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Research article

Tartary buckwheat transcription factor FtbZIP83 improves the drought/salt tolerance of Arabidopsis *via* an ABA-mediated pathway

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ABSTRACT

Plants are subjected to a variety of abiotic stresses during their lifetime, and drought and salt stress are some of the main causes of reduced crop yields. Previous studies have shown that AREB/ABFs within bZIP transcription factors are involved in plant drought and salt stress responses in an ABA-dependent manner. However, the properties and functions of AREB/ABFs in *Fagopyrum tataricum*, a cereal with good resistance to abiotic stresses, are poorly understood. In this study, a gene encoding an AREB/ABF, designated *FtbZIP83*, was first isolated from Tartary buckwheat. Expression analysis in Tartary buckwheat indicated that *FtbZIP83* was significantly induced by abscisic acid (ABA), NaCl and polyethylene glycol (PEG). The overexpression of *FtbZIP83* in Arabidopsis resulted in increased drought/salt tolerance, which was attributed not only to higher proline (Pro) contents and antioxidant enzyme activity in transgenic lines compared with controls but also to the lower reactive oxygen species (ROS) accumulation and malondialdehyde (MDA) content. In addition, we found that *FtbZIP83* was able to respond to drought and salt stress by upregulating the transcript abundance of downstream ABA-inducible gene. Furthermore, promoter sequence analysis showed that ABREs were present, and the activity of the *FtbZIP83* promoter in transgenic Arabidopsis after drought stress was significantly higher than that under normal conditions. Based on the potential signalling pathways involved in AREB/ABFs, we also screened for the interaction protein FtSnRK2.6/2.3, which may phosphorylate FtbZIP83. Collectively, these results provide evidence that FtbZIP83, as a positive regulator, responds to drought/salt stress *via* an ABA-dependent signalling pathway composed of SnRK2-AREB/ABF.

1. Introduction

Plants, as sessile organisms, are often affected by multiple biotic and abiotic stresses that inhibit their biological processes. Abiotic stress caused by drought and high salinity have a negative impact on the production of economic crops (Cutler et al., 2010; Deng et al., 2019). Studies on the mechanism of plant responses to osmotic stress have shown that the ABA-dependent signalling pathway is the main regulatory pathway for salt/drought stress (Raghavendra et al., 2010). Transcription factors, as direct regulators of stress-responsive genes, play a key role in signal transduction. Many transcription factors, such as WRKYs, NACs, bZIPs and MYBs, have been found to be involved in the plant response to osmotic stress in an ABA-dependent manner (Iuchi et al., 2010).

ABA response element-binding (AREB) proteins or ABRE binding factors (ABFs) interact with ABREs (PyACGTG/TC) by recognizing a conserved 8 bp *cis*-acting sequence, whose core sequence is ACGT. AREB/ABFs belong to the A subfamily of bZIP transcription factors and have 9 members in Arabidopsis (Jakoby et al., 2002). Previous studies on AREB/ABFs in Arabidopsis showed that AREB1/ABF2, AREB2/ABF4, ABF1, and ABF3 are mainly expressed in vegetative tissues in response to ABA and osmotic stress, while other members are expressed during the process of seed maturation and seed germination (Finkelstein and Lynch, 2000; Sandra et al., 2002). Compared to wild-type (WT), single mutants or double mutants, triple AREB/ABF mutants display a reduced resistance to drought and a decreased sensitivity to exogenous ABA (Takuya et al., 2010). In addition, AREB/ABF transcription factors in other species have been reported to improve the tolerance of transgenic plants to drought/salt stress in an ABA-dependent manner (Gao et al., 2011; Hossain et al., 2010).

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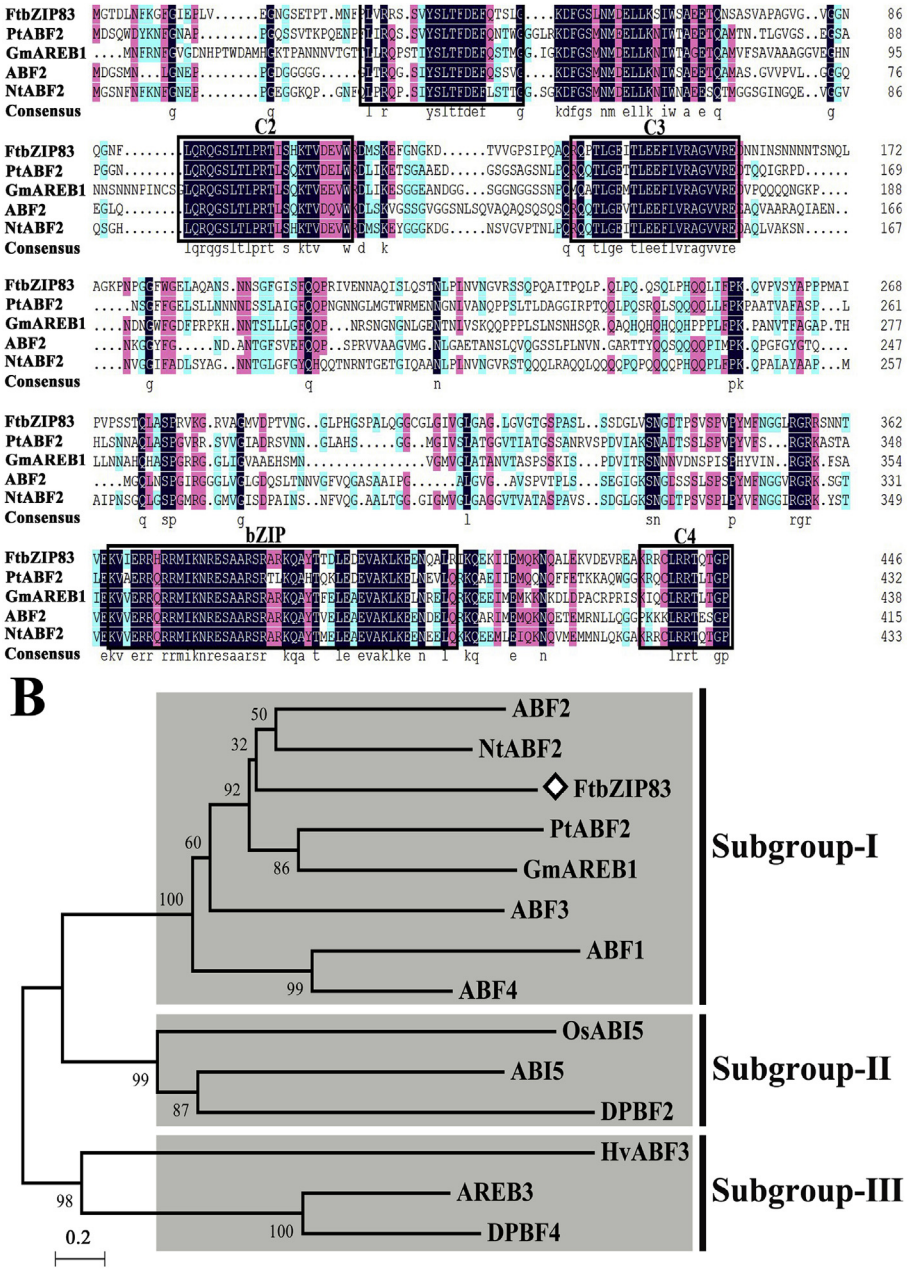

Fig. 1. Sequence characteristics and homology analysis of FtbZIP83. (A) The sequence alignment of the FtbZIP83 protein, its conserved domain and four phosphorylation groups are indicated by box. (B) Phylogenetic analysis of FtbZIP83 with bZIP family proteins classified in A group from other plants. The GenBank accession numbers of the bZIP proteins are as follows: NtABF2 (KF736850.1), ABF2 (NP_849777), PtABF2 (EF405964.1), GmAREB1 (EU418491.1), ABF3 (NM_179159), ABF1 (NP_564551), ABF4 (NP_566629), OsABI5 (EF199631.1), ABI5 (NP_565840), ABF1 (NM_001198254), DPBF2 (NM_114314), HvABF3 (DQ786410.1), AREB3 (NM_115544) and DPBF4 (NM_129672).

After binding to ABA, the soluble ABA receptor PYR/PYL/RCAR can inactivate protein phosphatase 2C (PP2C) *via* conformational changes what results in its release from the receptor/phosphatase/kinase complex and allows activation of ABA-dependent Sucrose non-fermenting 1 - Related protein Kinase 2 (SnRK2) (Hirayama and Umezawa, 2010; Nakashima and Yamaguchi-Shinozaki, 2013). The activation of AREB/ABFs requires phosphorylation of SnRK2 protein kinases to regulate the expression of downstream stress-related genes containing ABREs in their promoters (Mukherjee et al., 2006; Yoshida et al., 2015). However, the SnRK2 family in Arabidopsis is divided into three subgroups, of which the third subgroup includes three members: SnRK2D/SnRK2.2, SnRK2E/SnRK2.6/OST1 and SnRK2I/SnRK2.3. The members of this subgroup are strongly induced by ABA and osmotic stress (Kulik et al., 2011; Yoshida et al., 2014). Furthermore, studies have shown that the SnRK2s of subclass III can phosphorylate AREB/ABFs in *vitro* and can interact with each other in plant nuclei (Hiroaki et al., 2007; Umezawa et al., 2010). Compared with that in single mutants and double mutants, ABA-dependent phosphorylation of AREB/ABFs in the triple mutant *srk2d/e/i* is completely blocked, and most of the expression levels of AREB/ABF regulatory genes are decreased, resulting in reduced tolerance to drought stress (Hiroaki et al., 2011). These studies suggest that subclass III SnRK2s can facilitate the adaptation of plants to adverse environmental conditions by phosphorylating AREB/ABFs to regulate the expression of stress/ABA response genes under drought stress.

Tartary buckwheat (*Fagopyrum tataricum*), a type of small miscellaneous cereal, has advantages including cold resistance, nutrient infertility resistance, rich flavonoid contents and other production performance and economic characteristics (Hiroaki et al., 2011). In 2017, the first genome-wide analysis of high-quality Tartary buckwheat showed that the gene families involved in the abiotic stress response were significantly expansive (Zhang et al., 2017). These extra expansive genes cause functional redundancy, which is essential for the evolution of Tartary buckwheat and its adaptation to adversity. Therefore, this species can be used as a good research material for stress resistance. At present, on the basis of the identification of the functions of

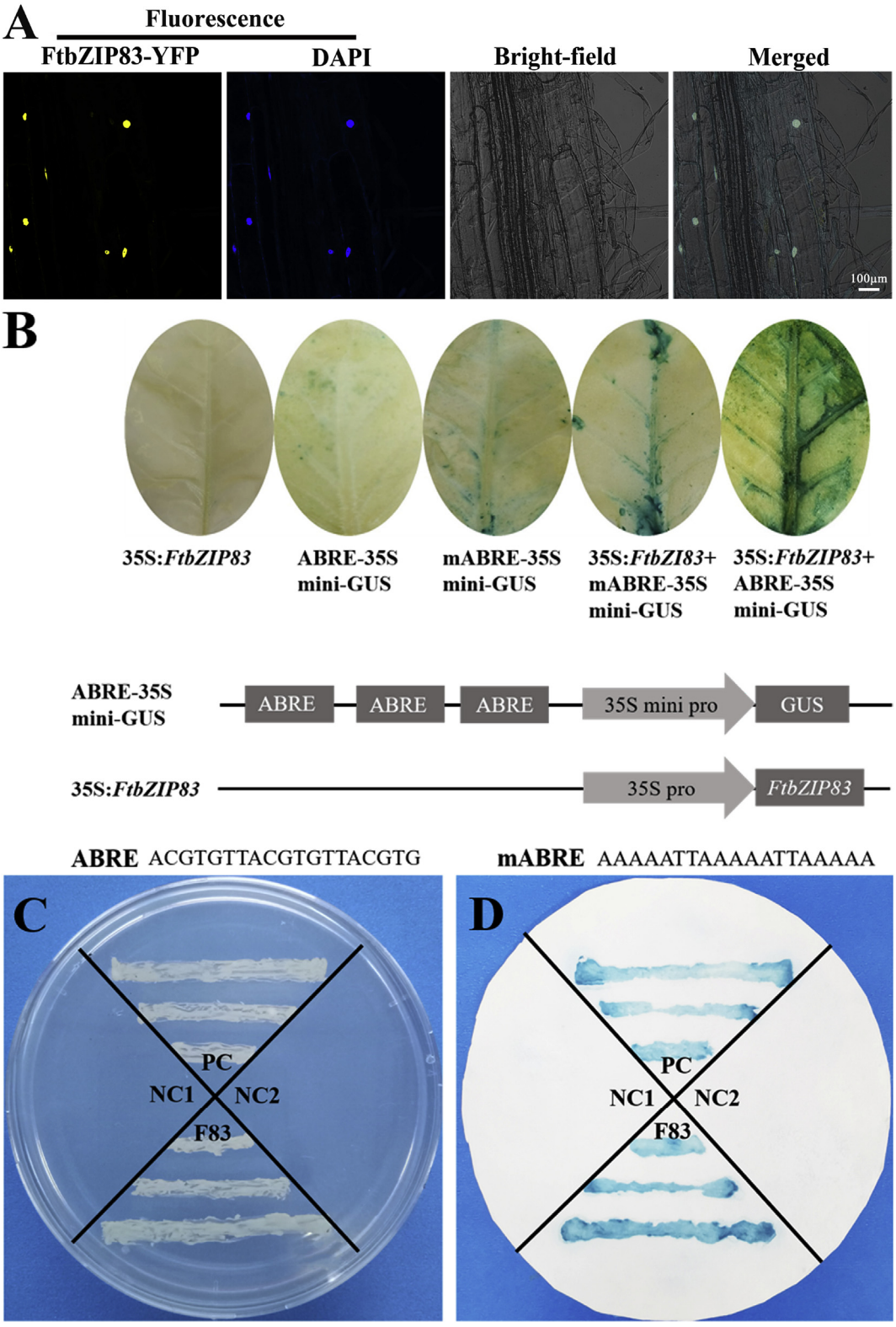

Fig. 2. Molecular characteristics of FtbZIP83 as a transcriptional regulator. (A) Subcellular localization of FtbZIP83 protein and DAPI nuclear localization markers. (B) 35S:*FtbZIP83* and 35Smini:ABRE co-transformed tobacco leaves when the concentration of *Agrobacterium* strain reached an OD_{600} of 0.6 and the corresponding negative control was set. (C) Culture of transformed yeast cells on SD/-His-Trp media. (D) Experiment with galactosidase activity by X-gal staining. NC1 (AH109 cells) and NC2 (pBridge empty plasmids) were used as negative controls. PC: pBridge-FtMYB13 as a positive control, reflecting the transcriptional activity of FtbZIP83-pBridge.

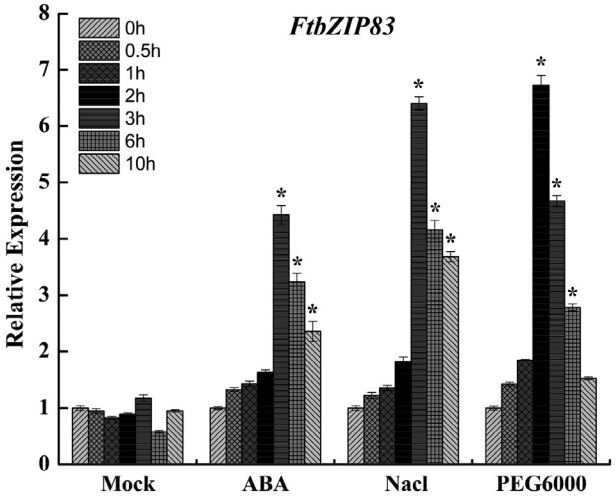

Fig. 3. Expression pattern of *FtbZIP83* in Tartary buckwheat under different abiotic stresses. The sampling time for each sample was 0, 0.5, 1, 2, 3, 6, and 10 h. The relative expression of *FtbZIP83* was analysed by qPCR, and the expression level of *FtbZIP83* was defined as 1 after 0 h of treatment under normal growth conditions. The error bars denote ± SDs, and each data value is from three replicate experiments. Significant differences are denoted by *, meaning $P < 0.05$.

transcription factors involved in Tartary buckwheat resistance, such as MYBs, NACs and bHLHs, it was found that the regulatory signalling pathways of Tartary buckwheat resistance are complex and diverse (Yao et al., 2017). The SnRK2-AREB/ABF signalling pathway in model plants has been used as the main regulator of ABA-dependent gene expression in response to abiotic stress, but the functional diversity of the members of the AREB/ABF subgroup makes it impossible to interpret their functions only from their sequence structure (Fujita et al., 2009; Kim et al., 2010). By applying a bioinformatics analysis of the *FtbZIP83* gene, an element analysis of its promoter, and a response analysis to abiotic stress in the early stage of Tartary buckwheat, we preliminarily determined that *FtbZIP83* is a member of the AREB/ABF subgroup and may be involved in plant responses to environmental stress (for subsequent studies). Here, to establish the role of *FtbZIP83* in the plant response to osmotic stress, we identified its function *via* transgenic Arabidopsis and confirmed the potential phosphorylation of the FtSnRK2 protein.

2. Materials and methods

2.1. Plant materials and treatment

The seeds of Tartary buckwheat "Xiqiao No. 2" were germinated on wet filter paper under long-day illumination (16 h/8 h, light/dark) at 23 °C. The germinated seeds were then cultivated in 1/2-strength Hoagland solution for two weeks, after which they were treated with 150 μM ABA, 20% PEG and 200 mM NaCl. Samples were collected after 0, 1, 1.5, 2, 3, 6 and 10 h of treatment and then stored at −80 °C until RNA was extracted. Untreated seedlings exhibiting similar growth and development were used as controls. Each sample consisted of three biological repeats.

2.2. Cloning of FtbZIP83 cDNA from Tartary buckwheat

Total RNA was extracted from the mixed tissues of Tartary buckwheat using an RNA OUT Kit (Tianze, Beijing, China) according to the instructions in the kit, and restorative first-strand cDNA was synthesized into the DNA using a synthesis kit (MBI). Based on the *Fagopyrum tataricum* transcriptome data in our laboratory (data not published), specific primers (forward primer 5′-ACACGGGGACGAGCTCATGGGAA CTGATCTGAACTTCA AG-3′, reverse primer 5′-CTTGCTCACCATGTCG ACCCCCCCGTCTGTTTTC-3′) were designed to amplify the full-length open reading frame (ORF) of the *FtbZIP83* gene by PCR. FtbZIP83 homologous protein sequences were retrieved from the NCBI database, and the multiple amino acid sequences obtained were calibrated and aligned by DNAMAN software. The neighbour-joining method was then used to build a phylogenetic tree *via* the Mega 7.0 program and, and their conserved domains were predicted *via* the MEME online website.

2.3. Subcellular localization and transcriptional activation of FtbZIP83

The localization of FtbZIP83 was determined by a previously described method (Zhao et al., 2007). The roots of 15-day-old homozygous transgenic Arabidopsis plants (35S:FtbZIP83:YFP) were cultured on 1/2-strength MS media. The subcellular localization of the FtbZIP83 protein was detected using DAPI as a nuclear localization marker. The fluorescence of roots was observed by confocal laser microscopy with magnification of 100 times. In the transcriptional activation experiment, the restriction sites *Eco*RI and *Sal*I were selected, and the following primers were designed: 5′-TGACTGTATCGCCGGAA TTCATGG GAACTGATCTGAACTTCAAG-3′ and 5′-TAGCTTGGCTGCAGGTCGACC CATGGA CCCGTCTGTGTTC-3'. The ORF of *FtbZIP83* was then amplified by PCR and inserted into a pBridge vector to generate pBridge-*FtbZIP83*. The pBridge-*FtbZIP83*, pBridge (empty vector as negative control) and pBridge-*FtMYB13* (positive control) vectors were subsequently transformed into yeast AH109 competent cells. The lacZ activity was determined by the galactosidase filter lift method after culture on SD/-His-Trp media for 2–3 days.

2.4. Analysis of yeast two-hybrid (Y2H) experiments

The full-length ORF of *FtbZIP83* was inserted into a pGADT7 vector using *Nde*I and *Eco*RI restriction sites as bait proteins. The full-length ORF of *FtSnRK2.6/2.3/2.2* was subsequently inserted into a pGBKT7 vector as a target protein by the same restriction endonuclease. The primers used for the PCR amplification are listed in Supplementary Table1. The combination of plasmid *FtSnRK2.6/2.3/2.2*-BD + *FtbZIP83*-AD was then co-transferred into yeast AH109, and a corresponding negative control was established. The transformed yeast colonies were mixed in YPDA liquid medium until the cell density (OD_{600}) was approximately 0.6. The yeast colonies were then diluted to 10^{-1}, 10^{-2} and 10^{-3} with sterilized double-distilled water. Six millilitres of the diluted yeast cell solution was transferred to SD/-Trp-Leu and SD/-Leu-Trp-His-Ade defective medium plates *via* a sterilized gun head. The cells were cultured at 30 °C for 2–4 days. Finally, X-β-Gal staining analysis was used to verify any possible interactions (Zhang et al., 2014).

2.5. Preparation of transgenic Arabidopsis thaliana overexpressing FtbZIP83

The ORF of *FtbZIP83* was inserted into the plant expression vector pCHF-YFP by using the restriction sites *Eco*RI and *BamH*I, and then the resultant fusion construct (driven by the CaMV promoter) 35S:*FtbZIP83*-YFP was transformed into wild-type Arabidopsis *via* Agrobacterium strain GV3101. The homozygous transgenic Arabidopsis lines of the T3 generation were screened by plate screening analysis with 1/2-strength MS media supplemented with 50 μg/mL *kanamycin* and detection at the molecular level *via* PCR, and subsequent experiments were carried out.

2.6. Stress tolerance assays of transgenic Arabidopsis

For seed germination experiments, sterilized WT and transgenic seeds were germinated on 1/2-strength MS agar plates containing 0.8 μM ABA, 150 mM NaCl and 200 mM mannitol. The germination rate

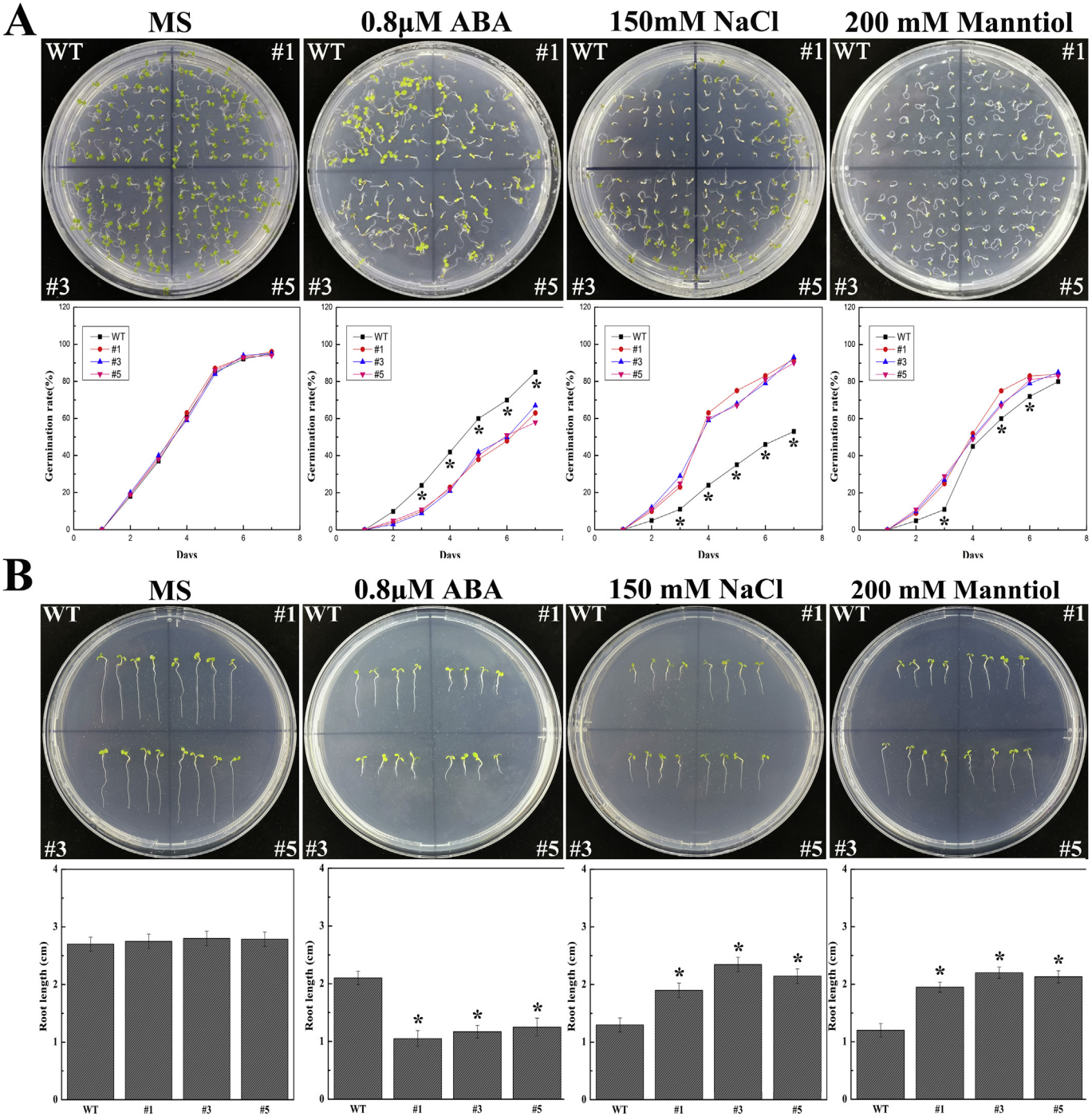

Fig. 4. Effects of drought, salt and ABA stress on the seed germination and root elongation of WT and transgenic Arabidopsis. (A) The phenotype and germination rate of seeds treated with drought, salt and ABA for 7 days. (B) Phenotypic and length analysis of the effects of drought, salt and ABA stress on the root elongation of WT and transgenic lines. The error bars represent ± SDs, and each data value is from three replicate experiments. Significant differences are denoted by *, meaning $P < 0.05$ (Student T-test).

of the seeds was recorded every 24 h for 7 days. For the root length experiments, seedlings aged 3 days and growing in 1/2-strength MS media were transplanted to the same stress plate as mentioned above, and the root length was measured on day 7. Three repeats were used for the transgenic lines.

For drought and salt stress experiments, the T3 homozygous transgenic lines and WT plants were cultivated in soil for three weeks under the same growth conditions. For drought treatment, seedlings aged 3 weeks were refused watering for 2 weeks, and their survival rate was calculated after 7 days of re-watering. For salt treatment, 3-week-old seedlings were treated with 200 mM NaCl for 3 weeks, and their phenotypic changes were recorded every 7 days. Survival rate was calculated after 3 weeks of NaCl treatment. After two weeks of drought/salt stress, the activities of three antioxidant enzymes (SOD, POD and CAT) were determined in transgenic and WT plants. Afterward, MDA and Pro were measured at 15 and 30 days after drought/salt treatment, respectively. The above physiological indicators were determined by the previous methods (Kotchoni et al., 2010). Moreover, DAB and NBT staining methods were used to evaluate ROS accumulation in the leaves (Tu et al., 2016). All of the above experimental treatments were

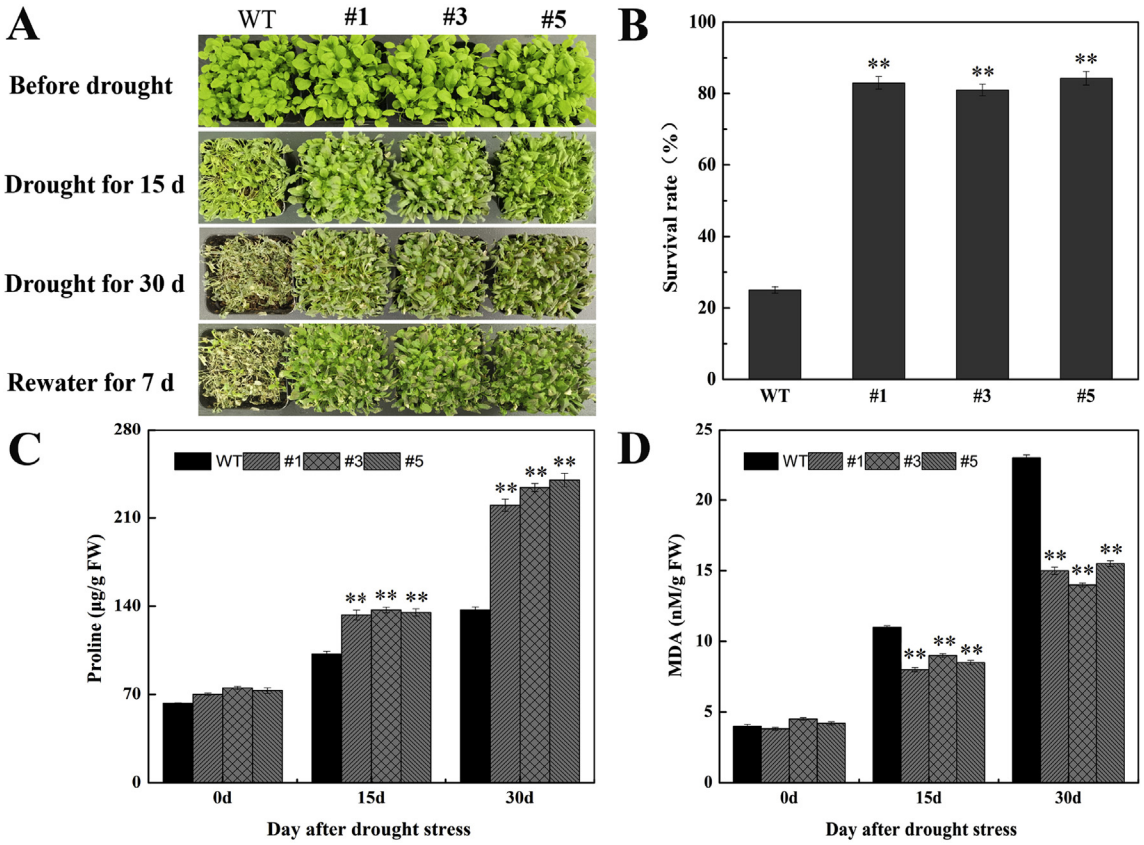

Fig. 5. Overexpression of *FtbZIP83* reduced the drought sensitivity of transgenic Arabidopsis. (A) Images of phenotypic changes caused by drought treatment. (B) The survival rates were calculated after rehydration for 7 days. (C–D) The contents of Pro (C) and MDA (D). Significant differences are denoted by **, meaning $P < 0.01$ (Student T-test).

repeated three times.

2.7. Functional analysis of the FtbZIP83 promoter

To study whether the promoter responds to drought stress, the promoter of the *FtbZIP83* gene was isolated and designated as $P_{FtbZIP83}$. The plasmids pBI101:$P_{FtbZIP83}$: GUS and pBI101:(CaMV)35S:GUS (positive control) were constructed from *Hind*III and *Sal*I restriction sites, and stable genetic transformation of transgenic Arabidopsis was obtained *via* the use of strain GV3101. The amplified primers and sequence element analysis results are listed in Supplementary Table 2. Under the same conditions, the positive plants and control plants were cultivated in soil for approximately 3 weeks and then treated without water for one week. The results were revealed by comparing the activity and the expression level of the GUS gene of positive plants before and after drought treatment.

2.8. Quantitative real-time PCR analysis

Total RNA from mixed tissues of plant seedlings was extracted using an RNA OUT Kit (Tiangen, China), and the synthesized first-strand cDNA was reverse transcribed *via* a Prime Script™ RT reagent kit (Takara, Beijing, China). Quantitative real-time PCR (qPCR) was performed by using a TB Green Premix Ex *Taq*II Kit (Tli RNaseH Plus) with a CFX96 RT-PCR machine (Bio-Rad, U.S.A.). The amplification programmes used were as follows: 98 °C for 45 s followed by 34 cycles of 98 °C for 15 s and 60 °C for 45 s. Each sample was analysed in triplicate to ensure the accuracy of the data. *Atactin2* was used as a housekeeping gene, and the primers involved in qPCR analysis were listed in Supplementary Table 3.

2.9. Data analysis

SPSS software (version 22.0) was used to analyse the outcomes of the different treatments. The results were considered to have statistical significance when $P < 0.05$.

3. Results

3.1. Isolation and characteristic analysis of FtbZIP83

To study the bZIP transcription factors involved in abiotic stress in Tartary buckwheat, we cloned and isolated *FtbZIP83* gene (GenBank accession No. MN_120689). The complete ORF contains a 1344 bp sequence encoding a 447 amino acid protein with the predicted molecular weight of 48 kDa. Sequence alignment analysis showed that FtbZIP83 contained not only the conserved domain of bZIPs but also the conserved C1, C2, C3 and C4 regions of the AREB/ABF protein kinase target sequence (Fig. 1A). Phylogenetic analysis revealed that the AREB/ABF transcription factors of the A family with identified functions in different species were classified into three subgroups (Fig. 1B). Moreover, FtbZIP83 and four osmotic stress-related transcription factors of AREB/ABF members in Arabidopsis were in the same subgroup. These results suggest that FtbZIP83 may have similar functions to AREB/ABFs involved in the plant response to osmotic stress.

To further study the characteristics of the FtbZIP83 transcription factor, we verified its subcellular localization, specific binding sequence and transcriptional activation. By observing the fluorescence of FtbZIP83-YFP and DAPI in the roots of transgenic *Arabidopsis*, we found that the fluorescence of YFP was consistent with that of the marker DAPI, indicating that FtbZIP83 was located in the nucleus (Fig. 2A). Additionally, we co-transformed tobacco leaves with 35Smini-G-box

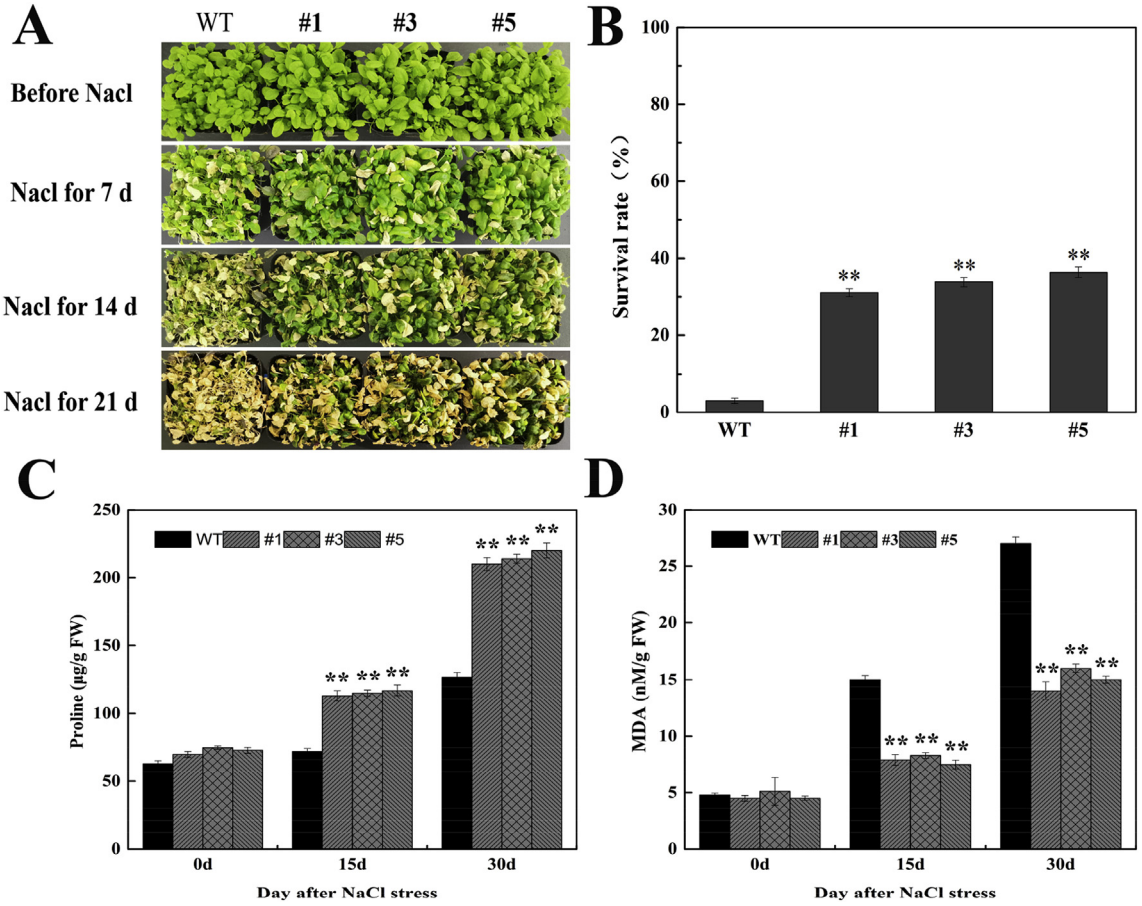

Fig. 6. Overexpression of *FtbZIP83* reduces the NaCl sensitivity of transgenic Arabidopsis. (A) Images of phenotypic changes caused by salt stress. (B) The survival rates after 3 weeks of salt stress were determined. (C–D) The contents of Pro (C) and MDA (D). The error bars represent ± SDs, and each data value is from three replicate experiments. Significant differences are denoted by **, meaning $P < 0.01$ (Student T-test).

+35S-*FtbZIP83* mediated by GV1301, and the negative controls were GV3101, 35Smini-ABRE, 35S-*FtbZIP83* and 35S-*FtbZIP83* + 35Smini-mABRE. In contrast, the GUS histochemical staining results of 35Smini-ABRE + 35S-*FtbZIP83* were the most significant, indicating that the combination of *FtbZIP83* and an ABRE enhanced the expression of the *GUS* gene (Fig. 2B). Moreover, the transcriptional activation ability of FtbZIP83 was determined *via* a yeast experimental system. All transformants were cultured on SD/-His-Trp plates. AH109 and AH109 yeast cells containing a pBridge empty vector did not grow, while the positive control (pBridge-*FtMYB13*) and pBridge-*FtbZIP83* grew well (Fig. 2C). The results of X-gal staining showed that FtbZIP83 had transcriptional activation in yeast (Fig. 2D).

3.2. Expression analysis of FtbZIP83 in Tartary buckwheat

To verify the results of previous bioinformatics analysis, we analysed the expression pattern of *FtbZIP83* after abiotic stress in Tartary buckwheat *via* qPCR. For the ABA and NaCl stress treatments, the peak appeared at 3 h and then began to decrease slowly. Moreover, after 2 h of PEG6000 treatment, there was a tendency to decrease immediately after the maximum value reached (Fig. 3). The results show that *FtbZIP83* may be involved in the response of Tartary buckwheat to ABA, salt and drought stress.

3.3. Overexpression of FtbZIP83 in Arabidopsis increased ABA sensitivity

To further confirm the role of *FtbZIP83* in plant resistance to osmotic stress, we analysed the effects of NaCl, drought and ABA treatments on the seed germination and root elongation of transgenic lines and WT plants. As shown in Fig. 4A and Fig. 4B, the germination rate of the seeds and root elongation of the transgenic lines (#1, #3, #5) increased significantly compared with those of WT plants. on 1/2-strength MS media containing 150 mM NaCl and 200 mM mannitol. However, the germination rate and root elongation of transgenic lines in 1/2-strength MS media containing 0.8 μm ABA were lower than those of the WT plants. These results indicated that overexpression of *FtbZIP83* increased the resistance of the transgenic plants to salt and drought stress while increasing their sensitivity to ABA.

3.4. Analysis of the effects of FtbZIP83 overexpression on drought/salt stress resistance in Arabidopsis

To determine the effect of *FtbZIP83* overexpression on plant responses to drought and salt stress, 4-week-old transgenic lines and WT were tested plants under the same growth conditions. For the analysis of the drought treatment, phenotypic changes were observed after 15 and 30 days of withholding water and after 7 days of re-watering, and the survival rate after 7 days of rehydration was determined. Fig. 5B shows that the survival rate of the transgenic lines was greater than 80%, while that of the WT plants was approximately 10%. For the analysis of the 150 mM NaCl stress treatment, phenotypic changes were observed after 7 days, 14 days and 21 days of treatment, and the survival rate after 21 days was determined. Fig. 6B shows that the survival rate of the transgenic lines was greater than 30%, while that of the WT plants was less than 10%. Furthermore, MDA and Pro contents were measured for each transgenic line (#1, #3, #5) and the WT plant at 10 and 30 days of drought/salt treatment, respectively. The contents of Pro in transgenic lines were significantly higher than that in WT plants

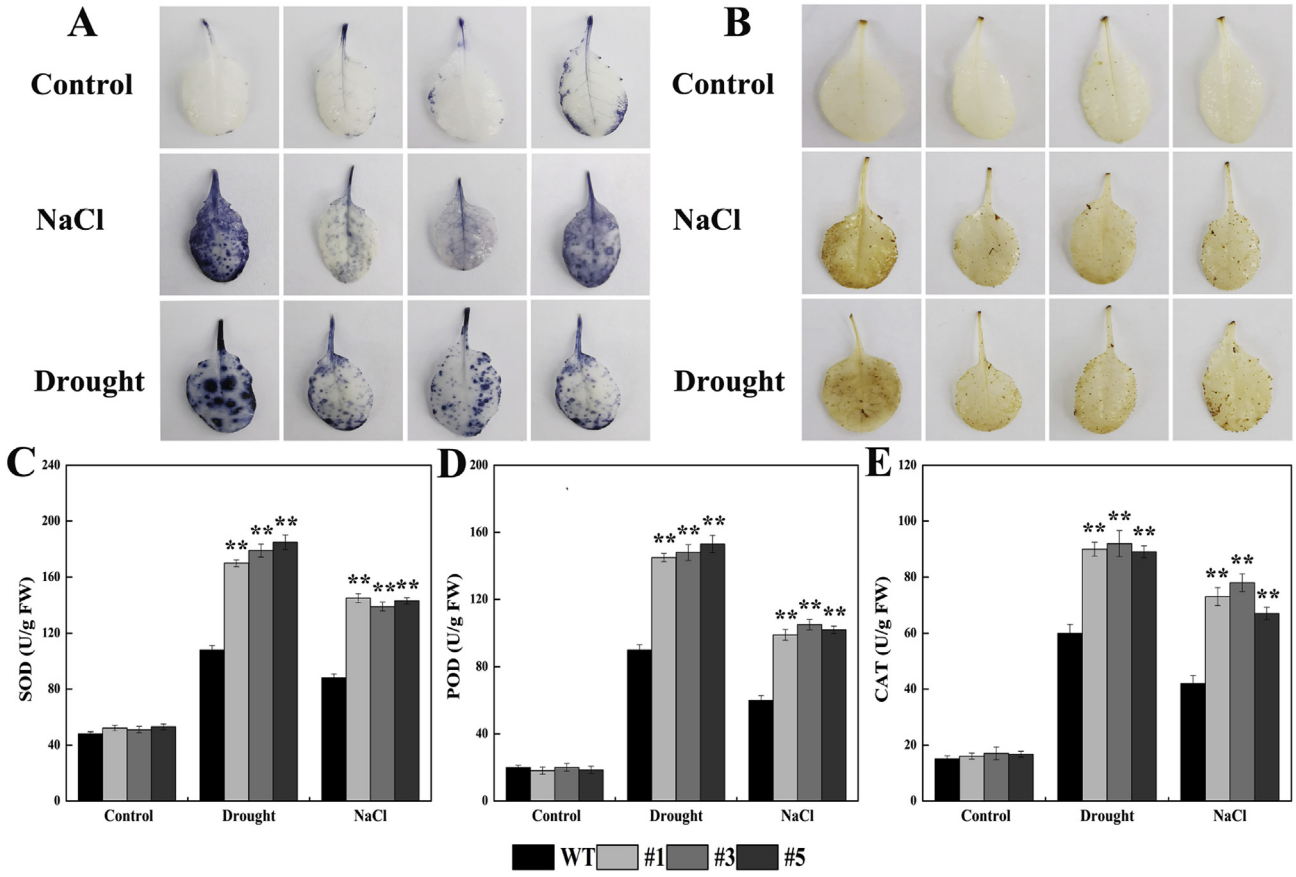

Fig. 7. Effects of *FtbZIP83* overexpression on ROS accumulation and antioxidative enzymes activity in Arabidopsis. (A–B) The accumulation of O_2^- and H_2O_2 in the leaves of WT and transgenic lines was analysed by histochemical staining with NBT (A) and DAB (B) under normal conditions and abiotic stress conditions, respectively. (C–E) SOD, POD and CAT activities of transgenic lines and WT plants under normal conditions and abiotic stress. The error bars denote ± SDs, and each data value is from three replicate experiments. At least 10 plants were used per experiment per line. ** represents T-test significance: $P < 0.01$.

($P < 0.01$) (Figs. 5C and 6C). Meanwhile, the MDA contents in transgenic lines were significantly lower compared with WT plants ($P < 0.01$) (Figs. 5D and 6D). The results of Fig. 7C, D and E shows that the activities of three antioxidant enzymes (SOD, POD, CAT) in transgenic lines were higher than that in the WT plants. Additionally, the results of the DAB and NBT staining showed that the contents of H_2O_2 and O_2^- in the leaves of the transgenic lines was lower than that in the WT plants after drought/NaCl treatment (Fig. 7A and B). Therefore, overexpression of *FtbZIP83* seems to increase the drought and salt tolerance of transgenic plants by reducing oxidative damage.

3.5. Overexpression of FtbZIP83 regulates the expression of genes involved in stress response in Arabidopsis

To further study the mechanism by which *FtbZIP83* regulates the downstream stress-related genes at the transcriptional level, the expression of nine stress/ABA-responsive genes were measured by qPCR in transgenic Arabidopsis. Under normal growth conditions, the transcript abundance of most of these response genes was similar (Fig. 8). Furthermore, after one week of drought and salt stress treatment, the expression levels of the response genes *AtRD29A*, *AtRD29B*, *AtRD20*, *AtAIL*, *AtRAB18*, *AtKIN2*, *AtABI1* and *AtABI2* in the transgenic lines were significantly induced compared with WT plants ($P < 0.05$). Our results suggest that FtbZIP83 may enhance stress resistance by increasing downstream stress/ABA-related gene expression.

3.6. Screening SnRK2 protein interactions with FtbZIP83 in yeast

Based on the transcriptome data and bioinformatics analysis, 11 protein kinases of the SnRK2 family of Tartary buckwheat were obtained, and a phylogenetic tree was established with the SnRK2s of Arabidopsis. FtSnRK2.6/2.3/2.2 belonged to the third subgroup of the SnRK2 family (Fig. 9A). To investigate whether FtbZIP83 interacts with the protein kinase FtSnRK2.6/2.3/2.2, validation was performed in yeast using a Y2H assay. All transformants grew well on SD/-Trp-Leu media. Only strains with *FtSnRK2.6/2.3* + *FtbZIP83* could grow on SD/-Trp-Leu-His-Ade media and showed blue colour on filter paper after x-β-Gal staining (Fig. 9B). Therefore, the results showed that the FtbZIP83 transcription factor could interact with the FtSnRK2.6/2.3 protein.

3.7. Analysis of the FtbZIP83 promoter

To further study the potential functions of the *FtbZIP83* gene in plant stress resistance, we cloned a 2086 bp promoter ($P_{FtbZIP83}$) region from the 5′-upstream region of *FtbZIP83* based on buckwheat genomic data (Fig. 10A). *Via* sequence analysis, it was found that there were many *cis*-acting elements, such as ABREs (ABA response elements), TC-rich elements (defence and stress response elements), and TCA-elements (salicylic acid response elements), that may respond to environmental stress. In addition, a large number of light response elements and binding elements of transcription factors (MYBs, MYCs) were found. The activity of FtbZIP83 promoter was confirmed by transferring Arabidopsis. Then, the effects of drought stress on report gene *GUS* were analysed in transgenic plant leaves. Seen from Fig. 10B, a more intense GUS staining was detected in transgenic lines after drought treatment. Furthermore, the *GUS* gene expression level after drought treatment was at least 6 times higher than that under normal growth

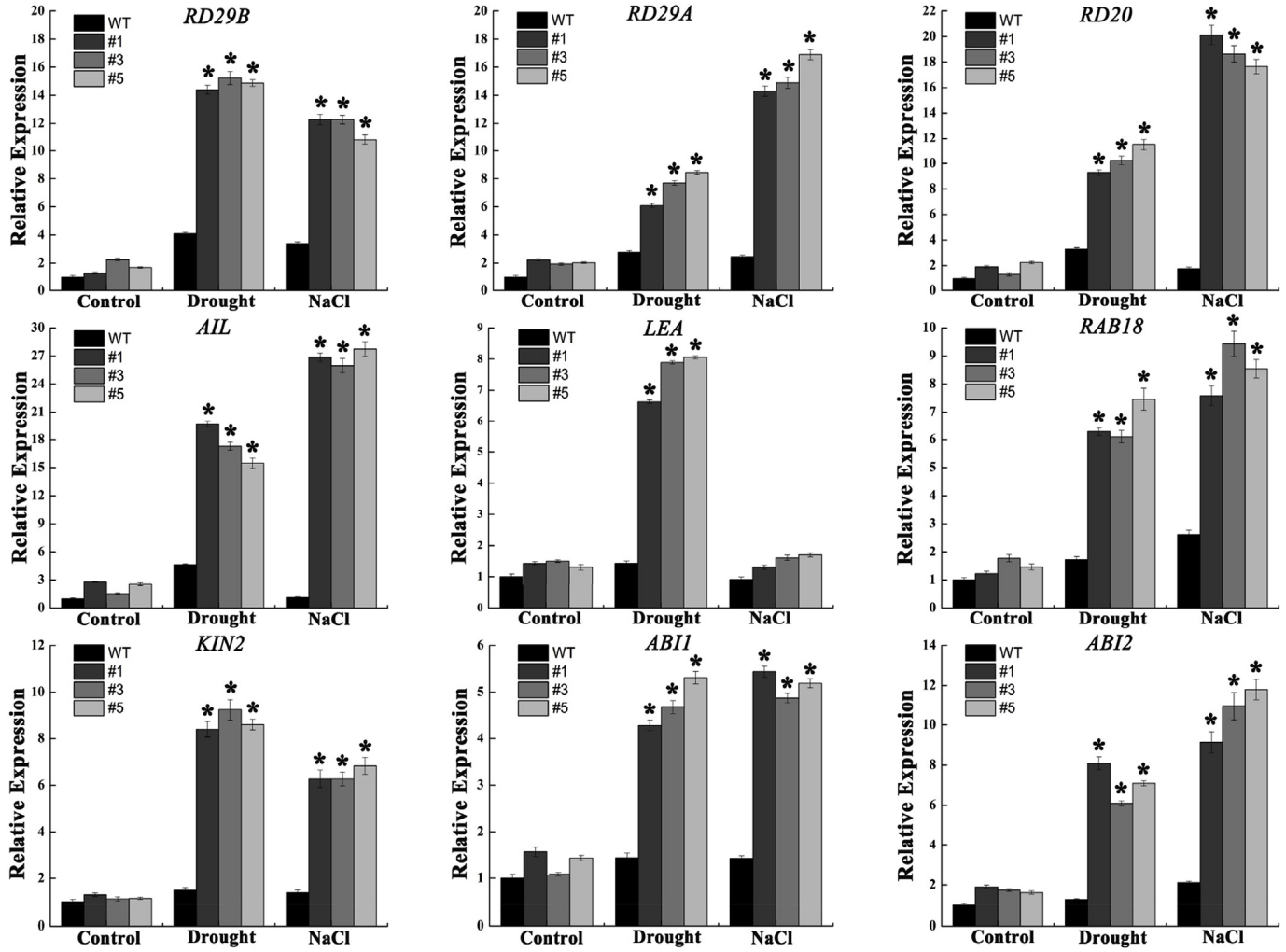

Fig. 8. Expression analysis of response genes in *FtbZIP83* transgenic lines and wild-type *Arabidopsis thaliana* after drought and salt treatment. Total RNA was extracted from transgenic and WT plants after 1 week of treatment under normal and stress conditions. The relative expression of all genes was analysed by qPCR, and the expression level of WT plants was defined as 1 under normal growth conditions. Three independent experiments were conducted, each of which involved at least three plants. The error bars denote ± SDs. Significant differences are denoted by *, meaning $P < 0.05$.

conditions ($P < 0.05$) (Fig. 10C). Overall, the results revealed that $P_{FtbZIP83}$ can respond to drought stress, which supported that FtbZIP83 had the function involved in physiological process of drought resistance.

4. Discussion

When facing various environmental stresses, such as salt and drought, plants can enhance their resistance *via* complex physiological and biochemical regulation in vivo (Gao et al., 2016; Lata and Prasad, 2011). The regulation of gene expression in plants under stress is well understood at the transcriptional level. Research on the resistance mechanism of Tartary buckwheat is also gradually intensifying. Recently, the 96 *FtbZIP* genes in Tartary buckwheat were identified by transcriptome analysis, and 20 *FtbZIP* genes, including *FtbZIP83*, were found to be highly expressed in leaves, roots, flowers and fruits(Liu et al., 2019). In this study, we analysed the expression of *FtbZIP83* in young Tartary buckwheat treated with drought, salinity and ABA by quantitative qPCR. It was found that the expression level of *FtbZIP83* increased sharply after 3 h of drought/salt treatment, and it decreased after reaching the maximum after several hours. Moreover, promoter element analysis showed that there are several stress response elements in its sequence. These analyses also indirectly indicate that the *FtbZIP83* gene may play a key role in the resistance and biological processes of Tartary buckwheat.

It is well known that different stress environments usually cause various physiological, biochemical and morphological changes in plants, which can damage plant homeostasis (Vierling and Kimpel, 1992). The accumulation of ROS in plants is crucial to abiotic stress tolerance (Parida and Das, 2005). Excessive accumulation of reactive oxygen species (O_2^- and H_2O_2) can lead to cell membrane leakage and chlorophyll degradation, while antioxidant enzymes can reduce oxidative damage to plants (Kurepa et al., 2010). In addition, MDA is a product of membrane lipid peroxidation and can be used as an index to evaluate the destructive effect of reactive oxygen species (Tu et al., 2016). In fact, high ROS accumulation and MDA contents are the main causes of oxidative damage. Most stress-related transcription factors, including AREB/ABFs, have been found to improve plant stress resistance by regulating plant physiological responses. For example, overexpression of *ABP9* (ABA-responsive-element-binding protein 9) in Arabidopsis leads to a decrease in MDA content and ROS (O_2^- and H_2O_2) levels in leaves and a dramatic increase in Pro content and antioxidant enzyme activities (SOD, POD and CAT) (Wang et al., 2017). Based on our experimental results, we suggest that the expression of *FtbZIP83* may be involved in the regulation of ROS accumulation and the mitigation of oxidative and lipid peroxidation damage to cells.

As previously studied, AREB/ABF regulates plant responses to abiotic stress by acting on downstream genes (Huang et al., 2010).

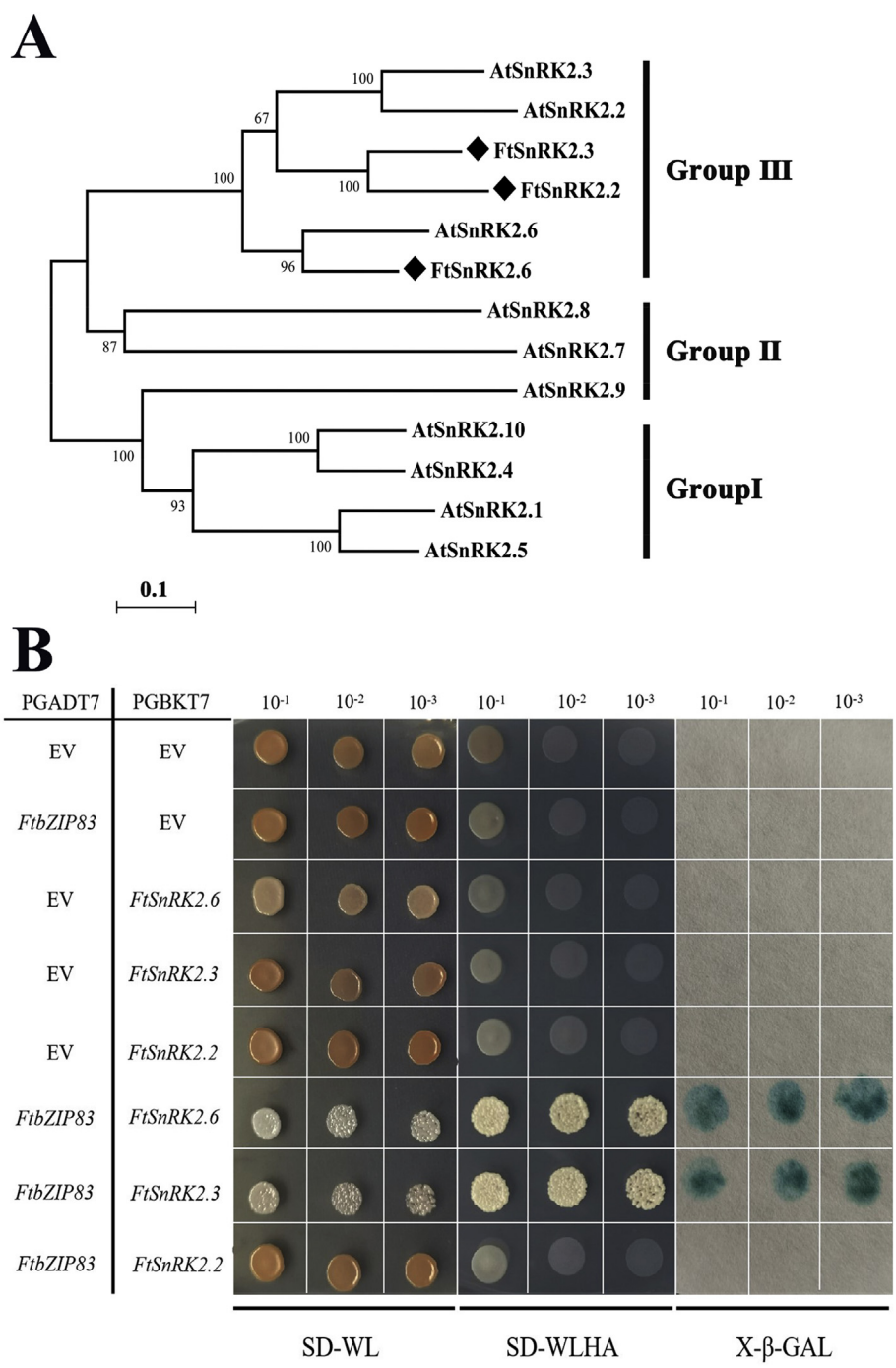

Fig. 9. Physical interaction between FtSnRK2.6/2.3/2.2 and FtbZIP83. (A) Phylogenetic trees of FtSnRK2.6/2.3/2.2 and the SnRK2 family in *Arabidopsis thaliana*. The GenBank accession numbers of the SnRK2 family in the phylogenetic tree are as follows: AtSnRK2.10 (NM_104774), AtSnRK2.1 (NM_120946), AtSnRK2.2 (NM_001203118), AtSnRK2.3 (NM_001345807), AtSnRK2.4 (NM_00103 5944), AtSnRK2.5 (NM_125760), AtSnRK2.6 (NM_119556), AtSnRK2.7 (NM_120165), AtSnRK2.8 (NM_202441), AtSnRK2.9 (NM_127867), FtSnRK2.6 (NM_120292), FtSnRK2.3 (NM_120691), and FtSnRK2.2 (NM_120690). (B) AH109 yeast cells transformed with plasmid combinations were orderly diluted 10^{-1}, 10^{-2} and 10^{-3} times, cultivated on SD/-Trp-Leu media and SD/-Trp-\Leu-His-Ade media, and then stained with x-β-Gal.

Many downstream genes of AREB/ABFs have been identified in Arabidopsis, including *RD29A*, *RD29B*, *AIL*, *RAB18*, *KIN2*, *RD20* and *LEA* (Yasunari et al., 2005). ABI1 and ABI2 encode homologous glutamate 2Cs, which regulate ABA signalling during the reaction to osmotic stress, and the mutants exhibit defective stomatal closure (Gao et al., 2011; Narendra, 2007). By further analysing the transcriptional regulatory function of FtbZIP83, we found that the expression of a large number of stress-regulated target genes in the overexpressed lines of *FtbZIP83* was significantly upregulated under abiotic stress. The difference is that the transcript abundance of LEA proteins in transgenic lines increased significantly only under drought stress compared with that in wild type. Previous studies have also found that LEA proteins in different species are widely involved in osmotic stress but not in response to each type of osmotic stress (Banerjee et al., 2016). We believe that these phenomenons may be caused by the complexity and diversity of plant signalling networks in response to different stresses. Furthermore, many developmental processes and adaptive stress responses of plants are regulated by ABA signals (Narendra, 2007). ABA promotes seed dormancy under abiotic stresses that are not conducive to germination or seedling growth (Mehrotra et al., 2014). There have also been studies showing that some AREB/ABFs are involved not only in plant resistance to stress but also in seed embryonic development and lateral root elongation. Our results revealed that the overexpression of *FtbZIP83* enhanced the sensitivity to exogenous ABA, which was characterized by slow seed germination and inhibited seedling development (Nakashima and Yamaguchi-Shinozaki, 2013). However, it needs to be further verified whether *FtbZIP83* is involved in development.

Different regulatory modes of plant transcription factors at the post-

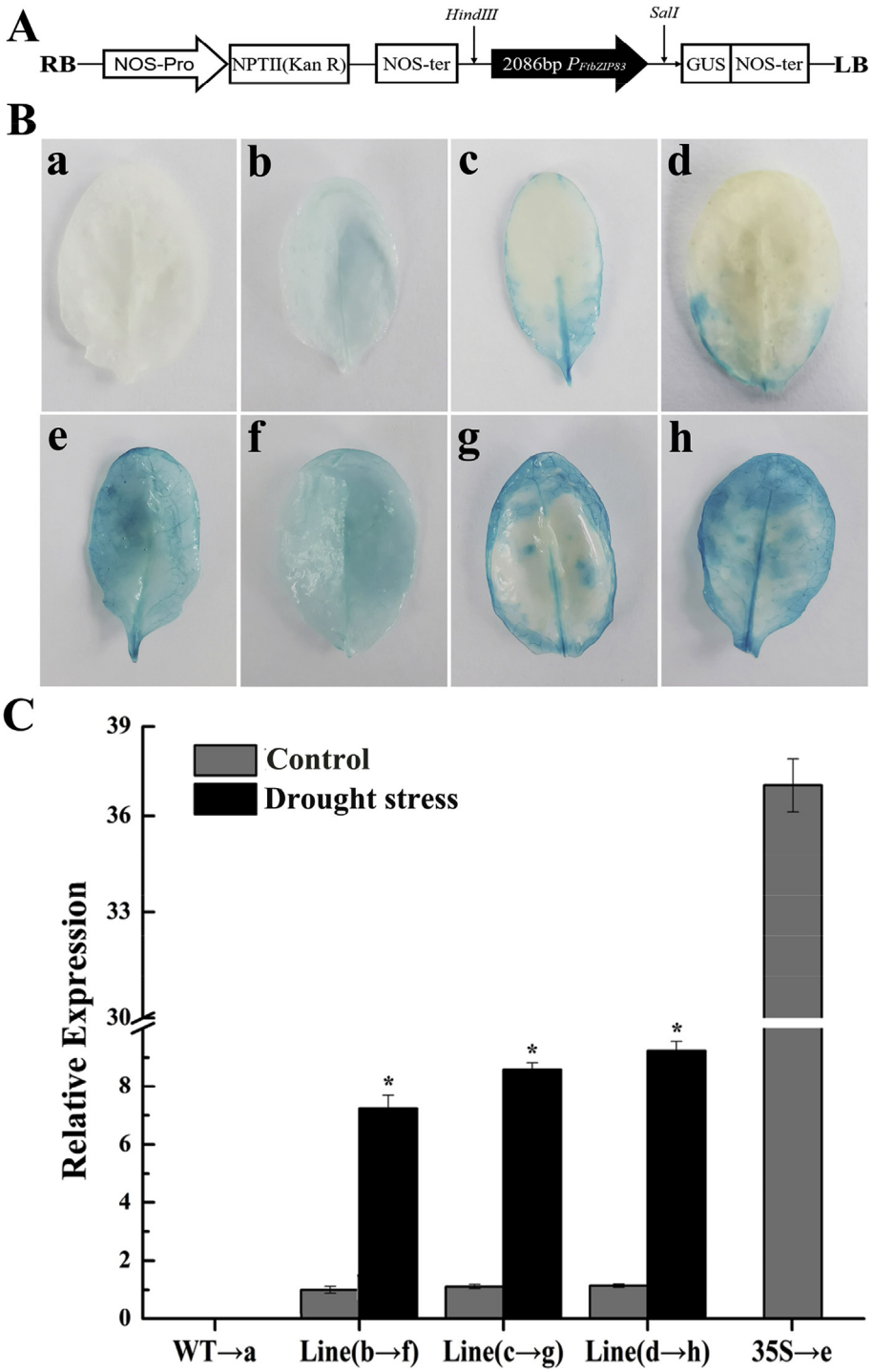

Fig. 10. Effects of drought on GUS gene expression in transgenic Arabidopsis with $P_{FtbZIP83}$. (A) A schematic diagram of the fusion expression vector of the *FtbZIP83* promoter and GUS gene. (B) Staining analysis of leaves of *Arabidopsis thaliana* transgenic lines and WT plants was carried out after a week of drought treatment. (a) Wild-type Arabidopsis as a negative control. (b–d) Positive lines with $P_{FtbZIP83}$ under normal conditions. (e) Transformed Arabidopsis with the CaMV 35S promoter as a positive control. (f–h) Positive lines b-d after one week of drought treatment. (C) Comparison of GUS gene expression between transgenic lines under normal growth and drought conditions. The relative expression was determined by the $2^{-\Delta\Delta CT}$ method, and the expression level of the *GUS* gene in the positive lines (b–f) before treatment was defined as "1". The error bars denote ± SDs, and each data value is from three replicate experiments. * represents *t*-test significance: $P < 0.05$.

transcriptional level affect their transcriptional activity, DNA-binding capability, intracellular distribution, ability to form dimers and ability to interact with other proteins (Schütze et al., 2008). The phosphorylation of AREB/ABF transcription factors by SnRK2 protein kinase has been reported in Arabidopsis (Hiroaki et al., 2007), and the most common target sequence for phosphorylation is R-X-X-S/T (Takashi et al., 2006). The previous studies have shown that SnRK2.2/2.3/2.6 protein kinases activated by ABA can phosphorylate the ABA response site of AREB/ABFs in Arabidopsis, and these proteins function redundantly in these processes (Fujita et al., 2009). Moreover, an analysis of the phosphorylation of StABF1 in different tissues showed that the transcription factor may involve phosphorylation and may differentially regulate transcription in a tissue-specific manner (García et al., 2012). Our Y2H experiment showed that FtbZIP83 could interact with FtSnRK2.6/2.3 but not with FtSnRK2.2. Recently, similar studies have shown that SmAREB1 can interact with SmSnRK2.6/2.3 to regulate the osmotic stress response of *Salvia miltiorrhiza* (Jia et al., 2017). In summary, we suggest that AREB/ABF transcription factors in plants are modified after translation and that they may be modified by a variety of different protein kinases and vary in different tissues.

In conclusion, we identified *FtbZIP83*, a Tartary buckwheat AREB/ABF transcription factor gene that is induced by ABA, salt and drought. Overexpression of *FtbZIP83* in Arabidopsis enhanced salt/drought tolerance in an ABA-dependent manner by reducing the cellular levels of

ROS and increasing the transcript abundance of downstream stress-related genes. FtbZIP83 may be involved in signal transduction via phosphorylation by FtSnRK2.6/2.3. Taken together, the results of this report on the function of FtbZIP83 enriches the study of AREB/ABFs and are beneficial to the understanding of the stress resistance mechanism of Tartary buckwheat.

Contributions

QL performed most of the experiments and all the data analysis. QL and Qi Wu conceived and designed the study. BBL and QXD performed the plasmid constructions. YJY and Qiong Wu assisted in Arabidopsis transformation. HXZ and HCanalyzed experimental results. CLL analysed sequencing data and developed analysis tools. AHW provided Tartary buckwheat seed materials. QL wrote the manuscript. Qi Wu and XLW provided theoretical guidance and financial support. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.003.

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