ARIA, an Arabidopsis Arm Repeat Protein Interacting with a Transcriptional Regulator of Abscisic Acid-Responsive Gene Expression, Is a Novel Abscisic Acid Signaling Component[1]

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Arabidopsis (*Arabidopsis thaliana*) genome contains more than 90 armadillo (arm) repeat proteins. However, their functions are largely unknown. Here, we report that an Arabidopsis arm repeat protein is involved in abscisic acid (ABA) response. We carried out two-hybrid screens to identify signaling components that modulate ABA-responsive gene expression. Employing a transcription factor, ABF2, which controls the ABA-dependent gene expression via the G-box type ABA-responsive elements, we isolated an arm repeat protein. The ABF2-interacting protein, designated as ARIA (arm repeat protein interacting with ABF2), has another conserved sequence motif, BTB/POZ (broad complex, tramtrak, and bric-a-brac/poxvirus and zinc finger) domain, in the C-terminal region. The physiological relevance of ABF2-ARIA interaction was supported by their similar expression patterns and similar subcellular localization. Plants overexpressing ARIA are hypersensitive to ABA and high osmolarity during germination and insensitive to salt during subsequent seedling growth. By contrast, an ARIA knockout mutant exhibits ABA and glucose insensitivities. Changes in the expression levels of several ABF2-regulated genes were also observed in ARIA overexpression lines, indicating that ARIA modulates the transcriptional activity of ABF2. Together, our data indicate that ARIA is a positive regulator of ABA response.

Armadillo (arm) repeat is a 42-amino acid protein-protein interaction motif (Peifer et al., 1994; Hatzfeld, 1999; Andrade et al., 2001). The repeat was first identified in the Drosophila segment polarity gene *armadillo* (Riggleman et al., 1989) and since then in many eukaryotic proteins involved in cell signaling or cellular architecture. Armadillo and its vertebrate homolog β-catenin are components of the Wingless and the Wnt signaling pathways, which determine the patterning of Drosophila embryo body segments and vertebrate cell fates, respectively (Polakis, 2000). When triggered by the Wingless or Wnt growth factor signal, otherwise unstable Armadillo/β-catenin becomes stabilized, translocates into the nucleus, and together with the TCF/LEF subfamily of transcription factors, activates the Wingless/Wnt target genes. β-Catenin also plays a structural role in cell-cell adhesion by linking the transmembrane adhesion molecules cadherins to actin cytoskeleton.

Pfam (http://www.sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/) protein databases enlist more than 90 Arabidopsis (*Arabidopsis thaliana*) arm repeat proteins. Based on their sequence homology, these proteins can be grouped into several different subfamilies such as impotin-α, kinesin, and U-box protein families (Coates, 2003). However, the functions of the Arabidopsis and other plant arm repeat proteins have not been characterized in detail except those of ARC1 and PHOR1. ARC1 interacts with an *S*-locus receptor kinase of Brassica (Gu et al., 1998) and has been demonstrated to be a positive regulator of the self-incompatibility response (Stone et al., 1999). A recent study shows that ARC1 promotes ubiquitination and proteasomal degradation of compatibility factors in pistil (Stone et al., 2003). The potato arm repeat protein PHOR1, on the other hand, is involved in GA signaling (Amador et al., 2001). Antisense suppression of its expression reduces GA sensitivity and plant height, whereas its overexpression increases GA sensitivity and internode length.

The BTB (broad-complex, tramtrak, and bric-a-brac) domain is another evolutionarily conserved protein-protein interaction domain (Bardwell and Treisman, 1994; Zollman et al., 1994). The approximately 120-amino acid motif, also known as poxvirus and zinc finger (POZ) domain, was first identified in a group of poxvirus proteins and in Drosophila zinc finger pro-

teins. Subsequently, it has been found that the BTB/POZ domain is present in 5% to 10% of zinc finger transcription factors and in some actin-binding proteins or ion channels (Aravind and Koonin, 1998; Collins et al., 2001). Arabidopsis genome contains approximately 80 BTB domain proteins. However, only three of them have been reported to date: NPH3 and RPT2, signal transducers of phototrophic response (Motchoulski and Liscum, 1999; Sakai et al., 2000), and NPR1/NIM1, a regulator of gene expression during systemic acquired response (Cao et al., 1997; Ryals et al., 1997).

Plant hormone abscisic acid (ABA) controls various aspects of plant growth and development (Finkelstein et al., 2002). It inhibits germination and postgermination growth at high concentrations, although it is necessary for normal seedling growth. It regulates seed maturation process and prevents embryos from precocious germination. During vegetative growth, ABA plays an essential role in adaptation to various abiotic stresses such as drought, high salinity, and cold (Xiong et al., 2002). Extensive genetic and biochemical studies have been done to identify the regulatory components of various aspects of ABA response. As a consequence, a large number of ABA-signaling components have been reported that include transcription factors, kinases, phosphatases, RNA-binding proteins, G-proteins, and secondary messengers (Finkelstein et al., 2002; Xiong et al., 2002).

During vegetative growth, ABA controls the expression of numerous genes associated with adaptive responses to drought and other abiotic stresses (Ramanulu and Bartels, 2002; Shinozaki et al., 2003). The ABA regulation of stress-responsive genes is largely mediated by cis-regulatory elements sharing the CACGTGGC consensus. Previously, we and others identified a small subfamily of basic Leu zipper (bZIP) class transcription factors that interact with the elements (Choi et al., 2000; Uno et al., 2000). We subsequently showed that the factors, named as ABFs (i.e. ABF1–ABF4) or AREBs (i.e. AREB1–AREB3), are involved in ABA and various abiotic stress responses (Kang et al., 2002; Kim et al., 2004). In particular, ABF2/AREB1, which will be referred to as ABF2 hereafter, regulates seedling growth rate and plays an essential role in Glc-induced developmental arrest process. Its overexpression phenotypes, such as altered ABA sensitivity and multiple stress tolerance, also suggest that it is involved in ABA and stress responses. We are interested in delineating the ABA-signaling pathway(s) that lead to the ABF-dependent ABA/stress-responsive gene expression in vegetative tissues. Toward this end, we carried out two-hybrid screens to isolate proteins that interact with ABF family members to modulate their activities. Here, we report that an arm repeat and BTB/POZ domain protein interacts with ABF2. In vivo analysis of its function revealed that the ABF2-interacting protein is a novel ABA-signaling component that regulates seed germination, seedling growth, Glc response, and ABA and/or stress responses.

RESULTS

Isolation of ABF2 Interacting Proteins by Yeast Two-Hybrid Screens

We carried out yeast (*Saccharomyces* cerevisiae) two-hybrid screens to isolate ABF2-interacting proteins (Chien et al., 1991; Gyuris et al., 1993). Since ABF2 has transcriptional activity (Choi et al., 2000), bait constructs were prepared employing partial fragments of ABF2 (Fig. 1A) to reduce the background activity. A cDNA expression library representing RNA from ABA-treated Arabidopsis seedlings (Choi et al., 2000) was then used to transform a yeast strain containing each bait construct. We recovered five positive clones that interacted with the variable region (amino acids 234–337) of ABF2. Insert analysis of the clones showed that two of them (Group 1) encoded a transcription factor, which will be reported elsewhere. The remaining three clones (Group 2) encoded an arm repeat protein (see below). The Group 2 clones did not interact with nuclear lamin or with the corresponding regions of ABF3 and ABF4 (Fig. 1B), indicating that they specifically interact with ABF2.

The longest open reading frame of the Group 2 clones encoded a protein containing 705 amino acid residues. The open reading frame was missing the initiation codon. Database search and subsequent isolation and sequencing of the full-length cDNA revealed that the protein consists of 710 amino acid residues with a predicted molecular mass of 78 kD (Fig. 1C). The ABF2-interacting protein, ARIA, has nine copies of arm repeat in the N-terminal half, with arm 1, 8, and 9 being less well conserved. Additionally, it has a BTB/POZ domain in the C-terminal region. The gene encoding ARIA (At5g19330) is composed of 19 exons, and ARIA exhibits the highest sequence identity (59%) to another Arabidopsis arm repeat protein (At5g13060) of unknown function.

ARIA Interacts with ABF2 in Vitro

The interaction between ARIA and ABF2 was confirmed by in vitro binding assay. Recombinant proteins (Fig. 1D, lanes 3–5) containing the entire ARIA coding region, the arm repeat region, or the BTB domain as a fusion to the glutathione-S-transferase (GST) were prepared. Their interaction with the full-length ABF2 was then determined by GST pulldown assay, using in vitro translated ABF2 labeled with ^{35}S. As shown in Figure 1D, ABF2 was retained by the GST full-length ARIA fusion protein (lane 7), whereas it was not retained by GST alone (lane 6). Thus, full-length ARIA interacted with ABF2. Similarly, the fragments containing the arm repeat region or the BTB domain also interacted with ABF2 (lanes 8 and 9). The stronger band intensity observed with the BTB domain (lane 9) suggested that ABF2 bound the domain more strongly.
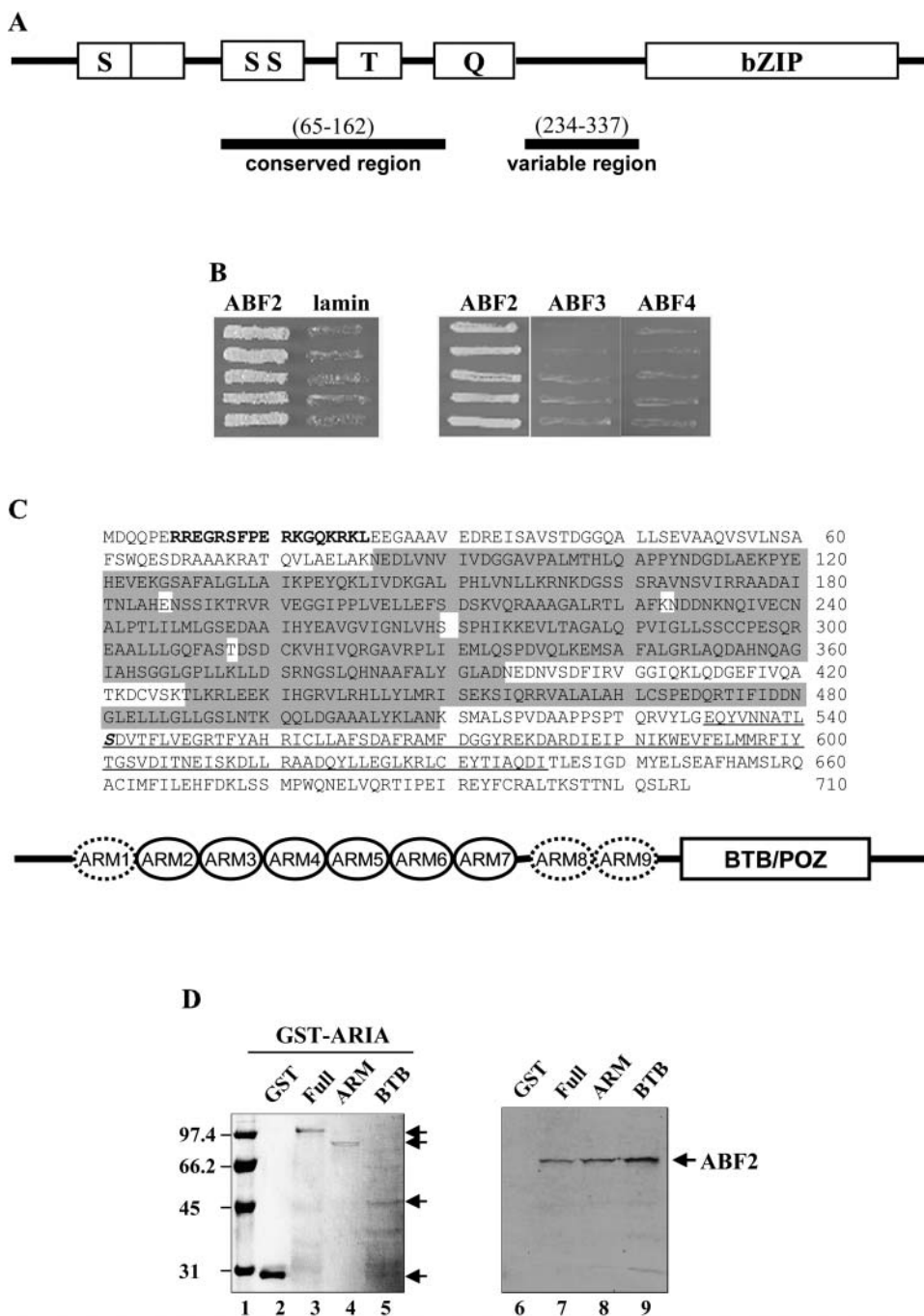
Figure 1. Summary of the two-hybrid screens to isolate ABF2-interacting proteins. A, Schematic diagram of ABF2 and the fragments used in the two-hybrid screens. The regions conserved among ABF family members are shown as boxes. S and T denote Ser and Thr residues, respectively, that are putative phosphorylation sites. The Gln-rich (Q) and the bZIP (bZIP) regions are also indicated. The thick bars indicate the fragments used for the bait constructs, with the amino acid position numbers in parentheses. The full-length ABF2 consists of 416 amino acid residues. B, Specificity of interaction. The interaction between a Group2 positive clone (clone 20) and ABF2 (amino acids 234–337), nuclear lamin, ABF3 (amino acids 274–373), or ABF4 (amino acids 265–352) was tested. Yeast containing each bait construct was transformed with the positive clone, transformants were patched on a Synthetic Complete-Leu medium, and growth was examined after 4 d to test the *LEU2* reporter activity. C, Deduced amino acid sequence of ARIA. The arm repeat region is shaded, and the BTB/POZ domain is underlined. The predicted nuclear localization signal in the N-terminal region is indicated in bold. Below, the conserved motifs are also shown schematically. arm repeats 1, 8, and 9 are less well conserved. D, In vitro interaction of ABF2 and ARIA. Left, Coomassie Blue-stained gel of GST alone (GST) and GST-ARIA fusion proteins containing the full-length ARIA (Full), the arm repeat region (ARM; amino acids 1–518), or the BTB domain (BTB; amino acids 511–710), respectively. Right, GST pulldown assay. An autoradiogram showing in vitro-translated ^{35}S-Met-labeled ABF2 retained by the GST-ARIA fusion proteins. The same amounts of recombinant proteins were used in the assay. The arrows indicate the position of protein bands.

Expression Patterns and Subcellular Localization of ARIA Are Similar to Those of ABF2

The ABA and stress inducibility of *ARIA* expression was examined by RNA gel-blot analysis. Like *ABF2*, whose expression is induced by ABA and high salt (Choi et al., 2000), *ARIA* transcript level was enhanced by ABA and high salt treatments (Fig. 2A). To investigate the temporal and spatial expression patterns of *ARIA* in detail, histochemical β-glucuronidase (GUS) staining of transgenic plants carrying an *ARIA* promoter-*GUS* fusion construct was conducted. Strong GUS activity was detected in the radicles of germinating seedlings (data not shown) and in the roots of young seedlings (Fig. 2B, a). In older seedlings (Fig. 2B, b), leaves exhibited stronger GUS activity than roots. In particular, the vascular tissues and the guard cells were stained strongly (Fig. 2B, c). In roots

of older seedlings, GUS activity was detected mainly in lateral roots rather than in the primary roots (Fig. 2B, d). The vascular region was more strongly stained than the epidermal tissues (Fig. 2B, e, upper section), and very strong GUS activity was observed in lateral root primordia and in the basal part of the lateral roots (Fig. 2B, e, lower section). Anthers, filaments, stigma, and the abscission zone of immature siliques exhibited strong GUS activity among the reproductive organs (Fig. 2B, f–h). Embryos were also stained strongly (Fig. 2B, a, inset). In summary, *ARIA* promoter activity was detected in embryos and most of the vegetative and reproductive organs. The temporal and spatial expression patterns of *ARIA* are very similar to those of *ABF2*. For instance, *ABF2* promoter is very active in most of the vegetative tissues, especially in lateral roots, leaf veins, and guard cells. Besides, *ABF2* is also

Figure 2. Expression patterns of ARIA. A, RNA gel-blot analysis. RNA was isolated from seedlings treated with 100 μM ABA, 250 mM NaCl, cold (24 h at 4°C), or dehydration (withholding from water for 2 weeks). Bottom section shows the ethidium bromide-stained gel. B, Histochemical *GUS* staining of transgenic plants transformed with a 2.1-kb *ARIA* promoter-*GUS* reporter construct. T2 or T3 generation plants were stained with X-gluc for 24 h. a, Three-day-old seedling. The inset shows a mature embryo from a dry silique; b, two-week-old seedling; c, leaf; d and e, root; f, flower; g, immature silique; h, mature silique. C, Subcellular localization of ABF2 and ARIA. The top section shows the light microscopy images of onion cells transiently transformed with a *35S-ABF2-GUS* fusion construct and stained with X-gluc (GUS) or 4′,6-diamino-2-phenylindole (DAPI). Middle and bottom sections show the confocal images of root cells of a plant transformed with *35S-ARIA-GFP* (ARIA-GFP) or *35S-GFP* (GFP). GFP, GFP channel; PI, cells stained with propidium iodide. The arrows denote nuclei.
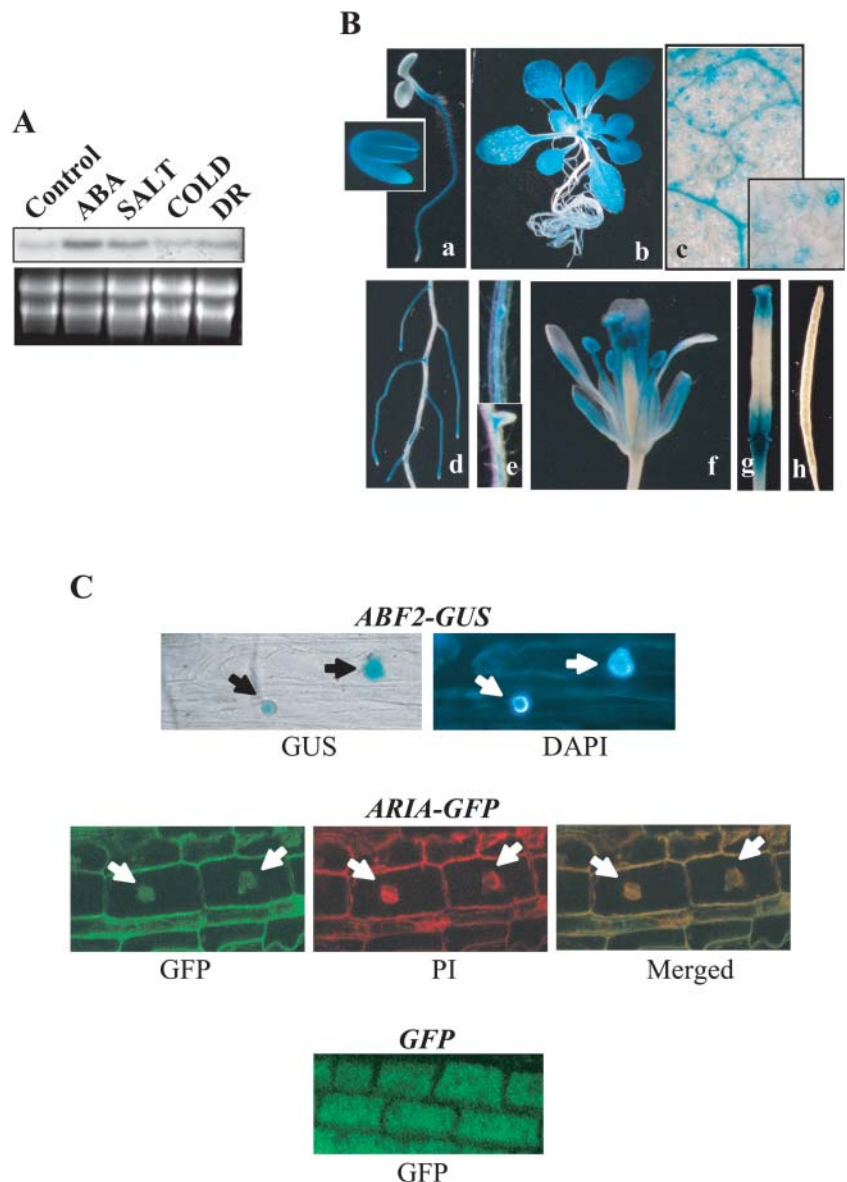
strongly expressed in anthers, filaments, and stigma among the floral organs.

ABF2 is a transcription factor and, as shown in Figure 2C (top section), is localized in the nucleus. We noticed that ARIA has a nuclear-localization signal near its N terminus (Fig. 1C), suggesting that it may be localized in the nucleus. To determine the intracellular localization of ARIA, transgenic plants harboring an *ARIA*-green fluorescent protein (*GFP*) fusion construct were generated, and the localization of the fusion protein was determined. Figure 2C (middle section) shows that GFP was localized in the nucleus, indicating that ARIA is nuclear localized. GFP was also detected in the periphery of cells. It appears that ARIA is localized in the cell membrane as well.

Overexpression of ARIA Affects ABA and Osmolarity Sensitivities during Germination

To investigate the in vivo function of ARIA, we generated and analyzed ARIA overexpression lines. Transgenic Arabidopsis plants expressing *ARIA* under the control of *35S* promoter were generated (see "Materials and Methods"), and after preliminary analysis of seven T3 homozygous lines, ABA and/or stress-related phenotypes of two representative lines were investigated in more detail.

ARIA overexpression lines did not exhibit significant growth phenotypes under normal conditions except slightly (approximately 1 h) delayed germination (data not shown). However, ARIA overexpression affected ABA sensitivity during germination. ABA
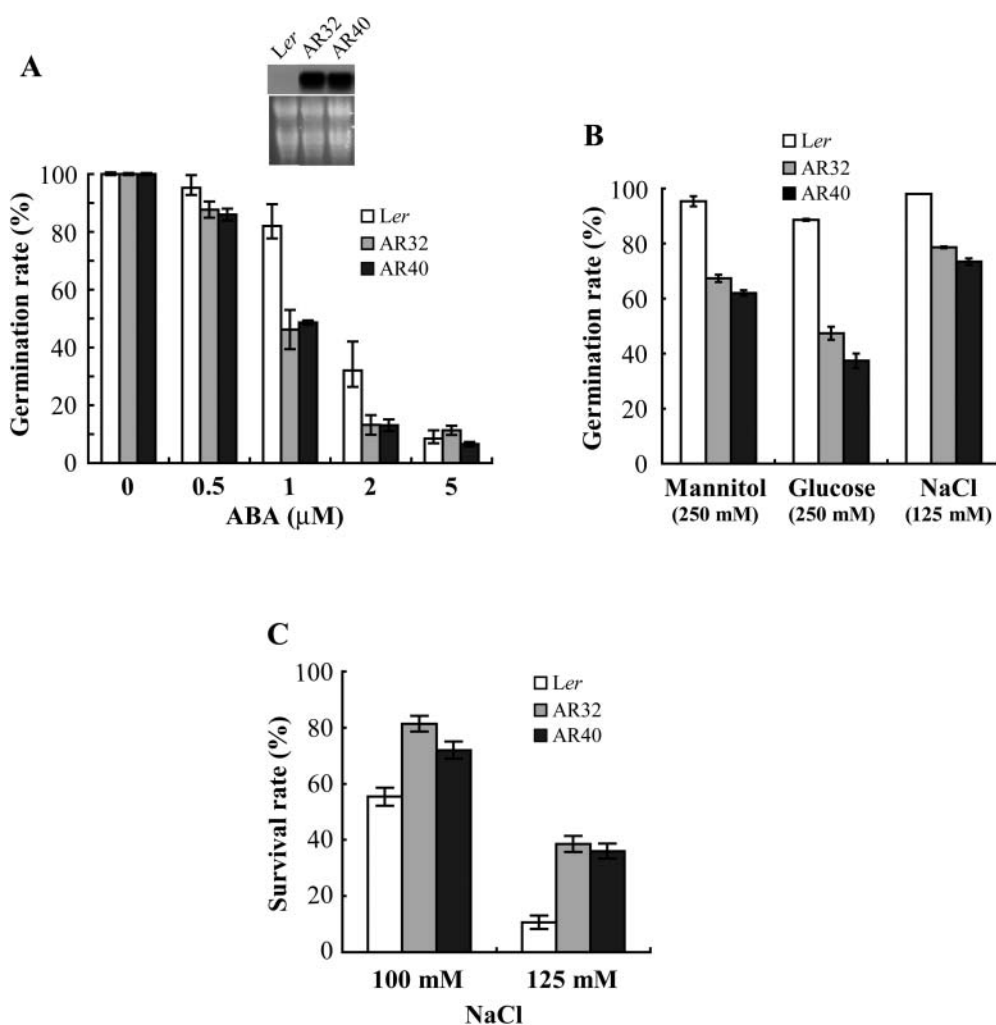

Figure 3. Phenotypes of *35S-ARIA* plants. A, ABA dose response of germination. Seeds were cold treated for 5 d at 4°C and plated on Suc-free MS medium containing various concentrations of ABA. Germination (full emergence of radicle) was scored after 3 d. Experiments were done in triplicates ($n = 36$ each), and the small bars indicate SEs. B, Osmolarity sensitivity of germination. Germination assay was performed as in A on MS media containing various concentrations of mannitol, Glc, or NaCl, and the germination rates at 250 mM mannitol, 250 mM Glc, and 125 mM NaCl are presented. Experiments were done in triplicates ($n = 36$ each). C, Salt tolerance. Seeds were germinated and grown on MS media containing 100 mM or 125 mM NaCl for 2 weeks, and survival rates were determined. The experiments were done in triplicates ($n = 50$ each).
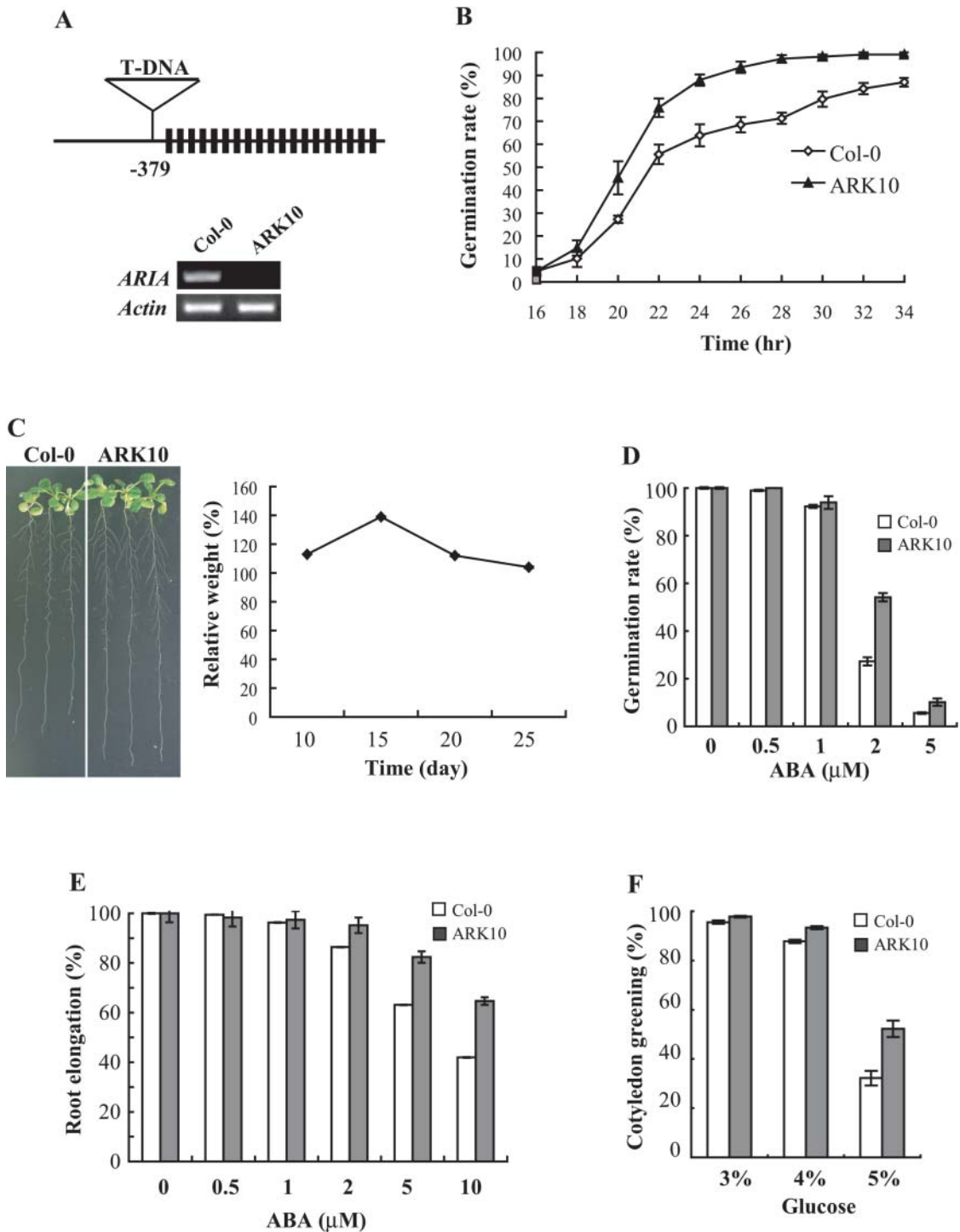
Figure 4. Phenotypes of an *aria* knockout mutant. A, Schematic diagram of T-DNA insertion mutant. Top, The position of T-DNA insertion is presented. Bottom, Expression levels of *ARIA* in wild type (Col-0) and the *aria* mutant (ARK10) plants determined by RT-PCR. B, Germination assay. Germination rates were determined as in Figure 3A on MS medium, (triplicates, $n = 36$ each). C, Growth of the *aria* mutant seedlings. Left section, seedlings grown on MS medium for 2 weeks. Right section, relative weight of aerial parts of soil-grown plants compared with that of Col-0 plants. The data point represents the mean of six determinations ($n = 6$ each). D, ABA dose response of germination. Germination assays were carried out on Suc-free MS media containing various concentrations of ABA (triplicates, $n = 50$ each). E, ABA dose response of primary root elongation. Seeds were germinated on ABA-free MS medium for 3 d, transferred to media containing various concentrations of ABA, and the primary root elongation after the transfer was measured 5 d after the transfer (triplicates, $n = 6$ each). The control growth rates of Col-0 and ARK10 on ABA-free medium are 24.1 and 32.6 mm, respectively. F, Glc response. Seeds were germinated and grown on MS media containing 3%, 4%, or 5% Glc for 6 d before counting plants with green cotyledons (triplicates, $n = 30$ each).

dose response analysis (Fig. 3A) showed that germination of *35S-ARIA* transgenic seeds was more severely inhibited by ABA than wild-type seeds, especially at medium concentrations (1 and 2 μM) of ABA. Thus, *ARIA* overexpression enhanced ABA sensitivity during seed germination. In addition, germination of the transgenic seeds was more sensitive to mannitol, Glc, and NaCl (Fig. 3B), indicating that *ARIA* overexpression resulted in hypersensitive response to high osmolarity.

We also investigated the responses of *35S-ARIA* seedlings to various abiotic stresses and found that they are less sensitive to high salt. For example, the survival rate of wild-type plants at 100 mM NaCl was 55%, whereas those of *35S-ARIA* plants were 81% (AR40) and 72% (AR32), respectively (Fig. 3C). At 125 mM NaCl, 38% (AR40) or 36% (AR32) of the transgenic plants survived, whereas the wild-type survival rate was 11%. Thus, ARIA overexpression lines were more tolerant to high-salinity conditions.

Phenotypes of an *aria* Mutant

To gain further insights into the in vivo function of ARIA, we analyzed the *aria* mutant phenotypes. A mutant, in which a T-DNA is inserted in the promoter region of *ARIA* (Fig. 4A), was obtained from the Arabidopsis stock center and, after the confirmation of T-DNA insertion (see "Materials and Methods") and the abolishment of *ARIA* expression (Fig. 4A), various phenotypes were scored.

Germination assay (Fig. 4B) showed that the mutant seeds germinated more efficiently than wild-type seeds under normal growth conditions, although the degree of difference was not high. Postgermination growth of the *aria* mutant was also more efficient; i.e. *aria* seedlings were larger than wild-type plants, as shown in Figure 4C. They developed normally, however, and the fully-grown mutant seedlings were of similar size to the wild-type plants, indicating that the mutation affected the growth of young seedlings only. Together, the observations demonstrate that ARIA is a negative regulator of seed germination and young seedling growth.

The *aria* mutant also exhibited altered ABA response. ABA dose response analysis of germination (Fig. 4D) revealed that the mutant seed germination was less sensitive to ABA inhibition than wild-type seeds at high concentrations of ABA (i.e. 2 and 5 μM), indicating that their germination was partially insensitive to ABA. Similarly, primary root elongation of *aria* plants was less sensitive to ABA inhibition than wild-type plants at higher ABA concentrations (i.e. 2, 5, and 10 μM; Fig. 4E).

Glc inhibits the shoot development (i.e. cotyledon greening, cotyledon expansion, and true leaf formation) at high concentrations, and the inhibition process is dependent on ABA (Jang et al., 1997; Leon and Sheen, 2003). To see whether ARIA is involved in the process, we determined the Glc sensitivity of *aria* plants. Figure 4F shows that cotyledon greening of
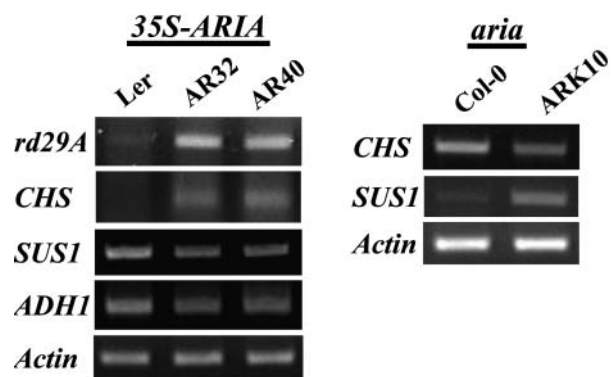

Figure 5. Expression of ABA-responsive genes in *35S-ARIA* and *aria* plants. RNA levels were determined by RT-PCR using total RNA isolated from 2-week-old seedlings. The number of amplification cycles is different for different genes and for overexpression and knockout lines.

wild-type plants was gradually inhibited as Glc concentration in the medium increased. The *aria* mutant plants were also responsive to Glc in a similar manner, but the degree of inhibition was lower than that of the wild-type plants. The differential response was not observed with mannitol, i.e. it was not osmotic response (data not shown). The result demonstrates that ARIA is a necessary component for the Glc inhibition of shoot development.

ARIA Affects the Expression of ABA-Responsive Genes

To examine whether ARIA affects ABF2-regulated gene expression, we determined the expression levels of a number of ABF2-responsive genes in *35S-ARIA* plants. Coupled reverse transcription and PCRs (RT-PCR; Fig. 5) showed that the RNA levels of *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994) and *CHS* (Feinbaum and Ausubel, 1988), which are down-regulated by ABF2 under normal conditions but up-regulated under high-salt conditions, were higher in *35S-ARIA* plants. On the other hand, *SUS1* (Martin et al., 1993) and *ADH1* (de Bruxelles et al., 1996) expression levels, which are down-regulated by ABF2 under normal conditions, were slightly lower than wild-type levels. In *aria* mutant plants, *CHS* RNA level was reduced, whereas *SUS1* RNA level was elevated, further suggesting the regulatory role of ARIA in their expression. Thus, over- or underexpression of ARIA altered the expression of several ABF2-regulated genes, suggesting that it may be involved in the ABF2-dependent gene regulation process.

DISCUSSION

We described an arm repeat protein designated as ARIA, which specifically interacts with ABF2. In animals, arm proteins are involved in a variety of cellular functions such as cell contact, signal transduction, tumor suppression, and nuclear import (Hatzfeld,

1999; Andrade et al., 2001). In plants, their functions are largely unknown except for a few genes. ARC1 and PHOR1 are regulators of self incompatibility and GA signaling, respectively, as mentioned earlier (Stone et al., 1999; Amador et al., 2001). A tobacco arm repeat protein, NtPUB4, which interacts with a receptor-like kinase, has been suggested to be a developmental regulator (Kim et al., 2003). The majority (approximately 40%) of Arabidopsis arm repeat proteins, including ARC1 and PHOR1, contain the U-box motif found in a subclass of E3 ubiquitin ligases (Coates, 2003; Mudgil et al., 2004). Many of them therefore may participate in the ubiquitin-dependent protein degradation process. ARIA, however, does not contain the U-box motif. Instead, it possesses another conserved sequence motif, BTB/POZ domain. Sequence comparison indicates that only two Arabidopsis proteins, including ARIA, have both arm repeat and BTB/POZ domain. The BTB/POZ domain is found in many transcription factors and in some actin-binding proteins in animals (Aravind and Koonin, 1998; Collins et al., 2001). Most recent studies show that some BTB domain proteins are substrate-specific adapters for the CUL-3-based E3 ubiquitin ligases (van den Heuvel, 2004). Although arm repeat and BTB domain proteins play diverse roles, the basic functions of the two motifs are to mediate protein-protein interactions. Thus, ARIA has the potential to form complexes with other proteins or to function as a scaffold.

The physiological relevance of the ABF2-ARIA interaction was supported by their similar expression patterns. Expression of both *ABF2* and *ARIA* is induced by ABA and high salt (Fig. 2; Choi et al., 2000). Both are highly expressed in vegetative tissues (especially in lateral roots, leaf vascular tissues, and guard cells) and in reproductive organs (i.e. anthers, filaments, stigma, and abscission zone). Furthermore, both proteins are localized in the nucleus (Fig. 2), although ARIA is also found in the cell membrane or cell wall region.

Our data on the in vivo function of ARIA further support the physiological significance of the ABF2-ARIA interaction. ARIA overexpression enhanced ABA and osmolarity sensitivities at the germination stage. During subsequent seedling growth, it enhanced salt tolerance. Disruption of its expression, on the other hand, promoted germination and/or seedling growth and impaired Glc response. Several of the *35S-ARIA* and *aria* mutant phenotypes are similar to those of *35S-ABF2* and *abf2* plants. For instance, delayed germination of overexpression lines, faster germination and/or growth of mutant seedlings, salt tolerance of overexpression lines, and Glc insensitivity of knockout mutants were also observed with ABF2 (Kim et al., 2004). Furthermore, we observe altered expression of several ABF2-responsive genes in *35S-ARIA* and *aria* plants (Fig. 5), suggesting that ARIA affects ABF2-regulated gene expression.

Our results indicate that ARIA is a positive component of ABA signaling. ABA sensitivity was enhanced by its overexpression and impaired by its knockout

mutation. Germination was delayed by its overexpression and promoted by its mutation. Also, other ABA-associated processes such as osmolarity sensitivity and sugar response were positively and negatively affected by ARIA overexpression and its mutation, respectively. Two observations are worthy to be mentioned regarding the role of ARIA in ABA response. First, most of the ARIA overexpression and knockout phenotypes are relatively weak or partial (Figs. 3 and 4), although they are consistently observed. This implies that the function of ARIA might be redundant. As mentioned before, there is an arm repeat/BTB domain protein in the Arabidopsis genome, which is highly homologous to ARIA not only in the amino acid sequence but also in its gene structure (data not shown), and thus, functional redundancy between the two proteins can be speculated. Another observation is that ARIA affects only a subset of ABA-dependent processes. ABA sensitivity during germination and young seedling growth was affected by ARIA. However, other ABA-dependent processes, such as stomatal closure and abiotic stress responses other than salt tolerance, were not significantly affected by it (data not shown).

The altered expression of several ABF2-regulated genes (Fig. 5) suggests that ARIA affects the ABF2-dependent gene expression. We do not know the biochemical mechanism of ARIA function at present. However, it can be speculated that it may function as a coactivator or repressor of ABF2. In animals, the arm protein, β-catenin, has been demonstrated to be a transcriptional coactivator; i.e. it translocates into the nucleus in response to a hormone signal and forms complexes with transcription factors to activate target gene expression (Polakis, 2000). The BTB/POZ domain, on the other hand, is known to mediate transcriptional repression by recruiting transcriptional corepressors, which, in turn, recruit histone deacetylase to suppress transcription (Collins et al., 2001). The BTB/POZ domain is also involved in protein degradation (van den Heuvel, 2004). Thus, ARIA might be involved in the stability control of ABF2 or other proteins that possibly might associate with it. Since ARIA possesses two protein-protein interaction domains, another possibility is that it may function as an adaptor for ABF2 to form a protein complex.

MATERIALS AND METHODS

DNA Manipulation and RNA Gel-Blot Analysis

DNA manipulation and RNA gel-blot analyses were performed according to the standard methods (Sambrook and Russell, 2001). DNA sequencing was done on ABI 310 Genetic Analyzer (PE-Applied Biosystems, Foster City, CA). RNA was isolated by the method of Chomczynski and Mackey (1995) and purified further by LiCl precipitation and ethanol precipitation. ABA, salt, cold, and drought treatments of Arabidopsis (*Arabidopsis thaliana*) seedlings were conducted as described (Choi et al., 2000). For RNA gel-blot analysis, 25 μg of total RNA was separated on 1.1% formaldehyde agarose gel, transferred to nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Buckinghamshire, UK), and fixed using Stratagene's UV Crosslinker (model 2400, La Jolla, CA). Hybridization was carried out at 65°C for 18 to 24 h in Rapid-hyb buffer (Amersham Pharmacia Biotech), using a ^{32}P-labeled DNA

fragment containing the less well-conserved region (amino acid position 336–554) of ARIA as a probe. Filters were washed sequentially as follows: twice in 2× SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 10 min at room temperature, twice in 0.2× SSC for 10 min at room temperature, twice in 0.2× SSC for 10 min at 65°C. Exposure was done at −70°C. RT-PCR was carried out by processing 0.5 μg of total RNA according to the manufacturer's instruction, employing the Access RT-PCR System (Promega, Madison, WI). Primer sets, including the actin primers used for control reaction (Arabidopsis actin-1 gene; GenBank accession no. M20016), were described previously (Kang et al., 2002). RNA samples were confirmed to be free of contaminating DNA by using the actin primer set that spans an intron and, when possible, also by using primer sets spanning an intron(s). The number of PCR cycles was variable depending on specific genes (generally 20–30 cycles), within the linear range of PCR amplification. The results of RT-PCR were confirmed by several independent reactions.

Yeast Techniques and Two-Hybrid Screening

Yeast (*Saccharomyces cerevisiae*) growth and transformation were according to the standard techniques (Guthrie and Fink, 1991). Two-hybrid screens were carried out employing the Matchmaker LexA Two-Hybrid System (CLON-TECH Laboratories, Palo Alto, CA), with some modifications. Bait constructs were prepared by cloning two partial fragments of ABF2 into pGilda (CLON-TECH Laboratories), which carries the LexA DNA-binding domain under the control of the *GAL1* promoter and the *HIS3* marker gene. The ABF2 fragments, spanning amino acid residues 65 to 162 (conserved region) and 234 to 337 (variable region), respectively, were prepared by PCR (primer sets, 5′-GC-TAGTGGTGTGGTTCCAGTTC-3′ and 5′-gagagctcgag CTGAGCTCTTGCAG-CAACCTG-3′, and 5′-CCAATCATGCCTAAGCAGCC-3′ and 5′-gagagctc-gagCTCTACAACTTTCTCCACAGTG-3′, respectively), and after digestion with *Xho*I, ligated with pGilda, which in turn was prepared by *Bam*HI digestion, Klenow fill-in reaction, and *Xho*I digestion. The bait constructs were then individually introduced into the reporter yeast, EGY48 (*MATα, his3, trp1, URA3::LexA$_{op(x8)}$-LacZ*, and *LexA$_{op(x6)}$-LEU2*), by transformation. The EGY48 strain carries two reporter genes, *LEU2* and *LacZ*, integrated into the chromosome. Large-scale transformation for the screening was carried out as described (Choi et al., 2000). The reporter yeast was transformed with library plasmid DNA representing cDNA of ABA/salt-treated Arabidopsis seedlings (Choi et al., 2000). Transformed yeast was grown on Gal/Raf/CM-His-Leu-Trp-Ura medium for 5 to 7 d, and positive colonies were identified by colony lift β-galactosidase assay. The Leu$^+$/LacZ$^+$ positive colonies were purified by streaking on the same selection medium followed by another round of β-galactosidase assay. For each reporter yeast, 6.6 million transformants were screened, and 5 positive clones were obtained from the variable region bait, whereas no positive clones were obtained from the conserved region bait. Specificity of the interaction of the positive clones was tested by retransforming the reporter yeast with the plasmid DNA rescued from the clones (see below).

Plasmid rescue and insert DNA analysis were carried out as described (Choi et al., 2000). Sequencing of the plasmid DNA rescued from the positive clones revealed that three of them (clones 12, 20, and 24) encoded an arm repeat protein (At5g19330) and two of them (clones 17 and 27) encoded a transcription factor. The longest arm protein clone was missing the first five amino acid residues. Full-length gene was isolated by PCR using the primer set 5′-GGA-TCGTCTTTTACTTTGTGAACG-3′ and 5′-CATTCAAGACCGATTGTGAT-CAG-3′ and 1 μg of library DNA. The PCR product, which contains the entire coding region and 5′ (208 bases) and 3′ (24 bases) additional sequences, was cloned into the Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, CA) and sequenced fully. The correctness of its nucleotide sequence was confirmed by comparing it with the genomic sequence on the Arabidopsis database.

In Vitro Binding Assay

GST-ARIA fusion constructs were prepared by cloning PCR fragments of various portions (full-length, amino acids 1–518, and amino acids 511–710) of ARIA into the *Sma*I site of pGEX-6P-2 (Amersham Pharmacia Biotech). Constructs were used to transform BL21 cells, and transformed cells were grown in 2× YT medium containing 50 μg/mL ampicillin overnight. The cultures were diluted 100-fold and grown to A_{600} of 0.6 at 30°C (BTB construct) or 37°C (full-length and ARM constructs). The expression of recombinant proteins was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h. At the end of the induction, cells were

pelleted down by centrifugation, resuspended in 6 mL of phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$, pH 7.3), and sonicated. The lysate was cleared of cell debris by centrifugation and further purified according to the supplier's instruction. For in vitro translation of ABF2, full-length ABF2 cloned into pCITE (Novagen, Madison, WI) was processed with the TNT in vitro translation kit (Promega) in the presence of ^{35}S-Met according to the manufacturer's instruction.

For binding assay, GST-ARIA fusion proteins (0.5 μg) were incubated with the glutathione-sepharose 4B resins for 1 h at 4°C in a binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). In vitro-translated, ^{35}S-labeled ABF2 was then added, and incubation was continued for 2 h with constant rotation. The resins were washed five times with the binding buffer and resuspended in SDS-PAGE sample buffer. The proteins were separated on 15% SDS-polyacrylamide gel and visualized by autoradiography.

Histochemical GUS Staining

A 2.1-kb promoter fragment was prepared by PCR using the primer set 5′-GATCCGAAGAAGAGGAGAGATC-3′ and 5-GCCACGCTGTCTTCTTT-CACTACACTAAAAAATACAGC-3′ and cloned into the *Hind*III-*Xba*I sites of pBI101.2. The construct was introduced into Arabidopsis (Landsberg *erecta*, L*er*) by transformation, and T2 or T3 generation plants were used for the analysis of GUS activity. GUS staining was performed according to Jefferson et al. (1987). Whole plants or tissues were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-β-GlcUA (X-gluc) solution in 100 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.1% Triton X-100 for 24 h at 37°C. Chlorophyll was cleared from the tissues by ethanol series: 35%, 50%, and 70%.

Subcellular Localization

To prepare the *35S-ARIA-GFP* fusion construct, the entire coding region of ARIA was prepared by PCR and, after digestion with *Nco*I-*Spe*I, cloned into the same sites of pCAMBIA1302 (CAMBIA). The construct was introduced into Arabidopsis (Columbia, Col-0) by transformation, and T1 plants were used for GFP localization analysis. Nuclei were visualized by propidium iodide (PI)-staining. Roots of 10-d-old transgenic seedlings were used for the green (GFP localization) and red (PI) fluorescence analysis using a confocal microscope (TCS-NT; Leica Microsystems, Wetzlar, Germany).

To investigate ABF2 localization, the coding region of ABF2 was inserted in front of the GUS-coding region of pBI221 in frame. Onion epidermal cells were then transiently transformed with the *ABF2-GUS* construct by particle bombardment using PDS 1000 (Bio-Rad, Hercules CA). GUS activity was determined by X-gluc staining after 24 h at 23°C. Nuclei were visualized by 4′,6-diamidino-2-phenylindole staining and observed under a fluorescence microscope (BX51, Olympus, Tokyo).

Overexpression and Knockout Mutant Lines

To prepare the *35S-ARIA* construct, the coding region of ARIA was prepared by PCR using primers 5′-cgcggatccATGGACCAACAACCGGAGAGG-3′ and 5′-gcgggatccCAACCTCAAGCTTTGCAGGTTTG-3′, and after digestion with *Bam*HI, cloned into the *Bam*HI site of pBI121 lacking the GUS-coding region. Transformation of Arabidopsis (L*er*) was according to the vacuum infiltration method (Bechtold and Pelletier, 1998), using *Agrobacterium tumefaciens* strain GV3101. Seven homozygous lines were recovered, and after preliminary analysis, two representative lines (T4) were chosen for detailed analysis.

To establish *aria* mutant lines, four putative ARIA knockout mutant lines were obtained from the Arabidopsis Stock Center. The stock seeds were sown and grown on soil, and seeds were harvested from individual plants. To choose T-DNA insertion lines with single integration, segregation ratio of kanamycin resistance (Kan^R) was tested, and homozygous sublines were established from those segregating at 3:1 ratio of Kan^R:Kan^S. Genomic DNA was isolated from the sublines and the integration of T-DNA at the annotated site was confirmed by the sequencing of PCR fragments. We were able to identify one insertion line (SALK_143439) with a single T-DNA insertion in the annotated site among the four putative lines. T-DNA is inserted at −379 from the translation start site. Expression analysis by RT-PCR showed that ARIA expression is abolished in the insertion line. For phenotype analysis, two

sublines (ARK5 and ARK10) were used. Same results were obtained from them, and those from AR10 are presented.

Phenotype Analysis

Arabidopsis ecotypes L*er* and Col-0 were used. Plants were grown under long-day conditions (16-h-light/8-h-dark cycle) at 22°C, on 1:1:1 mixture of vermiculite, perlite, and peat moss or on Murashige and Skoog (MS) plates. Soil-grown plants were irrigated with 0.1% Hyponex once every week. For general aseptic growth, seeds were sterilized with 70% ethanol for 5 min and then with 30% household bleach for 5 min, washed five times with sterile water, and plated on MS medium (Murashige and Skoog, 1962) supplemented with 1% Suc and solidified with 0.8% Phytoagar.

For germination test, seeds collected at the same time were plated, unless stated otherwise, on MS medium supplemented with 1% Suc and other supplements (i.e. ABA, mannitol, Glc, and NaCl), and radicle emergence was examined at various time points. For ABA dose response analysis of germination, Suc was omitted from the media.

Phenotype analyses other than germination assay were performed on MS medium supplemented with 1% Suc and also with ABA, Glc, or mannitol as specified in the figure legend. For root elongation assay, plants were grown at vertical position.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number NM121938.

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