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BRIEF COMMUNICATION

The promoter-elements of some abiotic stress-inducible genes from cereals interact with a nuclear protein from tobacco

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Abstract

In this communication, we report the binding of abscisic acid responsive elements (ABREs) of rice *Osem*, namely motif A and motif B, with a cognate trans-acting factor present in the nuclear extract of tobacco leaf. The binding is specific as both the complexes were disrupted with an excess of homologous non-radioactive DNA like motif A or motif B themselves or with cis-elements of rice *Rab16A*, motif I (ABRE) and motif IIa (non-ACGT ABRE-like sequences). Four tandem repeats of ABRE from wheat *Em* (4X ABRE) or two tandem repeats of *Em* ABRE, plus two copies of coupling element (CE1) from barley *HVA22* (2X ABRC), also showed specific complexes, that were competed out by an excess of homologous competitors like motif I, motif IIa, motif A, motif B, 4X ABRE and 2X ABRC, but not by the unrelated 4X DRE sequence. Elution of the protein from all the complexes showed a single 26 kDa polypeptide band. Introgression of two of the above synthetic promoters 4X ABRE and 2X ABRC, each fused with minimal promoter of cauliflower mosaic virus 35S (CaMV 35S), could induce the expression of the reporter gene β-glucuronidase (*gus*) in transgenic tobacco in response to high NaCl concentration, dehydration or abscisic acid, but not at the constitutive level, proving that they can be used as efficient stress-inducible promoters. Our work shows both *in vivo* and *in vitro* activity of the promoters from monocot genes in the model dicot plant tobacco.

Additional key words: abscisic acid responsive complex, abscisic acid responsive elements, barley, coupling elements, *Hordeum vulgare*, *Nicotiana tabacum*, *Oryza sativa*, rice, transgenic tobacco.

Plant responses to environmental stress including high salinity, drought and low temperature are accompanied by an increase in endogenous content of the phytohormone abscisic acid (ABA), which in turn induces characteristic sets of genes called late embryogenesis abundant (*lea*) in the embryo during the late phase of seed development or in the vegetative tissues in response to ABA, dehydration, salinity or cold stress (Mundy and Chua 1988, Marcotte *et al.* 1989, Hattori *et al.* 1995, Busk and Pages 1998, Cousson 2009, Huang *et al.* 2009). The *cis*-acting sequences called abscisic acid responsive elements (ABREs), having a core sequence of (T/G/C) ACGTG, have been identified in the promoters of *lea* genes like wheat *Em*, rice *Osem* and *Rab16A-D* and barley *HVA22* and *HVA1*, that are required for induction of transcription in response to stress. The wheat *Em* promoter is represented by box Em1 (ABRE) which is repeated as Em1a and Em1b and box Em2 (non-ACGT sequence), both conferring ABA inducibility (Marcotte *et al.* 1989). The promoter of *Osem*, a rice gene homologous to the wheat *Em*, has three ACGTG-containing sequences namely motif A, motif B and motif A', of which motif A plays a central role in contributing the responsiveness of the promoter to external stress (Hattori *et al.* 1995). Likewise, the promoter of *Rab16A-D* contains two consensus sequences motif I (ABRE) and motif II (non ACGT ABRE-like sequences), of which the latter is duplicated (motif IIa and IIb) in *Rab16A* (Yamaguchi-Shinozaki *et al.* 1990). An ABRE often functions in combination with a GC-rich second sequence element

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Abbreviations: ABRE - abscisic acid responsive element; ABRC - abscisic acid responsive complex; CaMV 35S - cauliflower mosaic virus 35S; CE - coupling element; DRE - dehydration responsive element; EMSA - electrophoretic mobility shift assay; *gus* - β-glucuronidase gene; PEG - polyethylene glycol.

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(Shen and Ho 1995, Shen *et al.* 1996) called the coupling element (CE), like the CE1 in barley *HVA22* and CE3 in barley *HVA1* and rice *Osem*. The two elements ABRE and CE together constitute an ABA responsive cis-element complex (ABRC), which can synergistically activate transcription, in response to ABA (Hattori *et al.* 2002). Multimerized ABREs are found to confer ABA-responsiveness to a heterologous, minimal promoter (Ishige *et al.* 1999). A hexamer of motif I, when fused with 90 bp CaMV 35S promoter could confer ABA-inducible gene expression (Skriver *et al.* 1991). The wheat *Em* promoter could drive reporter gene expression in embryo and aleurone tissues of transgenic barley and rice (Furtado and Henry 2005). Another cis-element called dehydration responsive element (DRE), containing 9 bp consensus TACCGACAT, found in addition to ABRE in *Arabidopsis rd29A* promoter, shows response to dehydration, high salt and low temperature but not through ABA-dependent pathway (Yamaguchi-Shinozaki and Shinozaki 1994).

The basic leucine zipper (bZIP)-class of factors, usually localized in the nuclei, binds to the core ACGT of ABRE or CE and plays a role in trans-regulation of plant *lea* genes (Kosova *et al.* 2007). The proteins EmBP-1 from wheat; OSBZ8, osZIP1a and TRAB1 from rice were initially shown to be ABA-inducible that bind to several ABRE or CE of stress-inducible genes (Guiltinan *et al.* 1990, Nantel and Quatrano 1996, Nakagawa *et al.* 1996, Hobo *et al.* 1999). The seed specific factor (OS) VP1 was shown to bind to the *Osem* ABREs in the embryonic tissues (Hattori *et al.* 1995) of rice. Our laboratory is interested in the characterization of trans-acting factors from monocot as well as dicot plants that display sequence-specific binding to different cis-acting elements from abiotic stress-inducible genes. We have previously shown that the trans-acting factor OSBZ8 from rice that binds to *Em* or *Rab16A* ABREs or ABRE-like sequences, is differentially expressed in salt sensitive and salt tolerant rice cultivars (Mukherjee *et al.* 2006, Roy-Choudhury *et al.* 2008). In another communication, we showed that a tobacco nuclear factor of approximately 26 kDa binds specifically to both motif I (an ABRE) and motif IIa (CE-like sequences) of rice *Rab16A* promoter (RoyChoudhury *et al.* 2007). Here, we were interested to find out whether any tobacco nuclear factor binds to the ABREs (motif A and motif B) of rice *Osem*, to the four tandem repeats of ABRE (4X ABRE) of Em1a sequence of wheat *Em* (considered as the strongest G-box element) and to the two tandem repeats of ABRE of *Em,* plus two copies of CE1 of barley *HVA22* (2X ABRC). We have also selected two of the above synthetically designed promoters 4X ABRE and 2X ABRC, hooked to the 5' end of CaMV 35S basal promoter, supplying TATA and CAAT box, to assess the expression of the reporter gene β-glucuronidase (*gus*) in the model dicot plant tobacco and hence measure the strength of the promoter activity.

Seeds of *Nicotiana tabacum* L. (cv. Petit Havana SR1) were used for our experiment. The germination and *in vitro* growth conditions for tobacco plants were essentially the same as described earlier (RoyChoudhury *et al.* 2007). The matured plants were finally brought to the field and watered with half-strength Hoagland nutrient solutions daily. Preparation of nuclei and nuclear extract from fresh and young leaves of two-month old tobacco plants were as described (RoyChoudhury *et al.* 2007) and the protein content estimated according to Bradford (1976). The sequences and the preparation of different probes for electrophoretic mobility shift assay (EMSA) like [^{32}P]-motif A, [^{32}P]-motif B, [^{32}P]-4X ABRE or [^{32}P]-2X ABRC and non-radioactive competitors were as described earlier (RoyChoudhury *et al.* 2008). Binding reactions were performed essentially as described (RoyChoudhury *et al.* 2007). Each assay reaction mixture contained tobacco nuclear extract (20 µg total protein), 5 µg of poly (dI-dC) and an equal amount of each of the probes. An excess of several non-labeled competitors were used for competition assays. Finally, the proteins were eluted from the DNA: protein complexes in the dried gel as described earlier (RoyChoudhury *et al.* 2008), run in a 12 % SDS poly-acrylamide gel and visualized after silver staining the gel (Blum *et al.*1987).

For tobacco transformation, synthetic promoters containing either four tandem copies of ABREs (CGAAAGCTTGCCACGTGGCGCCACGTGGCGCCACGTGGCGCCACGTGGCACCCTTCCTT) or two copies of ABRCs (GTCAGAAAGCTTGCCACGTGGCTGCAGTGCCATTGCCACCGGATCAGCCACGTGGCTCCAGTGCCATTGCCACCGGATGACGCACAATCCCAC), each fused with common 3'-ends providing the basal promoter upto -70 from the CaMV 35S promoter (AGGCTAGGATCCTCTAGATTCCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCAT), and supplying CAAT box and TATA box (70 LS), were designed. The two oligonucleotide-primer pairs (4X ABRE and 70 LS or 2X ABRC and 70 LS), which are complementary on their extreme 3' ends, were annealed followed by filling-in with Klenow enzyme to generate double-stranded DNA molecule. The filled-in products were amplified through PCR by using two other primers designed from the two ends (GAAAGCTTGCCACGT and AGG CTAGGATCCTCTA). Finally, the 4X ABRE: 35S and 2X ABRC: 35S inducible promoters were subcloned separately in the binary vector pBI121 at HindIII and BamHI site by replacing CaMV 35S constitutive promoter following standard procedures (Sambrook and Russell 2001). The two constructs were separately transferred into the competent cells of *Agrobacterium tumefaciens* LBA4404 strain (An 1987). Transformation of tobacco leaf discs was done according to RoyChoudhury *et al.* (2007) to generate T_0 plants, which were allowed to flower and set seeds under normal environmental conditions. Finally T_0 seeds were selected on kanamycin (200 µg cm^{-3}) medium to generate matured T_1 plants. Total RNA was isolated from the young leaf tissues of wild type (WT) and T_1 transgenic plants

following the method of Longhurst *et al.* (1994). About 30 µg of total RNA were blotted onto *Optitran* pure nitrocellulose membrane (*Schleicher and Schuell BioScience,* Keene, New Hampshire, USA) using 10× SSC as transfer buffer. The 1.8 kbp *gus*, released from pBI121 after BamHI and SacI digestion, was [^{32}P]-labeled using the Prime-It^{R} II random primer labeling kit (*Stratagene*, La Jolla, CA, USA), and the probe purified on *Sephadex G50* columns. Hybridization was performed at 42 °C in formamide buffer for 24 h following the standard protocol (Sambrook and Russell 2001).

Regulation of gene expression depends not only on the presence or strength of cis-acting elements but also on the availability and the specific interaction of the requisite trans-acting factor(s) with the cis-acting elements present in the promoter of the concerned gene (Singh 1998, Cherian *et al.* 2006). The 5' upstream regions of plant genes may contain binding sites for multiple nuclear protein factors. Even a single nuclear factor may interact with more than one promoter (Katagiri *et al.* 1989). Previously, *Osem* ABREs were shown to bind (OS) VP1 in rice embryo (Hattori *et al.* 1995) and Motif A or CE3 to rice TRAB1 (Hobo *et al.* 1999). Em1a ABRE was shown to interact with wheat EmBP1 present in the embryo nuclear extract (Guiltinan *et al.* 1990). However, whether these cis-acting elements from cereals interact with any DNA-binding protein homologue in dicot has been shown through our experimental analysis taking tobacco as the model plant. Since tetramers of Em1a or Em1b elements can confer ABA inducibility to a minimal CaMV 35S promoter, we have used 4X ABRE as probe in EMSA. Not only that, 2X ABRC containing the CE1 from *HVA22* has also been used as probe to check if the CE binds to a homologous or different factor in tobacco. EMSA with equal counts of either Motif A or Motif B synthetic oligo duplex probes (from *Osem* gene) and tobacco nuclear extract (Fig. 1) showed intense complex formation. The DNA: protein complex in each case totally disappeared on adding excess of homologous competitors like motif A, motif B, motif I and motif IIa. EMSA with equal counts of 4X ABRE and 2X ABRC DNA probes also showed distinct complexes (Fig. 2) each with 4X ABRE and 2X ABRC; however the intensity of the complex was more with 2X ABRC than with 4X ABRE. The complex formation was completely abolished after adding excess of non-radioactive homologous DNA like 4X ABRE, 2X ABRC, motif I, motif IIa, motif A or motif B, but not with excess of cold 4X DRE as competitor, because DRE contains unrelated or non-homologous sequences and operates through a separate ABA-independent pathway. To identify the polypeptide(s) that bind to [^{32}P]-motif A DNA, [^{32}P]-motif B DNA, [^{32}P]-4X ABRE DNA or [^{32}P]-2X ABRC DNA, the dried gel containing the complexes were cut by orienting the autoradiograph over the gel, polypeptide(s) were eluted from the dried gel and analyzed by 12 % SDS-PAGE followed by silver staining the gel. A single band of 26 kDa protein was eluted from each complex (Fig. 3), which means that this single nuclear protein from tobacco bound to all the upstream elements of monocot genes that we used for our analysis. RoyChoudhury *et al.* (2007) reported earlier that *Rab16A* promoter from rice bound to a tobacco trans-acting factor, which was competed out totally by excess of *Rab* promoter itself or 1X ABRE (Em1a monomer). They also showed that both motif I and motif IIa (which is a GC-rich, CE-like sequence) bound to a 26 kDa factor from tobacco. Our present communication shows that probably the same 26 kDa factor binds to *Osem* ABRE as well as 4X ABRE or 2X ABRC, because the complexes formed with *Osem* or *Em* ABREs and ABRCs were disrupted totally by an excess fold of non-radioactive motif I or

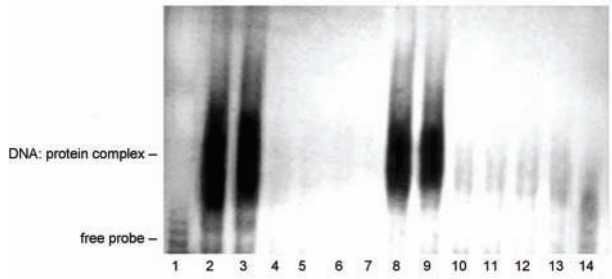

Fig. 1. EMSA of 26 mer [^{32}P]-motif A DNA (*lanes* 1 - 7) or 26 mer [^{32}P]-motif B DNA (*lanes* 8 - 14) with the transacting factor present in the nuclear extract (NE) prepared from tobacco leaves and the specificity of the binding reactions using excess of several competitors. *lanes* 2 and 3 - motif A: protein complex; *lanes* 8 and 9 - motif B: protein complex; *lanes* 4 and 11 - motif A competitor; *lanes* 5 and 10 - motif B competitor; *lanes* 6 and 12 - 40 mer motif I competitor; *lanes* 7 and 13 - 38 mer motif IIa competitor; *lane* 1 - free motif A probe; *lane* 14 - free motif B probe. About 20 µg of NE was incubated with the same amount of either of the probes. The photograph was made from the autoradiogram developed after 7 h of exposure.

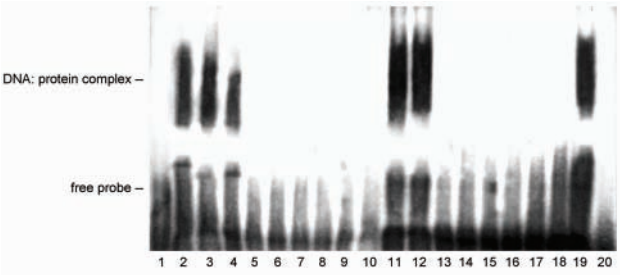

Fig. 2. EMSA of 69 mer [^{32}P]-4X ABRE DNA (*lanes* 1 - 10) or 93 mer [^{32}P]-2X ABRC DNA (*lanes* 11 - 20) with the trans-acting factor present in the nuclear extract (NE) prepared from tobacco leaves and the specificity of the binding reactions using excess of several competitors: *lanes* 2 and 3 - 4X ABRE: protein complex; *lanes* 11 and 12 - 2X ABRC: protein complex; *lanes* 4 and 19 - 95 mer 4X DRE competitor; *lanes* 5 and 14 - 4X ABRE competitor; *lanes* 6 and 13 - 2X ABRC competitor; *lanes* 7 and 15 - motif I competitor; *lanes* 8 and 16 - motif IIa competitor; *lanes* 9 and 17 - motif A competitor; *lanes* 10 and 18 - motif B competitor; *lane* 1 - free 4X ABRE probe*; lane* 20 - free 2X ABRC probe. About 20 µg of NE was incubated with the same amount of either of the probes. The photograph was made from the autoradiogram developed after 7 h of exposure.

motif IIa. The complex formed with 2X ABRC-promoter was more intense than with 4X ABRE-promoter, probably due to the presence of the coupling element in the former which acts together with ABRE to further strengthen the promoter activity and hence its binding. Oeda *et al.* (1991) first reported a tobacco nuclear factor TAF-1 (M_r ~26,000), having a close phylogenetic relationship with several other bZIP factors that bind to the G-box and related motifs conserved among rice *rab* genes and cotton *lea* genes, and regulating ABA-dependent signal transduction and gene expression. Our data predicts and concludes the binding of several cis-acting elements from stress-inducible genes with their cognate 26 kDa trans-acting factor, probably TAF-1, from tobacco. Our data also suggests that the transcriptional machinery of tobacco can recognize all of the control elements directing the expression of at least some of the stress-inducible genes from cereals.

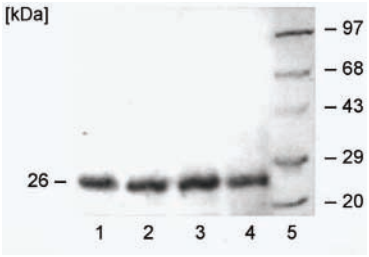

Fig. 3. Silver-stained, 12 % SDS-polyacrylamide gel showing the eluted DNA-binding protein (a single polypeptide band of 26 kDa) from motif A: protein complex (*lane 1*), motif B: protein complex (*lane* 2), 2X ABRC: protein complex (*lane* 3) and 4X ABRE: protein complex (*lane* 4) from the dried native gel. The marker protein has been shown in *lane* 5.

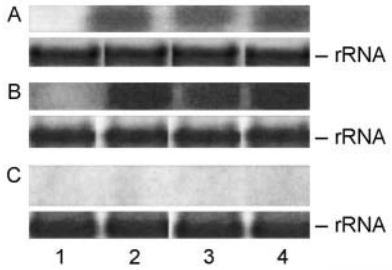

Fig. 4. RNA gel blot analysis detecting *gus* expression in the T1 transgenic tobacco seedlings, driven by either 4X ABRE (*A*) or 2X ABRC (*B*) synthetic promoters, in response to NaCl (200 mM, 6 h) (*lane* 2), PEG (20 %, 6 h) (*lane* 3), or ABA (50 µM, 6 h) (*lane* 4), but undetected under no-stress (control) condition (*lane* 1). The WT plants also showed no *gus* expression or hybridization signals even after NaCl (*lane* 2), PEG (*lane* 3) or ABA (*lane* 4) treatment (*C*). Equal amount of each RNA sample (30 µg) were probed with [^{32}P]-*gus* DNA (1.8 kbp). The ethidium bromide stained rRNA is shown below the RNA blot. The photographs were made from the autoradiograms developed after 15 d of exposure.

Inducible promoters are likely to get activated only when triggered by the appropriate environmental conditions but not at the background level. Previous observation by Ono *et al.* (1996) showed histochemical localization of *gus* expression under *Rab16B* promoter in the root, leaf and flower of ABA or NaCl treated or desiccated transgenic rice plants, but not from the untreated plants. In order to test whether synthetic promoters containing multiple copies of ABRE or ABRC in tandem repeats, along with minimal promoter sequences (-70 to +5) of CaMV 35S, can induce the expression of any gene downstream to them, we have engineered constructs like 4X ABRE:*gus* and 2X ABRC:*gus*, and monitored the expression of the reporter gene *gus* in 4X ABRE and 2X ABRC transgenics against WT plants in response to NaCl, PEG or ABA treatments. Since the basal transcription factors are required to assist RNA polymerase II to initiate transcription from the start site, CAAT and TATA boxes were included in the downstream of the inducible promoters. Actually, drought condition can be mimicked by treatment with PEG. Cells do not readily take up PEG; thus it reduces the external free water concentration without attaining the ionic composition of the cell. The transformed plants, containing pBI:4X ABRE:*gus* or pBI:2X ABRC:*gus,* were verified through PCR from the genomic DNA isolated from the healthy plants (data not shown). RNA gel-blot analysis with [^{32}P]-*gus* fragment showed abiotic stress-inducible expression of *gus* gene regulated by either 4X ABRE (Fig. 4*A*) or 2X ABRC (Fig. 4*B*) promoters in NaCl (200 mM, 6 h), polyethylene glycol (PEG, 20 %, 6 h) or ABA (50 µM, 6 h) treated young seedlings of the transgenic tobacco plants but not during control (unstressed) condition. The induction in *gus* expression was sharper and more pronounced with 2X ABRC promoter than 4X ABRE, as it appears from band intensity. The *gus* transcript was undetectable in WT (untransformed) plants even after NaCl, PEG or ABA treatment (Fig. 4*C*). This proved that 4X ABRE and 2X ABRC functioned efficiently as inducible promoters and they could be readily utilized to over express any desirable gene lying downstream to them in tobacco in response to abiotic stress to monitor the corresponding functional gene expression *in planta*. It is expected that these synthetic promoters will work equally well in monocots like rice. Moreover, such ABA or salinity-stress inducible genes like *Osem a*nd *Em* from monocots are likely to show expression or exert their physiological roles also in tobacco, since tobacco trans-acting factor targets their upstream elements. In future, rice *Osem* and wheat *Em* will be over expressed under their own promoters to check if these genes can contribute towards salt tolerance in tobacco or other dicot plants.

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