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A C2H2 ZINC FINGER PROTEIN GhZFP8 FROM COTTON (*GOSSYPIUM HIRSUTUM*) IS INVOLVED IN SALT STRESS TOLERANCE IN *ARABIDOPSIS*

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Abstract

C2H2-type zinc finger proteins (ZFP) function in many biological processes. In this study, *GhZFP8* encoding a C2H2 ZFP was cloned from cotton. Results of qPCR indicated that the transcripts of *GhZFP8* were detected in the leaves and fibers of 3, 6, and 30 days post anthesis, but not in the roots, stems, and flowers. The transcriptional level of *GhZFP8* was regulated by cold, salt, PEG, and ABA. Sub-cellular localization analysis showed that GhZFP8 was distributed in the nucleus of tobacco leaves. *GhZFP8* transgenic *Arabidopsis* exhibited improved tolerance to high salinity but less sensitivity to ABA. Meanwhile, overexpression of *GhZFP8* elevated the rate of water loss in leaves compared to that in wild types. All results suggested that GhZFP8 may function in the response of *Arabidopsis* to salt and ABA in cotton.

Key words: ABA, C2H2-type, *Gossypium hirsutum*, Salt stress, Zinc finger protein.

Introduction

Abiotic stress is an influential cause limiting the yield of crops and leading to enormous loss. Many factors can harm crops, including drought, salinity, high temperature, and cold, of which high salinity is one of the most serious stresses. When facing salt stress, plants can regulate the transcription of stress-related genes and translation and post-translational modification to change their physiology and metabolism, all of which help plant survival. Regulation of transcription is one of the most efficient ways to respond to salt stress. Transcription factors (TFs) such as MYB, NAC, bZIP, WRKY, and ZFPs have important roles in transcription.

ZFPs belong to a large family binding zinc ions. They have many functions in regulating the growth and development of plants, including the trichome formation (Gan *et al*., 2006; Gan *et al*., 2007; Zhou *et al*., 2011; Zhou *et al*., 2013), shoot development (Prigge & Wagner *et al*., 2001), flowering (Hake *et al*., 2004), pollen germination, and fruit setting (Han *et al*., 2011; Han *et al*., 2018). According to the amount of conserved Cysteine (C) and Histidine (H) in zinc finger domain, ZFPs can be divided into five classes, including C2H2, C2C2, C2HC, C2C2C2C2 and C2HCC2C2 (Ciftci-Yilmaz & Mittler, 2008). C2H2 ZFPs that are TFIIIA-type ZFPs have been thoroughly studied in prokaryote. Previous study has shown that ZFPs can regulate the response to salt stress by activating or increasing the transcription of stress-associated genes. *ZFP182* encoding a zinc finger protein was induced by cold, drought, and ABA. Overexpression of *OsZFP182* in tobacco and rice enhanced their tolerance to salt stress (Yang *et al*., 2007). Overexpression of *OsZFP179* induced by NaCl, PEG600, and ABA improved the elimination of ROS, which enhanced drought and salt stress tolerance (Sun *et al*., 2010). In cotton, several C2H2 ZFPs have been characterized. *CSTZ* was cloned from a cDNA library of petals and encoded a C2H2 ZFP. Salt stress can increase the transcripts of *CSTZ* in cotton seedlings, but the mechanism is unclear (Wang & Yang, 2002). *GhDi19-1* and *GhDi19-2* were induced by high salinity and drought stresses and encoded proteins containing two C2H2 domains. The expression of both genes in *Arabidopsis* improved their sensitivity to ABA and high salinity (Li *et al*., 2010). In our previous study, a gene named *GhSIZ1* was mainly expressed in cotton roots and stems. Meanwhile, it is regulated by PEG6000, NaCl, and cold (Zhang *et al*., 2015). In a word, C2H2-type ZFPs may have a crucial function in the response of plants to salinity stress.

In this study, another C2H2-type zinc finger protein was identified and named *GhZFP8*. The transcription of *GhZFP8* was significantly induced by cold, NaCl, ABA, and PEG6000 treatment. GhZFP8 fused with GFP or RFP was detected in the nucleus. Transgenic plants of *GhZFP8* showed reduced sensitivity to ABA and an increased ratio of water loss. Meanwhile, with overexpression of *GhZFP8*, they became much more tolerant to salt stress. Our findings further broaden our knowledge of the role of ZFPs in signal transduction.

Material and Methods

Plant materials, growth conditions and treatment: Seeds of cotton (*Gossypium hirsutum* L.) were sown in soil and grown under normal conditions for about twenty days. When three true leaf emerged, uniform seedlings were cultured in nutrient solution for two days and then treated by 0.1 mM ABA, 4ºC, 400 mM NaCl and 15% PEG6000 for 0h, 1h, 3h, 6h, 12h, and 24 h, respectively. The whole young seedlings were harvested and stored at -80ºC for total RNA extraction.

Cloning of the *GhZFP8* gene and sequence analysis: BLAST analysis was performed for screening the EST database in NCBI using a C2H2 zinc finger protein GIS (OAP02170.1) in *Arabidopsis* as prey. Four homologous

ESTs were found including ES802869.1, DT567237.1, ES825295.1, DW494174.1, and DW494173.1. According to the sequences, specific primers were designed for the gene clone. DW494174.1 containing a complete ORF was cloned using specific primers, 5'ATGGAGAAGAACG AAAGGGAGA3', and 5'CCTCATCTTGCAGAGC AAAGAT3'. MEGA6 and GeneDoc were used for sequence alignment and phylogenetic analysis.

Total RNA extraction and real-time qPCR: RNA was isolated from various organ, such as roots, stems, leaves, flowers, and fibers of cotton. The cDNA was synthesized using reverse transcriptase according to the manufacturer's instructions. Gene expression were detected by RT-qPCR with the specific primers 5'-CTT CATGGAACTCTAGCT-3' and 5'-GGCAAGGGAT GCGAGGATG-3'. The *GhUBI* gene was used for control with the primers 5'-CAGATCTTCGTCAAAACCCT-3' and 5'-GACTCCTTCTGGATGTTGTA-3'. PCR conditions were as follows: pre-incubation at 94ºC for 1 min was followed by 45 cycles of denaturing at 95°C for 15 s; annealing at 55ºC for 20 s; and extension at 72ºC for 30 s. Finally PCR mixture was incubated at 72°C for 10 min. Three repeats were set for each expression analysis, and the average value of the three independent repeats were calculated as the level of gene expression.

Subcellular localization of *GhZFP8*: To construct the *GhZFP8-GFP* (Green Fluorescent Protein) / *RFP* (Red Fluorescent Protein) vector, the coding sequence of *GhZFP8* was cloned into the pCanmbia1300-GFP/RFP vector with primers 5'- GGATCCATGGAGAAGAA CGAAAGGGAGA-3' (*Bam*HI site is underlined) and 5'-ACTAGTCAGATGT AGATCCAAACTCAC-3' (The *Spe*I site is underlined). Then the *GhZFP8-GFP/RFP* vectors was transferred into *Agrobacterium tumefaciens* strain EHA105. The strain were diluted using liquid cultures untile the OD600 value was 1.5, and then injected the EHA105 strain into the young leaves of *Nicotiana benthamiana*. After injection, the tobacco were trans-located to culture room for about three days. Then, the epidermis of the tobacco leaves were stripped off and observed using a fluorescence confocal microscopy.

The phenotype of transgenic plants treated with salinity and ABA: To investigate the role of *GhZFP8* in abiotic stress, transgenic plants were generated. Two transgenic lines and a wild type (WT) were used to test the response to salt and ABA in germination. Surface-sterilized and cold-treated seeds were plated on 1/2 MS medium supplemented with 100 mM and 125 mM NaCl; no NaCl-supplemented treatment was used for the control. Meanwhile, the response to ABA was tested with the same method. Seeds were germinated in a culture room under 22°C (light/dark, 12h/12h). The rate of seed germination was recorded for each twelve hours. After seven days, the ratio of green cotyledon on arabidopsis growing on 1/2 MS medium complemented with NaCl was counted. Experiments on response to salt and ABA were repeated three times. A water loss assay was conducted using detached rosette leaves. Five rosette leaves were harvested from 4-week-old transgenic *Arabidopsis* growing in soil, and the fresh weight was weighed immediately. All leaves were placed on a weighing paper and weighed every 30 min, and the results were recorded. For each genotype, three plants were used for the water loss assay.

Results

Isolation and sequence analysis of *GhZFP8*: The length of coding sequence of *GhZFP8* gene was 747 bp, encoding a polypeptide of 248 aa with a molecular weight of 27.52 kDa. In the N-terminal region, the *GhZFP8* protein contains a zinc finger domain in which Cys and His were conserved (Fig. 1A and B), so *GhZFP8* was a C2H2 type ZFP. Meanwhile, the QALGGH motif essential for DNA binding was found in the zinc finger domain of *GhZFP8*. Its protein contained an EAR/DLN-box, a putative transcription repression domain at the C-terminus. Phylogenetic analysis showed that all the proteins used in this study were divided into two subgroups, and *GhZFP8* was clustered into the same group with AtGIS, AtGIS2 and AtZFP8. *GhZFP8* is most like XP 017614237.1 and PPS02967.1 from *Gossypium arboreum* and *Gossypium barbadense*, and least like AtGIS, AtGIS2 and AtZFP8. GhSIZ1 and AtZFP5, two C2H2-type ZFPs from cotton and *Arabidopsis*, were classified into another subgroup (Fig. 1C).

The expression of *GhZFP8* in different organs and under various abiotic stress treatment: To detect the expression of *GhZFP8* under different abiotic stress, the expression profiles were examined in cotton under salt, PEG6000, cold and ABA treatment by qPCR. The results of qPCR indicated that the expression of *GhZFP8* was significantly induced by these treatments (Fig. 2A-C). For the ABA treatment, the transcription of *GhZFP8* began to increase and peaked after being treated for 3 h and then declined (Fig. 2C). After treatment by NaCl, the induction profiles of *GhZFP8* peaked at 6 h of treatment, and then decreased significantly (Fig. 2B). The expression pattern of *GhZFP8* under PEG and cold treatment markedly increased as the treatment time progressed, reaching the highest points at 12 h and 6 h treatment, respectively (Fig. 2A and D).

Subcellular localization of GhZFP8: In previous studies, many TFs were localized in the nucleus. To check this hypothesis, coding regions were fused with GFP and RFP reporter genes. *GhZFP8-GFP/RFP* fused genes were expressed and driven by a CaMV35S promoter into tobacco leaves. The signal was detected using a confocal microscope after three days. The result showed that both the *GhZFP8-GFP* and *GhZFP8-RFP* fusion proteins accumulated in the nuclei of leaf cells, thus demonstrating that *GhZFP8* is a nuclear-localized protein (Fig. 3).

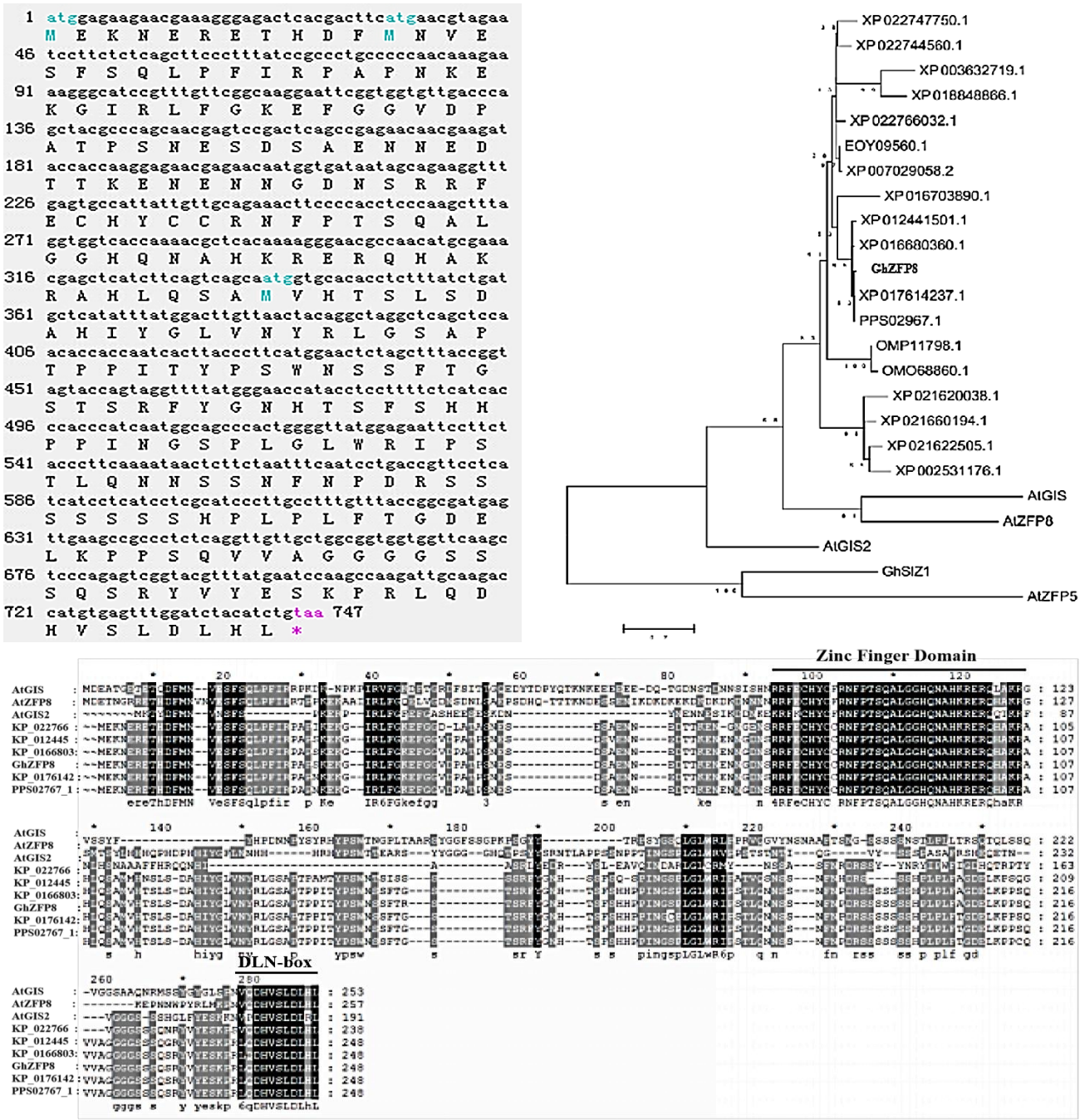

Fig. 1. Sequence analysis of GhZFP8 and other zinc finger proteins from different species
A. ORF and protein sequence predicted by ORF founder; B. Phylogenetic tree of GhZFP8 and other zinc finger proteins from other species; C. Amino acid alignments of the GhZFP8 with zinc finger proteins from other species.

Overexpression of *GhZFP8* is insensitive to ABA: To check if the *GhZFP8* was involved in the response of the plant to ABA, the seeds of WT and two transgenic lines were germinated to test the sensitivity to ABA in the germination stage. Both two transgenic lines showed a decreased sensitivity compared to the WT. In normal conditions, the germination rate of *GhZFP8* overexpression was the same as that of WT. Under ABA treatment, the germination rate of WT, OX-1 and OX-2 decreased significantly. After being grown on a medium containing 0.5 μM ABA for three days, the WT's germination rate was 52%, while both transgenic lines were more than 80%. When the concentration increased to 1 μM, the germination rate of the WT reduced to 40%, while nearly 48% of transgenic seeds germinated (Fig. 4A and B).

ABA is a key factor that controls the movement of stoma and water loss in leaves. The water loss ratio of WT and overexpressors was investigated. The leaves of both transgenic lines lost water much more quickly than that of the WT. Three hours later, transgenic lines lost 37% and 39% of the whole water content, respectively, while leaves of the control lost 32% (Fig. 4D). After being detached for five hours, most leaves of OX-1 and OX-2 wilted seriously, but the leaves of the WT seemed to be very well (Fig. 4C). The results of the germination and water loss ratio assay showed that *GhZFP8* negatively regulated the plant's response to ABA.

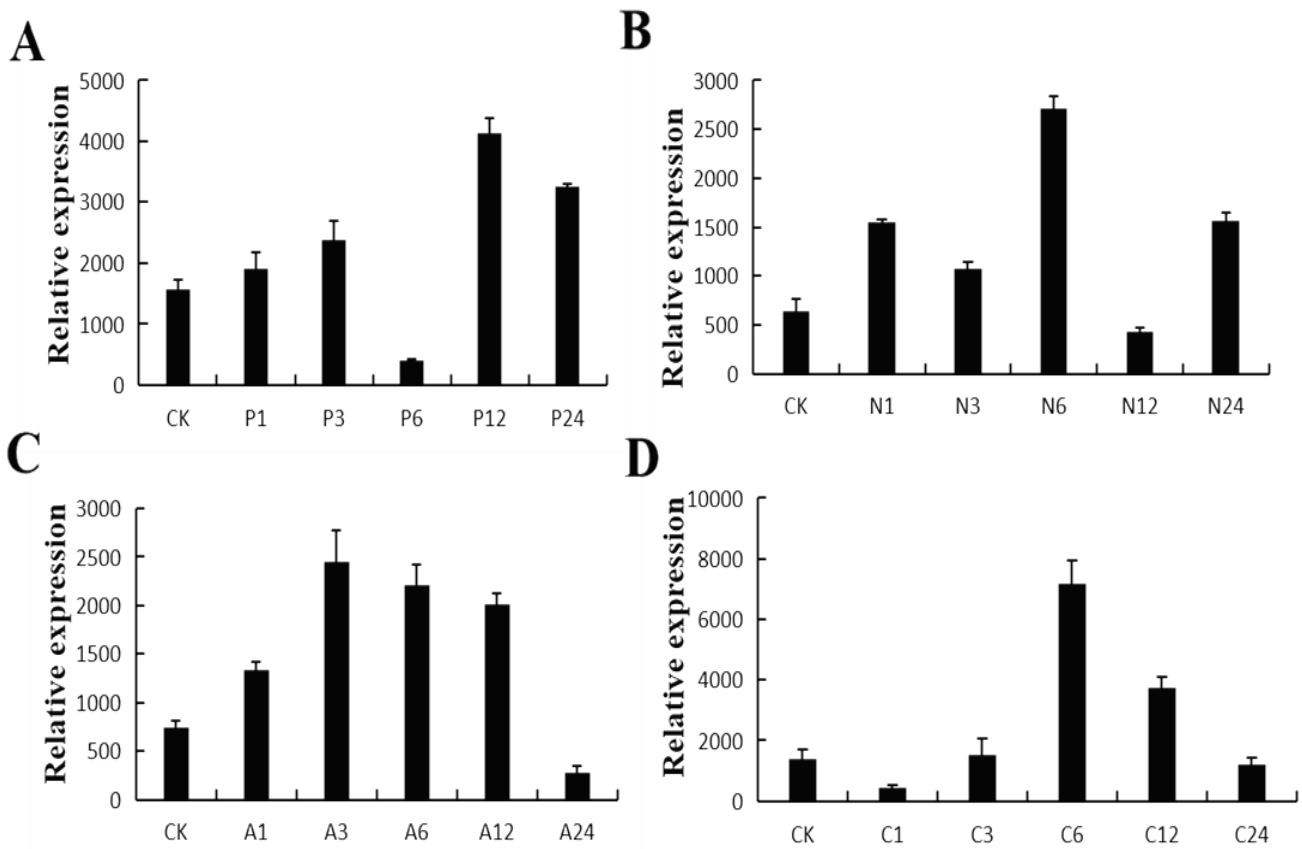

Fig. 2. Expression pattern of *GhZFP8* in different tissue and responses to abiotic stresses
A-D, Expression of *GhZFP8* in the seedlings under PEG (20% PEG 6000), salt (200 mM NaCl), abscisic acid (0.1 mM ABA), and cold (4°C) treatment, respectively.

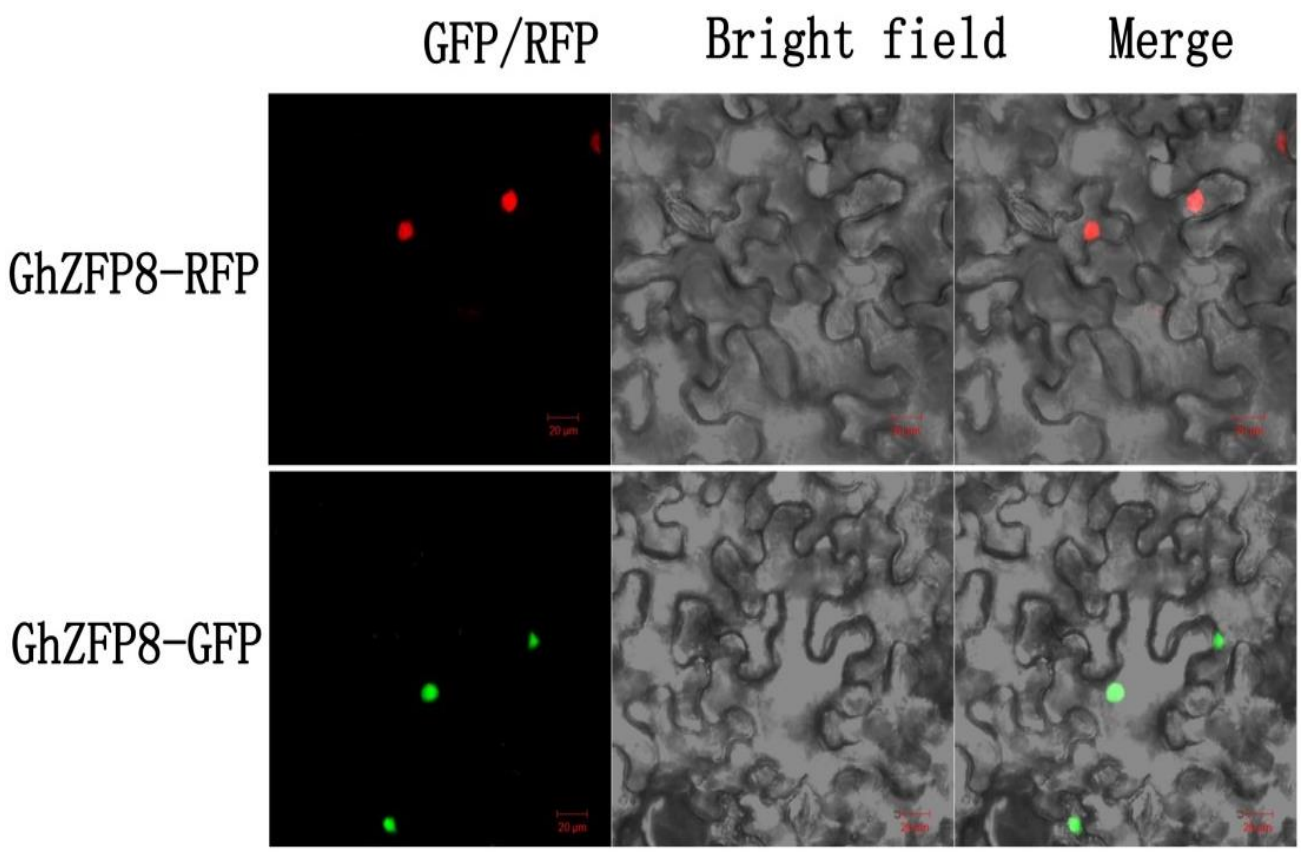

Fig. 3. The localization of GhZFP8 in the leaves of *Nicotiana benthamiana*
Detection of fluorescence in tobacco leaf expressing GhZFP8-GFP and GhZFP8-RFP respectively, Bar=20 μm.

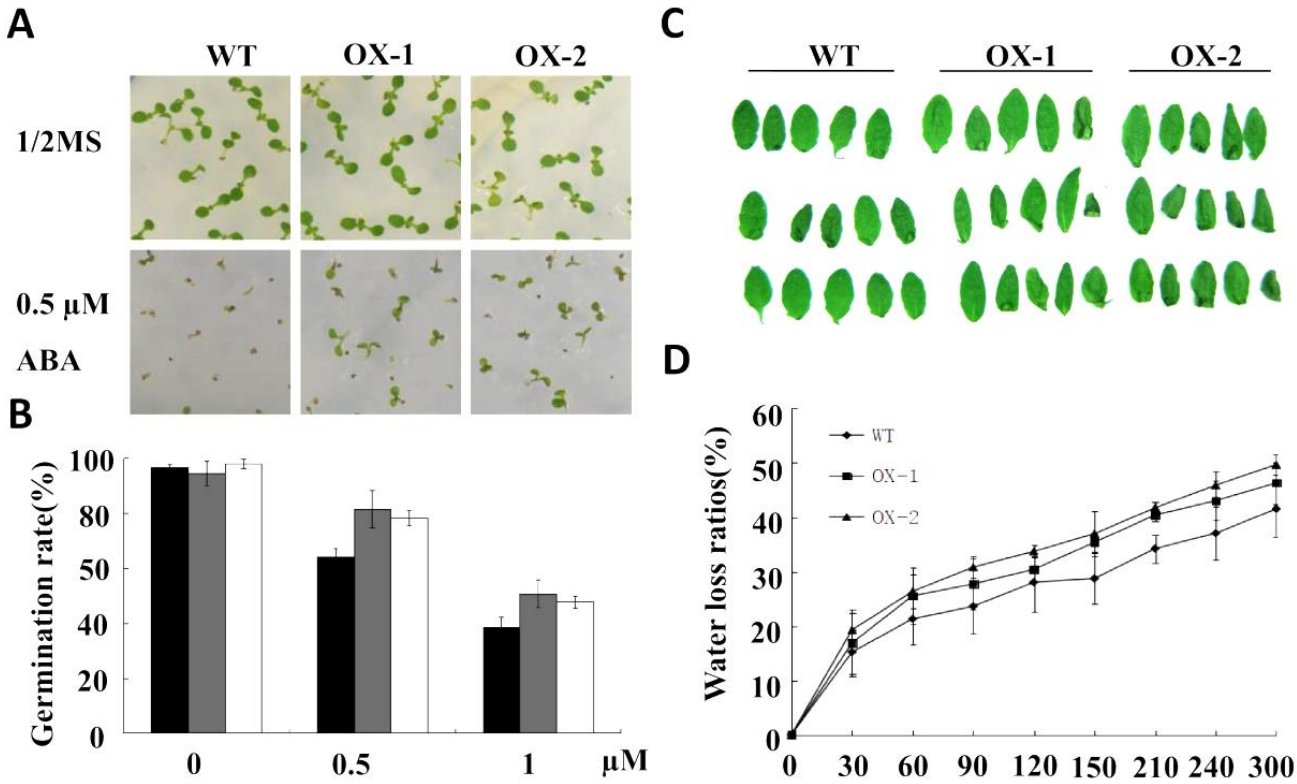

Fig. 4. Response of *GhZFP8* overexpression to ABA and water loss
A.The phenotype of transgenic plants treated with ABA; B. The rate of seed germination on 1/2 MS medium complemented with different concentration of NaCl for five days; C. The phenotypes of the leaves excised from transgenic plants after water loss for 5 h; D. Statistic analysis of water loss rate. All leaves were weighted at the time point (n = 15). The values were the meanings from three independent experiments, and bars presented standard errors.

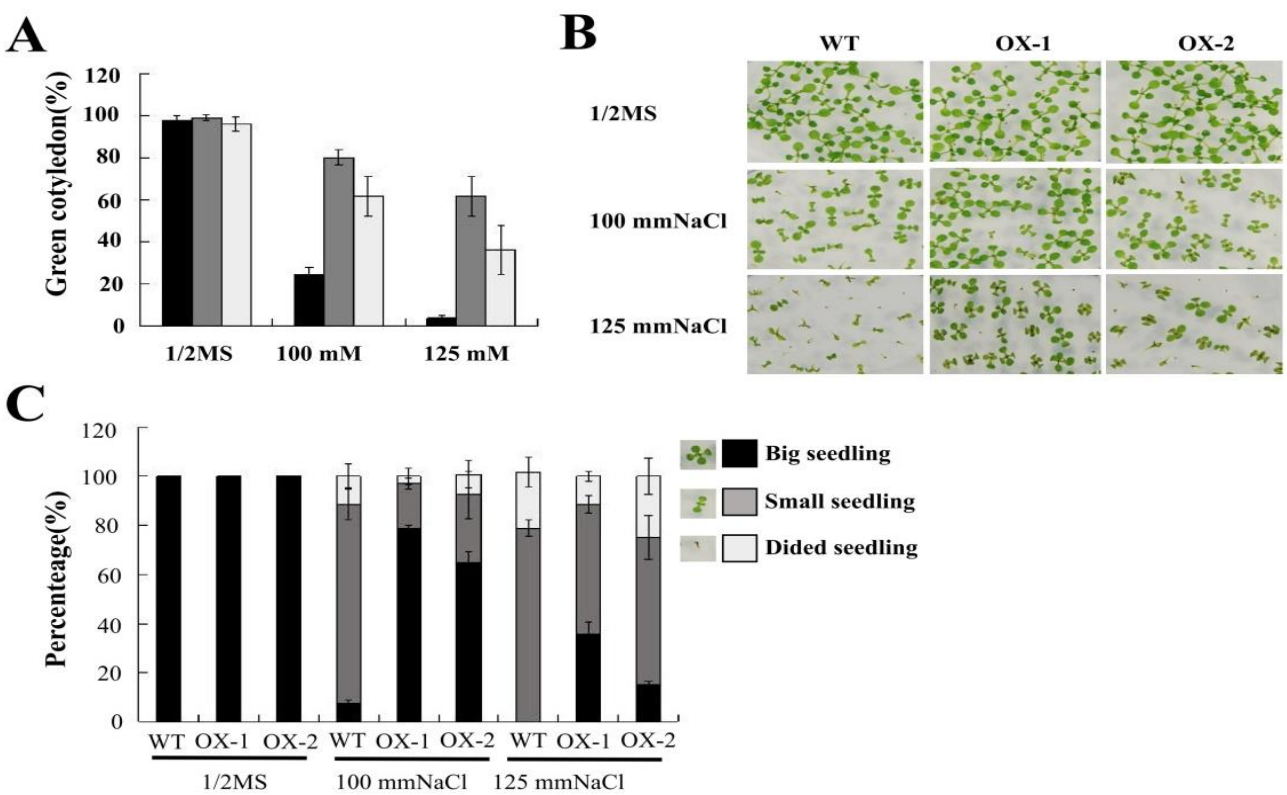

Fig. 5. Effect of *GhZFP8* overexpression on salt tolerance in transgenic plants
A. Green cotyledon ratio of *GhZFP8* overexpression under 100 mM and 125 mM NaCl treatment for two weeks; B; Performance of seedlings grown on solid medium containing NaCl; C. Quantitative analysis of different WT plant types and overexpression of *GhZFP8* shown in B.

Overexpression of *GhZFP8* enhance tolerance to salt stress: *GhZFP8* was induced by salt stress and PEG6000, which suggested that *GhZFP8* may respond to salt stress. To confirm our speculation, transgenic plants were generated. There was no visible difference between the WT and *GhZFP8* overexpressors without NaCl treatment. After being treated with 100 mM NaCl for seven days, the green cotyledon ratios of both transgenic lines (80.4% and 61.8%) were much higher than that of the WT (24.4%). When the NaCl concentration was increased to 125 mM, 3.8% of the WT cotyledons and 36–61% of the transgenic cotyledons turned green (Fig. 5A). Two weeks later, the number of different plants was recorded. Compared to the WT, there were many more big, overexpressed seedlings when they were grown on a medium containing 100 mM and 125 mM NaCl (Fig. 5B and C). These results indicated that heterologous expression of *GhZFP8* improved the tolerance of plant to salt stress.

Discussion

Most C2H2 zinc finger TFs have DNA binding activity and recognize more elements than any other TFs in human (Najafabadi *et al*., 2015). The conserved QALGGH motif was found in plant ZFPs, but not in the ZFPs of other eukaryotes. Previous study showed that changes in any amino acid decrease the activity of binding DNA (Kubo *et al*., 1998). Sequence analysis revealed that *GhZFP8* and other proteins used in this study also contain a QALGGH motif, which may function in DNA binding. In the C-terminus of *GhZFP8*, a DLN-box/EAR-motif was found that includes an SLDLHL motif (Fig. 1). Many ZFPs containing a DLN-box can active the transcription of many genes, such as *OsART1* (Yamaji *et al*., 2009), *OsC3H12* (Deng *et al*., 2012) and *ThZF1* (Xu *et al*., 2007), but most process the ability to inhibit the expression of many genes, such as *ZFP*, *AZF1*, and *AZF2* (Kodaira *et al*., 2011; Ren *et al*., 2018). Whether *GhZFP8* can activate or inhibit the expression of downstream gene or not is unclear.

In the world, high salinity and drought are major constraints on crop plant yields. Evidence from multiple sources showed that ZFPs function as the key transcriptional regulator related to the plant's response to various abiotic stress. Many zinc finger genes were induced by biotic stresses, including high salinity, drought, cold, and ABA. Meanwhile, increasing or decreasing their transcription affects the plant's response to biotic stresses. Overexpression of *OsZFP213* in rice enhanced the salt tolerance and the activity of some enzymes to scavenge reactive oxygen (Zhang *et al*., 2018). *OsDRZ1* was regulated by abiotic stresses and encoded a transcriptional repressor, overexpression of which enhanced the drought tolerance and accumulated much more proline and less reactive oxygen species (ROS) and elevating the activities of antioxidant enzymes (Yuan *et al*., 2018). STF-2 is a C2H2 zinc finger TF and function in drought tolerance of transgenic tobacco. Under drought condition, *OsCTZFP8* overexpressors processed significantly higher survival rate than that of the control (Song *et al*., 2019). In cotton, some ZFPs TF have been identified, such as GhZFP1, CSTZ, GhDi19-1, GhDi19-2 and GhSIZ1 (Wang and Yang., 2002; Li *et al*., 2010; Zhang *et al*., 2015; Guo *et al*., 2009). All of them were induced by multiple abiotic stresses. In the present study, the transcriptional level of *GhZFP8* was regulated by cold, high salinity, PEG, and ABA (Fig. 2). Transgenic plants of *GhZFP8* showed much more insensitivity to salt stress and ABA, and a much faster water loss ratio than WT (Figs. 3, 4, 5). All results indicated that the ZFPs known as TFs process important function in the response to stresses by enhancing or inhibiting the transcription of stress-related genes.

In addition to abiotic stress, several C2H2 TFs regulated the formation of trichomes in *Arabidopsis*, such ZFP5, ZFP6, ZFP8, GIS, GIS2, GIS3, etc, (Gan *et al*., 2006; Gan *et al*., 2007; Zhou *et al*., 2011; Zhou *et al*., 2013). In other species, ZFPs were involved in trichome development. *JcZFP8* encoding a C2H2 ZFP TF from *Jatropha curcas* L., can regulate trichome development of tobacco by regulating the expression of CycB2 and MYB genes (Shi *et al*., 2018). *Hair* and *Woolly* encoded two ZFPs that interacted with each other and functioned as a heterodimer. Both represses the formation of type-I trichomes in tomato (Chang *et al*., 2018). Although a zinc finger domain and DLN-box/EAR motif existed in GhZFP8, ZFP8, GIS, and GIS2, they share a low similarity (Fig. 1B and C). Although *GhZFP8* has the highest level of fibers at 3 DPA (Fig. 2), which indicates that *GhZFP8* may regulate the development of cotton fiber, overexpression of *GhZFP8* cause the increased number of trichomes in *Arabidopsis*. It is speculated that the developments of cotton fiber and trichomes were regulated by a similar mechanism.

Acknowledgments

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