized from 2 μg of total RNA using *Super-Script* III reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) and an *oligodT18* primer. This cDNA was used as template for PCR amplification using degenerate primers. Conditions for reverse transcription were as follows: 65 °C for 5 min, 50 °C for 50 min and 70 °C for 15 min. PCR amplification was performed as follows: 30 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min. The resulting PCR fragment was sequenced and compared with reported *AhAREB1* sequences present in the GenBank database. The missing 5' and 3' ends from the confirmed *AhAREB1* homologue were recovered by RACE-PCR using the *GeneRacer* kit and following manufacturer's instructions. The gene-specific primers for 5'-RACE were 5GSP1 (5'-ATCTTTGGC TCCGGCTCCAGTGCT-3') (nested) and 5GSP2 (5'-TTGGTGAGAGCTGGTGTTGTGAGA-3') (outer); the gene-specific primers for 3'-RACE were 3GSP1 (5'-AAGCCTCCTCCTCCTCCTCTGTCG-3') (outer) and 3GSP2 (5'-ATGATTGGAGTGCCGGAGCGTTCG-3') (inner). In all cloning experiments, PCR fragments were gel purified with an agarose gel DNA purification kit (*TaKaRa*, Otsu, Shiga, Japan) and ligated into the *pMD18-T* vector (*TaKaRa*). Plasmids were isolated and sequenced from both strands. Sequence analysis was performed by using *EditSeq* software (*DNASTAR*). Computer analyses of DNA and amino acid sequences were conducted using *BLAST* and *Clustal W* programs (*DNASTAR*) available at the National Center for Biotechnology Information Services (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was performed using neighbor-joining (NJ) methods implemented by the *DNAMAN* software.

The upstream *AhAREB1* DNA sequence was isolated by PCR based genomic walking procedure as described by Siebert *et al.* (1995). To amplify the upstream region of the coding sequence, two non-overlapping gene-specific primers were designed based on the genomic DNA sequence of *AREB* gene (Genebank acc. No. JF766571). The primary PCR was carried out using a gene-specific primer 5'-ATCTTTGGCTCCGGCTCC AGTGCT-3' and an adaptor primer 5'-GTAATA CGACTCACTATAGGGC-3', followed by a second PCR with a nested gene-specific primer 5'-TTGGTG AGAGCTGGTGTTGTGAGA-3' and a nested adaptor primer 5'-ACTATAGGGCACGCGTGGT-3'. The PCR conditions were as follows: 7 cycles (94 °C, 25 s; 68 °C, 3 min); 32 cycles (94 °C, 25 s; 63 °C, 3 min); and 1 cycle (63 °C for 7 min). Long and acccurate (LA) Taq DNA polymerase (*TaKaRa*) was used for amplification. The nested products were purified by 1.5 % agarose gel electrophoresis with an agarose gel DNA purification kit (*TaKaRa*) and cloned into the *pMD18-T* vector (*TaKaRa*). The cloned vectors were then sequenced from both strands. Promoter motif search of upstream DNA sequence was carried out to define putative *cis*-acting elements using the software programs *PLACE* (Higo *et al.* 1999) and *PlantCARE* (Lescot *et al.* 2002).

To analyze the expression of *AhAREB1* under high salinity, water deficit, and exogenous hormone treatments, 14-d-old seedlings of peanut were removed from the soil and immersed in the following solutions: 300 mM NaCl, 30 % PEG 6000, 100 μM ABA, 10, 50, 250 μM GA_3, 10, 50, 250 μM SA (all chemicals from *Genview*, Houston, USA), or deionized water (control). The plants were treated at 26 °C and irradiance of 200 μmol m^{-2} s^{-1} for 0.5 to 24 h (see Table 1) as our preliminary RT-PCR results showed that the expression of *AhAREB1* was apparently up-regulated after 5 h of treatments with 300 mM NaCl, 50 μM GA_3, or 50 μM SA. Leaves were collected after treatments, flash frozen immediately in liquid nitrogen, and stored at -80 °C for RNA extraction.

Total RNA was isolated by *TRIzol* reagent (*Invitrogen*) and genomic DNA was removed by adding RNase-free DNase I (*TaKaRa*). Approximately 2 µg of total RNA was reverse transcribed using random primer and *Super-Script* III reverse transcriptase (*Invitrogen*) following manufacturer's instructions. Quantitative real-time RT-PCR was performed using the *SYBR Premix Ex TaqTM* II kit (*TaKaRa*) following manufacturer's instructions and samples were run in a *LightCycler 480* (*Roche*, Mannheim, Germany) equipped with *Light-Cycler* software *v. 1.5* (*Roche*) (Alos *et al.* 2008). PCR conditions were as followed: 95 °C for 10 s, 45 cycles of 95 °C for 10 s, 62 °C for 10 s (depending on the optimal reaction temperature of the different primers), and 72 °C for 20 s. Data were collected during the annealing phase of the each amplification. Melting curve analysis was performed with temperature ranging from 62 to 95 °C by reading the fluorescence every 0.2 s. Specific *AhAREB1* primers were designed using *Primer v. 5.0* (*Premier Biosoft International*, Palo Alto, CA, USA). The 141-bp *AhAREB1* amplicon gene primers are: forward (5'-ACAAGGGCAACCAGCATTAGG-3') and reverse (5'-TCACCACCACCATACCAACCA-3'). The *18SrRNA* gene from peanut seed was used as an internal control for calculating relative transcript abundance. The 104-bp *18SrRNA* amplicon gene primers are: forward (5'-TACGTCCCTGCCCTTTGTAC-3') and reverse (5'-CCTACGGAAACCTTGTTACGAC-3'). Three technical replicates were performed for all real-time PCR reactions. The relative quantification of RNA expression was calibrated using the formula $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression levels were normalized using *18SrRNA* transcript as the internal control. The expression of *AhAREB1* in sample from untreated native leaves was used as the target calibrator.

One-way analysis of variance (*ANOVA*) was performed and the means were compared using Tukey's HSD *post hoc* comparisons. All tests were carried out at a 0.05 level of significance using *SPSS v. 11.5* (*SPSS Inc.*, Chicago, IL, USA).

Results and discussion

A number of conserved regions are present in *AREB* genes (Furihata *et al.* 2006) (Figs 1, 2). These conserved regions were used to design degenerate primers (DP1 and DP2) to amplify a new homologue of ABF2/AREB1 (NP_849777) and GmAREB (ACB46529) from *Arabidopsis thaliana* and soybean (*Glycine max*). A 955-bp fragment was amplified from cDNA of dehydrated (2 h) peanut leaves. The full-length cDNA obtained using RACE-PCR, named *AhAREB1*, consists of 1814-bp nucleotides including a 129-bp 5'-untranslated region and a 347-bp 3'-untranslated region. AhAREB1 has an open reading frame (ORF) encoding a polypeptide of 445 amino acids with a calculated molecular mass of 48.15 kDa and an isoelectric point of 9.51.

In the present study, the complete cDNA sequence of *AhAREB1* gene from *A. hypogaea* was reported. Conserved sequences and characteristic motifs, such as AREB family signatures, three N-terminal (C1, C2, and C3), as well as one C-terminal (C4) conserved domains characteristic of AREBs, were found in the AhAREB1 amino acid sequence (Fig. 2). A highly conserved bZIP domain was observed near the C-terminus of AhAREB1 indicating that AhAREB1 encodes a bZIP family protein. The bZIP domain is made up of a basic region for DNA binding and three heptad leucine repeats involving TF dimerization (Correa *et al.* 2008). Moreover, we found several conserved serine (S) and threonine (T) residues which are consistent with the structure of AREBs from other plants (Choi *et al.* 2000, Uno *et al.* 2000) and are presumably phosphorylation sites of different kinases, such as RyKXXSyT calmodulin-dependent protein kinase (CaMK), SyTXXDyE for casein kinase (CK) II, SyTXKyR for protein kinase C (PKC), and KyRXXXSyT for cGMP-dependent protein kinase. The activation of AhAREB1 might also be regulated by protein phosphorylation as has been reported in other AREBs/ABFs (Uno *et al.* 2000, Kobayashi *et al.* 2005, Furihata *et al.* 2006, Yoshida *et al.* 2010).

A phylogenetic analysis using the AhAREB1 full-length sequences and 19 AREB proteins showed that *AhAREB1* is different from other AREB family members (Fig. 1). Multiple sequence alignment of the deduced amino acids of AREB reveals that the AhAREB1 protein shares high identity with the GmAREB1 (*Glycine max*), SlAREB (*Lycopersicon esculentum*), and ABF2/AREB1 (*Arabidopsis thaliana*), respectively (Figs 1, 2). Peanut cDNA can be described as encoding a putative ABA response element binding protein based on the overall similarity with other reported *AREB* genes. The full-length cDNA sequence of *AhAREB1* has been deposited in the *EMBL* nucleotide sequence database (acc. No. JF766570).

Based on our phylogenetic analysis (Fig. 1), the AhAREB1 protein is most closely related to GmAREB1, SlAREB, and ABF2/AREB1. These proteins were reported to be mainly involved in ABA signaling under water stress conditions (Kim *et al.* 2004, Hsieh *et al.* 2010), and may have similar biological functions as they have similar conserved domains.

A number of *cis*-element motifs were found within the 787-bp upstream sequence of the *AhAREB1* gene ORF (Fig. 3). The *cis*-element motifs that followed transcriptional start site (+1 bp) included putative basic *cis*-elements TATA box (-267 to -262 bp) and CAAT box (-10 to -6 bp), four different MYB recognition sites (CNGTTR) motifs (-150 to -145, -401 to -395, -465

to -459, and -646 to -640 bp) (Solano *et al.* 1995), one ACGT recognition site motif (-94 to -89 bp), one MYC recognition site (CANNTG) motif (-121 to -115 bp) (Hartmann *et al.* 2005, Agarwal *et al.* 2006), and one WRKY binding site (-818 to -814 bp) (Rushton *et al.* 2002). All these *cis*-elements were reported to be involved in plant responses to various biotic (Eulgem and Somssich 2007) and abiotic stresses (Abe *et al.* 2003, Furihata *et al.* 2006).

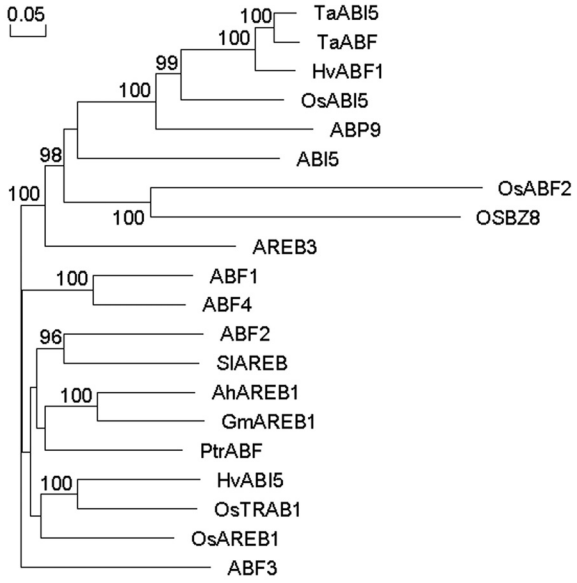

Fig. 1 Phylogenetic tree based on an alignment of AhAREB1 with other 19 AREB proteins. GenBank acc. Nos. and original species are: *Triticum aestivum* TaABI5 (AB238932), TaABF (AF519804); *Hordeum vulgare* HvABF1 (DQ786408), *Oryza sativa* OsABI5 (ABM90395); *Zea mays* ABP9 (ADA70308); *Arabidopsis thaliana* ABI5 (NP_565840); *Oryza sativa* OsABF2 (NP001051086), OSBZ8 (U42208); *Arabidopsis thaliana* AREB3 (NP_191244), ABF1 (NP_564551), ABF4 (NP_566629), ABF2(NP_849777); *Lycopersicon esculentum* SlAREB (AY530758); *Glycine max* GmAREB1 (ACB46529); *Poncirus trifoliata* PtrABF (HM171703); *Hordeum vulgare* HvABI5 (AY150676); *Oryza sativa* OsTRAB1 (BAA83740), OsAREB1 (NM_001063653), and *Arabidopsis thaliana* ABF3 (NP_849490). Data were analyzed using *DNAMAN v. 6.0* software. Bootstrap values from 1000 replicates were used to assess tree robustness.

AhAREB1 was constitutively expressed in all examined organs. Expression levels were high in leaves (1.32) and roots (1.22) but significantly lower in flowers (0.96) and stems (0.85) and especially low in seeds (0.14). We found that *AhAREB1* gene expression in peanut seedlings was changed under different abiotic stresses. Relative expression levels for control were similar from 0 to 10 h (Table 1). The 50 μM SA treatment for 5 h led to an 8-fold increase in expression (Table 1). However, expression at 10 h was only 1/4 of that after 5 h and was not significantly different from expressions at 0 h (Table 1). The 10 μM SA (5 h) yielded 10-fold increase in *AhAREB1* transcript expression compared to control. However, expression at 250 μM of SA for 5 h was not significantly different compared to controls. The transcription of *AhAREB1* increased significantly in response to NaCl treatments (Table 1). Expression increased 3.6- and 4.1-folds at 5 and 10 h treatment with 300 mM NaCl, respectively, compared to 0 h treatment. 50 μM GA_3 caused 8.5- and 8.1-folds increases in levels of *AhAREB1* transcripts at 5 and 10 h relative to control. In addition, *AhAREB1* transcripts at 5 h were significantly higher than the control at all concentrations of GA_3. A treatment with 100 μM ABA led to a peak expression of *AhAREB1* transcripts at 1 and 24 h (Table 1). The 30 % PEG treatment initially led to an increase in the expression of *AhAREB1* already at 0.5 h. The major increases in *AhAREB1* expression peaked at 2 and 3 h of PEG treatment followed by a marked decrease at 4 h of treatment but subsequently a significant increase at 8 h of treatment. Afterwards at 12 h, it was decreased to similar level to that at 0.5, 1, or 4 h of treatment (Table 1). These results clearly suggest that *AhAREB1* expression is inducible and certain stimuli such as SA, NaCl, GA_3, ABA, and PEG affect transcription levels.

Transcript levels of plant ABFs change under abiotic stresses (Choi *et al.* 2000, Uno *et al.* 2000, Yanez *et al.* 2009). Our real-time PCR analysis showed that specific treatments of ABA, PEG, SA, NaCl, and GA_3 can induce *AhAREB1* gene expression. These results suggest that similar to ABA-response bZIP transcription factors, the *AhAREB1* gene may also be involved in regulation of various stress signal pathways (drought, high salt, and ABA) (Choi *et al.* 2000, Uno *et al.* 2000, Fujita *et al.* 2005). The strong induction of *AhAREB1* under ABA and PEG treatments suggest that *AhAREB1* may play an important role in ABA-dependant signaling and drought tolerance in peanut, however, further investigation to elucidate the potential role of the *AhAREB1* gene is needed. In addition, we found that *AhAREB1* is also induced by GA_3 showing a coordinated response to GA_3 and ABA. Such a coordinated response has also been observed for nine genes in de-embryonated barley aleurone that were coordinately up-regulated by both GA_3 and ABA (Chen and An 2006) although these two hormones are well known to exert antagonistic effects at the level of gene expression and GA_3 reverses the ABA induction of expression of many genes (Chandler and Robertson 1994). The reasons for the coordinated response of *AhAREB1* to GA_3 and ABA in the present study might be that different dynamics are responsible for the simultaneous stimulation of these two hormones. First, exogenous ABA and GA_3 treatments were used in this study but actually the levels or the ratio of endogenous ABA and GA_3 might affect the expression of *AhAREB1*. Second, the expression levels of *AhAREB1* under 5 and 10 h of 100 μM ABA treatments were not significantly different from that of control but expression

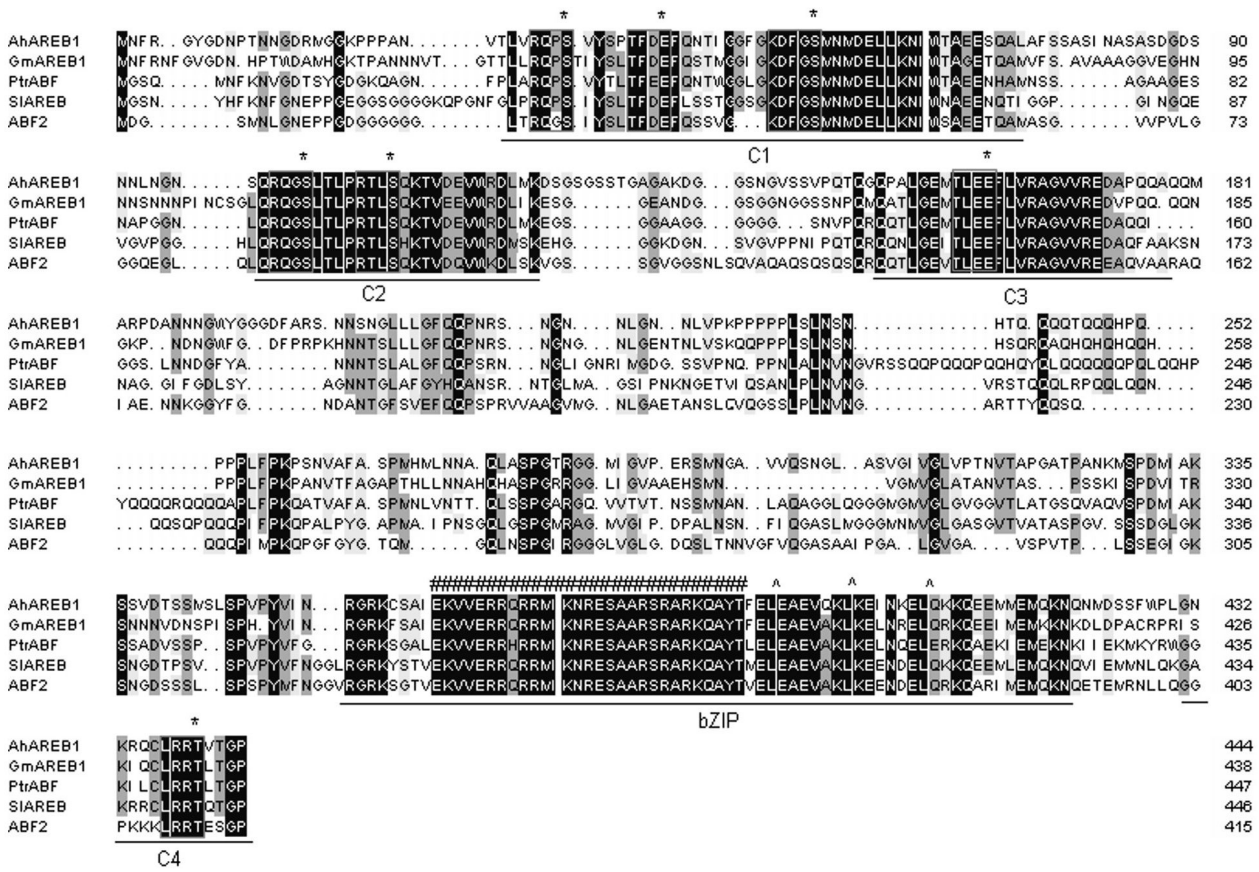

Fig. 2 AhAREB1 protein sequence alignment including other plant AREBs. Amino acid sequences used in the analysis are: *Glycine max* GmAREB1 (ACB46529); *Poncirus trifoliata* PtrABF (HM171703); *Lycopersicon esculentum* SlAREB (AY530758); *Arabidopsis* ABF2 (NP_849777). The conserved regions, C1, C2, C3, C4, and the bZIP signature are *underlined*. * denotes identical amino acid residues representing phosphorylation regions and indicates consensus sequences for various kinases (RyKXXSyT for calmodulin-dependent protein kinase (CaMK), SyTXXDyE for casein kinase (CK) II, SyTXKyR for protein kinase C (PKC), KyRXXXSyT for cGMP-dependent protein kinase which appear within the conserved regions. # denotes the basic region and ^ denotes leucine repeats. Dots represent gaps in the sequence alignment.

Table 1 The time courses of the relative expression of *AhAREB1* mRNA in leaves after H_2O (control), SA, NaCl, GA_3, ABA, and PEG treatments. Refer to the text for the procedure of the extraction of total RNA and quantitative RT-PCR analysis and the calculation of expression levels of *AhAREB1*. Values represent the mean of three replicates. Different letters indicate that the mean is significantly different at $\alpha = 0.05$ within the time courses of *AhAREB1* mRNA expression in leaves exposed to different treatments.

Treatment	Time of treatment [h]										
	0	0.5	1	2	3	4	5	8	10	12	24
H_2O	1.02^{a}	-	-	-	-	-	1.29^{a}	-	1.32^{a}	-	-
SA	1.02^{a}	-	-	-	-	-	8.07^{b}	-	1.89^{a}	-	-
NaCl	1.02^{a}	-	-	-	-	-	3.72^{b}	-	4.20^{b}	-	-
GA_3	1.02^{a}	-	-	-	-	-	8.62^{b}	-	8.27^{b}	-	-
ABA	1.02^{a}	-	8.81^{b}	4.39^{c}	-	-	3.11^{ac}	-	1.82^{a}	-	8.29^{b}
PEG	1.02^{a}	3.45^{ae}	2.19^{ae}	17.12^{b}	20.21^{c}	3.21^{ae}	-	8.45^{d}	-	4.82^{e}	-

at 5 and 10 h of 50 μM GA_3 treatments were significantly up-regulated. Third, the two types of phytohormones can not only be involved in the stress responses but act as the regulator of plant development. Therefore, it is possible that the expression of the *AhAREB1* gene can be induced by both ABA and GA_3 in the present study.

Cloning and predictive analysis of *AhAREB1* gene promoter reveals that a number of potential *cis*-acting elements are involved in environmental signaling and the product of AhAREB1 may be involved in different

aspects of the signal transduction. Four different MYB recognition sites and one MYC recognition site were detected at the promoter region which suggest that transcription factors bind to these sites and regulate the expression of *AhAREB1* (Abe *et al.* 2003). Furthermore, one ACGT recognition site was found. All these sites may be important for elucidating the regulatory mechanism of *AhAREB1* under ABA and PEG stresses. In addition, the induction of *AhAREB1* by GA_3 and NaCl may be possible due to the presence of MYB sites (Jung *et al.* 2008, Peng 2009). Similarly, the presence of one WRKY zinc finger motif binding site suggests that AhAREB1 can be induced by SA (Gao *et al.* 2011). Overall, our results suggest that the expression of *AhAREB1* gene is induced by varying stresses (*i.e.* SA, GA_3, ABA, drought, and high salinity) and provide the basis for further studies on transcriptional activity and regulation mechanisms of *AhAREB1*.

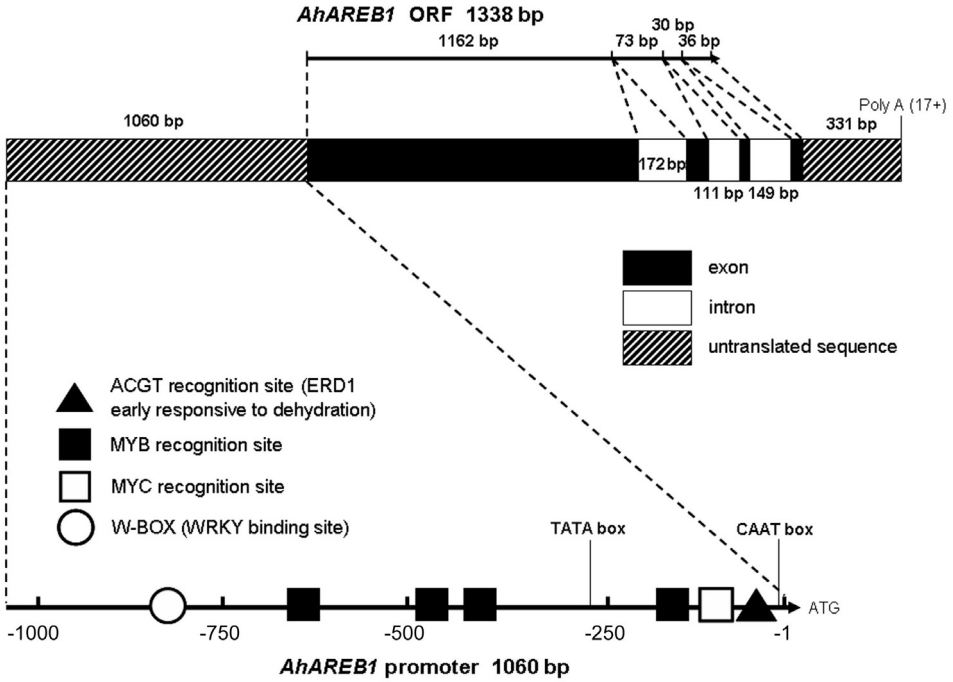

Fig. 3. Structure of the *AhAREB1* gene. Exons and introns are drawn schematically to indicate their relative position and size.

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