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OsAREB1, an ABRE-binding protein responding to ABA and glucose, has multiple functions in Arabidopsis

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Expression patterns of *OsAREB1* revealed that expression of *OsAREB1* gene can be induced by ABA, PEG and heat. Yeast one-hybrid assay demonstrated it can bind to ABA-responsive element (ABRE), which was found in most stress-induced genes. Transgenic Arabidopsis over-expressing *OsAREB1* had different responses to ABA and glucose compared to wild-type plants, which suggest OsAREB1 might have a crucial role in these two signaling pathways. Further analysis indicate that *OsAREB1* have multiple functions in Arabidopsis. First, *OsAREB1* transgenic plants had higher resistance to drought and heat, and OsAREB1 up-regulated the ABA/stress related gene such as *RD29A* and *RD29B*. Second, it delayed plant flowering time by down-regulating the expression of flowering-related genes, such as *FT, SOC1, LFY* and *AP1*. Due to the dates, OsAREB1 may function as a positive regulator in drought/heat stresses response, but a negative regulator in flowering time in Arabidopsis. [BMB reports 2010; 43(1): 34-39]

INTRODUCTION

Plants are constantly exposed to environmental stresses that impose on their growth and development, such as drought, high salinity and extreme temperature. In the signal transduction network from perception of stress signals to stress-responsive gene expression, ABA plays an important role, and the physiological responses to ABA are in large part brought about by changes in gene expression (1). Transcription factors, proteins which can bind to the *cis*-acting elements in the stress-responsive promoters, function for plant adaptation to environmental stresses (2-6). Many *cis*-elements known as ABA-responsive elements (ABREs) have been identified from the promoter analysis of ABA-regulated genes. Among them, those sharing a (C/T)ACGTGGC consensus sequence are found to be present in numerous ABA and/or stress-regulated genes. Till now, a set of experiments have reported that AREB/ABFs (ABRE binding factor/ ABRE-responsive elements binding protein), a distinct subfamily of bZIP proteins (7), can transactivate an ABRE-containing reporter gene in yeast (8) and plants (4, 9), and may function redundantly to mediate response to ABA or abiotic stresses (10). In Arabidopsis, Marc *et al*. divide the 75 AtbZIPs into 11 groups, and the ABFs/AREBs are classified to group A, which appear to function as important players in ABA signal transduction both in seeds and vegetative tissues, such as ABI5, ABF2/AREB1, ABF4/AREB2, AREB3, GBF4, ABF1 and ABF3 (11). These reported studies suggest that ABFs may function in different stress response pathways; i.e. ABF1 in cold signaling; ABF2 in salt, drought, heat and glucose signaling pathways (12, 13); ABF3 in salt signaling; ABF4 in cold, salt, and drought signaling pathways (12).

Recently, 89 bZIP transcriptional factors (OsbZIP01-OsbZIP89) are sorted into 11 groups according to their DNA binding specificity and amino acid sequences in basic and hinge regions (14). But till now, only 17 bZIP transcription factors have been identified or functionally characterized from rice (14-17). Compare with the intensive research of the group A subfamily in Arabidopsis, only 4 genes of this subfamily have been studied in rice. The first one is TRAB1 (18, 19), the second named OsABI5 encodes a protein that can bind to ABRE (G-box) and is suggested to be involved in ABA signal transduction and stress responses (15, 20), the third is OsbZIP23 which positively regulates ABA sensitivity and stress resistance in rice (16), and the last is OsbZIP72 which is a positive regulator of ABA response and drought tolerance in rice (17). To learn more about the functional role of this subgroup of bZIP transcriptional factors in rice, one member of this subgroup named OsAREB1 was cloned by PCR according to the sequence registered on NCBI (Os06g0211200) and the function of OsAREB1 was analyzed in this study.

RESULTS AND DISCUSSION

Expression patterns of *OsAREB1* gene

Expression patterns of the *OsAREB1* gene under various environmental stresses and hormones were analyzed by RT-PCR. *OsAREB1* gene was induced within 1 or 2 h under 100 μM ABA and 15% PEG 6,000 treatments, and maintained the ex-

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pression level for at least 8 hours. It's expression was induced by heat within 1 h, and rapidly reached the top expression level within 2 h, then declined to initial level (Fig. 1, Suppl). *OsAREB1* was not induced by KT, MeJA, NaCl and cold (date-not shown). These results indicated that *OsAREB1* was induced by exogenous ABA, water stress and heat. This result was consistent with the report of Lu *et al*. (17).

OsAREB1 has ABRE-binding activity in yeast

Blast result indicated that OsAREB1 belongs to ABF subfamily. Most members of this subfamily can bind to the ABRE *cis*-element with a core sequence ACGTGCC. Yeast one-hybrid system was used to determine the DNA-binding activity of OsAREB1 with ABRE element. The entire coding region of OsAREB1 was fused to the GAL4 transcription active domain (TA). The construct was transformed into yeast (EGY48) harboring ABRE sequence fused upstream of a *lacZ* reporter gene, and the growth status of transformants was observed. Yeast cells harboring pPC86 and G222 could grow on SD medium lacking Trp, while cells only with G222 could not grow on the selection medium (Fig. 1A). The colony-lift filter assay suggested that OsAREB1 can bind to the ABRE cis-element. Shown as Fig. 1B, when the colony grew on X-gal containing plate, only cells with pPC86-OsAREB1 and G222 turned blue, cells only with G222 or with both G222 and pPC86 did not turn blue. This result indicated that only OsAREB1 can bind to the ABRE cis-element and then active the expression of *lacZ* gene. Further, quantificational analysis for β-galactosidase activity was performed. Compared to the negative control, the relative β-galactosidase activity of the transformants was about four (Fig. 1C), which revealed there's a distinct enhancement for β-galactosidase activity.

Overexpression of *OsAREB1* alters seedling sensitivity to ABA and glucose

Transgenic Arabidopsis was generated to study the function of OsAREB1 in plant, and over-expressing of *OsAREB1* was analyzed by RT-PCR. Show as Fig. 2A, over-expressing of *OsAREB1* was detected in TP33, TP34 and TP36 plants, TP33 and TP34 almost had the same expression level, while TP36 had lower expression level, correspondingly.

ABA is an important substance in signal transduction and stresses response. To investigate the function of OsAREB1 in ABA signaling, WT and transgenic seeds were grown on Murashige and Skoog medium containing different concentrations of ABA, just as 0, 0.3, 0.5, 1.0, 2.0 μM, to observe their germination and development with exogenous ABA. There was no germination difference between WT and transgenic seeds. But when ABA concentration was 1 μM, almost all of the cotyledons of WT plants were turn yellow, while about 80% of the cotyledons of transgenic plants (TP33 and TP34) were green, which indicated that cotyledons of the transgenic plants were insensitive to ABA compaing WT plants (Fig. 2B, C). Kim *et al*. reported that ABF2 over-expressed plants were insensitive to ABA for leaves and hyper-sensitive to ABA for roots (12). OsAREB1 in our study is the most homologous gene to ABF2, OsAREB1 may have similar response. So root elongation assay was carried out to further investigate the response to ABA of roots. All seeds were germinated on medium without ABA for 2 days, then removed to ABA containing plates, and ABA-free plate as the control. The results after removal for 7 days were shown as Fig. 2D, E, as 0.5 μM ABA concentration, the relative root length of WT plants was 83.6% while TP33 and TP34 were 60.5% and 59.9%; as 1.0 μM ABA concentration, the dates were 79.0%, 47.0% and 32.5%, correspondingly (Fig. 2E). These results indicated that roots of transgenic plants were hypersensitive to ABA.

Earlier results indicate that ABA signaling related proteins also can participate in glucose signaling (12, 21-23). Glucose may modify plant growth and development in response to prevailing environmental conditions by regulating biosynthesis of

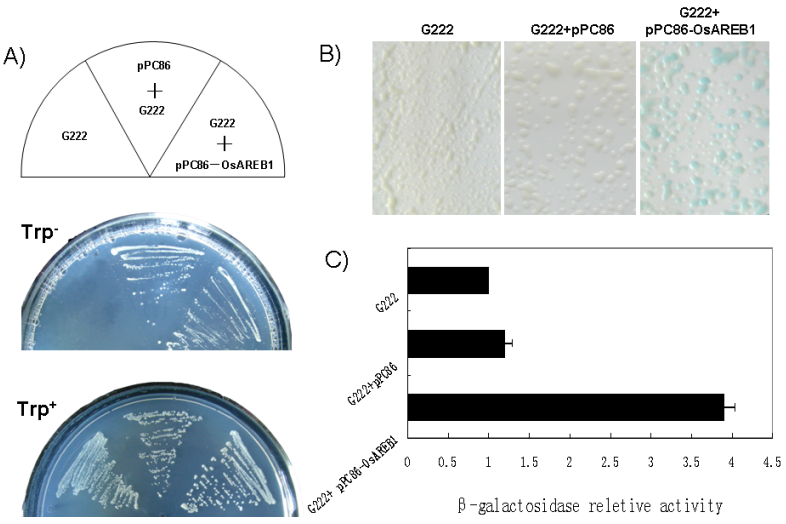

Fig. 1. DNA binding assay. (A) Growth ability on Trp^- and Trp^+ plates for each case as sketch map. Trp^-, SD medium plus glucose, adenine, casein, but not tryptophan; Trp^+, the same medium with tryptophan. (B) Colony-lift filter assay. The pictures were captured after 10 h incubation at 30°C with 80 μg/mL X-gal. (C) The relative activity of β-galactosidase.

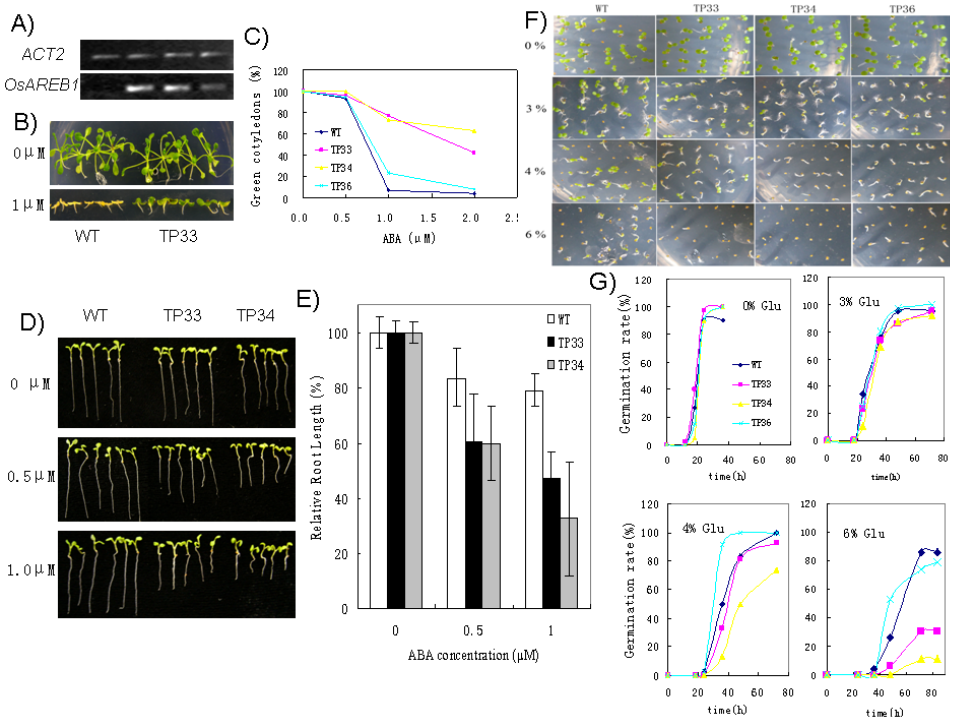

Fig. 2. ABA and glucose responses of *35S-OsAREB1* plants. (A) *OsAREB1* expression level in TPs. (B, C) ABA effect on cotyledon development. The photo was screened 20 d after grown on the medium, seedlings with green cotyledons were counted two weeks after grow on the medium. Green cotyledon rate was counted as: green cotyledon plants number/germinated seeds number×100%. (D, E) ABA effect on primary root elongation. The root length was counted after grown on ABA containing medium for 7 days ($n>=9$ each), and the relative root length was counted as root length on ABA containing plants compare with corresponding root length on MS plates. Error bars represent standard deviation. (F, G) Seeds germination on glucose plates. Seeds were germinated on MS medium containing 0, 3, 4, 6% exogenous glucose. The photos were screened 5 d after growing on the medium, and the germination rates of WT and TPs were counted as times showed.

the plant hormone abscisic acid (ABA) (24, 25). Here, WT and transgenic plants were germinated on MS plates containing different concentration of glucose (0, 3, 4, 6, 8%; M/V). The kinetics of germination was determined by using the method of Price *et al*. (25). Different from ABA response, OsAREB1 can influence seeds germination on glucose plates. For instance, on glucose-free medium, WT and transgenic seeds almost germinated completely. When added to 3% or 4% glucose concentration, TP33 and TP34 began to have lower germination rate than WT seeds. When the glucose concentration reached to 6%, TP33 and TP34 distinctly had severer delayed seed germination rates (Fig. 2G). Transgenic seeds were hypersensitive to glucose in germination period.

Enhanced tolerance of *35S-OsAREB1* plants to drought and heat

As *OsAREB1* was strongly induced by PEG and heat, we presumed that it would relate to drought and heat response. For drought experiment, seelings had grown in soil for about 4 weeks were treated without watering. Almost all the wild-type plants withered completely when water was withdrawn for 7 d, whereas most of the TP33, TP34 plants of *OsAREB1* lines survived when rewatered. To exclude growth-dependent effects in the drought tolerance test, we further did the dehydration test on filter paper. Three-week-old wild-type and transgenic plants were removed from agar plates and kept on filter paper for 1 h, and then rehydrated. During the first 10 min of dehydration, all wild-type plants had flopped, while TP33 plants remained standing. By 30 min, the wild-type plants had withered almost completely, while the TP33 plants withered only slowly. One hour later, both WT and *35S-OsAREB1* plants were all withered. Three hours after rehydration, about 75% of the TPs renewedly extended their wilted leaves, while the wild-type plants still had wilted and crinkled leaves (Fig. 3B). Water relation analysis found that TP33 had higher water content and lower water loss rate during this process (Fig. 3C), which indicated that TP33 plants can hold more water to stand against drought condition.

To testify the heat tolerance, three-week-old seedlings grow on MS medium were exposed to the lethiferous temperature (46°C) for 2 h, no difference was observed, but after 7 d recovery in normal environment, almost all of the WT plants turned scorch, while the transgenic seedlings were still green (Fig. 3D).

To further analyze the molecular mechanism of OsAREB1 in ABA and stresses response, expression patterns of stress-related genes, such as *RD29A, RD29B, RD22* and *COR15A* were detected. The results suggested that the expression levels of *RD29A* and *RD29B* were higher in TPs than in WT. But there were no expression differences for *RD22* and *COR15A* genes (Fig. 3E). Preovious studies show that *RD29A* and *RD29B* are closely located on the Arabidopsis genome, and *RD29A* has at least two *cis*-acting elements, one involved in the ABA-associated response to dehydration and the other induced by changes in osmotic potential, *RD29B* promoter region contains two ABREs (26, 27). *RD22* and *COR15A* don't have ABRE cis-element. Our results implicated that *OsAREB1* may

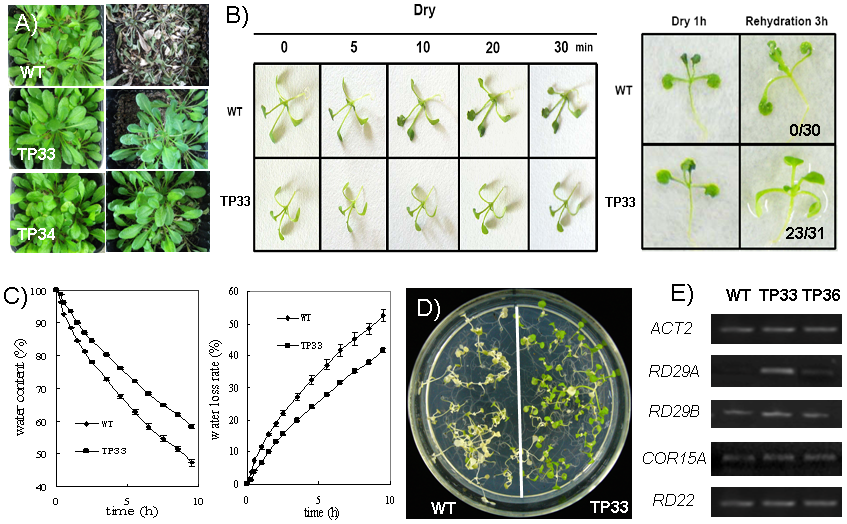

Fig. 3. Drought and heat tolerance of *OsAREB1* transgenic Arabidopsis. (A) Drought tolerance. Seelings grown in soil for about 4 weeks were treated without watering for about 7 d, and then rewatering. (B) Dehydration analysis. Three-week-old plants grown on MS plates were detached, and laid on the filter paper immediately. The photos were screened at the described times. The number at right of the rehydration picture represented the survival number. (C) Water content and water loss rate. (D) Heat tolerance of *35S-OsAREB1* plants. Three-week-old seedlings grow on MS medium were exposed to 46°C for 2 h, and the photo was taken after 7 d of recovery period at normal growth temperature. (E) Expression patterns of stress-related genes. Seedlings for RNA abstraction were a month old.

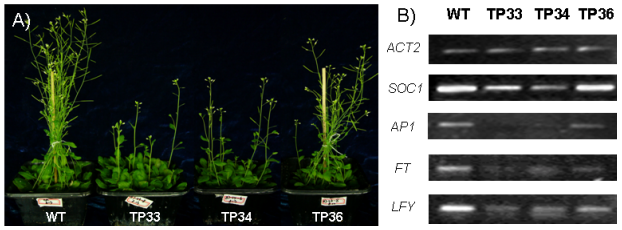

Fig. 4. OsAREB1 delay the flowering time. (A) Picture captured 40 d after transformed to soil. (B) Expression patterns of flowering-related genes.

regulate the transcription of *RD29A* and *RD29B* by binding to the ABRE *cis*-element. This result was consistent with the yeast one-hybrid experiment.

OsAREB1 delay the flowering time

By repetitious observation, *OsAREB1* transgenic plants had flowering delayed phenotype. TPs had no retardation before flowering suggesting OsAREB1 only influence flowering time. As showed in Fig. 4A, TP33, TP34, and TP36 all exhibit flowering delayed phenotype. RT-PCR results of *FT, SOC1, LFY* and *AP1* genes indicate OsAREB1 reduced the expression of these genes (Fig. 4B).

In Arabidopsis, SOC1 is directly activated by CONSTANS (CO) in long photoperiods and is repressed by FLC (28). Mutations in FT cause late flowering and 35S-FT plants show a phenotype of early flowering (29, 30). *LFY* encodes a transcription factor in plants, and upregulation of *LFY* in meristem make floral identity (31). AP1 is directly activated by LFY (32), and 35S-AP1 plants are early flowering (33). bZIP transcription factors have been reported to participate in regulating flowering time. The group A member flowering locus D (FD, synonym AtbZIP27), preferentially express in the shoot apex, could interact with both FT and terminal flower 1 (TFL1), and it recruit these floral regulators to promoters of floral identity genes to regulate flowering (29, 34). OsAREB1 down-regulated these flowering related genes, consequently restrained the plant to flowering.

Compare to other ABF proteins such as ABF2, an essential component of glucose signaling and its overexpression affects multiple stress tolerance (12, 13); OsbZIP23, a key player for conferring ABA sensitivity and salinity and drought tolerance in rice (16); OsbZIP72, a positive regulator of ABA response and drought tolerance in rice (17) and so on. We concluded that OsAREB1 is a classic ABA-responsible bZIP protein, and it is also a positive regulator in drought and heat stresses. Retarded growth was observed in 35S-ABF2 plants also, but this retardation was arosed by delayed germination and slower growth compared with wild-type plants (12). However, in our study, we neither discovered the germination difference in transgenic and wild-type seeds, nor observed the growth retardation before flowering. Based on the expression patterns of flowering related genes, OsAREB1 can be considered as a negative regulator in flowering control.

MATERIALS AND METHODS

Plant materials and treatments

For *OsAREB1* gene expression pattern analysis, rice seeds (*Oryza Sativa L. IR36*) were washed with flowing water, germinated on humid gauze at 30°C in darkness, then transferred to 12 h photoperiod cultivation with 1/4 MS liquid culture medium. Two-week-old seedlings were treated with stresses. For chemical stresses, seedlings were irrigated with double-distilled water, 200 mM NaCl, 100 μM ABA and 15% PEG 6,000 respectively. For cold and heat stresses, seedlings were transferred to a growth chamber at 4°C and 42°C respectively with constant illumination with cool-white fluorescent lamp (25-30 mol m^{-2} s^{-1}). All plants with different treatments were quickly

immersed in liquid N_2 and stored at -70^{o}C for RNA extraction. *A. thaliana* cv. Columbia and *35S-OsAREB1* over-expressed plants were grown at 22°C under 12 h photoperiod condition aseptically on MS or on soil (18 : 6 : 1 mixture of vermiculite, peat moss, and perlite). For ABA and Glucose treatments, different amount of ABA or glucose were added into the MS medium.

RNA isolation and RT-PCR analysis

Total RNA from rice and Arabidopsis were extracted by using the Trizol Kit (Watson biotechnology Inc.). DNase I was used (Promega, Msdison, WI, USA) to remove the genomic DNA contamination. The first strand of cDNA was synthesized by using 2 μg total RNA as template with Reverse Transcription System (TOYOBO, Japan) in a 20 μL reaction volume. The rice *actin* gene *RAc1* (GenBank No. ×16,280) and Arabidopsis *actin* gene *ACT2* (GenBank No. U41998) were used as the internal standard. The RT-PCR primers for each gene used in our study were listed in Table 1 in supplemental material. PCR conditions were as following: 94°C for 5 min, 30 cycles of 94°C for 15 s, 56°C for 15 s, 72°C for 20 s, and with a final extension at 72°C for 10 min. DNA intensity ratio of the target genes to *RAc1/ACT2* was analyzed with Shine Tech Gel Analyse (Shanghai Shine Science of Technology Co. Ltd., Shanghai, China).

DNA binding assay

Yeast one-hybrid system was used to analyze the DNA binding ability between OsAREB1 and ABRE *cis*-element. The entire *OsAREB1* coding region was fused into the *Bam* HI-*Sac* I site of the pPC86 MCS, which carried the Trp synthesize gene. pPC86-OsAREB1 was then transformed into the yeast strain EGY48, which already carried the vector G222 with the reporter gene *LacZ* under the control of ABRE (5'-ACCCTCGAG CGGATAACAATTTCACACAGGTACGTGGCTACGTGGCTAC GTGGCTACGTGGCGGATCCGGGCCC-3' Gene Bank NO. AF394912) tandem repeat sequence, as described previously (35). pPC86 vector alone was also transformed into EGY48 with G222 as a control. Transformation was confirmed by growth on SD medium without Trp at 30°C for 3 d. The β-galactosidase filter-lift assay and β-galactosidase activity mensuration were performed according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). Once the fusion protein interact with the ABRE element, the expression of *lacZ* gene will be induced and X-gal will be decompounded into blue substance.

Construction of *OsAREB1* transgenic plants

The cDNA of *OsAREB1* was cloned into the binary vector pYG8187 driven by the CaMV 35S promoter. The constructs were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. *A. thaliana* cv. Columbia was transformed by floral dip method as described previously (36). T1 Seeds were plated on MS medium containing 25 μg/L hygromycin (Roche, Mannheim, Germany). The homologous T3 generation seeds were used in our experiments.

Analysis of plant water relations

The water content was measured as described by Fujita *et al.* (13). Aerial parts from 4-week-old soil-grown plants were detached and weighed for fresh weight over time. Detached parts were then dried at 100°C for 2 h to determine the dry weight. Water loss rate was calculated as $[(FW_0\text{-}FWi)/(FW_0\text{-}DW)]\times 100$, where FWi and FW_0 are fresh weight for any given interal and original fresh weight, respectively, and DW is dry weight.

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