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A novel *abi5* allele reveals the importance of the conserved Ala in the C3 domain for regulation of downstream genes and salt tolerance during germination in *Arabidopsis*

Kenji Tezuka,[1,2] Teruaki Taji,[1,2] Takahisa Hayashi[1,2] and Yoichi Sakata[1,2,*]

[1]Department of BioScience; Tokyo University of Agriculture; Tokyo, Japan; [2]Department of Applied Biology and Chemistry; Faculty of Applied Biosciences; Tokyo University of Agriculture; Tokyo, Japan

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Abscisic acid (ABA) signal transduction during Arabidopsis seed development and germination requires a Group A bZIP transcription factor encoded by *ABA INSENSITIVE5* (*ABI5*). In addition to the basic leucine zipper DNA binding domain, Group A bZIPs are characterized by three N-terminal conserved regions (C1, C2 and C3) and one C-terminal conserved region (C4). These conserved regions are considered to play roles in ABI5 functions; however, except for the phosphorylation site, the importance of the highly conserved amino acids is unclear. Here, we report a novel *abi5* recessive allele (*abi5-9*) that encodes an intact ABI5 protein with one amino acid substitution (A214G) in the C3 domain. The *abi5-9* plants showed ABA insensitivity during germination and could germinate on medium containing 175 mM NaCl or 500 mM mannitol. *Em1* and *Em6*—both encoding late embryogenesis abundant (LEA) proteins and directly targeted by ABI5 regulation—were expressed at very low levels in *abi5-9* plants compared with the wild type. In yeast, the abi5-9 protein exhibited greatly reduced interaction with ABI3 compared with ABI5. These data suggest that Ala214 in ABI5 contributes to the function of ABI5 via its interaction with ABI3.

Introduction

The sesquiterpene abscisic acid (ABA) is produced in organisms across all kingdoms.[1] In land plants, it plays essential roles in adaptation to environmental stresses, such as drought,[2] and in the developmental regulation of maturation[3] and desiccation tolerance of seeds.[4] Exogenously applied ABA is known to prevent seed germination.[5,6] Genetic screening of mutagenized Arabidopsis has identified mutants that can germinate on media containing ABA.[7] These ABA-insensitive (*abi*) mutants exhibit defects in molecular components of the ABA signal transduction machinery. Subsequent isolation of causal genes revealed that *ABI1* and *ABI2* encode Group A protein phosphatases type 2C (PP2Cs),[8,9] while *ABI3*,[10] *ABI4*[11] and *ABI5*[12] encode transcription factors. *ABI5* encodes a bZIP transcription factor, and is dominantly expressed in seeds but not in vegetative tissues, indicating that these transcription factors specifically function in seed maturation and germination.[12,13] Like drought and high-salt stress, exogenous application of ABA induces ABI5 expression in germinating embryos.[14] In turn, ABI5 in concert with ABI3 regulates ABA-inducible expression of *Em1* and *Em6*,[15,16] which encode late embryogenesis abundant (LEA) proteins[17] that are suggested to protect cells from desiccation.[18] These ABA-induced events are greatly reduced in *abi5* mutant plants,[14] indicating that ABI5 is a key factor in monitoring environmental conditions upon seed germination.

ABI5 is a Group A bZIP. This group includes 13 genes present in Arabidopsis,[19] and can be further divided into two sub-groups based on the conserved N-terminal domains. Nine bZIPs form the ABI5/AREB/ABF family, which each contain three N-terminal conserved regions (C1, C2 and C3) and one C-terminal conserved region (C4); the other sub-group includes four bZIPs that lack the C1 domain.[20] The ABI5/AREB/ABF family is characterized by involvement in ABA signaling during seed development and germination (ABI5, EEL, DPBF2/AtbZIP67, DPBF4 and AREB3) or in vegetative tissues (AREB1/ABF2, AREB2/ABF4, ABF1 and ABF3).[21-23] These bZIPs bind to ABA-responsive elements (ABREs: PyACGTGG/TC)[24] of the ABA-regulated genes via the bZIP DNA-binding domain.[17,25,26]

*Correspondence to: Yoichi Sakata; Email: sakata@nodai.ac.jp
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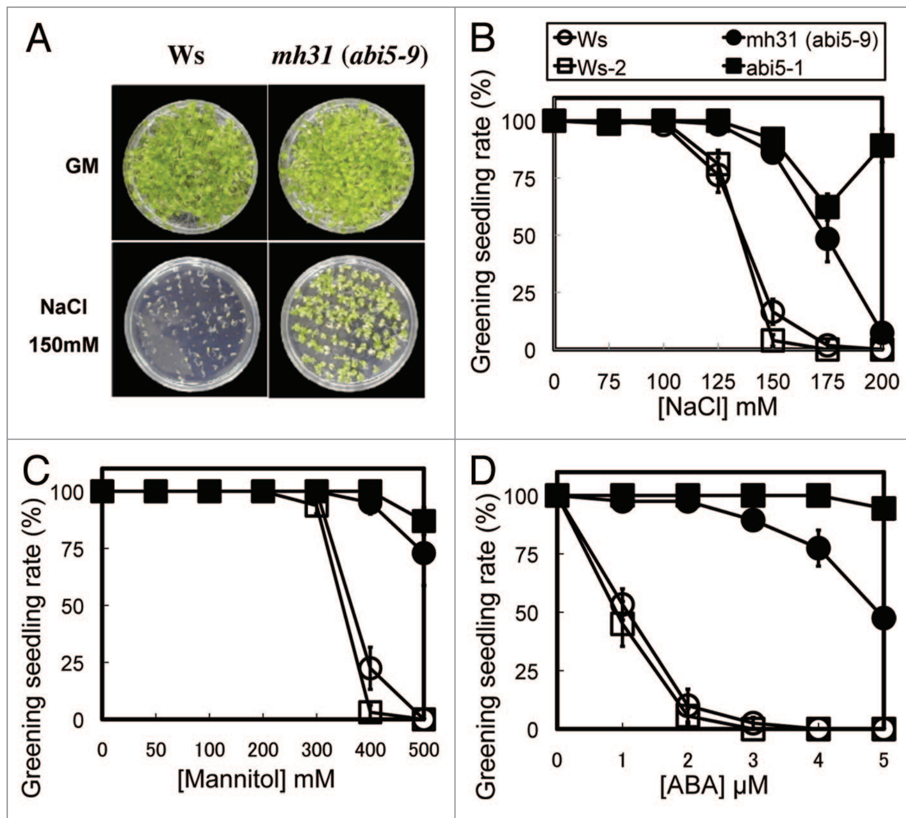

Figure 1. Physiological analysis of the salt-tolerant mutant *mh31*. (**A**) Phenotypes of Ws and *mh31* (Ws background); after stratification, seeds were sown on the medium, grown and then photographed. Upper left and upper right panels, respectively, show Ws and *mh31* grown on GM. Lower left and lower right panels, respectively, show Ws and *mh31* grown on GM supplemented with 150 mM NaCl. (**BD**) Greening rates of the plants grown on GM supplemented with the indicated concentration of NaCl (**B**), mannitol (**C**) or ABA (**D**). Stratified seeds were sown on the plate and incubated at 23°C for two weeks under continuous light (2,000 lx). After incubation, plates were photographed. The numbers of seedlings and greening seedlings were counted, and greening seedling rates were calculated. Data represent the average of values from quadruplicate plates of 20 seeds. Bars indicate standard errors. *abi5-1* is in a Ws-2 background. Open circles, closed circles, open squares and closed square indicate Ws, *mh31* (*abi5-9*), Ws-2 and *abi5-1*, respectively. Greening seedling rate is expressed as the percentage of seedlings with green cotyledons (n = 20). Mean ± SE; n = 4.

Compared with the bZIP domain, less is known about the functional significance of the conserved regions of the ABI5/AREB/ABF family. The C2 and C3 domains of ABI5 reportedly interact with ABI3.[27] The R-X-X-S/T motifs distributed in the conserved domains are the preferred recognition motifs for subclass III SnRK2s (SRK2D/SnRK2.2, OST1/SRK2E/SnRK2.6 and SRK2I/SnRK2.3),[28,29] which are activated in response to ABA[30] and are essential for ABA signal transduction in Arabidopsis.[31-34] In plants, OST1/SRK2E/SnRK2.6 phosphorylates T451 in the C4 domain of ABF3, and this phosphorylation appears to be important for ABF3 stabilization.[35] In contrast, the R-X-X-T motif in the C3 domain is apparently not essential for ABI5 function; expression of mutated abi5 protein, in which T201 within the R-X-X-T motif of the C3 domain was changed to Ala, can complement phenotypes of *abi5-4* plants,[15] indicating that the C3 domain does not mediate ABI5 function through phosphorylation.

All *abi5* alleles reported to date encode premature proteins terminated in front of the bZIP domain, or are obtained from T-DNA insertion lines—except *abi5-3*, which has a small rearrangement adjacent to the 5' splice site of the final exon.[12,17,36,37] In the present study, we report the first *abi5* recessive allele (*abi5-9*) that encodes a full-length ABI5 protein with one amino acid substitution in the conserved alanine (A214G) of the C3 domain. The mutant showed insensitivity to ABA and salinity, comparable to the *abi5-1* null mutant. In yeast, the abi5-9 protein showed reduced ability to interact with ABI3 compared with intact ABI5. Our data demonstrate the importance of the conserved Ala in the C3 domain for the in vivo function of ABI5.

Results

Physiological characterization of a salt-tolerant mutant. A salt-tolerant mutant (originally designated as *mh31*) was isolated from activation-tagged lines of *Arabidopsis thaliana* (Ws) that can develop green seedlings on medium supplemented with 150 mM NaCl (**Fig. 1A and B**). The *mh31* mutant could also grow on medium containing up to 500 mM mannitol, which arrested growth of wild-type germinated embryos (**Fig. 1C**). Since post-germinative developmental arrest involves ABA signaling,[14] we also tested the ABA sensitivity of the *mh31* mutant. As expected, the germination and subsequent seedling development of the *mh31* mutant was less inhibited by exogenous ABA compared with the wild type (**Fig. 1D**), suggesting that the *mh31* mutant was also ABA insensitive. In the absence of stress treatments or exogenous ABA, we observed no significant growth difference between the *mh31* mutant and the wild type (**Fig. 1A**).

***mh31* is a novel *abi5* allele with one amino acid substitution in the C3 domain.** We performed genetic analyses to identify the causal mutated gene of the *mh31* mutant. The *mh31* mutant was crossed with Col-0, and the resulting F_1 plants were self-pollinated to produce the next generation. This F_2 population was tested for ABA sensitivity and osmotolerance (**Table S1**). The F_2 population included 196 ABA-sensitive plants and 74 ABA-insensitive plants, suggesting the ABA-insensitive phenotype to be a recessive trait. On the other hand, an osmotolerance assay of the F_2 population revealed 61 osmotolerant plants and 14 osmosensitive plants. Moreover, F_1 seeds obtained by crossing *mh31* (male) with Ws (female) are NaCl tolerant

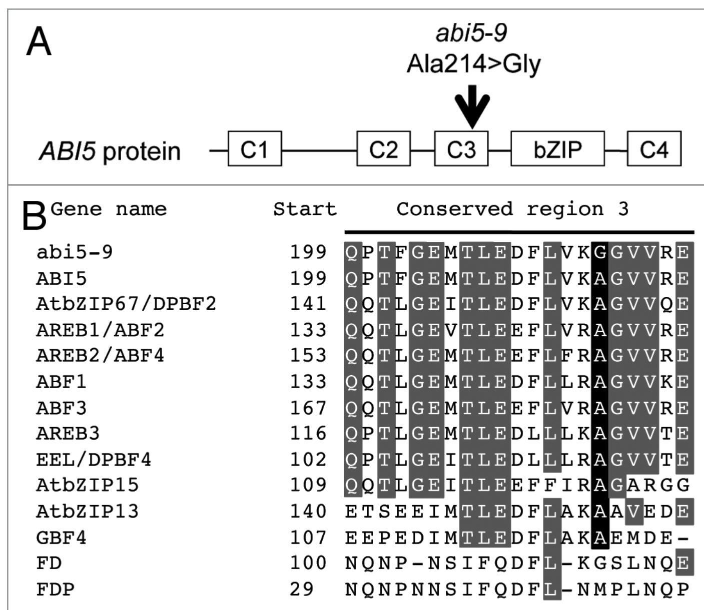

Figure 2. New *abi5* allele *abi5-9* and the conserved property of alanine 214 in the Arabidopsis Group A bZIPs. (**A**) Position of the *abi5-9* mutation. Schematic structure of the ABI5 protein is presented with boxes. C1, C2, C3, C4 and bZIP indicate conserved region 1, conserved region 2, conserved region 3, conserved region 4 and basic DNA binding domain, respectively. The *abi5-9* mutation, in which alanine 214 is substituted with a glycine, is located on C3. (**B**) Alignment of the Arabidopsis Group A bZIPs. The sequence of conserved region 3 is indicated. Gray shading indicates conserved amino acids, black shading indicates alanine 214. Arabidopsis Genome Institutive (AGI) code for the genes as follows: *ABI5* (At2g36270), *AREB1/ABF2* (At1g45249), *AREB2/ABF4* (At3g19290), *AREB3* (At3g56850), *ABF1* (At1g49720), *ABF3* (At4g34000), *AtbZIP67/DPBF2* (At3g44460), *EEL/DPBF4* (At2g41070), *GBF4* (At1g03970), *AtbZIP13* (At5g44080), *AtbZIP15* (At5g42910), *FD* (At4g35900) and *FDP* (At2g17770).

(**Table S2**), suggesting that NaCl tolerance was a dominant trait of the *mh31* plants. Since the *mh31* plants were not tolerant to LiCl (data not shown) but were tolerant to NaCl- and osomo-stresses (**Fig. 1B and C**), their NaCl tolerance was probably caused by osomotolerance. Because of the genetic nature of the *mh31* mutation, the ABA-insensitive plants and osmosensitive plants were rescued from F_2 seedlings and used for genetic mapping.

The *mh31* mutant was originally isolated from activation-tagged lines with the single-copy T-DNA inserted in the upper arm of chromosome 1 (**Fig. S1**). However, rough mapping of F_2 plants showed that both ABA insensitivity and osmo-intolerance had linkage to the SSLP markers CZSOD2 and NGA168 that are located on the bottom arm of chromosome 2 (**Fig. S1**). The chromosome region around the two markers contained *ABI4* and *ABI5*, two important loci for ABA signal transduction during seed germination. We amplified cDNAs of both genes. Sequencing revealed that compared with wild type, the *ABI4* sequence was the same in the *mh31* mutant, whereas the *ABI5* sequence in *mh31* contained one base substitution (C to G) that resulted in an amino acid substitution (A214G) in the conserved C3 region (**Fig. 2A and B**). This mutation was strongly associated with the mutant phenotype (**Fig. S1**).

To verify that *ABI5* was the causal gene of the *mh31* mutant, we performed an allelism test, crossing *mh31* plants (male) with *abi5-1* plants (female) to obtain F_1 progeny. The F_1 plants were tested for ABA sensitivity during germination. As shown in **Table 1**, the F_1 progeny showed ABA insensitivity comparable to the parental *mh31* and *abi5-1* plants, indicating that *mh31* and *abi5-1* did not complement each other. We concluded that *mh31* and *abi5-1* affect the same locus; therefore, the *mh31* mutant was re-named as *abi5-9* since *abi5-1* to *abi5-8* have been previously described.[12,17,36,38,39]

Molecular characterization of abi5-9 protein in yeasts. Regulation of *Em1* and *Em6* reportedly requires both ABI5 and ABI3,[16,27,40] which physically interact with each other through the ABI5 region containing the C2 and C3 domains in yeast.[27] We performed a yeast two-hybrid assay to test the physical interaction between abi5-9 and ABI3. The yeasts harboring the Gal4 DNA-binding domain (BD)-abi5-9 but not BD-ABI5 could grow on medium lacking histidine and adenine, suggesting stronger autoactivation of the reporter genes by abi5-9 (**Fig. 3A**). The full-length ABI3 fused to Gal4-BD showed strong autoactivation of the reporter genes (data not shown), as previously reported;[41] therefore, truncated ABI3 proteins were fused to Gal4-BD and used to evaluate the interaction with abi5-9 and ABI5 (**Fig. 3B**). As shown previously,[27] ABI5 interacted with truncated ABI3 proteins, including the B1 domain alone (B1S and B1L) and the B1 and B2 domains together (B1B2). On the other hand, abi5-9 showed weak interaction only with B1B2, as judged by the growth of yeasts harboring both AD-abi5-9 and BD-B1B2 constructs, and growth was completely inhibited by addition of 10 mM 3-AT, a competitive inhibitor of the HIS3 used to titrate the expression level of the *HIS3* reporter gene.[42] These results suggested that the conserved Ala in the C3 domain is important for the physical interaction of ABI5 with ABI3 in yeast.

Functional analysis of abi5-9 in plants. Experiments using yeast suggested that abi5-9 retained transactivation ability but lacked the ability to interact with ABI3, suggesting that failure of abi5-9 to interact with ABI3 affected the regulation of downstream genes. We investigated the expression profiles of *abi5-9*, *ABI5* and the downstream genes *Em1* and *Em6* in dry seeds and in imbibed seeds with or without ABA treatment (**Fig. 4**). We did not observe any significant changes in the expression profiles between *abi5-9* and *ABI5*, indicating that the *abi5-9* mutation affects *ABI5* function at the post-transcription level (**Fig. S2**). On the other hand, expressions of *Em1* and *Em6* were reduced in dry seeds and in ABA-treated imbibed seeds of *abi5-9* plants compared with wild type, and the reduction level was comparable to that seen in *abi5-1* plants (**Fig. 4**). These data clearly demonstrated that *abi5-9* mutation severely affected ABI5 function.

Discussion

In this study, we report a novel allele of *abi5* that encodes a full-length protein with one amino acid substitution in the conserved C3 region, and we prove that this domain is essential for ABI5

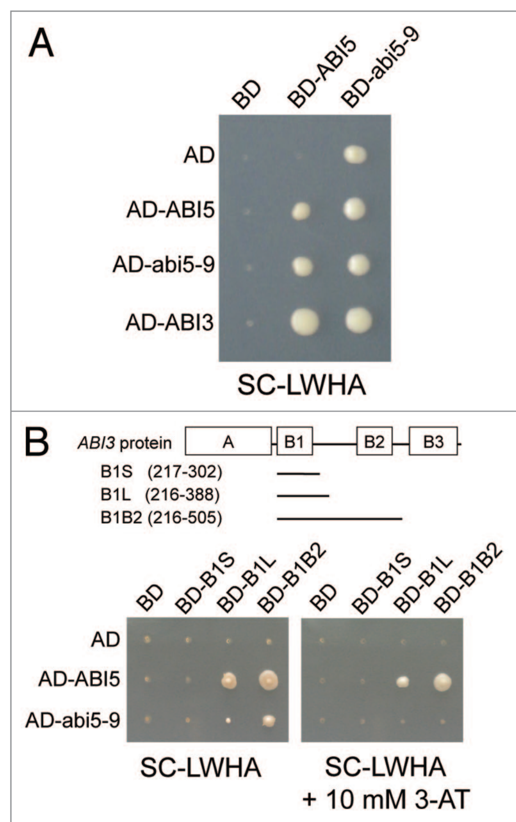

Figure 3. Interaction of abi5-9 with ABI3 in yeast two-hybrid system. Yeast strain PJ69-4 harboring constructs of the Gal4-Activation domain (AD) fusion and Gal4-DNA binding domain (BD) fusion, as indicated to the left and above, were grown for 4 d at 30°C on Synthetic Complete dropout medium lacking the amino acids leucine and tryptophan (SC-LW). Yeast cultures were replicated on the synthetic complete dropout medium lacking the amino acids leucine, tryptophan, histidine and adenine (SC-LWHA) with or without 3-amino-1,2,4-triazole (3-AT), and were grown for an additional 5 d (**A**) or 10 d (**B**) at 30°C. B1S, B1L and B1B2 are the partial regions of the ABI3, which interact with ABI5, as described by Nakamura et al.[27] Schematic representation of the full-length and truncated regions of ABI3 are indicated (**B**). A, B1, B2 and B3 are acidic domain, basic domain 1, basic domain 2 and basic domain 3, respectively. Numbers in parentheses indicate the position of amino acid residues from the first methionine of ABI3.

Table 1. Allelism test

Genotype	ABA sensitive	ABA insensitive
Ws	28	0
mh31	0	32
abi5-1	0	32
F_1 *abi5-1 x mh31*	5	26

After stratification, seeds were sown on the plate with 3 μM ABA and incubated at 23°C under continuous light (2,000 lx). After incubation for 2 weeks, the numbers of growth-arrested seedling and greening seedling were counted. "ABA sensitive" and "ABA insensitive" indicate the growth-arrested seedling and the greening seedling, respectively.

function. In the *abi5-9* mutant, the C to G mutation of a single nucleotide resulted in the substitution of an evolutionarily conserved alanine with a glycine at residue 214. This conserved alanine was not located in the C3 phosphorylation site and it has been previously shown that mutation of the C3 phosphorylation site does not affect ABI5 function in plants,[15] indicating that the A214G mutation affected ABI5 regulation mechanisms other than phosphorylation. Interactions have been previously shown between members of the ABI5/AREB/ABF and various proteins, including kinases (SnRK2s and CPKs),[43-46] DREB[47,48] and the Arm repeat protein interacting with ABF3 (ARIA).[49] Our present data clearly demonstrated that interaction between ABI5 and ABI3 was mediated through the C3 domain and that A214 had an important role in this interaction. The small plant-specific protein AFP1 interacts with the C3 domain of ABI5, promoting ABI5 degradation,[50] and the C3 domain might also be important in regulating ABI5 activity through interaction with other regulatory proteins.

Previous reports have suggested the autoregulation of ABI5 transcription.[12,14] Notably, the *abi5-1* and *abi5-9* mutations greatly affected the mRNA accumulation of *Em1* and *Em6* (**Fig. 4**); however, reduction of *ABI5* mRNA was only observed in the *abi5-1* plants, not the *abi5-9* plants (**Fig. S2**). We demonstrated that the *abi5-9* mutation, which produces the full-length of ABI5 protein with one amino acid substitution, did not affect *ABI5* mRNA accumulation. These results suggest that autoregulation of *ABI5* transcription may not require interaction with ABI3. Future studies, such as microarray analysis of the *abi5-9* plants, will enable differentiation between ABI3-dependent and ABI3-independent ABI5 targets.

We observed several effects of A214G mutation on ABI5 function; however, these differences could not explain why the *abi5-9* allele results in loss of function. The A214G mutation in the C3 domain could affect ABI5 protein stability, resulting in a variety of phenotypes; however, we do not think that this is likely because we observed GFP expression in the nucleus of the transgenic Arabidopsis plants in which *abi5-9* cDNA translationally fused to the 3' end of the *GFP* gene was introduced under control of the CaMV 35S promoter (data not shown). Moreover, in a transient assay using mesophyll protoplasts, the *Em6* promoter was significantly activated by abi5-9, comparable to that by ABI5 (**Fig. S3**). In contrast, *Em6* transcript accumulation was drastically reduced in *abi5-9* seedlings (**Fig. 4**). These differences may be partly attributed to the heterologous assay systems we employed: a yeast system and a transient assay system using mesophyll protoplasts, which do not express ABI5. We also speculate that chromatin modification of the *Em6* promoter is important for the regulation by ABI5 and ABI3. The seed-specific *phaseolin* promoter is repressed by histone modification, and PvALF, a *Phaseolus vulgaris* ABI3 ortholog, is involved in the chromatin remodeling to bring the phaseolin promoter to the potentiated state, which is followed by activation by ABA.[37,51,52] Since ABI3 and ABI5 interact with each other and the A214G mutation reduced this interaction, ABI5 may also participate in chromatin remodeling with ABI3. It is possible that abi5-9 affects the regulation of *Em6* at the chromatin remodeling level, but is not defective in ABRE binding and/or transactivation. This hypothesis may explain why abi5-9 can activate the *Em6* promoter in a transient assay.

The Ala214 in the C3 domain is highly conserved among AREB/ABF proteins (**Fig. 2B**). It is possible that Ala214 is also involved in the regulation of AREB/ABF activity in vegetative tissues. Our data demonstrated that abi5-9 increased autoactivation ability in yeasts compared with ABI5 (**Fig. 3A**). This improved transactivation ability was also observed in the mesophyll protoplasts gene expression assay, in which the *Em6* promoter was activated more strongly by abi5-9 than by ABI5 (**Fig. S3**). Tang et al.[53] recently demonstrated that the OsbZIP46 lacking the C3 domain shows constitutive transactivation activity in yeasts and plants, and proposed that the C3 domain might have a role in transactivation repression. These observations are in good agreement with our data in yeasts and mesophyll protoplasts. In contrast to these results, we found that in seedlings and dry seeds—in which the *ABI5* gene is expressed—*Em6* transcript accumulation was drastically reduced in the *abi5-9* mutant compared with wild type. These data suggested that the C3-mediated transactivation activity is also important for the expression of ABI5-regulated genes such as *Em6* in germinating seeds.

Our findings highlight the importance of the Ala, which is strictly conserved in the C3 domains but not the direct target motif for phosphorylation of AREB/ABF proteins. It would be interesting to see whether substitutions of the alanine to various types of amino acids affect activities of AREB/ABF proteins, which may eventually lead to the genetic engineering of drought-tolerant crops, since these proteins play critical roles in regulating ABA responses of vegetative tissues.

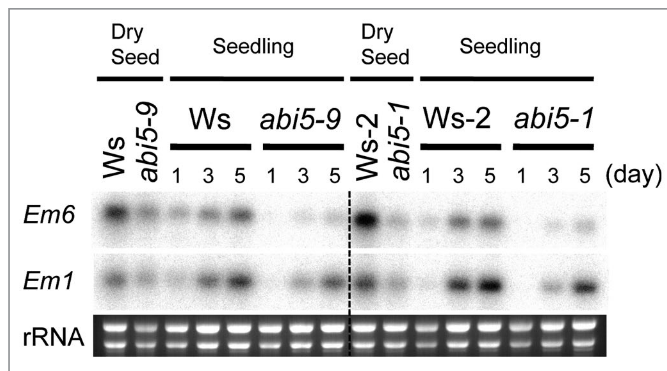

Figure 4. RNA expressions of *Em1* and *Em6* in *abi5-9* mutant. RNA expression levels determined by northern blot. After stratification, seeds were sown on the plate with or without 3 μM ABA and incubated at 23°C for the indicated duration under 24-h continuous light (2,000 lx). Total RNA was extracted and 2 μg total RNA was used per lane. The rRNA bands stained with ethidium bromide in the gel were used to verify equal loading.

Materials and Methods

Plant materials and growing conditions. The *Arabidopsis thaliana* ecotypes Col-0 (Columbia-0), Ws (Wassilewskija) and Ws-2 and the ABA-insensitive Arabidopsis mutant *abi5-1* (Ws-2 background) were obtained from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis plants were grown at 23°C under light conditions of 2,000 lx for 16 h/day. For the greening assay, after stratification at 4°C for 3–4 d, seeds were sown on germination medium (GM)[54] containing 0.8% (w/v) agar supplemented with different concentrations of NaCl, mannitol, or ABA. The plates were incubated for two weeks at 23°C under 24-h continuous light (2,000 lx). For RNA expression assays, stratified seeds were sown on filter paper placed on top of 0.8% agar, and grown at 23°C under continuous light (2,000 lx). For 2-d-old seedlings, the seedlings were transferred to a 0.8%-agar plate containing ABA or dimethylsulfoxide (DMSO) and incubated for indicated periods.

Isolation of a salt-tolerant mutant. Calluses were produced from the hypocotyl of Ws. Generation of callus, transformation of callus using *Agrobacterium tumefaciens* strain GV3101::pMP90RK containing pPCVICEn4HPT, and plant regeneration were performed according to the methods described by Kakimoto.[54] After transformation by *A. tumefaciens*, calluses derived from Ws hypocotyl were grown on shoot-inducing media (SIM) supplemented with 20 mg/L hygromycin and 150 mM NaCl. The *mh31* mutant was isolated as salt-tolerant shoots regenerated from callus.

Genetic mapping. The F_1 generation was obtained by crossing the *mh31* mutant with the wild-type Col-0; it was then self-fertilized to establish the F_2 generation. The F_2 mapping populations were screened based on the ABA-insensitive phenotype or mannitol-sensitive phenotype, and these plants were used for genetic linkage analysis with simple sequence length polymorphism (SSLP) DNA markers. SSLP mapping for Arabidopsis was performed as described by Bell and Ecker.[55] SSLP DNA markers were amplified by polymerase chain reaction (PCR) using oligonucleotide sequences registered as specific primers at the Arabidopsis information resource (TAIR; www.arabidopsis.org/). DNA extraction was performed as previously described.[56]

Sequencing analysis of *ABI4* and *ABI5*. The cDNAs of *ABI5* and *ABI4* were amplified by PCR and sequenced. The oligonucleotide sequences of the primer pairs were as follows: *ABI5* cDNA forward primer, 5'-TCT CTT TCT CAA AAC CTT TCA GTC-3', reverse primer, 5'-TTC TAT AAC CTC ATT CCT CAA AGA CA-3'; and *ABI4* cDNA forward primer, 5'-AAG TGA GTG AGA AGA GAG TGT AAG T-3', reverse primer, 5'-ACC GTA ATC TCT TTT ACG AAT TCC-3'.

Yeast strain and construction. Yeast strains PJ69-4A and PJ69-4α[57] were used for the yeast two-hybrid assay. These strains have nutritional requirements for histidine, adenine, tryptophan and leucine and harbor the reporter genes *GAL1-HIS3* and *GAL2-ADE2*. Plasmids pGBTK[58] and pGAD424 (Clontech) were used to construction the Gal4 DNA-binding domain (BD) fusion and Gal4-activation domain (AD) fusion, respectively. The plasmid pGBTK contained the *TRP1* nutritional marker and Gal4-BD. The plasmid pGAD424 contained the *LEU2* nutritional marker and Gal4-AD.

ABI5 was amplified by PCR with the following primer pair: forward primer, 5'-ATC ATC gaa ttc ATG GTA ACT AGA GAA ACG AAG-3' and reverse primer, 5'- ATC ATC gga tcc TTA GAG TGG ACA ACT CGG-3'. The amplified *ABI5* ORF was cloned into the *Eco*RI and *Bam*HI sites of pGBTK or pGAD424. *ABI3* was amplified by PCR with the following pair of primers:

forward primer, 5'-ATC ATC gga tcc GTA TGA AAA GCT TGC ATG-3' and reverse primer, 5'-ATC ATC gtc gac TCA TTT AAC AGT TTG AGA AG-3'. The *ABI3* ORF was cloned into the *Bam*HI and *Sal*I sites of pGBTK and pGAD424. The coding regions of the B1S, the B1L and the B1B2, which have been indicated to physically interact with ABI5,[27] were amplified by PCR with following pair of primers: B1S forward, 5'-ATC ATC gga tcc GTG AAG ACC AGG TCG TTG-3' and reverse, 5'-ATC ATC gtc gac TCA TTG GAC CCA TTC AAG AA-3'; B1L forward, 5'-ATC ATC gga tcc GTC AAG AAG ACC AGG TCG T-3' and reverse, 5'-ATC ATC gtc gac TCA TTC AAG TAA AGG AAG GA-3'; and B1B2 forward, 5'-ATC ATC gga tcc GTC AAG AAG ACC AGG TCG T-3' and reverse, 5'-ATC ATC gtc gac TCA GTT AAG TTG TGG AGC CA-3'. These amplified fragments were cloned into BamHI and SalI sites of pGBTK. Insertions of the resultant plasmids were verified by sequencing.

Yeast two-hybrid analysis. The Gal4-BD fusion constructs based on the plasmid pGBTK and the Gal4-AD fusion constructs based on pGAD424 were used for transformation of yeast strains PJ69-4A and PJ69-4α, respectively, using the LiCl/ssDNA/PEG method.[59] Methods described by Yoshimura et al.[58] were used to acquire diploid yeast strains harboring the Gal4-BD fusion construct with the Gal4-AD fusion construct by mating PJ69-4A with PJ69-4α, as well as to perform the plate assay on synthetic complete (SC) dropout medium lacking the amino acids leucine (L), tryptophan (W), histidine (H) and adenine (A) (SC-LWHA). 3-Amino-1,2,4-triazole (3-AT) was used as a histidine synthesis inhibitor.

Constructions for transient assay. The overexpression vector pGHX—which is basically the same as pGKX[60] but with the resistance marker changed to hygromycin—was used for construction of pGHX-ABI5 and pGHX-abi5-9. *ABI5* cDNA was amplified by PCR with the following pair of primers: forward primer, 5'-ATC ATC gga tcc ATG GTA ACT AGA GAA ACG AA-3' and reverse primer, 5'-ATC ATC gcg gcc gcC CAA AGA TTG ATG ATG TTG A-3'. Amplified *ABI5* was cloned into the *Bam*HI and *Not*I sites of pGHX. For the pGHX-ABI3 plasmid, pGBTK-ABI3 was digested with *BamH*I and *Sal*I. After blunting using the DNA Blunting kit (Takara, Japan), the DNA fragment containing the *ABI3* ORF was subcloned into the *Sma*I site of pGHX. For construction of the *Em6* promoter-*GUS* plasmid, the pGreen0029-GUS-NosT plasmid was constructed by subcloning GUS-NosT from pBI221 into pGreen0029.[61] The promoter region of *Em6* was amplified by PCR with the following pair of primers: forward, 5'-ATC ATC tct aga TAA TAA TGA TGT ATA GAT GAT TGG AG-3' and reverse, 5'-ATC ATC cag ctg AGC TGC TTC TTC TCT TGT TG-3'. The *Xba*I-*Pvu*II fragment of the amplified *Em6* promoter was cloned into the *Xba*I and *Sma*I sites of pGreen0029-GUS-NosT. The cDNAs and *Em6* promoter of the constructed plasmids were verified by sequencing analysis. p35S-Emerald luciferase (Eluc) was created by digesting pBI221 with *Sma*I and *Sac*I to release the *GUS* gene. The vector was ligated to the 1896-base *Eco*RV-*Sac*I fragment of pEluc-test (Toyobo, Japan).

Isolation of protoplasts and transient gene expression. The transient gene expression assay was performed as previously described.[62] GUS and LUC activities were determined as described by Komatsu et al.[63] Pica Gene luciferase assay reagent (Toyo-Ink, Japan) was used for the luciferase-luciferin reaction.

RNA gel blot analysis. Total RNA extractions from dry seeds and seedlings were performed using methods described by Vicient et al.[64] and Shirzadegan et al.,[65] respectively. RNA gel blot analysis was performed as previously described.[63] Probe DNAs for *Em1* and *Em6* were acquired by RT-PCR. The probe DNA for *ABI5* was PCR-amplified from pGHX-ABI5. The oligonucleotide sequences for the primer pairs were as follows: *Em1* forward, 5'-CAG AGA AGA GCT TGA TGA GA-3' and reverse, 5'-GCT ACA TTA GAC CCT AGT TA-3'; *Em6* forward, 5'-CTC AAC AAG AGA AGA AGC AGC TGG-3' and reverse, 5'-GGT CTT GGT CCT GAA TTT GGA TT-3'; *ABI5* forward, 5'-ATC ATC gga tcc ATG GTA ACT AGA GAA ACG AA-3' and reverse, 5'- ATC ATC gcg gcc gcC CAA AGA TTG ATG ATG TTG A-3'.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/psb/article/23455

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