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N-terminal domains of AhAREB1 protein are necessary for transcriptional characteristics and negative regulation of the *AhNCED1* gene

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Abstract

A transcription factor gene *Arachis hypogaea* abscisic acid (ABA) responsive element binding protein 1 (*AhAREB1*) has been isolated from peanut previously. Here, the function of different domains from *AhAREB1* was investigated using construct series containing *AhAREB1* full-length and truncated fragments to transform peanut hairy roots and *pAhNCED1* (promoter of *Arachis hypogaea* 9-*cis*-epoxycarotenoid dioxygenase 1) *GUS/Col Arabidopsis thaliana*, respectively. The results of real-time quantitative PCR, transient expression, and chromosome immunoprecipitation (ChIP) assay all showed that *AhAREB1* negatively regulated the expression of the *AhNCED1* gene. β -Glucuronidase (GUS) staining shows that *AhAREB1* and the *AhAREB1* gene truncated fragment A1 may be bound to ABA responsive element motifs in the promoter region of *AhNCED1* and involved in the negative regulation of the upstream *AhNCED1* gene promoter, reflected by the inhibited expression of the *AhNCED1* promoter reporter gene and significantly reduced GUS activity in transgenic *A. thaliana* plants. Furthermore, only the full variant of *AhAREB1* and a fragment without a C1 domain had repression activity on the *AhNCED1* promoter. On the contrary, the *AhAREB1* gene truncated fragments A2 and A3 variant without a C2 domain had no such repression activity. Moreover, the negative regulation of *AhNCED1* was detected only when the C2 domain was present suggesting that the C2 domain was required for *AhAREB1* activity. Subcellular localization analysis shows that the deletion of conserved domains C1, C2, C3 had no effects on the nuclear localization of *AhAREB1*. In addition, ChIP analysis indicates that the deletion of domains C1 and C3 significantly affected the binding of the *AhAREB1* transcription factor to the *AhNCED1* promoter. Taken together, the results indicate that the different N-terminal domains of the *AhAREB1* protein, which played different roles in the negative regulations of *AhNCED1*, were necessary for *AhNCED1* transcription.

Additional key words: ABA responsive element, 9-*cis*-epoxycarotenoid dioxygenase, chromosome immunoprecipitation, negative regulation, peanut,

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Abbreviations: ABA - abscisic acid; ABF - ABRE binding factor; ABRE - ABA responsive element; AF - *AhAREB1* full-length cDNA; AREB - ABA-responsive element binding protein; ATAF - *Arabidopsis thaliana* activating factor; bZIP - basic leucine zipper; CAMV - cauliflower mosaic virus; cDNA - complementary DNA; ChIP - chromosome immunoprecipitation; GFP - green fluorescent protein; LUC - luciferase; NAC - petunia NAM and *Arabidopsis* ATAF1, ATAF2, and CUC2; NCED - 9-*cis*-epoxycarotenoid dioxygenase; TF - transcription factor.

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Introduction

Absciscic acid (ABA) is an important plant stress hormone crucial for modulating physiological responses to drought stress and adaptation to unfavourable environments (Zhang *et al.* 2012). Nine-*cis*-epoxycarotenoid dioxygenase (NCED) is a key rate-limiting enzyme in ABA biosynthetic pathway (Xiong *et al.* 2001, Zhu 2002). The high expression of *NCED* gene is one of the important factors promoting ABA biosynthesis under drought conditions (Burbidge *et al.* 1999, Qin and Zeevaart 1999, Chernys and Zeevaart 2000, Iuchi *et al.* 2000, 2001).

During ABA signalling-mediated stress responses, many transcription factors have crucial regulatory roles in activating ABA-dependent stress-responsive gene expression (Tang *et al.* 2012). For example, increased *NCED3* expression and ABA content in *Arabidopsis thaliana* is correlated with an *A. thaliana* NAC transcription factor, *A. thaliana* activating factor 1 (ATAF1), which binds to the *NCED3* promoter (Jensen *et al.* 2013). Yang and Tan (2014) found that a distal ABA responsive element (ABRE) is required for ABA-induced *AtNCED3* expression. Absciscic acid responsive element binding protein (AREB)/ABRE binding factors (ABF) transcription factors are basic domain/leucine zipper (bZIP) transcription factors. They bind to the ABA responsive element (ABRE) motifs in the promoter region of ABA-inducible genes and hence enhance the ability of plant resistance to environmental stresses. The AREB proteins contain four conserved domains; three N-terminals (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).

Arachis hypogaea L. (peanut) is an important economic crop and source of oil. Low rainfall or seasonal

drought are the major factors that constrain its quality and yield (Wan and Li 2006). The *Arachis hypogaea* 9-*cis*-epoxycarotenoid dioxygenase 1 (*AhNCED1*) gene (GenBank accession No. AJ574819) was characterized in dehydrated peanut plants and found to exhibit a typical and significant response to dehydration (Liang *et al.* 2009). Indeed, overexpression of *AhNCED1* can promote ABA biosynthesis in transgenic *Arabidopsis thaliana* under drought stress and hence improve its ability to tolerate these unfavourable conditions (Wan and Li 2006). Analysis of the *AhNCED1* promoter (Genbank registration number: EU497940) shows that it consists of four ABRE motifs indicating that there may be AREB transcription factors that combine with the *AhNCED1* promoter to regulate its expression (Liang *et al.* 2009).

We have also isolated *AhAREB1* (*Arachis hypogaea* ABA responsive element binding protein 1) from peanut and found that the *AhAREB1* gene is specifically induced by ABA, dehydration, and drought (Hong *et al.* 2013). As for the possible role of the *AhAREB1* transcription factor in the regulation of *AhNCED1*, Liu *et al.* (2016a) demonstrated that the *AhAREB1*/*AhNAC2* protein complex could negatively regulate the expression of *AhNCED1*. However, the regulation of *AhAREB1* on *AhNCED1* is not clear and the domain function of the *AhAREB1* transcription factor also needs to be further confirmed. Therefore, in this study, we examined how *AhAREB1* might regulate *AhNCED1* expression. Using serial constructs containing *AhAREB1* full-length and truncated fragments to transform peanut hairy roots and *pAhNCED1::GUS/Col Arabidopsis*, we tried to identify what is the definite function of each domain of *AhAREB1* and which domain is essential to regulate the expression of *AhNCED1*.

Materials and methods

Plants and cultivation: Seeds of *Arabidopsis thaliana* L. were surface sterilized in 70 % (v/v) ethanol for 2 min and then in 1.0 % (m/v) sodium hypochlorite for 2 min. After five washes with sterile water, the seeds were sown on a half strength Murashige and Skoog (MS) medium supplemented with 2.0 % (m/v) sucrose and 1.0 % (m/v) agar. After 2 d of cold treatment at 4 °C, the seeds were germinated and transferred to growth chambers with a 16-h photoperiod, an irradiance of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a temperature of 20 ± 1 °C, and an air humidity of 65 - 70 %. Seedlings of different developmental stages were planted in plastic pots in a mixture of *Vermiculite*, peat moss, and *Perlite* (1:2:1, v/v/v) and grown in the same growth chamber. Plants in different growth stages were selected according to experimental requirements.

Plasmid construction: The overexpression construct of series of the *AhAREB1* coding region, *AhAREB1* full-length cDNA (AF), and three different *AhAREB1* gene truncated fragments (A1, A2, and A3) was generated by real-time PCR with forward primers (AF-F, A1-F, A2-F, and A3-F) and reverse primers (AF-R, A1-R, A2-R, and A3-R) (Table 1 Suppl.) for each gene (Fig. 1). Under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter, all PCR products, confirmed by DNA sequencing, were cloned into a modified binary vector pBI121-GUSless between the Xba I and BamHI sites (Liu *et al.* 2011) to generate the 35S:AF frame (encoding a full-length protein), 35S:A1 (encoding the protein N-terminal deletion of 82 amino acids), 35S:A2 (encoding the protein N-terminal deletion of 146 amino

acids), and 35S:A3 (encoding the protein N-terminal deletion of 174 amino acids) (Fig. 1).

The subcellular localization vector was constructed by replacing the GUS fragment of pBI121 (*Clontech*, Mountain View, CA, USA) with a green fluorescent protein (GFP) cDNA following the CaMV 35S promoter. The AF, A1, A2, and A3 coding regions were amplified as before and inserted into the upstream multiple cloning sites of the GFP frame for creating 35S:AF-GFP, 35S:A1-GFP, 35S:A2-GFP, and 35S:A3-GFP fusions. The empty *p35S:GFP* was preserved in our laboratory and used as a control for cellular localization.

To further confirm the role of the motifs of AhAREB1 in regulating *AhNCED1* expression, we designed AhAREB1, A4, A5, and A6 section carriers. Full-length cDNA and *AhAREB1* gene truncated fragments (A4) were generated by real-time PCR with forward primers (AREB1-F and A4-F) and reverse primers (AREB1-R and A4-R) (Table 1 Suppl.) for each gene. Individual *AhAREB1* gene truncated fragments (A5-C1, A5-C(3+4), A6-C(1+2), and A6-C4) were generated by real-time PCR with forward primers (A5-C1-F, A5-C(3+4)-F, A6-C(1+2)-F, and A6-C4-F) and reverse primers (A5-C1-

R, A5-C(3+4)-R, A6-C(1+2)-R, and A6-C4-R) (Fig. 1, Table 1 Suppl.) for each fragment (Fig. 1). A PCR purification kit (*Axygen*, Tewksbury, MA, USA) was used to purify and recover the PCR products. The amplified products (Fig. 1) were used to perform an overlap extension PCR reaction to connect the two segments. The second amplification was done with forward primers (A5-A-F and A6-A-F) and reverse primers (A5-B-R and A6-B-R) (Table 1 Suppl.) for each gene (Fig. 1). All recycled PCR products confirmed by DNA sequencing were ligated into a pMD-18T vector (*TaKaRa*, Tokyo, Japan), transformed into DH5 α , and screened on Luria Bertani (LB) plates containing 50 mg dm⁻³ ampicillin using a blue-white selection. The selected recombinant cloning vectors were digested with Xba I and Sma I and cloned into a modified binary vector pBI121-GUSless between the Xba I and Sma I sites (Liu *et al.* 2011) under the control of the CaMV 35S promoter to generate the 35S:A4 frame (encoding the protein N-terminal deletion of 87 amino acids), 35S:A5 frame (encoding the protein N-terminal deletion of 53 amino acids), and 35S:A6 frame (encoding the protein N-terminal deletion of 23 amino acids) (Fig. 1).

Fig. 1. The structure of different lacking fragments of AhAREB1.

Stable *Arabidopsis thaliana* transformation and GUS histochemical staining: To test whether the AhAREB1 series regulates the expression of *AhNCED1* by binding to the ABRE *cis*-acting elements in its promoter, the four overexpression constructs (*p35S:AF-GFP*, *p35S:A1-GFP*, *p35S:A2-GFP*, and *p35S:A3-GFP*) were introduced into the *Agrobacterium tumefaciens* strain GV3101 and transformed into *pAhNCED1:GUS/Col Arabidopsis* (Liang *et al.* 2009), respectively, following the floral dip method described by Clough and Bent (1998). The seeds of *pAhNCED1:GUS* were preserved in our laboratory (Liang *et al.* 2009). The *p35S:AF/AhNCED1p:GUS*, *p35S:A1/AhNCED1p:GUS*, *p35S:A2/AhNCED1p:GUS*, and *p35S:A3/AhNCED1p:GUS* transgenic *A. thaliana* plants were generated and after the constructs namely abbreviated as af, a1, a2, and a3, respectively.

Primers for PCR used to confirm the recombinant transgenes in transgenic plants are shown in Table 1 Suppl. At least 10 lines carrying each construct described above were obtained. The transgenic lines expressing transgenes

at similar levels were selected and used for further analyses. Three independent T3 transgenic lines were used for the following experiments, and the experiments were repeated three times. The *pAhNCED1:GUS/Col Arabidopsis* was used as a control.

At least 10 independent plants from each individual transgenic line were analyzed by histochemical GUS staining. Two developmental stages were observed: 2-d-old seedlings and 2-week-old seedlings during the reproductive stage. β -Glucuronidase staining was carried out according to Jefferson (1987), modified by Liang *et al.* (2009). Tissues were observed and photographed with a scanner (EPSON) and a SZX12 microscope (Olympus, Tokyo, Japan) equipped with a DP70 digital camera system. The GUS stained tissues and plants shown in this paper represent the typical results of at least three independent lines for each construct.

***Agrobacterium* strain and induction of peanut hairy roots:** *Agrobacterium* strain and induction of peanut hairy

roots were carried out as described by Liu *et al.* (2016b). The resulting binary constructs *p35S::A4-GFP*, *p35S::A5-GFP*, *p35S::A6-GFP*, and the *p35S::GFP* control were transformed into *A. rhizogenes* strain K599 using the freeze-thaw method. The neomycin phosphotransferase gene (NPTII) under the control of the NOS promoter located within T-DNA enables positive transformants to be selected by kanamycin. Transgenic *AhAREB1* peanut hairy roots were obtained from our laboratory preservation (Liu *et al.* 2016b). The *A4*, *A5*, and *A6* peanut hairy roots identified as GFP-positive were selected, and total RNA was extracted using a *TRIzol* reagent (Takara) according to the manufacturer's instructions. Primers for PCR used to confirm transgenic *A4*, *A5*, and *A6* peanut hairy roots are shown in Table 1 Suppl.

Real-time quantitative PCR assay: Five different lines of peanut hairy roots with over-expressed *AhAREB1* were used to analyze how *AhAREB1* regulates the expression of *AhNCED1*. The RNA was extracted as described by Wan and Li (2005). Reverse transcription was carried out using a *PrimeScript*TM reagent kit with a gDNA eraser (*Perfect Real Time*) (TaKaRa). Quantitative real-time reverse transcription PCR was performed with *SYBR*[®] *Premix Ex Taq*TM (TaKaRa) and a *LightCycler 480* (Roche, Mannheim, Germany) equipped with the *Light-Cycler v.1.5* software based on the manufacturer's instructions (Alos *et al.* 2008). Amplification reactions were carried out in a total volume of 0.02 cm³. Cycling was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s (depending on the optimal reaction temperature of the different primers), and 72 °C for 20 s. Data collection was performed during the annealing phase of each amplification. Then processing a melting curve was from 60 °C to 95 °C by reading the intensity of fluorescence every 0.2 s. All real-time PCR reactions were technically repeated three times. Relative expressions were calibrated using the formula $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The peanut gene *ACTIN* (Genbank: DQ873525.1) was used as an internal control gene. The primers used for real-time PCR analysis are listed in Table 1 Suppl.

Subcellular localization: To examine subcellular localization of all transcription factors (TFs)-GFP fusion proteins, isolation of *A. thaliana* mesophyll protoplasts and transformation were conducted as described by Yoo *et al.* (2007). Recombinant expression plasmids were introduced into *A. thaliana* mesophyll protoplasts by polyethylene glycol mediated transformation. The expression of the series truncated *AhAREB1*-GFP fusion protein in *A. thaliana* mesophyll protoplasts was observed at 12 h after transformation. The empty *p35S::GFP* was preserved in our laboratory and used as a control.

To further explore the localization of the series truncated *AhAREB1*-GFP fusion protein, the root elongation zone of 6-d-old seedlings of af, a1, a2, and a3

were also used for GFP expression. Empty *p35S::GFP/pAhNCED1::GUS/Col* was preserved in our laboratory and used as a control. A GFP fluorescence was observed with a confocal laser scanning microscope (*LSM710*, Carl Zeiss, Germany). A GFP emission was observed between 515 and 530 nm to avoid crosstalk with chloroplast autofluorescence. The GFP was excited at 488 nm using an argon laser.

Transient transactivation assay in *Arabidopsis thaliana* protoplasts: To further study *AhAREB1* function on *AhNCED1* gene activity, the effector and reporter plasmids were purified for a transient expression assay. Transient expression assays using protoplasts from wild-type *Arabidopsis thaliana* plants were performed following the method of Yoshida *et al.* (2010). The series *pBI121-p35S::AhAREB1* plasmids and *pCAMBIA1301-pAhNCED1::LUC* plasmid were co-transfected with each effector plasmid as a reporter and an internal control, respectively. The *pCAMBIA1301-pAhNCED1::LUC* vector was preserved in our laboratory.

Chromatin immunoprecipitation (ChIP) analysis: To examine whether *AhAREB1* series execute their function by binding to the *AhNCED1* promoter, a ChIP assay was performed. Transgenic *AhAREB1*, *A4*, *A5* and *A6* peanut hairy roots were used for the ChIP assay and a *p35S::GFP* peanut hairy root was used as a negative control. The ChIP assay was performed as described by Liu *et al.* (2016a) with minor modifications. In brief, anti-GFP antibody (Abcam) and protein A/G agarose beads (Roche) were used to precipitate the chromatin complex. The ChIP products were dissolved in 0.5 cm³ of Tris-EDTA (TE) and 5 mm³ of each primer was used for real-time PCR. According to the core ABRE sequence position of the *AhNCED1* promoter, five pairs of specific primers were designed, in which pN2, pN3, and pN4 contained one ABRE *cis*-element, and pN1 and pN5 did not contain any ABRE *cis*-element. Each sample was assayed in triplicate by quantitative real-time PCR as described above, and the peanut gene *60s* (Brand and Hovav 2010) was used as an internal control to calculate the relative fold-enrichment of target DNA fragments. Primers used for real-time PCR analysis in ChIP assays are listed in Table 1 Suppl.

Statistical analysis: All tests were carried out at $\alpha = 0.05$ level of significance using SPSS v. 11 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to determine statistical significance for relative gene expression among different transgenic peanut hairy roots, for fold change among different regions of *AhNCED1* promoters, and for the effects of transient expressing fragmented-*AhAREB1* on luciferase (LUC) activity of *AhNCED1* gene promoters. Means were compared at $\alpha = 0.05$ using Tukey honestly significant difference (HSD) *post hoc* comparisons.

Results

Over-expressed *AhAREB1* peanut hairy roots were used to analyze how *AhAREB1* regulates the expression of *AhNCED1*. Real-time PCR shows that the *AhAREB1* expression in all the five different lines of *AhAREB1* over-expressed hairy roots was significantly higher than in the control (Fig. 2A). The expression of *AhNCED1* in

AhAREB1 over-expressed peanut hairy roots was significantly lower than in the control under normal conditions (Fig. 2B). These show that *AhAREB1* may negatively regulate the expression of the *AhNCED1* gene in transgenic peanut hairy roots.

To examine the role of *AhAREB1* series in regulating

Fig. 2. The relative expression of *AhAREB1* (A) and *AhNCED1* (B) genes in different transgenic peanut hairy roots. Control: *p35S::GFP* hairy roots. 1-5: five different lines of *AhAREB1* over-expressed peanut hairy roots. Means $\pm$ SEs, $n = 10$. Within each graph, different letters indicate significantly different means at $P < 0.05$ according to Tukey HSD *post hoc* comparisons.

AhNCED1 in transgenic *A. thaliana* plants, 10 individual plants from each line were analyzed by histochemical GUS staining. In 2-d-old seedlings, GUS expression was found in veins, stems, and root conducting tissues in the control (Fig. 3a-e), a2 (Fig. 3p-t), and a3 (Fig. 3u-y), but it was significantly inhibited in these parts in af (Fig. 3f-j) and a1 (Fig. 3k-o). Moreover, GUS expression occurred only in the apical parts of af and a1 (Fig. 3f,g,k,l). During the reproductive stage, GUS activity was found in leaves (Fig. 4b,n,r), siliques (Fig. 4c,o,s), and sepals (Fig. 4d,p,t) of control, a2, and a3 transgenic plants, but expressed little in those parts of af (Fig. 4e-h) and a1 (Fig. 4i-l). The results of GUS staining show that *AhAREB1* and *A1* may be bound to ABRE motifs in the promoter region of *AhNCED1* and involved in the negative regulation of the upstream *AhNCED1* gene promoter inhibiting expression of the *AhNCED1* promoter reporter gene and significantly reducing GUS activity in transgenic *A. thaliana*.

Fluorescence nuclear localization experiments show that all the TF-GFP fusion proteins were located in the nucleus (Fig. 2 Suppl.) and combined with the nuclear AREB localization sequence (NLS)-located bZIP region (Yoshida *et al.* 2010). These results suggest that the deletion of conserved domains C1, C2, and C3 had no effects on the nuclear localization of *AhAREB1* and may not be involved in the transforming process of *AhAREB1* into the nucleus.

The TF activity was assessed in transient expression analysis in *A. thaliana*. Under normal conditions, the

relative activity of LUC was about 0.3 when the control plasmid-empty vector pBI121 was transformed. However, the relative activity of LUC was significantly reduced when the effectors *p35S::AF*, *p35S::A4*, and *p35S::A6* were transformed (Fig. 5A). These findings indicate that *AhAREB1* negatively regulated the gene expression of *pAhNCED1::Fluc*. In addition, the results of the effector *p35S::AF* show that the deletion of C2 significantly affected the regulation of *AhNCED1* gene expression, whereas C1 and C3 did not play a significant role in this regulation.

In order to analyze the combination of the *AhAREB1* fragments and *AhNCED1* promoter, ChIP assay was used to detect the binding of *AhAREB1* fragments to the promoter of *AhNCED1* in control, AF, A4, A5, A6 transgenic peanut hairy roots (Fig. 5A). In the full-length aspect (Fig. 5B), the expression of AF was significantly higher than of control in *pN2*, *pN3*, and *pN4* regions of *AhNCED1* promoters, whereas no obvious enrichment of AF was found in *pN1* and *pN5* regions. This suggests that *AhAREB1* negatively regulated the expression of *AhNCED1* by binding to the ABRE *cis*-elements on the *AhNCED1* promoter. From the conserved domain aspect, the enrichment of A5 in *pN2*, *pN3*, and *pN4* regions was similar to that in AF suggesting that the deletion of conserved domain C2 did not affect the binding of *AhAREB1* to the *AhNCED1* promoter. The enrichments of A4 and A6 in *pN2*, *pN3*, and *pN4* regions were similar to those of the control, which was significantly lower than

that of AF indicating that the deletion of conserved domains C1 and C3 significantly affected the binding of

the *AhAREB1* transcription factor to the *AhNCED1* promoter.

Fig. 3. Histochemical analysis of β -glucuronidase (GUS) expression (blue color) in 2-d-old *Arabidopsis thaliana* transgenic seedlings *p35S:AF/AhNCED1p:GUS* (af), *p35S:A1/AhNCED1p:GUS* (a1), *p35S:A2/AhNCED1p:GUS* (a2), and *p35S:A3/AhNCED1p* (a3). a, f, k, p, u - seedlings (bar = 500 μ m); b, g, l, q, v - leaves (bar = 200 μ m); c, h, m, r, w - stems (bar = 400 μ m); d, i, n, s, x - roots (bar = 200 μ m); e, j, o, t, y - root tips (bar = 200 μ m).

Discussion

The AREB-like transcription factors play an important role in the ABA signaling pathway, which activates ABA

dependent gene expression under drought stress. They bind to ABRE, thereby regulating plant drought and

osmotic stress tolerance (Uno *et al.* 2000). In this study, *AhAREB1* might inhibit the expression of the *AhNCED1* promoter (Figs. 3 and 4). In over-expressed *AhAREB1* transgenic peanut hairy roots, *AhAREB1* might negatively regulate expression of *AhNCED1* (Fig. 2B). Specifically, ChIP assay and transient expression analysis identified that *AhAREB1* negatively regulated the expression of

AhNCED1 by binding to the ABRE *cis*-elements on the *AhNCED1* promoter (Fig. 6A-C). Combined with above results, we assume that *AhAREB1* may negatively regulate the expression of *AhNCED1* by binding to the ABRE element in the *AhNCED1* promoter. It is also consistent with what has been reported by Liu *et al.* (2016a).

Fig. 4. Histochemical analysis of β -glucuronidase (GUS) expression (blue color) in 2-week-old *Arabidopsis thaliana* transgenic seedlings and leaves, as well as silique and flowers of 4-week-old transgenic seedlings [control; *p35S:AF/AhNCED1p:GUS* (af), *p35S:A1/AhNCED1p:GUS* (a1), *p35S:A2/AhNCED1p:GUS* (a2), and *p35S:A3/AhNCED1p* (a3)]: a, e, i, m, q - seedlings (bar = 4 mm); b, f, j, n, r - leaves (bar = 2 mm); c, g, k, o, s - silique (bar = 0.5 mm); d, h, l, p, t - flowers (bar = 2 mm).

The results of GUS staining show that *AhAREB1* and *A1* significantly reduced GUS activity in transgenic *A. thaliana* (Figs. 3 and 4). This finding suggests that *AhAREB1* and *A1* proteins likely specifically bind to *cis*-acting elements (ABRE) of the upstream promoter DNA fragments of *AhNCED1* and may negatively regulate the *AhNCED1* gene. We also found that only the full variant of *AhAREB1* and the fragment without C1 domain had repression activity on the *AhNCED1* promoter. However, A2 and A3 variants without C2 domain had no such repression activity. In other words, the deletion of conserved domain C1 did not affect the transcription

activity of *AhAREB1*, but the deletion of conserved domain C2 led to a reduction in the transcription activity of *AhAREB1*. It is consistent with the results of transient expression analyses, which show that the deletion of C2 significantly affected the regulation of *AhNCED1* gene expression by *AhAREB1*, whereas C1 and C3 did not play a significant role in this regulation (Fig. 6A). That is to say, the negative regulation of the transactivation activity of *AhNCED1* was detected only when C2 domain was present suggesting that C2 domain is an essential motif and may play a prominent role in *AhNCED1* activity.

Fig. 5. Transient expression analysis and chromosome immunoprecipitation (ChIP) assay of fragmented-*AhAREB1* protein binding to the *AhNCED1* gene promoter. **A** - Effect of transient expressing fragmented-*AhAREB1* on luciferase (LUC) activity of *AhNCED1* gene promoters. Control - *p35S::GFP* hairy roots, AF- *p35S::AF GFP* hairy roots, A4- *p35S::A4 GFP* hairy roots, A5- *p35S::A5 GFP* hairy roots, A6 -- *p35S::A6 GFP* hairy roots. Means $\pm$ SEs, $n = 3$ and different letters indicate significant differences at $P < 0.05$ according to Tukey honestly significant difference (HSD) *post hoc* comparisons. **B** - The design of the ChIP analysis. **C** - Gene expressions detected by real-time PCR. *60s* - internal control gene, *pN1* to *pN5* - the regions examined by ChIP. Means $\pm$ SEs, $n = 3$. Within each region of *AhNCED1* promoters, different letters indicate significantly different means at $P < 0.05$ according to Tukey HSD *post hoc* comparisons.

Fluorescence nuclear localization experiments indicate that conserved domains C1, C2, or C3 were likely not involved in the transforming process of *AhAREB1* into the nucleus (Fig. 5). These results are consistent with what has been reported by Fujita *et al.* (2005), where a nuclear localization signal is located in the bZIP of AREB1.

The results from ChIP assays show that conserved domains C1 and C3 of *AhAREB1* affected the binding of the *AhAREB1* transcription factor to the ABRE element on the *AhNCED1* promoter and play an important role in a negative regulation of *AhNCED1* expression (Fig. 5B,C). It is in agreement with the results of transient expression analysis (Fig. 5A).

However, our results are inconsistent with those of Furihata *et al.* (2006), where conserved domain C1 of the N-terminal plays a key role in regulating transcriptional

activation, and conserved domains C2 and C3 also influence transcription activation. Thus, we compared the amino acid sequences of conserved domains of *A. thaliana* ABF1-ABF4 and *AhAREB1*, soybean SIAREB, tomato GmAREB, and poplar PtrABF (Hong *et al.* 2013) (Fig. 1 Suppl.). Phosphorylation sites in C1 domain of *A. thaliana* are R-Q-G/N/S-S, whereas we found that these sites in C1 domain of *AhAREB1* are R-Q-P-S. Phosphorylation of key amino acid sites R and S did not change, but the mutation of G/N/S into P may cause structural inactivation. Moreover, phosphorylation sites in conserved domain C2 in *A. thaliana* are L-Q-R-Q, but these sites in C2 domain of peanut *AhAREB1* are S-Q-R-Q; L is mutated into S and then it becomes as a new phosphorylation site. Phosphorylation sites in conserved domain C3 in *A. thaliana* are R-Q-Q-T, but these sites in

AhAREB1 are G-Q-P-A (Fig. 1 Suppl.). It is speculated that a change in the amino acid sequence at these critical sites may result in a difference between the peanut AhAREB1 protein region and the *A. thaliana* AREB1/ABF2 protein region, but this needs further experimental verification.

Absciscic acid could be synthesized and accumulated rapidly by a rate limiting enzyme NCED in peanut plants in the early stage of drought stress (Hu *et al.* 2013). In this

study, we demonstrated that AhAREB1 could down-regulate the expression of *AhNCED1* through binding to ABRE *cis*-elements on the *AhNCED1* promoter, and the different domains of AhAREB1 protein play different roles in this regulation. These results provide a theoretical basis for our further studies on transcriptional activity and regulation mechanisms of AhAREB1 in complex transcriptional networks.

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