ABFs, a Family of ABA-responsive Element Binding Factors*  

Hyung-in Choi, Jung-hee Hong, Jin-ok Ha, Jung-youn Kang, and Soo Young Kim‡  

From the Kumho Life and Environmental Science Laboratory, Kwangju 500-712, South Korea

Abscisic acid (ABA) plays an important role in environmental stress responses of higher plants during vegetative growth. One of the ABA-mediated responses is the induced expression of a large number of genes, which is mediated by cis-regulatory elements known as abscisic acid-responsive elements (ABREs). Although a number of ABRE binding transcription factors have been known, they are not specifically from vegetative tissues under induced conditions. Considering the tissue specificity of ABA signaling pathways, factors mediating ABA-dependent stress responses during vegetative growth phase may thus have been unidentified so far. Here, we report a family of ABRE binding factors isolated from young Arabidopsis plants under stress conditions. The factors, isolated by a yeast one-hybrid system using a prototypical ABRE and named as ABFs (ABRE binding factors) belong to a distinct subfamily of bZIP proteins. Binding site selection assay performed with one ABF showed that its preferred binding site is the strong ABRE, CACGTGGC. ABFs can transactivate an ABRE-containing reporter gene in yeast. Expression of ABFs is induced by ABA and various stress treatments, whereas their induction patterns are different from one another. Thus, a new family of ABRE binding factors indeed exists that have the potential to activate a large number of ABA/stress-responsive genes in Arabidopsis.

* This paper is Kumho Life and Environmental Science Laboratory publication no. 32. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF093544 (ABF1), AF093545 (ABF2), AF093546 (ABF3), and AF093547 (ABF4).  
† To whom correspondence should be addressed: Kumho Life and Environmental Science Laboratory, 1 Oryong-Dong, Puk-Gu, Kwangju 500-712, South Korea. Tel.: 82-62-970-2647; Fax: 82-62-972-5085; E-mail: sykim@ksc.kumho.co.kr.

1 The abbreviations used are: ABA, abscisic acid; ABRE, abscisic acid-responsive element; bZIP, basic leucine zipper; PCR, polymerase chain reaction; RT-PCR, coupled reverse transcription and PCR; GST, glutathione S-transferase; EMSA, electromophoretic mobility shift assay; ORF, open reading frame; DPBF, De3 promoter binding factors.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
has been identified in a tobacco leaf nuclear extract by in vitro binding study (25). Furthermore, it has been well established by genetic studies that different ABA signaling pathways operate in seeds and in vegetative tissues, respectively (2), and tissue-specific ABRE binding activities have been demonstrated (24). None of the source materials used in the previous protein-DNA interaction clonings, however, were ABA- or stress-treated young plant tissues, and thus, inducible factors that may be critical for the ABA-mediated stress response during vegetative growth phase may have been missed so far.

We are interested in ABA-regulated gene expression during environmental stress response and set out to isolate relevant transcription factors. Here, we report a family of ABA-inducible bZIP proteins (designated as ABFs) that can bind to both G/ABREs and C/ABREs. ABFs are also inducible by various stress treatments, and each ABF exhibited unique induction pattern, suggesting that they are probably involved in different ABA-mediated stress-signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Plant Materials—**Arabidopsis thaliana (ecotype Columbia) was grown in either natural soil or in a 1:1 mixture of soil (a 1:1 combination of loam and moss) irrigated with mineral nutrient solution (0.1% Hypoxen) in 8 h light/16 h dark cycles. For RNA isolation, 4–5-week-old plants were subject to various treatments, flash-frozen in liquid nitrogen, and kept at −70 °C until needed. For ABA treatment, roots of plants were submerged after the removal of soil in a 100 μM ABA (Sigma, A 1012) solution for 4 h with gentle shaking. ABA solution was sprayed intermittedly during the incubation period. Salt treatment was performed in the same way, except that 250 mM NaCl solution was employed. For drought treatment, plants were withheld from water for 2 weeks before harvest and left on the bench after removing the soil for 1 h just before collection. For cold treatment, plants were placed at 4 °C for 24 h under dim light before harvest.

**Yeast Techniques, DNA Manipulation, and RNA Gel Blot Analysis—**Standard methods (25–27) were used in manipulating DNA and yeast. DNA sequencing was performed on ABI 310 genetic analyzer according to the manufacturer’s instructions. DNA sequence analysis was done with DNA Strider® and Generum®, and BLAST algorithm (28) was used for a data base search. Multiple sequence alignment and phylogenic netic tree construction were performed with CLUSTAL W program (29) available on the web.

RNA was isolated according to Chomczynski and Mackey (30) and further purified by LiCl precipitation followed by ethanol precipitation. For RNA gel blot analysis, 25 μg of total RNA was fractionated on 1.1% agarose gels (2% lund agarose, 5% formaldehyde-agarose gel, transferred to nylon membrane (Hybond N+, Amersham Pharmacia Biotech) by the “downward capillary transfer” method (25) and fixed using the Stratagene UV Crosslinker (Model 2400). After the DNA was digested by HindIII, or EcoRI, it was then subjected to electroblotting. Hybridization was at 42 °C in 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt’s solution (1× Denhardt’s solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 1% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide for 24–30 h. RNA was visualized by autoradiography. To prepare DNA gel, filters were washed twice in 2× SSC, 0.1% SDS at room temperature and three times in 0.2× SSC, 0.1% SDS for 10 min each at 65 °C. Exposure time was 7–8 days. RT-PCR was performed using the GeneAmp PCR System (Promega) using 0.5 μg of total RNA according to the manufacturer’s instruction. Amplification after the first strand cDNA synthesis was 45, 35, 40, and 45 cycles for ABF1, 2, 3, and 4, respectively. ABF primers (sequences are available upon request) were from variable regions between the bZIP and the conserved regions. The actin primers used in the control reaction were from the Arabidopsis actin-1 gene (accession number M20016). A lack of contaminating DNA in RNA samples was confirmed by using primer sets (ABF3 and actin) that flank introns.

**cDNA Library Construction and Yeast One-hybrid Screening—**Poly(A)+ RNA was isolated from total RNAs prepared from ABA- or salt-treated Arabidopsis seedlings. cDNA was synthesized from an equal mixture (6 μg total) of poly(A)+ RNA prepared from the two sources of total RNAs employing the Stratagene cDNA synthesis kit. cDNA was fractionated on a Sephacrose CL-2B column, peak fractions containing cDNAs larger than 500 base pairs were pooled, and pooled cDNAs were ligated with pYESTrp2 (Invitrogen) predigested with EcoRI/Xhol. The ligation mixture was electroporated into Escherichia coli DH10B cells. The titer of this original library was 5.4 × 107 colony-forming units. Portion of the library (2 × 107) was plated on 15-cm plates at a density of 150,000 colony-forming units/plate. Cells were suspended in LB after overnight growth at 37 °C on plates and pooled together. Finally, plasmid DNA was purified from the collected cells using Wizard® plasmid purification kit (Promega). pYCT7-I and pSK1 (14, 15) were used as HIS3 and LacZ reporter plasmids, respectively. The G/ABRE reporter construct was prepared by inserting a trimer of Em1a element (GGACACGTGGCG) into the Smal site of pYCT7-I and the XbaI site of pSK1. To prepare reporter yeast, YPH 500 was transformed with the Stratagene-digested pYCT7-I reporter construct. Resulting yeast colonies were transformed with pSK1 construct and maintained on a SC-Leu-Ura medium. Screening of the library was performed as described (14) except that transformed reporter yeast was grown on Gal/Raf/CM-His-Leu-Trp plates instead of Glu/CM-His-Leu-Trp plates. Putative positive clones from the screen were streaked on fresh Gal/Raf/CM-His-Leu-Trp plates to purify colonies. To confirm reporter growth pattern, mini-plates were patched on Glu/CM-Leu-Ura-plates to be kept as master plates. Galactose dependence of the His ‘lacZ’ phenotype of the purified isolates was examined subsequently by comparing their growth pattern and β-galactosidase activity on Gal/Raf/CM-His-Leu-Trp and Glu/CM-His-Leu-Trp dropout plates.

**Analysis of Positive Clones—**Yeast DNA was prepared from 5 μl of overnight cultures and digested with HindIII and BamHI (for clone 19) or EcoRI and HaelIII or AluI (in group to group the cDNAs. For library plasmid rescue, yeast DNAs from representative clones were introduced into DH10B E. coli cells by electrotransporation. Plasmid DNAs used in DNA sequencing and confirmation experiments were isolated from these E. coli transformants by the alkaline lysis method. For the confirmation experiment shown in Fig. 1, plasmid DNAs thus isolated were re-introduced into the yeast containing pSK1 or ABRE-pSK1, transformants were kept on Glu/CM-Leu-Trp plates, and their growth was tested after spotting 5 μl of overnight cultures (1/50 dilutions) on Gal/Raf/CM-His-Leu-Trp or Glu/CM-His-Leu-Trp plates containing 2.5 mg/ml 3-aminotriazole.

**Isolation of Full-length ABF3 and ABF4—**A PCR approach was used to isolate the missing 5′ portions of clone 11 and clone 19. A data base search revealed that clone 11 was part of the BAC clone F28A23 of the Arabidopsis chromosome IV. On the other hand, the 5′ portion of the clone 19 sequence was identical to the 3′ region of an EST clone, 178F17T7. Based on the sequence information, 5′ PCR primers (5′-GTACCTCTTCTCCCTGAGTGGC-3′ for clone 11 and 5′-ATTTCACAAGGTTTTAGGC-3′ for clone 19′) were synthesized. 3′ primers (5′-TTCAATTACACCAAGACACATGCC-3′ and 5′-GATTGTGTTGC- CACTTCTAAGG-3′, which are complementary to the 3′-most sequences of clones 11 and 19, respectively) were prepared using our sequence information. PCR was performed with Pwo polymerase (Roche Molecular Biochemicals) using the primer sets and 1 μg of our library plasmid DNA in a 50-μl volume of reaction. After 30 cycles of reaction, the DNA fragments corresponding to the expected size of the full-length clones were gel-purified and cloned into the PCRI-Script vector (Stratagene). Several clones from each PCR product were then sequenced in their entirety. The fidelity of the full-length sequences was confirmed by comparing their sequences with each other and with those of the original partial clones and the genomic clones deposited later by the Arabidopsis Genome Initiative Project.

**Plasmid Constructs—**To prepare GST-ABF fusion constructs, entire coding regions and the 3′-untranslated regions of ABF1 and ABF3 were amplified by PCR using Pfu polymerase (Stratagene). After XhoI digestion followed by gel purification, the fragments were cloned into the SmaI-SulI sites of pGEX-5X-2 (Amersham Pharmacia Biotech). The constructs used in the transactivation assay were also prepared in a similar way. The coding regions were amplified by PCR. The resulting fragments were digested with XhoI, gel-purified, and cloned into pXY243. pXY243 was prepared by Ncol digestion, Klenow fill-in reaction, SalI digestion, and gel purification. Intactness of the junction sequences was confirmed by DNA sequencing. **Preparation of Recombinant ABFs and Mobility Shift Assay—**Recombina ABF1 and ABF3 were prepared employing a GST-purification scheme. In order to facilitate the in vitro mobility shift assay, E. coli BL21 cells were transformed with the GST-ABF constructs by electroporation. To prepare bacterial extract, single colony of transformed bacteria was inoculated in 2YT/Amp medium and grown overnight. The culture was diluted (1:100) into 250 ml of fresh media. Isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.1 mM when A600 reached 0.7. Cells were
Abscisic Acid-responsive Element Binding Factors

plants. The vector contains the B42 activation domain (31) whose expression is under the control of yeast GAL1 promoter. Thus, expression of cDNAs, which are inserted as a fusion to the activation domain, is inducible by galactose and repressed by glucose. The library DNA was used to transform a reporter yeast that harbors the ABRE-containing HIS3 and lacZ reporters. From a screen of 4 million yeast transformants, ~40 His− blue colonies were obtained, among which 19 isolates were characterized further. Analysis of the cDNA inserts of the positive clones indicated that they could be divided into four different groups according to their restriction patterns. Representative clones with longer inserts from each group were analyzed in more detail.

First, binding of the cDNA clones to the G/ABRE in yeast was confirmed. The G/ABRE-HIS3 reporter yeast was retransformed with the library plasmid DNAs isolated from the representative clones. The growth pattern of the transformants on media lacking histidine was then examined to measure the HIS3 reporter activity. The result in Fig. 1 showed that transformants obtained with all four clones could grow on a galactose medium lacking histidine but not on a glucose medium. In the same assay, the transformed yeast containing a control reporter construct lacking the ABRE could not grow on the same galactose medium. Thus, the clones could activate the HIS3 reporter gene reproducibly, indicating that they bind to the ABRE in yeast.

Next, nucleotide and deduced amino acid sequences of the representative clones were determined. Clone 1, which represents two isolates, contained a cDNA insert of 1578 base pairs including a poly(A)+ tail (GenBank accession number AF093544). An open reading frame (ORF) that is in-frame with the B42 domain was present within the sequence. The ORF, referred to as ABF1, contains an ATG initiation codon near the B42-cDNA junction, suggesting that it is a full-length clone. The amino acid sequence starting from the initiation codon is shown in Fig. 2. The insert of clone 2, which represents 8 isolates, is 1654 base pairs long (GenBank accession number AF093545), and the longest ORF including an initiation codon near the B42-cDNA junction encodes a protein of 416 amino acids (ABF2, Fig. 2).

The insert of clone 11, representing 6 isolates, encoded a protein containing 434 amino acids. An ORF containing 366 amino acids was found in clone 19 cDNA. The clones were partial, however, and the missing 5′ portions were isolated using the available partial sequence information on data bases (see “Experimental Procedures”). Sequencing of the full-length clones (GenBank accession number AF093546 and AF093547) showed that the original clone 11 was missing the first 20 amino acids, and thus, full-length clone 11 encodes a protein containing 454 amino acids (ABF3, Fig. 2). The longest ORF of clone 19 is composed of 431 amino acids (ABF4, Fig. 2).

RESULTS

Isolation of ABRE-binding Protein Factors—We employed a modified yeast one-hybrid system (14, 15) to isolate ABRE binding factor(s) using the prototypical ABRE, Em1a element (GGACACGTGGCG). A cDNA expression library representing 2 × 107 colony-forming units was constructed in a yeast expression vector pYESTrp2 using a mixture of equal amounts of mRNAs isolated from ABA- and salt-treated Arabidopsis

harvested by centrifugation after further growth (1.5 h). The bacterial pellet was resuspended in 12.5 ml of phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM NaHPO4, 1.8 mM KH2PO4, pH 7.3) and sonicated on a Branson Sonifier 250 (4 × 40-s burst at setting 5 at 50% duty cycle). The lysate was cleared of cell debris by centrifugation, and the supernatant was loaded onto a column packed with 0.125 ml (bed volume) of glutathione-Sepharose 4B resin. Wash and elution was performed as suggested by the supplier. Protein concentration was determined using the Bio-Rad protein assay kit. Production of GST-ABF1 fusion protein was confirmed by Western blotting using GST antibody.

Mobility shift assay was performed as described (15). To prepare probe oligonucleotide sets shown in Fig. 4, the oligonucleotides were annealed by boiling 100 pmol each of complementary oligonucleotides for 5 min and slowly cooling to room temperature. Portions of the annealed oligonucleotides (4 pmol of each set) were labeled by Klenow fill-in reaction in the presence of [32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C.

Binding Site Selection Assay—A pool of 58-base oligonucleotides, R58, containing 18 bases of random sequence was synthesized: CATTGGACGGAGATCTGTCG(N)18, where N is a nucleotide. The random sequence is flanked by BamHI and EcoRI sites for the convenience of cloning after selection. R58 was made double strand by annealing a primer, RANR (AGTTGCACTGAATTCGCCTC) and then by extending it using Klenow fill-in reaction. For the first round of selection, 0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-[32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C. Binding Site Selection Assay—A pool of 58-base oligonucleotides, R58, containing 18 bases of random sequence was synthesized: CATTGGACGGAGATCTGTCG(N)18, where N is a nucleotide. The random sequence is flanked by BamHI and EcoRI sites for the convenience of cloning after selection. R58 was made double strand by annealing a primer, RANR (AGTTGCACTGAATTCGCCTC) and then by extending it using Klenow fill-in reaction. For the first round of selection, 0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-[32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C. Binding Site Selection Assay—A pool of 58-base oligonucleotides, R58, containing 18 bases of random sequence was synthesized: CATTGGACGGAGATCTGTCG(N)18, where N is a nucleotide. The random sequence is flanked by BamHI and EcoRI sites for the convenience of cloning after selection. R58 was made double strand by annealing a primer, RANR (AGTTGCACTGAATTCGCCTC) and then by extending it using Klenow fill-in reaction. For the first round of selection, 0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-[32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C. Binding Site Selection Assay—A pool of 58-base oligonucleotides, R58, containing 18 bases of random sequence was synthesized: CATTGGACGGAGATCTGTCG(N)18, where N is a nucleotide. The random sequence is flanked by BamHI and EcoRI sites for the convenience of cloning after selection. R58 was made double strand by annealing a primer, RANR (AGTTGCACTGAATTCGCCTC) and then by extending it using Klenow fill-in reaction. For the first round of selection, 0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-[32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C. Binding Site Selection Assay—A pool of 58-base oligonucleotides, R58, containing 18 bases of random sequence was synthesized: CATTGGACGGAGATCTGTCG(N)18, where N is a nucleotide. The random sequence is flanked by BamHI and EcoRI sites for the convenience of cloning after selection. R58 was made double strand by annealing a primer, RANR (AGTTGCACTGAATTCGCCTC) and then by extending it using Klenow fill-in reaction. For the first round of selection, 0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-0.125 ml of 40 mM stock solution of chlorophenylred-[32P]dATP. Binding reactions were on ice for 30 min, and electrophoresis was performed at 4 °C.
**ABFs Are bZIP Proteins**—Analysis of the deduced amino acid sequence of ABF1 revealed that it has a basic region near its C terminus (Fig. 2). The region immediately downstream of it contains four heptad repeats of leucine, indicating that ABF1 is a bZIP protein (32). Similarly, other ABFs also have a basic region followed by a leucine repeat region (Fig. 2). The basic regions of ABF1 and ABF3 are identical to each other, and those of ABF 2 and ABF4 are also identical. The two shared basic regions are same except that one of the lysine residues of ABF1 and ABF3 is replaced by arginine in ABF 2 and ABF4 (Fig. 2). The analysis shows that a family of bZIP proteins with conserved basic regions interacts with the G/ABRE.

ABFs also share several highly conserved regions outside the basic domains. As shown in Fig. 2, the conserved regions are clustered in the N-terminal halves. Invariably, they contain one or two potential phosphorylation sites. The N-most region, for example, contains one multifunctional calmodulin-dependent protein kinase II site (RRXXYS*) (33) followed by a casein kinase II phosphorylation site (XXD/EY). One or two calmodulin-dependent protein kinase II or casein kinase II phosphorylation sites are also present in other conserved regions. The middle portions of ABFs are highly variable and rich in glutamine commonly found in transcriptional activation domains.

**In Vitro Binding Activity of ABFs**—To test in vitro DNA binding activity of ABFs, we performed EMSA using recombinant ABF1 or ABF3 and a probe DNA containing a G/ABRE. Similar results were obtained with both proteins, and the assay result of ABF1 is shown in Fig. 3. A major shifted band was observed with a weaker minor band (Fig. 3A, lane 2). The band formation was abolished by the addition of excess amounts of unlabeled probe DNA to the reaction mixture (Fig. 3B, lanes 3 and 4) and by a mutated oligonucleotide (lanes 5 and 6) as competitors, respectively. Sequences of oligonucleotides are shown at the bottom of each figure, and shifted bands are indicated by arrowheads.

**Abscisic Acid-responsive Element Binding Factors**
Competing with 3' and 4 lanes, the competition was not observed with a mutated probe DNA (lanes 5 and 6), demonstrating that the binding was specific to the C/ABRE. Thus, ABF1 and ABF3 could bind to a C/ABRE as well.

**Binding Site Preference of ABF1**—Our in vitro binding assay indicated that ABF1 and ABF3 can interact with both G and C/ABREs, although mutual competition assay (not shown) showed that they have higher affinity to the G/ABRE. To investigate ABF binding sites further, we performed a random binding site selection assay (35) (see “Experimental Procedures”) using the recombinant ABF1. Shifted bands were visible on a mobility shift assay gel after three rounds of selection (Fig. 4A, top panel). After confirming the binding of ABF1 to the probe DNAs from the final round of selection (Fig. 4A, bottom panel), the DNAs were cloned and sequenced.

The 44 selected sequences are presented in Fig. 4B. The sequences could be divided into four groups (groups I, IA, IB, and II) according to their consensus sequences. All of the group I sequences except one (sequence 49) contain an ACCTG element, whereas the group II sequences contain the C/ABRE core. The most frequently selected sequences (30 of 44) are those sharing a strong G/ABRE, CACGTGGC (6): gACACGTGGC (group IA) or CCACGTGGC (group IAA). The group IA element is similar to the prototypical ABRE, Em1a (GGA-CACGTGGC), whereas the group IAA consensus is the same as the palindromic G/ABREs present in many ABA-inducible genes such as maize rab28, Arabidopsis kin1, cor6.6, and Adh1 genes (reviewed in Ref. 36). In some of the group IA sequences (sequences numbers 38, 45, and 42), the GGC following the ACGT core is replaced by GTC, forming another palindromic consensus sequence, GACACGTGTC. The group IB sequences share a GNTGACGTGGC consensus or its variants, differing in one or two bases flanking the ACGT core. Although the conserved element differs from those of group IA and IAA in the bases preceding the ACGT core, it contains the same ACGTG(G/t)C, where the lower case t represents a less frequent nucleotide. Hence, the preferred binding sites of ABF1 can be represented as ACGTG(G/t)C, with AC, CC, or TG preceding it.

One of the selected sequences (number 24 of group II) contains the C/ABRE core sequence (CGCGTG). The three other group II sequences also contain the C/ABRE core. The element in them, however, is flanked by one of the group I consensus sequences, and thus, they contain both types of ABREs. Another sequence (number 49 of group IB) does not contain the ACGT core; the C of the ACGT is replaced by A. The resulting AAGTGGA sequence is similar to the half G-box (CCAAGTGG) of Arabidopsis Adh1 promoter, which is required for high level ABA induction of the gene (37). Thus, ABF1 interacts with sequences without the ACGT core, which includes the C/ABRE. The low selection frequency, however, suggests that affinity of the ABF1 to the C/ABRE is lower.

**Expression of ABFs Is ABA-inducible**—Since we are interested in ABA-inducible stress-responsive factors, we investigated ABA inducibility of ABF expression by RNA gel blot analysis (Fig. 5A). With the ABF1 probe, no hybridization signal was detectable with RNA isolated from untreated plants, whereas a clear signal was detected with RNA from 3B, lanes 3 and 4). The competition was not observed with a mutated probe DNA (lanes 5 and 6), demonstrating that the binding was specific to the C/ABRE. Thus, ABF1 and ABF3 could bind to a C/ABRE as well.

**Fig. 4. Binding site selection assay.** A, binding site selection assay. Top, probes (P0 to P5) after each round of selection were amplified and used in EMSA. 1.5 μg of ABF1 was used. Only the top part of the gel containing the shifted bands is shown. Bottom, EMSA of P5 probe DNA. P5 probe DNA was employed in EMSA and titrated with increasing amounts (μg) of ABF1. Arrowheads denote shifted bands. The band shown by the asterisk is probably an artifact resulting from secondary structure formation of palindromic sequences in the selected sequences. B, selected sequences. The selected sequences are aligned and grouped according to their consensus sequences shown in parentheses. The nucleotides highly conserved within each group are in bold, and those 100% conserved are underlined. G/ABRE elements flanking the C/ABRE core of group II sequences are in italics and underlined. The number of selected sequences in each group is indicated in the parentheses on the right.
ABA-treated plants. Similar results were obtained with other ABF probes; although hybridization signals were weak (ABF2 and -4) or undetectable (ABF-3) with the RNA from untreated plants, distinct signals were observed with the RNA sample from ABA-treated plants. Thus, expression of ABFs is ABA-inducible.

Although all are induced by ABA, the time course of ABA-induced expression of ABFs was not identical to each other (Fig. 5B). ABF1 RNA level reached a peak approximately 2 h after ABA treatment started, remained the same up to 12 h, and decreased to the uninduced level after 16 h. ABF2 and ABF4 expression appeared to be induced faster, reaching a plateau after 30 min of ABA treatment. Afterward, their RNA level remained relatively same until 24 h. The induction pattern of ABF3 was similar to those of ABF2 and ABF4, except that it reached the peak level later, i.e., after 2 h.

We also examined the effect of various environmental stresses on the expression of ABFs. The results (Fig. 5A) showed that ABF1 expression was induced by cold treatment but not by other stress treatments. On the other hand, ABF2 and ABF3 were not induced by cold but by high salt treatment. ABF4 expression was induced by all three treatments, although the induction level after cold treatment was relatively low. Expression of ABFs is, thus, inducible also by various environmental stresses, and their induction patterns are different, suggesting that they function in different stress-responsive pathways.

ABFs Can Transactivate an ABRE-containing Reporter Gene in Yeast—Our result so far demonstrates that ABF1 and probably other ABFs, too, can bind to various ABREs and that their expression is both ABA- and stress-dependent. Thus, ABFs have the potential to activate a large number of ABA/stress-responsive genes if they have transactivation capability. We therefore investigated whether ABFs can activate an ABRE-containing reporter gene. Coding regions of ABFs were cloned into a yeast expression vector, and the constructs were individually introduced into a yeast strain that harbored a G/ABRE (a timer of the Em1a element)-containing lacZ reporter gene. The β-galactosidase activity was then assayed and indicated as Miller units. For each construct, 5 different transformants were assayed in duplicates. YX243, control vector without any inserts. Bars represent S.D. values.

With the ABF1 construct, β-galactosidase activity was six times higher than that obtained with the control construct (Fig. 6, top panel). No enzyme activity was detectable with the same ABF1 construct when a reporter lacking the ABRE was used. Thus, ABF1 can transactivate the reporter gene, and the activation is ABRE-dependent. With the ABF2 construct, reporter enzyme activity two times higher than the background activity was detected, indicating that the factor also can transactivate the reporter gene (Fig. 6, top panel). Likewise, ABF3 and 4 could transactivate the reporter gene (Fig. 6, bottom panel). The activation level of ABF3 was higher than that of the ABF1, whereas ABF4 showed weaker activation. The result of our transactivation assay demonstrates that ABFs can activate an ABRE-containing gene in yeast.

**DISCUSSION**

Numerous studies, both genetic and biochemical, show that ABA mediates stress response in vegetative tissues, although not all stress responses are ABA-dependent (2–5). Central to the response is the ABA regulation of gene expression through G/ABREs or C/ABREs. Transcription factors mediating ABA-independent cold and drought responses have been reported recently (38, 39). However, those regulating ABA-dependent stress response via the G/ or the C/ABREs have yet to be identified. Among the ABRE binding factors mentioned earlier, TAF-1 is known not to be directly involved in ABA-responsive gene expression (11), whereas EmBP-1 and DPBFs are highly embryo-specific (15, 40). GF3 and a homology-based cloned factor OSBZ8, although inducible by ABA, are from cultured cells or from embryos (13, 19). Taken together with the lack of data demonstrating their role in ABA or stress response, it is likely that unknown factors may mediate ABA-responsive gene expression in vegetative tissues.

In a search for such transcription factors, we isolated a family of G/ABRE-binding proteins from young *Arabidopsis* plants treated with ABA or high salt. The factors, referred to as...
Abscisic Acid-responsive Element Binding Factors

**Fig. 7.** Phylogenetic analysis of ABRE binding bZIP factors. A, bZIP regions of the ABRE binding factors mentioned in the text are aligned together. mlp15 is a maize bZIP factor induced by low temperature (Kusano et al. (18)). Conserved amino acids are highlighted, and the leucine residues in the “zipper” regions are underlined. B, unrooted phylogenetic tree diagram. The bZIP regions shown in A were aligned, and a tree diagram was constructed using CLUSTAL W algorithm.

ABFs, are ABA/stress-inducible bZIP class transcription factors with shared basic regions. Sequence comparison with known ABRE-binding factors indicated that, although they do not show any significant homology to other factors, they are similar to the DPBFs (14, 15). DPBFs have been isolated from a seed-specific library based on their interaction with a lea gene promoter containing both G/- and C/ABREs. The two family members are nearly identical in their basic regions (Fig. 7A), and their DNA-binding properties are similar in that they can interact with both types of ABREs. Some of the conserved phosphorylation sites within the N-terminal halves of ABFs are also conserved in DPBFs. However, ABFs diverge from the DPBFs outside the basic regions and their immediate flanking sequences, overall identity being in the range of 30–40%. As a result, they form a subfamily distinct from DPBFs and also from other known factors, as shown in Fig. 7B. Furthermore, their expression patterns are different from those of DPBFs; i.e., DPBF expression is embryo-specific. Cloning of ABFs shows that two related subfamilies of ABRE binding factors are present in seed and in vegetative tissues, respectively. The presence of distinct factors in the tissues that have similar ABRE binding affinity has been demonstrated in maize (24).

ABFs contain regions highly conserved among them apart from the basic regions. Thus, ABFs appear to share some properties other than DNA binding activity. The conserved regions, however, do not have any easily recognizable motifs, except that two of them can form α-helix, and thus, their function remains to be identified. They may be involved in nuclear translocation, DNA binding, transcriptional activation, or interaction with other regulatory proteins. Whatever their function may be, the conservation of potential phosphorylation sites within the regions suggests that it is probably modulated by post-translational modification.

Our in vitro binding assay showed that the most preferred binding site of ABF1 in vitro can be represented as CACGT-GGC (Fig. 4B). The element first identified as EmBP-1 recognition site (7) is highly conserved among ABA/stress-inducible promoters and strongly affects ABA inducibility in vivo (6). Together with the fact that ABF1 is ABA/stress-inducible and has transcriptional activity, this suggests that ABF1 can potentially activate a large number of ABA/stress-responsive genes (see below). Also, ABF1 can bind to other ABREs including the C/ABREs, further supporting the broad spectrum of potential ABF1 target genes. The affinity to C/ABREs, however, was relatively low. It cannot be ruled out, therefore, that factors other than ABFs interact with C/ABREs with higher affinity.

The expression pattern of ABFs implies that they are involved in the regulation of ABA-responsive genes whose expression requires protein synthesis. Depending on the requirement of de novo protein synthesis, ABA-responsive gene activation events can be divided into two classes: one involving protein synthesis and the other independent of protein synthesis (reviewed in Ref. 3). Expression of rice rabi16A, Arabidopsis rd29B and rice Osem genes, for example, requires protein synthesis, whereas ABA induction of the wheat Em gene does not. Our result (i.e. ABA inducibility of ABF expression) thus suggests that ABFs are likely to regulate the expression of the former class of genes.

Each ABF may function in different ABA-dependent stress-signaling pathways. Although all are ABA-inducible and can bind to same ABREs, they are differentially regulated by various environmental stresses (Fig. 5A). ABF1 expression is induced by cold, ABF 2 and ABF3 by high salt, and ABF4 by cold, high salt and drought. The simplest interpretation of the result would be that ABF1 is involved in cold signal transduction, whereas ABF2 and ABF3 function in osmotic stress signaling. ABF4, on the other hand, appears to participate in multiple stress responses. In addition, ABFs differ in their ABA induction patterns. Expression of ABF1 was induced rather slowly (Fig. 5B), and the accumulation of its RNA was transient, whereas induction of other ABFs appeared faster, and their RNA levels remained relatively stable once a plateau was reached. The multiplicity of ABA-dependent stress-signaling pathways has been demonstrated in Arabidopsis by genetic analysis (2, 41). Our result suggests further that multiple transcription factors are likely to function in these signal transduction cascades through common ABREs.

ABA-dependent stress-responsive gene expression is critical to plant growth and productivity. Here, we reported a family of transcription factors that interact with cis-regulatory elements mediating this process. Although their specific roles in planta remain to be determined, our data presented here suggest that they are likely to be involved in various ABA-mediated stress responses. They can bind to ABREs highly conserved among stress-responsive promoters. They can transactivate an ABRE-containing reporter gene. Their expression is induced by ABA and by various environmental stresses.

**Acknowledgments**—We thank Drs. Somi Kim, Moon Soo Soh, Ohkmae Kim, Jungmook Kim, and Pill-Soon Song for their critical reading of the manuscript. We also thank In-Taek Hwang for his technical help during initial stages of this work.

**REFERENCES**

1. Zeevaart, J. A. D., and Creelman, R. A. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 439–473

2. Leung, J., and Giraudat, J. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 199–222
Abscisic Acid-responsive Element Binding Factors

3. Shinozaki, K., and Yamaguchi-Shinozaki, K. (1996) Curr. Opin. Biotechnol. 7, 161–167
4. Thomashow, M. F. (1998) Plant Physiol. 118, 1–7
5. Ingram, J., and Bartels, D. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 377–403
6. Busk, P. K., and Pages, M. (1998) Plant Mol. Biol. 37, 425–435
7. Guiliano, G., Pichersky, E., Malik, V. S., Timko, M. P., Seolnik, P. A., and Cashmore, A. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7089–7093
8. Guiliano, G, Pichersky, E., Malik, V. S., Timko, M. P., Scolnik, P. A., and Cashmore, A. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7089–7093
9. Menkens, A. E., Schindler, U., and Cashmore, A. R. (1995) Trend Biochem Sci. 20, 506–510
10. Izawa, T., Foster, R., and Chua, N.-H. (1993) J. Mol. Biol. 230, 1131–1144
11. Oeda, K., Salinas, J., and Chua, N.-H. (1991) EMBO J. 10, 1793–1802
12. Schindler, U., Menkens, A. E., Beckmann, H., Ecker, J. R., and Cashmore, A. R. (1992) EMBO J. 11, 1261–1273
13. Lu, G., Paul, A.-L., McCarty D. R., and Ferl, R. J. (1996) Plant Cell 8, 847–857
14. Chung, H.-J. (1996) Analysis of the 5’ Upstream Region of the Carrot Dc3 Gene: Bipartite Structure of the Dc3 Promoter for Embryo-specific and ABA-inducible Expression. Doctoral Dissertation, Texas A & M University