

# The Arabidopsis *AtbZIP1* transcription factor is a positive regulator of plant tolerance to salt, osmotic and drought stresses

Xiaoli Sun · Yong Li · Hua Cai · Xi Bai ·  
Wei Ji · Xiaodong Ding · Yanming Zhu

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**Abstract** According to the AtGenExpress transcriptome data sets, *AtbZIP1* is an Arabidopsis gene induced by several abiotic stresses, such as salt, cold and drought. Here, we isolated *AtbZIP1*, and used semi-quantitative reverse transcription-PCR to verify that *AtbZIP1* expression was indeed significantly induced by salt, osmotic, and cold stresses in Arabidopsis. *AtbZIP1* knockout mutants showed a reduced tolerance to salt and osmotic stresses, coinciding with a suppression of the expression of several stress-responsive genes, such as *COR15A*, *RD17* and *RD29A*. Consistently, the restoration of *AtbZIP1* in the knockout lines restored the plants ability to tolerate salt and osmotic stresses. Furthermore, overexpressing *AtbZIP1* in

the wild type Arabidopsis resulted in an enhanced tolerance to salt and drought stresses. Sequence analysis shows that *AtbZIP1* belongs to the S subfamily of basic leucine zipper transcription factors (TFs). The transient expression of green fluorescent protein-*AtbZIP1* in tobacco leaf cells showed that *AtbZIP1* localizes in nuclei. A transactivation assay further suggested that *AtbZIP1* functions as a transcriptional activator in yeast and the two protein motifs (aa 13–38 and 92–118) are indispensable for transactivation activity. These results indicate that the TF *AtbZIP1* is a positive regulator of plant tolerance to salt, osmotic, and drought stresses.

**Keywords** Arabidopsis · *AtbZIP1* · Drought stress · Osmotic stress · Salt stress

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X. Sun · Y. Li · H. Cai · X. Bai · W. Ji · X. Ding · Y. Zhu (✉)  
Plant Bioengineering Laboratory, Northeast Agricultural  
University, Harbin 150030, China  
e-mail: ymzhu2001@neau.edu.cn

X. Sun  
e-mail: sunxiaoli2008@yahoo.cn

Y. Li  
e-mail: Yong@neau.edu.cn

H. Cai  
e-mail: caihua@neau.edu.cn

X. Bai  
e-mail: baixi@neau.edu.cn

W. Ji  
e-mail: iwei\_ji@neau.edu.cn

X. Ding (✉)  
Department of Neurology, The University of Texas  
Southwestern Medical Center, Dallas, TX 75390, USA  
e-mail: xiaodong.ding@utsouthwestern.edu

## Introduction

The proper regulation of gene expression permits normal growth and development in all organisms. One important means of modulating gene expression is through the control of transcription factors (TFs) (Beckett 2001). The basic leucine zipper (bZIP) family is one of the largest TF families, controlling many fundamental processes, particularly seed formation and abiotic stress responses.

The bZIP TFs are characterized by a conserved domain (the bZIP domain) composed of a basic region, responsible for the specific binding of the TFs to their targets, and a leucine zipper, required for dimerization (Wingender et al. 2001). It has been demonstrated that bZIPs interact with the G-box element 5'-CACGTG-3', regulating the expression of downstream genes, and participating in plant responses to various abiotic stresses including salt, drought, and cold (Choi et al. 2000; Kim et al. 2002; Kang et al.

2002; Lu et al. 2009; Xiang et al. 2008; Zou et al. 2007). In plants, bZIPs bind to DNA *cis*-elements as dimers, and specific dimerizations can alter the affinity of these proteins for certain *cis*-element subsets, altering the transcriptional activity of the associated gene (Weltmeier et al. 2006, 2009; Schutze et al. 2008; Alonso et al. 2009). An example of this can be found with group C and group S members of the C/S1 network of bZIP TFs, which functionally interact, and are cooperatively involved in plant development and responses to stress (Kaminaka et al. 2006; Weltmeier et al. 2009; Kang et al. 2010).

The Arabidopsis gene *AtbZIP1* (At5g49450) belongs to the S subfamily of bZIP TFs. Previous studies have demonstrated that, owing to the upstream open reading frame (uORF) contained in its 5' untranslated region (UTR), *AtbZIP1*, like the Arabidopsis bZIPs *AtbZIP9* and *AtbZIP63*, is involved in sugar repression at the transcriptional and post-transcriptional level. The expression of *AtbZIP1* was strongly repressed by glucose treatment, where transcript levels became almost nonexistent (Kang et al. 2010). *AtbZIP1* was also reported to participate in the C/S1 network, forming heterodimers, with *AtbZIP10* and *AtbZIP63*, which could improve its binding affinity to G-box and C-box *cis*-elements (Ehlert et al. 2006; Kang et al. 2010).

*AtbZIP1* expression is largely induced by salt, cold, and drought stresses, according to the AtGenExpress transcriptome data sets. We chose to investigate the expression pattern of *AtbZIP1* under salt, osmotic, and cold stress, using semi-quantitative reverse transcription (RT)-PCR, as well as determine its subcellular localization, using a green fluorescent protein (GFP)-fusion protein. We also explored the importance of two *AtbZIP1* protein domains, the basic region (aa 13–38) and a fragment close to the C-terminus (aa 92–118), which we found to be indispensable to its transactivation activity. Furthermore, by comparing the phenotypes of wild type (WT), *AtbZIP1* knockout (KO), rescued *AtbZIP1* KO (CO) and *AtbZIP1* overexpression (OX) Arabidopsis, under a variety of environmental conditions, we demonstrate that *AtbZIP1* plays a crucial role in abiotic stress tolerance.

## Materials and methods

### Plant materials, growth conditions and stress treatments

The WT *Arabidopsis thaliana* (Columbia ecotype), and the *A. thaliana AtbZIP1* knockout mutants, SALK\_069489C (KO-1) and SALK\_059343 (KO-2), were obtained from the European Arabidopsis Stock Centre (NASC; <http://arabidopsis.org.uk/>). Arabidopsis seeds were germinated at 22°C with 100 μmol photons/m<sup>2</sup> s, 60% relative humidity, and an 18 h light/6 h dark cycle. For developmental stage

analysis, total RNA was isolated from 4-week-old Arabidopsis at the rosette stage, and 10-week-old Arabidopsis at the flowering stage. For the expression pattern analysis of *AtbZIP1*, WT Arabidopsis seeds were germinated and grown on filter paper (Whatman 3MM) saturated with ½ Murashige and Skoog (MS) solution, and then the 21-day-old seedlings were treated with 300 mM NaCl, 30% (w/v) PEG6000, or 4°C. The total RNA was separately isolated, from the leaves and roots of Arabidopsis seedlings harvested at 0, 0.5, 1, 3, 6, and 12 h after treatments. For the analysis of abiotic stress response genes, total RNA was isolated from WT and KO mutant Arabidopsis seedlings, which were treated with 300 mM NaCl or 30% PEG6000 for 3 h.

### Semi-quantitative reverse transcription-PCR

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Using the Superscript III Kit (Invitrogen, Carlsbad, CA, USA), the first-strand cDNA was synthesized with the oligo d(T)<sub>18</sub> reverse primer according to the manufacturer's instructions. Semi-quantitative RT-PCR was carried out with 0.5–2 μL of cDNA in a 25 μL reaction volume. The actin gene was used as an internal control. The primer pairs used in the RT-PCR are presented in Table S1. The PCR products were resolved by electrophoresis on a 2% agarose gel.

### Transcription activation assays

The full-length *AtbZIP1* was amplified using the following primer pairs: 5'-TTTCCATGGTTATGGCAAACG-3' and 5'-AAACTGCAGCTTGTCTTAAAGGACG-3'. Four truncated forms of *AtbZIP1* were also amplified: *AtbZ1*, encoding aa 13–145 (5'-AAACCATGGACATAGATGA GAAGA-3' and 5'-AAAGGATCCATGTCTTAAAGGAC C-3'), *AtbZ2*, encoding aa 38–145 (5'-AAACCATGGAGTT AATGGAAGAC-3' and 5'-AAAGGATCCATGTCTTAA AGGAC-3'), *AtbZ3*, encoding aa 1–118 (5'-TTTCCATG GTTATGGCAAACG-3' and 5'-AAAGGATCCCCGCGT TTG-3'), and *AtbZ4*, encoding aa 1–92 (5'-TTTCCATGG TTATGGCAAACG-3') and (5'-AAAGGATCCTCTCTA AATCGCTAAC-3').

The yeast strain AH109 (*LYS2::AL1*, *MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ<sub>UAS</sub>-GAL1-TATA-HIS3*, *GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2*, *URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>-lacZ*, *MEL1*) was used in the transcription activation assays. Yeast growth and transformation were carried out according to the BD Yeastmaker™ Transformation System 2 User Manual (PT1172-1).

For the transcription activation assays, pGBKT7-*AtbZIP1*, pGBKT7-*AtbZ1*, pGBKT7-*AtbZ2*, pGBKT7-*AtbZ3*, pGBKT7-*AtbZ4*, and pGBKT7-*AtDREB1A* were transformed into yeast strain AH109 cells. The transformants

were grown and selected on synthetic defined (SD)/-Trp and SD/-Trp-His media. Preparation of yeast protein extracts was conducted according to the Yeast Protocols Handbook (PT3024-1), using the TCA method. LacZ activity was assessed using the  $\beta$ -galactosidase filter lift assay. The quantitative  $\beta$ -galactosidase activity assays were performed according to the Yeast Protocols Handbook, using chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) as a substrate.

#### Subcellular localization of AtbZIP1 protein

To determine the subcellular localization of AtbZIP1, the full-length *AtbZIP1* was amplified (primers: 5'-TTTCCATGGTTATGGCAAACG-3' and 5'-AAAGAGCTCCTTGTCTTAAAGGACG-3') and inserted into the *NcoI/SacI*-digested pCEG vector to generate pCEG-AtbZIP1:GFP. The pCEG-AtbZIP1:GFP vector was introduced into *Agrobacterium tumefaciens* strain EHA105, with pCEG as the negative control. The intact leaves of 4-week-old *Nicotiana benthamiana* were infiltrated with *A. tumefaciens* harboring pCEG-AtbZIP1:GFP or pCEG as described (Kapila et al. 1997). Green fluorescence from the transiently expressed constructs was observed at 488 nm using a confocal laser scanning microscope (SP5; Leica, Germany).

#### Transformation of Arabidopsis

To generate transgenic plants, the ORF of *AtbZIP1* was cloned into the *SwaI* and *BamHI*-digested plant transformation binary vector pFGC1008, placed under the control of the CaMV 35S promoter, with *HPT* as the selectable marker gene. The ORF was amplified using the primer pair: 5'-ATATTTAAAATGGCAAACG-3' and 5'-AAAGGATCCCTTGTCTTAAAGGACG-3'. The construct, pFGC1008-AtbZIP1, was introduced into *Agrobacterium tumefaciens* strain LBA4404, and transformed into the KO mutants and WT Arabidopsis as previously described (Clough and Bent 1998), producing the *AtbZIP1* complementation (CO) and overexpression (OX) transgenic lines, respectively. The transformants were selected on 1/2 MS media containing 20  $\mu$ g/mL hygromycin, then transplanted into soil and grown in the greenhouse. The T<sub>1</sub> seeds were harvested and sown again to generate T<sub>2</sub> plants for further characterization. The homozygous transgenic lines from the T<sub>4</sub> generation were used in the following phenotypic analysis.

#### Phenotypic analysis of knockout mutant and transgenic Arabidopsis plants

In all phenotypic examinations, Arabidopsis seeds were surfaced-sterilized as described (Ho et al. 2010), and grown on 1/2 MS media. For salt tolerance, the plants were grown

on the media supplemented with 100 mM NaCl, for 21 days, or 125 mM NaCl, for 14 days. For the osmotic stress tolerance test, seeds were grown on 1/2 MS media with 200 or 300 mM mannitol, for 14 days.

For the measurement of the lengths of rosettes and roots, the seeds from WT, KO, and CO lines were germinated and grown on 1/2 MS media for 7 days. The plants were then transferred to fresh medium (with and without 125 mM NaCl, or 300 mM mannitol), to be grown for another 12 days before rosette size and root lengths were measured.

For the salt tolerance test, 3-week-old seedlings of WT and OX transgenic (T<sub>4</sub>) Arabidopsis were irrigated with a 300 mM NaCl solution, every 3 days. Photos were taken on the 15th day, and chlorophyll content was determined spectrophotometrically. For the drought tolerance test of WT and OX transgenic plants, the plants were grown under normal conditions for 3 weeks. Water was then withheld from the plants for 15 days, followed by a resumption of regular watering. All experiments were repeated at least 3 times, and the results from one representative experiment are shown. The data were subjected to statistical analyses using SPSS statistical software 13.0 (SPSS, <http://www.spss.com>).

## Results

#### Isolation and bioinformatics analysis of *AtbZIP1* gene

Previous studies have demonstrated that bZIP TFs are involved in regulating plant responses to various biotic and abiotic stresses. The publicly available AtGenExpress transcriptome data sets (Kilian et al. 2007), indicate that *AtbZIP1* is up-regulated in responses to abiotic stress. Therefore, we isolated the full-length *AtbZIP1* gene, including the 5' and 3' UTRs, from *Arabidopsis thaliana* genomic DNA.

Sequence alignments revealed that *AtbZIP1* contained a conserved bZIP domain (Fig. S1b), which includes a basic region at the N-terminus, responsible for sequence-specific DNA binding, and a less conserved leucine zipper at the C-terminus, responsible for protein dimerization. Further analysis revealed that the *AtbZIP1* transcript contains a uORF of 78 bp (Fig. S1a), which is also present in other group S bZIPs (e.g., *AtbZIP2*, *AtbZIP44*, and *AtbZIP53*) that are involved in post-transcriptional repression and sugar signalling (Jakoby et al. 2002; Weltmeier et al. 2009; Kang et al. 2010).

#### Expression profile of *AtbZIP1* under abiotic stresses

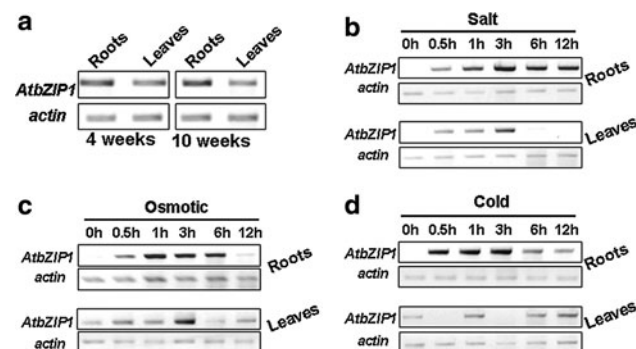
To better understand the function of *AtbZIP1*, we used semi-quantitative RT-PCR to investigate its transcript levels, in leaves and roots, under various environmental

conditions. As shown in Fig. 1a, *AtbZIP1* transcript levels, under normal growth conditions, were greater in roots than in shoots. No obvious difference in transcript levels was observed between 4- and 10-week-old Arabidopsis, indicating that *AtbZIP1* expression was independent of plant development.

Under abiotic stress conditions, *AtbZIP1* expression in different organs varied greatly. Transcript levels were much higher in the roots when the plants were salt stress (300 mM NaCl), osmotic stress (30% PEG6000), or cold stress (4°C). For each of these treatments, *AtbZIP1* expression reached maximal levels at 3, 1, and 3 h, respectively (Fig. 1b–d). Increases in *AtbZIP1* transcription were also detected in shoots after 0.5 h of salt and osmotic stress treatments, reaching a maximum after 3 h of exposure (Fig. 1b, c). Cold treatments, on the other hand, resulted in fluctuations in *AtbZIP1* transcripts in the shoots where *AtbZIP1* level dropped to almost zero at 0.5 h and 3 h but recovered at 1 h and 6 h and thereafter (Fig. 1d). These changes in *AtbZIP1* transcription levels, under different abiotic stress conditions, indicate that *AtbZIP1* might be an important gene involved in plant abiotic stress response.

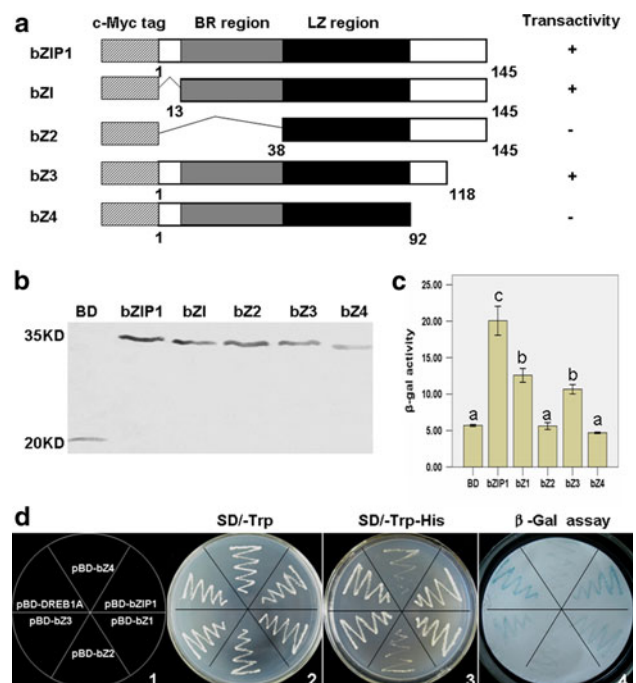
#### Identification and characterization of the AtbZIP1 transactivation domain

We fused the full-length *AtbZIP1*, and serially truncated fragments thereof, to the GAL4 DNA-binding domain in the pGBKT7 vector (Fig. 2a), and introduced the constructs to the yeast reporter strain AH109. The fusion



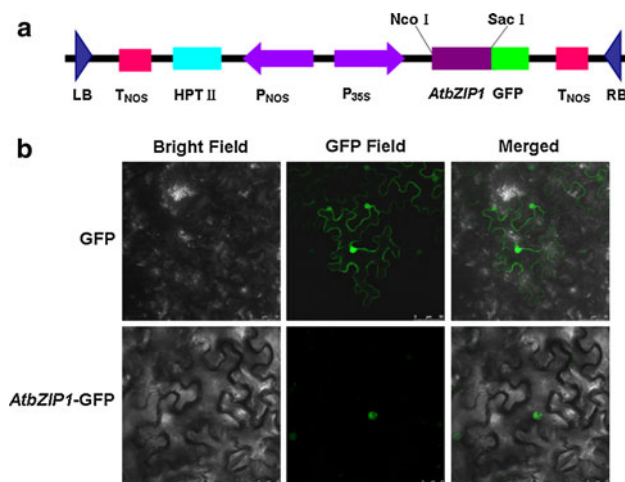
**Fig. 1** *AtbZIP1* expression patterns in response to age and stress treatments. **a** The organ-specific expression of *AtbZIP1* is independent of developmental stage. **b** The expression of *AtbZIP1* demonstrated by RT-PCR in shoots and roots, respectively, under salt treatment. **c** The expression of *AtbZIP1* under drought treatment. **d** The expression of *AtbZIP1* under cold treatment. Seeds were sown and grown on filter paper (Whatman 3MM) saturated with  $\frac{1}{2}$  MS solution. Total RNA was extracted from 3-week-old seedlings and analyzed by semi-quantitative RT-PCR. Actin gene was used as an internal standard. Thirty-one PCR cycles were used for *AtbZIP1* and only 26 for *actin* gene. The time of the treatments are indicated. Cold: 4°C; osmotic: 30% PEG6000; salt: 300 mM NaCl

protein expression was detected by Western blot (Fig. 2b). LacZ activity was assessed using a  $\beta$ -galactosidase filter lift assay. The pGBKT7-AtDREB1A vector was employed as a positive control. Yeast cells harboring pGBKT7-AtbZIP1, pGBKT7-AtbZ1, pGBKT7-AtbZ2, pGBKT7-AtbZ3, pGBKT7-AtbZ4 and pGBKT7-AtDREB1A, were capable of growth on the SD/-Trp media, however, as shown in Fig. 2d, only the yeast cells harboring pGBKT7-AtbZIP1, pGBKT7-AtbZ1, pGBKT7-AtbZ3 and pGBKT7-AtDREB1A grew well on SD/-Trp-His media, indicating that only these constructs were capable of promoting transcription. The yeast bearing pGBKT7-AtbZ2 and pGBKT7-AtbZ4 could not grow on SD/-Trp-His, nor did they demonstrate any  $\beta$ -galactosidase activity, thus indicating a lack of transactivation activity. Quantitative  $\beta$ -galactosidase activity assays showed that the full-length *AtbZIP1* had the highest transactivation activity, and that the deletion of its N-terminus (aa 1–13, AtbZ1) or



**Fig. 2** *AtbZIP1* transactivity assays. **a** Schematic representation of *AtbZIP1* serially truncated fragments (*AtbZIP1*, *AtbZ1*, *AtbZ2*, *AtbZ3* and *AtbZ4*). **b** Western blot analysis of the full-length and serially truncated fragments in yeast cells. The yeast proteins were extracted and used for Western blot with the c-Myc antibody. Two independent experiments were performed and one representative example is shown. **c**  $\beta$ -Galactosidase activity in yeast strain AH109 carrying different constructs as described in **a**. **d** Transactivation assays of *AtbZIP1* in yeast cells. The transformed yeast grown on SD/-Trp media and SD/-Trp-His media are shown. LacZ activity was assessed by  $\beta$ -galactosidase filter lift assay. pGBKT7-AtDREB1A was used as the positive control. Twelve measurements obtained from 3 independent yeast transformants were used for statistical analysis. A Student's *t* test was used to determine statistical significance; significant differences ( $P < 0.05$ ) are indicated by different lowercase letters





**Fig. 3** Nuclear localization of AtbZIP1 protein. **a** A schematic showing the construction of pCEG-AtbZIP1:GFP. **b** AtbZIP1 protein in the plant cell nucleus. Images showing *N. benthamiana* cells expressing GFP (upper lane) or AtbZIP1:GFP (bottom lane) fusion protein, examined under bright-field illumination (left) and under fluorescent-field illumination (middle, 488 nm) to detect GFP fluorescence, and by confocal microscopy (right) for an overlay of bright and fluorescent illumination

C-terminus (aa 118–145, AtbZ3) would decrease AtbZIP1 transactivation activity. However, these deletions were not as serious as the deletion of larger N-terminal (aa 1–38, AtbZ2) and C-terminal (aa 92–145, AtbZ4) fragments, whose deletion significantly decreased AtbZIP1 transactivation activity ( $P < 0.01$ ). This indicates that the basic region of the N-terminus, and the fragment close to the C-terminus (aa 92–145), are indispensable for transactivation activity (Fig. 2c).

AtbZIP1 protein targets to the nuclei

To determine the subcellular localization of AtbZIP1, a fusion of AtbZIP1 and GFP, under the control of a 35S promoter (Fig. 3a), was transiently expressed in the leaves of *N. benthamiana*. Three days after infiltration, GFP fluorescence could be efficiently detected under the confocal microscope at 488 nm. *N. benthamiana* leaves infiltrated with the pCEG vector (negative control) displayed fluorescence throughout the entire cell (Fig. 3b), but fluorescence in the *N. benthamiana* leaves infiltrated with pCEG-AtbZIP1:GFP was observed exclusively in the nuclei, demonstrating that AtbZIP1 is a nuclear-localized protein.

Knockout of AtbZIP1 reduces plant tolerance to salt and osmotic stress

We used WT Arabidopsis and two KO mutants (SALK\_069489C and SALK\_059343) to examine the role

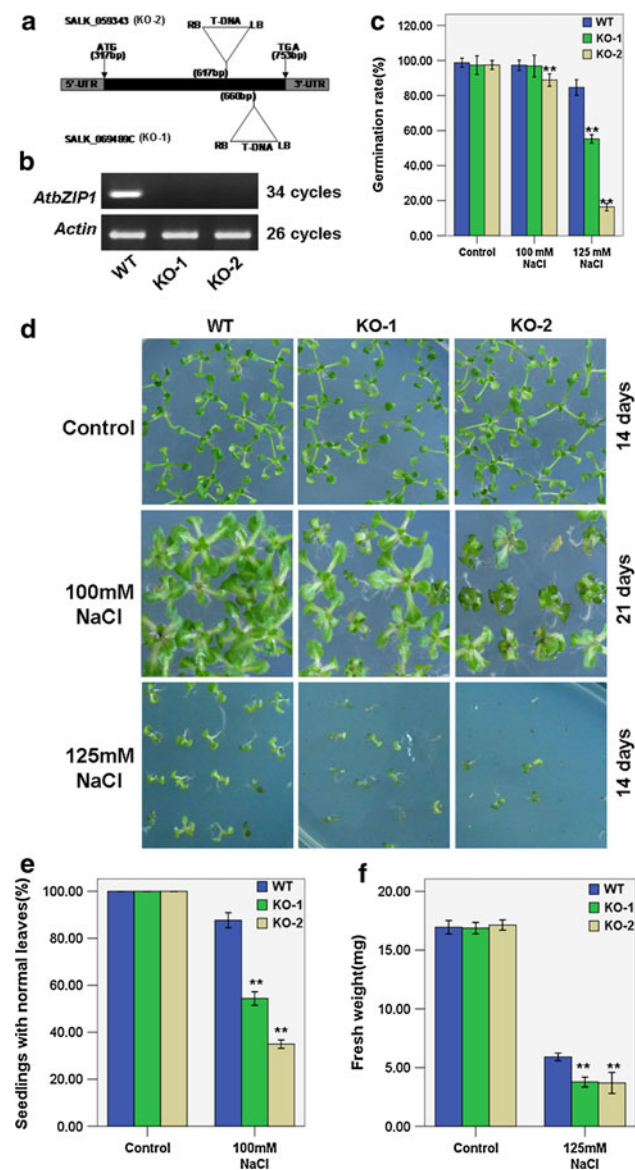
AtbZIP1 plays in abiotic stress response. The KO mutants were generated by T-DNA insertion (Fig. 4a), and, using RT-PCR, we verified that AtbZIP1 was not expressed in the two KO mutants, in contrast to WT Arabidopsis, indicating that AtbZIP1 was effectively silenced in the two KO mutant (Fig. 4b).

To determine whether the AtbZIP1 knockout affected the plants tolerance to salt, we exposed seedlings to increasing concentrations of NaCl. When treated with 150 mM NaCl, the germination of WT and KO seeds was greatly inhibited (data not shown). When treated with 100 mM NaCl, KO-1 had similar but KO-2 had significantly lower germination rate than the WT Arabidopsis ( $P < 0.05$  by Student's *t* test; Fig. 4c). On the 14th day of the 100 mM NaCl treatment, WT and mutant seedlings exhibited similar growth performance, showing no significant differences in fresh weight. On day 21, WT plants displayed better growth with more normal true leaves, while the KO lines exhibited dwarfed and vitrified leaves (Fig. 4d, e). When treated with 125 mM NaCl, the germination rate and fresh weight of WT plants was greater than that of the two KO mutants (Fig. 4c–e). On the 21st day, most plant leaves (WT and KOs) were dwarfed and vitrified.

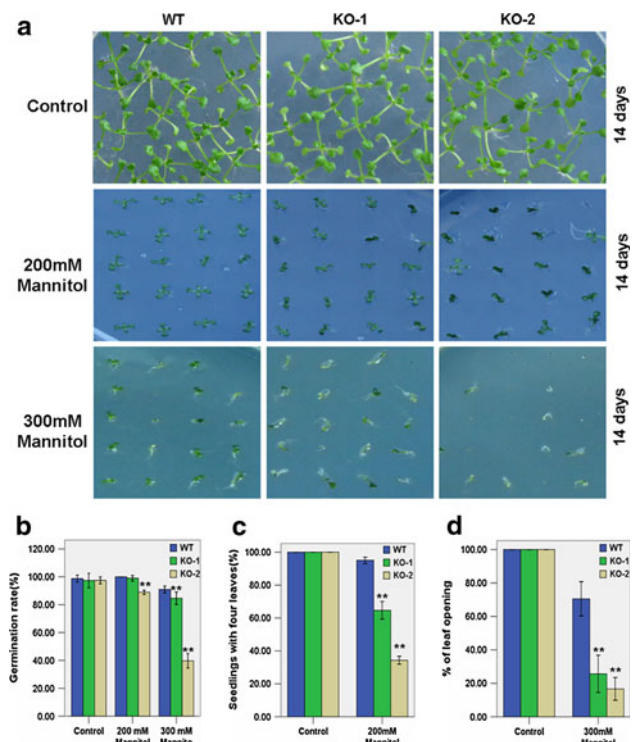
To determine whether the AtbZIP1 knockout affected the plants tolerance to osmotic stress, we exposed seedlings to increasing concentrations of mannitol. On  $\frac{1}{2}$  MS media supplemented with 400 mM mannitol, only WT seeds could germinate, although they could not develop healthy true leaves (data not shown). When treated with 200 mM mannitol, the KO-2 plants exhibited a decreased germination rate, compared with WT, while the germination and leaf-opening rate of the KO-1 mutant remained similar to that of WT Arabidopsis. About 95, 65 and 35%, of WT, KO-1, and KO-2 seedlings, respectively, displayed 4 normal true leaves, however, many more KO mutant leaves were vitrified (Fig. 5a, c). When exposed to 300 mM mannitol, the WT plants had higher germination rate and more opening leaves than the KO lines (Fig. 5b, d). However, whether in WT or KO lines, most of the seedlings had only 2 leaves.

AtbZIP1 restores salt and osmotic stress resistance in the mutant knockout lines

To verify that the decreased resistance of KO plants to abiotic stresses was due to the knockout of AtbZIP1, we generated AtbZIP1 complementation lines (CO), which expressed AtbZIP1, under the control of the strong constitutive CaMV35S promoter, in KO mutant lines (Fig. 6a). Two independent,  $T_4$  generation, transgenic lines were identified by semi-quantitative RT-PCR (Fig. 6b), where



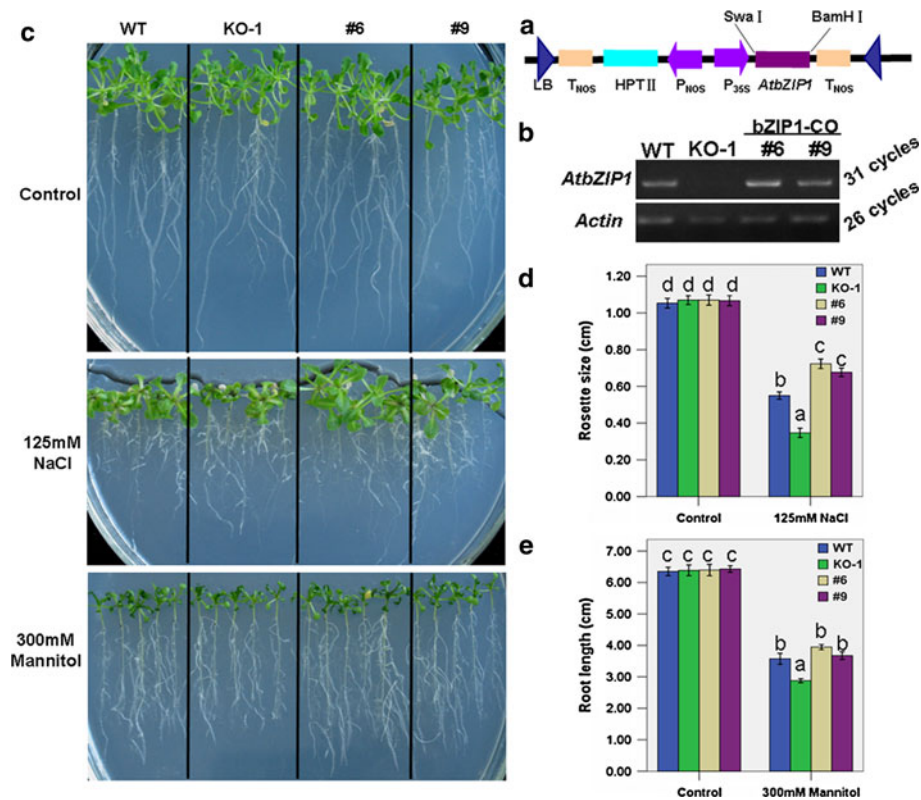
**Fig. 4** Seed germination assays of WT Arabidopsis and two KO mutant lines in response to salt stress. **a** T-DNA and its flanking structure in *AtbZIP1*. **b** RT-PCR analysis of the two KO mutants. Total RNA extracted from 3-week-old seedlings was analyzed by semi-quantitative RT-PCR. Actin gene was used as an internal standard. Thirty-four PCR cycles were used for *AtbZIP1* and only 26 for actin gene. **c** Seed germination ratio of WT plants and KO mutants on 1/2 MS media in the presence of 100 mM NaCl or 125 mM NaCl. Germination was recorded when radicles completely penetrating the seed coat. The germination rate was measured on the 8th day post-stratification. **d** Growth performance of WT and KO seedlings germinated and grown on 1/2 MS media with and without 100 mM NaCl or 125 mM NaCl. Photographs were taken 21 days (for 100 mM NaCl) and 14 days (for 125 mM NaCl) after germination. **e** WT and KO seedlings with normal true leaves on 1/2 MS media with and without 100 mM NaCl. The percentage of normal true leaves was scored 21 days after germination. **f** Fresh weight of WT and KO plants on 1/2 MS media with and without 125 mM NaCl. The fresh weight was measured 14 days after germination. Data are means ( $\pm$ SE) of 3 replicates (30 seeds each).  $**P < 0.05$  by Student's *t* test



**Fig. 5** Seed germination assays of WT Arabidopsis and KO mutants in response to osmotic stress. **a** Growth performance of WT and KO seedlings germinated and grown on 1/2 MS media with and without 200 or 300 mM mannitol. Photographs were taken 14 days after germination. **b** Seed germination ratio of WT Arabidopsis and KO mutants on 1/2 MS media in the presence of 200 mM mannitol or 300 mM mannitol. **c** WT and KO seedlings with four normal true leaves on 1/2 MS media with and without 200 mM mannitol. **d** Leaf opening rate of WT and KO plants under control conditions and drought stress (300 mM mannitol). Data are means ( $\pm$ SE) of 3 replicates (30 seeds each).  $**P < 0.05$  by Student's *t* test

results showed that *AtbZIP1* expression was restored to the KO plants.

Under standard culture conditions, we did not observe any noticeable difference in the germination of the two CO lines (lines #6 and #9), compared with WT plants. To test the resistances to salt and osmotic stresses at the seedling stage, 7-day-old WT, KO, and CO seedlings, germinated and grown under normal conditions, were transferred to 1/2 MS media supplemented with either 125 mM NaCl or 300 mM mannitol, and grown for 12 days. As shown in Fig. 6d, under 125 mM NaCl, the maximum rosette size of both WT and CO plants was larger than measured in KO lines. When grown in 300 mM mannitol, the growth of primary roots was significantly inhibited in the KO plants compared to that of WT and CO lines (Fig. 6c, e). All of these results demonstrate that the decreased stress resistance exhibited by the KO plants was due to the *AtbZIP1* knockout, and that the re-insertion of *AtbZIP1* could restore salt and osmotic stress resistance to the KO mutant lines.



**Fig. 6** The wild type *AtbZIP1* gene could restore stress tolerance to the knockout mutant. **a** A schematic showing the structure of the p35S: *AtbZIP1* construct. **b** RT-PCR analysis of WT, KO and CO plants. *Actin* gene was used as an internal standard. 31 PCR cycles were used for *AtbZIP1* and only 26 for *actin* gene. **c** Phenotypes of WT, KO and two CO seedlings grown in either 1/2 MS medium with and without 125 mM NaCl or 300 mM mannitol. 7-day-old seedlings grown on 1/2 MS were transferred to new solid agar plates

supplemented with 125 mM NaCl or 300 mM mannitol. Photographs were taken after 12 days growth. **d** Measurements of maximum rosette size under normal conditions and salt stress (125 mM NaCl). **e** Measurements of root lengths under normal conditions and osmotic stress (300 mM mannitol). All values are means (±SE) from three independent experiments (15 seedlings per experiment). \*\*\**P* < 0.05 by Student's *t* test

*AtbZIP1* enhances salt and drought resistance when overexpressed

To further characterize the function of *AtbZIP1*, we generated transgenic plants (OX) by overexpressing *AtbZIP1*, under the control of a CaMV35S promoter, in WT Arabidopsis (Fig. 7a). RT-PCR analysis revealed more *AtbZIP1* transcripts in the transgenic plants than in the WT plants (Fig. 7b).

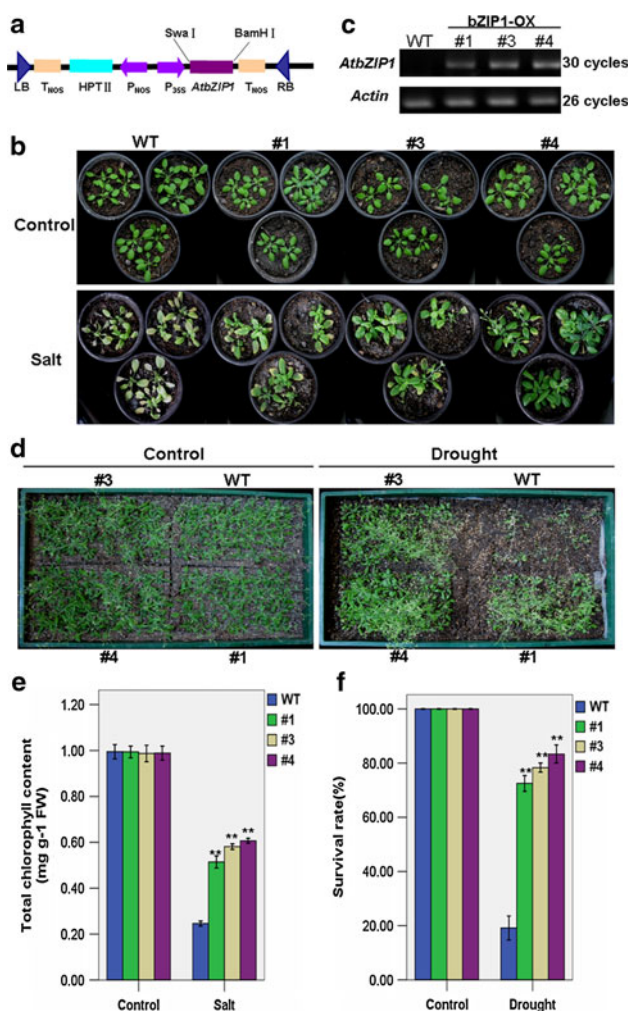
We tested the salt and osmotic stress tolerance of WT Arabidopsis and transgenic OX lines at the adult seedling stage. After irrigating with 300 mM NaCl for 15 days, the transgenic plants displayed greater salt tolerance than WT, with many more green leaves (Fig. 7c). Quantitative data showed that the decrease in total chlorophyll content was less in OX lines than in WT plants (Fig. 7e). As shown in Fig. 6d, after the drought treatment, most WT Arabidopsis died, while the OX plants survived and bloomed. These results were further quantified in an analysis of survival rates (Fig. 7f). Altogether, overexpressing *AtbZIP1*

significantly improved the salt and drought resistance of the transgenic plants.

Expression analysis of abiotic stress response marker genes

Previous studies have suggested that several genes are good markers of abiotic stress responses. In this project, we chose *NCED3*, *COR15A*, *COR15B*, *LEA14*, *RD17*, *RD29A*, *RD29B*, and *COR47* as marker genes, and examined their expression in WT Arabidopsis and the KO-1 mutant (SALK\_069489C) under different conditions, in an attempt to determine the possible regulatory relationships between *AtbZIP1* and these genes. Our RT-PCR results showed that all the genes responded to salt and osmotic stress in WT and KO plants, however, their expression in the KO-1 mutant was reduced in comparison to WT (Fig. 8). Expression analysis of these stress response markers suggests that they have a close relationship with *AtbZIP1* in the plants response to abiotic stresses.

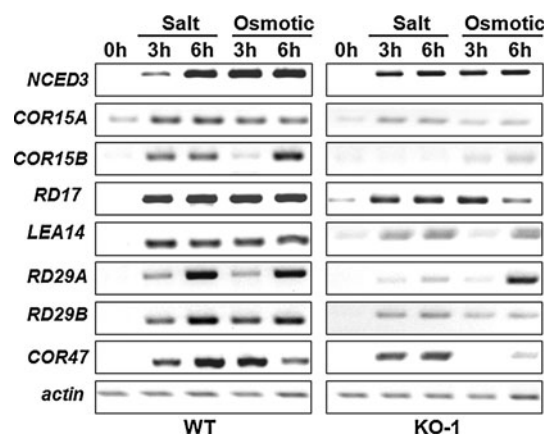




**Fig. 7** Phenotypic observations of plants under salt and drought stress. **a** A schematic showing the structure of the p35S: *AtbZIP1* construct. **b** RT-PCR analysis of WT and transgenic OX plants. Actin gene was used as an internal standard. 30 PCR cycles were used for *AtbZIP1* and only 26 for actin gene. **c** Phenotypes of WT and OX seedlings in response to salt stress. Three-week-old soil-grown plants were irrigated with 300 mM NaCl solution (salt stress) every 3 days for 15 days. Pictures were taken on day 15. **d** Phenotypes of WT and OX seedlings in response to drought stress. WT and OX seedlings grown under normal conditions, were, at 22 days, exposed to water deficit for 18 days. Watering was then resumed, and photographs were taken 10 days later. **e** The total chlorophyll content of WT and OX seedlings under salt stress. For the salt tolerance test, 2 or 3 plants were grown in one bowl, and 15 bowls of each line were used for salt treatment. The total chlorophyll content was measured on the 15th day after salt treatment. **f** The survival rate of WT and OX seedlings under drought stress. For the drought assay, 40 plants of WT and 3 transgenic lines were grown in one basin, and 4 independent experiments were conducted. As above, measurements were taken 10 days after watering had resumed. Data are means ( $\pm$ SE) of 4 replicates.  $**P < 0.05$  by Student's *t* test

## Discussion

bZIPs play important roles in diverse biological processes such as stress signalling, energy metabolism, and



**Fig. 8** Comparative RT-PCR analyses of stress-responsive gene expression in WT Arabidopsis and KO-1 under salt and drought stress. Total RNA was prepared from WT and KO-1 plants after 3 h of treatment with and without 300 mM NaCl (salt), or 30% PEG6000 (drought). The induction of stress-responsive genes (*NCED3*, *COR15A*, *COR15B*, *RD17*, *LEA14*, *RD29A*, *RD29B*, and *COR47*) was measured by semi-quantitative RT-PCR analysis. Expression of *Actin* gene was used as an internal control. Twenty-six cycles was used for actin gene, 29 for *COR15A*, *COR15B*, *RD29A* and *RD29B*, and 31 for *NCED3*, *RD17*, *LEA14*, and *COR47*

light response (Liu et al. 2007; Uno et al. 2000). In this study, we isolated Arabidopsis gene *AtbZIP1*, which belongs to the S subfamily of bZIP TFs. It was reported that *AtbZIP1* was repressed by glucose, and involved in the glucose response by affecting sugar-mediated gene expression (Kang et al. 2010). Previous studies have revealed that plant cells can sense sugar signals, triggering convergent gene activation in response to diverse and seemingly unrelated stress signals (Baena-González et al. 2007). Our results showed that *AtbZIP1* expression is induced by salt, osmotic, and cold stresses, and that this gene has an important physiological function in plant stress resistance.

In the present study, *AtbZIP1* was demonstrated to show self-transactivity in yeast cells (Fig. 2). Due to differences in cellular conditions, differences in expression levels, and factors specific to the individual plant, this transactivation property might behave differently in plants. However, according to Ehlert et al. (2006), *AtbZIP1* can also transactivate GUS reporter gene in plant cells. It has been reported that the N-terminal regions of some other bZIPs (e.g., *CAbZIP1* and *AtbZIP68*) are also required for their transactivity (Lee et al. 2006; Shen et al. 2007a, b). Interestingly, after examining the effects of series of peptide deletions, we found that, in addition to the basic region (aa 13–38) of *AtbZIP1*, the C-terminus (aa 92–118) is also necessary for the transactivity of *AtbZIP1*. Nearby deletions (aa 1–13 and aa 118–145) simply reduced transactivation activity (Fig. 2c, d). One possible mechanism



making the basic region responsible for transactivity is an interaction with the fragment in the N-terminus (aa 1–13).

According to Satoh et al. (2004), *AtbZIP11/ATB2*, another member of the bZIP S1 subfamily, localizes to the nucleus. *AtbZIP44*, *AtbZIP2/GBF5*, and *AtbZIP53* are also considered to localize to the nucleus, owing to their highly conserved basic domain, which includes a putative nuclear localization signal. Although *AtbZIP1* shares minimal similarity with other S1 bZIPs, our results also suggest that it localizes to the nucleus. Among the C subfamily bZIPs (the heterodimerization partners of S1 bZIPs), *AtbZIP63* is targeted to the nucleus and *AtbZIP10* shuttles between the nucleus and the cytoplasm (Walter et al. 2004; Kaminaka et al. 2006).

bZIP TFs play important roles in plant responses to various abiotic stresses. The *CAbZIP1* gene is induced by abiotic stress, and its overexpression increased the drought and salt tolerance of transgenic plants during all growth stages (Lee et al. 2006). Overexpression of *GmbZIP132*, *GmbZIP44*, *GmbZIP62*, and *GmbZIP78* significantly increased the tolerance of transgenic plants to salt stress (Liao et al. 2008), while overexpression of *OsbZIP23* significantly improved the tolerance of transgenic rice to drought and salt stress, increasing their sensitivity to abscisic acid (Xiang et al. 2008).

In our research, we demonstrate that the knockout of *AtbZIP1* results in a reduced resistance to salt and osmotic stress (Figs. 4, 5), and that the expression of abiotic stress response markers, such as *RD29A*, *COR15A* and *RD17*, were likewise reduced (Fig. 8). According to our results, the KO-1 mutant showed better growth performance than KO-2 under the same stress conditions. One possible reason for this difference may be the additional expression of some other genes due to the T-DNA insertion. The observed sensitivity to stress observed in these KO lines could be eased when the KO's were complemented with a functional copy of *AtbZIP1* (Fig. 6), and the overexpression of *AtbZIP1* in WT plants resulted in an enhanced tolerance to salt and drought stress (Fig. 7). Given that the two KO mutants showed similar phenotypes, compared to WT, that the CO lines were of similar or better health than WT, and that OX transgenic lines exhibited an increased tolerance to salt and drought stress, our results suggest that *AtbZIP1* is an important TF controlling plant tolerance to environmental stress.

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