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RESEARCH PAPER

Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice

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Abstract

The Cys2/His2-type zinc finger proteins have been implicated in different cellular processes involved in plant development and stress responses. Through microarray analysis, a salt-responsive zinc finger protein gene *ZFP179* was identified and subsequently cloned from rice seedlings. *ZFP179* encodes a 17.95 kDa protein with two C2H2-type zinc finger motifs having transcriptional activation activity. The real-time RT-PCR analysis showed that *ZFP179* was highly expressed in immature spikes, and markedly induced in the seedlings by NaCl, PEG 6000, and ABA treatments. Overexpression of *ZFP179* in rice increased salt tolerance and the transgenic seedlings showed hypersensitivity to exogenous ABA. The increased levels of free proline and soluble sugars were observed in transgenic plants compared to wild-type plants under salt stress. The *ZFP179* transgenic rice exhibited significantly increased tolerance to oxidative stress, the reactive oxygen species (ROS)-scavenging ability, and expression levels of a number of stress-related genes, including *OsDREB2A*, *OsP5CS* *OsProT*, and *OsLea3* under salt stress. Our studies suggest that ZFP179 plays a crucial role in the plant response to salt stress, and is useful in developing transgenic crops with enhanced tolerance to salt stress.

Key words: Rice, salt stress, transcription factor, zinc finger protein.

Introduction

High salt is one kind of environmental stress affecting plant growth and crop productivity in some areas. Plants can initiate a number of molecular, cellular, and physiological changes to respond and adapt to salt stress. During these responses and adaptations, many salt stress-related genes are induced (Moons *et al.*, 1997; van der Krol *et al.*, 1999; Garg *et al.*, 2002; Sakamoto *et al.*, 2004). Among them, transcription factors (TFs) play critical roles in plant responses to salt stress via transcriptional regulation of the downstream genes responsible for plant tolerance to salt challenges. The Cys2/His2-type zinc finger proteins constitute one of the largest transcription factor families in eukaryotes (Kubo *et al.*, 1998). A number of stress-responsive C2H2-type zinc finger proteins have been identified in various plant species. Several studies have reported that overexpression of some C2H2-type zinc finger protein genes resulted in both the activation of some stress-related genes and enhanced tolerance to salt, dehydration, and/or cold stresses (Sakamoto *et al.*, 2000, 2004; Kim *et al.*, 2001; Sugano *et al.*, 2003). However, the roles of the C2H2-type zinc finger proteins in plant stress responses are still not well known.

Rice is one of the most important crops in world, and it is also the model for molecular biological research in monocots (Cantrell and Reeves, 2002). Several genes for this type of zinc finger protein have previously been identified in rice (Huang *et al.*, 2005*a*, 2007; Xu *et al.*, 2008). ZFP245, the first C2H2-type zinc finger protein identified in rice was

Abbreviations: ABA, abscisic acid; DREB, dehydration responsive element binding protein; ABRE, abscisic acid responsive element; CBF, C-repeat binding factor; P5CS, pyrroline-5-carboxylate synthetase; ROS, reactive oxygen species; POD, peroxidase; SOD, superoxide dismutase.

induced by cold and drought stresses (Huang *et al.*, 2005*a*). A salt inducible zinc finger gene *ZFP182* could improve salt tolerance in transgenic tobacco and rice plants (Huang *et al.*, 2007). Overexpression of *ZFP252*, a salt- and drought-inducible zinc finger protein gene conferred salt and drought tolerance (Xu *et al.*, 2008). In this study, the isolation and functional characterization of a novel salt-responsive zinc finger protein gene, *ZFP179* from rice, is reported here. In addition to salt stress, *ZFP179* was induced by PEG 6000, H_2O_2, and ABA treatments. Transgenic rice plants overexpressing *ZFP179* showed enhanced salt tolerance. Furthermore, it was suggested that ZFP179-mediated salt tolerance was involved in the ABA-dependent and -independent signalling pathways and the scavenging of reactive oxygen species in plants.

Materials and methods

Plant materials and stress treatments

Rice seeds (*Oryza sativa* L. subsp. *japonica* cv. Jiucaiqing) were sterilized with 0.1% $HgCl_2$, germinated, and grown in the incubator according to Zhou *et al.* (2006). The seedlings at the 3-leaf stage were transferred from the basal nutrient solution to nutrient solution containing 200 mM NaCl, 20% PEG 6000 (providing an osmotic potential of –0.54 MPa), 0.1 mM H_2O_2 or 0.1 mM ABA for abiotic stresses. The seedlings were sampled at 0, 1, 3, 6, 12, 24, and 48 h after each treatment and immediately stored at –80 °C. The roots, leaves, culms, immature spikes, and flowering spikes at the adult stage were also harvested.

RNA isolation and first strand DNA synthesis

Total RNA from stress-treated and untreated rice seedlings were extracted using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA was sequentially treated with DNaseI(Promega, USA) at 37 °C for 15 min in order to remove the remaining genomic DNA. The first strand cDNA was synthesized with 2 μg of purified total RNA using the RT-PCR system (Promega, USA) according to the manufacturer's protocol.

Cloning of ZFP179

By microarray analysis (our unpublished data), it was discovered that an EST probe (probe ID: Os.10411.1.S1_at) encoding a putative zinc finger protein was induced approximately 4.5-fold by salt stress. This EST sequence was used to search the rice genome database (http://www.ncbi.nlm.nih.gov/) and the genomic sequence containing such an EST was downloaded for gene prediction. Then *ZFP179* gene was amplified from total RNA prepared from rice seedlings by RT-PCR using the primers as follows: 5′-AGAGAAGAAGCGGAGAGCAA-3′ and 5′-TACAGACGCCAATTCAAT TC-3′.

Semi-quantitative RT-PCR

The PCR conditions are as follows: 0.5 μl RT product was amplified in a 25 μl volume containing 2.5 μl of 10× PCR buffer with $MgCl_2$, 0.5 μl of 10 mM dNTPs, 1 μl of DMSO, and 1 μl *Taq* polymerase (Tiangen, Beijing). The constitutively expressed rice gene, *OsRAC1*, was amplified as the internal control (Huang *et al.*, 2005*b*). The primers for *OsRAC1* are as follows: sense: 5′-GGAACTGGTATGGTCAAGGC-3′; anti-sense: 5′-AGTCTCATGGATAACCGCAG-3′.

Quantitative real-time RT-PCR

Quantitative real-time PCR was performed as previously described by Huang *et al.* (2008). The *ZFP179* primers (5′-ATGTGAATTGAATTGGCGTCT-3′ and 5′-ATCATCACGCTCCAAAATTTA-3′) were used for quantitative PCR analysis. The *18S-rRNA* primers (5′-ATGGTGGTGACGGGTGAC-3′ and 5′-CAGACACTAAAGCGCCCGGTA-3′) were used for the normalization of the quantitative PCR analysis. For quantitative RT-PCR of stress-related genes in transgenic plants, the expression of four stress-related genes was analysed, including *OsDREB2A* (Dai *et al.*, 2007), *OsLea3* (Moons *et al.*, 1997), *OsP5CS* (Igarashi *et al.*, 1997), and *OsProT* (Igarashi *et al.*, 2000). Relative quantification relates the PCR signal of the target transcript in the transgenic plants to that of the wild-type (WT) plants.

Assay of GUS expression

Based on the rice genome information deposited in GenBank (http://www.ncbi.nlm.nih.gov), the putative promoter sequence of *ZFP179* was determined. Then a fragment of approximate 1.5 kb upstream of the translation start site of *ZFP179* was amplified from rice genomic DNA and cloned into pCAMBIA1301 at the sites of *Hin*dIII and *Bgl*II. The construct was subsequently transformed into rice callus via *Agrobacterium*-mediated transformation. For stress treatments, rice callus were kept in 150 mM NaCl solution or 20% PEG 6000. The rice callus kept in double distilled water (ddH_2O) served as a control. After 12 h of treatment, rice callus was rapidly washed with ddH_2O. Histochemical analysis of GUS expression was performed by incubating the rice callus in X-gluc buffer at 37 °C overnight (Couteaudier *et al.*, 1993).

Transcriptional activation analysis in yeast cells

The transcriptional activation activity of ZFP179 was determined as previously described (Zheng *et al.*, 2009). DNA fragments containing the whole ORF of *ZFP179* was inserted into the *Eco*RI/*Sma*I sites of the pGBKT7 vector to create the pGBKT7-ZFP179 construct. According to the protocol of the manufacturer (Stratagene, USA), pGBKT7-ZFP179, the positive control pGBKT7-53+pGADT7-T, and the negative control pGBKT7 plasmids were used to transform the AH109 yeast strain. The transformed strains were streaked onto SD/–Trp or SD/–Trp–His–Ade plates, and the *trans*-activation activity of each protein was evaluated according to their growth status and the activity of β-galactosidase.

Generation of transgenic rice plants

The full-length ORF of *ZFP179* was inserted into the plant binary vector pCAMBIA1301 to construct pCAMBIA1301-*ZFP179*. Then the *ZFP179* gene under the control of the CaMV 35S promoter was transformed into rice (*Oryza sativa* L. subsp. *japonica* cv. Zhonghua11) by the *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994).

Analysis of transgenic plants for salt and oxidative stress tolerance

To evaluate the performance of the transgenic rice plants under salt stress, the seeds of the T_3 transgenic lines and the WT were germinated and grown in half-strength Murashige and Skoog (1/2MS) medium plus 0 mM, 150 mM or 250 mM NaCl for 2 weeks under a long-day photoperiod (16/8 h light/dark) (Huang *et al.*, 2007). The shoot length and fresh weight of each *ZFP179* transgenic plant or wild-type plant were measured. For survival experiments, the seeds of the T_3 transgenic lines and the WT were germinated and grown in basal nutrient solution. 20-d-old rice plants were treated by 100 mM or 150 mM NaCl solution for 6 d and allowed to recover for 7 d. Seedlings which did not grow were considered as dead (Xiong and Yang, 2003). For oxidative stress, the seeds of transgenic and WT were geminated and grown on 1/2MS medium supplied with 0 mM, 30 mM or 50 mM H_2O_2 for 2 weeks under a long-day photoperiod. The shoot length of each *ZFP179* transgenic plant and wild-type plant was measured.

Measurement of proline and soluble sugar contents

The seedlings at the four-leaf stage were used for biochemical analysis. WT and *ZFP179*-ox plants were transferred from the basal nutrient solution to nutrient solution containing 100 mM NaCl. After 6 d of salt treatment, the content of proline and soluble sugars in WT and transgenic lines without or with salt treatment were determined by the sulphosalicylic acid method (Troll and Lindsley, 1955) and the anthrone method (Morris, 1948).

ABA sensitivity test of transgenic plants

The T_3 generation of *ZFP179* transgenic plants were used in the ABA sensitivity test. For the ABA sensitivity test of transgenic rice at the seedling stage, the seeds were germinated on 1/2MS medium plates. Geminated rice plantlets at the same stage as the transgenic rice and WT control were transformed to 1/2MS medium with different concentrations of ABA (0 μM, 5 μM. and 10 μM ABA). Twelve days after growth in the greenhouse with the temperature at 26 °C, root length was measured (Lu *et al.*, 2009).

Measurements of POD and SOD activities

One gram of frozen leaves of the T_3 transgenic lines or the wild type was homogenized in 1 ml of ice-cold solution containing 50 mM phosphate buffer (pH 7.8), 20% glycerol, and 1% PVP. The homogenates were then centrifuged at 10 000 *g* for 30 min. The aliquots of supernatants were used for the analysis of POD and SOD activities, as previously described by Yang *et al.* (2009).

In vivo detection of H_2O_2 deposition

In vivo H_2O_2 deposition was detected in the control and salt-treated leaves. *In vivo* infiltration of 3, 3-diaminobenzidine (DAB) was performed to detect H_2O_2 accumulation sites in the leaf tissues caused by salt. The protocol was followed according to Orozco-Cardenas and Ryan (1999).

Results

Cloning and sequence analysis of ZFP179

The *ZFP179* gene containing a complete ORF of 516 bp was cloned by RT-PCR from total RNA prepared from rice seedlings. The predicted protein product of *ZFP179* comprises 171 amino acids with the calculated molecular mass of 17.95 kDa. The ZFP179 protein contains two C2H2-type zinc fingers, with a plant-specific QALGGH motif in each zinc finger domain. A homology search against the GenBank database showed that ZFP179 was homologous to many plant C2H2-type zinc finger proteins, especially in finger domains (Fig. 1A). Like most reported C2H2-type zinc finger proteins, ZFP179 contains a DLN-box/EAR-motif with a consensus of DLN at the C-terminus, but it lacks a B-box functioning as a putative nuclear localization signal (NLS) (Fig. 1A).

To investigate the evolutionary relationship among plant C2H2-type zinc finger proteins involved in stress responses, a phylogenetic tree was constructed using Neighbor–Joining method with the full-length amino acid sequences (Fig. 1B). The result revealed that ZFP179 was clustered with ZFP182, ZFP150, and ZAT12, whereas other stress responsive zinc finger proteins were categorized into another big branch.

Expression analysis of ZFP179

The expression profiles of *ZFP179* were investigated in various rice tissues at the adult stage by real-time RT-PCR. The *ZFP179* transcripts were detected in all organs tested, and the highest level was found in immature spikes and the lowest in leaves (Fig. 2A).

In addition, real-time RT-PCR was performed to examine the expression pattern of *ZFP179* in rice seedlings under different stress conditions. Under salt stress, the transcripts of ZFP*179* began to increase 3 h after NaCl treatment and gradually accumulated up to 24 h (Fig. 2B). For PEG 6000 stress, it was observed that *ZFP179* was up-regulated 1 h after treatment and was maintained constant up to 24 h (Fig. 2C). The expression of *ZFP179* was also induced by an exogenous 0.1 mM ABA treatment (Fig. 2D).

The promoter sequence of *ZFP179* was analysed through the MatInspector program (http://www.genomatix.de). The promoter sequence contains many putative stress-related *cis*-acting elements, such as ABRE, CRT/DRE, MYBRS (MYB recognition sites) and W-box (Fig. 2E). These stress-related *cis*-acting elements may be responsive to the stress-regulated expression of *ZFP179*. To examine whether the *ZFP179* promoter is actually responsive to abiotic stress, ~1.5 kb promoter region of *ZFP179* was cloned to the upstream region of the β-glucuronidase (*GUS*) gene and then introduced into rice callus. Histochemical analysis showed strong GUS expression in rice callus under 150 mM NaCl and 20% PEG 6000, whereas no GUS expression was observed under H_2O treatment (Fig. 2F).

ZFP179 functions as a transcriptional activator in yeast

The transcriptional activity of ZFP179 was examined using a yeast hybrid system. A GAL4 DNA binding domain-ZFP179 fusion protein was expressed in yeast cells, which were assayed for their ability to activate transcription from the GAL4 sequence. ZFP179 promoted yeast growth in the absence of histidine and adenine, and showed β-galactosidase activity, while the vector control pGBKT7 did not (Fig. 3). The data confirmed that ZFP179 functions as a transcriptional activator in yeast.

Overexpression of ZFP179 increases salt tolerance in rice

To study the physiological function of ZFP179, transgenic rice plants that overexpressed *ZFP179* (*ZFP179*-ox plants) were generated. The positive transgenic plants were confirmed by genomic DNA PCR using hygromycin specific primers (data not shown) and RT-PCR using *ZFP179* specific primers. With *OsRAC1* as the reference gene, the T_2 generation of the transgenic lines showed higher expression levels of *ZFP179* than the WT control (Fig. 4B).

The effect of salt on the seedling development of the *ZFP179* overexpression (*ZFP179*-ox) lines was investigated. Homozygous transgenic lines, S1, S2, S5, and S6 of the T_3 generation were used to measure their performance under salt stress. The wild-type and *ZFP179*-ox transgenic rice seeds were germinated and grown on media containing 0,

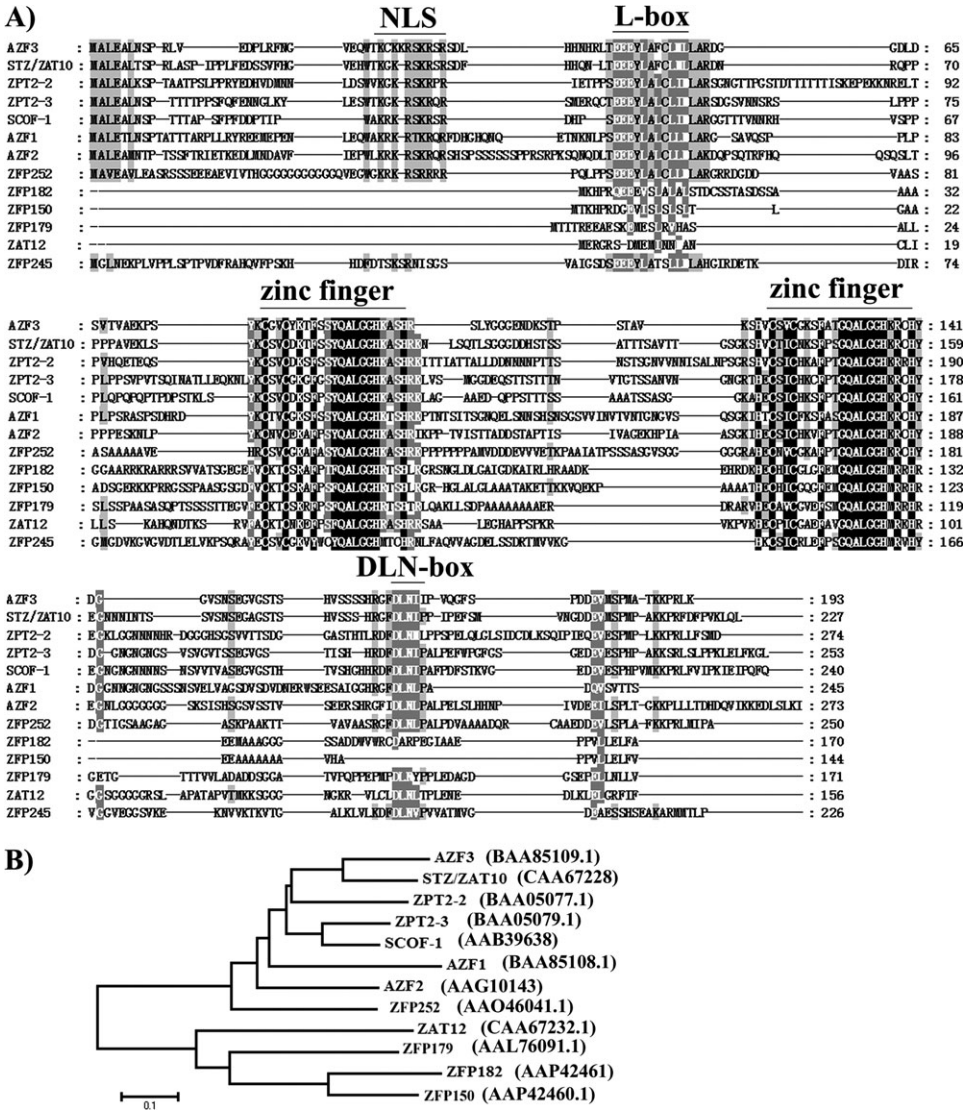

Fig. 1. Multiple sequence alignment and phylogenetic tree analysis of amino acid sequences of ZFP179 with the other stress-responsive C2H2-type zinc finger proteins. (A) Multiple sequence alignment of amino acid sequences of ZFP179 with the other stress-responsive C2H2-type zinc finger proteins. Black boxes indicate the positions at which the residues are identical and grey boxes highlight the residues that are similar. AZF3 is included to show the positions in the domains of NLS, L-box, zinc finger, and DLN-box. (B) The phylogenetic tree of plant stress-responsive C2H2-type zinc finger proteins. The Neighbor–Joining tree was constructed with MEGA (version 4.1). Branch numbers represent a percentage of the bootstrap values in 1000 sampling replicates and the scale bar indicates branch lengths. The references for the amino acids sequence are *Arabidopsis* STZ/ZAT10, AZFs, ZAT12 (Lippuner *et al.*, 1996; Sakamoto *et al.*, 2000, 2004); soybean SCOF-1 (Kim *et al.*, 2001), petunia ZPTs (van der Krol *et al.*, 1999; Sugano *et al.*, 2003), rice ZFP245 (Huang *et al.*, 2005*a*), ZFP150 (Huang *et al.*, 2005*b*), ZFP182 (Huang *et al.*, 2007), and ZFP252 (Xu *et al.*, 2008).

150, and 250 mM NaCl for 2 weeks. It was observed that transgenic rice plants grew better than wild-type plants (Fig. 4C), as reflected by comparisons of shoot length (Fig. 4D) and fresh weight (Fig. 4E). For the survival rates assay, 20-d-old transgenic and WT seedlings cultured in liquid medium were transferred to liquid medium with 100 mM or 150 mM NaCl for 6 d and then allowed to recover under normal conditions for 7 d. Plants that could not grow any more after the recovery were considered to be dead. After recovering for 7 d, more transgenic seedlings survived than WT plants which appeared to be mostly withered (Fig. 4F). The survival rates of transgenic lines were significant higher than those of WT plants (Fig. 4G). These results indicated that overexpression of *ZFP179* in rice seedlings enhanced plant tolerance to salinity.

Overexpression of ZFP179 increases proline and soluble sugar contents under salt stress

Plant adaptation to environmental stresses is often associated with metabolic adjustment, such as the accumulation of proline and soluble sugars (Abraham *et al.*, 2003). To investigate the physiological basis for the improved stress tolerance of transgenic rice, proline and soluble sugar levels

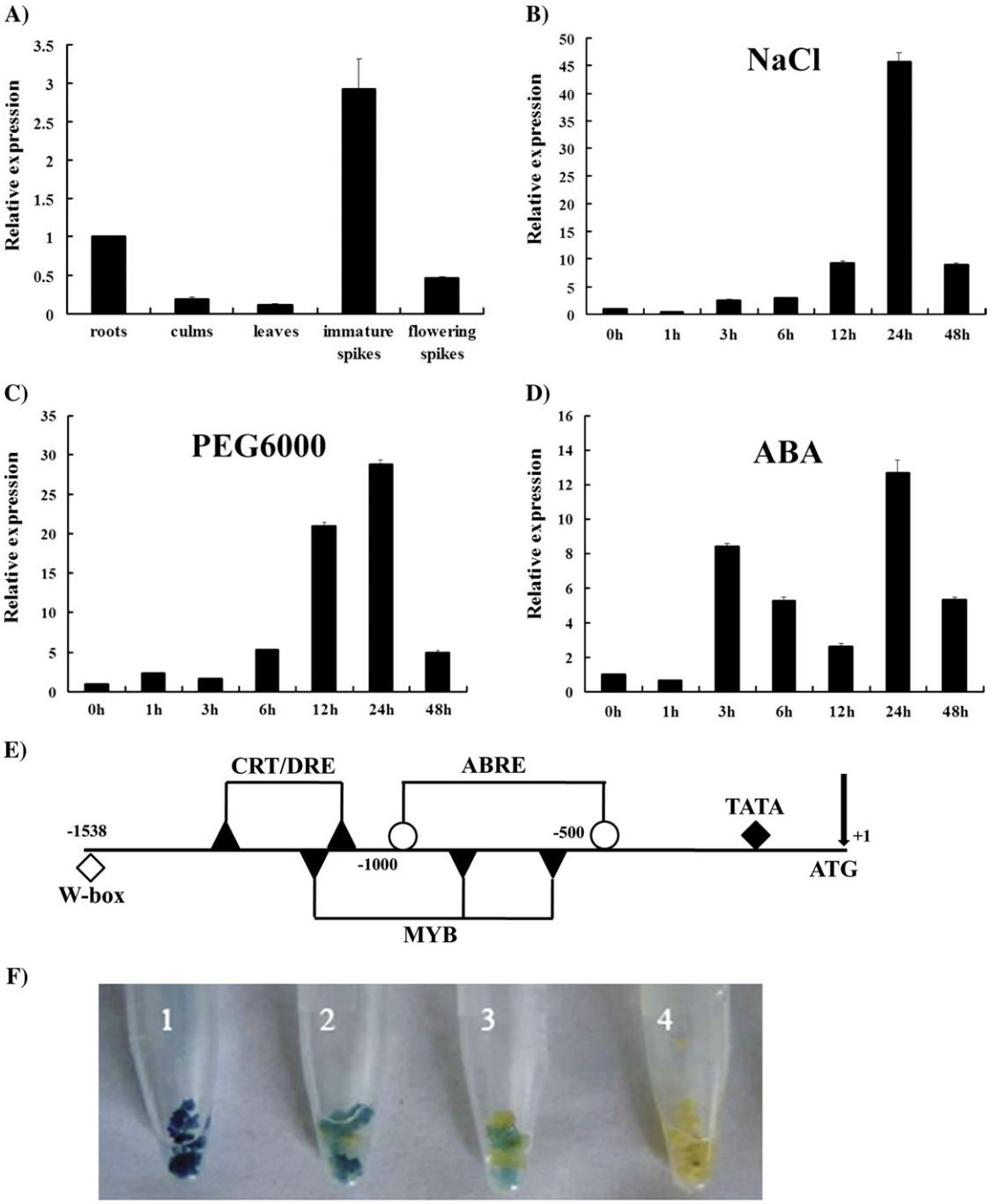

Fig. 2. Real-time PCR analysis for the expression patterns of *ZFP179* in rice. (A) The mRNA expression of *ZFP179* in various rice tissues. (B–D) Expression of *ZFP179* in the seedlings under salt stress (200 mM NaCl), osmotic stress (20% PEG 6000) and exposed to 0.1 mM ABA, respectively. *18S-rRNA* was used as an internal control. Data represent means and standard errors of three replicates. (E) Distribution of *cis*-elements in the promoter region of *ZFP179*. DNA sequences similar to the stress-related *cis*-elements are indicated as follows: open circles, ABRE; closed triangles, CRT/DRE; closed inverted triangles, MYB recognition site; open diamonds, W-box; closed diamonds, TATA. (F) *ZFP179* promoter is responsive to salt stress in rice callus. GUS expressed under the control of CaMV 35S promoter (1) and *ZFP179* promoter (2, 3, and 4) in rice callus. For salt and osmotic stresses, rice callus were put in 150 mM NaCl (2) and 20% PEG 6000 (3). The rice callus kept in water served as control (4).

were measured in the WT and *ZFP179*-ox plants under unstressed and salt-stressed conditions. After salt treatment for 6 d, *ZFP179*-ox plants accumulated an approximately 4-fold higher content of proline (Fig. 5A) and a 5-fold higher content of soluble sugars (Fig. 5B) than the plants did before salt stress, while the WT plants accumulated an approximately 3-fold higher content of proline and a 4-fold higher content of soluble sugars, respectively. The salt stress-induced increase of proline and soluble sugar content in the transgenic plants were significantly higher

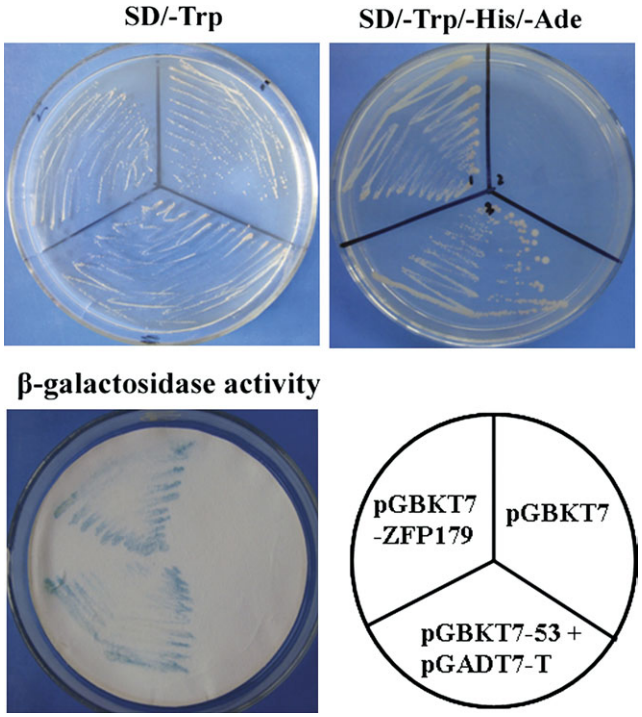

Fig. 3. Transcriptional activation of ZFP179 in yeast. A fusion protein of the GAL4 DNA binding domain and ZFP179 was expressed in yeast strain AH109. The vector pGBKT7 or pGBKT7-53 and pGADT7-T were expressed in yeast as a negative or a positive control. The culture solution of the transformed yeast was dropped onto SD plates with or without histidine and adenine. The plates were incubated for 3 d and subject to β-galactosidase assay.

than those in wide-type plants, indicating that ZFP179 may regulate the accumulation of free proline and soluble sugar levels in rice seedlings under salt stress.

Expression of stress-related genes in ZFP179 transgenic rice plants

To elucidate the possible role of ZFP179 in response to salt stress further, the expression of several stress-related genes in *ZFP179* transgenic lines and WT were analysed, including *OsDREB2A* encoding a DREB-type transcription factor, *OsP5CS* encoding pyrroline-5-carboxylate synthetase, *OsProT* encoding *a* proline transporter, and *OsLea3* encoding a group 3 late embryogenesis abundant protein. Expression levels of all the genes analysed were induced by salt treatment both in WT control and transgenic rice plants (Fig. 6). Under salt treatment, the expression levels of all four stress-related genes in *ZFP179*-ox plants were increased more than those in WT plants. It is suggested that ZFP179 might be one upstream regulator of these genes and play an important role in regulating the stress-responsive genes.

Overexpression of ZFP179 makes seedlings hypersensitive to ABA

The effect of ABA on seedling development of the *ZFP179* overexpression (*ZFP179*-ox) lines was investigated. There were no significant differences between WT and the transgenic rice plants when shoot length and root length were compared in medium without ABA. However, as shown in Fig. 7A, the root length of WT growing on medium with 5 μM ABA was reduced to 40.6% of the control growing on medium without ABA, while root lengths of transgenic rice lines were arrested to 25.19%, 22.83%, 23.70%, and 16.91%, respectively. When the ABA concentration in the medium was elevated to 10 μM, the root lengths of WT rice were reduced to 24.81% of the control, while the root lengths of transgenic plants were arrested to 15.26%, 10.23%, 5.92%, and 12.5% of the control (Fig. 7B). The severity of ABA-induced growth arrest of root length was more significant in the transgenic lines than in the WT control (*t* test, $P < 0.01$). The results indicated that ZFP179 increases the sensitivity of rice to ABA treatment. It was also found that the expression levels of *OsP5CS*, *OsProT*, and *OsLea3* in *ZFP179*-ox plants had increased more compared with WT plants (Fig. 7C–E),while there was no significant difference between WT and transgenic rice for the expression level of *OsDREB2A* under ABA treatment (Fig. 7F).

ZFP179 enhances the ROS scavenging ability and the tolerance to oxidative stress

H_2O_2 production was first visualized by a histochemical method with 3, 3-diaminobenzidine (DAB). After the 15-d-old rice seedlings of transgenic and WT plants were immersed in 150 mM NaCl for 2 d, the presence of H_2O_2 could be detected in all the experimental plants. Figure 8A showed that the generation of H_2O_2 was much less in *ZFP179*-ox than in WT plants. Abiotic stresses may increase the level of reactive oxygen species (ROS), and the accumulation of ROS is toxic to plant cells. To analyse whether the ROS scavenging enzymes contribute to the plant's tolerance to abiotic stress, the enzyme activity assays for peroxidase (POD) and superoxide dismutase (SOD) were conducted under unstressed and salt conditions. It was found that the POD activities of two transgenic lines were more increased compared with those in WT plants under salt stress (Fig. 8B). There was no significant difference of SOD activities between the two transgenic lines and WT plants under unstressed or salt conditions (Fig. 8C). Further, in the culture media containing 30 mM or 50 mM hydrogen peroxide (H_2O_2), *ZFP179*-ox plants showed more resistance to H_2O_2 than the WT plant (Fig. 8D). The relative shoot lengths of *ZFP179*-ox plants were significant higher than WT plants (Fig. 8E). This indicated that ZFP179 might mediate in the rice response to oxidative stress caused by H_2O_2. Furthermore, it was observed that the transcript level of *ZFP179* was induced after 1 h treatment of H_2O_2 which was maintained constant for up to 48 h (Fig. 8F).

Discussion

ZFP179 contains two typical C2H2 zinc finger domains and a DLN-box/EAR-motif located at its C-terminus. Several

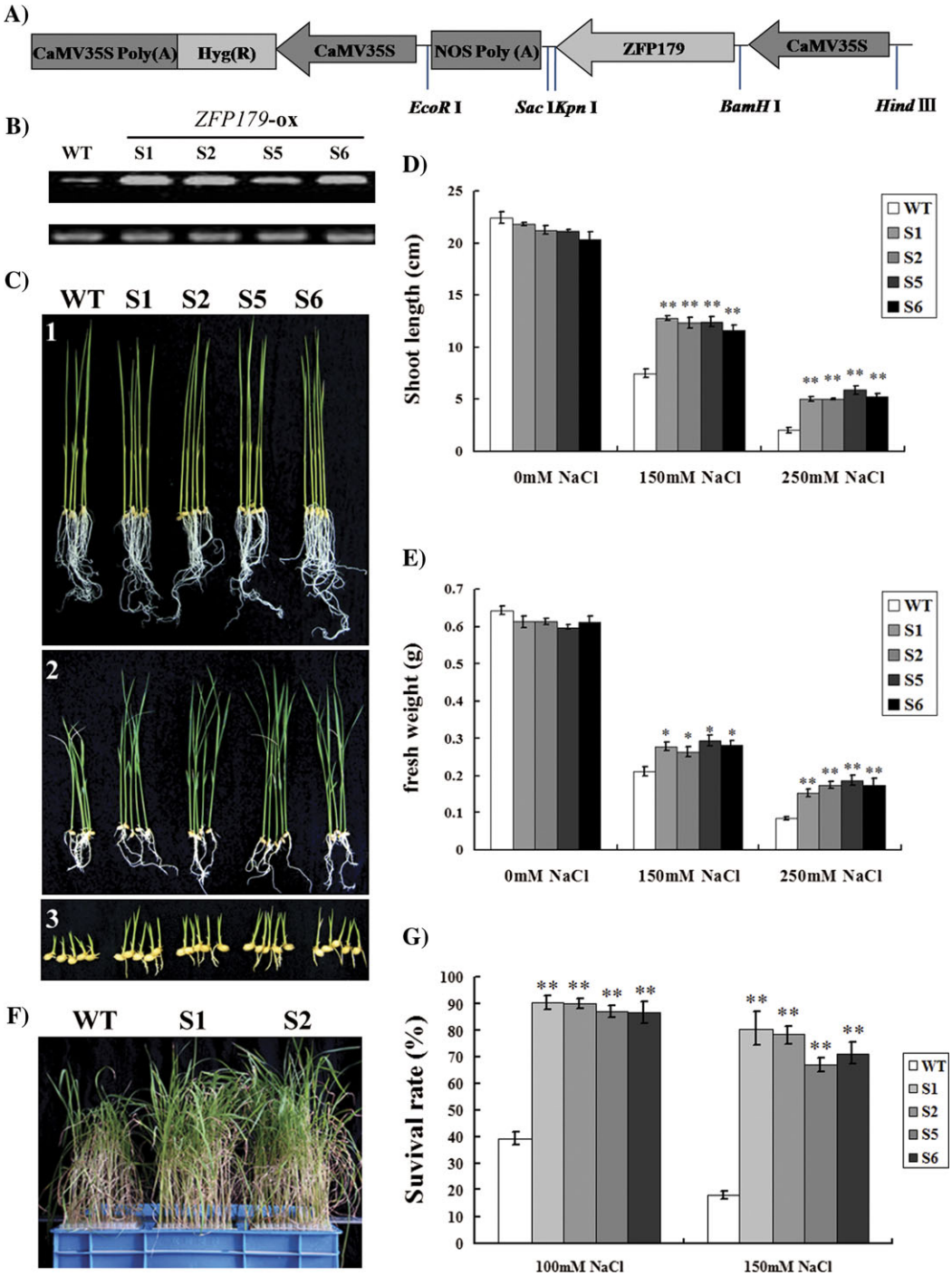

Fig. 4. Effect of *ZFP179* overexpression on salt tolerance in transgenic rice plants. (A) The construction of transgenic vector of *ZFP179*. (B) The mRNA expression analysis of *ZFP179* in the T_2 generation of transgenic rice plants by semi-quantitative RT-PCR. (C) The seedlings of WT and transgenic lines geminated and grown on 1/2MS medium supplemented with 0 mM, 150 mM, and 250 mM NaCl (1–3). (D, E) The shoot length and fresh weight were measured. (F) Performance of WT and transgenic lines S1 and S2 after 6 d of 150 mM NaCl treatment and 7 d recovery. (G) Survival rates of WT and four transgenic lines in recovery after 100 mM or 150 mM NaCl treatment. Error bars indicate three replicates.

zinc finger proteins containing a DLN-box/EAR-motif have exhibited transcription repressive activities by transient analysis in plants, such as petunia ZPT2-3 (Sugano *et al.*, 2003) and *Arabidopsis* STZ/ZAT10 (Sakamoto *et al.*, 2004). ZFP179 showed transcriptional activation activity in yeast cells although it contains a DLN-box/EAR-motif. Similarly, the zinc finger proteins ThZF1 from salt cress (Xu *et al.*, 2007) and CaZF from chickpea (Jain *et al.*, 2009), both containing the DLN-box/EAR-motif, exhibited their transcriptional activation activities. Altogether, it is suggested that some unknown domains may affect the transcriptional activity of C2H2-type zinc finger proteins.

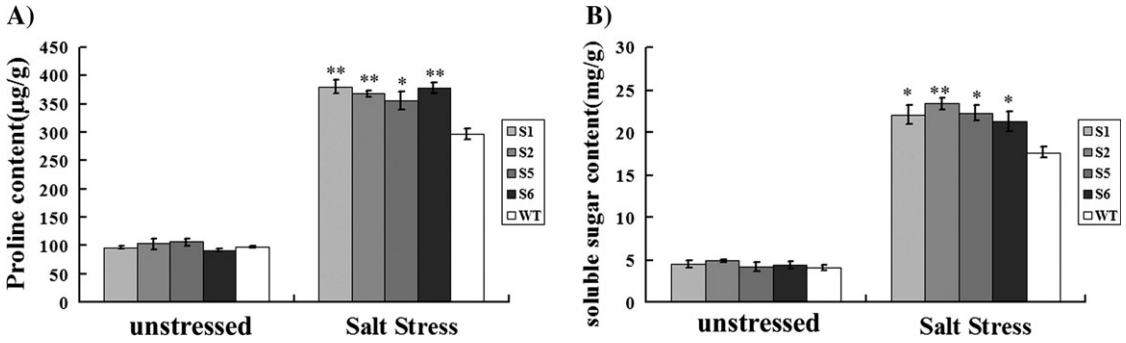

Fig. 5. The contents of free proline (A) and soluble sugars (B) in the *ZFP179*-ox and WT rice under 100 mM NaCl stress conditions.

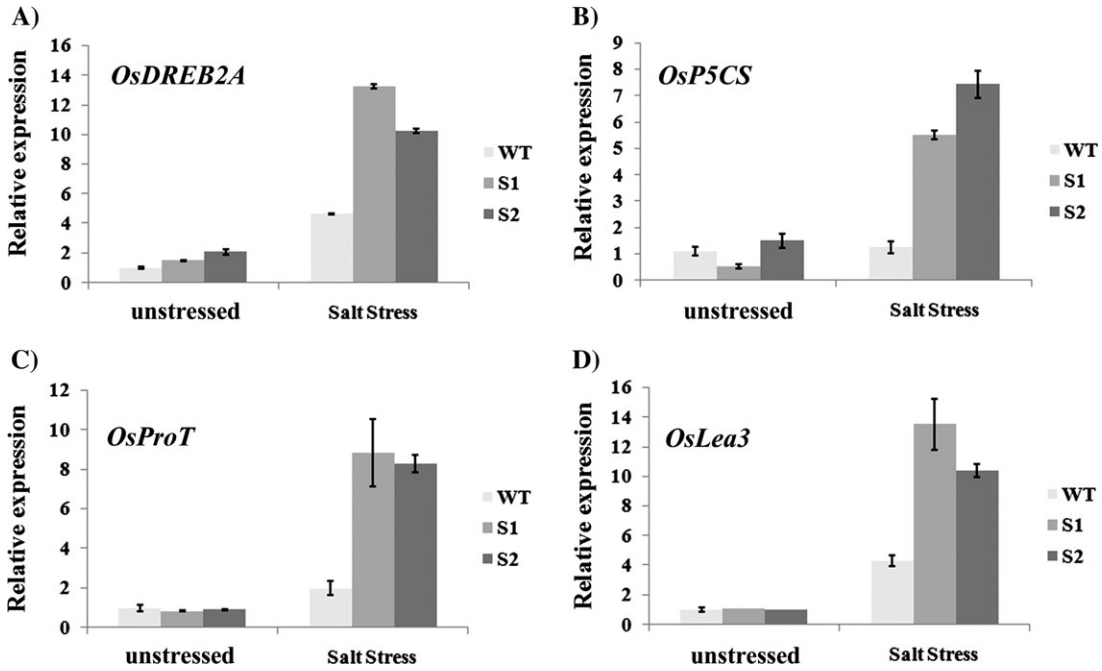

Fig. 6. Expression of stress-related genes in *ZFP179*-ox and WT rice. Total RNA was extracted from the rice seedlings at the four-leaf stage grown under control and salt treatments. (A–D) The transcript levels of *OsDREB2A*, *OsP5CS*, *OsProT*, and *OsLea3* were measured by quantitative real-time PCR under unstressed conditions or 150 mM NaCl for 24 h, respectively. *18S-rRNA* was used as an internal control. Data represent means and standard errors of three replicates.

Several stress-related *cis*-acting elements, including ABREs, MYBREs, W-boxes, and CRT/DRE were found in the 1.5 kb promoter region of *ZFP179* (Fig. 2G). These *cis*-acting elements and their corresponding transcription factors are important for ABA signalling or abiotic stress responses in plants (Abe *et al.*, 1997; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006; Nakashima *et al.*, 2007). These *cis*-acting elements may be responsive for the stress-regulated expression of *ZFP179*. In addition to responses to abiotic stress, it was found that *ZFP179* was also highly expressed in immature spikes, indicating that ZFP179 might also play a role in spike growth or development in rice.

In response to abiotic stress, many plants can accumulate compatible osmolytes, such as free proline and soluble sugars, to protect their subcellular structures from damage by adjusting the intracellular osmotic potential (Garg *et al.*, 2002; Armengaud *et al.*, 2004). To address the possible mechanism of the enhanced stress tolerance of the *ZFP179*-ox plants, the contents of proline and soluble sugars were ascertained in transgenic plants under normal growth and stress conditions. The data showed that *ZFP179*-ox plants accumulated more free proline and soluble sugars than WT under salt-stress conditions (Fig. 5). Quantitative real-time PCR results also showed that the proline synthetase gene (*OsP5CS*) and the proline transport gene (*OsProT*) had significantly higher expression levels in the *ZFP179*-ox lines than in the WT (Fig. 6B, C). However, overexpression of *ZFP179* could not enhance the expression of stress-related genes, including *OsP5CS*, *OsProT*, and *OsLea3* in transgenic rice plants under unstressed conditions. One possible explanation is that ZFP179 may mediate the activation of such stress-related genes accompanied by other stress-responsive regulators.

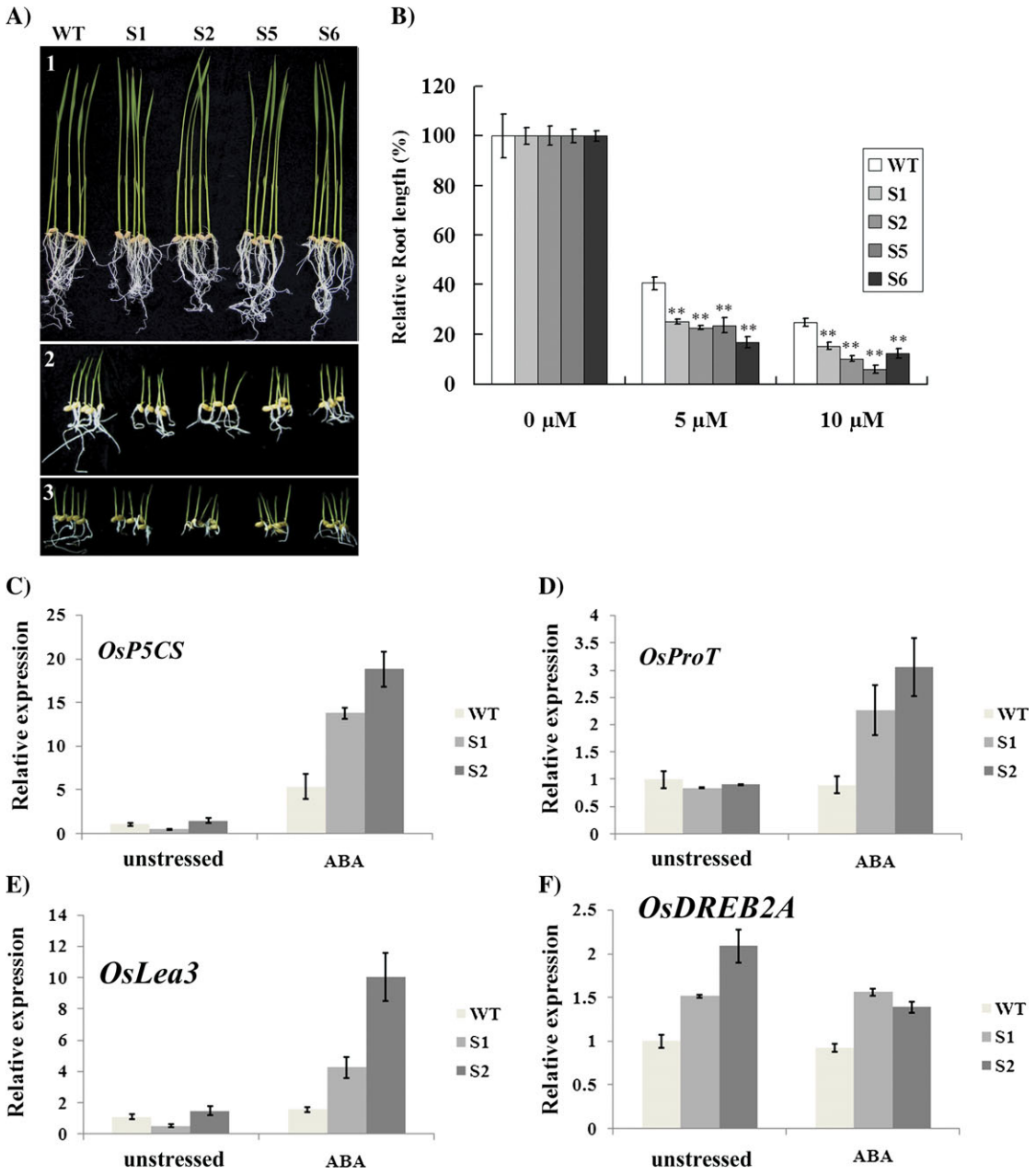

Fig. 7. Seedling development of *ZFP179*-ox transgenic rice plants under ABA treatment. (A) The WT control and transgenic lines were grown under 0 μM, 5 μM, and 10 μM ABA (1–3). (B) The relative root length of each WT and transgenic lines was measured and the error bars are based on three replicates. The values with significant difference according to the *t* test are indicated by asterisks (**, $P \leqslant 0.01$). (C–F) The transcript levels of *OsP5CS*, *OsProT OsLea3,* and *OsDREB2A* were measured by quantitative real-time PCR under unstressed conditions and 0.1 mM ABA for 24 h, respectively. *18S-rRNA* was used as an internal control. Data represent means and standard errors of three replicates.

ABA plays a critical role in the osmotic stress response in plants. Under osmotic stress, the plant always initiates the ABA signalling transduction pathway to activate the expression of stress defence genes (Chinnusamy *et al.*, 2004). Overexpression of some transcription factor genes, such as *OsABI5* and *OsZIP72* made the transgenic plants hypersensitive to exogenous ABA and resulted in abiotic stress tolerance (Zou *et al.*, 2008; Lu *et al.*, 2009). Our data showed that *ZFP179*-ox plants significantly increased plant sensitivity to exogenous ABA (Fig. 7), indicating that ZFP179 might play a role in the ABA signal transduction pathway during the stress responses. It was shown that the *OsP5CS*, *OsProT*, and *OsLea3* genes were all more highly expressed in *ZFP179*-ox plants compared with WT plants under ABA treatment, suggesting that the regulation of these stress defence genes by ZFP179 under salt stress might be ABA dependent. Further, it was found that *OsDREB2A* encoding a DREB protein in rice was responsive to *ZFP179* overexpression (Fig. 6A), suggesting that ZFP179 might be an upstream regulator of *OsDREB2A* under salt stress.

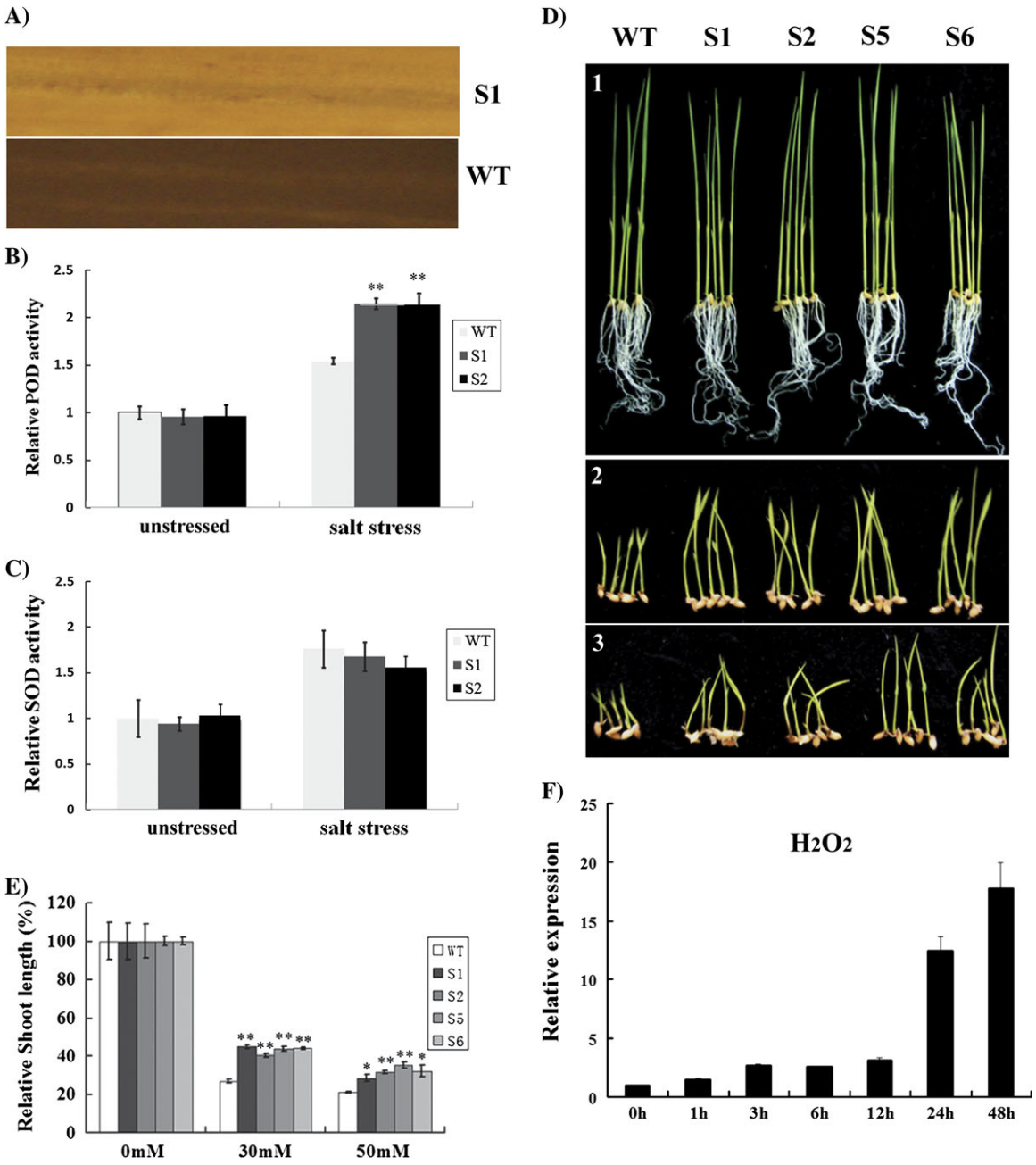

Fig. 8. ZFP179 decreases H_2O_2 production and enhances POD activity and oxidative stress tolerance. (A) *In vivo* detection of H_2O_2 accumulation in plants exposed to salt stress via the DAB method. (B, C) The POD and SOD activities of transgenic plants under salt and unstressed conditions. (D, E) The seedlings and corresponding relative shoot length of WT and *ZFP179*-ox lines were germinated and grown on 1/2MS plus 0 mM, 30 mM, and 50 mM H_2O_2 for 14 d, respectively. Error bars are based on three replicates. (F) Expression of *ZFP179* in the seedling under oxidative stress (H_2O_2).

Furthermore, it has been demonstrated that *OsDREB2A* was gradually induced within 24 h after salt treatment, but not induced by ABA (Dubouzet *et al.*, 2003). Under ABA treatment, it was found that *ZFP179*-ox plants did not promote a higher expression of *OsDREB2A* compared with WT plants (Fig. 7F). Thus, OsDREB2A-regulated expression of some stress defence genes may be controlled by ZFP179 in an ABA-independent manner. Consistently, the *Arabidopsis* DREB2A has been shown to have a role in the regulation of some stress defence genes in plant responsiveness to drought and salt stresses through an ABA-independent signal transduction pathway (Liu *et al.*, 1998). Altogether, it is suggested that ZFP179 may play important roles in response to salt stress both in ABA-dependent and -independent pathways.

Many stress conditions lead to the accumulation of reactive oxygen species (ROS) which are toxic to plant cells. ZFP179 may enhance ROS scavenging activity to reduce the accumulation of H_2O_2 induced by salt stress. In line with this idea, our data showed that the expression of

ZFP179 was also induced by H_2O_2 treatment and overexpression of *ZFP179* enhanced oxidation tolerance (Fig. 8), suggesting that ZFP179 might activate the antioxidant system in rice. Peroxidase (POD) is a very important ROS scavenging enzyme in plants. Our data showed that ZFP179 increased POD activity in rice seedlings under salt stress, however, SOD activity was not enhanced by ZFP179, indicating that ZFP179 might specifically regulate the activities of some ROS scavenging enzymes, such as POD. To understand further the interaction of ZFP179 and the ROS scavenging system, the activities of more ROS scavenging enzymes need to be measured.

In conclusion, when plants suffer from salt stress, ZFP179 might either enhance the expression of stress-defence genes, such as *OsP5CS*, *OsProT*, and *OsLEA3*, and the subsequent accumulation of free proline, soluble sugars, and Group 3 late embryogenesis abundant proteins through an ABA-dependent manner, or activate the expression of *OsDREB2A* through an ABA-independent pathway. Both types of response may increase the salt tolerance of plants. In addition, ZFP179 may enhance ROS-scavenging activity to remove the toxic ROS in plants induced by salt stress.

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