Plant Biotechnology 23, 249–258 (2006)

Original Paper

Characterization of AtbZIP2, AtbZIP11 and AtbZIP53 from the group S basic region-leucine zipper family in *Arabidopsis thaliana*

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Received March 27, 2006; accepted April 20, 2006 (Edited by T. Hashimoto)

Abstract We have previously described the *lip19* subfamily of basic region-leucine zipper protein (bZIP)-encoding genes from plants. Phylogenetic analysis of bZIP-encoded genes of *Arabidopsis thaliana* revealed that *AtbZIP53* belongs to the *lip19* subfamily and that four other members, *AtbZIP1*, *AtbZIP2*, *AtbZIP11* and *AtbZIP44* have close relationships to this subfamily. Here, we further characterized *AtbZIP53* and two additional bZIP genes, *AtbZIP2* and *AtbZIP11*. All three gene products are localized to the nucleus and show strong DNA binding activity to hexamer and C/G box hybrid sequences. Transactivation activities of the proteins were examined in plant cells. The expression of the genes upon exposure to various abiotic stresses and to hormone treatments was examined in *Arabidopsis*. We found that *AtbZIP11* and *AtbZIP53* were responsive to cytokinin and high salt stress, respectively. Based on these data, the physiological roles are discussed.

Key words: Abiotic stress, *Arabidopsis thaliana*, bZIP gene, plant hormone, salt stress.

Gene expression is regulated by transcription factors (TFs). Comprehensive analysis of the *Arabidopsis thaliana* genome disclosed that this organism contains more than 1,500 genes that may code for TFs (Riechmann and Ratcliffe 2000; The Arabidopsis Genome Initiative 2000). Based on their domain structures and their DNA-binding motifs, TFs have been classified into several families such as Myb, EREBP/AP2, basic-helix-loop-helix, NAC, Cys_2/His_2-type zinc finger, basic region-leucine zipper (bZIP), and WRKY. The bZIP-family proteins are present in all eukaryotes, and some bZIP members in animals and yeast have been studied extensively, revealing that the amino distal subdomain, the basic region, is involved in DNA binding and that the leucine-zipper region mediates dimerization (Landschulz et al. 1988; Hurst 1994). In *Arabidopsis*, there are 75 bZIP-encoding genes (*AtbZIP1-AtbZIP75*) which, based on criteria of associated common domains, have been subdivided into 10 groups designated A to I and S (S stands for small) (Jakoby et al. 2002). The group S proteins are 145–186 amino acids in length, and have an unusually long heptad leucine repeat (Jakoby et al. 2002; Rook et al. 1998a; b).

We have characterized rice *lip19* (Aguan et al. 1993; Shimizu et al. 2005) and its homologous genes in other plants, maize *mlip15* (Kusano et al. 1995), tobacco *tbz17* (Kusano et al. 1998), radish *rdlip* (Ito et al. 1999) and tobacco *tbzF* (Yang et al. 2001). *lip19*, the gene encoding a low-temperature induced protein, clone number 19, codes for a 148 amino acid protein with a bZIP motif and 9 heptad leucine motifs. LIP19 protein shares these structural features, as well as expression patterns with the other small bZIP proteins described above. Therefore, we have proposed that they should collectively be called the *lip19* subfamily. In addition to the above members, maize *OBF1* (Singh et al. 1990), *Antirrhinum majus 910* and *911* (Martínez-García et al. 1998), and tobacco *BZI-2* and *BZI-4* (Strathmann et al. 2001) are judged to be members of the *lip19* subfamily by sequence homology, and were found to be predominantly expressed in flowers, with the exception of *OBF1*. Expression of maize *mlip15*, tobacco *tbz17* and *tbzF* is associated with leaf- and/or flower senescence (Berberich et al. 1999; Yang et al. 2001). Expression profiles of this subfamily suggest that they may play certain roles in the cold stress response, leaf aging and/or flower organ development. In the model plant *A. thaliana*, bZIP encoding group S genes show high similarity to the *lip19* subfamily.

To further investigate the function of the *lip19* subfamily members, we chose three *AtbZIP* genes from the *Arabidopsis* group S, *AtbZIP2* (*GBF5*), *AtbZIP11* (*ATB2*) and *AtbZIP*53, and characterized them. We

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discuss their physiological roles within the framework of previous data reported in the literature.

Materials and methods

Plant materials and treatments

A. thaliana (ecotype Columbia Col-0) was used in all experiments. Plants were grown in soil at 22°C under a 16 h light/8 h dark photoperiod cycle. Stress treatments used the detached rosette leaves from 3-week-old plants. To minimize wounding effects, the detached rosette leaves were floated on half-strength Murashige and Skoog (MS) liquid medium and maintained for 24 h under same temperature and photoperiod conditions before starting any treatments. The detached rosette leaves were treated in half-strength MS liquid medium with 160 mM NaCl, 40 mM LiCl, 320 mM sorbitol, 100 μM abscisic acid (ABA), 10 μM 6-benzylaminopurine (BA), 100 μM indole acetic acid (IAA), 50 μM jasmonic acid (JA), 10 μL/L ethylene gas, 10 μM *t*-zeatin (Zea), 10 μM kinetin (Kin), or 10 μM isopentenyl adenine (iPA) for various periods and were frozen at −80°C until analyzed. For ethylene gas treatment, special gas-tight glass vessels were used. Low-temperature treatment was performed by transferring plants to a growth chamber set at 4°C.

Construction of GFP-fusion plasmids and fluorescence microscopy

The coding regions of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* were amplified by polymerase chain reaction (PCR) with the appropriate primer pairs, namely, *AtbZIP2* forward, 5′-GCTCTAGAATGGCGTCATCTAGCAGCAC-3′ and reverse, 5′-CGGTACCATACATATTGATATCATTAGC-3′; *AtbZIP11* forward, 5′-GCTCTAGAATGGAATCG-TCGTCGTCGGG-3′ and reverse, 5′-GCGGTACCA-T-ACATTAAAGCATCAGA-3′; *AtbZIP53* forward, GC-TCTAGAATGGGGTCGTTGCAAATGC-3′ and reverse, 5′-GCGGTACCGCAATCAAACATATCAGCAG-3′. The restriction enzyme sites in the primers, *Xba*I and *Kpn*I, were underlined. The PCR fragments were digested with *Xba*I and *Kpn*I and subcloned into the corresponding sites of pGFP2 (kindly provided by Dr. N.-H. Chua), resulting in pAtbZIP2-GFP, pAtbZIP11-GFP and pAtbZIP53-GFP, respectively. Onion bulbs were biolistically bombarded as described previously (Yang et al. 2001), with tungsten particles (Bio-Rad, Hercules, CA) coated with 2.5 μg each of the above plasmids. After more than 6 h incubation at 22°C in complete darkness, the nuclei of epidermal cell layers were stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) solution (1 μg ml^{-1}), and fluorescence derived from GFP and DAPI was observed by epifluorescence microscopy (Axioskop, Zeiss, Germany) with 40× magnification of PLAN-NEOFLUAR lens.

Electrophoretic mobility shift assay (EMSA)

The following oligonucleotides, C/G-hybrid (60 mer, 5′-TCTGCCACGTCCCCAATCCG-3′ ×3) (Yang et al. 2001), Hex (60 mer, 5′-TCGGCCACGTCACCAATC-CG-3′ ×3) (Nakayama et al. 1992) and ProDH *cis*-element (60 mer, 5′-ATCATCCACTCATCCTTCAT-3′ ×3) (Satoh et al. 2004) and their complementary oligonucleotides were synthesized, end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase, annealed and used as probes. The respective core sequences were marked by underlines. EMSA was performed as described (Kusano et al. 1995), using similar amounts of glutathione S-transferase (GST)-fused AtbZIP2, AtbZIP11 and AtbZIP53 proteins.

Production of GST, GST-AtbZIP2, GST-AtbZIP11 and GST-AtbZIP53 proteins in E. coli

The coding regions of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* were amplified by PCR with the gene-specific primer pairs: *AtbZIP2* forward, 5′-GCGGATCCATGGCGTC-ATCTAGCAGCAC-3′ and reverse, 5′-GCGAATTCT-CAATACATATTGATATCATT-3′; *AtbZIP11* forward, 5′-GCGGATCCATGGAATCGTCGTCGTCGGG-3′ and reverse, 5′-GCGAATTCTTAATACATTAAAGCATCA-G-3′; *AtbZIP53* forward, 5′-GCGGATCCATGGGGTC-GTTGCAAATGC-3′ and reverse, 5′-GCGAATTCTCA-GCAATCAAACATATCAGC-3′. The amplified fragments were cut with *Bam*HI and *Eco*RI, purified and cloned into the respective sites of the pGEX 4T-1 vector (Amersham Bioscience, Piscataway, NJ). The *Escherichia coli* JM109 cells carrying pGEX 4T-1 or its AtbZIPs fusion constructs were grown until the optical density at 600 nm of the culture solution reached to ca. 0.3, then 0.1 mM IPTG was added to the culture and further incubated for 2 h at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation, rinsed, and resuspended into 500 μl each of the extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol, 1 mM PMSF, 1 μM pepstatin), then disrupted by sonication on ice. Unbroken cells were removed by centrifugation and aliquots (1 μl each) of the supernatants were used for EMSA.

Transcription activity assay in yeast and plant cells

To examine transcriptional activity in yeast, DNA fragments encoding AtbZIP2, AtbZIP11, and AtbZIP53 were inserted into the pBD-GAL4 Cam phagemid vector, resulting in fusions with the GAL4 DNA-binding domain (Stratagene, La Jolla, CA, USA). These constructs, termed pBD-AtbZIP2, pBD-AtbZIP11 and pBD-AtbZIP53, were introduced to yeast strain Y190 cells, and the transformants were tested for histidine autotrophy and also subjected to β-galactosidase filter assays, according to the method described in the supplier's protocol (Stratagene). pBD-TBZ17 was used

as a positive control plasmid (Yang et al. 2001).

To investigate transcriptional activity *in planta*, the coding regions of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* were amplified by PCR with gene-specific primer pairs; *AtbZIP2* forward, 5′-TTCCCGGGAATGGCGTCATCTAGCAGC-3′ and reverse, 5′-GTTGTCGACTCAATACATATTGATATC-3′; *AtbZIP11* forward, 5′-TTCCCGGGAATGGAATCGTCGTCGTCG-3′ and reverse, 5′-GTTGTCGACTTAATACATTAAAGCATC-3′; *AtbZIP53* forward, 5′-TTCCCGGGAATGGGGTCGTTGCAAATG-3′ and reverse, 5′-GTTGTCGACTCAGCAATCAAACATATC-3′. The underlined restriction enzyme sites, *Sal*I and *Sma*I, were added for cloning purpose. The amplified fragments were digested with the above enzymes and cloned into the corresponding sites of GAL4DB basal plasmid (Ohta et al. 2001), resulting in 35S-GAL4DB-AtbZIP2, 35S-GAL4DB-AtbZIP11 and 35S-GAL4DB-AtbZIP53, respectively (Uehara et al. 2005). The reporter plasmid GAL4-LUC cloned into pUC19 vector was described previously (Hiratsu et al. 2002). To normalize the efficiency of particle bombardment, the reference plasmid pPTRL (Ohta et al. 2001), in which a luciferase gene from *Renilla* is placed under the control of the 35S promoter, was also co-bambarded. Relative luciferase (LUC/RLUC) activities were measured according to the protocol of the Dual-Luciferase Reporter Assay System (Promega) by using a luminescence reader (Lumat LB9507, Berthold Japan, Tokyo).

Northern blot analysis

Total RNA (20 μg each), isolated from unbolted whole plants by a standard method (Nagy et al. 1988), was separated by electrophoresis on 1.0% agarose gels containing formaldehyde. The gel was blotted onto Hybond N+ membrane (Amersham Bioscience) in 20× SSC. The entire coding regions of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* cDNA fragments were labeled with [α-^{32}P] dCTP (Hungary Academy Science) using a random primer DNA-labeling kit (TaKaRa, Otsu, Japan) and used as probes. Under the hybridization conditions used, no cross hybridization was observed among the *AtbZIP1*, *AtbZIP2*, *AtbZIP11*, *AtbZIP44* and *AtbZIP53* probes (data not shown). Hybridization and washing of filters was performed as described previously (Berberich et al. 1999).

RT-PCR analysis

Total RNA from 2-week-old seedlings, extracted by the procedure of Plant RNA Purification Reagent (Invitrogen), was subjected to DNase (Cloned DNase I, TaKaRa) treatment. Reverse transcription (RT) was initiated in the presence of oligo (dT) primers at 42°C for 30 min, and the reverse transcriptase was heat inactivated at 95°C for 5 min, then the appropriate primer pairs were added for PCR cycling. One each of rehydrated beads (Ready-To-Go RT-PCR bead, Amersham) was added per reaction consisting of 50 μl. The following primer pairs were used: *AtbZIP53* forward, 5′-GCATGGGGTCGTTGCAAATGC-3′ and reverse, 5′-CGTCAGCAATCAAACATATC-3′; *ProDH* forward, 5′-CCGGGGAATGATGGCCACCGGAGCTCATG-3′ and reverse, 5′-CGTAAAAAGCATTTTTATTGATAAGGTGA-3′; *GDH2* (glutamate dehydrogenase 2) (Oono et al. 2003) forward, 5′-GCTAATCTCAGATCTCAAGCTTTATTGTGT-3′ and reverse, 5′-CCTTCACATCTCCAGCATTTTCCTTGTTCA-3′; *rd29A* forward, 5′-GGTGAAGCCAGAATCGCCACATTCTGTTG-3′ and reverse, 5′-CCAATCCAAAGCCGAACAATTTATTAACC-3′; *rd22* forward, 5′-GCCAGGGACCGTTCCGGTCTGCCACTTCC-3′ and reverse, 5′-CCGAAATGGTAACATTTCAGCTCTAATTT-3′; *AtP5CS* forward, 5′-CCGAGTGTCTGTTTGTGTATTTGGTTGAG-3′ and reverse, 5′-GAAAAGTAGTAGTATCTTGATAAAGATCA-3′; *ERD14* (Nylander et al. 2001) forward, 5′-GGAGAAGAAAGGGATTCTTGAGAAGATTAA-3′ and reverse, 5′-CCATATCACTTAATCAAATGAAAATATTCA-3′. The constitutively expressed *tubulin* was also amplified by PCR as an internal control of the assays. Primers for *tubulin*, forward 5′-CGTGGATCACAGCAATACAGAGCC-3′ and reverse, 5′-CCTCCTGCACTTCCACTTCGTCTTC-3′.

Generation of AtbZIP53 promoter::GUS transgenic plants and GUS activity assay

The *AtbZIP53* genome DNA fragment spanning from −919 to +552 of the transcription start site was amplified by PCR using a primer pair, (forward) 5′-GCCTGCAGGAAATTGCCAAGTCTTG-3′ and (reverse) 5′-GGATCCAGGACTTGTTTGCATTTGC-3′, in which restriction enzyme sites were added for cloning purposes (underlined). The sequence-verified fragment was digested with *Pst*I and *Bam*HI, and subcloned into the respective restriction enzyme sites of the binary vector pBI101 (Clontech, Palo Alto, CA), yielding pAtZIP53G. The recombinant plasmid was a GUS-translational fusion construct. This plasmid was introduced into *Agrobacterium tumefaciens* EHA105 cells (Hood et al. 1993) by electroporation (Shen and Forde 1989). *A. thaliana* ecotypes Col-0 plants were transformed by the floral dip-method (Clough and Bent 1998) using the *A. tumefaciens* culture. Transformants were selected on MS agar medium containing 50 μg ml^{-1} kanamycin.

Quantitative and histochemical GUS activity assays

Seedlings (T2 generation) of transgenic plants were treated with 160 mM NaCl and 40 mM LiCl for 6 h and 24 h, respectively, and subjected to the quantitative GUS activity assay and GUS histochemical staining assay. The quantitative GUS activity was measured by fluorometric determination of production of 4-methyl-umbelliferone

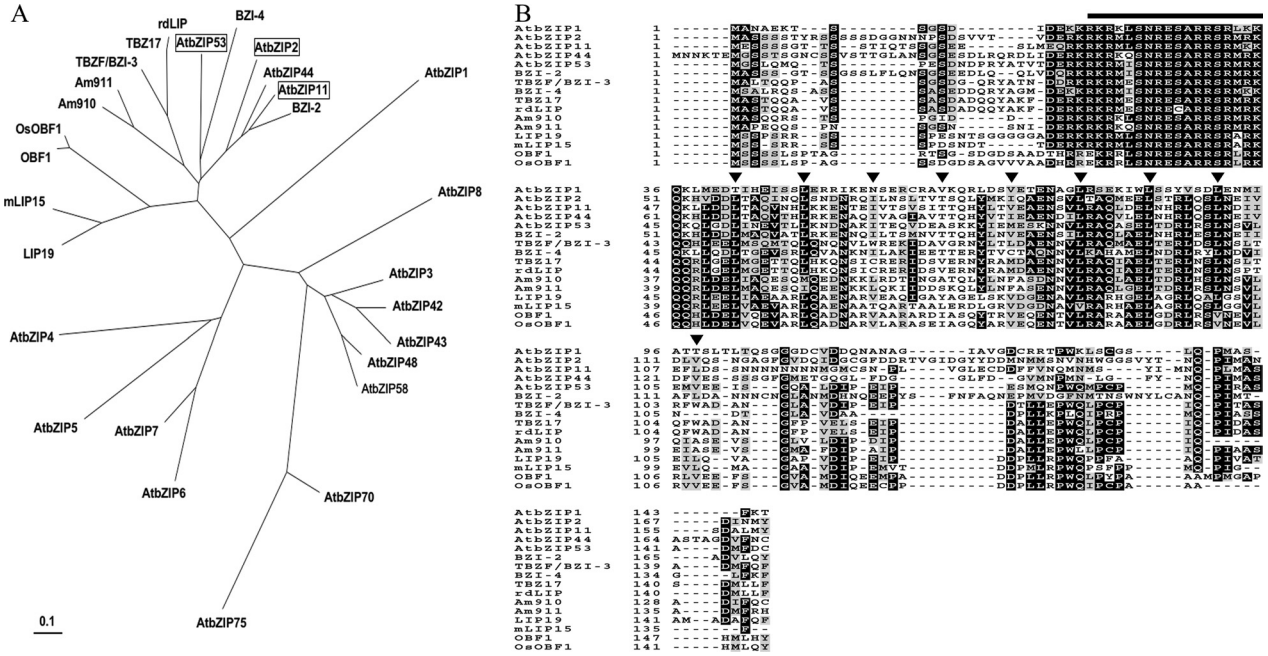

Figure 1. Phylogenetic relationship between 17 bZIP proteins of group S in *Arabidopsis* and the bZIP members of *lip19* subfamily, and the comparison of their amino acid sequences. (A) Phylogenetic relationship of 17 bZIP proteins of group S (Jakoby et al. 2002) and LIP19 subfamily members. The amino acid sequence alignment was constructed by the ClustalW program and the relationship was visualized by TREEVIEW program (Page 1996). AtbZIP2, AtbZIP11 and AtbZIP53 were boxed. (B) Amino acid sequence alignment of AtbZIP1, AtbZIP2, AtbZIP11, AtbZIP44, AtbZIP53 and LIP19 subfamily members. Arabic numerals indicate the amino acid positions from their amino-termini. Identical residues and similar residues are highlighted in black and in gray background, respectively. Dashes were introduced to maximize the alignment. The basic region and the positions of heptad leucine residues are indicated by the black bar and arrowheads, respectively. AtbZIP1 (At5g49450), AtbZIP2 (GBF5, At2g18160), AtbZIP11 (ATB2, At4g34590), AtbZIP44 (At1g75390), AtbZIP53 (At3g62420), Am910 (Y13675), Am911 (Y13676), BZI-2 (AY045570), BZI-4 (AY045572), LIP19 (X57325), mLIP15 (D26563), OBF1 (X62745), rdLIP (AB015187), TBZ17 (D63951), TBZF (identical to BZI-3, AB032478), OsOBF1 (AB185280).

(4MU) from 4-MUG (4-methyl umbelliferyl β-D-glucuronide) according to the fluorogenic assay protocol (Jefferson 1987). For histochemical GUS assays, NaCl- and LiCl-stressed seedlings and non-stressed mature plants at the flowering stage were stained with an X-Gluc (5-bromo-4-chloro 3-indolyl-β-D-glucuronide) solution, as described (Jefferson et al. 1987).

Results

Five Arabidopsis group S bZIP proteins are closely related to members of the lip19 subfamily of bZIP proteins

Rice *lip19*, maize *mlip15* and tobacco *tbzF* are responsive to low temperature, as we showed in earlier reports (Aguan et al. 1993; Kusano et al. 1995; Yang et al. 2001). Other research groups have reported that *A. majus 910* and *911*, tobacco *BZI-2*, -3 (*tbzF*), and *-4* are abundant in flower organs (Hurst 1994; Strathmann et al. 2001). The expression of *mlip15*, *tbz17* and *tbzF* has also been associated with senescence (Berberich et al. 1999; Yang et al. 2001). These genes are all intron-less and share high sequence similarity. They encode small bZIP proteins and contain conserved upstream open-reading frames (uORFs) in their 5′-untranslated regions (Wiese et al. 2004). We have proposed that this group of genes be designated the *lip19* subfamily of the plant bZIP-encoding gene family. Of 75 *Arabidopsis* bZIP-encoded genes, we classified *AtbZIP53* into the *lip19* subfamily and found that, of other 16 members of group S (Jakoby et al. 2002), four other members, *AtbZIP1*, *AtbZIP2*, *AtbZIP11* and *AtbZIP44* are structurally quite similar to the *lip19* subfamily (Figure 1). With this background, we focused on the *AtbZIP2*, *AtbZIP11* and *AtbZIP53*, and their gene products in this study.

Characterization of the AtbZIP2, AtbZIP11 and AtbZIP53 proteins

Using a GFP-fusion method, cellular localization of AtbZIP2, AtbZIP11 and AtbZIP53 proteins were determined in onion epidermal cells. As shown in Figure 2, all the three bZIP proteins localized to the nucleus, which is consistent with their role as transcription factors.

Then, we tested whether these proteins have DNA binding activity. Satoh et al. (2004) recently reported that this subset of bZIP proteins primarily bound to the *cis*-element identified in the *ProDH* (proline dehydrogenase) gene promoter. In addition to the ProDH *cis*-element sequence, we used the C-box/G-box hybrid (C/G-hybrid) sequence and the hexamer (Hex) sequence for DNA

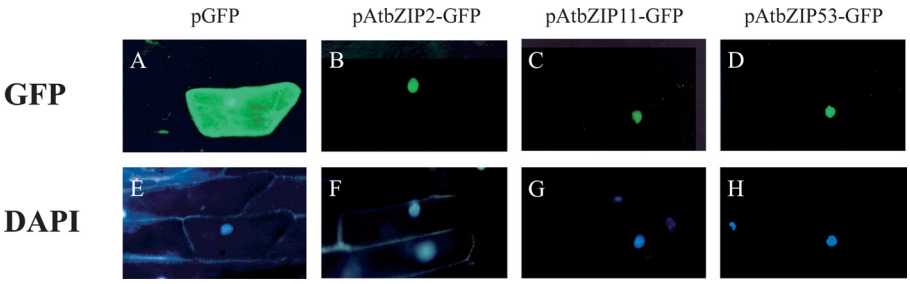

Figure 2. AtbZIP2, AtbZIP11 and AtbZIP53 proteins localized to nuclei in onion epidermal cells. Onion bulbs were bombarded with tungsten particles coated with pGFP2 (A, E), pAtbZIP2-GFP (B, F), pAtbZIP11-GFP (C, G) and pAtbZIP53-GFP (D, H) plasmids. GFP-fusion proteins were transiently expressed. Epidermal cell layers were stained with DAPI to ensure nucleus position and GFP- (A-D) and DAPI- (E-H) fluorescence was observed by fluorescence microscopy.

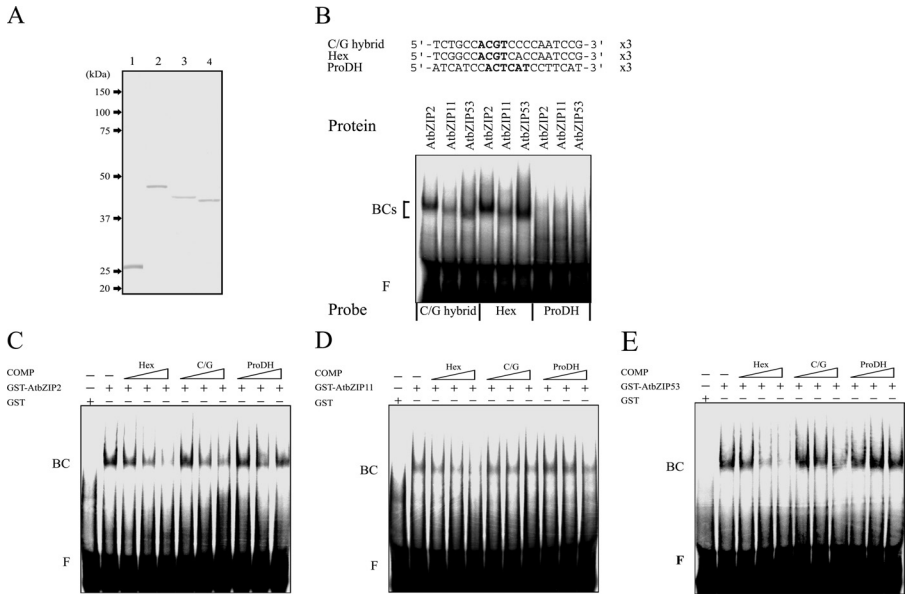

Figure 3. AtbZIP2, AtbZIP11 and AtbZIP53 proteins bound to DNA sequences containing C/G-hybrid and Hex motifs. (A) Production of GST, GST-AtbZIP2, GST-AtbZIP11 and GST-AtbZIP53 proteins in *E. coli*. Same volumes (2.5 μl) of the supernatants (See Materials and methods) were loaded to SDS-PAGE, blotted onto a PVDF membrane, and detected with anti-GST antibody. Lanes 1, GST; 2, GST-AtbZIP2; 3, GST-AtbZIP11; 4, GST-AtbZIP53. Protein sizes (in kDa) were indicated in the left margin. (B) EMSA analysis of AtbZIP2, AtbZIP11 and AtbZIP53 proteins. C/G-hybrid, Hex and ProDH fragment sequences used for EMSA were presented. The DNA fragments were 60 bp-long, containing three tandem-repeats of each 20 bp sequence. The positions of the free probes (F) and binding complexes (BCs) were indicated. (C), (D) and (E) Competition assay on DNA binding of AtbZIP2 (C), AtbZIP11 (D), and AtbZIP53 (E). Equal amounts of AtZIP2, AtbZIP11 and AtbZIP53 proteins were used in each assay. Hex oligonucleotide shown in A was used as a radiolabelled probe in EMSAs. The competitors (COMP) used were Hex, C/G hybrid, and ProDH. The competitors were added at 2-, 15-, and 30-fold molar excess (from left to right) as indicated by triangles. Plus and minus signs indicate presence and absence of each of proteins and competitors, respectively.

binding assays (Shimizu et al. 2005). Under the assay conditions, AtbZIP2, AtbZIP11 and AtbZIP53 proteins bound to the C/G-hybrid and Hex sequences well, but to a much lesser extent to the ProDH *cis*-sequence (Figure 3A, B). To test whether the DNA binding of AtbZIP2, AtbZIP11 and AtbZIP53 proteins is sequence-specific, we performed competition assays using the unlabeled oligonucleotides comprising C/G-hybrid, Hex and ProDH sequences to compete out the binding to the Hex-sequence (Figure 3C, D, E). In case of AtbZIP2, the Hex-binding was competed out by all three sequences, most weakly by the ProDH sequence (Figure 3C). This shows that the DNA-binding was specific, and that *AtbZIP2* prefers the binding to ACGT core-containing sequences rather than that to the ProDH *cis*-element. The similar result was obtained with AtbZIP53 in a stronger tendency, in which ProDH could not compete out the binding to the Hex-sequence at all even at a 30-fold molar excess (Figure 3E). In contrast, AtbZIP11 binds most strongly to the Hex-sequence and this binding was only weakly suppressed by the C/G-hybrid which also contains an ACGT core. Even at a 30-fold excess, the ProDH-sequence only partly competed out the binding to the Hex-sequence (Figure 3D). These EMSA data suggest that these three bZIP proteins recognize different promoter regions of yet unidentified target genes.

Next, the transactivation activities of these three proteins were assayed in yeast cells. As judged by

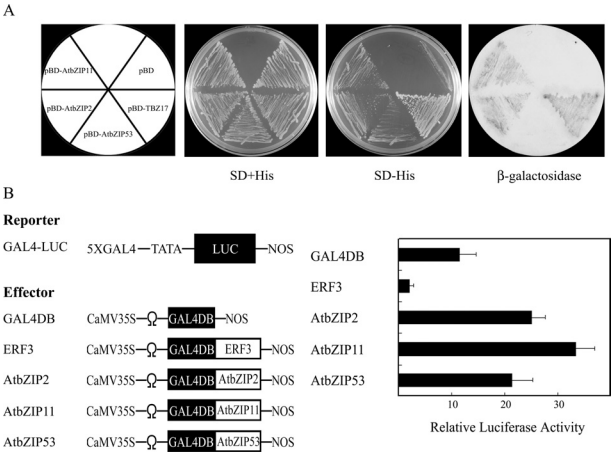

Figure 4. Transcription activity assay of AtbZIP2, AtbZIP11 and AtbZIP53. (A) Transactivation assay of AtbZIP2, AtbZIP11 and AtbZIP53 in yeast cells. pBD, negative control; pBD-TBZ17, positive control for transactivation (Yang et al. 2001). Photographs were taken at 5 days after streaking on SD (synthetic dropout) medium, with or without histidine. β-galactosidase filter assays were also performed. (B) Transcription activity assay of AtbZIP2, AtbZIP11 and AtbZIP53 in *Arabidopsis* cells. The scheme of constructs used for the transcription activity assay. The reporter gene, GAL4-LUC, contained five tandem-copies of the GAL4 binding site, TATA region of the CaMV 35S minimal promoter, and the luciferase gene (LUC). A GAL4 DNA binding domain (GAL4DB) is the basal effector and ERF3 is used as a control for a transcriptional repressor (Ohta et al. 2001). Effector plasmids are under the control of the CaMV 35S promoter and omega (Ω), a translation enhancer of tobacco mosaic virus. NOS denotes the terminator signal of the gene for nopaline synthase. The reference plasmid, pPTRL, was also used to normalize the efficiency of particle bombardment. The relative luciferase (LUC/RLUC) activities after bombardment to *Arabidopsis* rosette leaves. Experiments were performed three times in triplicate.

expression of two reporter genes (*HIS3* and *lacZ*), AtbZIP2 and AtbZIP11 have transactivation activity, while AtbZIP53 showed no such activity in yeast (Figure 4A). We, then, examined the potential of those bZIP proteins on transcription activity in *Arabidopsis* leaf cells. All the three proteins activated the expression of the reporter gene in *Arabidopsis* cells. The result indicated that not only AtbZIP2 and AtbZIP11 but also AtbZIP53 function as transcriptional activators in host plant cells (Figure 4B).

Abiotic-stress response of AtbZIP2, AtbZIP11 and AtbZIP53 genes

The *AtbZIP2*, *AtbZIP11* and *AtbZIP53* genes were expressed in plants under non-stressed conditions (Figure 5). Upon exposure to ionic stress (160 mM NaCl), *AtbZIP53* was strongly and *AtbZIP11* only slightly up-regulated, whereas *AtbZIP2* was down-regulated. *AtbZIP53* was also up-regulated by 40 mM LiCl (Figure 5). High osmoticum, low temperature and drought treatments did not change the transcript levels significantly for any of the three genes (Figure 5).

Salinity response of *AtbZIP53* was assessed by RT

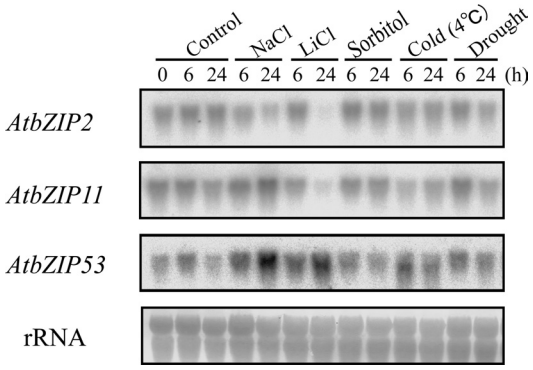

Figure 5. Expression analyses of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* upon exposure to various abiotic-stresses. The detached rosette leaves were exposed to 160 mM NaCl, 40 mM LiCl, 320 mM sorbitol, cold (4°C), and drought stresses for 6 h and 24 h.

(reverse transcription)-PCR. The levels of *AtbZIP53* transcripts were gradually increased upon the treatment with 160 mM NaCl, while those of *ProDH* and *GDH2* (Oono et al. 2003) were down-regulated or not affected (Figure 6A). Under the same condition, drought-responsive genes such as *rd29A*, *rd22*, *P5CS* (the gene encoding Δ^1-pyrroline-5-carboxylate synthetase) and *ERD14* (Nylander et al. 2001) were up-regulated (Figure 6A). To further confirm the responsiveness of *AtbZIP53* to NaCl and LiCl, *Arabidopsis* plants carrying an *AtbZIP53* promoter::GUS reporter were generated. Two independent transgenic lines #1–2 and #1–3 were grown on half-strength MS medium, and their two-week-old seedlings were treated with 160 mM NaCl and 40 mM LiCl for 6 h and 24 h, respectively, followed by measurement of the GUS activity of their cell extracts. Even without any ionic stress, the two transgenic plants showed certain levels of GUS activity, indicating that its expression is constitutive (Figure 6B). However, after both NaCl- and LiCl-treatment, the transgenic plants showed higher GUS activity (Figure 6B). To visualize the NaCl- and LiCl-responsiveness of *AtbZIP53*, seedlings of line #1-2 were subjected to histochemical GUS staining. The seedlings were more intensely stained after NaCl- and LiCl-treatments (Figure 6C). The other line #1-3 showed similar results (data not shown). These data indicate that the induction of *AtbZIP53* by NaCl and LiCl is controlled at the transcriptional level.

Phytohormone response of AtbZIP2, AtbZIP11 and AtbZIP53 genes

AtbZIP2 was rather constitutively expressed on transcript level, and down-regulated by cytokinin (BA) and jasmonic acid (JA). *AtbZIP11* and *AtbZIP53* transcripts were detected at low levels under normal conditions. *AtbZIP53* was slightly up-regulated by JA (Figure 7A), being consistent with the information presented by Genevestigator (http://www.genevestigator.ethz.ch/~w3pb/genevestigator/index.php). When BA was exogenously

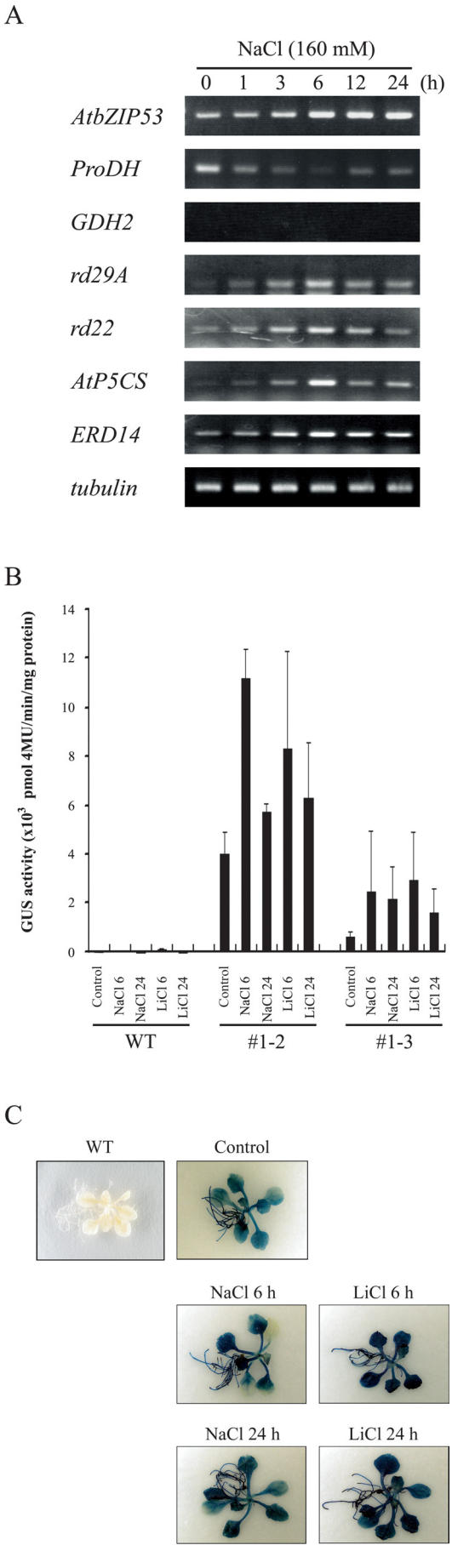

Figure 6. Ionic stress response of *AtbZIP53*. (A) RT-PCR analysis of *AtbZIP53* and some of high salinity-responsive and hypoosmotic-responsive genes upon 160 mM NaCl treatment. Two-week-old seedlings were floated on half-strength MS liquid medium and incubated for 12 h under the same conditions to eliminate the wounding effect. Seedlings were treated with 160 mM NaCl for certain periods. *tubulin* was also amplified and displayed as a loading control. (B) The quantitative GUS activity assay of *AtbZIP53* promoter::*GUS* transgenic *Arabidopsis* plants. Seedlings of two independent transgenic lines #1–2 and #1–3 were treated with 160 mM NaCl and 40 mM LiCl for 6 h and 24 h, respectively, then GUS activities were measured. The mean values of GUS activity were obtained from three plants of wild type (WT) and two independent transgenic lines. (C) Histochemical analysis of GUS activity in *AtbZIP53* promoter::*GUS* transgenic *Arabidopsis*. The seedlings of #1–2 were subjected to NaCl and LiCl treatment, and then stained with X-Gluc as described (Shen and Forde 1989; Clough and Bent 1998).

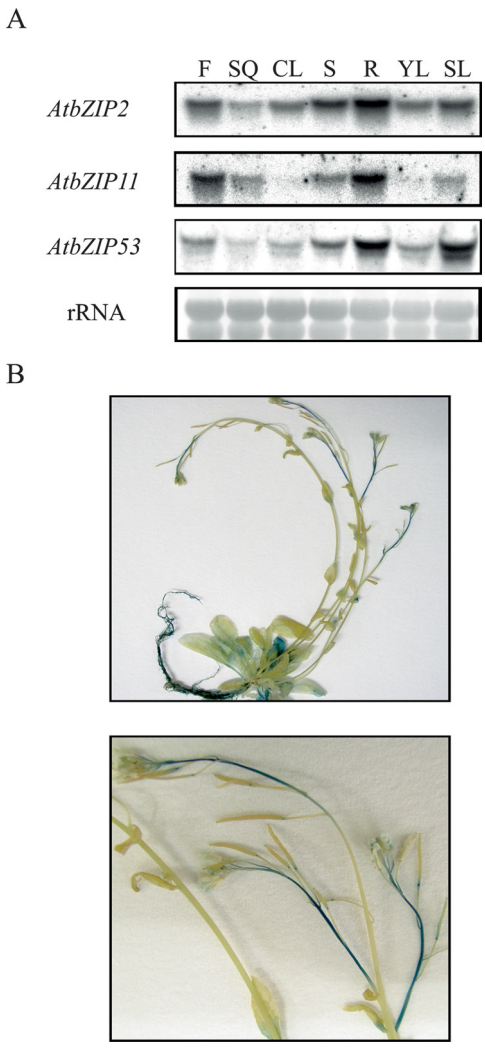

Figure 8. Organ specificity of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* expression in *A. thaliana* mature plants. (A) An RNA blot was stained by 0.04% methylene blue solution and rRNA patterns were photo-recorded to confirm equal loading of the samples. The blot was sequentially hybridized with the specific probes. F, flowers; SQ, siliques; CL, cauline leaves; S, stems; R, roots; YL, young rosette leaves; SL, senescing rosette leaves. (B) Histochemical analysis of *AtbZIP53* promoter::*GUS* transgenic *Arabidopsis* plants. The whole plant at flowering stage was subjected to GUS staining (left panel) and its close-up view was also shown (right panel).

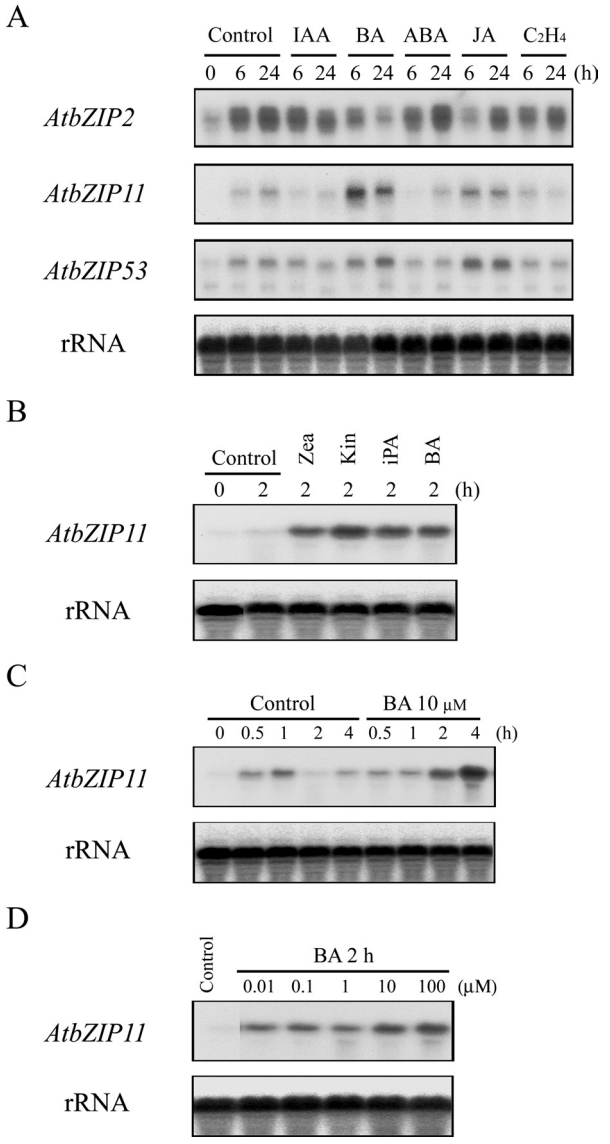

Figure 7. (A) Effect of phytohormones on *AtbZIP2*, *AtbZIP11* and *AtbZIP53* expression. (B) Effect of several cytokinins on *AtbZIP11* expression. Zea, *t*-zeatin; Kin, kinetine; iPA, isopentenyl adenine; BA, 6-benzyl adenine. Time-course (C) and dose response (D) of BA on *AtbZIP11* expression. For time-course analysis, 10 μM BA was applied to detached rosette leaves. For dose response, rosette leaves were treated with 0, 0.01, 0.1, 1, 10 and 100 μM of BA for 2 h.

applied, *AtbZIP11* transcripts accumulated (Figure 7A). To confirm the BA response of *AtbZIP11*, several cytokinins were tested (Figure 7B). All the tested compounds enhanced the levels of *AtbZIP11* transcripts. Furthermore, the response to BA was time and dose dependent (Figure 7C, D).

Organ specificity of AtbZIP2, AtbZIP11 and AtbZIP53 genes

AtbZIP2 was ubiquitously expressed in all plant organs. In contrast, *AtbZIP11* was primarily expressed in roots and flower organs, but less efficiently in leaves. *AtbZIP53* expression was abundant in roots and senescing leaves (Figure 8A). The expression profile of *AtbZIP53* was confirmed with the use of the *AtbZIP53* promoter::*GUS* transgenic plants described above. Strong β-glucuronidase activity was detected in roots and rosette leaves. Interestingly, flower stalks were also strongly and uniquely stained (Figure 8B).

Discussion

Of the *A. thaliana* bZIP protein classes, group S is the largest, consisting of 17 members in total. To date, however, except for AtbZIP11/ATB2 (see below), no in-depth analysis of the group has been performed. The *lip19* subfamily of bZIP genes of higher plants shows certain links to abiotic stress responses (especially cold stress), leaf senescence, and flower development. We found that *AtbZIP53* of the group S genes is a member of the *lip19* subfamily and that four other group S members also show high sequence similarity to *lip19* subfamily members (Figure 1). In this study, we investigated *AtbZIP53*, and two other group S members, *AtbZIP2* and *AtbZIP11*.

The basal level of *AtbZIP2* transcripts was totally unaffected by abiotic stresses and exogenously applied hormones. Furthermore, *AtbZIP2* transcripts were detected in all organs tested. The localization of AtbZIP2 to the nucleus and the *in vitro* DNA binding data suggest that it acts as a transactivator *via* binding to Hex- or C/G-hybrid sequences. The functional role of AtbZIP2, however, remains elusive.

The expression of *AtbZIP11*/*ATB2* is up-regulated by light, and its transcripts are abundant in flowers of mature plants (Rook et al. 1998b). This study showed that *AtbZIP11* transcripts are rich in flowers and roots, but fairly low in leaf tissues; i.e., photosynthetic organs. These observations support the hypothesis that the gene plays a role in carbohydrate-consuming (i.e. sink) tissues, which was originally proposed by Rook et al. (1998a). These authors also clearly showed that translation of *AtbZIP11*/*ATB2* transcripts was repressed via a conserved uORF (upstream open reading frame) in a sucrose-specific manner (Wiese et al. 2004). They designated this phenomenon as sucrose-induced repression of translation. The uORFs were observed in some members (*AtbZIP1*, *AtbZIP2*, *AtbZIP44* and *AtbZIP53*) of the group S and also in all *lip19* subfamily members (Wiese et al. 2004). In future studies it would be interesting to determine whether the translation of all the *lip19* family members is repressed by sucrose. Here, we found that the expression of *AtbZIP11* was up-regulated at the mRNA level by exogenously applied cytokinins including BA (Figure 7). The BA response was time- and dose-dependent (Figure 7C, D). Cytokinin has been implicated in many aspects of developmental and physiological processes of plants, including cell division, shoot formation and leaf senescence. Sweere et al. (2001) revealed that ARR4, a

response regulator in cytokinin signaling, specifically interacted with phytochrome B and this interaction kept phytochrome B active. It strongly suggests that cytokinin-signaling pathway, in which ARR4 is involved, cross-talked with phytochrome B-mediated light signaling (Fankhauser 2002). It is plausible that the induction of *AtbZIP11* by light might be mediated by cytokinin or *vice versa*. Furthermore, interactive effects of cytokinins, light and sucrose on the phenotypes and anthocyanin- and lignin-syntheses in cytokinin overproducing transgenic plants were described (Guo et al. 2005). It may be worth to investigate the participation of this bZIP factor on the expression of the pigment- and lignin-synthesis pathway genes.

Satoh et al. (2004) recently demonstrated that AtbZIP2, AtbZIP11/ATB2, AtbZIP44 and AtbZIP53 function as transcriptional activators via binding to the *cis*-element, ACTCAT (Satoh et al. 2002), identified in the *ProDH* gene promoter. Activation of *ProDH* leads to the decomposition of proline, which occurs during adaptation to rehydration or hypoosmotic conditions (Yoshiba et al. 1997). *In vitro* DNA binding assays, however, showed that, under our assay conditions, AtbZIP2, AtbZIP11 and AtbZIP53 proteins bind preferably to the ACGT core-containing Hex sequence rather than to the ACTCAT sequence and in case of AtbZIP2 and AtbZIP53 also to the hybrid sequence of the C-box/G-box motif (Figure 3). This result suggests that those bZIP members play roles in developmental process and/or other stress responses, in which C-box/G-box hybrid and Hex sequences are involved, rather than in hypoosmotic response. Consistent with this, *AtbZIP53* and *ProDH* responded oppositely to high salt treatment (Figure 6A), suggesting that AtbZIP53 itself could not drive *ProDH* transcription with its transactivation activity which displayed in plant cells (Figure 4) (Satoh et al. 2004).

Here, we demonstrated that three *Arabidopsis* genes, *AtbZIP2*, *AtbZIP11* and *AtbZIP53*, sharing high similarity to members of the *lip19* subfamily, respond differentially to abiotic stresses and hormone treatments, especially to high salinity and to cytokinin. To fully understand the regulatory roles of *AtbZIP2*, *AtbZIP11* and *AtbZIP53* in gene transcription, extensive analysis of gene-knockout plants will be required, as well as gene expression analyses of *AtbZIP*-overexpressing plants.

Acknowledgements

The authors thank Drs. N.-H Chua (Rockefeller University), M. Ohme-Takagi (Advanced Institute of Science and Technology) and E. Hood (National Science Foundation) for providing the GFP plasmid and *Agrobacterium* strain, respectively. SS Lee was supported by a fellowship from the Hirose International Scholarship Foundation of Japan.

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