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A WRKY transcription factor, PcWRKY33, from *Polygonum cuspidatum* reduces salt tolerance in transgenic *Arabidopsis thaliana*

Wenqi Bao[1] · Xiaowei Wang[1] · Mo Chen[1] · Tuanyao Chai[1,2] · Hong Wang[1]

Abstract

Key message **PcWRKY33 is a transcription factor which can reduce salt tolerance by decreasing the expression of stress-related genes and increasing the cellular levels of reactive oxygen species (ROS).**

Abstract WRKY transcription factors play important roles in the regulation of biotic and abiotic stresses. Here, we report a group I WRKY gene from *Polygonum cuspidatum, PcWRKY33*, that encodes a nucleoprotein, which specifically binds to the W-box in the promoter of target genes to regulate their expression. The results from qPCR and promoter analysis show that expression of *PcWRKY33* can be induced by various abiotic stresses, including NaCl and plant hormones. Overexpression of *PcWRKY33* in *Arabidopsis thaliana* reduced tolerance to salt stress. More specifically, several physiological parameters (such as root length, seed germination rate, seedling survival rate, and chlorophyll concentration) of the transgenic lines were significantly lower than those of the wild type under salt stress. In addition, following exposure to salt stress, transgenic plants showed decreased expression of stress-related genes, a weakened ability to maintain Na^+/K^+ homeostasis, decreased activities of reactive oxygen species- (ROS-) scavenging enzymes, and increased accumulation of ROS. Taken together, these results suggest that *PcWRKY33* negatively regulates the salt tolerance in at least two ways: by down-regulating the induction of stress-related genes and by increasing the level of cellular ROS. In sum, our results indicate that *PcWRKY33* is a group I WRKY transcription factor involved in abiotic stress regulation.

Keywords *Polygonum cuspidatum* · *PcWRKY33* · Salt stress · Transcription factor · WRKY

Introduction

Saline water covers about 75% of the earth's surface, and approximately 3% of land is affected by salt under normal circumstances (FAO 2005). In addition, human activities, such as intensive agriculture and irrigation, are increasing

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the degree of salinization of arable soils. Hence, soil salinity has become a primary environmental risk for plant cultivation and markedly reduces the yield and quality of crops. It is predicted that more than 50% of the productivity of the world's cultivated land will be lost by 2050, as a result of soil salinity, exacerbated by global climate change (Mahajan and Tuteja 2005). Growing in such an environment, salt-adapted plant species have evolved multifaceted mechanisms to tolerate salt stress, including morphological, physiological, and biochemical changes. Although the molecular mechanisms underlying plant salt tolerance are still not fully clarified, there is no doubt that transcription factors play important roles. Over the past few decades, increasing attention has been paid to the regulation of plant abiotic stress tolerance by transcription factors, such as AP2/ERF, WRKY, MYB, NAC, and bHLH (Cui et al. 2013; Xu et al. 2015; Zhou et al. 2009).

The WRKY transcription factor family is one of the largest transcription factor families in higher plants (Ulker and Somssich 2004). WRKY is named after the

conserved WRKYGQK sequence which contains about 60 amino acid residues at the N-terminal end. Members of this family contain one or two WRKY domains at the N-terminus and a zinc-finger motif at the C-terminus, and are divided into three groups, namely group I, II, and III. Group I contains two WRKY domains and a C_2H_2 ($C–X_{4–5}–C–X_{22–23}–H–X–H$) zinc-finger motif; group II includes one WRKY domain and a C_2H_2 zinc-finger motif and can be further split into five subgroups (IIa–e) (Rushton et al. 2008; Zhang and Wang 2005); and group III comprises one WRKY domain and a C_2HC ($C–X_7–C–X_{23}–H–X–C$) zinc-finger motif (Eulgem et al. 2000).

WRKY transcription factors are reported to be involved in responses to biotic and abiotic stresses (Li et al. 2004; Zhou et al. 2008), and in metabolic and developmental regulation (Devaiah et al. 2007; Kato et al. 2007), through specifically binding to the W-box (TTGACC/T) in the promoter region of target genes (Rushton et al. 1996). Thus far, most studies on the function of group I WRKY transcription factors have focused on their roles in disease resistance and in response to abiotic stresses. In grape, *VvWRKY3* induction and the increased expression of the *VvPR10.1* pathogenesis-related gene in the leaves of the mildew-resistant cultivar 'Regent' after infection with *Plasmopara viticola* were correlated, whereas, in the susceptible cultivar 'Lemberger', *VvWRKY33* and *VvPR10.1* were not induced. The results of ectopic expression studies showed that *VvWRKY33* could strongly enhance resistance to *P. viticola* in the susceptible cultivar 'Shiraz' and reduce pathogen sporulation by about 50–70% (Merz et al. 2015). In *Arabidopsis thaliana*, the overexpression of a poplar (*Populus trichocarpa*) salicylic acid- (SA-) inducible *WRKY* gene, *PtrWRKY73*, increased resistance to biotrophic pathogens but reduced resistance against necrotrophic pathogens (Duan et al. 2015). A series of experiments on *AtWRKY34* overexpressing plants showed that this gene negatively regulated cold-sensitivity of mature *Arabidopsis* pollen by participating in the CBF signal cascade (Zou et al. 2010). In addition, *CsWRKY2*, a WRKY transcription factor gene from tea (*Camellia sinensis*), was reported to play an important role in cold stress response via the ABA signaling pathway (Wang et al. 2016).

A growing number of studies have demonstrated that many group I *WRKY* genes can play either positive or negative roles in response to salt stress. For instance, overexpression of *GmWRKY54* from soybean in *Arabidopsis* improved salt and drought tolerance, possibly by regulating the expression of *DREB2A* and *STZ/Zat10*, whereas *GmWRKY13*-transgenic *Arabidopsis* plants showed an increase in salt and mannitol sensitivity, and a reduction in ABA sensitivity (Zhou et al. 2008). Both the *Atwrky33* null mutants and the *Atwrky25Atwrky33* double mutants showed a greater sensitivity to NaCl and ABA, and genetic complementary experiments confirmed that overexpression of either *AtWRKY25* or

AtWRKY33 was sufficient to increase *Arabidopsis* NaCl tolerance (Jiang and Deyholos 2009). Studies on *GhWRKY25*, a group I *WRKY* gene from cotton, indicated that its heterologous expression in *Nicotiana benthamiana* reduced the tolerance to drought stress but increased the tolerance to salt stress (Liu et al. 2016). Transformation with *RtWRKY1*, from the recretohalophyte *Reaumuria trigyna*, was also reported to confer salt tolerance to the transgenic *Arabidopsis* by regulating plant growth, osmotic balance, Na^+/K^+ homeostasis, and the antioxidant system (Chao et al. 2017). In chrysanthemum (*Dendranthema grandiflorum*), *DgWRKY1, DgWRKY3, DgWRKY4*, and *DgWRKY5* were reported to increase tolerance to salt stress in transgenic tobacco or chrysanthemum (Liang et al. 2017; Liu et al. 2014, 2013; Wang et al. 2017). On the contrary, *CmWRKY17* negatively regulated salt tolerance in transgenic chrysanthemum, *Chrysanthemum morifolium* (Li et al. 2015). The above findings indicated that *WRKY* genes regulate salt stress tolerance through multiple processes, mainly by regulating the cellular levels of reactive oxygen species (ROS), controlling the ABA signaling pathway, altering the expression of stress-related genes, maintaining the Na^+/K^+ homeostasis, and maintaining membrane stability.

Biotic and abiotic stresses can cause changes in gene expression within a short time. Overexpression of *GhWRKY34* enhanced salt tolerance in *Arabidopsis*, largely by up-regulating the expression of the stress-related genes *AtRD29A, AtRD29B, AtABF4, AtSOS2, AtSOS1*, and *AtCBL1* (Zhou et al. 2015). On the contrary, exposure to salt stress reduced the expression of the stress-related genes *AtRD29A, AtDREB2B, AtSOS1, AtSOS2, AtSOS3*, and *AtNHX1* in *CmWRKY17* transgenic *Arabidopsis* plants and enhanced the sensitivity to salt (Li et al. 2015). In addition, salt stress also causes the accumulation of ROS in plants. For example, overexpression of *GhWRKY17* in *Nicotiana benthamiana* significantly reduced plant tolerance to salt stress through reduced expression of genes encoding ROS-scavenging enzymes, increased accumulation of ROS, increased malondialdehyde (MDA) content, caused by the interaction between ROS and the lipids of cell membranes, reduced proline content and antioxidant enzyme activities [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX)], and increased electrolyte leakage (Yan et al. 2014). *WRKY* genes were reported to affect salt tolerance by regulating the expression of stress-related genes and the cellular level of ROS simultaneously. For example, overexpression of a novel cotton gene *GhWRKY6-like* in *Arabidopsis* enhanced salt tolerance by reducing the accumulation of ROS, increasing the levels of ROS-scavenging enzymes, and increasing the expression of stress-related genes (*AtSOS2, AtRD29a*, and *AtRD29b*) (Ullah et al. 2017).

Polygonum cuspidatum Sieb. et Zucc. is a perennial plant widely used in herbal medicine in Asian countries, such as China and Japan, because of its pharmacological effects with respect to antifungal (Agarwal et al. 2000), antioxidant (Yen et al. 2000), antiviral (Barnard et al. 1995), and antineoplastic activities (Su et al. 2005), with the active organs being the roots and rhizomes. *P. cuspidatum* has also become a serious invasive alien plant in Europe and part of the US. In large-scale planting of *P. cuspidatum*, soil salinity is one of the important factors influencing yield and quality. Thus far, most Asian studies on *P. cuspidatum* have dealt with its pharmacodynamic effects, with apparently no reports on the regulation of abiotic stress by *P. cuspidatum* transcription factors. In the present study, we report the isolation and molecular characterization of a group I WRKY gene, *PcWRKY33*, from *P. cuspidatum*. Our results showed that *PcWRKY33* overexpression in *A. thaliana* can negatively regulate salt stress through decreasing the expression of stress-related genes and regulating cellular levels of ROS.

Materials and methods

Plant materials, growth conditions, and treatments

Polygonum cuspidatum plants were maintained in the medicinal plant garden and in a greenhouse at the Institute of Botany, The Chinese Academy of Sciences, Beijing, China. Seeds were surface-sterilized and sown on Murashige and Skoog (MS) agar medium in a growth chamber at 24 °C with a 16/8 h light/dark cycle. One-month-old seedlings were used in the subsequent treatments. For plant hormone treatment, the seedlings were uniformly sprayed with 0.1 mM methyl jasmonate (MeJA), 0.1 mM ABA, or 500 µM gibberellic acid (GA₃), and the solutions had been filter-sterilized before use. For salt treatment, the 1-month-old seedlings were transplanted to MS agar medium containing 100 mM NaCl. For drought treatment, seedlings, transplanted from agar to a 1:1 (v/v) mixture of soil and vermiculite in pots, and grown in greenhouse at 24 °C with a 16/8 h light/dark cycle, were used. All the samples were frozen in liquid nitrogen at the appropriate time and stored at -80 °C before RNA extraction. Each treatment was repeated at least twice and the results were expressed as the mean ± standard deviation (SD) of two replicate experiments.

Seeds of wild-type *A. thaliana* Columbia ecotype (Col-0) (and of transgenic lines derived from the wild type) were potted in a 1:1 (v/v) mixture of soil and vermiculite, and grown in a greenhouse at 22 ± 1 °C with a 16/8 h light/dark cycle and a relative humidity of 60%.

Gene cloning, promoter cloning, and sequence analyses

Total RNA was isolated from *P. cuspidatum* leaves using the Plant Total RNA Purification Kit (GeneMark). The first-strand cDNA was synthesized using 5 µg total RNA, and TransScript®One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) in a reaction volume of 20 µL. The primers used are listed in Table S1. The full-length sequence of *PcWRKY33* was cloned into the pCloneEZ-TA-Amp/HC (ThBio, Beijing, China) for sequencing.

Genomic DNA was isolated from seedling leaves using Plant Genomic DNA Kit (TIANGEN, Beijing, China), and was then digested by *Eco*RV, *Stu*I, *Dra*I, and *Pvu*II, respectively. After purification, a 1:1 (v/v) mixture of GW-AP1 and GW-AP2 was used as an adapter to connect with the treated DNA (Siebert et al. 1995). Two rounds of PCR were carried out and the products were cloned into the pCloneEZ-TA-Amp/HC (ThBio, Beijing, China) for sequencing.

The PcWRKY33 amino acid sequence was aligned with its homologs using the ClustalX program (http://www.clustal.org) and BLAST online software (http://www.ncbi.nlm.gov/blast). The phylogenetic relationships of the WRKY proteins were analyzed with the neighbor-joining method using MAGE 7.1 (http://www.megasoftware.net/). Physicochemical property analysis was carried out using the ProtParam program (http://au.expasy.org/tools/protparam.html). Identification of protein domains and significant sites was performed with the ScanProsite tool (http://prosite.expasy.org/scanprosite/). Analysis of the promoter sequence of *PcWRKY33* was performed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The localization of the PcWRKY33 protein was predicted by the ProtComp program (http://linux1.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc).

Quantitative real-time PCR

Total RNA was isolated by the Plant Total RNA Purification Kit (GeneMark). The first-strand cDNA was synthesized using 3 µg total RNA and HiScript II Q RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, Nanjing, China) in a reaction volume of 20 µL. Quantitative real-time PCR (qPCR) was performed using TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) and the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR mix was composed of 5 µL 2×TransStart®Tip Green qPCR SuperMix, 1.0 µL of 1:10 diluted cDNA, 0.2 µL of each primer (10 mM), and 3.6 µL PCR grade water in a final volume of 10 µL. The reactions were incubated under the following conditions: 1 cycle at 94 °C for 30 s; 40 cycles at 94 °C for 5 s, 55 °C for

15 s, and 72 °C for 10 s; and then a single melt cycle from 65 to 95 °C. The primers used in qPCR are listed in Table S1 and the expression levels were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method (Livak and Schmittgen 2001). The results were expressed as the mean ± standard deviation (SD) of three replicate experiments.

Subcellular localization analysis

The coding sequence of *PcWRKY33* without the termination codon and the sequence of GFP were fusion expressed by overlapping PCR. Then, the fragment was cloned into the binary vector pBI121 and driven by the CaMV35S promoter, using seamless cloning technology. The recombined plasmid and the positive control *35S::GFP* plasmid were, respectively, transferred into living onion epidermal cells via the biolistic bombardment transformation method (Shi et al. 2010), using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) with gold particles (1.0 µL) and a helium pressure of 1,350 psi. Then, the treated onion epidermal cells were cultured on MS medium under dark conditions at 28 °C for 24 h. Before observation, the nuclei were stained with 0.1 µg/mL 4′6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS) for 15 min. The fluorescence was observed using an inverted fluorescence microscope (TCS SP5: Leica, Wetzlar, Germany).

Yeast one-hybrid and co-transfection experiments

For yeast one-hybrid experiments, triple tandem copies of the W-box (TTGACC) and mutant W-box (TAGACC) were, respectively, inserted into the pLacZi vector at the *Eco*RI and *Kpn*I sites, using T4 DNA Ligase (NEB, Ipswich, MA, USA), and the coding sequence of *PcWRKY33* was inserted into the pB42AD vector using the One-Step Cloning Kit (Vazyme, Nanjing, China). Both vectors were then co-transformed into the yeast strain EYG48, and the transformed yeast cells were grown on tryptophan (Trp) and uracil (Ura)-deficient synthetic dextrose (SD) medium (SD/-Trp/-Ura), to confirm the success of the transformation. Then, the confirmed yeast strains were smeared on SD/-Trp/-Ura medium with 20 mg/L X-gal and cultivated at 30 °C for less than 12 h.

For co-transfection experiments, the effector plasmid was obtained by replacing GUS in the binary vector pBI121 with the ORF of *PcWRKY33* using the One-Step Cloning Kit (Vazyme, Nanjing, China), while the reporter vector was obtained by replacing CaMV35S in pCAMBIA1301 with 3×W-box-35Smini and 3×mW-box-35Smini, respectively, using the same method. Both recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 at a concentration equivalent to an OD_{600} of 0.2. The assay of

Agrobacterium-mediated transient transformation was performed as previously described (Yang et al. 2010). GUS histochemical staining was detected using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as the substrate at 2 days after transformation. The leaf samples were soaked in GUS staining solution (2 mL 0.5 M Na_2EDTA, 3.8 mL 1 M $NaH_2PO_4·H_2O$, 12.4 mL 0.5 M $Na_2HPO_4·12H_2O$, 0.5 mL 0.1 M $K_4Fe(CN)_6$, 0.5 mL 0.1 M $K_3Fe(CN)_6$, 25 mL methanol, 60 µL Triton X-100, and 100 mg X-gluc (dissolve in 500 µL DMFO), and distilled H_2O to make the volume up to 250 mL, pH 7.0) and vacuumized at − 800 Pa for 5 min. After incubation overnight at 37 °C, the leaf samples were immersed in 70% (v/v) ethanol until the chlorophyll was removed.

Genetic transformation

The *35S::PcWRKY33-pBI121* recombinant plasmid was transferred into *Agrobacterium tumefaciens* strain GV3101, and *A. thaliana* was transformed with *Agrobacterium*-mediated DNA transfer by the floral dip method (Clough and Bent 1998). Transformed *Arabidopsis* seeds were selected on half-strength Murashige and Skoog (1/2×MS) medium containing 40 mg/L kanamycin.

Semi-quantitative RT-PCR

For the semi-quantitative RT-PCR (RT-PCR) detection of transgenic *A. thaliana*, total RNA from the leaves of seven kanamycin resistance transgenic plants and control plants were isolated using the Plant Total RNA Purification Kit (GeneMark). First-strand cDNA synthesis was performed on total RNA using TransScript®One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The resultant first-strand cDNA was used as a template, *AtActin2* was used as an internal control, and its program was: 1 cycle at 94 °C for 10 min; 24 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and then followed by 1 cycle at 72 °C for 5 min. After PCR, the products were detected using 2% (w/v) agarose gels. RT-PCR experiments were repeated at least three times using independent cDNA samples. All primers used in RT-PCR are listed in Table S1.

Salt sensitivity analyses

In the germination assay, seeds of three independent over-expressed *PcWRKY33* lines (OE1, OE2, and OE3) from the T_1 generation, and the wild-type (WT) seeds were surface-sterilized and spread on 1/2×MS medium supplemented with different concentrations of NaCl (0, 100, or 125 mM), and the germination rates were counted daily. For the root elongation assay, the seeds were germinated on 1/2×MS medium for 3 days before being transferred to 1/2×MS medium containing

different concentrations of NaCl (0, 100, or 125 mM), and the plates were placed vertically in the chamber. There were six independent samples of each of the four lines, and the root lengths were measured after 10 days treatment.

For salt stress treatment, 4-week-old transgenic and wild-type plants were irrigated every 3 days with 100 mM NaCl solution for 10 days and maintained under the same growth conditions as described above to record survival rates. The chlorophyll concentration was determined as previously described (Lichtenthaler and Wellburn 1983). In addition, the concentrations of Na$^+$ and K$^+$ were measured by inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer, Waltham, MA, USA); before measurement, the samples were nitrated with 8 mL concentrated nitric acid and 3 mL concentrated hydrogen peroxide. The salt stress treatment experiments were repeated at least three times and the results were expressed as the mean ± standard deviation (SD) ($n = 6$).

Oxidative stress analyses

To detect the accumulation of H_2O_2 and O_2^-, a histochemical staining procedure was performed using 3, 3′-diaminobenzidine (DAB) or nitrotetrazolium blue chloride (NBT), respectively, according to the method previously described (Hu et al. 2013). The content of MDA was detected using a maleic dialdehyde assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plant tissue was extracted in a phosphate buffer (pH 7.8) as previously described (Li et al. 2013), and the ROS-scavenging enzymes activities of SOD, POD, and CAT were measured with kits produced by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein concentration was quantified with the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China), and specific enzyme activity was expressed on a protein basis.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) using the GraphPad software (La Jolla, CA, USA) and multiple comparisons were carried out using Tukey's test. The results were expressed as the mean ± standard deviation (SD) of at least three independent replicate experiments, and the significance threshold was set at $P < 0.05$.

Results

Identification and sequence analysis of *PcWRKY33*

A cDNA fragment encoding *PcWRKY33* was isolated using gene-specific primers designed from the sequences of the *P.*

cuspidatum transcriptome database (SRA038892), with the rapid amplification of cDNA ends PCR (RACE-PCR) being used to amplify the 5′-untranslated region (UTR) and the 3′-UTR. The full-length cDNA sequence was 1572 bp and contained a complete open reading frame (ORF) of 1449 bp. The gene encoded a 482-amino-acid protein, with a predicted relative molecular mass of 53.354 kDa and a theoretical isoelectric point of 6.33. Due to the highest degree of homology with *AtWRKY33*, this new WRKY protein was designated as *PcWRKY33* (MG779503). Multi-sequence alignment showed that *PcWRKY33* showed 53.93, 48.79, and 47.11% homology with *VvWRKY33* (AHG99400), *MsWRKY33* (AKM20827), and *AtWRKY33* (AAM34736), respectively (Fig. 1a). Structural analysis showed that the PcWRKY33 protein contained two WRKY domains and two zinc-finger motifs (C–X$_4$–C–X$_{23}$–H–X$_1$–H), indicating that *PcWRKY33* belongs to group I of the WRKY family. In Fig. 1a, the conserved WRKY sequence is outlined by a box, and the C and H residues in the zinc-finger motif are marked by triangles. A phylogenetic tree was constructed to evaluate the evolutionary position of PcWRKY33 within the WRKY protein family, using the MEGA 7.1 software, based on the amino acid sequences of different WRKY members from different species (Fig. 1b).

Characterization of *PcWRKY33* as a transcription factor

The presence of putative nuclear localization signals in the PcWRKY33 protein sequence predicted its potential location in the nucleus like most other transcription factors. To test this prediction, we constructed *35S::PcWRKY33-GFP* and *35S::GFP* vectors, with the green fluorescent protein (GFP) gene acting as the reporter gene in the subcellular localization experiment. The plasmids carrying *35S::PcWRKY33-GFP* and *35S::GFP* were introduced, respectively, into onion epidermal cells using a gene gun. Onion epidermal cells carrying the *35S::PcWRKY33-GFP* plasmid emitted fluorescence only in the nucleus, which was further confirmed by DAPI staining, whereas the *35S::GFP* control exhibited GFP signals in both the cytoplasm and the nucleus (Fig. 2a). These findings confirmed that the PcWRKY33 protein was localized in the nucleus.

On the basis of reports that WRKY transcription factors modulate protein expression by interacting with the *cis*-element W-box to regulate transcription, we performed the yeast one-hybrid assay to verify this characterization of the PcWRKY33 protein. Three tandem repeats of the W-box (TTGACC) and the mW-box (TAGACC) were, respectively, inserted into the pLacZi vector, and *PcWRKY33* was cloned into the pB42AD vector (Fig. 2b). These two vectors were then co-transformed into the yeast strain EYG48, and the transformed yeast cells were grown on tryptophan- (Trp)

a

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AtWRKY33              MAASELTMDNSRTRQ NMNGSANWSQQSGRTSTSSL EDLEIPKERSFAESSISISP -SLVSPSTC-----ESPSL  68
MsWRKY33              MTSSFESDLLNSPTDQ NL---KDTERSHHQVLLDQN RSGLPPKEKSTPESLPISP TPLFSPSYFSIPPGESLSE  73
PcWRKY33  MSSMASLNNSLNNDSGFSEQ FMNSPFTDFEATDDDHHAMQ RLD-SSGWSVFEHQNAITNH DFTEQPKEKSMAESL--AP VSSSTSSYLPTSPLSESPSV  97
VvWRKY33  MASSAASFNTSSHNSQDDES ESTQLMSSSETDLLSSGGDM DHSAPFAWALSDPLSHTT-- ---EFPKEKSLPESLPISP -PPVSFSSYFAFSSGESATD  94

AtWRKY33  FLDSEAFVSSSANVLASPIT CALITN-----VTNQKGINE GDKSNNNNFNLFDESFHTQG VSAPTTTTTT---TTTTTTT NSS----------------  143
MsWRKY33  LLDSEVLLNSS-HILESPIT CSFANNEGFNWKNNYEEKQE NVKAEEETISSS-ESFQAQP AGFQSSTTTVQNGWNMVKTE NSS------------SMQ  157
PcWRKY33  FLDSELLFPNSNNFL-SPIV CDFNEQ-----KPDVFSFQA PIRPSAVSSSSSIEQSVNST EDGVWRNLD-----QAHQTE NQN--------------KQ  171
VvWRKY33  LLDSELLLSSS-NVLESPIA CNFAAP-GFNWRSNSNEHQQ AFNDTDRKFSDLLEQSQTRP SGGEALKTQAP--WNSDKPE KQTHFPQEKTGVKSEFASLQ  190

                                                          WRKY
AtWRKY33  --IFQSQEQQKKNQSEQWSQ TETRPNNCAVSYNGREQRKG EDGYNWRKYGQKQVKGSENE RSYYKCTFPNCPTKKKVERS IDGQITEIVYKCSENHPKPQ  241
MsWRKY33  SFPPESNSTSEQNNKSGSQS NYNNYQSCPQVQIL--SRRS DDGYNWRKYGQKQVKGSENE RSYYKCTYPNCPTKKKVEPG IDGQITEIVYKGSENHPKPV  255
PcWRKY33  TTLPEKPKIEQGTQSISAAP QPLRSGSCPSYQFIRSSKTS EDGYNWRKYGQKQVKGSENE RSYYKCSYPNCPTKKKVEMS IDGHVTEIVYKGSENHPKPQ  271
VvWRKY33  SLSPEIASIQTNMQSNNIPQ SGRSHHAQPS-ESYREQRRS DDGYNWRKYGQKQVKGSENE RSYYKCTFPNCPTKKKVEPS IDGHITEIVYKGTESHPKPQ  289
                                                                  ▲       ▲                      ▲

AtWRKY33  STRRSSSSSSTFHSAVYNAS LDHNRQASSDQPNSNNSFHQ SDSFGMQQEDNTTSLSVGDI E-EQGSSIVSRDEED-CGS EPEAKRMKGDNETNGGN-GG  338
MsWRKY33  ANKRNTNSMSSSSS------ -------LANPPPLNNFGS EIQMDLVATPENSSISIGLD D-SEQTSHKSGGDHDQFCED EPDSKKWRIEGENEGISLGA  340
PcWRKY33  PKRSSYDSLDA-------- ----------PVPAVGSSQ SMEPSPNLTPETSSVSMELD DEEDHTSALSMTRPAK--ED EPESKRWKAENETEAMS-AY  348
VvWRKY33  PKRSSSQSFPSAST---N-- -------HGHSMPIG NPYMDSMTTSENSSVSIGED D-EDQNSPMSRSGGDD--EN EREAKRWKGEYENEAIS-AS  371

                                                 WRKY
AtWRKY33  GSKTVREPPRIVVQTTSDIDI LDDGYRWRKYGQKVVKGNPN PRSYYKCTITGCPVRKHVER ASHDMRAVITTYEGKHNHDV PAARGSG-YATNRAPQDSSS  437
MsWRKY33  GNRTVREPPRIVVQTTSDIDI LDDGYRWRKYGQKVVKGNPN PRSYYKCTNPGCPVRKHVER ASQLIRAVITTYEGKHNHDV PAFRGSGNHSINKPLPMQTT  440
PcWRKY33  GSRAVKEPPRIVVQTTSEIDI LDDGYRWRKYGQKVVKGNPN PRSYYKCTISTGCPVRKHIER ASNDIRAVITTYEGKHNHDV PAARGSGS-YTINRPGARDAA  447
VvWRKY33  ESRTVKEPPRIVVQTTSDIDI LDDGYRWRKYGQKVVKGNPN PRSYYKCTISTGCPVRKHVER SSKDIRAVITTYEGKHNHDV PAARGSGSHFVTKPLPNNST  471
                                                       ▲       ▲                    ▲   ▲

AtWRKY33  V----PIRPAAIAGHSNYTT SSQAPYTLQMLHNNNTNTGP FGYAMNNNNNNS--N--LQT QQNFVGGGFSRAKEBNEET SFEDSEMP  517
MsWRKY33  T-------------NNTTS ANNS---INNNNNVSLHNGF FNHEMMQNNGFTVDSYMMMN QQRVNNILRGRAKEBVEDD SPEESELS  511
PcWRKY33  I----PVRPSA----YNPSQ LNSAPATLEWLSNPAGFGYS SGF                                               483
VvWRKY33  TTVPAPIRPSVMTNHSNYTT TNANP---QTRPPTSASQAP FTLEMLQSPGSFGFS--GFG RMSHPDGVFSRTKEEEKDDL --EQSEFC  552
```
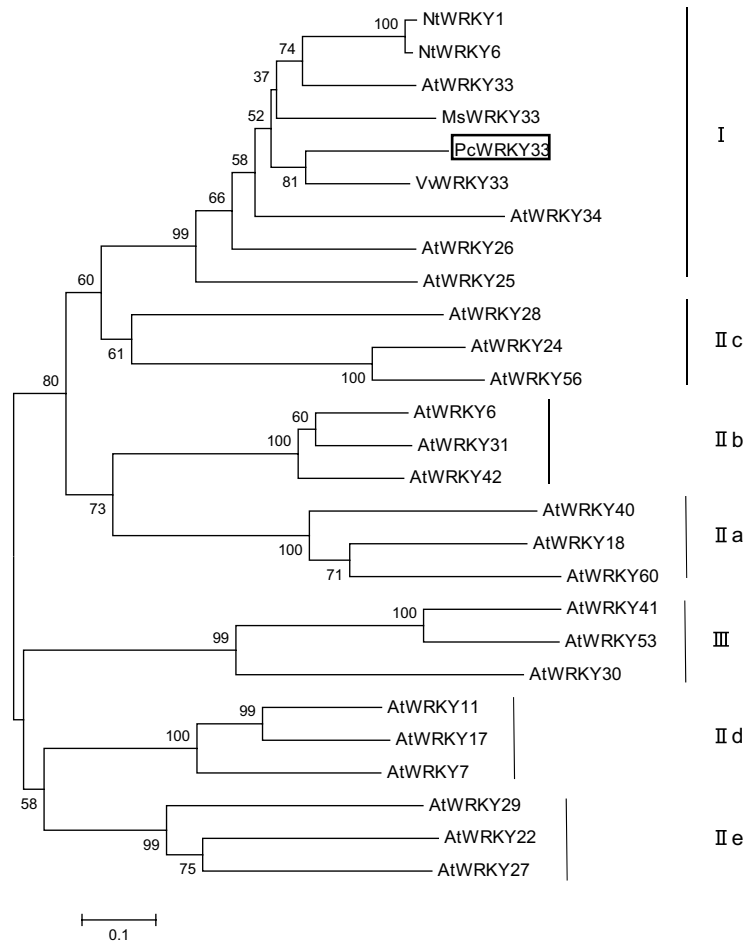
b

0.1

◀**Fig. 1** Sequence and phylogenetic analysis of PcWRKY33. **a** Alignment of the putative PcWRKY33 protein with its homologous proteins. Identical amino acids are shaded in black. The WRKYGQK conservative sequence is outlined by a box and the C and H residues in the zinc-finger motif are marked by triangles. **b** Phylogenetic analysis of PcWRKY33 and other WRKY proteins from different species. The amino acid sequences were subjected to Clustal W using the neighbor-joining method in MEGA 7.1. PcWRKY33 is boxed. At, *Arabidopsis thaliana*; Nt, *Nicotiana tabacum*; Pc, *Polygonum cuspidatum*; Ms, *Medicago sativa*; Vv, *Vitis vinifera*

and uracil- (Ura) deficient synthetic dextrose (SD) medium (SD/-Trp/-Ura), to test the success of transformation. After growth on SD/-Trp/-Ura medium containing 20 mg/L X-gal for less than 12 h, only the yeast strain carrying both the pLacZi-W-box and pB42AD-PcWRKY33 plasmids turned blue (Fig. 2c). These results demonstrated that PcWRKY33 specifically bound to the W-box element and functioned as a transcription factor in this yeast system.

For a further study on the transcriptional activities of *PcWRKY33* in the plant cell, a transient co-expression experiment was performed in tobacco leaves. The W-box-35Smini and mW-box-35Smini sequence, respectively, replaced the promoter of GUS in the pCAMBIA1301 vector, and *PcWRKY33* was inserted into the pBI121 vector. We transformed W-box-35Smini-GUS or mW-box-35Smini-GUS alone or with 35S::*PcWRKY33* into *Nicotiana benthamiana* leaves using the *Agrobacterium*-mediated transient expression system, and used the pCAMBIA1301 vector as a positive control. After the GUS histochemical staining assay, we observed that tobacco leaves transformed with pCAMBIA1301 were stained dark blue and those co-transformed with W-box-35Smini-GUS and 35S::*PcWRKY33* were stained light blue. In contrast, leaves co-transformed with mW-box-35Smini-GUS and 35S::*PcWRKY33* were stained only slight blue, similar to that achieved in leaves transformed with only W-box-35Smini-GUS. As expected, the leaves transformed with only 35S::*PcWRKY33* did not stain blue (Fig. 2d). These findings proved once again that PcWRKY33 could combine with the W-box to regulate gene expression, in a specific manner.

Promoter analysis of *PcWRKY33*

To clarify which biotic and abiotic stresses regulated the expression of *PcWRKY33*, we isolated the 1061 bp promoter region of this gene, using the genome walking method. Using the PlantCARE database for bioinformatics analysis, we found many response elements associated with biotic and abiotic stresses, and with pathogen- and light-induced, and with tissue-specific expression (Table 1). The presence of abiotic stress response elements, such as ARE, CGTCA-motif, ERE, HSE, P-box, TC-rich repeats, TCA-element, and WUN-motif, suggested that the expression

level of *PcWRKY33* might be regulated by methyl jasmonate (MeJA), ethylene (ET), heat, gibberellic acid (GA), salicylic acid (SA), and wounding. The presence of the W-box in the promoter of *PcWRKY33* indicated that *PcWRKY33* might have interactions with other WRKY transcription factors.

PcWRKY33 expression in *P. cuspidatum* influenced by various stresses

Quantification of the expression level of *PcWRKY33* using qPCR revealed that expression was higher in the root than in either the stem or the leaf (Fig. 3a). Having identified *cis*-acting elements in the promoter of *PcWRKY33*, we used qPCR to analyze transcriptional changes in *P. cuspidatum* seedlings exposed to various abiotic stresses and hormone treatments. Results showed that ABA, GA, and salt markedly induced the expression of *PcWRKY33*, but its expression was significantly reduced under drought condition, while there was no significant change after MeJA treatment (Fig. 3b–f). Under GA and salt treatments, the expression profiles of *PcWRKY33* showed two peaks, at 4 and 12 h, whereas, following ABA treatment, the two peaks appeared at 8 and 24 h. The greatest increases in expression level of *PcWRKY33* under GA, ABA, and salt treatment were fourfold, 40-fold, and fivefold, respectively. Under drought treatment, the expression of *PcWRKY33* initially increased at 2 days, but decreased rapidly at 3 days and then maintained a steady rise to the control level. These findings demonstrated that the expression of *PcWRKY33* responded to various stress conditions.

Increased salt sensitivity of transgenic *Arabidopsis* following *PcWRKY33* overexpression

We constructed transgenic *A. thaliana* plants that overexpressed *PcWRKY33* under the control of the CaMV35S promoter via *Agrobacterium*-mediated transformation, to further investigate the function of *PcWRKY33* in plants. Seven transgenic lines were obtained using kanamycin selection (40 mg/L) and then confirmed by PCR. Then, we performed the semi-quantitative RT-PCR detection of kanamycin resistance *Arabidopsis* and the control plants (WT). The result showed that *PcWRKY33* was expressed at the transcription level, and the expression was different in different transgenic lines (Fig. S1). Based on the result of RT-PCR detection, we selected line1, line3, and line4, which showed the highest expression of *PcWRKY33*, named as OE1, OE2, and OE3, respectively, for the subsequent experiments.

The results of promoter analyses and quantification of expression levels under the different treatments showed that *PcWRKY33* might be involved in various stress defense responses, especially the salt stress response. To identify the function of *PcWRKY33* during early plant development,

a

| | GFP | DAPI | Merge | Bright |

35S::GFP

35S::PcWRKY33-GFP

b

c

W-box: **GAATTC**TTGACCCCTTTTTGACCCGTTTTTGACCTCCA**GGTACC**

mW-box: **GAATTC**TAGACCCCTTTAGACCCGTTTAGACCTCCA**GGTACC**

d

35S::GUS   35S::PcWRKY33 +W-box-35Smini-GUS   35S::PcWRKY33+ mW-box-35Smini-GUS   W-box-35Smini-GUS   35S::PcWRKY33
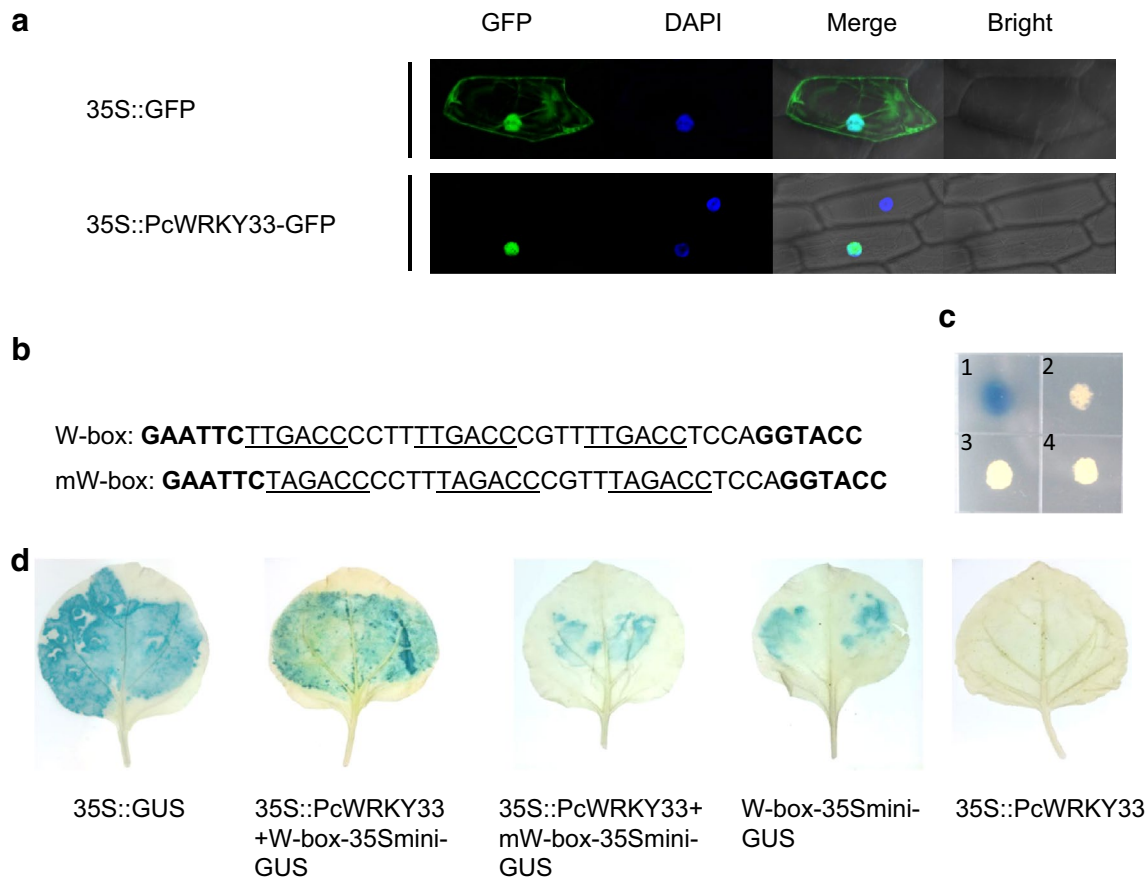
Fig. 2 Characterizations of PcWRKY33 as a transcription factor. **a** Subcellular localization of PcWRKY33. The images of fluorescence were visualized using an inverted fluorescence microscope. **b** Sequences of the triple tandem repeats of the W-box and mW-box. Restriction enzyme cutting sites are in bold font and the W-box and mW-box are underlined. **c** Transactivation activity analysis of PcWRKY33 by the yeast one-hybrid assay using the $3 \times$ W-box or $3 \times$ mW-box as bait. 1, pLacZi-W-box/pB42AD-PcWRKY33; 2, pLacZi-mW-box/pB42AD-PcWRKY33; 3, pLacZi-W-box/pB42AD; 4, pLacZi-mW-box/pB42AD. **d** GUS histochemical staining of co-transfected *N. benthamiana* leaves

the transgenic *Arabidopsis* seeds were surface-sterilized and germinated on $1/2 \times$ MS agar medium containing 0, 100, or 125 mM NaCl to test the germination capacity. Under control conditions, there was no significant difference in germination rate between *PcWRKY33* transgenic lines and the wild type (Fig. 4a, b). However, the *PcWRKY33* transgenic lines showed a lower germination rate than the wild type under 100 and 125 mM NaCl treatments. In particular, on $1/2 \times$ MS medium containing 125 mM NaCl, the seed germination rate of the wild type was 83.3%, while those of the three *PcWRKY33* transgenic lines were only 54.8% (OE1), 66.7% (OE2), and 59.5% (OE3), respectively. Seedlings of the transgenic lines and wild type were cultured vertically on $1/2 \times$ MS medium containing different NaCl concentrations (0, 100, or 125 mM) for 10 days, before the root lengths of the seedlings were measured. The root lengths of the wild-type and transgenic lines were similar on $1/2 \times$ MS medium lacking NaCl, but the root lengths of the three transgenic lines were shorter than those of the wild type growing on

medium containing NaCl (Fig. 4c, d). Statistical analysis confirmed that there was a significant difference in root length between the transgenic lines and wild type under conditions of salt stress.

To confirm the function of *PcWRKY33* during vegetative growth, 1-month-old plants grown in soil were watered with 100 mM NaCl solution for 10 days. The transgenic plants showed serious leaf curling and chlorosis, compared with wild-type plants under NaCl treatment (Fig. 4e). The average survival rate of transgenic plants under salt stress was only 37.4%, which was considerably lower than that of the wild-type plants (58.3%) (Fig. 4f). In addition, the leaves of transgenic and wild-type plants grown under control or salt stress were detached to measure chlorophyll concentrations. Compared with the values under control conditions, the chlorophyll concentrations of both transgenic and wild-type plants growing under salt stress declined, but the decrease in transgenic plants was greater than that in the wild-type plants (Fig. 4g).

Table 1 Putative *cis*-acting elements of the promoter of *PcWRKY33*

Cis-element	Position	Sequence (5′–3′)	Function
Abiotic stress-responsive element			
ARE	148 (−)	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
	257 (−)		
CGTCA-motif	220 (−)	CGTCA	*cis*-acting regulatory element involved in the MeJA responsiveness
ERE	421 (+)	ATTTCAAA	Ethylene-responsive element
HSE	1006 (−)	AGAAAATTCG	*cis*-acting element involved in heat stress responsiveness
P-box	345 (+)	CCTTTTG	Gibberellin-responsive element
TC-rich repeats	456 (+)	GTTTTCTTAC	*cis*-acting element involved in defense and stress responsiveness
	1009 (+)	ATTTTCTTCA	
TCA-element	1010 (−)	GAGAAGAATA	*cis*-acting element involved in salicylic acid responsiveness
WUN-motif	932 (+)	AAATTTCCT	Wound-responsive element
Pathogen/elicitor-related elements			
W-box	892 (+)	TTGACC	Fungal elicitor responsive element
Light-responsive elements			
3-AF1-binding site	282 (+)	TAAGAGAGGAA	Light-responsive element
Box 4	293 (+)	ATTAAT	Part of a conserved DNA module involved in light responsiveness
	297 (+)		
Box I	422 (+)	TTTCAAA	Light-responsive element
G-Box	231 (+)	TCCACATGGCA	*cis*-acting regulatory element involved in light responsiveness
Sp1	938 (+)	CC(G/A)CCC	Light-responsive element
TCT-motif	460 (+)	TCTTAC	Part of a light-responsive element
Tissue-specific and development-related elements			
as-2-box	755 (−)	GATAatGATG	Involved in shoot-specific expression and light responsiveness

K$^+$/Na$^+$ homeostasis disruption and down-regulation of stress-responsive gene expression by *PcWRKY33* overexpression in *Arabidopsis* under salt stress treatment

Long-term exposure to salt stress can cause accumulation of Na$^+$ in plant cells, inhibiting the absorption of K$^+$. Since the K$^+$/Na$^+$ ratio is an important indicator of salt tolerance (Zhu 2003), to clarify the possible mechanism of the reduced salt tolerance exhibited by plants overexpressing *PcWRKY33*, we measured the concentrations of Na$^+$ and K$^+$ ions by ICP-OES in transgenic and wild-type plants under control conditions and under 100 mM salt treatment. The results showed that there was no obvious difference in the concentrations of Na$^+$ and K$^+$ between transgenic and wild-type plants under control conditions. Under the 100 mM salt treatment, however, transgenic plants accumulated more Na$^+$, while the K$^+$ concentration remained largely unchanged. As a consequence, the Na$^+$/K$^+$ ratio in transgenic plants was significantly higher than that in wild-type plants under salt stress (Fig. 5a–c).

To further elucidate the molecular mechanism involved in the increased salt sensitivity in *PcWRKY33* overexpressed (OE) plants, the expression levels of stress-related genes (*AtRD29A, AtDREB2B, AtSOS1, AtSOS2, AtSOS3*, and *AtNHX1*) were determined by qPCR in the transgenic and wild-type plants exposed to 100 mM salt stress. There was no significant difference in the expressions of these genes between transgenic and wild-type plants under control conditions (Fig. 5d–i). However, under salt stress, the expressions of these genes were induced in both the transgenic and wild-type plants, but the magnitude of the induction in the transgenic plants was notably lower than that in the wild-type plants. The maximum inhibition (in the transgenic relative to the wild type) of expression of *AtDREB2B, AtSOS1, AtSOS2, AtSOS3*, and *AtNHX1* occurred at 6 h after salt treatment commenced, while that of *AtRD29A* occurred at 4 h after treatment. The expression in the overexpressed plants of *AtSOS3*, the most inhibited of the stress-related genes, decreased by about 16.6 times, compared with that in the wild type (Fig. 5d–i). These findings indicated that overexpression of *PcWRKY33* negatively regulated salt tolerance, possibly through decreasing the expression of stress-related genes.

The increased accumulation of ROS and oxidative damage associated with *PcWRKY33* overexpression

Salt stress leads to the accumulation of ROS in plants, mainly H$_2$O$_2$ and O$_2^-$ (Mittler 2002). We treated the detached leaves of transgenic *PcWRKY33*-overexpressed and wild-type plants with 3, 3′-diaminobenzidine (DAB: to
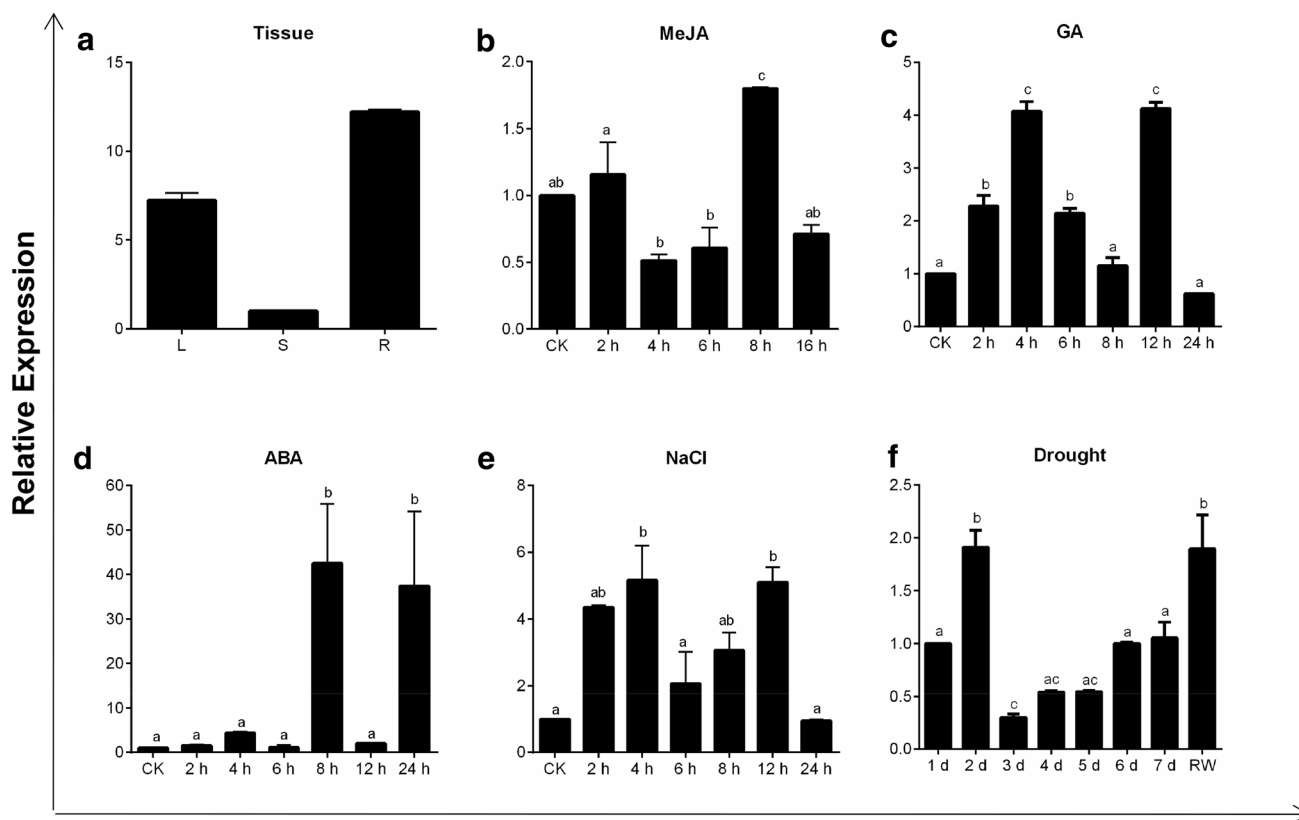
Fig. 3 Expression levels of *PcWRKY33* in *P. cuspidatum* under various stresses. **a** Tissues expression, **b** MeJA, **c** GA, **d** ABA, **e** NaCl, and **f** Drought. The plants without any treatment were used as control, and *PcActin2* was the reference gene. Each column stands for a point of time after treatment, and each experiment was repeated at least twice. Any two bars within a graph with a common letter mean they are not significantly different ($P > 0.05$). *L* leaf, *S* shoot, *R* root, *CK* under normal conditions, *RW* rewater

detect hydrogen peroxide) or nitroblue tetrazolium (NBT: to detect the superoxide anion) to visualize the accumulation of ROS in *PcWRKY33*-overexpressed plants. There was no obvious staining in wild-type or transgenic plants under control conditions. However, under salt stress treatment, staining in the transgenic lines was darker than that in the wild-type plants (Fig. 6a, b). The previous studies have shown that excessive accumulation of ROS results in oxidative damage to plant cells, with the concentration of MDA being an important physiological index of oxidative damage to cell membranes. After the salt stress treatment in the present work, the MDA concentration increased in both transgenic and wild-type plants, but the increase was higher in transgenic plants than in wild-type plants (Fig. 6c).

Increasing the activities of ROS-scavenging enzymes is one of the main ways to alleviate oxidative damage (Bartels and Sunkar 2005). Therefore, we analyzed the activities of SOD, POD, and CAT under control and salt stress conditions. There was no significant difference between the enzyme activities of the transgenic and wild-type plants under control conditions (Fig. 6d–f). However, after exposure to salt stress, the activities of SOD, POD, and CAT

in both transgenic and wild-type plants increased considerably, with the increases in activities of the ROS-scavenging enzymes being higher in the wild-type plants than in the transgenic plants (Fig. 6d–f).

Discussion

As a unique transcription factor family in plants, the WRKY transcription factor family has attracted an increasing attention in recent years. Although WRKY transcription factors have been reported to be involved in various activities in many plants, such as growth and senescence (Miao et al. 2004), responses to biotic and abiotic stresses (Li et al. 2004; Zhou et al. 2008), and development and metabolism (Devaiah et al. 2007; Kato et al. 2007), there have been no reports of WRKY transcription factors in *P. cuspidatum*. In the present study, the results of gene structure and phylogenetic analysis showed that *PcWRKY33* belongs to the group I WRKY family (Fig. 1b). Further studies of multi-sequence alignment showed that *PcWRKY33* showed the greatest similarity to *VvWRKY33,*
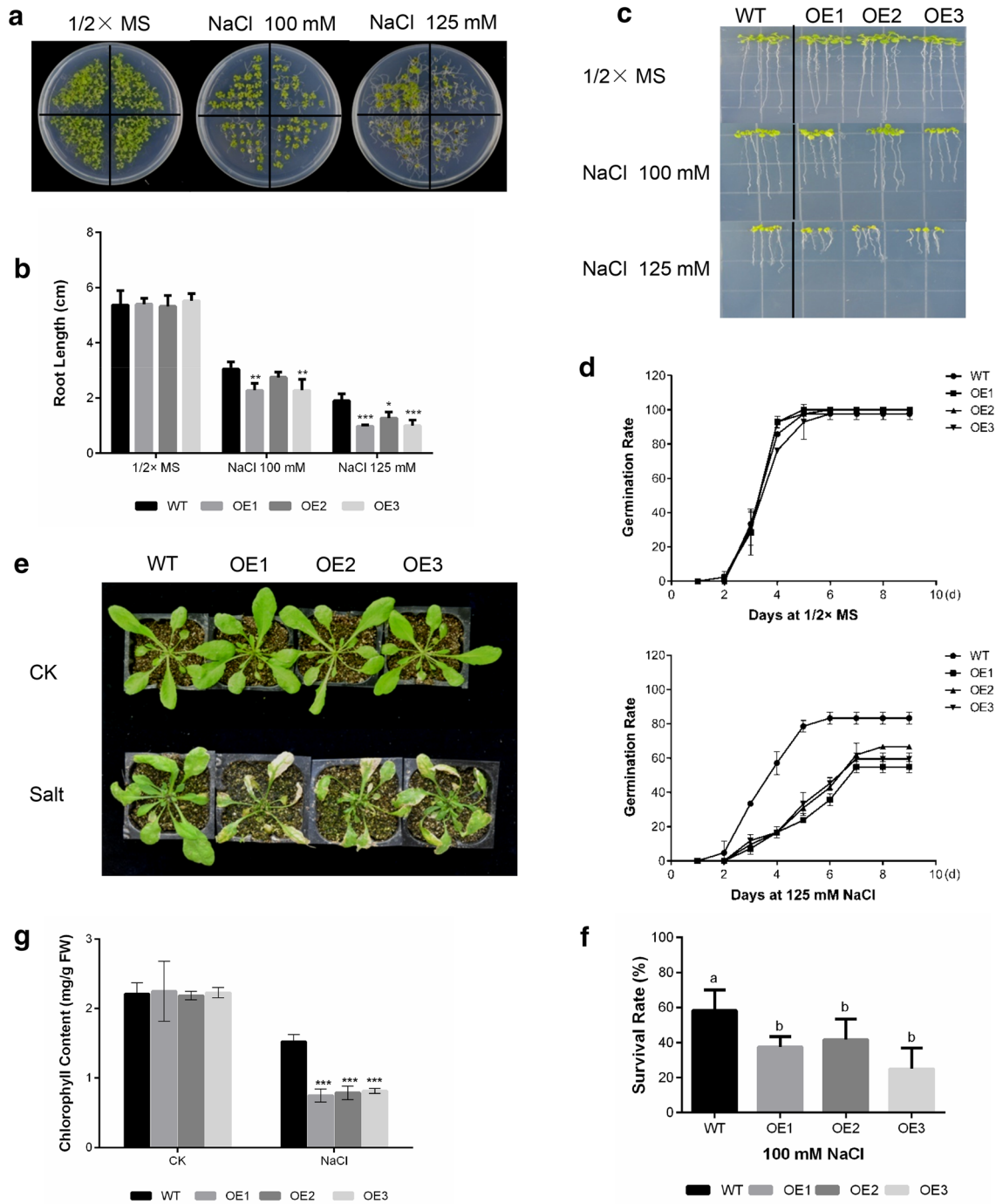
Fig. 4 Expression of *PcWRKY33* enhanced the salt sensitivity of transgenic plants. **a** Germination of transgenic and wild-type plants grown on 1/2×MS medium containing 0, 100, 125 mM NaCl. **b** Statistical analysis of the seed germination rate in **a**. **c** Root elongation of transgenic and wild-type plants grown on 1/2×MS medium containing 0, 100, and 125 mM NaCl. **d** Graphical representation of the root length in **c**. **e** Phenotype of 4-week-old transgenic and wild-type plants after salt treatment (100 mM NaCl) for 10 days. **f** Statistical analysis of the survival rate in **e**. **g** Chlorophyll content of 4-week-

old transgenic and wild-type plants under normal conditions and salt treatment. The mean values and standard errors were calculated based on the results of three independent experiments. One, two, and three asterisks represent significance level $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Any two bars within a graph with a common letter mean that they are not significantly different ($P > 0.05$). *WT* wild-type plants, *OE* overexpressed plants, *CK* under normal conditions, *NaCl* salt treatment
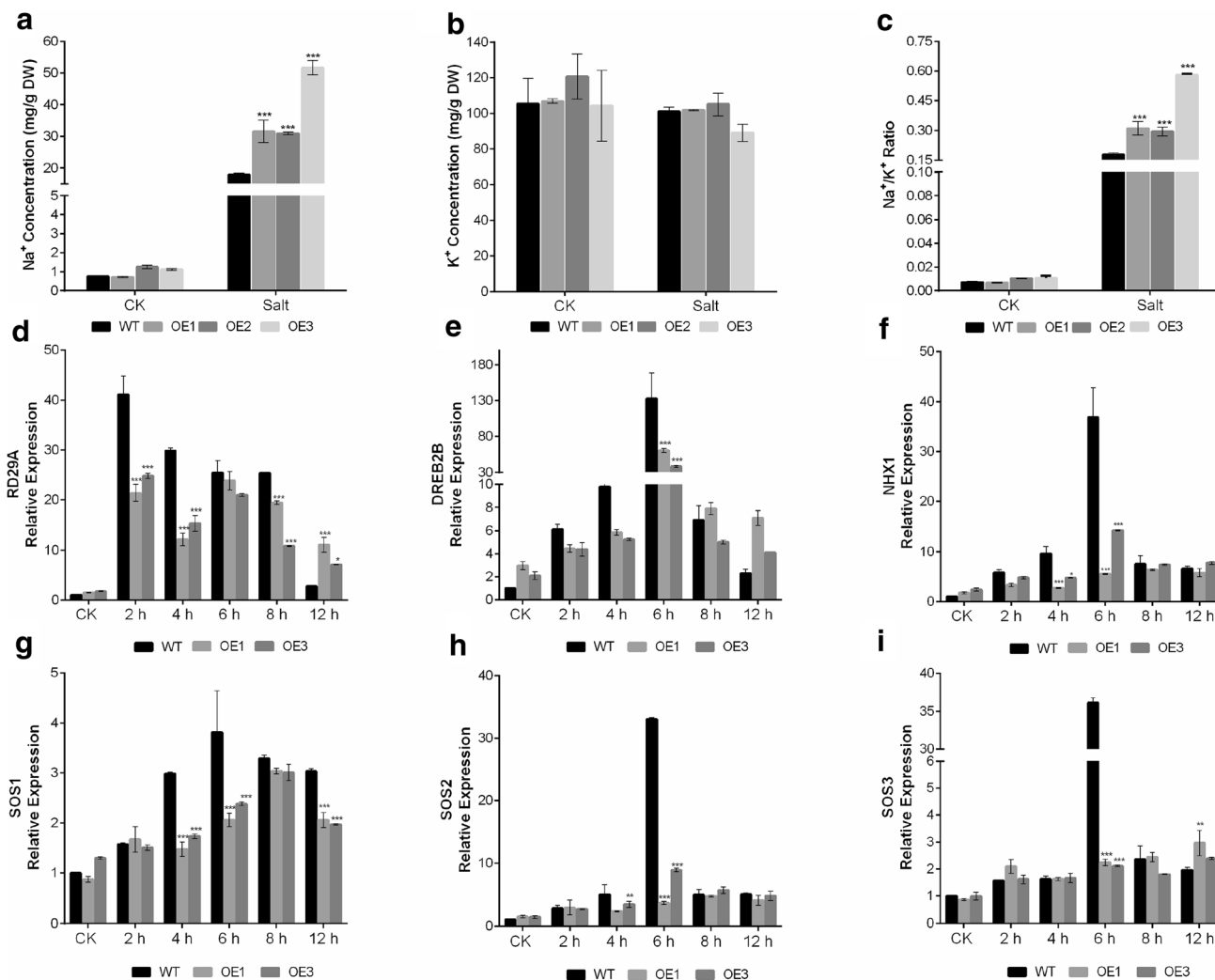
Fig. 5 Salt tolerance of transgenic and wild-type plants in the vegetative stage. **a–c** Na⁺ content, K⁺ content, and Na⁺/K⁺ ratio of transgenic and wild-type plants under normal conditions and salt treatment. The Na⁺/K⁺ ratio calculated from the rate of Na⁺ content and K⁺ content. Four-week-old transgenic and wild-type plants were irrigated every 3 days with 100 mM NaCl solution for 10 days. **d–i** Expression levels of stress-related genes in transgenic and wild-type plants under normal conditions and salt treatment. The mean values and standard errors were calculated based on the results of three independent experiments. One, two, and three asterisks represent significance level $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. *WT* wild-type plants, *OE* overexpressed plants, *CK* under normal conditions, *Salt* after salt treatment

MsWRKY33, and *AtWRKY33* (Fig. 1a). These results confirmed that *PcWRKY33* is a group I WRKY transcription factor from *P. cuspidatum*.

Members of the WRKY family have been reported to bind specifically to the W-box (TTGACC/T) *cis*-acting elements (Eulgem et al. 2000). In our studies, the results of the yeast one-hybrid assay and the transient co-expression experiment in tobacco leaves showed that *PcWRKY33* has the ability to specifically bind to the W-box and to regulate the expression of downstream reporter genes (Fig. 2c, d). In addition, the PcWRKY33 protein was localized to the nucleus, also confirming its role as a transcription factor (Fig. 2a). These results suggested that *PcWRKY33* might

activate the expression of target genes in the nucleus by binding to the W-box in the promoters of these genes.

It has been reported that the expression of *WRKY* genes can be induced by various abiotic stresses (Matsui et al. 2008). Using northern hybridization, the expression profiles of *AtWRKY26, AtWRKY25*, and *AtWRKY33* were monitored (Fu and Yu 2010), and their results showed that expression of these three genes was induced by a range of abiotic stresses, such as temperature, high salinity, and osmotic stress, as well as by ABA. In the present study, promoter analysis and expression pattern detection under various treatments indicated that the expression profiles of *PcWRKY33* and *AtWRKY33* were very similar, being induced by a number of
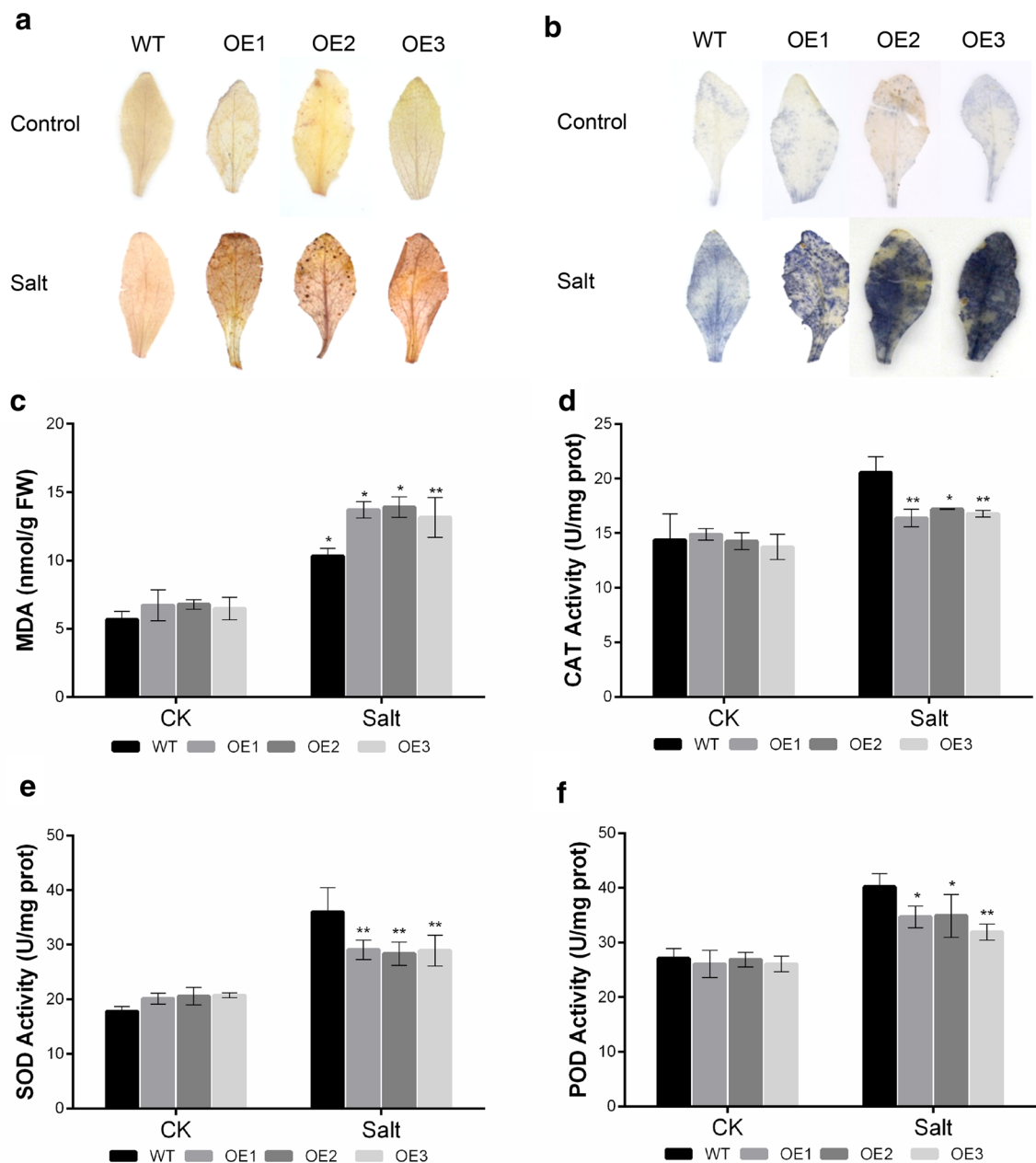
Fig. 6 Overexpression of *PcWRKY33* increased the accumulation of ROS and reduced the activities of antioxidant enzymes. **a** Leaves of transgenic and wild-type plants after DAB staining for H_2O_2 detection under normal conditions and salt treatment. **b** Leaves of transgenic and wild-type plants after NBT staining for O_2^- detection under normal conditions and salt treatment. **c** MDA content of transgenic and wild-type plants under normal conditions and salt treatment. **d–f** Enzyme activities of SOD, POD, and CAT. The results came from three independent experiments ($n = 6$). The mean values and standard errors were calculated based on the results of three independent experiments. One and two asterisks represent significance level $P < 0.05$ and $P < 0.01$, respectively. *Control and CK* under normal conditions, *salt* under salt treatment, *WT* wild-type plants, *OE* overexpressed plants

abiotic stresses (Fig. 3b–f). In addition, the presence of the W-box in the promoter of *PcWRKY33* suggested that transcription of *PcWRKY33* might be mediated by other WRKY transcription factors to regulate gene expression (Table 1).

Salinity has become one of the most important environmental threats to plants, and its most obvious effect is on plant growth and development. In the present study, under salt stress treatment, several growth parameters (such as root length, seed germination rate, seedling survival rate, and chlorophyll concentration) in the overexpressing transgenic lines were significantly lower than those in the wild type (Fig. 4). These results indicated that *PcWRKY33* might play

a negative role in a plant's response to salt stress. A high salt concentration in the soil usually disrupts K^+/Na^+ homeostasis in the plant. Since an environment with a high concentration of Na^+ forces plants to absorb large amounts of Na^+, instead of K^+, which is toxic to plant growth, and which is an essential nutrient for plant cells (Hasegawa et al. 2000), the K^+/Na^+ ratio is an important indicator of plant tolerance to salt. Under salt stress, the extremely high Na^+/K^+ ratio in transgenic plants (compared with wild-type plants), caused by excessive accumulation of Na^+ instead of K^+, indicated that the increase in salt sensitivity associated with overexpression of *PcWRKY33* was probably caused by its relatively weak ability to maintain K^+/Na^+ homeostasis (Fig. 5a–c).

One of the important aspects of plant stress response is the modification of the temporal and spatial expression patterns of stress-related genes (Rushton and Somssich 1998). The salt overly sensitive (SOS) signaling pathway is an important regulatory system for plant survival under salt stress. In the SOS signaling pathway, SOS3 and SOS2 interact to form a complex, and then activate SOS1. *SOS1* encodes a Na^+/H^+ antiporter located on the plasma membrane, which can eliminate the Na^+ from the cell, and transport it to various tissues of the plant simultaneously (Shi et al. 2000). Similarly, overexpression of *AtNHX1* can sharply improve salt tolerance (Apse et al. 1999). There are two ways that salt stress induces the expression of stress-related genes; one is via an ABA-dependent pathway and the other is via an ABA-independent pathway (Zhu 2002). In the ABA-dependent pathway, *RD29A* is usually considered to be a marker gene for plant adaptation to stress, and it was reported to function in the detoxification or alleviation of stress-related damage (Zhu 2001). In the ABA-independent pathway, the expression of the *DREB* transcription factor could simultaneously regulate the expression of multiple stress-related genes, and participate extensively in the physiological and biochemical processes of plants (Zhu 2001).

In our study, the qPCR results showed that the increased expressions of *AtRD29A, AtDREB2B, AtSOS1, AtSOS2, AtSOS3*, and *AtNHX1*, when the plant was exposed to salt stress, were inhibited in transgenic plants (Fig. 5d–i). Under salt stress treatment, *AtRD29A* exhibited the most marked and rapid inhibition effect, with its expression peaking at 2 h after exposure to salt stress, while the greatest inhibition of *AtDREB2B, AtSOS1, AtSOS2, AtSOS3*, and *AtNHX1* occurred at 6 h. The time course of the induced expressions of these genes indicated that the regulation of expressions of stress-related genes may occur through both the ABA-dependent and ABA-independent pathways, with the ABA-dependent pathway responding first. In addition, after promoter analysis of these genes, we found four W-box *cis*-elements in the *AtSOS2* promoter region, suggesting that *PcWRKY33* might bind directly to the W-box in the promoter of *AtSOS2* in response to salt

stress. All these findings suggest that overexpression of *PcWRKY33* can increase salt sensitivity through both the ABA-dependent and ABA-independent pathways, to regulate the expressions of stress-related genes. In *A. thaliana, AtWRKY33* increased salt tolerance, via a route which depends not on the SOS signaling pathway, but partially on the ABA signaling pathway (Jiang and Deyholos 2009). These results indicate that the same member of the WRKY family from different plants may have different functions in response to salt stress.

Note that our reported generally decreased expression effects of stress-related genes in *PcWRKY33* transgenic plants do not necessarily mean that PcWRKY33 directly inhibits the expression of genes; it is a result of complex multi-stage interaction. At this stage, whether PcWRKY33 plays a direct activation or inhibition role in transgenic plants is still an open issue, and warrants further investigation in the future.

Another mechanism that increased salt sensitivity was the accumulation of ROS and the associated decrease in the activities of ROS-scavenging enzymes. High salt concentrations in the soil affect plants through not only ionic pressure but also osmotic stress, which results in the production of ROS, mainly H_2O_2 and O_2^- (Zhu 2002). In response to drought and salt stress, ROS act as crucial signal molecules (Bhattacharjee 2005). The accumulation of ROS causes severe oxidative damage to plants, especially peroxidation of the lipids in cell membranes, resulting in a large number of degradation products, most importantly the accumulation of MDA (Moore and Roberts 1998). Our results of DBA and NBT staining in detached leaves from transgenic plants and wild-type plants showed that the transgenic plants accumulated higher levels of O_2^- and H_2O_2 than did the wild-type line (Fig. 6a, b). In addition, MDA accumulation in transgenic plants was also significantly higher than that in the wild type (Fig. 6c). All these results demonstrate that salt stress inflicts greater osmotic stress on the transgenic plants, as evidenced by the observation that the transgenic leaves accumulated more ROS. To remove excessive ROS, plants have an antioxidant enzyme system composed of multiple ROS-scavenging enzymes, such as SOD, CAT, and POD. The superoxide anion radicals in the cell are catalyzed by SOD to form O_2 and H_2O_2, with CAT degrading H_2O_2 into water and molecular oxygen, while POD oxidizes co-substrates such as phenolic compounds and/or antioxidants to break down H_2O_2 (Gill and Tuteja 2010). In the present study, there was no obvious difference in the activity of SOD, POD, and CAT under control conditions, but, under salt stress, the activities of these enzymes increased considerably in both transgenic and wild-type plants, with the increase in the wild-type plants being higher than in the transgenic plants (Fig. 5d–f). Higher concentrations of ROS and lower activities of ROS-scavenging enzymes in

transgenic plants under salt stress indicated that *PcWRKY33* might alter salt sensitivity via regulation of ROS levels.

In brief, our findings suggest that *PcWRKY33* may increase salt sensitivity through decreasing the expression of stress-related genes and increasing the cellular levels of ROS. Our present work seems to be the first report of WRKY transcription factors from *P. cuspidatum*, and provides some new insights into the functionality of WRKY transcription factors.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest regarding the publication of this paper.

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