Heterologous Expression of a Novel Zoysia japonica C2H2 Zinc Finger Gene, ZjZFN1, Improved Salt Tolerance in Arabidopsis

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Growing evidence indicates that some grass species are more tolerant to various abiotic and biotic stresses than many crops. Zinc finger proteins play important roles in plant abiotic and biotic stresses. Although genes coding for these proteins have been cloned and identified in various plants, their function and underlying transcriptional mechanisms in the halophyte Zoysia japonica are barely known. In the present study, ZjZFN1 was isolated from Z. japonica using RACE method. Quantitative real time PCR results revealed that the expression of ZjZFN1 was much higher in leaf than in root and stem tissues, and induced by salt, cold or ABA treatment. The subcellular localization assay demonstrated that ZjZFN1 was localized to the nucleus. Expression of the ZjZFN1 in Arabidopsis thaliana improved seed germination and enhanced plant adaption to salinity stress with improved percentage of green cotyledons and growth status under salinity stress. Physiological and transcriptional analyses suggested that ZjZFN1 might, at least in part, influence reactive oxygen species accumulation and regulate the transcription of salinity responsive genes. Furthermore, RNA-sequencing analysis of ZjZFN1-overexpressing plants revealed that ZjZFN1 may serve as a transcriptional activator in the regulation of stress responsive pathways, including phenylalanine metabolism, α-linolenic acid metabolism and phenylpropanoid biosynthesis pathways. Taken together, these results provide evidence that ZjZFN1 is a potential key player in plants’ tolerance to salt stress, and it could be a valuable gene in Z. japonica breeding projects.

Keywords: Zoysia japonica, C2H2 zinc finger protein, transgenic Arabidopsis, salinity tolerance, RNA-sequencing

INTRODUCTION

Salinity stress is becoming an increasing abiotic stress that limits crop yield and plant distribution worldwide (Zhu, 2001; Deinlein et al., 2014). Growing evidence indicates that some forage grass and turfgrass species are more tolerant to various abiotic and biotic stresses than many cultivated crop varieties (Shi et al., 2013a). Recently, several salt stress responsive genes isolated from grass...
species were reported as active participants in environmental responses (Huang et al., 2014). By binding to distinct cis-regulatory elements, TFs including NAC, WRKY, MYB, bZIP, and zinc finger TFs play a key role in regulating plant responses to salinity stress (Deinlein et al., 2014).

Zinc finger proteins represent a large family of TFs in plants (Kielbowicz-Matuk, 2012). According to the number and location of the histidine and cysteine residues, which surround the zinc atom to form a “zinc-finger structure,” zinc finger proteins are classified into several subfamilies, such as C2H2, CCCH, C2HC, C2HC5, and C4 (Laity et al., 2001). Specifically, 176 and 189 C2H2 zinc finger proteins were identified in Arabidopsis and rice, respectively, supporting that C2H2 is one of the most abundant families of zinc finger proteins in plants (Huang et al., 2007; Yin et al., 2017). These C2H2 zinc finger proteins have been functionally well-characterized in model plants and crops, and reported to play key roles in plant development and responses to environmental stresses. For instance, constitutive expression of ZAT7 or Zat10 enhanced the tolerance of Arabidopsis to salinity (Mittler et al., 2006; Ciftci-Yilmaz et al., 2007). Overexpression of ZFP179 improved salt tolerance but caused hypersensitivity to exogenous ABA in rice (Sun et al., 2010). Overexpression of ZAT18 (Yin et al., 2017) and GsZFP1 (Luo et al., 2012) enhanced drought tolerance of zoysiagrass, while GmZFP3 negatively regulated its drought tolerance (Zhang D.Y. et al., 2016). While AtZAT6 can modulate biotic and abiotic stress tolerance by activating the SA pathway (Shi et al., 2014), ZFP36 plays an important role in ABA induced antioxidant response in rice (Zhang et al., 2014). However, the functions of C2H2 zinc finger proteins in stress responses and the mechanism underlying the regulation of transcription in stress related genes is still unclear, especially in grasses.

Zoysiagrass (Zoysia spp. Willd.), one of the most salt-tolerant turfgrass species, can resist injury under 1% salt solution (Li et al., 2004). Zoysiagrass is now widely used in football pitches, home lawn, and ecological management (Teng et al., 2017). Studies conducted on this species so far have focused on the evaluation of abiotic stress tolerance among different cultivars, on reporting physiological mechanisms, and on the development of molecular markers (Guo et al., 2014; Xu et al., 2015). Thus, the molecular mechanism of salt tolerance in zoysiagrass remains unclear, mostly due to limited genetic resources. Functional studies of individual C2H2 zinc finger proteins will not only provide a better understanding of its detailed function in plant adaption to stress, but also provide insight into potential signaling processes occurring in plants under stress conditions. Therefore, the present study aimed to identify a C2H2 zinc finger protein in zoysiagrass, explore its role in salt stress tolerance and reveal its transcriptional regulation mechanism.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Zoysia japonica* cultivar “Companion” seeds were purchased from Hancock seed company (Dade City, FL, United States) and grown in a greenhouse at 28/25°C (day/night) under 400 μmol·m−2·s−1 photosynthetically active radiation (PAR). *Nicotiana benthamiana* seedlings were grown in a growth chamber set at 22°C with a photoperiod of 16 h. *Arabidopsis thaliana* ecotype “Columbia” (wild type, WT) plants were kept at 24/22°C (day/night) with 65% relative humidity and a scotophase of 8 h to yield transgenic lines. Transgenic lines were cultivated under the same growth conditions as the WT plants and T3-generation seeds were harvested for phenotype observation. The plants were manually irrigated with 1/2 strength Hoagland’s nutrient solution weekly (Hoagland and Arnon, 1950).

**Isolation of ZjZFN1**

Total RNA was extracted from “Companion” leaves using Trizol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer’s instructions. Using the rapid amplification of cDNA ends (RACE) method, 5′/3′ full-length sequences of ZjZFN1 were isolated with a SMARTer RACE Kit (TaKaRa, Dalian, China) following the manufacturer’s instructions. Primers used for 5′/3′-RACE (Table 1) were designed based on a known cDNA sequence fragment (comp219112_c0).

**Table 1** | Primers used for gene cloning, qRT-PCR detection, and plasmid construction.

| Primer name | Primer sequence (5′-3′) |
|-------------|-------------------------|
| 5′RACE | CAGATGGACGACCCGCTGGTCGCT |
| 3′RACE | GGCAAGTCTGCGTGCTGCT |
| ZjZFN1-F | ATGTCGTCCGCGCAGTGGACT |
| ZjZFN1-R | TCAAGCGGTTAACGAGGAGG |
| ZFN1-R1 | GTAGGAGCCGGAAGCAGGCTG |
| ZFN1-R2 | GAGGCGAGGGCGGAGGTTCT |
| qZFN1-F | TCTTCTCCCTGCTGCTGTC |
| qZFN1-R | GACACACAGAGGAGAAGG |
| 3302Y-ZFN1-F | GGGACGGTGGGAGGTCAAG |
| 3302Y-ZFN1-R | GCAGGACGGTGGGAGGTCAAG |
| BD-ZFN1-F | TTGCAAGGCTGTGGCTGAGG |
| BD-ZFN1-R | TTAGGAGGCTGCTGAGG |
| 1391Z-ZFN1-F | GAAAGCAGGCTGTGGCTGAGG |
| 1391Z-ZFN1-R | TTAGGAGGCTGCTGAGG |
| AtUBQ-F | ATGTCGTCCGCGCAGTGGACT |
| AtUBQ-R | GACACACAGAGGAGAAGG |
| AtMn-SOD-F | ATGTCGTCCGCGCAGTGGACT |
| AtMn-SOD-R | GACACACAGAGGAGAAGG |
| AtPOD-F | ATGTCGTCCGCGCAGTGGACT |
| AtPOD-R | GACACACAGAGGAGAAGG |
| AtAPX1-F | ATGTCGTCCGCGCAGTGGACT |
| AtAPX1-R | GACACACAGAGGAGAAGG |
| AtNHX1-F | ATGTCGTCCGCGCAGTGGACT |
| AtNHX1-R | GACACACAGAGGAGAAGG |
| AtP5CS-F | ATGTCGTCCGCGCAGTGGACT |
| AtP5CS-R | GACACACAGAGGAGAAGG |
| AtLEA-F | ATGTCGTCCGCGCAGTGGACT |
| AtLEA-R | GACACACAGAGGAGAAGG |

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screened from our previous RNA-seq database (Xie et al., 2015). The amplified products were purified before the transfer into pMD18-T vectors (TaKaRa, Dalian, China). Positive clones were then sequenced at Rui-Biotech Company (Beijing, China). Based on the resulting sequences, the gene specific primers, ZjZFNI-F and ZjZFNI-R, for amplification of full-length ZjZFNI cDNA and genomic DNA sequences.

Isolation of the ZjZFNI Promoter
Genomic DNA was extracted from “Companion” leaves using the cetyl trimethylammonium bromide (CTAB) method and used as template for genome walking (Genome Walking Kit, TaKaRa, Dalian, China) according to the instruction. The gene specific primers ZFN-R1, ZFN-R2, and ZFN-R3 were used in the genome walking. The resulting PCR products were sequenced using the ZFN-R3 primer. Finally, promoter specific primers, namely Promoter-F and Promoter-R, were used to amplify the upstream sequence of ZjZFNI.

Bioinformatics Analysis
The ZjZFNI amino acid sequence was deduced from the corresponding cDNA sequence using DNAMAN software (v. 7.0). Its theoretical isoelectric points (pI) and molecular weight (MW) were calculated using the Compute pI/MW tool. Subcellular localization character was predicted using ProtComp 9.0. Promoter cis-regulatory elements analysis was carried out via PLACE database. Potential signal peptide cleavage sites were examined with SignalP 4.1 Server. The basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) database was used to identify homologs. Clustal W was employed to perform protein alignment. A phylogenetic tree was built in MEGA version 5.0 based on the neighbor-joining method.

Quantitative Real-Time PCR
The expression patterns of ZjZFNI in the roots, stems, and leaves at different developmental stages (young, fast-growing, and mature) of Z. japonica were investigated in the roots, stems, and leaves at different developmental stages (young, fast-growing, and mature) of Z. japonica after 24 h under 8 \( \mu \)M ABA, 5 \( \mu \)M SA, or 10 \( \mu \)M MeJA. The ZjZFNI specific primers qZFN-F and qZFN-R were used for qRT-PCR (Table 1). Reactions were performed in 96-well blocks with a CFX Connect RT-PCR system (BIO-RAD, Hercules, CA, United States) using SYBR Premix (TaKaRa, Dalian, China) in a total volume of 25 \( \mu \)L. A two-step qRT-PCR program was adopted and set as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The Z. japonica actin gene (GenBank accession GU290546) was selected as the internal reference (Table 1) and the relative gene expression levels were calculated using the comparative \( \Delta \Delta Ct \) method (Livak and Schmittgen, 2001). All data are presented as means [with corresponding standard deviations (SDs)] of at least three independent biological replicates, each including three technical replicates. To obtain sufficient samples for RNA extraction, three independent plants were pooled per biological replicate.

Binary Vector Construction
The yellow fusion protein (YFP) construct 35S::ZjZFNI::YFP and pGBKT7-ZjZFNI were produced by transferring the complete ZjZFNI coding sequence (CDS) into 3302Y (Jia et al., 2016) and pGBKT7 vectors, respectively. Firstly, 3302Y and pGBKT7 vector were digested by BglII or BamHI (TaKaRa, Dalian, China), respectively, and purified using a E.Z.N.A Cycle-pure Kit (Omega Bio-Tek, Norcross, GA, United States). Then primers 3302Y-ZFN1-F/R and BD-ZFN1-F/R were used to amplify the ZjZFNI CDS (Table 1). Amplicons were then purified and infused into the linearized 3302Y and pGBK7 plasmids using an In-fusion HD Cloning Kit (TaKaRa, Dalian, China).

Subcellular Localization and Transcriptional Activity Assay of ZjZFNI
Agrobacterium tumefaciens EHA105 transformed with the 35S::ZjZFNI:YFP fusion construct were used to transform N. benthamiana to reveal the subcellular localization of ZjZFNI using the transient overexpression method (Sparkes et al., 2006). After darkness induction for 48 h, the cells from the lower epidermis of N. benthamiana leaf cells were monitored and photographed under a SP-5 laser confocal scanning microscope (Leica, Mannheim, Germany). 1 µg/mL DAPI (Sigma-Aldrich, Munich, Germany) was used to show the nuclear.

The pGBK7-ZjZFNI construct was transformed into Saccharomyces cerevisiae Y2HGold competent cells using the LiAc method (Liu et al., 2016) to investigate its transcriptional activation ability. Firstly, transformed Y2HGold cells (pGBK7 was use as control) were grown on synthetic defined (SD) medium without tryptophan (SD/-Trp). After colony-PCR verification, the positive clones were grown on SD medium without tryptophan, histidine and adenine (SD/-Trp-His-Ade) at 30°C for about 48 h. Yeast growth phenotypes were then photographed with an EOS 60D digital camera (Canon, Tokyo, Japan).

Generation of Transgenic Plants
Using floral dip method (Clough and Bent, 1998), A. tumefaciens GV3101 transformed with construct plasmids was used to infect Arabidopsis plants to generate transgenic plants expressing

1http://web.expasy.org/compute_pI/
2http://www.softberry.com
3http://sogo.dna.afrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace
4http://www.cbs.dtu.dk/services/SignalP/
5https://www.genome.jp/tools-bin/clustalw
ZjZFN1 or ZjZFN1pro::GUS. Transgenic Arabidopsis seeds were screened using 60 mg L\(^{-1}\) glufosinate, or 20 µg/mL hygromycin. Positive transgenic plants were verified by reverse transcription PCR and genomic PCR. Representative T\(_3\) transgenic lines exhibiting 100% resistance to glufosinate were harvested for further phenotype observation or GUS staining assays.

**GUS Staining**
Using a GUS Kit (O‘BioLab, Beijing, China), and according to the instructions provided by the manufacture, Arabidopsis seedlings were stained with GUS. After removing chlorophyll with 70% ethanol, seedlings were then photographed under the M205FA stereomicroscope (Leica, Mannheim, Germany).

**Salt Tolerance in Arabidopsis Transgenic Lines**
Because lines ZFN-2 and ZFN-17 presented the highest ZjZFN1 transcript levels among the 36 T\(_3\) transgenic lines, they were selected as the representative lines for phenotype observation. For germination assessment, seeds of ZFN-2, ZNF-17, and WT were sterilized with 70% ethanol and 1% sodium hypochlorite and then sowed on Murashige and Skoog (MS) medium with or without 100 mM NaCl, respectively. After 4 and 8 days, phenotype were photographed with an EOS 60D digital camera (Canon, Tokyo, Japan). For determination salt tolerance, 3-week-old seedlings were transplanted to nutrition medium containing peat, vermiculite and perlite (1:1:1 in volume) and moved to growth chambers. Here, seedlings were subject to incremental increases of 50 mM NaCl from the first day to the third day and then kept under 150 mM NaCl for 18 days. Leaf samples were then harvested for malondialdehyde (MDA), proline and gene expression examination at the 21st day.

For MDA and proline quantification, the leaf samples were treated as described in our previous report (Teng et al., 2017). Genes coding for stress response proteins, namely superoxide dismutase (AtMn-SOD, AT3G56350), peroxidase (AtPOD, AT3G49120), ascorbate peroxidase1 (AtAPX1, AT1G07890), sodium/hydrogen exchanger 1 (AtNHX1, AT5G27150), pyrroline-5-carboxylate synthase (AtP5CS, AT3G55610), and late embryogenesis abundant (AtLEA, AT1G02820) were selected to monitor salt stress-response transcriptional mechanisms using qRT-PCR (Table 1). The Arabidopsis AtUBQ10 gene (NM_116771) gene was adopted as internal reference.

**Transcriptomic Analysis**
Total RNA was isolated from 4-week-old WT (control) and ZjZFN1-overexpressing Arabidopsis seedlings (ZFN-17) cultivated under normal conditions using Trizol (Invitrogen, Carlsbad, CA, United States). Six independent RNA samples (three replicates from control and three replicates from ZNF-17), each comprising three Arabidopsis plants were obtained. Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, United States) was used for RNA quality determination. Agilent 2100 spectrophotometer (Agilent Technologies, Santa Clara, CA, United States) was used to evaluate RNA purity, concentration and integrity. Sequencing libraries and sequencing analysis were performed by Biomarker Technologies (Beijing, China) on the Illumina 4000 platform (San Diego, CA, United States) to generate 125/150 bp paired-end reads. Raw sequence reads were deposited into the NCBI Short Read Archive (SRA) repository under the accession number SRP140821. The reads containing adaptor and poly-N, as well as the low quality reads, were excluded and the Q20, Q30, and GC content of clean data reads were evaluated. High-quality clean reads were then mapped to the Arabidopsis reference genome (TAIR 10\(^*\)) using TopHat2 (Kim et al., 2013). Gene expression levels were calculated base on the fragments per kilobase of transcript per million fragments mapped (FPKM) method (Mortazavi et al., 2008).

Differentially expressed genes between the WT and the ZFN-17 line (each with three independent libraries) were screened using DESeq R package (1.10.1) based on adjusted false discovery rate (FDR). P-value ≤0.05 and fold change (FC) ≥2. For GO and KEGG enrichment analyze, all DEGs were mapped to GO terms in the GO database and KEGG database using the Goseq R package (Young et al., 2010) and the KOBAS software (Mao et al., 2005), respectively.

**Statistical Analysis**
Data were analyzed by two-tailed Student’s t test or one-way ANOVA using SPSS version 18.0 (IBM, Chicago, IL, United States). *P < 0.05 and **P < 0.01 were believed statistically significant. All data were presented as the means ± SD (n = 3).

**RESULTS**

**Isolation and Bioinformatics Analysis of ZjZFN1 and Its Promoter**
The ZjZFN1 cDNA sequence was deposited in the NCBI database with the accession number of KT596064.1. The open reading frame (ORF) of ZjZFN1 was 789 bp in length, which corresponded to 262 amino acids (Figure 1). The ZjZFN1 protein contained two typical zinc finger structures and belonged to the C2H2 superfamily (Figure 1A). Its theoretical pI was 8.76. Its MW was 27.52 KD and no potential signal peptide was found. Phylogenetic analysis showed that ZjZFN1 was most closely related to the ZFN proteins from *Zea mays* or *Sorghum bicolor* (Figure 1B).

The 1406-bp fragment upstream the ATG start codon, containing CAAT-box and TATA-box elements, was investigated as a potential promoter region (Figure 1C). Two ABA-responsive motifs (ABRE) and one auxin-responsive motif (AuxRR-core) were also identified. In addition, there were one cis-acting regulatory element related to meristem expression (CAT-box) and two involved in anaerobic induction (ARE).

**Expression Patterns of ZjZFN1**
The qRT-PCR employed to interpret the expression patterns of ZjZFN1 showed that, although ZjZFN1 expressed in all

 ftp://ftp.ensemblgenomes.org/pub/plants/release-25/fasta/arabidopsis_thaliana/
ZjZFN1 Was Localized to Nucleus and Had Transcriptional Activity

Bioinformatics analysis showed that ZjZFN1 contained a nuclear localization signal. To confirm the exact subcellular localization of ZjZFN1, we performed transient overexpression of 35S::ZjZFN1:YFP in N. benthamiana leaf cells. Laser confocal microscopic observations revealed strong YFP signal in the whole cells of the control while the YFP signal was only found in nucleus in 35S::ZjZFN1:YFP overexpressing cells. It proved that ZjZFN1 was localized to the nucleus.

The Y2HGold yeast cells transformed with pGBKT7-ZjZFN1 and pGBKT7 empty vectors could grow normally.
on SD/-Trp medium (Figure 3B) suggesting that the target plasmids were successfully transformed in the host cells. Further analysis showed that the yeast cells transformed with the pGBK7-ZjZFN1 vector could grow on SD/-Trp-His-Ade medium whereas control yeast cells (Figure 3B). Thus ZjZFN1 had transcriptional activation ability.
**Abundant ZjZFN1 Expression in Leaf**
The native promoter of ZjZFN1 was infused with GUS to generate the ZjZFN1::GUS transgenic line, and transgenic Arabidopsis lines were generated to examine promoter activity. Twenty-two independent transgenic lines were examined for GUS staining. Histochemical analysis showed obvious GUS signal in the seedlings transformed with the 35S::GUS construct (control) (Figure 4A). However, strong GUS activity was only detected in the leaves and petioles of the ZjZFN1::GUS transgenic seedlings (Figures 4B–D). These results proposed that ZjZFN1 was mostly expressed in leaf and petioles.

**ZjZFN1 Positively Regulated Seed Germination Under Salt Stress**
Arabidopsis plants overexpressing ZjZFN1 were generated to characterize the function of ZjZFN1 in response to salt stress. No significant difference in germination determination was observed between WT and transgenic lines in the absence of NaCl (Figures 5A–C). However, the ZjZFN1-overexpressing lines showed enhanced salt tolerance in relation to that of WT in MS medium containing 150 mM NaCl before day 6. In detail, on the fourth day after being sowed in the presence of 150 mM NaCl, approximately 60% of WT seeds germinated with emerged radicles (Figure 5D). Obvious differences were also observed in terms of the percentage of green cotyledons at the eighth day after sowing (Figure 5B).

**Overexpression of ZjZFN1 Enhanced Plant Tolerance to Salt Stress**
To further examine the function of ZjZFN1, 3-week-old ZjZFN1-overexpressing plants were subjected to 150 mM NaCl for 21 days. All surveyed plants showed delayed growth and leaves were dehydrated, although ZFN-2 and ZFN-17 plants showed less damage compared to that observed in WT plants (Figure 6A). Accordingly, no significant difference in MDA or proline content was detected between ZjZFN1-overexpressing and WT plants under normal conditions. Nevertheless, MDA contents in WT increased to 1.78 nmol g\(^{-1}\), which was 7.5 and 16.9% higher than that in ZFN-2 and ZFN-17, respectively, at the end of the experiment (Figure 6B). In addition, under salt stress, ZFN-2 and ZFN-17 showed 15.6 and 27.7% increased proline content in relation to that of WT, respectively (Figure 6C).

To interpret the underlying transcriptional mechanism, a qRT-PCR was performed to evaluate gene expression differences between ZjZFN1-overexpressing and WT plants. Six salinity responsive genes involved in antioxidation (AtSOD, AtPOD, and AtAPX), ion transport (AtNHX1), and osmotic regulation pathways were examined.
FIGURE 4 | β-Glucuronidase (GUS) staining in transgenic Arabidopsis seedlings. (A) 35S::GUS (control), (B) 2-week-old ZjZFN1::GUS-overexpressing Arabidopsis seedlings, (C,D) Mature ZjZFN1::GUS-overexpressing Arabidopsis seedlings.

FIGURE 5 | Germination assay of ZjZFN1-overexpressing lines in 150 mM NaCl. Growth of the transgenic and WT plants in the presence of 150 mM NaCl for 4 days (A) and 8 days (B), respectively. Rates of emerged radicles of control and the transgenic plants in MS medium (C) and MS medium containing 150 mM NaCl (D).
(AtP5CS and AtLEA) were selected to monitor transcriptional variation. No obvious difference in gene expression was found between WT and transgenic plants under normal growth conditions. However, under salinity stress, the transcription of all genes was up-regulated, particularly in ZjZFN1-overexpressing plants (Figures 6D–I).

### Global Expression Analysis of Abiotic Stress-Related Genes in ZjZFN1-Overexpressing Plants

To provide a comprehensive landscape of the transcriptional regulation network of ZjZFN1 and gain insight into the performance of ZjZFN1-overexpressing plants, we carried out an RNA-seq assessment to screen DEGs between WT and transgenic plants (ZFN-17). Among the 289 DEGs identified, 274 were up-regulated and 15 were down-regulated in ZFN-17 (Figure 7A and Supplementary Table S1). The DEGs were classified into 51 groups based on their allocated GO terms (Supplementary Figure S1). Further classification of the DEGs within the “biological process” group revealed a large number of abiotic stress responsive DEGs including “response to stress,” “response to abiotic stimulus,” and “response to salt stress” (Figure 7B). All the 21 DEGs classified within the “response to salt stress” term were up-regulated, and these included genes SOS2, WRK33, MYB15, and peroxidase (isoforms 22 and 23), among other genes (Table 2).

All DEGs were analyzed regarding the pathways underlying the regulation of ZjZFN1. The 36 enriched KEGG pathways included 33 metabolic pathways (Supplementary Figure S1). Among these, the “phenylalanine metabolism” and “phenylpropanoid biosynthesis” pathways, both containing 10 annotated DEGs, were the most abundant pathways. Statistics of pathway enrichment revealed that “phenylalanine metabolism,” “alpha-linolenic acid metabolism,” “phenylpropanoid biosynthesis,” and “indole alkaloid biosynthesis” pathways were significantly enriched (Corrected P < 0.05) (Figure 7C).

### DISCUSSION

Genetic studies on halophytic species lag far behind those in other species and their potential remain unexplored (Orsini et al., 2010). Zinc finger proteins, a large gene family
in plants, plays a key role in environmental adaption. Here, we isolated a novel zinc finger protein, ZjZFN1, from *Z. japonica* aiming to better understand its function and underlying transcriptional regulation mechanism. Homology analysis showed that ZjZFN1 belonged to the C2H2 zinc finger protein family and contained two typical zinc finger structures, suggesting its potential for high DNA binding affinity (Kielbowicz-Matuk, 2012). The localization of ZjZFN1 to the nucleus was consistent with that of ZAT6 (Shi et al., 2014) and ZAT18 (Yin et al., 2017) in Arabidopsis, OsMSR15 in *Oryza sativa* (Zhang X. et al., 2016), and VvZFP11 in *Vitis vinifera* (Yu et al., 2016). The yeast assay provided evidence that ZjZFN1 has transcriptional activation ability, which was consistent with the results obtained for VvZFP11 and OsMSR15. As extensively reported, the nuclear localization attribute together with transcriptional activation ability suggest that ZjZFN1 might function as a typical plant TF.

Although ZjZFN1 was widely expressed in different tissues of *Z. japonica*, the highest expression levels were found in leaves. Additionally, ZjZFN1 transcript abundance was highest in mature leaves. In agreement, GUS staining results corroborated those of the qRT-PCR, suggesting that ZjZFN1 might be related with leaf development. The motifs exhibited in the ZjZFN1 promoter suggested that its expression might be regulated by different abiotic and hormone factors. In the present study, the transcription of ZjZFN1 was induced by cold and salt treatments but not by drought stress, which is consistent with that found for ZAT6, ZAT10, and ZAT18 (Yin et al., 2017), except...
for drought stress. Hormone induction experiments revealed that ZjZFN1 expression changed in response to SA, which is consistent with the results obtained for AtZAT6 (Shi et al., 2014) and VvZFP11 (Yu et al., 2016). The expression level of ZjZFN1 was also up-regulated by exogenous ABA, in agreement with the attributes of GmZFP3 (Zhang D.Y. et al., 2016), StZFP1 (Tian et al., 2010), and TaDi19A (Li et al., 2010). These results indicate that ZjZFN1 might play several roles in plants’ response to environmental stresses and might be an important factor for signal transduction.

C$_2$H$_2$ zinc finger proteins have key roles in the response to salinity stress in different plant species. In the present study, the germination and growth of ZjZFN1-overexpressing lines was obviously improved under salinity stress compared with that of WT plants. MDA is regarded as a typical indicator of salinity stress (Shi et al., 2013b). Additionally, increase in proline content is a primary metabolic defense for protecting plants from abiotic stresses (Zhang et al., 2011). Consistent with phenotype observations, transgenic lines showed lower MDA but higher proline content than WT plants under salt stress, supporting previous observations.

In plant responses to environmental stresses, transcriptional regulation plays a dominant role (Teng et al., 2017). Under salt stress, plants employ reactive oxygen species (ROS) scavenging enzymes, such as SOD, POD, and APX, to eliminate ROS accumulation. The improved transcript abundance of SOD, POD, and APX reflected an enhanced ROS-scavenging ability in ZjZFN1-overexpressing lines, which was consistent with that reported for ZFP179 (Sun et al., 2010). As an important ion transporter, the overexpression of NHX1 improves plant resistance to salinity stress (Shi et al., 2003). P5CS activates proline biosynthesis to protect protein integrity and enhance the activities of different enzymes under osmotic stress (Szabados and Savouré, 2010). LEA contributes to plant responses to abiotic stresses (Olvera-Carrillo et al., 2010). In the present study, the overexpression of ZjZFN1 induced the transcription of all selected genes under salinity stress in relation to shown by control (WT) plants, suggesting that ZjZFN1 may regulate salt tolerance via multiple pathways, in agreement with phenotype observations.

| AGI       | Log$_2$ FC | P-value | Annotation                                      |
|----------|------------|---------|------------------------------------------------|
| AT1G03220| 1.50151849 | 0.01388 | Aspartyl protease-like protein                   |
| AT2G38980| 2.589680215| 0.000644| Peroxidase 2                                    |
| AT3G08720| 1.439202055| 0.010174| Serine/threonine protein kinase 2                |
| AT2G38470| 1.488299735| 0.002867| Putative WRKY transcription factor 33           |
| AT5G26340| 1.37007294 | 0.022802| Sugar transport protein 13                      |
| AT3G48380| 1.404194641| 2.43E−05| TAC1-mediated telomerase activation pathway protein BT2 |
| AT3G57530| 1.120060074| 0.005128| Putative lipid transfer protein                  |
| AT4G12480| 2.158267439| 5.76E−05| Putative lipid transfer protein                  |
| AT3G23250| 1.963947163| 0.001117| MYB domain protein 15                           |
| AT5G49480| 1.462536757| 0.028422| Ca$^{2+}$-binding protein 1                      |
| AT4G11650| 2.097296486| 0.029137| Osmotin-like protein OSM34                       |
| AT2G33380| 1.933768292| 0.015409| Calmodulin binding protein 2                     |
| AT3G61890| 1.553503187| 0.022474| Homeobox-leucine zipper protein ATHB-12          |
| AT2G38980| 3.806701278| 0.004963| Peroxidase 23                                   |
| AT4G19810| 1.750323814| 0.011371| Class V chitinase                               |
| AT2G41010| 1.122992269| 0.030247| Cystine lyase protein CORI3                     |
| AT2G15390| 1.32321753 | 0.019627| Probable fucosyltransferase 4                   |
| AT4G34710| 1.141893898| 0.03177 | Arginine dehydrogenase 2                        |
| AT4G26300| 1.188045432| 0.021944| Cystine lyase protein CORI3                     |
| AT3G25780| 1.51296793 | 0.034314| Allene oxide cyclase 3                          |
| AT5G59820| 1.459308538| 0.014216| Zinc finger protein ZAT12                       |

For the gene expression data, we used the Student’s t-test to determine whether the expression of the genes was significantly different between the control (WT) and overexpression lines. The results showed that 237 genes were significantly differentially expressed (FDR < 0.05) between the two groups, with 21 genes up-regulated and 15 genes down-regulated. The gene expression data were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE123456.
ZjZFN1-overexpressing plants, indicating that ZjZFN1 could modulate stress responsive pathways, which in turn contribute to the improved adaptive capacity of transgenic plants.

**CONCLUSION**

In summary, we isolated ZjZFN1 and interpreted its functions in salinity stress responses. The overexpression of ZjZFN1 improved stress responses in seed germination and enhanced salt tolerance of transgenic lines. The ZjZFN1 transgene decreased leaf MDA content and improved proline content, and improved transcriptional activities of several salt-stress-related genes under salinity stress conditions. The RNA-seq results indicated that ZjZFN1 could function as a transcriptional activator, actively participating in the improvement of stress responsive genes’ expression and regulating stress-responsive pathways, including phenylalanine metabolism, ω-linolenic acid metabolism, and phenylpropanoid biosynthesis pathways. Overall, our results suggest that ZjZFN1 might be a valuable gene in *Z. japonica* breeding projects.

**AUTHOR CONTRIBUTIONS**

KT and JW conceived the study and designed the experiments. KT and PT performed the experiments. KT and WG analyzed the data with suggestions by YY, XF, and JW. KT wrote the manuscript.

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**FUNDING**

This research was supported by the China Postdoctoral Science Foundation (2017M620677), Beijing Postdoctoral Research Foundation (No. 2017ZZ-087), Postdoctoral Fund of the Beijing Academy of Agriculture and Forestry Sciences, Scientific Funds of Beijing Academy of Agriculture and Forestry Sciences (KJCX20161502-1 and KJCX20180101), and Beijing Technology Plan Project (No. 171100007217001).

**ACKNOWLEDGMENTS**

We are very grateful to Dr. Ning Jia (Institute of Food Science and Technology, Chinese Academy of Agricultural Science) for providing the pGBK-T7 vector and Dr. Chuanjing An (School of Pharmaceutical Sciences, Peking University) for technical assistance with the microscopy.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01159/full#supplementary-material

**FIGURE S1 |** Gene ontology and KEGG annotation of the identified DEGs. (A) GO classification. (B) KEGG annotation.

**TABLE S1 |** List of differently expressed genes between ZjZFN1-overexpressing line (ZFN-17) and WT screened by RNA-sequencing.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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