

Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions

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The induction of the dehydration-responsive *Arabidopsis* gene, *rd29B*, is mediated mainly by abscisic acid (ABA). Promoter analysis of *rd29B* indicated that two ABA-responsive elements (ABREs) are required for the dehydration-responsive expression of *rd29B* as cis-acting elements. Three cDNAs encoding basic leucine zipper (bZIP)-type ABRE-binding proteins were isolated by using the yeast one-hybrid system and were designated AREB1, AREB2, and AREB3 (ABA-responsive element binding protein). Transcription of the *AREB1* and *AREB2* genes is up-regulated by drought, NaCl, and ABA treatment in vegetative tissues. In a transient transactivation experiment using *Arabidopsis* leaf protoplasts, both the AREB1 and AREB2 proteins activated transcription of a reporter gene driven by ABRE. AREB1 and AREB2 required ABA for their activation, because their transactivation activities were repressed in *aba2* and *abi1* mutants and enhanced in an *era1* mutant. Activation of AREBs by ABA was suppressed by protein kinase inhibitors. These results suggest that both AREB1 and AREB2 function as transcriptional activators in the ABA-inducible expression of *rd29B*, and further that ABA-dependent posttranscriptional activation of AREB1 and AREB2, probably by phosphorylation, is necessary for their maximum activation by ABA. Using cultured *Arabidopsis* cells, we demonstrated that a specific ABA-activated protein kinase of 42-kDa phosphorylated conserved N-terminal regions in the AREB proteins.

The phytohormone abscisic acid (ABA) plays important roles in the adaptation of vegetative tissues to abiotic environmental stresses such as drought and high salinity, as well as in seed maturation and dormancy. ABA promotes stomatal closure in guard cells, mediating by solute efflux, and regulates the expression of many genes, the products of which may function in dehydration tolerance in both vegetative tissues and seeds (1–3). Many ABA-inducible genes contain a conserved, ABA-responsive, cis-acting element named ABRE (ABA-responsive element; PyACGTGGC) in their promoter regions (1–3). ABRE functions as a cis-acting DNA element involved in ABA-regulated gene expression. ABREs were first identified in the wheat *Em* gene, which functions mainly in seed during late embryogenesis (4), and in the rice *rab16* gene, which is expressed in both dehydrated vegetative tissues and maturing seeds (5). A cDNA for the ABRE-binding protein EmBP-1 was first shown to encode a basic leucine zipper (bZIP) protein containing a basic DNA-binding domain linked to a leucine zipper domain (4). However, a single copy of ABRE is not sufficient for ABA-responsive transcription. Furthermore, the G-box resembles the ABRE motif and functions in the regulation of plant genes in a variety of environmental conditions, such as red light, UV light, anaerobiosis, and wounding (6). G-box-binding proteins also contain a bZIP motif (6). Nucleotides around the ACGT core motif are important for determining the binding

specificity of bZIP proteins. Furthermore, a coupling element is required to specify the function of ABRE as an ABRE. ABRE and a coupling element constitute an ABA-responsive complex in the regulation of the wheat *HVA22* gene (7). Most of the known coupling elements have similarity with ABREs and contain an A/GCGT motif (8). Several genes for bZIP that bind to ABREs *in vitro* or are inducible by ABA have been isolated (1–3). However, none of their functions have been clearly determined in ABA signaling, because of a lack of genetic analysis in these studies.

The regulatory machinery in ABA-responsive gene expression in vegetative tissues is thought to be different from that in seeds. Mutations that alter the sensitivity to ABA in seeds have been described in maize and *Arabidopsis* (9, 10). The maize *VPI* and *Arabidopsis* *ABI3* genes play key roles in ABA-dependent seed maturation, and both genes encode seed-specific, homologous transcription factors that mediate ABA-regulated gene expression in seeds (10, 11). The existence of *VPI* homologues in vegetative tissues has not been reported thus far. In addition, some reports concerning the promoter analysis of the ABA-responsive genes showed that different cis-acting elements of the promoters regulate the expression of the genes by ABA between vegetative tissues and seeds (12, 13). These suggest that different members of transcription factors regulate the ABRE-dependent gene expression in response to dehydration stress.

On the other hand, the involvement of protein phosphatase 2C and farnesyltransferase in ABA signaling were revealed by cloning of the *ABI1*, *ABI2*, and *ERA1* genes in *Arabidopsis* (14–17). The *abi1* and *abi2* mutants show decreased sensitivities to ABA inhibition of seedling growth and defects in molecular responses to applied ABA in vegetative tissues (9). The *era1* mutant shows prolonged seed dormancy owing to an enhanced response to ABA (17). The *ERA1*, *ABI1* and *ABI2* genes are expressed in vegetative tissues and are thought to be essential for

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; AREB, ABA-responsive element binding protein; bZIP, basic leucine zipper; GUS, β -glucuronidase; 3-AT, 3-aminotriazole; DPBF, Dc3 promoter-binding factor.

Data deposition: The nucleotide sequences of the *AREB1*, *AREB2*, and *AREB3* cDNAs reported in this paper have been deposited in the GenBank database (accession nos. AB017160, AB017161, and AB017162, respectively).

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negative regulation of ABA signaling during vegetative growth (14–17). To understand the ABA signaling pathway during dehydration stress, it is critical to identify the transcription factors that function downstream of ABI1, ABI2, and ERA1 proteins in ABA signaling in *Arabidopsis*.

Two genes, *rd29A* and *rd29B*, which are located close to each other on the *Arabidopsis* genome, are differentially regulated by drought stress and ABA treatment (18). The promoter region of *rd29A* contains at least two cis-acting elements, DRE (dehydration responsive element) and ABRE, involved in ABA-independent and ABA-responsive gene expression under drought stress. In contrast, the *rd29B* promoter contains ABRE but not DRE (18). *rd29B* mRNA did not accumulate during dehydration stress in the ABA-deficient *aba1* mutant (19), and its level was very low in the ABA-insensitive *abi1* mutant of *Arabidopsis* (9), indicating that the drought-inducible expression of *rd29B* is mainly controlled by ABA. We analyzed the *rd29B* promoter in transgenic plants and found that two ABREs function as cis-regulatory elements in ABA-dependent expression of *rd29B*. We cloned three different cDNAs encoding ABRE-binding proteins (AREB1, AREB2, and AREB3) of *Arabidopsis* by using the yeast one-hybrid screening method. Each AREB protein contained a single bZIP-type DNA-binding domain, and genes encoding the AREB1 and AREB2 proteins were up-regulated by ABA. We analyzed the function of the AREB1 and AREB2 proteins as trans-acting factors by using transient expression in leaf protoplasts prepared from wild-type or ABA-related mutants. We also analyzed the ABA-specific phosphorylation of the AREB1 and AREB2 proteins by using cultured *Arabidopsis* cells. We discuss the functions of the AREB proteins in the ABA-dependent signaling pathways in vegetative tissues under dehydration stress conditions.

Materials and Methods

Plant Materials and Stress Treatments. Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 3 weeks as described (18). Various stress treatments and treatment with ABA were applied as described (18). T87 cells, derived from *Arabidopsis*, were grown and treated as described (20).

Base Substitution Analysis of the 77-bp Region of the *rd29B* Promoter. Six 77-bp fragments with base substitutions (M1–M6) between positions –169 and –93 of the *rd29B* promoter with *Hind*III sites at both ends were prepared by PCR (Fig. 1). The products were cloned into the *Hind*III site of pBluescript II SK[–] (Stratagene), and the resulting plasmids were confirmed by sequencing. The tandemly repeated, dimeric, 77-bp fragments with or without base substitution were ligated to the *Hind*III site of the –51 *rd29B* minimal TATA promoter-GUS (β -glucuronidase) fusion construct (18). Confirmation of the fusion constructs, transformation of *Nicotiana tabacum* cv SR1, and measurement of GUS activity were performed as described (18).

Yeast One-Hybrid Screening of *Arabidopsis* cDNA Libraries. Construction of reporter plasmids and selection of the yeast reporter strain were performed as described (21). *Arabidopsis* cDNA libraries were prepared from dehydrated and unstressed *Arabidopsis* plants as described (21). We screened 1.0×10^6 yeast transformants from each library according to the manufacturer's protocol (CLONTECH Matchmaker one-hybrid system). We obtained 230 positive colonies from selective-medium plates [15 mM 3-aminotriazole (3-AT)]. Growth of these clones was examined at 30, 45, and 60 mM 3-AT. The β -galactosidase activities of the clones then were further analyzed. Finally, 29 clones, which grew normally on the 30-mM 3-AT plate and had β -galactosidase activity, were selected. The cDNA isolation, sub-cloning, and sequencing of these 29 clones were performed as

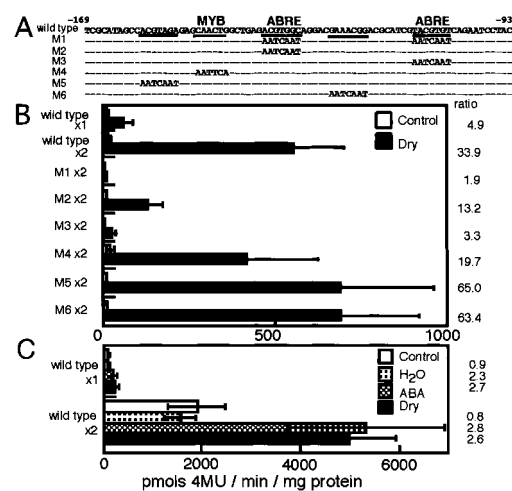


Fig. 1. Base substitution analysis of the 77-bp region of the *rd29B* promoter involved in ABA- and dehydration-responsive expression in transgenic tobacco. GUS activity before and after treatment was measured in 20 independent transformants for each construct and is shown as average values. MU, 4-methyl-umbelliferone. (A) Upper-strand sequences of the 77-bp fragment (wild type) and its mutated sequences (M1–M6). The monomer (x1) or tandemly repeated dimer (x2) of each 77-bp fragment containing different mutations was ligated to the –51 *rd29B* minimal TATA promoter-GUS construct, respectively. Dashes indicate the wild-type sequence. (B) Effect of base substitutions in the ABRE and MYB recognition sites for dehydration-responsive expression of the *rd29B*. Half of the leaf from T1 tobacco plant was used immediately for the assay of GUS activity (control), and the other half was dehydrated for 24 h (dry). (C) Effect of ABA treatment on the induction of the GUS reporter gene driven by the 77-bp fragment. T2 tobacco seedlings were used immediately for the assay of GUS activity (control), transferred from agar plates for hydroponic growth in water (H₂O) or 100 μ M ABA (ABA) solution, or dehydrated for 24 h (dry).

described (21). To analyze the binding specificity of isolated cDNA clones, four tandemly repeated copies of the mutated 77-bp fragment containing two ABRE sequences replaced with AATCAAT (Fig. 1A) were used for the construction of the reporter plasmids.

RNA Gel Blot Analyses. RNA gel blot hybridization was performed as described (18).

Transactivation Experiment with Protoplasts. Effector plasmids used in the transient transactivation experiment were constructed with DNA fragments containing the *AREB1*, *AREB2*, or *GBF3* coding regions, which were cloned into *Not*I sites of the plant expression vector pBI35S Ω . The pBI35S Ω vector was constructed as described (20). To construct a reporter plasmid, the 35S promoter of pBI221 was replaced with the *rd29B* minimal TATA promoter, and the 77-bp fragment of the *rd29B* promoter then was ligated into the *Hind*III site located upstream from the *rd29B* minimal TATA promoter. A transactivation experiment with *Arabidopsis* mesophyll protoplasts was performed as described (20).

In-Gel Kinase Assay. The ABA-dependent phosphorylation of the AREB proteins was analyzed by an in-gel protein kinase assay as described (22). GST-fused AREB proteins expressed in *Escherichia coli* were used as substrates in 10% SDS-polyacrylamide gel (50 μ g/ml). *Arabidopsis* T87 cells were incubated in the presence or absence of 50 μ M ABA for various periods and ground with mortar and pestle in liquid nitrogen; then proteins were extracted in 1 ml/g (fresh weight) extraction buffer as described (23). Cell extract proteins (10 μ g) were denatured and electrophoresed in 10% SDS-polyacrylamide gel

containing the GST-fused AREB proteins, and then kinase activity was assessed (22).

Reverse Transcription-PCR. Reverse transcription-PCR was performed according to the manufacturer's protocol (Amersham Pharmacia).

Results

Identification of Cis-Acting Elements Involved in Dehydration-Responsive Expression of *rd29B*. A promoter analysis with *rd29B*-*GUS* fusion genes indicated that the 77-bp region between -169 and -93 contains cis-acting elements that are involved in the dehydration- and ABA-responsive expression of *rd29B* (Fig. 1). The 77-bp region contains two closely located ABRE motifs (ACGTGGC and TACGTGTC) and one putative recognition site (CAACTG) for MYB-related transcription factors. First, we determined which motifs are the cis-acting elements involved in the dehydration-induced transcription of *rd29B*. We prepared the 77-bp fragments with or without base substitutions in two ABREs and the MYB recognition sites (Fig. 1A). The monomer or tandemly repeated dimer forms of these fragments were fused upstream of the -51 *rd29B* minimum promoter-*GUS* fusion construct and introduced into a tobacco chromosome by *Agrobacterium*-mediated transformation.

We analyzed 20 independent transgenic tobacco plants for the expression of each fusion gene (Fig. 1). The monomer form of the wild-type 77-bp fragment induced a 4.9-times increase in GUS (Fig. 1B). The dimer form of the wild-type 77-bp sequence induced a 33.9-times increase in GUS activity after dehydration treatment (Fig. 1B, wild type). The dimer forms of the 77-bp fragment with base substitutions in both ABREs did not function in the dehydration-induced GUS expression (Fig. 1B, M1). The 77-bp fragment with base substitution in either ABRE showed considerably reduced GUS induction (Fig. 1B, M2 and M3). In contrast, the 77-bp fragment with base substitutions in the MYB recognition site (Fig. 1B, M4) or those outside the ABRE and MYB sites (Fig. 1B, M5 and M6) responded to dehydration stress at the same level as or higher than the wild-type 77-bp fragment did (Fig. 1B). These results demonstrate that at least two ABREs function as positive, cis-acting elements in dehydration-responsive expression of *rd29B*. As tobacco plants could not absorb ABA easily we analyzed ABA-responsive expression of the 77-bp promoter region by using T2 tobacco seedlings of the transgenics. The level of GUS induction by ABA was similar to that by dehydration in the seedling (Fig. 1C).

Isolation of cDNAs Encoding DNA-Binding Proteins that Recognize ABRE in the 77-bp DNA Fragment of the *rd29B* Promoter. To isolate cDNAs encoding DNA-binding proteins that interact with the ABRE motif, we carried out yeast one-hybrid screening. We first constructed a parental yeast strain carrying as reporter genes integrated copies of *HIS3* and *lacZ* with four-time tandemly repeated, 77-bp DNA fragments of the *rd29B* promoter. The resulting yeast cells then were separately transformed with two expression libraries prepared from *Arabidopsis* rosette plants that had been dehydrated for 2 h or not dehydrated. The cDNA fragments were fused to the transcriptional activation domain of yeast *GAL4*. We isolated 23 3-AT-resistant clones from a library prepared from dehydrated plants and six clones from a library prepared from undehydrated plants. All of the isolated cDNA clones induced *lacZ* activity and formed blue colonies on filter papers containing 5-bromo-4-chloro-3-indolyl β -D-galactoside. The cDNA fragments of the isolated plasmids were analyzed by restriction enzyme digestion and DNA sequencing, which led to the classification of these 29 cDNA clones into five distinct cDNA groups. Two were the same as previously identified clones GBF1 and GBF3 (24), and three were novel clones that were designated AREB1, AREB2, and AREB3 (Fig. 2A).

When the five isolated plasmids were transformed into yeast

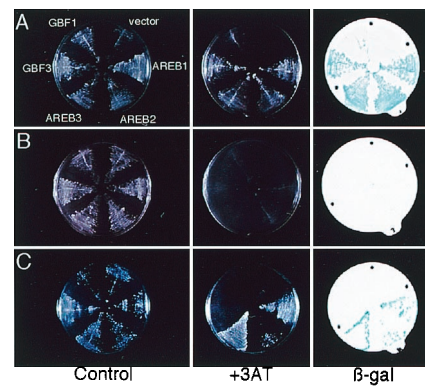


Fig. 2. Isolation of cDNAs encoding ABRE-binding proteins by the yeast one-hybrid system. (A) Confirmation of five isolated cDNAs encoding ABRE-binding proteins by the yeast one-hybrid system. Five plasmids containing insert DNA from AREB1, AREB2, AREB3, GBF1, and GBF3 were retransformed into yeast strains carrying the reporter genes *HIS3* and *lacZ* under the control of the 77-bp fragment containing two ABREs. The transformants were examined for growth in the presence of 3-AT and β -galactosidase (β -gal) activity. (B) Binding specificity of proteins encoded by isolated cDNAs. Five plasmids were used for transformation into yeast carrying the reporter genes under the control of the 77-bp fragment containing mutated ABREs. (C) Activation of reporter genes in yeast by proteins encoded by isolated cDNAs. The insert DNA fragments of the five isolated cDNA clones were cloned into the yeast expression vector YepGAP and used for transformation into yeast carrying the reporter genes under the control of the 77-bp fragment containing two ABREs.

strains carrying the reporter genes fused to the 77-bp DNA fragment with base substitution in the ABRE sequences, the yeast strains neither grew on medium lacking histidine in the presence of 3-AT nor induced *lacZ* activity (Fig. 2B). To select cDNAs that encode transcriptional activators in the five isolated clones, the insert cDNA fragments were cloned into the yeast expression vector YepGAP. Plasmids containing each insert DNA were transformed into yeast strains carrying the reporter genes, which had been fused to the 77-bp DNA. Yeast cells carrying the plasmid containing the cDNAs of AREB1, AREB2, and AREB3 grew on a medium lacking histidine in the presence of 3-AT, but yeast cells carrying the plasmid containing the cDNAs of GBF1 and GBF3 did not (Fig. 2C). All of the 3-AT-resistant yeast strains also formed blue colonies. These data indicate that cDNAs of AREB1, AREB2, and AREB3 encode polypeptides that specifically bind to the ABRE sequence and activate the transcription of the reporter genes in yeast. These three clones were analyzed further.

Structural Analysis of the AREB cDNAs. To examine the structures of the *AREB1*, *AREB2*, and *AREB3* cDNA clones, we sequenced inserted DNA fragments of 1.6, 1.6, and 0.7 kb, respectively. The *AREB1* cDNA contained a single ORF of 416 aa and encoded a putative protein with a predicted molecular mass of 44.2 kDa (Fig. 3). The *AREB2* cDNA contained an ORF of 431 aa and encoded a putative protein with a predicted molecular mass of 46.5 kDa. Analysis of the *AREB3* cDNA revealed that the 0.7-kb DNA insert was not full length. A full-length cDNA library was screened with the *AREB3* insert (25), and then a cDNA clone containing the entire coding region of this gene was isolated. The full-length *AREB3* cDNA contained a single ORF of 297 aa and encoded a putative protein with a predicted molecular mass of 32.4 kDa.

Homology search of DNA and protein databases indicated that the *AREB1*, *AREB2*, and *AREB3* proteins have a bZIP-type DNA-binding domain (Fig. 3). Four or three heptad repeats of leucine or isoleucine exist near the C terminus in the three AREB proteins. The basic domains of the AREB proteins are

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AREB1 1 M--DGSNMLNVEPPDDGG---G---GG-C-ARCOGLYSIFDFEQ---SSVQKDFGSMNDLLEK 54
AREB2 1 MGTHTFNLNLGGGGHPGGEGSSNQMKTPTGSHR-ARCOGLYSIFDFEQNTLGGPGCDFGSMNDLLEK 70
AREB3 1 M--DSQRGIIVEQAKSQ---S---SLNRCSSLSYSIFDFEQNTLGGPGCDFGSMNDLLEK 52
DBPF1 1 M--NMDFLNSIWAIBENQAH-QAHPPTAA-TIG--GGI-S-T--S-AAAMATNG--AS--SSGFL- 52

AREB1 55 IWSAE--TQARAS--GVVPV-LGG---GQEGEL-C-RGCGSLILRLISQNVVQVWLDLSKVGSGS VGG 116
AREB2 71 TWVALEADAMAMTSAPAAATAVQPGAGIIPPGG-ARCOGLYSIFDFEQNTLGGPGCDFGSMNDLLEK 139
AREB3 53 Y-CGE--AN-QFS--S-MAY-NGRGA--NQSGL--ESRGLILELHLSRSDVQVWVDT-C-QRKA-SG- 107
DBPF1 53 --NGINAN--S--AEP--NHIA-R--QV---S-LRSRGSLLRGLPESRSTVDEKQWISRTRTQRHQDQPS 106
*****

AREB1 117 NLSQVAQAQSQSQSQSCNCTGGEVHLKLEFLFRAGVVRREACQAVAAQAIENNKGQVYFG-NDANTGFSVEPQ 185
AREB2 140 S-G--GGESNVPPGRCNCTGGEVHLKLEFLFRAGVVRREACQAVAAQAIENNKGQVYFG-NDANTGFSVEPQ 206
AREB3 108 --S--AH-E-R-R-DKQNLGEMHLELLKAGVVVTE-T-IPGSNH--DGPVGG--G-S-A-G----- 153
DBPF1 107 N-DNHCNCEQVPGAQCENCTGGEVHLKLEFLFRAGVVRREACQAVAAQAIENNKGQVYFG-NDANTGFSVEPQ 162
*****

AREB1 186 QPSPRVA-AGVMNGLGAETANSIQQVQ--GSSFLPLVNGARTTYQQSQSQPQVIMPCKPGVYGTQMGQLNS 253
AREB2 207 QPNQNSITFTNGTDSMILNPQGLGLRGGITMQQQQC--QQQLQQQQQQMQL-NQPH-PC-QRLPTOTIF 272
AREB3 154 -S-----GR-G-LG-QNT-T-QV---G---PW-I--Q--Y---RQLPMP-QPQ-AF--M-PY-- 186
DBPF1 163 -PYNGNRIIG-PPSSAHRVRM-LG---LSTG-----G-Q-ASVTP--Y-Y--S--FLIRET 206
*****

AREB1 254 PGTIRGGGLVGLGDSITMNVGQSGAALPGLGVGA-VSPVTFLLSSEIGKSKGDSISSLSPSPYMFN- 321
AREB2 273 P--KQNVAFSAFVNITNK-GEFAGANNISINNNGLASYGTVTAATSPGTSSAQRNLSVPEVYLN- 318
AREB3 187 P-V--S--DM-QM--VS--Q--S--SL--MG-G-----L-SD--TQTPG-RRRV-AS-- 237
DBPF1 207 PG--YPGKRAGNTQQ-QPPYGGIIGNAGVAGYQ-Q-GLGISPPSPVSSDGLATYQDQSGNQYALEH 272
*****

AREB1 322 QGVR-GRKS-GT--VEKVERRQRMKIKRESAARSARKQAYTVLEAEVAKLEENDELQKQARIME 387
AREB2 359 ---R-GRRS-NTG-LEKVIERRQRMKIKRESAARSARKQAYTVLEAEVAKLEENDELQKQARIME 402
AREB3 218 GEV-----VEKVERRQRMKIKRESAARSARKQAYTVLEAEVAKLEENDELQKQARIME 276
DBPF1 273 QGIRGGRKRRIIDGVEKVKVRRQRMKIKRESAARSARKQAYTVLEAEVAKLEENDELQKQARIME 342
*****

AREB1 388 MQK-NQETE-----MRLNGLQGPQKCKRRESFHW 416
AREB2 403 MQK-NELKE-----TSRPRWGSKROCLRLNGLFHW 431
AREB3 277 -----LPSVPEFPKRLRRESFHW 297
DBPF1 343 KRKQFSEIRMKGVTKCQVRDKSRMLRRESFHW 378
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Fig. 3. Comparison of deduced amino acid sequences of the AREB 1, AREB2, and AREB3 proteins and sunflower DBPF1 (21). Symbols denote identical (*) and conserved (-) amino acid residues in the four sequences. The boxes represent conserved regions, and dashes indicate gaps introduced to maximize alignment. Inverted characters indicates consensus sequences for various kinases (R/KXXS/T for CDPK, S/TXXD/E for CK II, S/TXK/R for PKC, K/RXXS/T for cGMP-dependent protein kinase) appeared within the conserved regions.

nearly identical to each other, which suggests that these DNA-binding proteins recognize the same target sequence. The three AREB proteins contain the three conserved sequences in their N-terminus half regions and a short amino acid sequence near their C terminus. Two of the conserved amino acid sequences of the AREB proteins showed significant sequence identity in their N-terminus regions to the sunflower DBPF1 protein, which binds to ABA-responsive and embryo-specification elements in the carrot Dc3 promoter (ref. 26; Fig. 3).

Expression of the AREB Genes. The expression patterns of the *AREB1*, *AREB2*, and *AREB3* genes were analyzed by RNA gel blot hybridization to compare them with that of the *rd29B* gene (Fig. 4). The *rd29B* gene was induced within 2 h after dehydration, high-salt treatment, or exogenous ABA treatment. The *AREB1* gene expression was induced within 2 h after dehydration began, and the level of the *AREB1* mRNA increased over 24 h. The induction of *AREB2* was detected within 24 h after dehydration stress. The induction of the *AREB1* and *AREB2* mRNAs also was observed within 2 h after high-salt or exogenous ABA

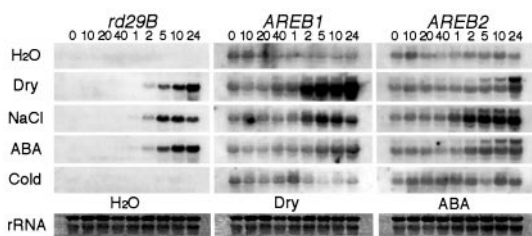


Fig. 4. (A) Expression of the *AREB1*, *AREB2*, and *rd29B* genes in response to dehydration, low temperature, high salt, or ABA. Each lane was loaded with 40 μ g of total RNA from 3-week-old *Arabidopsis* plants that had been dehydrated (Dry), transferred to 4°C (Cold), transferred to hydroponic growth in 250 mM NaCl (NaCl), transferred to hydroponic growth in 100 μ M ABA (ABA), or transferred to water (H₂O), as described in *Materials and Methods*. The number above each lane indicates the number of minutes or hours after the initiation of the treatment. rRNAs blotted on the membrane were visualized by staining with methylene blue.

treatment. The *AREB3* mRNA was not detected in any stressed or unstressed plants (data not shown). These data indicate that *AREB1* and *AREB2* gene products most likely function as transcription factors involved in the induction of *rd29B*. The weak expression of both *AREB1* and *AREB2* was detected in untreated plants. Tissue-specific expression also was analyzed and found that both *AREB1* and *AREB2* were expressed in roots and leaves but not in seeds (data not shown).

The AREB1 and AREB2 Proteins Transactivate the *rd29B* Promoter-GUS Fusion Gene in Leaf Protoplasts. The *rd29B* gene expression under various stress conditions was analyzed by RNA gel blot hybridization in wild-type or ABA-related mutants (Fig. 5A). The *rd29B* gene was induced by dehydration, high-salt treatment, or exogenous ABA treatment in wild-type plants (Columbia and Landsberg) and in the *abi3* mutant. However, the *rd29B* mRNA did not accumulate during dehydration or high-salt stress in the *aba1* mutant, and its level was very low in the *abi1* mutant. The *rd29B* gene was not induced by ABA in the *abi1* mutant. These observations suggest that the dehydration- and high-salt-responsive expression of *rd29B* is regulated mainly by ABA, and that this ABA-dependent expression is mediated by the ABI1 protein but not by ABI3. Previously, Nordin *et al.* (27) also have shown similar results analyzing the expression of *lfi65/rd29B*.

To determine whether the AREB1 and AREB2 proteins are capable of transactivating ABRE-dependent transcription in plant cells, we performed transactivation experiments using protoplasts prepared from *Arabidopsis* leaves. Protoplasts were cotransfected with a *GUS* reporter gene fused to the five tandem copies of the 77-bp fragment containing two ABRE motifs and an effector plasmid (Fig. 5B). The effector plasmid consisted of the CaMV 35S promoter fused to the *AREB1* or *AREB2* cDNAs. Coexpression of the AREB1 or AREB2 proteins in protoplasts derived from wild-type *Arabidopsis* plants transactivated the expression of the *GUS* reporter gene, but that of GBF3 protein did not (Fig. 5C). These results suggest that AREB1 and AREB2 function as transcription activators involved in the ABRE-dependent expression of *rd29B*. Addition of ABA into protoplasts further activated the expression of the *GUS* reporter gene (Fig. 5C). To investigate activation of a native ABA-inducible promoter by AREB1 and AREB2, leaf protoplasts were cotransfected with a *GUS* reporter gene fused to the monomer of the 77-bp fragment of the *rd29B* promoter (Fig. 5B). We obtained similar activation of *GUS* expression by using the monomer form of the promoter region of *rd29B* (Fig. 5D).

We isolated leaf protoplasts from several ABA-related mutants. In the *aba2* mutant background, transactivation of AREB1 and AREB2 was decreased to the basal level but was strongly increased by the addition of ABA (Fig. 5C). Transactivation of the AREB proteins was significantly decreased in protoplasts derived from the *abi1* mutant (Fig. 5C). We obtained similar results by using protoplasts prepared from the *abi2* mutant (data not shown). By contrast, the transactivation of the AREB proteins was strongly activated by ABA in the *era1* mutant (Fig. 5C). These results suggest that overproduction of the AREB1 and AREB2 proteins alone is not sufficient for the expression of *rd29B*. Activation of AREB1 by ABA was repressed by the addition of a protein kinase inhibitor, staurosporine (Fig. 5C). We obtained similar results by adding K252a (data not shown). Modification of the AREB proteins, such as phosphorylation in response to ABA, may be necessary for their activation.

ABA-Specific Phosphorylation of AREB1 and AREB2 Proteins. ABA-dependent phosphorylation of the AREB proteins was examined by using an in-gel protein kinase assay. The protein kinase activities were measured in polyacrylamide gels containing the recombinant AREB protein by using cell extracts from ABA-treated *Arabidopsis* T87 culture cells. We detected ABA-

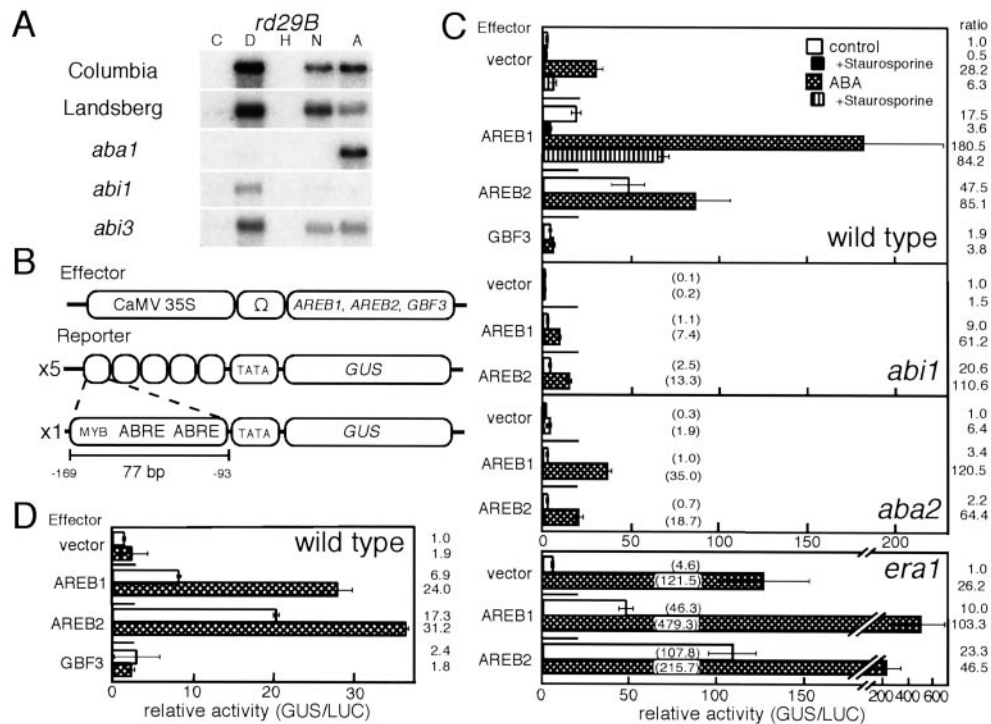


Fig. 5. Transactivation of the *rd29B* promoter-GUS fusion gene by AREB1 and AREB2 proteins using *Arabidopsis* protoplasts. (A) Expression of *rd29B* in response to dehydration, low temperature, high salt, or ABA in wild type (Columbia & Landsberg) or ABA-related mutants (*aba2*, *abi1*, and *abi3*). Plants were untreated (C), dehydrated for 10 h (D), or treated with water (H), 250 mM NaCl (N) or 100 μ M ABA (A) for 5 h. (B) Schematic diagram of the effector and reporter constructs used in cotransfection experiments. The effector constructs contain the CaMV 35S promoter and TMV Ω sequence fused to *AREB1*, *AREB2*, or *GBF3* cDNAs. The reporter constructs contain the 77-bp fragments of the *rd29B* promoter connected tandemly five times ($\times 5$) or single ($\times 1$). The promoters were fused to the -51 *rd29B* minimal TATA promoter-GUS construct. (C) Transactivation of the *rd29B* promoter-GUS fusion gene by AREB1, AREB2, and GBF3 proteins. The reporter gene driven by the 77-bp fragments tandemly repeated five times was transfected with each effector plasmid or the vector as control treatments. Transactivation experiments using protoplasts prepared from wild type or ABA-mutant (*abi1*, *aba2*, and *era1*) *Arabidopsis* leaves. To normalize for transfection efficiency, the CaMV 35S promoter-luciferase (LUC) plasmid was cotransfected in each experiment. Bars indicate the standard error of three replicates. Ratios indicate the multiples of expression compared with the value obtained with the pBI35S Ω vector, and ratio in parentheses indicates the multiples of expression compared with value obtained with the wild type transformed with pBI35S Ω vector. (D) Transactivation using the reporter construct containing the single 77-bp fragment.

dependent phosphorylation of the AREB proteins by 42-kDa protein kinases when we used a recombinant polypeptide of the AREB proteins containing the first conserved sequences in their N-terminal regions as substrates (Fig. 6A, AREB1a and AREB2a). Polypeptides containing the second and third conserved regions also were phosphorylated, but those of unconserved regions (data not shown) and the DNA-binding domain (Fig. 6A) were not. This protein kinase activity was activated by ABA within 2 min and reached a maximum level at 1 h. The *rd29B* mRNA accumulated in response to ABA in *Arabidopsis* culture cells, whereas the expression of the *AREB1* and *AREB2* genes was constant during 2 h (Fig. 6B).

Discussion

The base substitution experiments demonstrated that at least two ABREs in the 77-bp region of the *rd29B* promoter are involved in dehydration- and ABA-responsive expression (Fig. 1). It has been shown that polymerized copies of ABREs can confer ABA responsiveness to a minimal promoter, whereas a single copy of ABRE was not sufficient for the full ABA response (28). In the case of the wheat *Em* promoter, both the *Em1a* and *Em1b* ABRE motifs contribute to the promoter activity (4). In the barley *HVA1* promoter, the typical ABRE motifs and the coupling element CE3 directly upstream of ABRE are shown to be sufficient for ABA induction of gene expression. The CE3 sequence (ACGCGTGTCTC) is similar to a typical ABRE (ACGTGG/TC; ref. 29), and other coupling elements also contain an A/GCGT core sequence (8). The

existence of more than two ABREs at appropriate positions in the promoter regions seems to be sufficient for the ABA-inducible transcription.

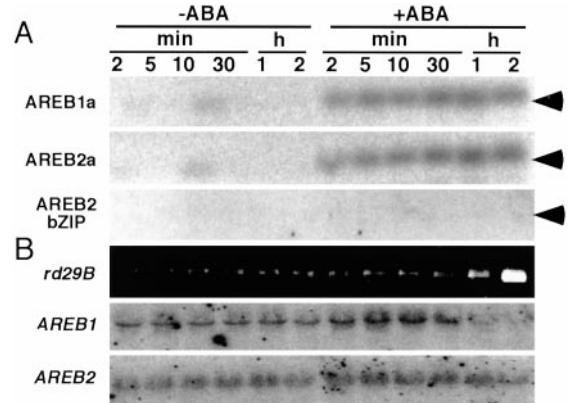


Fig. 6. ABA-dependent phosphorylation of recombinant AREB proteins by in-gel kinase activity assay. (A) T87 cell extracts treated with or without 100 μ M ABA treatment were resolved on 10% polyacrylamide gel containing recombinant AREB proteins (AREB1a: 73G-131Q; AREB2a: 84S-133D; AREB2 bZIP domain: 335Y-401V). The protein kinase activities were analyzed as described in *Materials and Methods*. (B) Accumulation of the *rd29B*, *AREB1*, and *AREB2* mRNAs in T87 culture cells. The *rd29B* mRNA was detected by reverse transcription-PCR, and the *AREB1* and *AREB2* mRNAs were detected by RNA gel blot analysis.

We identified three distinct cDNAs (*AREB1*, *AREB2*, and *AREB3*)-encoding DNA-binding proteins that specifically interact with ABREs involved in dehydration- and ABA-responsive gene expression of *rd29B*. These three AREB genes encode novel bZIP-type proteins. The *AREB1* and *AREB2* genes were shown to be expressed weakly in unstressed plants and to be induced by dehydration, high-salt treatment, and endogenous ABA treatment, whereas the *AREB3* mRNA was not detected in either stressed or unstressed plants. Both the *AREB1* and *AREB2* proteins functioned as transcriptional activators for ABRE-dependent transcription, not only in yeast cells (Fig. 2), but also in *Arabidopsis* leaf protoplasts (Fig. 5). Other bZIP factors, GBF-1 and GBF-3, also were cloned with the yeast one-hybrid screening. However, neither GBF protein transactivated the reporter genes fused to the *rd29B* promoter in yeast (Fig. 2). The GBF-3 protein did not function as a transcriptional activator for the ABA-inducible expression of *rd29B* in *Arabidopsis* leaf protoplasts (Fig. 5C). These results strongly suggest that the *AREB1* and *AREB2* proteins are specific bZIP transcription factors that are involved in ABRE-dependent expression of *rd29B*.

The expression analysis of *rd29B* in ABA-related mutants indicated that the dehydration- and high-salt-responsive gene expression of *rd29B* is mediated mainly by ABA (Fig. 5A). We carried out transactivation experiments using leaf protoplasts prepared from ABA-related *Arabidopsis* mutants. The overexpression of the AREB proteins was not sufficient for the induction of *rd29B*, and ABA treatment was required for the activation of AREBs (Fig. 5C). Posttranscriptional activation of the AREB proteins in response to ABA may be necessary for their activities. Transactivation by AREBs was significantly decreased in the ABA-insensitive *abil* and ABA-deficient *aba2* mutants, but was increased extremely in the ABA-hypersensitive *era1* mutant. These results suggest that the *ABI1*, *ABI2*, and *ERA1* proteins are involved in the upstream signal transduction pathway of the ABRE-dependent gene expression of *rd29B*.

Protein phosphorylation of the AREB proteins was suggested to be involved in their activation (Fig. 5C). So we analyzed posttranscriptional activation of AREB proteins by ABA using *Arabidopsis* T87 cultured cells and showed that N-terminal conserved regions of the *AREB1* and *AREB2* proteins were rapidly phosphorylated by a 42-kDa protein kinase (Fig. 6A). The AREB proteins have three conserved sequences in their N-terminal regions and a conserved sequence in their C-terminal regions (Fig. 3). These conserved regions contain possible target

sequences for Ser/Thr protein kinases (Fig. 3). Polypeptides containing the other conserved sequences also were phosphorylated, whereas other polypeptides were not (data not shown). Phosphorylation of these conserved regions may be involved in the activation of the AREB proteins by ABA.

To identify the target sequence of the *AREB1* and *AREB2* proteins, the *AREB1* and *AREB2* DNA-binding domains were expressed as glutathione *S*-transferase fusion proteins in *E. coli*. The ability of the *AREB1* and *AREB2* fusion proteins to bind the wild-type or mutated sequences was examined by the gel retardation method. Both the recombinant *AREB1* and *AREB2* fusion proteins specifically bound to the ABRE sequence (data not shown). The ABRE sequence contains a symmetrical ACGT core element, and most plant bZIP proteins recognize sequences that have this element. Some exceptions are reported, such as maize Opaque-2, which binds to the TGACTCA element and several other sequences with a CATG core (30). The consensus binding site of the sunflower Dc3 promoter-binding factor (DPBF), ACAC-NNG, differs from that of the typical bZIP proteins. The sequence similarity between the AREB and DPBF proteins is high among bZIP proteins. However, the DNA-binding specificity of these proteins and the tissue-specific expression of these genes are quite different. The expression of the DPBF genes is seed-specific, whereas the *AREB1* and *AREB2* genes express only in vegetative tissues. Recently, Hobo *et al.* (31) reported that a rice bZIP transcription factor, *TRAB1*, has high sequence similarity with the DPBF proteins and interacts with VP1. These genes may function in VP1-dependent gene expression that controls maturation and dormancy in plant embryos. These results suggest that transcriptional regulation in ABA-responsive gene expression in vegetative tissues is different from that in seeds. Further analysis of the function of the *AREB1* and *AREB2* proteins will lead us to an understanding of the linkage between ABA-dependent gene expression and upstream signaling molecules, including *ABI1/2* and *ERA1*, in vegetative tissues.

Note Added in Proof. While our manuscript was under review, cDNAs related or identical to our *AREB1* and *AREB2* were reported (32). The *ABI5* gene involved in ABA-insensitive phenotype was shown to encode AREB-related transcription factor (33).

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