Arabidopsis Cys2/His2 Zinc-Finger Proteins AZF1 and AZF2 Negatively Regulate Abscisic Acid-Repressive and Auxin-Inducible Genes under Abiotic Stress Conditions[1][W][OA]

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In plants, abiotic stresses induce various physiological changes and growth inhibition that result in adaptive responses to these stresses. However, little is known about how such stresses cause plant growth inhibition. Many genes have been reported to be repressed in plants under abiotic stress conditions. ZPT2 (for petunia [*Petunia hybrida*] zinc-finger protein 2)-related proteins with two Cys2/His2-type zinc-finger motifs and an ethylene-responsive element binding factor-associated amphiphilic repression motif are thought to function as transcriptional repressors. To characterize the roles of this type of transcriptional repressor under abiotic stress conditions, we analyzed the functions of two Arabidopsis (*Arabidopsis thaliana*) *ZPT2*-related genes that were induced by osmotic stress and abscisic acid: *AZF1* (for Arabidopsis zinc-finger protein 1) and *AZF2*. The nuclear localization of these two proteins was observed in the roots under control conditions, and the accumulation of AZF2 was clearly detected in the nuclei of leaf cells under stress conditions. Transgenic plants overexpressing *AZF1* and *AZF2* were generated using stress-responsive promoters or the GVG chemical induction system. The overexpression of these genes caused severe damage to plant growth and viability. Transcriptome analyses of the transgenic plants demonstrated that AZF1 and AZF2 repressed various genes that were down-regulated by osmotic stress and abscisic acid treatment. Moreover, many auxin-responsive genes were found to be commonly down-regulated in the transgenic plants. Gel mobility shift assays revealed that both the AZF1 and AZF2 proteins bound to the promoter regions of these down-regulated genes. These results indicate that AZF1 and AZF2 function as transcriptional repressors involved in the inhibition of plant growth under abiotic stress conditions.

Plants are exposed to various environmental stress conditions, such as drought, high salt, and low temperature. In response to these stresses, plants have evolved a number of mechanisms to achieve an optimal adaptation to adverse conditions. Transcriptional modulation is thought to be one of the most important ways that plants respond and adapt to stress conditions. A number of genes have been reported to be induced or repressed in plants under stress conditions (Kreps et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Various transcription factors have been shown to be involved in stress responses, such as those from the dehydration-responsive element-binding protein (DREB), ethylene-responsive element binding factor (ERF), zinc-finger, WRKY, MYB, basic helix-loop-helix, and basic domain-leucine zipper (bZIP) families. These transcription factors function as transcriptional activators or repressors and control downstream gene expression in stress signal transduction pathways (Chen et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006).

The plant hormone abscisic acid (ABA) plays important roles in the acquisition of dehydration and desiccation tolerance in vegetative tissues and during seed development (Finkelstein et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). ABA treatment

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induces not only stress tolerance but also growth inhibition in plants. Many of the drought- or salt stress-inducible genes are also regulated by ABA (Yamaguchi-Shinozaki and Shinozaki, 2006). A conserved cis-element known as the ABA-responsive element (ABRE) has been identified in the promoters of ABA-inducible genes (Busk and Pagès, 1998; Leung and Giraudat, 1998). Although various genes are induced by ABA, many ABA-down-regulated genes have also been reported (Hoth et al., 2002; Seki et al., 2002; Takahashi et al., 2004).

Recently, transcriptional repressors have been found to play important roles in modulating plant defenses against biotic and abiotic stresses (Kazan, 2006). In plants, the ERF-associated amphiphilic repression (EAR) motif (L/FDLNL/FXP) containing a DLN box was first identified in the C-terminal regions of the class II ERF transcription factors and the Cys2/His2-type zinc-finger proteins. This motif was shown to be essential for the repressive activity of these transcription factors (Ohta et al., 2001). Recently, it was also reported that the EAR motifs of the Novel Interactor of JAZ transcriptional repressor and the Auxin/Indole-3-Acetic Acid (AUX/IAA) proteins are responsible for the interaction between those proteins and the Groucho/Tup1-type corepressor TOPLESS (Szemenyei et al., 2008; Pauwels et al., 2010). Many Cys2/His2-type zinc-finger proteins have functional EAR motifs and are thought to function as transcriptional repressors (Ohta et al., 2001).

The Cys2/His2-type zinc finger, which is also referred to as the classical or TFIIIA-type finger, is one of the best-characterized DNA-binding motifs among the eukaryotic transcription factors (Laity et al., 2001). This motif can be represented as $CX_{2-4}CX3FX5LX2HX_{3-5}H$ and consists of approximately 30 amino acids and two pairs of conserved Cys and His residues that bind tetrahedrally to a zinc ion (Pabo et al., 2001). Many Cys2/His2-type zinc-finger proteins in plants have structural features that are unique to plant zinc-finger proteins (Takatsuji, 1999). Most fingers have a highly conserved QALGGH motif in the zinc-finger helices. In multiple-fingered proteins, each zinc-finger motif is separated by long spacers with a variety of lengths and sequences; in yeast and animals, Cys2/His2-type fingers are mostly clustered and separated by a short spacer (six to eight amino acids) known as an HC link (Klug and Schwabe, 1995). The plant zinc-finger proteins are thought to recognize target sequences and to regulate gene expression in a plant-specific manner (Takatsuji, 1999; Sakamoto et al., 2004).

The first Cys2/His2-type zinc finger protein found in plants as the DNA-binding protein was ZPT2-1 (previously named EPF1) in petunia (*Petunia hybrida*; Takatsuji et al., 1992). *ZPT2*-related genes encode two-fingered proteins and include 14 members in petunia (Kubo et al., 1998; Takatsuji, 1999). Many other ZPT2-related zinc-finger proteins have been reported in different plant species, including wheat (*Triticum aestivum*), petunia, Arabidopsis (*Arabidopsis thaliana*), and rice (*Oryza sativa*). One of the Arabidopsis *ZPT2*-related genes, *STZ/ZAT10* (for salt tolerance zinc finger/zinc finger of Arabidopsis 10), was cloned by complementation of the salt-sensitive phenotype of a yeast calcineurin mutant, and its expression conferred salt tolerance in wild-type yeast (Lippuner et al., 1996). The expression of *STZ* has been shown to respond to drought, salt, cold, and ABA (Sakamoto et al., 2000; Gong et al., 2001; Lee et al., 2002). The constitutive expression of *STZ* was found to result in growth suppression and an enhanced adaptation of plants to drought and osmotic stresses (Sakamoto et al., 2004; Mittler et al., 2006). *AZF1* (for Arabidopsis zinc-finger protein 1) and *AZF2* belong to a subset of the Arabidopsis *ZPT2*-related genes and are closely related to *STZ* in structure (Englbrecht et al., 2004). Their expression is induced by drought, salt, and ABA (Sakamoto et al., 2000). AZF1 and AZF2 both contain a functional EAR motif and appear to function as transcriptional repressors, as does STZ (Ohta et al., 2001; Lee et al., 2002; Sakamoto et al., 2004). These proteins bind to A(G/C)T repeats within an EP2 sequence that is known to be a target sequence of several petunia ZPT2 proteins (Sakamoto et al., 2004). The preceding reports have suggested potential roles for AZF1 and AZF2 under abiotic stress conditions, thus necessitating further characterization of their functions as transcriptional repressors.

In this study, we analyzed the roles of AZF1 (At5g67450) and AZF2 (At3g19580) during abiotic stresses using transgenic plants expressing *AZF1* and *AZF2* under the control of stress-responsive or glucocorticoid-inducible promoters. We found that AZF1 and AZF2 repressed the expression of various genes, including osmotic stress- and ABA-repressive genes and auxin-inducible genes.

RESULTS

STZ Family Genes Are Conserved across Divergent Plant Species and Are Differentially Expressed under Various Stress Conditions

STZ/ZAT10 shares a high level of homology with five proteins among the 18 members of the *ZPT2*-related genes that encode proteins with two Cys2/His2-type zinc fingers in Arabidopsis: AZF1, AZF2, AZF3, ZAT6, and ZAT8 (Supplemental Fig. S1, A and B; Englbrecht et al., 2004; Sakamoto et al., 2004; Ciftci-Yilmaz and Mittler, 2008). We collectively named these six genes the *STZ* family. To determine whether *STZ*-related genes are conserved in plants other than Arabidopsis, we searched for orthologous sequences in angiosperms, gymnosperms, pteridophytes, and bryophytes using the Phytozome (http://www.phytozome.net/index.php) and National Center for Biotechnology Information BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) services. Many genes that were orthologous to the *STZ* family were found in these plants, and a neighbor-joining tree analysis was performed based on the sequences of three protein regions: the L box,

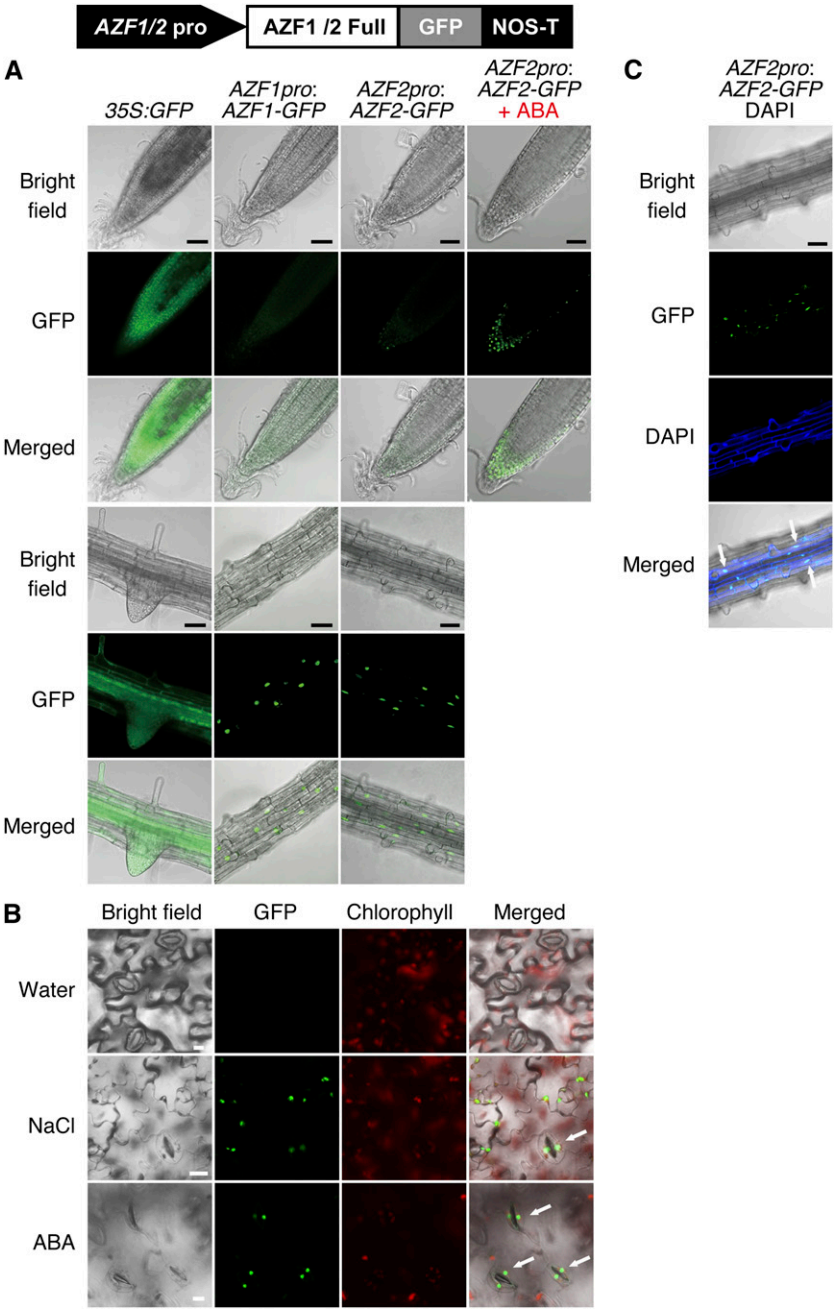

Figure 1. Induction and cellular localization of the AZF1-GFP and AZF2-GFP proteins in the roots and leaf epidermis. A, Confocal images of GFP fluorescence in the roots of transgenic Arabidopsis plants harboring the *35Spro:GFP*, *AZF1pro:AZF1-GFP*, and *AZF2pro:AZF2-GFP* constructs. Two-week-old plants were treated with or without 100 μM ABA for 5 h. Bars = 50 μm. B, Confocal images of GFP fluorescence in the leaf epidermis of *AZF2pro:AZF2-GFP* plants. Two-week-old plants were treated with liquid MS medium with or without 150 mM NaCl or 100 μM ABA for 30 and 10 h, respectively. Arrows indicate the guard cells. Bars = 10 μm. C, Root cells of *AZF2pro:AZF2-GFP* plants stained with DAPI were photographed using differential interference contrast, GFP fluorescence, and DAPI fluorescence microscopy. The arrows indicate the nuclei. Bar = 50 μm.

two zinc fingers, and the DLN box (Supplemental Figs. S1A and S2). The tree showed that the *STZ* family-type genes were conserved across divergent plant species, especially in angiosperms, and that they were likely to be evolutionarily separated from the other homologs in Arabidopsis and their orthologs (Supplemental Fig. S2).

In this study, we initially focused on *AZF1*, *AZF2*, and *ZAT8* because we had already reported the functions of *STZ/ZAT10* under stress conditions (Sakamoto et al., 2004). We used northern-blot analysis to compare the expression patterns of the six genes of the *STZ* family in response to various stresses (Supplemental Fig. S3). In agreement with previously reported results (Sakamoto et al., 2004), the expression of *AZF1* and *AZF2* was induced by drought, salt, and ABA treatment, although the amount of *AZF1* transcripts was relatively small. However, the expression level of *ZAT8* was extremely low, and we could not detect a clear induction of *ZAT8* under stress conditions. Therefore, we performed further research on *AZF1* and *AZF2*.

Subcellular Localization Analysis of the AZF1 and AZF2 Proteins

The AZF1 and AZF2 proteins have been shown to localize to the nucleus (Sakamoto et al., 2004). We

confirmed that all of the STZ-group proteins localized to the nucleus in Arabidopsis protoplasts (Supplemental Fig. S4). To identify the tissue specificity of the AZF1 and AZF2 proteins under nonstress and stress conditions in plants, we generated transgenic Arabidopsis plants that contained the *AZF1-GFP* and *AZF2-GFP* fusion genes driven by unique promoters (*AZF1pro*:*AZF1-GFP* and *AZF2pro*:*AZF2-GFP*) and analyzed the resulting fluorescence following exposure to nonstress and stress conditions. In both transgenic plants, the fluorescence was localized to the nuclei of the roots under nonstress conditions and was not detected in any aerial plant parts (Fig. 1A). The fluorescent signals of each protein were predominantly detected in the zones of maturation and were not observed in the zones of division and elongation in roots. In contrast, the AZF2-GFP signals were detected in the root tips when the plants were treated with exogenous ABA. The AZF2-GFP signals were also observed in leaves when the plants were treated with high salt and ABA (Fig. 1B). When the plants were exposed to high-salt stress, nuclear localization of the AZF2-GFP protein was observed in both epidermal and guard cells. However, in response to ABA treatment, the nuclear localization was observed mainly in guard cells. In root epidermal cells, colocalization of the AZF2-GFP signals with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI)-stained nuclear DNA was observed (Fig. 1C).

We also examined the tissue specificity of *AZF1* and *AZF2* expression by evaluating the expression of a *GUS* reporter gene driven by the *AZF1* and *AZF2* promoters in transgenic plants in response to drought and ABA treatment. A histochemical analysis of GUS activity in rosette plants revealed that *AZF1* and *AZF2* were expressed mainly in roots, excluding the zones of division and elongation, under normal growth conditions (Supplemental Fig. S5A). *AZF1* expression was restricted to roots under both stress and nonstress conditions. The expression of *AZF2* was weakly detected in the cotyledons, even in the absence of stress, and was induced in the rosette leaves in response to drought stress. *AZF2* expression was also detected in the flowers, specifically in the petals and stamens, irrespective of drought stress (Supplemental Fig. S5B). *AZF2* promoter activity was induced in the roots and aerial plant parts by the application of exogenous ABA (Supplemental Fig. S5C). In the roots, a marked induction of *AZF2* expression by ABA was detected in the zones of division. In the leaves, ABA enhanced the expression of *AZF2* in the guard cells to a greater extent than did the other treatments.

Effects of *AZF1* and *AZF2* Gene Overexpression in Transgenic Plants

To investigate the roles of AZF1 and AZF2 in plants under stress conditions, we previously attempted to generate transgenic Arabidopsis plants overexpressing *AZF1* or *AZF2* under the control of an enhanced 35S promoter (Sakamoto et al., 2004). However, the transformation efficiency of these plants was extremely low, and we obtained neither *AZF1*- nor *AZF2*-overexpressing plants (Sakamoto et al., 2004). Therefore, we decided to generate transgenic plants overexpressing *AZF1* or *AZF2* driven by a stress-inducible *RD29A* promoter (Kasuga et al., 1999). To evaluate the expression levels of *AZF1* and *AZF2* in the transgenic plants under high-salt conditions, 16 independent transgenic lines for each transgenic plant were subjected to RNA gel-blot analyses. Three lines from each transgenic plant in the T2 generation that demonstrated elevated salt-induced expression levels of *AZF1* or *AZF2* were selected for further analyses (Fig. 2A). Moreover, we generated transgenic plants that overexpressed *AZF2* under the control of its own promoter. From 24 independent lines, we selected T2 lines that exhibited higher expression levels of *AZF2* under salt-stress conditions compared with the vector control. Finally, we selected three stable lines from the T3 generation of the *AZF2* transformants for further studies (Fig. 2B). Under nonstress conditions, these *RD29Apro*:*AZF1*, *RD29Apro*:*AZF2*, and *AZF2pro*:*AZF2* plants displayed similar growth and morphological phenotypes (Supplemental Fig. S6). The salt sensitivities of these three transgenic plants were compared with those of the vector control plants. All of the transgenic plants displayed salt-hypersensitive phenotypes (Fig. 2, C–E). Whereas more than 60% of the vector control plants survived after salt-stress treatments, the survival rates of the *RD29Apro*:*AZF1* and *RD29Apro*:*AZF2* plants were less than 35% (Fig. 2, F and G). Up to 25% of the *AZF2pro*:*AZF2* plants survived, an approximately 40% decrease compared with the vector control plants (Fig. 2H). These phenotypes appeared to depend on the levels of *AZF1* and *AZF2* expression. We also generated transgenic *AZF1pro*:*AZF1* (T3 generation) plants in a manner similar to that described for the *AZF2pro*:*AZF2* plants. However, the levels of salt-induced *AZF1* expression in these transgenic plants were not significantly higher than those detected in the vector control plants (data not shown), which is consistent with the low inducibility of *AZF1* observed under various stress conditions, as shown in Supplemental Fig. S3. We were unable to detect clear differences in the extent of sensitivity to salt stress between the *AZF1pro*:*AZF1* and vector control plants (data not shown).

Next, we generated transgenic plants carrying *AZF1* and *AZF2* under the control of a glucocorticoid-inducible promoter (Aoyama and Chua, 1997). Treatment with 10 μM dexamethasone (DEX) was used to induce expression in these plants. Three independent lines that demonstrated high DEX-induced *AZF1* (*pTA7002*:*AZF1*) and *AZF2* (*pTA7002*:*AZF2*) expression were selected for further analyses (Fig. 2I). The growth of the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants on germination medium (GM) agar plates supplemented with or without 1 μM DEX after sowing was compared with that of the vector control plants. Both of the transgenic lines on DEX-containing plates displayed

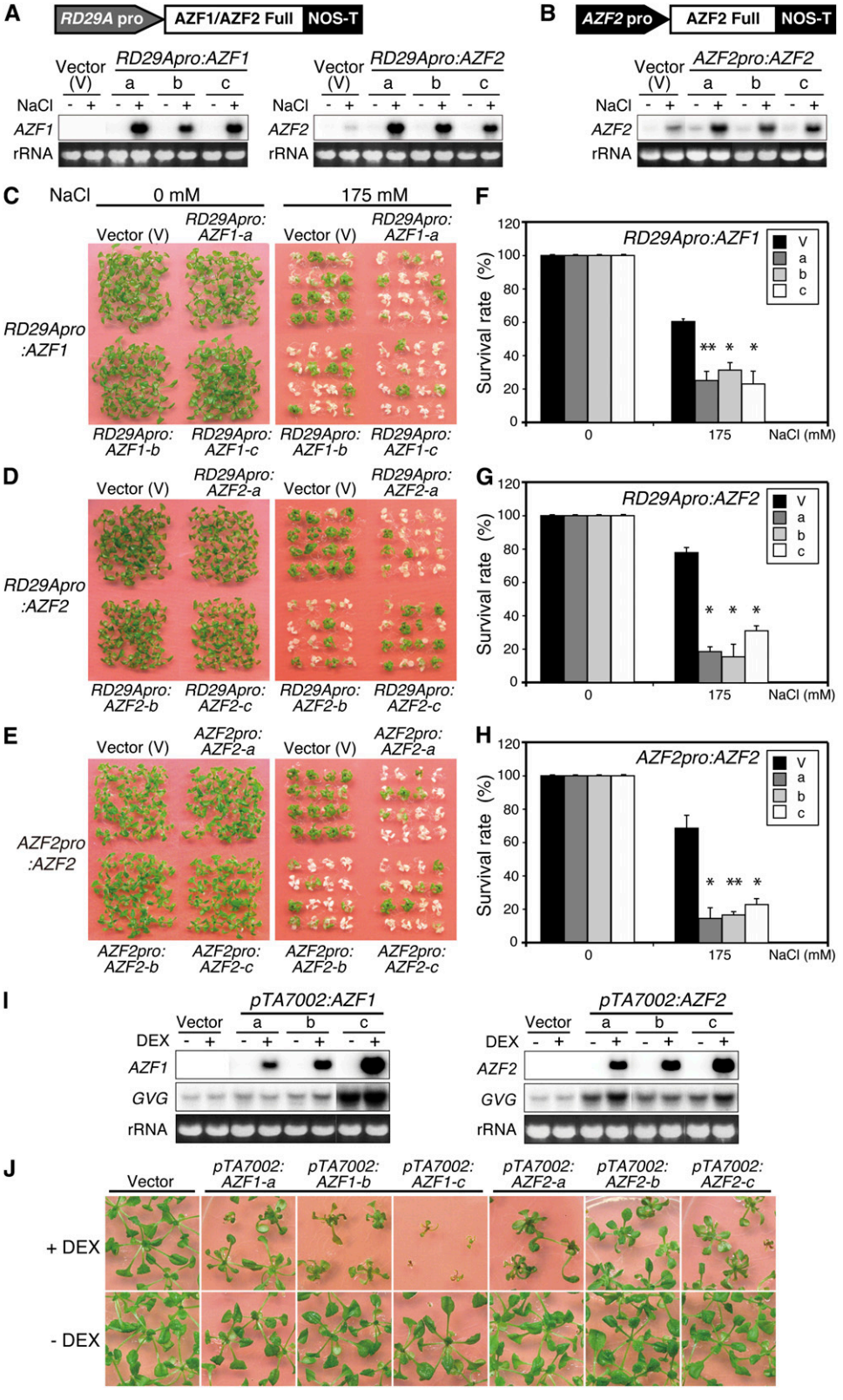

Figure 2. Morphological phenotypes and high-salt sensitivity of plants overexpressing *AZF1* or *AZF2*. A and B, Expression of *AZF1* in *RD29Apro:AZF1* and vector control plants and expression of *AZF2* in *RD29Apro:AZF2*, *AZF2-pro:AZF2*, and vector control plants. Each lane contained 20 μg of total RNA from 2-week-old plants that had been treated with or without 200 mM NaCl. rRNAs are shown as equal loading controls. C to E, Evaluation of salinity stress in plate conditions. Plants grown on GM plates were transferred onto a MS medium plate supplemented with or without 175 mM NaCl. The plates were observed for approximately 9 to 10 d after the transfer. F to H, Percentages of the plants that survived and SD (error bars) calculated from three independent experiments ($n > 30$). The asterisks indicate significantly lower survival rates compared with the vector control as determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$). I, Expression of *AZF1* and *AZF2* in the *pTA7002:AZF1*, *pTA7002:AZF2*, and vector control plants. Each lane contained 20 μg of total RNA from 3-week-old plants that had been treated with or without 10 μM DEX. rRNAs are shown as equal loading controls. J, Growth phenotypes of *pTA7002:AZF1*, *pTA7002:AZF2*, and vector control plants after 2 weeks of growth on GM agar plates supplemented with or without 1 μM DEX.

dwarfed growth with small curled leaves, but no phenotypes that were clearly different from the wild type were observed in the absence of DEX (Fig. 2J). For the salt-sensitivity tests, plants grown on GM agar plates were transferred to 0.5× Murashige and Skoog (MS) agar plates containing 1 μM DEX or 0.01% ethanol

with or without NaCl. In the absence of DEX, no clear differences between the vector control and the transgenic plants were observed, irrespective of the application of NaCl treatment. In contrast, these transgenic plants exhibited severe salt-sensitive phenotypes following treatment with a combination of DEX and 175 mM NaCl, which indicated that the overexpression of *AZF1* and *AZF2* enhanced salt sensitivity in the transgenic plants (Supplemental Fig. S7).

AZF1 and AZF2 Down-Regulate Many ABA- and Osmotic Stress-Repressive Genes

To unravel the transcriptional networks of *AZF1* and *AZF2*, we first compared the expression profiles of the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants with those of the vector control plants using the Arabidopsis 3 Oligo Microarray (Agilent Technologies), which contains more than 44,000 genes. For the microarray analyses, we used RNA extracted from plants treated with or without 10 μM DEX for 24 h. In the *pTA7002*:*AZF1* plants, a total of 468 and 1,076 genes were down-regulated and up-regulated with a ratio of greater than 2, respectively. In the *pTA7002*:*AZF2* plants, 1,672 and 1,774 genes were down-regulated and up-regulated with a ratio of greater than 2, respectively. Because these zinc-finger proteins have been reported to function as transcriptional repressors (Ohta et al., 2001; Sakamoto et al., 2004), we focused on the genes for which expression was repressed in the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants. Using Genevestigator (https://www.genevestigator.com/gv/index.jsp), we determined the expression profiles of the down-regulated genes in the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants. The top 100 down-regulated genes in the respective overexpressors included many genes that demonstrated reduced expression levels in response to osmotic stresses and/or ABA (Fig. 3A). Thus, many stress-repressive genes were down-regulated in both transgenic plants overexpressing *AZF1* and those overexpressing *AZF2*. In contrast, we were unable to detect any distinct relationship between the up-regulated genes in the *AZF1* and *AZF2* overexpressors and osmotic stress and/or ABA-responsive genes (Supplemental Fig. S8A).

We also performed microarray analyses to identify genes that were down-regulated in response to high-salt stress in the *AZF2pro*:*AZF2* plants. Both the *AZF2pro*:*AZF2* and vector control plants were treated with a high concentration of salt (200 mM NaCl) or water for 5 h prior to the microarray experiments. Overall, 89 and 70 genes were down-regulated with a ratio of greater than 2 in the high-salt- and water-treated *AZF2pro*:*AZF2* plants, respectively, compared with the vector control plants (Supplemental Table S1). The group of 89 genes included many osmotic stress- and ABA-repressive genes (Fig. 3A). Overall, 31 and 27

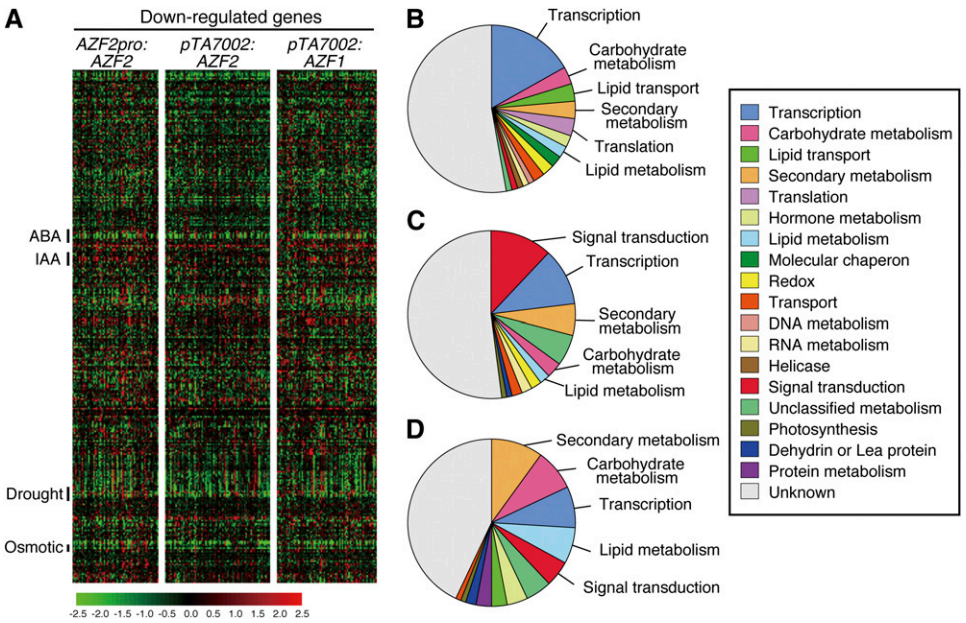

Figure 3. Expression and functional categorization of genes that were down-regulated in transgenic plants overexpressing *AZF1* or *AZF2*. A, The heat map for *AZF2pro*:*AZF2* (left) indicates the responsiveness to stimuli of all 89 genes that demonstrated a reduced expression in *AZF2pro*:*AZF2* plants compared with the vector control after 5 h of treatment with 200 mM NaCl. The heat maps for *pTA7002*:*AZF2* (center) and *pTA7002*:*AZF1* (right) show the responsiveness of the top 100 down-regulated genes in *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants, respectively, compared with the vector control plants under 10 μM DEX-supplemented conditions for 24 h. The results were based on data collected from Genevestigator (https://www.genevestigator.com/gv/html.jsp). The data for several genes were not available for analysis. B to D, Functional categorization of all 89 genes that were down-regulated in *AZF2pro*:*AZF2* plants (B) and of the top 100 genes that were down-regulated in the *pTA7002*:*AZF2* (C) and *pTA7002*:*AZF1* (D) plants.

genes were down-regulated more than 2-fold in the plants that were treated with ABA and high salt, respectively (Fujita et al., 2009). Moreover, among the 89 genes, two sets of 24 genes were down-regulated in the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants, respectively, and 18 of these 89 genes were down-regulated in both types of transgenic plants (Supplemental Table S1). In contrast, the repressed genes in the water-treated transgenic plants did not exhibit clear, common expression patterns following exposure to the various stimuli (Supplemental Fig. S8B).

The functional characteristics of these down-regulated genes were analyzed using The Arabidopsis Information Resource Gene Ontology annotation search tool (http://www.arabidopsis.org/tools/bulk/go/index.jsp) and the Pfam batch sequence search tool (http://pfam.janelia.org/search#tabview=tab1). We obtained the ontological profiles of the 89 genes that were down-regulated in the *AZF2pro*:*AZF2* plants and the top 100 genes that were down-regulated in the *pTA7002*:*AZF2* and *pTA7002*:*AZF1* plants (Fig. 3, B–D). We found that many genes known to be involved in transcription and various metabolic pathways, such as carbohydrate, lipid, and secondary metabolism, were down-regulated in these transgenic plants.

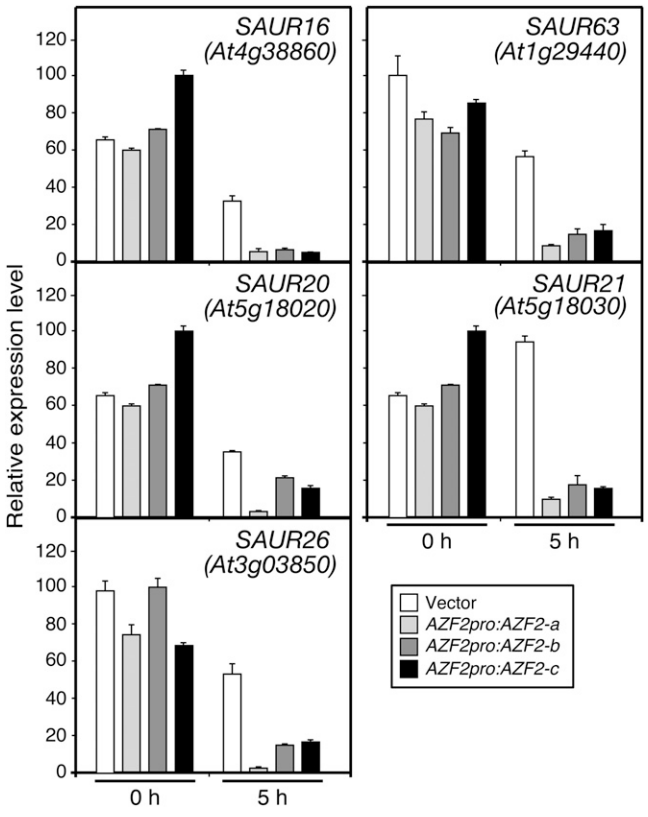

Figure 4. Experimental validation of selected down-regulated genes in *AZF2pro:AZF2* plants. The relative expression levels of the down-regulated genes were determined in the vector control and in three independent transgenic lines that were treated with or without 200 mM NaCl using qRT-PCR. The highest expression level of each gene was designated as 100. The data represent means ± SD of triplicate experiments.

AZF1/AZF2-Regulated *SAUR* Genes in Plants

It is notable that many of the auxin-related genes were down-regulated in the *AZF2pro*:*AZF2* plants that were treated with high salt, and most of these genes were *small auxin-up RNA* (*SAUR*) genes (Supplemental Table S1). Among the 18 genes that were commonly down-regulated in the *pTA7002*:*AZF1* and *pTA7002*:*AZF2* plants, 15 were *SAURs*, and almost all were repressed in response to stress. To validate the microarray results, we selected five genes (*SAUR16*, *-20*, *-21*, *-26*, and *-63*) whose expression was down-regulated due to the overexpression of *AZF1* and *AZF2*. Using quantitative real-time (qRT)-PCR, we found that the expression levels of these *SAUR* genes were significantly reduced in the *AZF2pro*:*AZF2* plants under high-salt conditions; the lowest expression for most of these genes was found in the transgenic lines displaying the highest expression of the *AZF2* transcript (Fig. 4).

The expression analyses using the transgenic plants overexpressing *AZF1* and *AZF2* suggest that AZF1 and AZF2 control the expression levels of the *SAUR* genes. The *SAUR* genes form a large family in Arabidopsis that includes over 70 members (Hagen and Guilfoyle, 2002). A total of 27 *SAURs* were down-regulated by either AZF1 or AZF2, a number that constitutes approximately 40% of the *SAUR* family members. The *SAUR* gene family can be phylogenetically classified into three clades, and we determined the positions of these 27 genes in a phylogenic tree (Fig. 5). Interestingly, 25 of the 27 *SAUR* genes were included in clades I or II. Clade I contains 22 genes, 11 of which were down-regulated in *AZF2pro*:*AZF2*, *pTA7002*:*AZF1*, or *pTA7002*:*AZF2* plants. A total of 12 of the 22 clade I genes, including *SAUR62*, *-63*, *-64*, *-65*, *-66*, *-67*, and *-68*, are auxin-up-regulated genes (Redman et al., 2004; Nemhauser et al., 2006). The second clade consists of 16 genes, including 14 that were down-regulated in *AZF1* or *AZF2* overexpressors: *SAUR13*, *-15*, *-23*, *-27*, and *-28* are up-regulated by auxin. Although the third clade consists of 28 members and is the largest of the three clades, it contains only two genes (*SAUR46* and *-58*) that were down-regulated by AZF1 or AZF2 and only four genes (*SAUR34*, *-35*, *-45*, and *-46*) that were up-regulated by auxin.

The expression profiles of all the *SAUR* genes in the roots and vegetative rosettes were obtained from the eFP browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The genes in clades I and II had a tendency to display higher expression levels in the leaves and lower expression levels in the roots under nonstress conditions (Supplemental Fig. S9A). In contrast, the genes in clade III demonstrated expression patterns opposite to those of the genes in the other two clades. Our Web-based transcriptome analyses of the *SAURs* indicated that many of the genes in clades I and

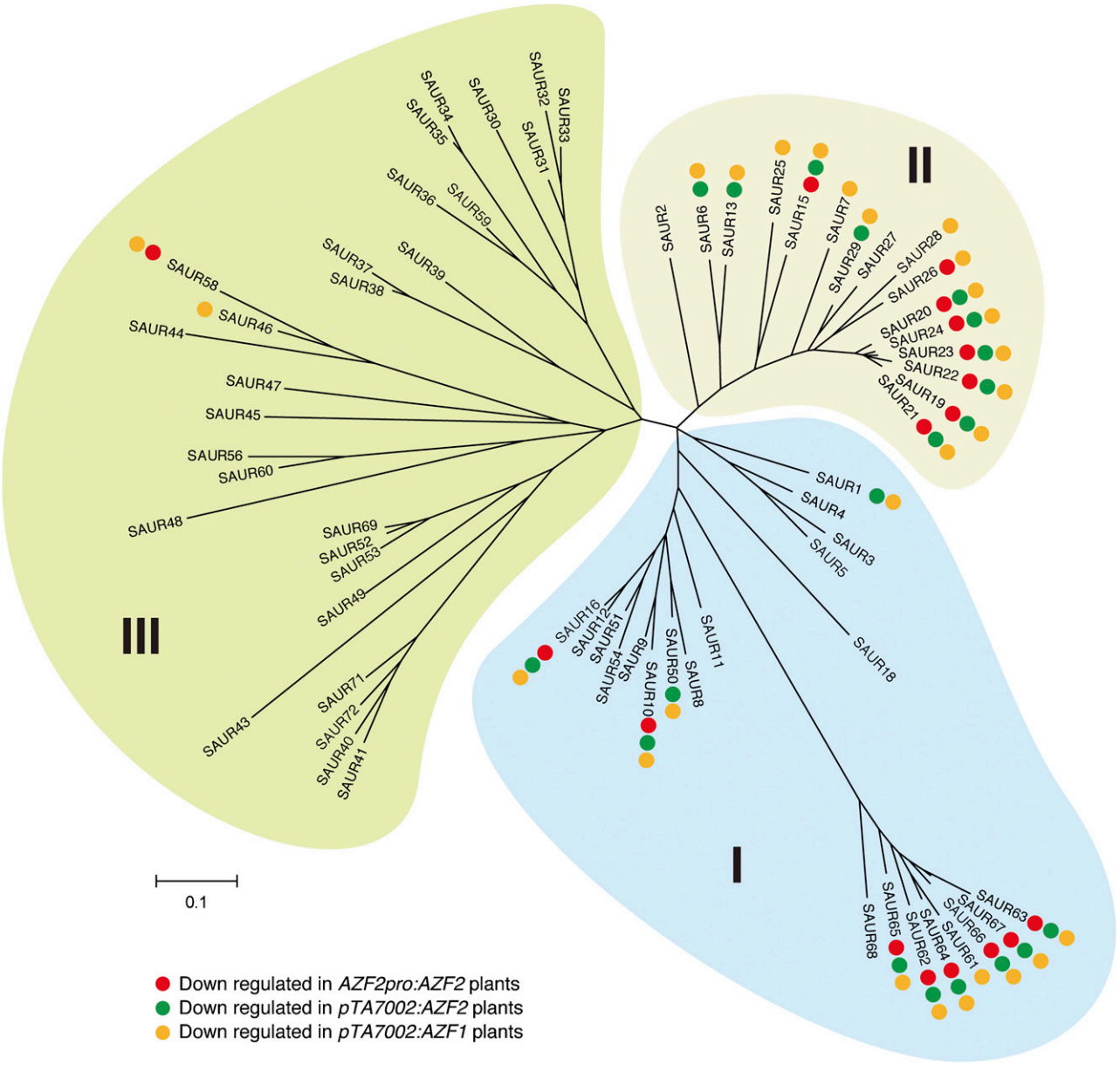

Figure 5. Phylogenetic analysis of the *SAUR* family in Arabidopsis. The sequence alignment and phylogenetic tree were prepared using the ClustalX program (version 2.0) and MEGA4 software as described in Supplemental Experimental Procedures S1. The genes that were down-regulated in *AZF2pro:AZF2*, *pTA7002:AZF2*, and *pTA7002:AZF1* plants are indicated by red, green, and yellow circles, respectively.

II were repressed by ABA and down-regulated by osmotic stress, but the genes in clade III did not demonstrate this tendency (Supplemental Fig. S9B). This classification of the *SAUR* genes revealed that AZF1 and AZF2 could specifically down-regulate the expression levels of the members of clades I and II. Both clades are likely to contain genes that respond to auxin, that show lower expression levels in the roots and higher expression levels in the leaves under nonstress conditions, and that are down-regulated by osmotic stress and ABA.

Analyses of Mutant Plants under Salt-Stress Conditions

Our microarray results indicated that the expression levels of many *SAUR* genes were down-regulated in the *pTA7002:AZF2* and *pTA7002:AZF1* plants. To further examine the effects of AZF1 and AZF2 on the expression levels of the *SAUR* genes, we obtained T-DNA insertion mutants of *azf1* and *azf2* in a Columbia background and generated double mutants. A semiquantitative reverse transcription-PCR analysis confirmed that *AZF1* and *AZF2* expression was com-

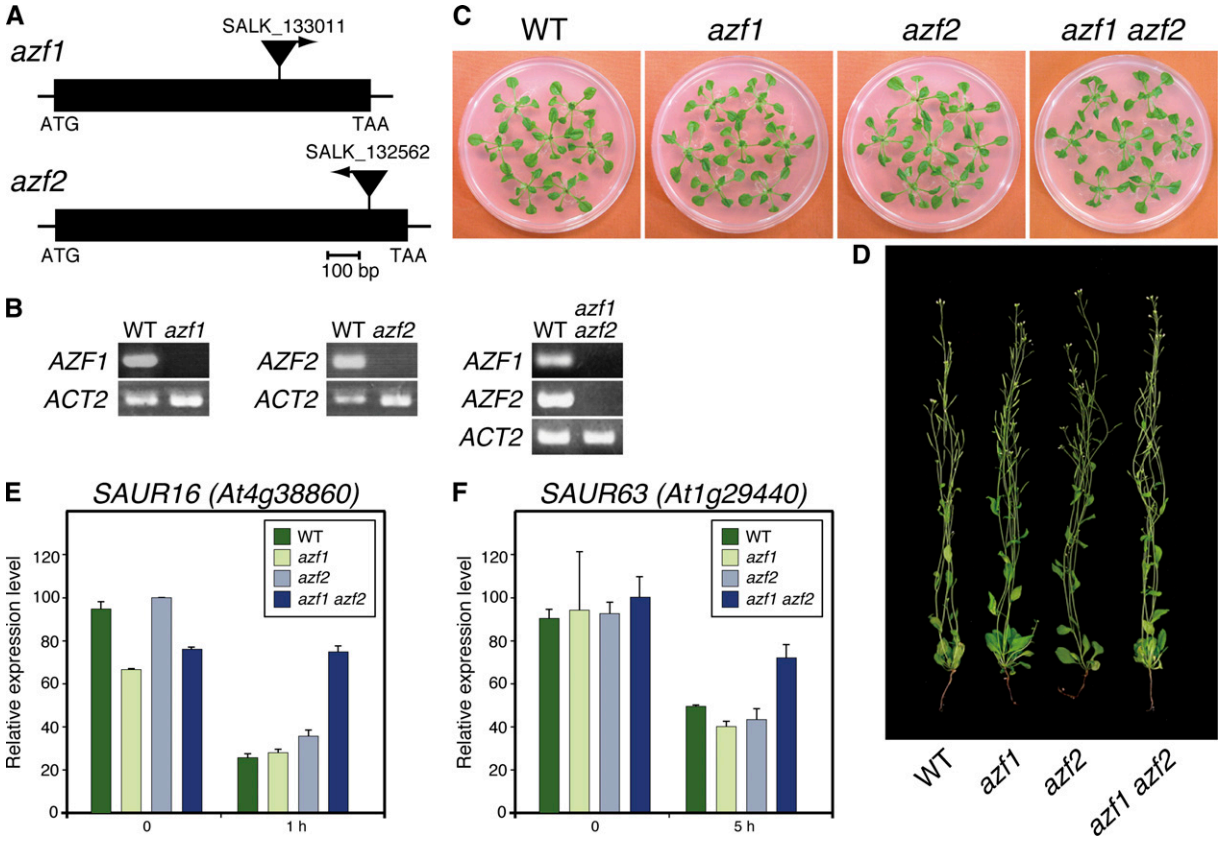

Figure 6. Morphological phenotypes and gene expression analysis of the *azf1* and *azf2* single mutants and the *azf1 azf2* double mutant. A, Schematic representation of the genomic organization of *AZF1* and *AZF2*, with each exon depicted as a black box. The positions of the T-DNA insertions are indicated by triangles. B, RT-PCR analysis showing the expression of *AZF1* in *azf1*, *AZF2* in *azf2*, and *AZF1* and *AZF2* in *azf1 azf2* mutants and the expression of each gene in wild-type (WT) plants exposed to drought conditions for 2 h. Actin cDNA was amplified as a control. C, The plants were grown on GM plates for 3 weeks. D, Three-week-old plants were transferred from GM plates to soil and grown for an additional 3 weeks. E and F, qRT-PCR analyses of the transcript levels of the selected *AZF1/AZF2* downstream genes in mutant and wild-type plants. The highest expression level of each gene was designated as 100. The data represent means ± SD of triplicate experiments.

pletely eliminated by the T-DNA insertion in the homozygous mutants (Fig. 6, A and B). Compared with the wild-type plants, all of the mutants displayed similar growth phenotypes on the GM agar plates and soil pots under normal conditions (Fig. 6, C and D). Double mutant plants tended to show delayed chlorosis or enhanced root growth compared with wild-type plants under high-salt conditions. However, those phenotypic differences were unstable and were difficult to evaluate statistically. We then analyzed the expression of *SAURs* under control and high-salt-stress conditions in the mutants. The lack of *AZF1* and *AZF2* transcripts in the *azf1 azf2* mutant resulted in increased levels of *SAUR* gene expression under high-salt conditions, and these expression patterns were not detected in the absence of salt stress (Fig. 6, E and F). However, the expression levels of the *SAURs* in the *azf1* and *azf2* single mutants were similar to those detected in wild-type plants. These results suggest that AZF1 and AZF2 coordinately repress the expression of these *SAUR* genes in response to osmotic stresses.

Binding of the AZF1 and AZF2 Proteins to the Promoter Regions of *SAUR* Genes

As described above, many genes were down-regulated in the transgenic plants overexpressing *AZF1* or *AZF2*, and the expression of many *SAUR* genes was commonly repressed in the transgenic plants. Using gel mobility shift assays, we examined whether AZF1 and AZF2 proteins could recognize the promoter regions of *SAUR* genes. Because it was very difficult to prepare full-length recombinant AZF1 and AZF2 proteins in bacterial expression systems, we decided to use the truncated forms of recombinant AZF1 and AZF2. Truncated AZF1 and AZF2 proteins including two canonical zinc-finger motifs were expressed as in-frame fusion proteins with the maltose-binding protein in *Escherichia coli* (Fig. 7A). We divided the 1,030-bp promoter regions of *SAUR20* and *SAUR63* into four 280-bp fragments that contained 30-bp overlap sequences at their 3′ ends. Then, we examined whether the recombinant AZF1 and AZF2 proteins

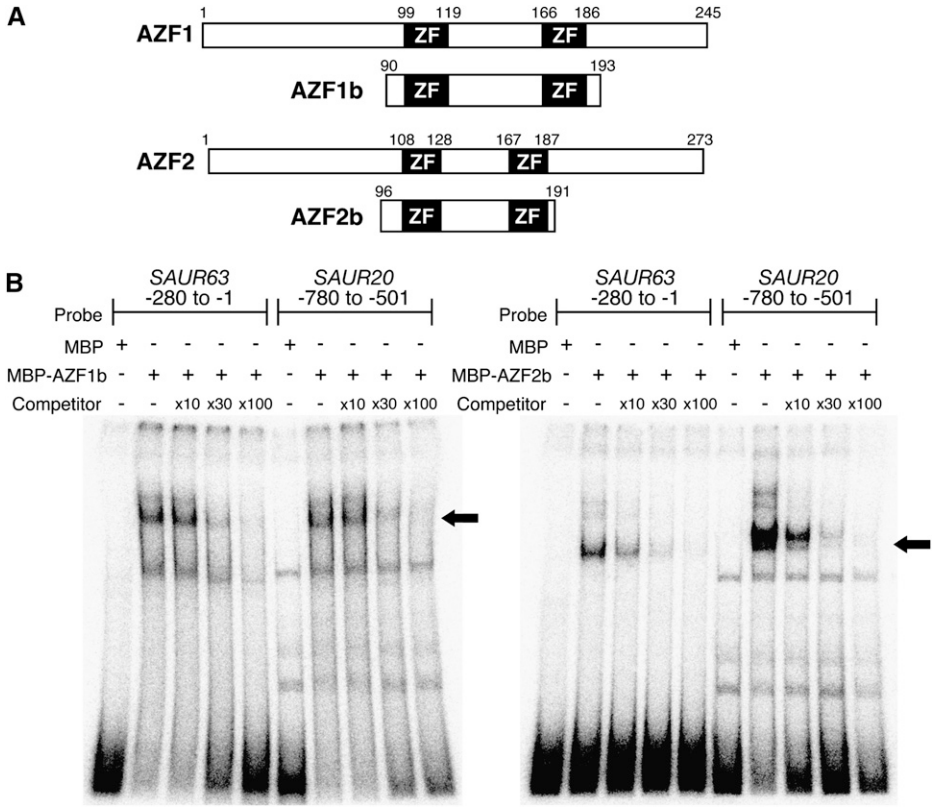

Figure 7. Interactions between the recombinant AZF1 and AZF2 proteins and the promoter regions of the *SAUR* genes. A, Schematic representation of full-length and truncated AZF1 and AZF2. The black boxes indicate zinc-finger (ZF) motifs. Amino acid numbers are from the full-length proteins. B, DNA-binding activities were analyzed by gel shift assays. ^{32}P-labeled 280-bp probes (0.2 ng) containing promoter fragments of *SAUR63* (−280 to −1 bp) and *SAUR20* (−780 to −501 bp) were incubated with maltose-binding protein (MBP; 11 ng), MBP-AZF1b (200 ng), or MBP-AZF2b (13 ng). Unlabeled probes were used as competitors. The amounts of the competitors were 2 ng (10×), 6 ng (30×), and 20 ng (100×). The shifted bands are indicated by arrows.

could interact with the *SAUR63* and *SAUR20* promoter regions. Gel shift assays showed that the recombinant AZF1 and AZF2 proteins bound to the bp −280 to −1 region of the *SAUR63* promoter and to the bp −780 to −501 region of the *SAUR20* promoter (Fig. 7B). Competition experiments showed that interactions between the proteins and the DNA fragments were competed gradually, depending on the concentration of unlabeled oligonucleotides, and disappeared following the addition of a 100-fold excess of competitors.

DISCUSSION

Our study indicates that two Cys2/His2-type zinc-finger proteins with EAR motifs, AZF1 and AZF2, can function as transcriptional repressors under osmotic stresses. Using transgenic plants overexpressing *AZF1* or *AZF2*, we empirically demonstrated that the overexpression of *AZF1* or *AZF2* repressed the expression of many osmotic stress- and ABA-repressive genes in plants and severely affected seedling growth. Below, we discuss possible downstream targets shared by the AZF1 and AZF2 proteins.

Under abiotic stress conditions, such as drought, high salt, and low temperature, many genes have been reported to be repressed in plants (Hoth et al., 2002; Seki et al., 2002; Takahashi et al., 2004). ABA is a key regulator that mediates abiotic stress signaling pathways and induces not only stress tolerance but also growth inhibition (Finkelstein et al., 2002). Previously, we revealed that the transcription levels of *AZF2* were markedly decreased in both *areb1 areb2 abf3* and *srk2d srk2e srk2i* triple mutants using microarray analyses (Fujita et al., 2009; Nakashima et al., 2009; Yoshida et al., 2010). In this work, northern-blot analysis indicated that the induction of *AZF2* expression by ABA was strong and persisted over time (Supplemental Fig. S3). The AZF2 protein accumulated in the leaf guard cells following ABA treatment (Fig. 1B). Considering these results together with the presence of the ABREs in the *AZF2* promoter, the expression of *AZF2* appears to be regulated mainly by ABA, and this gene is one of the downstream targets of the AREB/ABF bZIP-type transcription factors.

Transgenic plants overexpressing *AZF1* and *AZF2* under the control of a glucocorticoid-inducible promoter showed severe growth retardation with morphological defects (Fig. 2J). This dwarf phenotype is consistent with the results obtained from the overexpression of Cys2/His2-type zinc finger genes, such as *STZ* and *ZAT7* (Sakamoto et al., 2004; Mittler et al., 2006; Ciftci-Yilmaz et al., 2007). The reduced expression of genes involved in carbohydrate and lipid metabolism might lead to the growth inhibition observed in *AZF1* and *AZF2* overexpressors (Fig. 3, B–D). Although the number of photosynthesis-related genes with reduced expression was small, these genes' reduced expression may have also affected the growth of the plants. Previous microarray gene expression anal-

ysis has shown that the expression of many genes involved in carbohydrate metabolism and photosynthesis was down-regulated under drought, high-salt, and cold stress conditions (Seki et al., 2002). Growth suppression following a reduction in these types of proteins is a critical phenomenon in plant stress responses.

Surprisingly, the plants that overexpressed *AZF1* and *AZF2* under the control of stress-responsive promoters showed a hypersensitive phenotype to salt stress (Fig. 2). Moreover, the high overexpression of *AZF1* and *AZF2* induced by DEX treatment was lethal to the plants even under nonstress conditions (Supplemental Fig. S7). Because the enhanced sensitivity to high salt was also promoted by the overproduction of *AZF2* transcripts under the control of the native promoter of *AZF2* (Fig. 2E), these results were probably not caused by the ectopic expression of *AZF1* and *AZF2*; instead, they probably reflect functions of these proteins. The difficulty in generating transgenic plants that constitutively express *AZF1*, *AZF2*, and *AZF3* under the control of the cauliflower mosaic virus 35S promoter (Sakamoto et al., 2004) suggests that the overexpression of these genes may have a harmful effect on plant growth or development. However, the overexpression of *STZ* has been shown previously to enhance the adaptation of a plant to drought stress (Sakamoto et al., 2004). Another group has reported that both the knockout and the overexpression of *STZ* improved the plant's adaptation to osmotic and salinity stresses (Mittler et al., 2006). The varying results between the overexpression of these two *AZFs* and *STZ* may reflect partial differences in their sets of downstream genes. In fact, the constitutive expression of *STZ* enhanced the expression of the oxidative stress-responsive genes *ascorbate peroxidase2* (*APX2*), *Fe-superoxide dismutase1*, and *APX1* (Mittler et al., 2006). However, the transcript levels of these reactive oxygen species defense genes were not altered in the *AZF1* and *AZF2* overexpressors used in this study, and few *SAUR* genes were down-regulated in the DEX-induced *STZ* overexpressors (data not shown). The expression of many stress-inducible genes, such as *DREB1A*, *RD29A*, *KIN2*, *cold-regulated15A* (*COR15A*), and *COR15B*, was also suppressed in the transgenic plants that had enhanced expression of *AZF1* and *AZF2* under the control of a glucocorticoid-inducible promoter. Therefore, we suspect that these types of suppression had a causal influence on the enhanced salt sensitivity of the transgenic plants. The short-term salt treatment and mild enhancement of *AZF2* transcript levels might lead to almost no down-regulation of stress-inducible genes in plants that overexpress *AZF2* under the control of its own promoter. We observed the stress responses of *RD29Apro*:*AZF1*, *RD29Apro*:*AZF2*, and *AZF2pro*:*AZF2* plants under drought stress. We also analyzed the effects of ABA treatment on *AZF2pro*:*AZF2* seedlings. However, these transgenic plants showed no clear differences from the control plants (data not shown). These observations suggest the strong influence of the overproduction of *AZF1* and *AZF2* transcripts under high-salt conditions.

The analysis of microarray data from the Genevestigator database revealed that the expression levels of many osmotic stress- and ABA-repressive genes were down-regulated in both the *AZF1* and *AZF2* overexpressors (Fig. 3A). Although the transcript abundance of a large number of genes was both induced and repressed in the transgenic plants that overexpressed *AZF1* and *AZF2* under the control of a glucocorticoid-inducible promoter, the enhanced expression of *AZF2* using its native promoter resulted in a much lower number of up-regulated than down-regulated genes. Moreover, the stress-responsive expression patterns of the up-regulated genes were ambiguous compared with those observed for the down-regulated genes (Supplemental Fig. S8A). The moderate induction of *AZF2* using its own promoter might influence the clear appearance of gene suppression in plants. In agreement with the gain-of-function study, the suppression of gene expression caused by osmotic stress was partially attenuated in the *azf1 azf2* double mutant plants (Fig. 6, E and F). These results indicate that *AZF1* and *AZF2* function as repressors to regulate the expression of specific genes under stress conditions.

Although many osmotic stress- and ABA-repressive genes were down-regulated by AZF1 and AZF2, some were auxin response components (Fig. 3A). Many auxin-responsive genes were down-regulated in the transgenic plants that overexpressed *AZF2* using its native promoter (Supplemental Table S1). These genes include *IAA5*, *IAA29*, *auxin-regulated gene involved in organ size* (*ARGOS*), and many *SAUR* genes. Aux/IAA proteins are proposed to be transcriptional repressors that play crucial roles in auxin signaling by interacting with auxin response factors (Guilfoyle and Hagen, 2007). The *IAA5* gene is thought to be involved in the growth acceleration in response to auxin and brassinosteroids (Goda et al., 2002). The *IAA29* gene has been shown to be a component of auxin-mediated elongation growth in shade avoidance responses (Tao et al., 2008). *ARGOS* is thought to transduce auxin signals to regulate cell proliferation and organ growth during organogenesis (Hu et al., 2003). *SAUR* genes are the early auxin-responsive genes that constitute a large multigene family in plants (Paponov et al., 2008). It is noteworthy that almost all of the *SAUR* genes commonly repressed by AZF1 and AZF2 belong to the groups indicating relatively high auxin responsiveness (Fig. 5; Supplemental Fig. S9). Several of these *SAUR* genes have been shown to be expressed mainly in the elongation tissues of maize (*Zea mays*) and soybean (*Glycine max*), which suggests that they have important roles in auxin-mediated cell elongation in plants (Gee et al., 1991; Knauss et al., 2003). The high expression levels of the *SAUR* genes from clades I and II in shoots and their up-regulation in response to auxin also support the view that these genes are important for the stimulation of shoot elongation (Paponov et al., 2008; Supplemental Fig. S9). It has

been reported that the transcript levels of *AZF1* and *AZF2* were minimally increased by the synthetic auxin 2,4-dichlorophenoxyacetic acid (Sakamoto et al., 2004). Using the eFP browser, we found that the expression of *AZF1* or *AZF2* was relatively constant, regardless of auxin (IAA) treatment. These findings indicate that *AZF1* and *AZF2* repress the expression of several genes that may be involved in auxin signaling and auxin-mediated plant growth under osmotic stress conditions.

Although *AZF2* transcription was clearly induced by osmotic stress and ABA treatment, the induction of *AZF1* was slight (Supplemental Fig. S3). Using transgenic plants that contained the *AZF1pro*:*AZF1-GFP* and *AZF2pro*:*AZF2-GFP* fusion genes, we detected osmotic stress-induced AZF2 protein in leaves under high-salt conditions, but the induction of AZF1 was not clear (Fig. 1B). Hence, the repression activity of AZF1 under osmotic stresses may be much weaker than that of AZF2. In contrast to our expectations, the suppression of gene expression was much more reduced in the double mutant than in any single mutant (Fig. 6, E and F). Therefore, we speculate that the AZF1 and AZF2 proteins share several downstream genes and regulate gene expression in a coordinated manner under osmotic stresses. However, the *azf1* and *azf2* single and double mutant seedlings did not show obvious phenotypes in response to ABA treatment or osmotic stresses, such as drought, high salt, and cold, which suggests a high degree of functional redundancy among the various gene family members.

According to the data from the eFP database, the expression levels of the *SAURs* from clades I and II were low in roots and relatively high in shoots (Supplemental Fig. S9A), which was opposite to the expression patterns observed for *AZF1* and *AZF2* under normal growth conditions. Moreover, the expression of most of the *SAUR* genes, which belong to clades I and II, was down-regulated under drought, high salt, and ABA treatment conditions (Supplemental Table S1; Supplemental Fig. S9B). Because expression analyses using the overexpressors and the mutant plants indicated that the expression of certain *SAUR* genes was at least partially regulated by both *AZF1* and *AZF2*, it was intriguing to analyze whether the *SAUR* genes were the direct downstream targets of the AZF1 and AZF2 proteins. Gel shift assays showed that the AZF1 and AZF2 proteins interacted with promoter fragments of the *SAUR63* and *SAUR20* genes, respectively (Fig. 7). AZF1 and AZF2 contain the EAR repression domain and are thought to function as transcriptional repressors (Ohta et al., 2001; Sakamoto et al., 2004; Kazan, 2006). Therefore, our data suggest that the AZF1 and AZF2 proteins bind to the *SAUR* promoter regions to repress the expression of these genes.

It has been reported that the AZFs and STZ recognize the tandemly repeated A(G/C)T core sequences and that the sequences around the A(G/C)T repeats might influence binding (Sakamoto et al., 2004). The importance of the spaces and DNA sequences between the two core sites for the DNA-binding affinities of the petunia ZPT2-type proteins has also been reported (Takatsuji, 1999). We searched for the A(G/C)T core sequences within the promoter fragments of *SAUR63* and *SAUR20* and found a few sets of the A(G/C)T repeats in the fragments. Although it is possible that AZF1 and AZF2 recognize the promoter regions of *SAUR63* and *SAUR20* through those A(G/C)T repeats, further study is required to determine the binding sequences of the STZ family proteins within the native promoter regions in Arabidopsis.

The optimal binding affinities of the ZPT2-type proteins are greatly affected by the spacing between the units of the DNA-binding sites, whereas the recognition of the spacing and sequences can be partially relaxed, depending on the character of each protein (Takatsuji, 1999; Sakamoto et al., 2004). Therefore, it could be assumed that the STZ-related proteins have a dual nature in DNA recognition: (1) each member has several optimal, high-affinity binding sequences; and (2) there is combinatorial redundancy between the binding sites and STZ-related members, which leads to the complex overlapping of target genes. Thus, we propose that the overexpression of AZF1 and AZF2 preferentially repressed the expression of genes that had relatively ideal binding sequences for them and that this type of gene repression largely affected the gain-of-function phenotype. In contrast, the functional

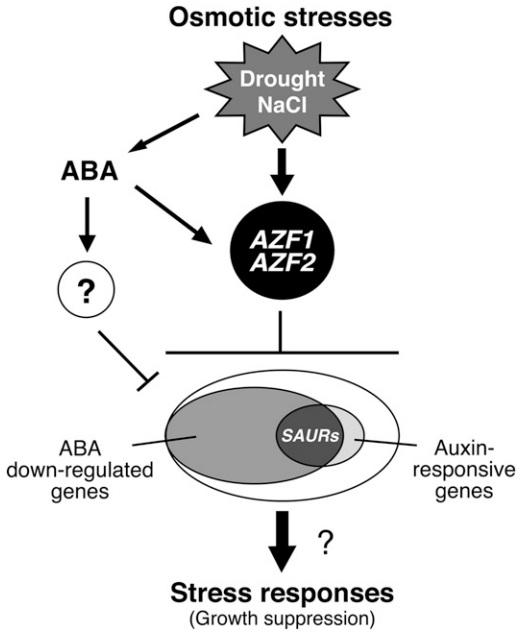

Figure 8. A model showing the putative functions of AZF1 and AZF2 during osmotic stress conditions. Osmotic stress induces the expression of *AZF1* and *AZF2* via ABA-dependent or -independent pathways. The accumulation of AZF1 and AZF2 during stress results in the suppression of many ABA-repressive genes, including several auxin-responsive genes, such as *SAURs*. Consequently, AZF1 and AZF2 are assumed to partially regulate ABA and auxin signaling and to be involved in plant responses to osmotic stress.

redundancy and compensatory functions among the homologous proteins might be expected to prevent the presentation of phenotypic differences in the mutants. We propose that when *AZF1* and *AZF2* are overexpressed in plants, they preferentially down-regulate many osmotic stress-repressive genes that are shared by both zinc fingers. Few of these shared genes are under the control of *STZ*. Despite the ability of AZF2 and STZ to bind to the same cis-acting element within an EP2 sequence (Sakamoto et al., 2004), differences in the downstream genes for which expression was preferentially regulated in the overexpressors can account for the different roles of *AZF2* and *STZ* under specific stress conditions.

The phytohormone auxin has been recognized as a key player in the regulation of plant growth and development and in plant responses to environmental changes (Benjamins and Scheres, 2008; Tromas and Perrot-Rechenmann, 2010). Recent studies have suggested that there is a coordinated, generic "stress-induced morphogenic response" that is stimulated by abiotic stresses and that controls plant morphology (Potters et al., 2007). ABA and auxin are thought to play important roles in stimulating the stress-induced morphogenic response (De Smet et al., 2006; Zolla et al., 2010). It is proposed that AZF1 and AZF2 partially regulate cross talk between the ABA and auxin signal transduction pathways under stress conditions.

In this work, phylogenetic analyses revealed the existence of genes orthologous to *AZF1* and *AZF2* in angiosperms and many other plant species (Supplemental Fig. S2). Because those orthologs in bryophytes, pteridophytes, and gymnosperms belong to the clade in which almost all the proteins have three or more conserved fingers, ZPT2-related proteins might have evolved from zinc-finger proteins that have three or four fingers, such as At4g35280 and At5g56200. Therefore, it is suggested that the *STZ*-related genes, which consist of 18 members in Arabidopsis, have developed specific functions as transcriptional regulators.

In summary, we demonstrated that *AZF1* and *AZF2* can function as transcriptional repressors to inhibit the expression of osmotic stress- and ABA-repressive genes under abiotic stress conditions (Fig. 8). The highly induced expression of *AZF2* in plants using its own promoter showed clear evidence that *AZF2* represses gene expression under osmotic stress. AZF1 and AZF2 interacted with the promoter regions of several *SAUR* genes. These findings indicate the possibility that these proteins directly bind to the promoter regions of their target genes to regulate transcription. The gain-of-function phenotype suggests that the overexpression of *AZF1* and *AZF2* severely inhibits plant growth and development, leading to enhanced salt sensitivity in seedlings. In addition, *AZF1* and *AZF2* down-regulated the expression of many auxin-responsive genes, suggesting that the repression of these types of auxin-related genes plays a role in plant responses to abiotic stresses. To cope with adverse environmental conditions, such as high salinity, drought, and low temperature, plants adjust by conserving energy and resources and reducing their growth. Therefore, the repression of gene expression, salt sensitivity, and inhibition of growth caused by the overexpression of *AZF1* and *AZF2* may be related to an adaptive response of plants to osmotic stresses. Because the seedlings of the *AZF1* and *AZF2* mutants showed no clear phenotype under osmotic stress, it is likely that functional redundancy and compensatory functions exist among the *STZ*-related gene family members. In the future, elucidating the combination of overlapping downstream targets between the *AZF* and *ZAT* genes will shed light on their complex interplay under various stress conditions.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All of the Arabidopsis (*Arabidopsis thaliana*) lines described herein were derived from the Columbia wild-type line. The T-DNA insertion lines of *azf1* (SALK_133011) and *azf2* (SALK_132562) were obtained from the Arabidopsis Biological Resource Center. The homozygous *azf1* and *azf2* mutants were isolated, and the *azf1 azf2* double mutants in the same backgrounds as the single mutants were constructed by genetic crosses. The primers that were used to screen homozygous mutants were designed according to http://signal.salk.edu/isect.2.html and are listed in Supplemental Table S2. Unless otherwise stated, the plants were grown on GM agar plates, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Constructs and Generation of Transgenic Plants

The primers used in this analysis are listed in Supplemental Table S2. To generate the *AZF1pro:AZF1* and *AZF2pro:AZF2* constructs, 1.6- and 2.2-kb fragments containing the *AZF1* and *AZF2* upstream sequences, respectively, were amplified from wild-type genomic DNA by PCR using *Kpn*I-*Sma*I linker primers. The resulting fragments were digested with *Kpn*I and *Sma*I and cloned into the *Kpn*I and *Sma*I sites of pGK-El2-35S-CsGFP (Qin et al., 2008) to produce pGK-AZF1pro:CsGFP and pGK-AZF2pro:CsGFP, respectively. The entire 0.7-kb coding region of *AZF1* and 0.8-kb coding region of *AZF2* were amplified by PCR using *Bam*HI-*Eco*RV linker primers. The PCR products were digested with *Bam*HI and *Eco*RV and cloned into the *Bam*HI-*Eco*RV sites of pGK-AZF1pro:CsGFP and pGK-AZF2pro:CsGFP to produce pGK-AZF1pro:AZF1 and pGK-AZF2pro:AZF2. To prepare the *AZF1pro:AZF1-sGFP* and *AZF2pro:AZF2-sGFP* constructs, the coding regions of *AZF1* and *AZF2* without stop codons, respectively, were amplified from *AZF1pro:AZF1* and *AZF2pro:AZF2*, respectively, using the primers containing the *Sma*I-*Bam*HI linkers. The generated fragments were inserted into the *Sma*I-*Bam*HI sites of pGK-AZF1pro:CsGFP and pGK-AZF2pro:CsGFP to produce pGK-AZF1pro:AZF1-sGFP and pGK-AZF2pro:AZF2-sGFP, respectively. To construct the PBI-RD29Apro:AZF1 and PBI-RD29Apro:AZF2 plasmids, the *AZF1* and *AZF2* coding sequences were amplified with the *Xba*I-*Sma*I linker primers and inserted into the *Xba*I and *Sma*I sites of the pBIRD29AAP-Not vector (Kasuga et al., 1999). To generate the glucocorticoid-inducible gene expression constructs (*pTA7002:AZF1* and *pTA7002:AZF2*), the binary transformation plasmid pTA7002, which contains the two-component glucocorticoid system (Aoyama and Chua, 1997), was digested with *Spe*I. The *Xba*I fragments of the *AZF1* and *AZF2* coding regions were then cloned into the *Spe*I sites of the pTA7002 vectors. All of the PCR products that were cloned into the vectors were sequenced to confirm the absence of PCR errors. These constructs were used to transform Arabidopsis plants using the vacuum infiltration method with *Agrobacterium tumefaciens* C58 cells (Bechtold and Pelletier, 1998). The T2 or T3 seeds were used for the subsequent experiments.

GFP Fluorescence

Two-week-old transgenic plants harboring the *AZF1pro:AZF1-sGFP* or *AZF2pro:AZF2-sGFP* construct were removed from GM agar plates and treated with liquid MS medium with or without 100 μM ABA to observe

GFP fluorescence in the roots. The plants were also treated with MS medium containing 150 mM NaCl or 100 μM ABA to observe GFP fluorescence in the leaves. To visualize the nuclei, the plants were incubated with 10 μg mL^{-1} DAPI for 15 min. The subcellular localization of GFP and DAPI was then visualized using a confocal laser-scanning microscope (LSM510; Zeiss).

RNA Preparation and Real-Time PCR

Total RNA was isolated from 2-week-old plants using the TRIzol reagent (Invitrogen). cDNA synthesis and real-time PCR analyses were performed as described previously (Qin et al., 2008). For each overexpressing transgenic line, at least two independent lines were analyzed, which represented biological replicates. For the T-DNA insertion lines, representative results from several independent samples were obtained. The primers used for the qRT-PCR are listed in Supplemental Table S2. To obtain quantitative data, three replicates were analyzed to characterize the expression data for each gene.

High-Salt Sensitivity of Transgenic Plants

For salt stress, 9- to 10-d-old transgenic plants grown on GM agar plates were transferred onto 0.8% agar plates (0.5× MS medium) with or without the addition of 175 mM NaCl. The plates were maintained at 22°C under a 16-h-light/8-h-dark cycle (60 ± 10 μmol photons m^{-2} s^{-1}) until visual symptoms were observed. For the stress assays, three independent lines for each transgenic plant were analyzed. The statistical significance of the values was determined using Student's *t* test.

Microarray Analysis

Genome-wide expression analyses were performed using the Arabidopsis 3 Oligo Microarray (Agilent Technologies), as described previously (Qin et al., 2008). Two-week-old *AZF2pro*:*AZF2* and vector control plants were treated with water as the control or 200 mM NaCl for 5 h. Three-week-old *pTA7002*:*AZF1*, *pTA7002*:*AZF2*, and vector control plants were treated with 10 μM DEX solution for 24 h. The gene expression levels were compared between the transgenic and control plants, using two different lines for each transgenic plant. The reproducibility of the microarray analysis was assayed by including biological and technical (dye-swap) replicates in each experiment. The microarray data were analyzed statistically as described previously (Qin et al., 2008). All of the microarray data are available at the Web site http://www.ebi.ac.uk/arrayexpress/ under accession number E-MEXP-2922.

Preparation of Probes and Recombinant Proteins and Gel Mobility Shift Assays

The primers used in this analysis are listed in Supplemental Table S2. The probes for the gel mobility shift assays were prepared from the promoter regions of the *SAUR20* and *SAUR63* genes. DNA fragments of 280 bp were amplified by PCR from wild-type genomic DNA using *Xho*I or *Hin*dIII linker primers and were subcloned into the pSK vector. The DNA fragments digested from the plasmids were labeled with or without the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. Fragments encoding the truncated proteins containing the two canonical zinc-finger motifs of AZF1 and AZF2 were PCR amplified using *Bam*HI-*Xba*I linker primers. The PCR fragments excised with *Bam*HI and *Xba*I were cloned into pMAL-c2X (New England Biolabs). The resulting plasmids were transformed into *Escherichia coli* BL21 (DE3). The production and purification of the fusion proteins and the gel shift assays were performed according to the methods described by Sakamoto et al. (2004), with minor modifications. DNA-binding reactions were performed in 25 mM HEPES-KOH (pH 7.6), 40 mM KCl, 0.01 mM $ZnCl_2$, 40 μg μL^{-1} poly(dIdC), 1% bovine serum albumin, and 1 mM dithiothreitol. After incubation for 20 min at the ambient temperature, the mixtures were subjected to electrophoresis on a 6% polyacrylamide gel in 0.5× Tris-borate/EDTA at 150 V for 90 min and visualized by autoradiography.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree and alignment of the two-fingered Cys2/His2-type zinc-finger proteins in Arabidopsis.

Supplemental Figure S2. Conservation of AZF/ZAT family proteins among plant species.

Supplemental Figure S3. RNA gel-blot analysis of *AZF* and *ZAT* expression in response to different treatments.

Supplemental Figure S4. Nuclear localization of the AZF and ZAT proteins.

Supplemental Figure S5. Patterns of *AZF1* and *AZF2* promoter-driven GUS expression in transgenic Arabidopsis plants.

Supplemental Figure S6. Morphological phenotype of plants overexpressing *AZF1* or *AZF2*.

Supplemental Figure S7. High-salt sensitivity of plants overexpressing *AZF1* or *AZF2* under the control of a DEX-inducible promoter.

Supplemental Figure S8. Responsiveness to stimuli of genes with altered expression levels in *AZF2pro*:*AZF2*, *pTA7002*:*AZF2*, and *pTA7002*:*AZF1* plants.

Supplemental Figure S9. Expression patterns of *SAUR* genes in Arabidopsis seedlings.

Supplemental Table S1. Transcripts down-regulated in *AZF2pro*:*AZF2* plants under high-salt conditions.

Supplemental Table S2. Primers used in this study.

Supplemental Experimental Procedures S1. Additional methods.

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