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Arabidopsis Basic Leucine Zipper Proteins That Mediate Stress-Responsive Abscisic Acid Signaling

Joung-youn Kang, Hyung-in Choi, Min-young Im, and Soo Young Kim[1]

Kumho Life and Environmental Science Laboratory, Korea Kumho Petrochemical Co., Ltd., Kwangju 500-712, South Korea

The phytohormone abscisic acid (ABA) plays an essential role in adaptive stress responses. The hormone regulates, among others, the expression of numerous stress-responsive genes. From various promoter analyses, ABA-responsive elements (ABREs) have been determined and a number of ABRE binding factors have been isolated, although their in vivo roles are not known. Here we report that the ABRE binding factors ABF3 and ABF4 function in ABA signaling. The constitutive overexpression of ABF3 or ABF4 in Arabidopsis resulted in ABA hypersensitivity and other ABA-associated phenotypes. In addition, the transgenic plants exhibited reduced transpiration and enhanced drought tolerance. At the molecular level, altered expression of ABA/stress-regulated genes was observed. Furthermore, the temporal and spatial expression patterns of ABF3 and ABF4 were consistent with their suggested roles. Thus, our results provide strong in vivo evidence that ABF3 and ABF4 mediate stress-responsive ABA signaling.

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INTRODUCTION

Being sessile, plants have the capability to adapt to adverse environmental conditions such as drought, cold, and high salt. Under these stress conditions, the plant hormone abscisic acid (ABA) level increases in vegetative tissues, triggering adaptive responses that are essential for their survival and productivity (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998). Under water deficit conditions, for example, ABA induces stomatal closure, minimizing water loss through transpiration. The ABA-controlled process is vital for plant survival, and ABA-deficient and ABA-responsive mutants are susceptible to water stress. On the other hand, high levels of ABA inhibit overall plant growth (Himmelbach et al., 1998).

Underlying the ABA-mediated stress responses is the transcriptional regulation of stress-responsive gene expression (Giraudat et al., 1994; Busk and Pages, 1998). Numerous genes have been reported that are upregulated under stress conditions in vegetative tissues (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). These include a class of genes known as *LEA* (for *LATE EMBRYOGENESIS ABUNDANT*) genes, which are expressed abundantly in developing seed under normal conditions, osmolyte biosynthetic genes, and genes of general cellular metabolism. In general, the gene products are considered to have protective or adaptive roles under stress conditions. In addition, the expression of many regulatory genes, including various kinase/phosphatase and transcription factor genes, also is induced by abiotic stresses. Not all stress-inducible genes are regulated by ABA. However, a large number of them also are responsive to exogenous ABA, and in many cases, their induction is impaired in ABA-deficient mutants. Meanwhile, the expression of some genes, such as *rbcS* and *CAB* genes, is suppressed by ABA and stress (Bartholomew et al., 1991; Wang et al., 1996; Weatherwax et al., 1996).

ABA-responsive elements (ABREs) that control ABA- and/or stress-responsive gene expression have been determined by numerous studies (Giraudat et al., 1994), and their putative cognate *trans*-acting factors have been isolated (Busk and Pages, 1998). Most ubiquitous among the *cis* elements is a group of sequences sharing the (C/T)ACGTGGC consensus. Many of these elements contain the G-box (CACGTG) sequence (Giuliano et al., 1988), which also is present in numerous genes regulated by other environmental cues (Menkens et al., 1995). Another group of ABREs, known as coupling element, *hex3*, or motif III (Busk and Pages, 1998), shares the CGCGTG core sequence. On the basis of their interactions with these two types of ABREs, which are referred to as ABREs hereafter, a number of putative *trans*-acting factors have been isolated (Busk and Pages, 1998). Also, their homologs and numerous other G-box binding factors, all belonging to the bZIP class proteins (Landschulz et al., 1988), are able to interact with the ABREs in vitro (Foster et al., 1994). However, the evidence showing that any of these biochemically identified factors play a role in ABA or stress signaling in planta is still lacking.

In an effort to identify transcription factors that control ABA-responsive gene expression during vegetative growth,

[1] To whom correspondence should be addressed. E-mail sykim@ksc.kumho.co.kr; fax 82-62-972-5085.

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we recently isolated four ABRE binding bZIP factors by yeast one-hybrid screening of an Arabidopsis cDNA expression library (Choi et al., 2000). Expression of the factors, referred to as ABF1 through ABF4 (ABRE Binding Factors 1 to 4), is ABA and stress inducible, and they can transactivate an ABRE-containing reporter gene in yeast. ABF2 and ABF4 also were reported by Uno et al. (2000), who named them AREB1 and AREB2, respectively, and showed that the factors can activate an ABA-responsive promoter in protoplasts. To investigate the in vivo functions of ABFs, we generated transgenic Arabidopsis plants constitutively overexpressing them. Here we show that ABF3 and ABF4 transgenic lines are hypersensitive to ABA and that they exhibit several other ABA/stress-associated phenotypes, including enhanced drought tolerance. The expression patterns of the two ABFs correlated well with their overexpression phenotypes.

RESULTS

Growth Phenotypes of ABF3 and ABF4 Overexpression Lines

To investigate the in vivo functions of ABF3 and ABF4, we used an overexpression approach. The coding region of *ABF3* or *ABF4* was fused to the 35S promoter of *Cauliflower mosaic virus*, and each construct was used to transform Arabidopsis (ecotype Landsberg *erecta* [L*er*]) plants. Thirty-eight and 12 T3 homozygous lines were recovered from the *35S-ABF3* and *35S-ABF4* constructs, respectively, and after preliminary analysis, transgenic lines with higher ABF expression levels were selected for more detailed analysis.

Compared with wild-type plants, *35S-ABF3* transgenic plants exhibited mild growth retardation in the aerial parts: petioles were slightly shorter, and leaves were rounder in shape (Figure 1A). The degree of retardation was not severe, however, and overall growth patterns were similar to those of wild-type plants except that siliques were somewhat shorter and thicker (Figure 1A, inset). In contrast, *35S-ABF4* transgenic plants exhibited severe growth retardation (Figure 1A), which was dependent on the *ABF4* expression level (Figure 1B). Petioles were shorter, leaves were smaller, flowering was delayed, and plants were shorter. Also, the germination of *35S-ABF3* plants was delayed several hours compared with that of wild-type plants in the absence of ABA (Figure 1C). *35S-ABF4* plants, on the other hand, germinated normally (data not shown); thus, the growth retardation observed with *35S-ABF4* plants was a postgermination process.

ABA Response of *35S-ABF* Plants

To determine whether ABF3 or ABF4 overexpression affected ABA sensitivity, *35S-ABF3* and *35S-ABF4* transgenic plants were germinated and grown on media containing various concentrations of ABA. When ABA concentration was 0.5 μM or greater, the growth of *35S-ABF* plants was arrested completely after radicles emerged; that is, cotyledon greening/expansion and root growth were inhibited severely, and none of the transgenic seedlings developed to have true leaves (Figure 2A). Under the same conditions, wild-type plants continued to grow and develop, although at slower rates than on ABA-free medium. The ABA hypersensitivity of *35S-ABF* transgenic plants also was observed at 0.25 μM ABA (Figure 2B), although the transgenic seedlings eventually grew to have true leaves (data not shown).

To determine the stage specificity of the ABA response, ABA dose response was examined during and after germination. As shown in Figure 2C, *35S-ABF* transgenic plants were hypersensitive to ABA at the germination stage: 0.5 μM ABA was sufficient to inhibit their germination efficiencies to 8% (ABF3) or 28% (ABF4), whereas wild-type plants retained 80% germination under the same conditions. Likewise, root growth of the *35S-ABF* transgenic lines was hypersensitive to ABA (Figure 2D). At 0.5 μM ABA, wild-type root growth was 84% of its control rate, whereas that of *35S-ABF4* and *35S-ABF3* plants was 35 and 51% of their control rates, respectively. At 1 μM ABA, root growth of the *35S-ABF4* plants was reduced to 6% and that of the *35S-ABF3* plants was reduced to 37%. In addition, lateral root and aerial part growth of *35S-ABF* transgenic plants was inhibited significantly at this concentration (data not shown). Wild-type plants grew at 62% of the control rate at the same ABA concentration. Transgenic root growth was arrested almost completely when ABA concentration was >5 μM, whereas wild-type plants still continued to grow. These results indicate that both the germination and postgermination growth of *35S-ABF* transgenic plants are hypersensitive to ABA.

Salt Response of *35S-ABF* Plants

High concentrations of salts inhibit the germination of Arabidopsis (Werner and Finkelstein, 1995; Leon-Kloosterziel et al., 1996; Quesada et al., 2000; Zhu, 2000). Several studies show that ABA plays a role in the inhibition process. Although not all salt-insensitive mutants are ABA insensitive (Werner and Finkelstein, 1995), all ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants exhibit salt insensitivity during germination (Leon-Kloosterziel et al., 1996). This is probably because ABA, whose level increases under high salt conditions, promotes the inhibition process. Because the expression of both ABF3 and ABF4 is salt inducible (Choi et al., 2000), they may participate in salt response (in this case, salt-induced germination inhibition). To test this possibility, *35S-ABF* transgenic plants were germinated on media containing various concentrations of NaCl. Figure 3A shows that the germination of wild-type and *aba1*, *abi1*, and *abi2* mutant plants was not affected by NaCl <100 mM. In contrast, germination and growth of the *35S-ABF* plants were affected significantly by 100 mM NaCl (Figures 3A and 3B):

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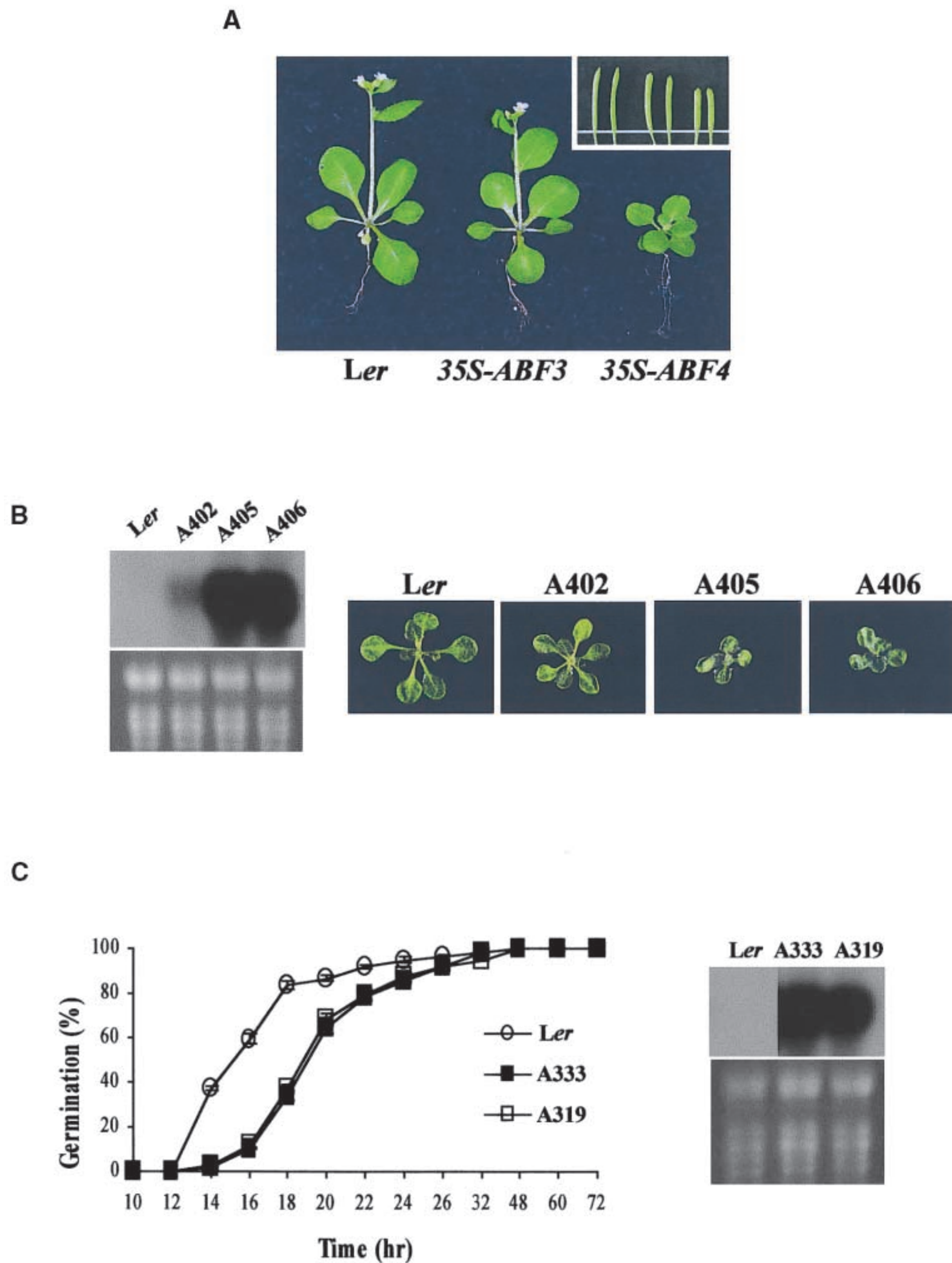

Figure 1. Growth Phenotype of *35S-ABF3* and *35S-ABF4* Plants.

(A) Growth on soil. *35S-ABF3* (line A319) and *35S-ABF4* (line A405) transgenic plants were grown for 3 weeks on soil. The inset shows fully grown siliques, two each from L*er*, *35S-ABF3*, and *35S-ABF4* plants (left to right).

(B) Relationship between ABF4 expression level and the severity of the growth phenotype. Left, RNA gel blot analysis of *ABF4* expression in L*er* and the *35S-ABF4* transgenic lines A402, A405, and A406. The bottom panel shows ethidium bromide staining of the RNA gel. Each lane contained 25 μg of total RNA. Right, *35S-ABF4* transgenic lines with varying degrees of ABF4 expression. Only the aerial parts of the plants are shown for clarity.

(C) Left, germination of *35S-ABF3* transgenic seed on ABA-free medium. Ten-week-old seed of L*er*, line A333, and line A319 were plated on ABA-free medium after 4 days of cold treatment, and germination (fully emerged radicle) was scored at various times. Each data point represents the mean of triplicate experiments ($n = 50$ each). Standard errors are smaller than the symbols. Right, RNA gel blot analysis of ABF3 expression in L*er* and transgenic lines A333 and A319. The bottom panel shows ethidium bromide staining of the RNA gel. Each lane contained 25 μg of total RNA. The ABF3 expression levels in the A333 and A319 lines are comparable to the ABF4 expression levels in the A405 and A406 lines.

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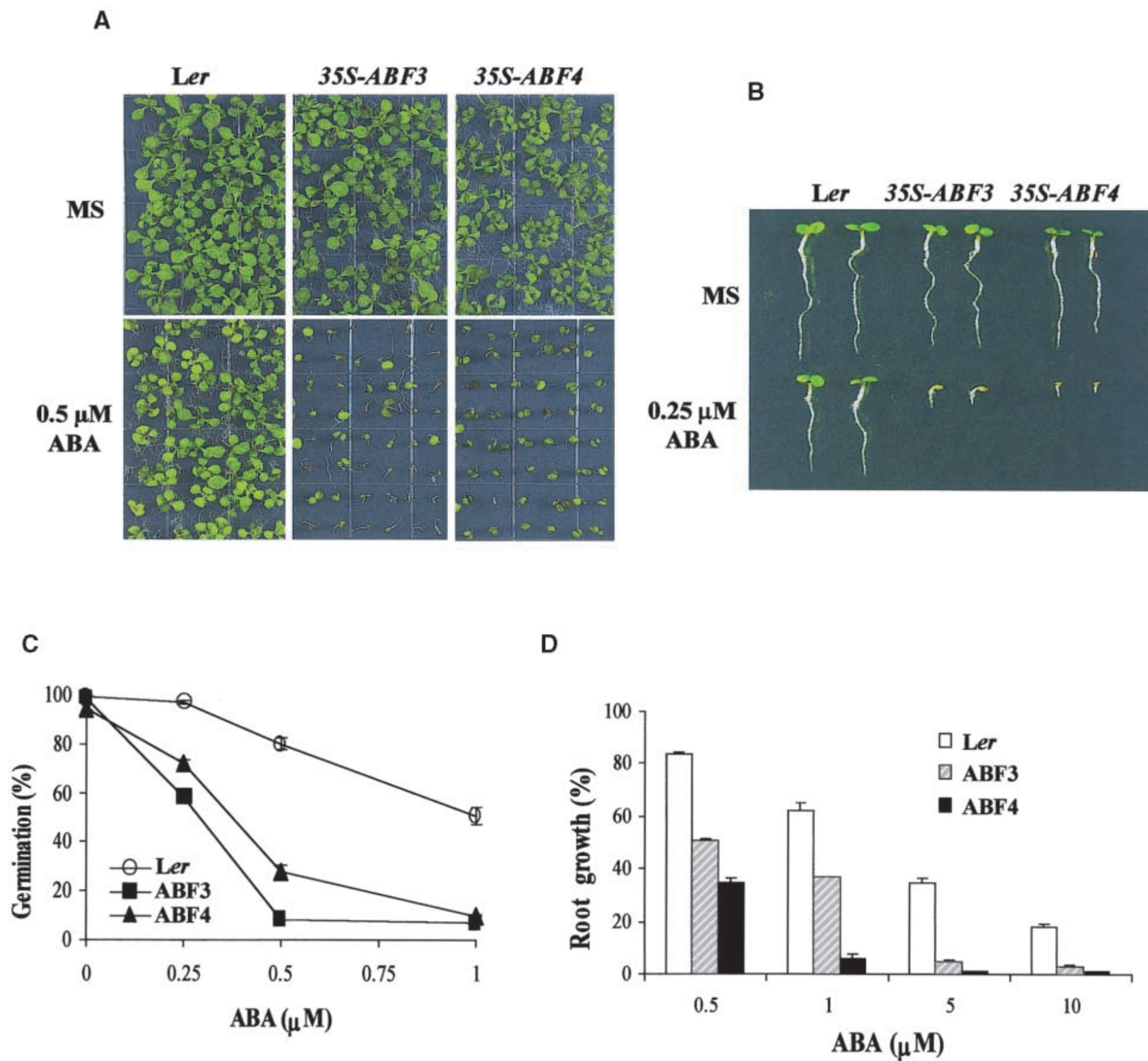

Figure 2. ABA Sensitivity of *35S-ABF3* and *35S-ABF4* Plants.

(A) Growth of transgenic plants on Murashige and Skoog (1962) medium (MS) containing 0.5 μM ABA. Seed were germinated and grown for 12 days.

(B) Growth of transgenic plants on MS medium containing 0.25 μM ABA. Seed were germinated on the medium for 3 days, and representative plants are shown.

(C) ABA dose response of germination. Seed, 6 months old after harvest and prechilled at 4°C for 4 days, were germinated on media containing various concentrations of ABA, and seedlings with fully emerged radicles were counted after 3 days. Experiments were performed in triplicate ($n = 50$ each), and the bars show standard errors.

(D) ABA dose response of root growth. Seed were germinated for 4 days on ABA-free medium, and the seedlings ($n = 6$) were transferred to media containing various concentrations of ABA. Root elongation was measured 5 days after the transfer. The experiments were performed more than four times, sometimes using different transgenic lines, and the results were consistent. The small bars represent standard errors. Transgenic lines A319 (ABF3) and A405 (ABF4) were used.

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radicle emergence, root growth, and cotyledon opening/expansion were inhibited severely. In a parallel experiment, the transgenic plants responded to KCl in a similar manner (Figure 3B). On the other hand, their response to the same concentration (Figure 3B) or twice the concentration (data not shown) of mannitol, which gives the same osmotic pressure, was normal. Thus, in contrast to the salt-insensitive phenotype of ABA-deficient or ABA-insensitive mutants, both ABF3 and ABF4 overexpression resulted in salt hypersensitivity at the germination/young seedling stage, and the hypersensitivity appeared to be ionic rather than osmotic in nature.

Sugar Response of *35S-ABF* Plants

At higher concentrations, sugars inhibit the development of young seedlings; that is, they inhibit cotyledon greening/expansion and shoot growth (Jang et al., 1997). According to studies performed by other researchers, ABA plays an essential role in glucose or sucrose signal transduction (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). For example, the ABA-deficient *aba2* mutation is allelic to the sugar-insensitive *sis4* mutation, and the glucose- or sugar-insensitive mutations *gin6*, *sis5*, and *sun6* are allelic to the ABA-insensitive mutation *abi4*. Also, these studies show that other *aba* mutants, and to some degree *abi5* mutants, are insensitive to glucose. Thus, ABF overexpression might have affected sugar sensitivity as well, if in fact ABF3 and ABF4 mediate ABA signaling. We addressed this possibility by examining their response to glucose, which exerts more severe growth inhibition than do other sugars (Jang et al., 1997). Under our experimental conditions, wild-type seedlings showed growth defects such as inhibition of cotyledon greening and true leaf development when glucose concentration was >4% (data not shown). The aerial part growth of *35S-ABF* transgenic lines, on the other hand, was arrested completely at 3% glucose, at which level wild-type plants developed fully (Figure 3C). Thus, *35S-ABF* transgenic plants were hypersensitive to glucose. This enhanced response of the transgenic plants was not observed with the same concentration of mannitol, which inhibited the growth of both wild-type and *35S-ABF* transgenic plants significantly but similarly. Thus, the hypersensitivity was glucose specific rather than osmotic.

Epinasty and Obstacle-Touching Response of 35S-ABF4 Plants

Recent genetic studies show that ABA signaling pathways interact with those of ethylene. According to these studies, ABA-mediated inhibition of germination is regulated negatively by ethylene, whereas ethylene signaling components are required for the ABA inhibition of root growth (Beaudoin et al., 2000; Ghassemian et al., 2000). The involvement of auxin in ABA-dependent stress response also has been demonstrated (Vartanian et al., 1994). When exposed to progressive drought, new lateral roots take a short and tuberized form. This process, known as drought rhizogenesis, is impaired not only in the ABA-deficient *aba-1* and ABA-insensitive *abi1-1* mutants but also in the auxin-resistant *axr1-3* mutant. To determine whether the overexpression of ABF3 or ABF4 affected ethylene or auxin sensitivity, we compared the effects of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid and indole-3-acetic acid on the growth of *35S-ABF* and wild-type plants. We did not see any differences in their responses. However, leaves of 35S-ABF4 plants have a tendency to have epinastic curvature when grown on plates (Figure 4A), which generally is attributed to ethylene action under stress conditions (Jackson, 1997). Also, roots of *35S-ABF4* transgenic plants exhibited abnormality in the obstacle-touching response (Okada and Shimura, 1990; Simmons et al., 1995). As shown in Figure 4B, roots of wild-type plants grow in a wavy pattern when grown on a hard agar surface at an inclined position. The wavy pattern of root growth, however, was diminished significantly in *35S-ABF4* plants. The obstacle-touching response is impaired in several auxin-resistant mutants, indicating that auxin signaling components are involved in the process (Okada and Shimura, 1992). Thus, this result implies that auxin signaling pathway(s) might have been perturbed in *35S-ABF4* plants. Together, our results suggest that ABF4 overexpression might have affected some aspects of ethylene and auxin responses.

Drought Tolerance of *35S-ABF* Plants

One of the key ABA-controlled processes is stomatal closure under water stress conditions, which minimizes water loss through transpiration (Leung and Giraudat, 1998). ABA biosynthesis mutants and some of the ABA response mutants (i.e., *abi1* and *abi2*), therefore, are very susceptible to drought because of the impaired stomatal aperture regulation (Leung and Giraudat, 1998; Schroeder et al., 2001). Thus, ABF3 and ABF4 overexpression lines are expected to exhibit an altered response to water deficit conditions if the factors are involved in ABA/stress signaling. To address this possibility, we examined the drought tolerance of *35S-ABF* plants. As shown in Figure 5A, wild-type plants withered completely when withdrawn from water for 11 days, and only 16% of them survived to maturity when rewatered afterward. *35S-ABF3* plants, however, were not affected noticeably, and all survived the treatment to set seed. In a similar experiment, *35S-ABF4* plants also exhibited higher survival rates under water deficit conditions; all of them survived a 12-day drought treatment, whereas 33% of the wild-type plants survived to set seed (Figure 5B). Thus, both *35S-ABF3* and *35S-ABF4* plants survived the drought conditions better than did the wild-type plants.

The enhanced drought tolerance of the transgenic plants could be attributed, at least in part, to their lower transpiration rates. When measured by the fresh weight loss of detached rosette leaves, the water loss rates of *35S-ABF3* and *35S-ABF4* transgenic lines were less than half and ~70% of

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those of the wild-type plants, respectively (Figures 5C and 5D). Consistent with this result, the stomata of the transgenic plants had smaller openings than did the wild-type plants (Figure 5E). Under normal growth conditions, ~85% (89 of 104) of the wild-type stomata were open, whereas ~20% of them were open in *35S-ABF3* (15 of 71) and *35S-ABF4* (17 of 71) plants when observed in the middle of the watering period. Thus, constitutive overexpression of ABF3 or ABF4 resulted in partial stomatal closure, reduced transpiration, and enhanced drought tolerance.

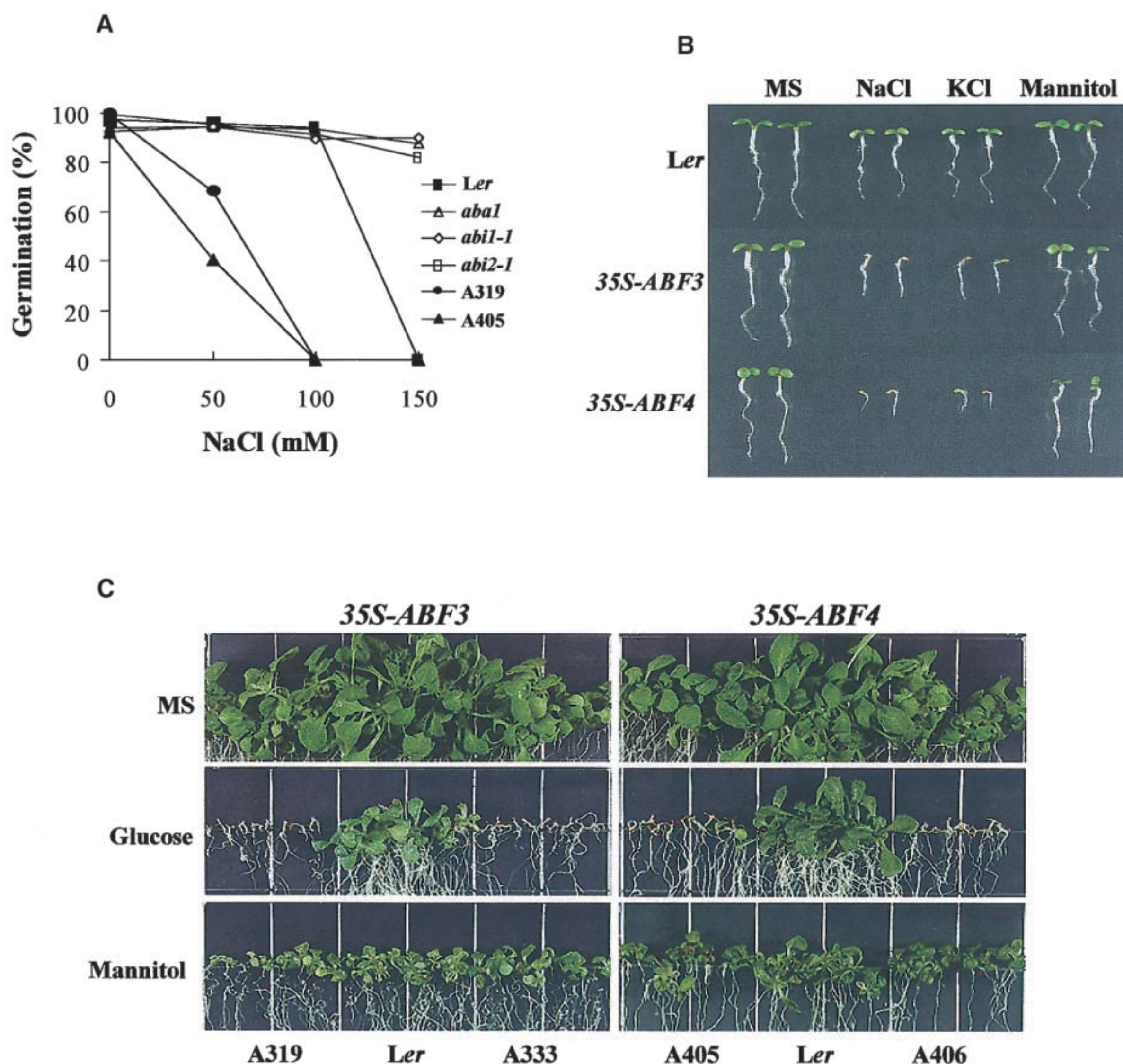

Figure 3. Salt and Glucose Sensitivity of *35S-ABF* Plants.

(A) Salt effect on germination. Seed of L*er*, *aba1-1*, *abi1-1*, *abi2-1*, *35S-ABF3* (line A319), and *35S-ABF4* (line A405) were plated after 4 days of cold treatment on media containing 50, 100, or 150 mM NaCl, and germination (fully open cotyledons) was scored after 4 days. Experiments were performed in triplicate ($n = 50$ each), and standard errors are smaller than the symbols.

(B) Salt effect on newly germinated seedling growth. Seed of the same transgenic lines were germinated for 4 days on MS medium containing 100 mM NaCl, KCl, or mannitol, and representative seedlings are shown.

(C) Glucose response of *35S-ABF* transgenic plants. Seed were germinated and grown for 14 days on MS medium or the same medium supplemented with 3% glucose or mannitol in the vertical position. A319, A333, A405, and A406 indicate transgenic lines.

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Expression of ABA-Responsive Genes in *35S-ABF* Plants

To investigate the transcriptional regulatory roles of ABF3 and ABF4 in planta, the expression of various ABA/stress-responsive genes in *35S-ABF* plants was determined. As shown in Figure 6, the transcript levels of a number of ABA-regulated genes (group I) were enhanced in *35S-ABF3* and *35S-ABF4* transgenic lines. These include *LEA* class genes *rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1994) and *rab18* (Lang and Palva, 1992), whose expression is induced by ABA and abiotic stresses. Expression of the ABA-inducible cell cycle regulator gene *ICK1* (cyclin-dependent kinase inhibitor) (Wang et al., 1998) also was increased in the *35S-ABF* transgenic lines. The *ICK1* gene has been suggested to mediate cell division arrest by ABA. In the *35S-ABF3* lines, strong enhancement of *ABI1* (Leung et al., 1994; Meyer et al., 1994) RNA level was observed, and *ABI2* (Leung et al., 1997) transcript level was increased, although the degree of increase was lower. An increase in the *ABI1* RNA level also was observed in the *35S-ABF4* line with higher *ABF4* expression (A405). The expression of *ABI1* and *ABI2*, which encode homologous protein phosphatase 2Cs and whose mutations result in defective stomatal closing and a wilty phenotype (Schroeder et al., 2001), is enhanced by ABA and water stress (Leung et al., 1997).

Meanwhile, the RNA level of the ABA-repressible gene *SKOR* (Gaymard et al., 1998) was reduced significantly or was undetectable in *35S-ABF4* transgenic lines (group II). This gene encodes a root-specific K^+ outward rectifying channel, and ABA repression of its expression has been suggested to be part of an adaptive water stress response. Similarly, guard cell ion channel genes *KAT1* and *KAT2* (Anderson et al., 1992; Pilot et al., 2001) were regulated negatively in the *35S-ABF3* line with higher *ABF3* levels (A319). The two ion channels normally mediate K^+ influx, enabling stomatal opening, but their activity is inhibited by ABA. Also, stress-responsive biosynthetic genes (group III), the chalcone synthase gene *CHS* (Feinbaum and Ausubel, 1988) and the alcohol dehydrogenase gene *ADH1* (de Bruxelles et al., 1996), were down-regulated. The transcript levels of these two genes, however, were higher in the *35S-ABF* transgenic lines when plants were treated with high salt, suggesting that stress-induced post-translational modification of ABF3 and ABF4 may be required for the regulation of these genes. In summary, overexpression of ABF3 or ABF4 resulted in the modulation of ABA/stress-responsive gene expression, and the two factors played positive or negative roles depending on specific genes and environmental conditions.

Expression Patterns of ABF3 and ABF4

The phenotypes described so far indicated that the overexpression of ABF3 or ABF4 enhanced various aspects of ABA response. To assess the physiological relevance of the results and to obtain further clues about their functions, we investigated their temporal and spatial expression patterns. We first investigated the tissue specificity of their uninduced, basal expression. Because the basal expression levels of ABF3 and ABF4 are very low (Choi et al., 2000), we used coupled reverse transcription (RT) and polymerase chain reaction (PCR) for the analysis. As shown in Figure 7A, relatively higher expression of both *ABF3* and *ABF4* was detected in roots. Also, lower *ABF3* expression was observed in flowers but not in leaves and siliques under the same conditions. On the other hand, weak *ABF4* expression was detectable in leaves, flowers, and siliques.

More detailed temporal and spatial expression patterns of *ABF3* and *ABF4* were determined by histochemical β-glucuronidase (GUS) staining of transgenic plants that harbored an *ABF* promoter–*GUS* reporter construct. With the *ABF3*

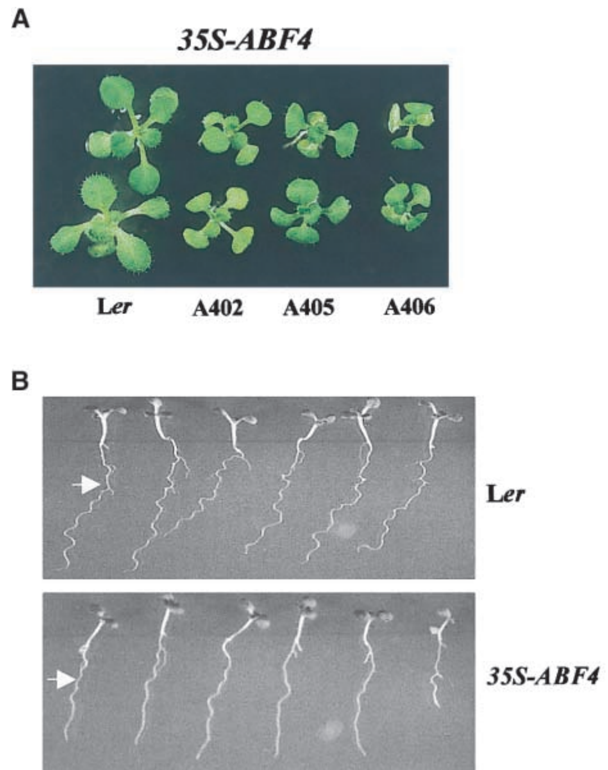

Figure 4. Epinasty and Obstacle-Touching Response of *35S-ABF4* Plants.

(A) Epinastic curvature of *35S-ABF4* transgenic leaves. L*er* and transgenic plants were grown on MS plates for 2 weeks. *ABF4* expression level is highest in the A406 line and lowest in the A402 line (Figure 1B).

(B) Obstacle-touching response of *35S-ABF4* plants. Plants (line A405) were grown on 1.5% agar plates for 4 days in the vertical position and then for 5 days at an angled (45°) position. Arrows indicate the root tip positions before changing to the slanted orientation.

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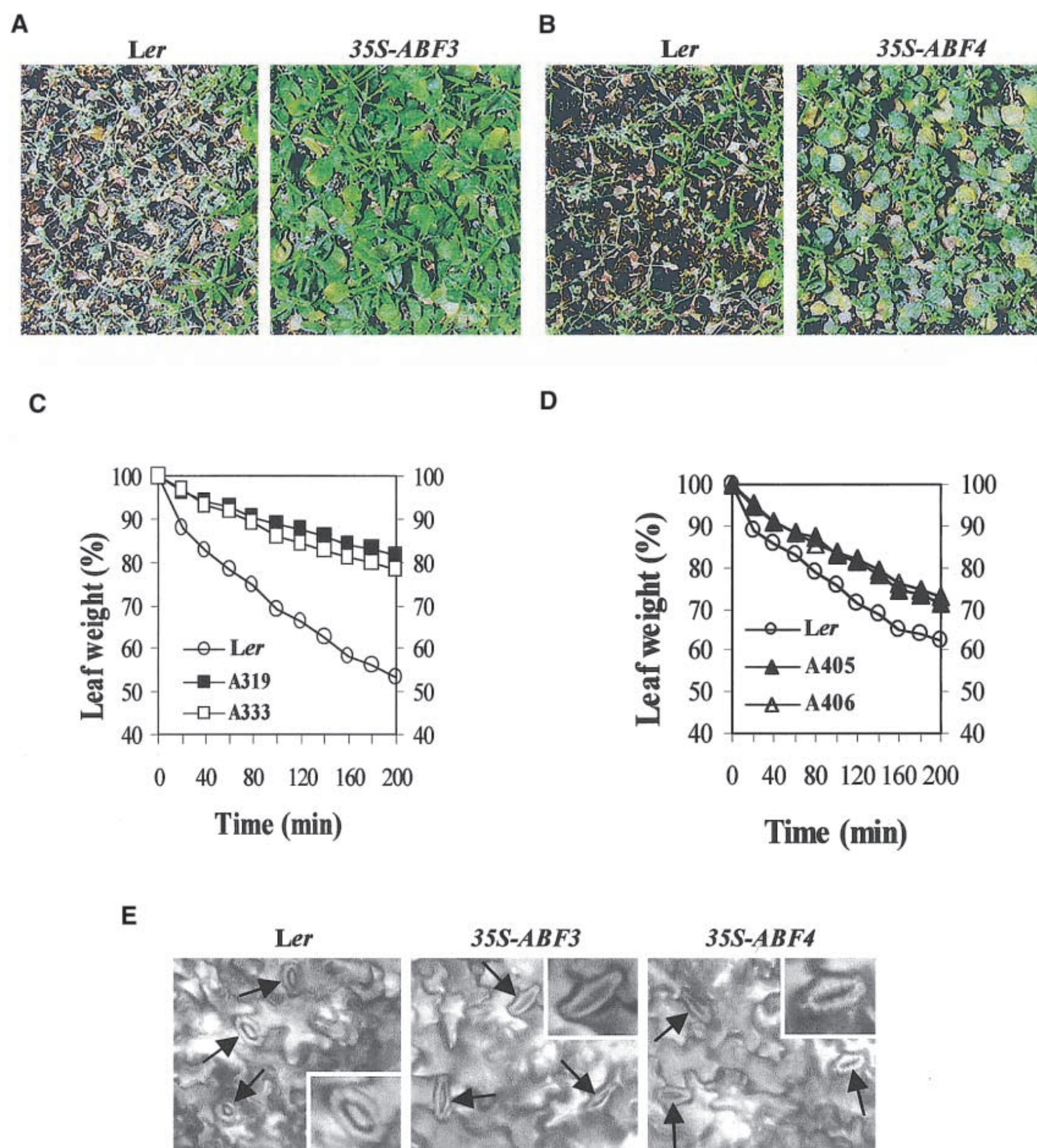

Figure 5. Drought Tolerance of *35S-ABF3* and *35S-ABF4* Plants.

(A) Drought tolerance of *35S-ABF3* transgenic plants (line A319). Transgenic and wild-type plants ($n = 100$ each) were grown on soil in the same container for 2 weeks, withheld from water for 11 days, and then rewatered. The photographs were taken 3 days after the rewatering.

(B) Drought tolerance of *35S-ABF4* transgenic plants (line A405). Plants at similar developmental stages (2-week-old wild-type plants and 3-week-old ABF4 plants) were withheld from water for 12 days and then rewatered. The photographs were taken 3 days after the rewatering.

(C) and **(D)** Transpiration rates of *35S-ABF3* and *35S-ABF4* transgenic plants, respectively. Leaves of similar developmental stages were excised and weighed at various times after the detachment. Each data point represents the mean of duplicate measurements ($n = 9$ each). Standard errors are smaller than the symbols.

(E) Stomatal aperture of ABF transgenic plants (lines A319 and A405). Stomatal guard cells were observed in the middle of the watering period. Arrows indicate guard cells, and the insets show representative stomata.

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promoter (2.1 kb) construct, GUS activity was undetectable in embryos, but it was observed in the emerging radicles at the germination stage (Figure 7B, a) and in most of the vegetative tissues at later stages (Figure 7B, b and c). Roots were stained most strongly except the tip area, and petioles, leaf vascular tissues, and guard cells exhibited relatively strong GUS activity (Figure 7B, b to f). Emerging shoots and younger leaves (Figure 7B, b and c), on the other hand, exhibited GUS staining only after ABA, salt, or mannitol treatment (Figure 7B, d). In mature plants, GUS staining was detected also in anthers, stigma, and siliques (abscission zone, replum, and funiculi) (Figure 7B, g and h).

The *ABF4* promoter was active in embryos from green siliques (Figure 7C, a), but its activity decreased as embryos became mature, and it was not detected in newly germinated seedlings except in some limited regions (radicle tip, shoot meristem region, and cotyledon tips) (Figure 7C, a). At later stages, starting from the stage of fully expanded cotyledons (Figure 7C, b), *ABF4* promoter activity was observed in all vegetative tissues (Figure 7C, c to f) and also in floral organs and siliques (abscission zone, replum, and funiculi) (Figure 7C, g and h). The *ABF4* promoter was most active in roots, especially in the growing regions (meristem, elongation zone, and lateral root primordia) (Figure 7C, e), suggesting its role in growth regulation. Also, it exhibited strong activity in petioles and guard cells (Figure 7C, c and f). Salt treatment of seedlings enhanced the *ABF4* promoter activity somewhat (Figure 7C, d).

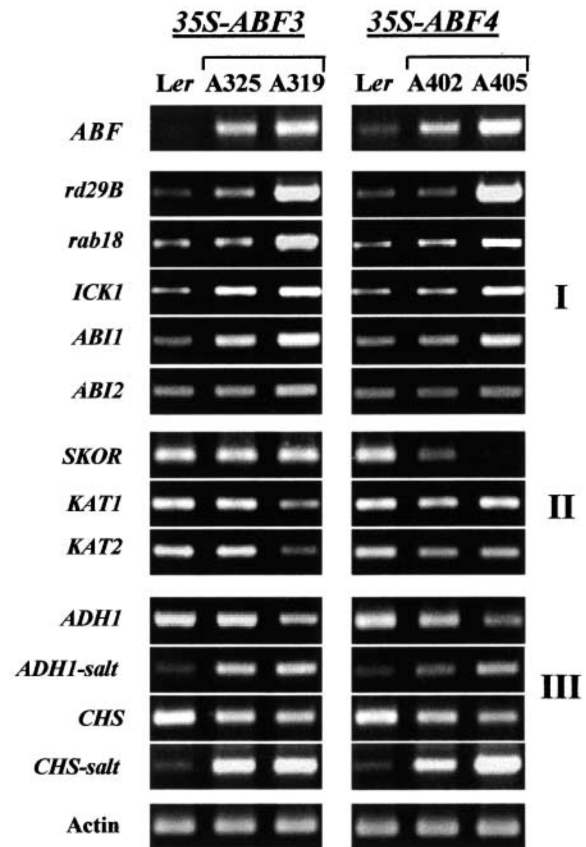

Figure 6. Expression of ABA-Regulated Genes in *35S-ABF* Transgenic Lines.

RNA levels of ABA-responsive genes were determined by coupled RT and PCR using total RNAs isolated from 2-week-old plants grown on MS plates. Lines A319 and A405 represent transgenic lines with higher *ABF* expression, whereas lines A325 and A402 represents transgenic lines with lower *ABF* expression.

DISCUSSION

ABA-regulated gene expression plays a central role in ABA signaling, and numerous ABA/stress-responsive genes are regulated by the (C/T)ACGTGGC- or CGCGTG-containing ABREs. Thus, identifying relevant transcription factors is critical for the delineation of ABA signal transduction cascades. Many studies show that ABA signaling pathways are tissue specific (Giraudat et al., 1994; Leung and Giraudat, 1998), and several seed-specific ABA signaling components (*ABI3*, *ABI4*, and *ABI5*) have been identified by genetic screens. *ABI3* and *ABI4* encode transcription factors (Giraudat et al., 1992; Finkelstein et al., 1998) whose binding sites and immediate target genes are unknown. Recently, *ABI5* has been shown to encode a bZIP factor that belongs to a seed-specific subfamily of ABF-related factors (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000), and its role in postgermination developmental arrest also has been demonstrated (Lopez-Molina et al., 2001). However, ABRE binding factors whose major function is to mediate ABA signaling during vegetative growth have not been reported, although numerous bZIP factors are known to interact with the ABREs in vitro (Foster et al., 1994).

ABFs are unique among the ABRE binding bZIP factors in that, unlike most of the other plant bZIP factors, they can interact with both the G-box type and the CGCGTG-containing ABREs (Choi et al., 2000). The broad binding specificity, together with the transactivation capability of an ABRE-containing reporter gene and the stress inducibility of their expression, suggested that ABFs have a potential to regulate a large number of ABA/stress-responsive genes and thus are likely to participate in stress-responsive ABA signaling. To address this question, we used an overexpression approach. Considering the potential functional redundancy of ABFs and numerous other bZIP factors interacting with ABREs (Foster et al., 1994), this approach would be better than loss-of-function approaches such as antisense, knockout, and RNA interference. The overexpression of ABFs (we estimate that *ABF3* and *ABF4* levels in the *35S-ABF* transgenic lines used in our study range from approximately two- to 10-fold of their

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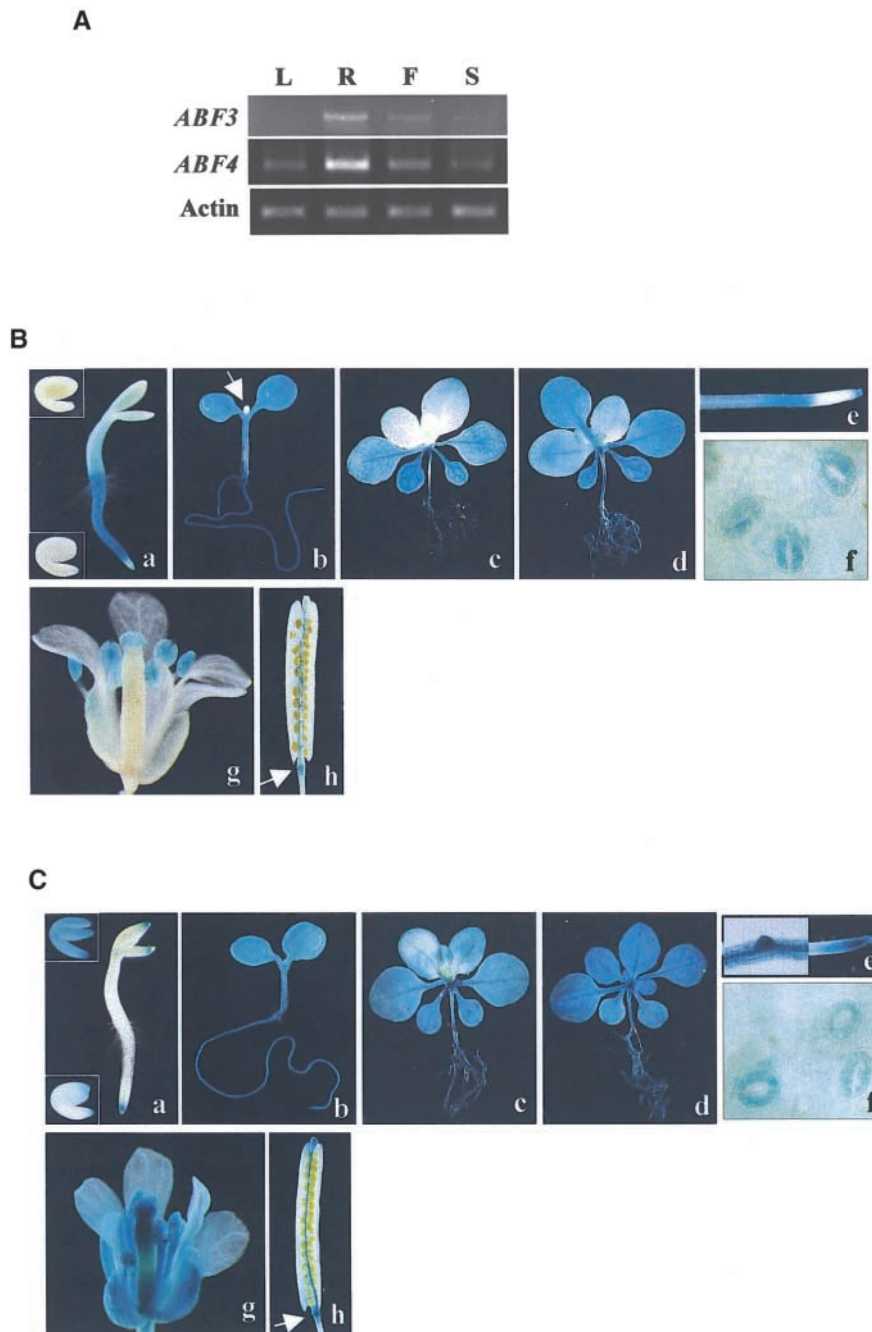

Figure 7. Expression Patterns of *ABF3* and *ABF4*.

(A) Tissue specificity of *ABF3* and *ABF4* expression. RNA was isolated from various tissues of wild-type plants grown under normal conditions, and the expression of *ABF3* and *ABF4* was determined by coupled RT and PCR. L, leaves from 3-week-old plants; R, roots from 3-week-old plants; F, flowers; S, immature siliques.

(B) and **(C)** Histochemical GUS staining of ABF3 and ABF4 promoter activity, respectively. T3 homozygous plants were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid for 6 hr (**[e]** and **[f]**) or 24 hr (other panels).

(a) Two-day-old seedlings. Insets show embryos from immature siliques (top) or dry seed (bottom).

(b) Five-day-old seedlings. The arrow in **(B)** shows the newly emerging shoot.

(c) Two-week-old seedlings.

(d) Two-week-old seedlings treated with 100 μM ABA (ABF3) or 200 mM NaCl (ABF4).

(e) Root tips. The left half of the image in **(C)** shows a lateral root primordium.

(f) Guard cells.

(g) Flowers.

(h) Siliques. Arrows indicate the silique abscission zone.

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ABA-induced levels), however, might have caused unnatural conditions. For example, genes that are not normally regulated by ABFs might have been turned on or off. Also, it may have affected the functions of other ABFs or potentially other bZIP factors by titrating them out via nonnatural heterodimerization. Thus, the overexpression phenotypes need to be interpreted with caution, and their roles can be further confirmed by other experimental means. Nevertheless, our results show that ABF3 or ABF4 overexpression conferred several ABA-associated phenotypes, such as ABA hypersensitivity, sugar hypersensitivity, and enhanced drought tolerance, with altered expression of ABA/stress-responsive genes. Thus, our data provide a strong in vivo case for the involvement of ABF3 and ABF4 in stress-responsive ABA signaling.

Whereas the ABA hypersensitivity conferred by ABF3 or ABF4 overexpression was very distinct and observed at both the germination and later growth stages (Figure 2), the overexpression effects on growth in the absence of exogenous ABA were either moderate (ABF3) or developmental stage dependent (ABF4) (Figure 1). ABF3 exerted an inhibitory effect on both germination and seedling growth. However, the low degree of inhibition compared with that in the presence of exogenous ABA suggests that ABF3 alone is not sufficient for the inhibitory function. On the other hand, ABF4 overexpression had little effect on germination but had a severe effect on seedling growth, suggesting that ABF4 activity is modulated developmentally. Alternatively, this result may indicate that ABA inhibition of seedling growth is mediated by a mechanism that differs from the germination inhibition mechanism. Also, the developmental stage dependence of the ABF4 effects implies that the growth retardation of *35S-ABF4* plants probably results from the constitutive operation of part of the ABA signal transduction cascades rather than from the pleiotropic effects of ABF4 overexpression. Except for the varying degrees of growth retardation, neither *35S-ABF4* nor *35S-ABF3* plants showed any abnormality in general development. Thus, their overexpression affected growth rate but not developmental processes.

Other ABA- or stress-associated phenotypes of *35S-ABF3* and *35S-ABF4* transgenic plants include their hypersensitivities to salt and glucose, and *35S-ABF4* plants exhibited additional phenotypes (i.e., epinasty of leaves and abnormal obstacle-touching response) that can be related to altered ethylene or auxin response. The salt and glucose hypersensitivities may reflect the increased sensitivity to high osmolarity, because both high salt and high sugar accompany increases in osmolarity. However, *35S-ABF* plants responded normally to mannitol, indicating that osmotic sensitivity was not affected. Thus, it appears that ABF3 and ABF4 are involved only in the nonosmotic branches of salt and glucose signaling pathways. The sugar-mediated developmental arrest is confined to a narrow window of developmental stages (∼2 days after germination) and is mediated by increased ABA level via an ABI4-dependent signaling cascade (Gazzarrini and McCourt, 2001). Also, it has been reported that ABA mediates developmental arrest at a similar stage and that the inhibition process requires ABI5 (Lopez-Molina et al., 2001), whose mutations result in weak sugar insensitivity. Thus, our results suggest that ABF3 and ABF4 have overlapping functions with ABI4 and ABI5 in mediating sugar- and ABA-induced developmental arrest. This is particularly so in the case of ABF3, because the onset of its expression coincides with the early developmental stage (Figure 7B, a).

Stomatal closure is a key ABA-controlled process in coping with water deficit conditions. Our data indicate that ABF3 and ABF4 are involved in this process. Their overexpression resulted in lower transpiration and enhanced drought tolerance (Figure 5), which are reminiscent of the phenotypes of the ABA-hypersensitive mutant *era1* (Pei et al., 1998). Furthermore, the stomatal openings of *35S-ABF* transgenic plants were smaller than those of wild-type plants, and altered expression of several genes involved in stomatal aperture regulation has been observed in transgenic plants (Figure 6). Among the genes we investigated, *ABI1* was the most strongly affected, especially in ABF3 overexpression lines. ABI1 is known to be a negative regulator of ABA signaling (Gosti et al., 1999), although its expression is enhanced by ABA and high osmolarity and reduced in the *aba1* and *abi1* mutants (Leung et al., 1997). It is not clear, though, whether the increased *ABI1* level played a positive or a negative role in the stomatal closing. Whatever ABI1's role might be, our results indicate that *ABI1* expression is subject to ABF3 regulation and that ABF3 and ABF4 overexpression affected the expression of genes involved in stomatal movement and/or guard cell ABA signaling (Schroeder et al., 2001), the net result of which was enhanced stomatal closure.

The transcript level changes of ABA-responsive genes in *35S-ABF* transgenic lines demonstrate that ABF3 and ABF4 function as transcriptional regulators in planta. As shown in Figure 6, both positive and negative changes were observed depending on specific genes. This result suggests that different subsets of ABF3 and ABF4 target genes are regulated by different mechanisms. Also, the negative regulation of some genes under normal growth conditions and the positive regulation of the same genes after salt treatment (Figure 6, group III) suggest that stress-induced modification of ABF activities is required for the regulation of these genes. The modifying activity may be limiting under normal conditions. Thus, the negative regulation of some genes can be explained, for example, by the binding of transcriptionally inactive, unmodified ABFs, whose proportion increases with higher ABF levels. The modification may involve phosphorylation of ABFs. The involvement of kinase/phosphatases in ABA/stress signaling is well known (Leung and Giraudat, 1998), and several phosphorylation sites are highly conserved among ABFs (Choi et al., 2000). More recently, Uno et al. (2000) reported an ABA-activated kinase activity in cultured cells that phosphorylates AREB1 (ABF2) and AREB2 (ABF4). Alternatively, the modification may involve interaction with other regulatory proteins.

The expression patterns of ABF3 and ABF4 were consistent with the functions suggested by their overexpression

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phenotypes. Spatially, both promoters were most active in roots and guard cells, consistent with their roles during water stress response. Also, ABF3, which exerted only minor growth inhibition, was expressed weakly in the growing tissues (root tips, new shoots, and new leaves), whereas ABF4 was expressed strongly in the growing regions of roots (meristem, elongation zone, and lateral root primordia). Temporally, strong ABF3 promoter activity was observed in the newly germinated seedlings, consistent with its more pronounced effect on germination (Figures 1C and 2C). On the other hand, the ABF4 promoter exhibited major activity at the onset of seedling growth, in agreement with its severe effect on seedling growth. Our results also show that both ABF3 and ABF4 might function during reproductive stages and seed abscission. Both promoters exhibited strong activity in the abscission zone, replum, and funiculi of siliques, and relatively strong activity was detected in stigma and anthers.

The isolation of multiple factors with similar binding activities but different expression patterns suggested that the ABA/stress signaling involving the ABREs is likely to be mediated by multiple factors (Choi et al., 2000). Our current results further support this observation. Although their overexpression phenotypes and expression patterns were similar, ABF3 and ABF4 were different from each other in several respects. The details of their temporal and spatial expression patterns differed (Figure 7). Growth retardation, root growth inhibition, and impaired stimulus-touching response were more prominent in ABF4 transgenic lines. Also, minor differences were observed at the molecular level. *ABI1* and *ABI2* expression levels were higher in *35S-ABF3* transgenic lines, whereas downregulation of *SKOR* expression was observed only in *35S-ABF4* plants. The functions of other ABFs (ABF1 and ABF2) remain to be determined, but their roles appear to be quite different from each other and from those of ABF3 and ABF4, according to our preliminary data. Thus, ABRE-dependent ABA/stress signaling in vegetative tissues appears to be mediated by multiple factors with overlapping but distinct functions.

As mentioned above, extensive genetic studies have been performed and a number of ABA signaling components have been identified (Leung and Giraudat, 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). However, none of ABFs has been isolated in these genetic screens, although they are highly homologous with ABI5, and our results strongly suggest that ABF3 and ABF4 are involved in ABA signaling. This is probably because of the selection criteria used in the mutant screenings. In most of the genetic studies, mutants were selected based on their altered sensitivities to ABA during germination. Thus, mutants that specifically affect vegetative ABA signaling might have been bypassed. Our observations that ABF3 and ABF4 are expressed mainly in vegetative tissues and that their overexpression effects on germination are moderate or insignificant support this hypothesis. Functional redundancy among ABFs (see above) also may have contributed to the negative results.

Table 1. Coupled RT and PCR Primers

Gene Name	Sequence (5′ to 3′)
Actin	F: cat cag gaa gga ctt gta cgg
	R: gat gga cct gac tcg tca tac
ABF3	F: aga acc tca acc ggt gga gag tg
	R: gga gtc aga tca ggt gac atc tgg
ABF4	F: aac tgt gtt caa cag atg ggt cag
	R: ggt tcc tcc gta act agc taa tcc
rd29B	F: gtg aag atg act atc tcg gtg gtc
	R: gcc taa ctc tcc ggt gta acc tag
rab18	F: atg acg agt acg gaa atc cga tgg
	R: tat gta tac acg att gtt cga agc
ICK1	F: acg cac acg taa cct aaa tcg
	R: gca tct ccg tca tca att tcg
ABI1	F: tca aga ttc cga gaa cgg aga tc
	R: gag gat caa acc gac cat cta ac
ABI2	F: gtt ctt gtt ctg gcg acg gag c
	R: cca tta gtg act cga cca tca ag
rd29A	F: gat aac gtt gga gga aga gtc ggc
	R: cag ctc agc tcc tga ttc act acc
SKOR	F: atg gga ggt agt agc ggc ggc
	R: gat tct ctg gta atc ccc tga ag
KAT1	F: ttc tgc gtc gag gaa tac aat ata g
	R: ctt agg gtc aac tag aag ata g
KAT2	F: aca caa gac caa tgt caa tct ctt g
	R: gtc gac tag aag ata tga gtg gc
ADH1	F: tcc acg tat ctt cgg cca tg
	R: tag cac ctt ctg cag cgc c
CHS	F: tca cca aca gtg aac aca tga cc
	R: gag tca agg tgg gtg tca gag g

F, forward primer; R, reverse primer.

METHODS

Arabidopsis Growth

Arabidopsis thaliana ecotype Landsberg *erecta* (L*er*) was used in this study. *aba1-1*, *abi1-1*, and *abi2-1* seed (Koornneef et al., 1982, 1984) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus), and their phenotypes were confirmed before use.

Plants were grown at 22°C under long day conditions (16-hr-light/8-hr-dark cycle) aseptically or on soil. For soil growth, seed were sown on a 1:1:1 mixture of vermiculite, perlite, and peat moss irrigated with 0.1% Hyponex (Hyponex Co., Marysville, OH) placed at 4°C for 4 days in the dark to break residual dormancy, and transferred to normal growth conditions. Unless stated otherwise, the plants were watered once per week. For aseptic growth, seed were treated 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) solidified with 0.8% phytoagar. The MS medium was supplemented with 1% sucrose and, as described in Results, with abscisic acid, salts, glucose, or mannitol as needed. For the germination test, seed collected at the same or

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similar times were used. For root growth measurements, plants were germinated and grown in the vertical position.

Constructs and Arabidopsis Transformation

The *35S* promoter–*ABF* coding region constructs (*35S-ABF3* and *35S-ABF4*) were prepared by replacing the β-glucuronidase (GUS) coding region of pBI121 (Jefferson et al., 1987) with the coding region of *ABF3* or *ABF4*. The *GUS* sequence was removed after BamHI–SacI digestion, and after T4 DNA polymerase treatment to remove the 3′ overhang, the remaining portion of pBI121 was ligated with the *ABF3* or *ABF4* coding region, which was prepared by polymerase chain reaction (PCR) followed by BamHI digestion. The *ABF* coding regions included their entire coding regions with the stop codons, and a BamHI linker sequence was attached in front of the initiation codons for cloning. The *ABF* promoter–*GUS* reporter fusions were prepared by inserting 2.1 kb (ABF3) or 1.2 kb (ABF4) of their 5′ flanking sequences from the initiation codons in front of the GUS reporter gene of pBI101.2 (Jefferson et al., 1987).

The promoter fragments were prepared by PCR using Arabidopsis (ecotype Columbia) genomic DNA as a template and the primer sets 5′-caaacttaccctgttgttgcaact-3′ and 5′-ctagtctagaaggatcaagcttctggatatttac-3′ for *ABF3* and 5′-gatcaatttgaatttttgatatacatc-3′ and 5′-ctagtctagattcaatgaaaacaaagcatccaag-3′ for *ABF4*. The PCR fragments were digested with XbaI and ligated with pBI101.2, which was prepared by HindIII digestion followed by Klenow treatment and XbaI digestion. DNA manipulation was according to standard procedures (Sambrook et al., 1989; Ausubel et al., 1994), and the intactness of the *ABF* coding regions and the junction sequences was confirmed by DNA sequencing.

Transformation of Arabidopsis was according to the vacuum infiltration method (Bechtold and Pelletier, 1998) using *Agrobacterium tumefaciens* strain GV3101. For the phenotypic investigation, T3 or T4 homozygous lines were used. GUS staining patterns were confirmed by observing at least five different transgenic lines, and T3 homozygous lines were used for detailed analysis.

RNA Isolation, Coupled Reverse Transcription and PCR, and RNA Gel Blot Analysis

RNA was isolated by the method of Chomczynski and Mackey (1995), with a minor modification (Choi et al., 2000). RNA gel blot analysis and coupled reverse transcription (RT)-PCR were performed as described (Choi et al., 2000) with the following modifications. For RNA gel blot analysis, hybridization was performed at 65°C in the Rapid-hyb buffer from Amersham Pharmacia Biotech. Exposure time was 6 hr (ABF3) or 20 hr (ABF4). RT-PCR was performed using the Access RT-PCR System from Promega or the Superscript One-Step RT-PCR System from Gibco BRL. Each RT-PCR result was confirmed by several independent reactions, and RNA preparations were confirmed to be free of contaminating genomic DNA using primer sets spanning introns whenever possible. Primers used in the RT-PCR reactions are listed in Table 1.

Histochemical GUS Staining

In situ assay of GUS activity was performed as described by Jefferson et al. (1987). Whole plants were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid 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, and after applying vacuum for 5 min, they were incubated at 37°C for the indicated times (see Figure 7). Chlorophyll was cleared from the plant tissues by immersing them in 70% ethanol.

Drought Treatment and Measurement of Transpiration Rate

For drought treatment, 3-week-old soil-grown plants were withheld completely from water for the specified times. To minimize experimental variations, the same numbers of plants was grown on the same tray. With *35S-ABF4* plants, which show growth retardation, two batches of plants, one of the same age and the other of similar developmental stages (i.e., wild-type seed were sown 7 days later so that they were at similar developmental stages at the end of the treatment) were tested, and similar results were obtained. The entire test was repeated at least four times, sometimes using different arrangements of plants (i.e., test plants on different containers, etc.), and the results were consistent. The transpiration rate of detached leaves was measured by weighing freshly harvested leaves placed abaxial side up on open Petri dishes on the laboratory bench. Leaves of similar developmental stages (third to fifth true rosette leaves) from 3-week-old soil-grown plants were used.

Guard Cells

To examine guard cells, leaves were excised from 3-week-old soil-grown plants in the middle of the watering (3 days after watering) and light periods. Leaves of similar developmental stages (third to sixth true rosette leaves) from 20 different plants of wild-type and transgenic lines were placed on slides abaxial side up immediately after excision, and photographs were taken. The number of guard cells then was counted in the randomly chosen fields, usually six to seven.

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