



RESEARCH PAPER

# *Populus euphratica* WRKY1 binds the promoter of H<sup>+</sup>-ATPase gene to enhance gene expression and salt tolerance

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## Abstract

Plasma membrane proton pumps play a crucial role in maintaining ionic homeostasis in salt-resistant *Populus euphratica* under saline conditions. High levels of NaCl (200 mM) induced *PeHA1* expression in *P. euphratica* roots and leaves. We isolated a 2022 bp promoter fragment upstream of the translational start of *PeHA1* from *P. euphratica*. The promoter–reporter construct *PeHA1-pro::GUS* was transferred to tobacco plants, demonstrating that  $\beta$ -glucuronidase activities increased in root, leaf, and stem tissues under salt stress. DNA affinity purification sequencing revealed that PeWRKY1 protein targeted the *PeHA1* gene. We assessed the salt-induced transcriptional response of PeWRKY1 and its interaction with *PeHA1* in *P. euphratica*. PeWRKY1 binding to the *PeHA1* W-box in the promoter region was verified by a yeast one-hybrid assay, EMSA, luciferase reporter assay, and virus-induced gene silencing. Transgenic tobacco plants overexpressing *PeWRKY1* had improved expression of *NtHA4*, which has a *cis*-acting W-box in the regulatory region, and improved H<sup>+</sup> pumping activity in both *in vivo* and *in vitro* assays. We conclude that salt stress up-regulated *PeHA1* transcription due to the binding of PeWRKY1 to the W-box in the promoter region of *PeHA1*. Thus, we conclude that enhanced H<sup>+</sup> pumping activity enabled salt-stressed plants to retain Na<sup>+</sup> homeostasis.

**Keywords:** DNA affinity purification sequencing, electrophoretic mobility shift assay, H<sup>+</sup>-ATPase, luciferase reporter assay, NaCl, non-invasive microtest technique, PeWRKY1, virus-induced gene silencing, W-box.

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Abbreviations: AbA, Aureobasidin A; DAP-seq, DNA affinity purification sequencing; EMSA, electrophoretic mobility shift assay; GUS,  $\beta$ -glucuronidase; LRA, luciferase reporter assay; MDA, malondialdehyde; NMT, non-invasive microtest; PM, plasma membrane; TCA, trichloroacetic acid; VC, vector control; VIGS, virus-induced gene silencing; WT, wild type; Y1H, yeast one-hybrid.

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## Introduction

Salinity and secondary soil salinization cause serious ecological and environmental problems worldwide (Pitman and Läuchli, 2002; Hasegawa, 2013). For example, perennial trees, such as *Populus euphratica*, have to undergo long-term ionic stress in salt-affected soils. *Populus euphratica* is a model species for assessing the physiological mechanisms underlying salt tolerance in woody plants (Chen and Polle, 2010; Chen *et al.*, 2014; Polle and Chen, 2015). Its ability to retain ionic homeostasis under salt stress is mostly related to H<sup>+</sup>-ATPases in the plasma membrane (PM) (Sun *et al.*, 2009a, b). Proton pumps in *P. euphratica* have been shown to promote Na<sup>+</sup> extrusion via Na<sup>+</sup>/H<sup>+</sup> antiport across the PM (Sun *et al.*, 2009a, b, 2010a, b, 2012). In addition to rapid post-translational modulations, particularly by the 14-3-3 protein (Palmgren *et al.*, 1991; Svennelid *et al.*, 1999; Camoni *et al.*, 2000) and the negative modulator PKS5 kinase (Yang *et al.*, 2010), PM H<sup>+</sup>-ATPases may undergo transcriptional regulation under salt stress (Gévaudant *et al.*, 2007; Zhang *et al.*, 2017).

Up-regulation of the PM H<sup>+</sup>-ATPase gene enabled *Aeluropus litoralis* to cope with salt stress (Olfatmiri *et al.*, 2014). Constitutively expressed wild-type (WT) PM H<sup>+</sup>-ATPase 4 (*wtPMA4*) or a *PMA4* mutant lacking the autoinhibitory domain enhances seed germination and plant growth under salt stress (Gévaudant *et al.*, 2007). Ectopic expression of *PeHA1*, a gene encoding the PM H<sup>+</sup>-ATPase in *P. euphratica*, has been shown to significantly increase ATP hydrolysis and proton pumping activity in isolated PM vesicles from Arabidopsis (Wang *et al.*, 2013). In addition, Song *et al.* (2017) demonstrated that *G<sub>2</sub>J11* overexpression increases transcription of H<sup>+</sup>-ATPase genes after alkaline treatment. We previously showed that *PeJ3* overexpression suppressed *AtPKS5* transcription in Arabidopsis. It is likely that *PeJ3* enhances PM H<sup>+</sup>-ATPase activity by suppressing transcription of *AtPKS5* in Arabidopsis (Zhang *et al.*, 2017). However, the direct activation of PM H<sup>+</sup>-ATPase genes by transcription factors has not been thoroughly investigated in tree species under salt stress.

WRKY transcription factors constitute a large class of regulatory proteins in plants under adverse conditions (Eulgem *et al.*, 2000; Rushton *et al.*, 2010; Ishihama *et al.*, 2011; Chen *et al.*, 2012; Hu *et al.*, 2013; Wang *et al.*, 2014). Previous studies have demonstrated that WRKY transcription factors, such as Arabidopsis WRKY8 (*AtWRKY8*), *AtWRKY18*, *AtWRKY40*, and *AtWRKY60*, are involved in salt stress (Seki *et al.*, 2002; Chen *et al.*, 2010; Hu *et al.*, 2013). *AtWRKY8* is a positive regulator of salt stress (Hu *et al.*, 2013). In transgenic wheat plants, *TaWRKY79* enhances tolerance to salt stress (Qin *et al.*, 2013). We have also shown that salinity induces *WRKY1* in *P. euphratica* and that *PeWRKY1* enables transgenic plants to extrude Na<sup>+</sup> and retain K<sup>+</sup> under NaCl stress (Shen *et al.*, 2015a). The salt-induced Na<sup>+</sup> efflux is thought to be promoted by H<sup>+</sup>-ATPase at the PM, as an inhibitor of H<sup>+</sup> pumps (vanadate) significantly reduces the Na<sup>+</sup> flux rate (Shen *et al.*, 2015a).

The WRKY domain is defined by its N-terminal conserved amino acid sequence WRKYGQK. The WRKY protein specifically binds to the *cis*-acting element W-box, and its DNA sequence motif is (T)(T)TGAC(C/T) (Rushton *et al.*, 1996, 2010; Eulgem *et al.*, 2000). *AtWRKY8* can bind to the

W-boxes in the promoters of *RD29A*, *RD29B*, *RD20*, and *ADH1* (Hu *et al.*, 2013). We also found that the *PeHA1* promoter regions harbored a *cis*-acting W-box (see Supplementary Fig. S1 at JXB online), implying that the transcription factor WRKY1 mediates *PeHA1* expression.

In the present study, the objective was to investigate the interaction between *PeWRKY1* and *PeHA1* and its relevance to salt stress tolerance. Transcription factor binding protocols, such as DNA affinity purification sequencing (DAP-seq), yeast one-hybrid (Y1H) assay, EMSA, luciferase reporter assay (LRA), and virus-induced gene silencing (VIGS), were used to explore the binding of *PeWRKY1* to the *cis*-acting W-box in the *PeHA1* promoter. Salt-induced expression of the *PeHA1* promoter was investigated in root and shoot tissues of transgenic tobacco. Furthermore, after overexpressing *PeWRKY1* in tobacco plants, we examined *in vivo* and *in vitro* H<sup>+</sup> pumping activities and Na<sup>+</sup> fluxes under salt stress. The aim was to confirm whether the increased salt tolerance was due to *PeWRKY1*-up-regulated gene expression of H<sup>+</sup>-ATPase in transgenic plants.

## Materials and methods

### Plant material and salt treatment

One-year-old *P. euphratica* seedlings were obtained from the Xinjiang Uygur Autonomous Region of Northwest China. In April, seedlings were planted in 10 liter pots containing 1:1 sand and soil and kept at 20–25 °C in a greenhouse at Beijing Forestry University. Potted seedlings were irrigated and fertilized every 2 weeks with 1 liter of full-strength Hoagland's nutrient solution. The photosynthetically active radiation (PAR) was 150–600 μmol m<sup>-2</sup> s<sup>-1</sup> during the culture period. The relative humidity was maintained at 40–70%. For salt treatment, 120 uniform seedlings were subjected to moderate salt stress by watering with 2 liters of NaCl (200 mM) in full-strength Hoagland's nutrient solution. The salt-resistant *P. euphratica* did not exhibit symptoms of salt injury during salt exposure (Chen *et al.*, 2001). Total RNA was extracted from the roots and upper mature leaves (leaf index numbers 4–20 from the shoot apex) at 0, 1, 3, 6, 12, 24, 48, and 72 h after salt treatment.

### RNA isolation and cloning full-length *PeWRKY1*

Total RNA was extracted from leaves using the EASYspin Plus Complex Plant RNA Kit (Aidlab Biotech, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesized in a reverse transcription reaction including 1 μg of total RNA, oligo(dT) primer, and M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR cloning of full-length *PeWRKY1* was performed in a total volume of 25 μl containing 1 μl of cDNA product, 1 U of Ex Taq polymerase (Takara, Dalian, China), 2.5 μl of 10× Ex Taq buffer, 2 μl of dNTP mixture (2.5 mM), and 0.5 μl of forward and reverse primers (10 μM) designed based on the homologous *WRKY1* sequence from *P. euphratica*. The 5' to 3' primer sequences were: forward, 5'-ATGGCTGCTTCTTCAGGGAG-3'; and reverse, 5'-CTACCAAAAACCTCTCTACTTCC-3'. The PCR product was gel purified and then ligated to the pMD18-T vector (Takara) for DNA sequencing.

### Quantitative real-time PCR analysis of *PeWRKY1* and *PeHA1* in *P. euphratica*

Total RNA was extracted from *P. euphratica* roots and upper leaves using an EASYspin Plus Complex Plant RNA Kit (Aidlab Biotech,

Beijing, China) according to the manufacturer's instructions. The *P. euphratica* leaves (leaf index numbers 4–20 from the shoot apex) and roots were sampled after 0, 1, 3, 6, 12, 24, 48, and 72 h of salt treatment (200 mM NaCl). The isolates then were treated with RNase-free DNase I (Takara) for 30 min at 37 °C. First-strand cDNA was synthesized using 1.0 µg of total RNA with the SuperScript™ First Strand Synthesis System (Promega). The cDNA product (1 µl) was used for SYBR Green-based real-time PCR (RT-PCR) analysis in the 7500 Fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Three biological replicates were established in each treatment group and *PeACT7* was used as an endogenous reference. Three technical replicates were analyzed for each biological sample. **Supplementary Table S1** lists the forward and reverse primers for *PeHA1*, *PeWRKY1*, and *PeACT7*. The expression of the target gene was normalized to that of the reference gene (*PeACT7*) using the  $2^{-\Delta\Delta C_t}$  method. The PCR running conditions were as follows: 95 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finally 72 °C for 10 min (Shen *et al.*, 2015a, b).

#### DNA affinity purification sequencing

DAP-seq binding assays were performed as described by O'Malley *et al.* (2016) and Bartlett *et al.* (2017), with modifications as described briefly herein.

#### Nuclear isolation and genomic DNA extraction

Fresh *P. euphratica* leaves were ground to fine powder using liquid nitrogen. The powder was resuspended in 2 ml of cold nuclear isolation buffer. The solution was filtered through a 30 µm mesh cell strainer and centrifuged at 2000 g for 4 min at 4 °C, after which the supernatant was discarded. The nuclear DNA was resuspended in 100 µl of phosphate-buffered saline (PBS). Then 1 µl of RNase was added at 37 °C for 30 min, followed by 3 µl of proteinase K at 55 °C for 30 min, and 100 µl of 2× cetyltrimethylammonium bromide (CTAB) at 65 °C for 30 min. Finally 200 µl of phenol/chloroform/isoamyl alcohol was added and centrifuged at 11 000 g for 10 min at room temperature. The supernatant was transferred into a new 1.5 ml Eppendorf tube, to which 2.5× volumes of ethanol were added, and incubated at –80 °C for 1 h, followed by centrifuging at 11 000 g for 10 min at room temperature. The supernatant was discarded, and the tube was dried at room temperature. The DNA was dissolved in 50 µl of Tris-EDTA (TE) buffer.

#### DAP-seq genomic DNA library preparation

Genomic DNA (gDNA; 5 µg in 130 µl of TE buffer) was fragmented to an average of 200 bp using a Covaris M220 (Woburn, MA, USA) as per the manufacturer's recommended settings. The fragmented gDNA then was purified using AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA) at a DNA to beads ratio of 0.7–1.1. The beads were incubated with the gDNA for 5 min at room temperature, placed on a magnet to immobilize the beads, and then the supernatant was removed. The beads were washed twice with 200 µl of 80% ethanol and allowed to dry. Once dry, they were resuspended in 22 µl of resuspension buffer, incubated at room temperature for 5 min, and placed on the magnet. The DNA-containing supernatant was then transferred to a new tube. Libraries were constructed using the NEXTFLEX Rapid DNA-Seq Kit (PerkinElmer, Inc., Austin, TX, USA) according to the instruction manual.

#### DAP-seq protein expression

The coding sequencing of *PeWRKY1* was cloned into a pFN19K HaloTag T7 SP6 Flexi expression vector. Halo-PeWRKY1 fusion protein was expressed using the TNT SP6 Coupled Wheat Germ Extract System (Promega) following the manufacturer's specifications for expression in a 50 µl reaction with a 2 h incubation at 37 °C. Expressed proteins were directly captured using Magne Halo Tag Beads (Promega).

#### DAP-seq binding assay and sequencing

The protein-bound beads were incubated with 50 ng of adaptor-ligated gDNA fragments on a rotator for 1 h at room temperature in 50 µl of wash/bind buffer. Beads were washed three times using the same wash buffer to remove unbound DNA fragments. The HaloTag beads were resuspended in 30 µl of elution buffer and heated to 98 °C for 10 min to denature the protein and release the bound DNA fragments into solution. The supernatant was transferred to a new well, and 25 µl were used in a 50 µl PCR employing the KAPA HiFi HotStart ReadyMix PCR Kit (Roche, Basel, Switzerland) for 10 cycles. PCR primers consisted of the full-length Illumina TruSeq Universal primer (5'-AATGATACGGGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3') and an Illumina TruSeq Index primer (5'-CAAGCAGAAGACGGCATAACGAGAT-NNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'), where NNNNNN represents the 6 bp sequence index used for sample identification. The PCR product was purified and selected using AMPure XP beads (Beckman), as previously described, and resuspended in 20 µl of nuclease-free water. DNA concentrations were determined using a Qubit (Life Technologies, Burlington, Ontario, Canada). Eluted DNA fragments were sequenced on an Illumina NavoSeq. Negative control mock DAP-seq libraries were prepared without the addition of protein to the beads.

#### DAP-seq data processing

Reads were mapped to the *P. euphratica* genome sequence (<https://www.ncbi.nlm.nih.gov/genome/13265>; Ma *et al.*, 2013) using BOWTIE2 (Langmead *et al.*, 2012). Peak calling was done using Macs2 (Zhang *et al.*, 2008). Association of DAP-seq peaks located upstream or downstream of the transcription start site (TSS) within 3.5 kb were analyzed using Homer (Heinz *et al.*, 2010), based on the General Feature Format (GFF) files. Gene function annotation was blasted from NR, NT, Swissprot, and Pfam databases. FASTA sequences were obtained using BEDTools for motif analysis (Quinlan *et al.*, 2010). Motif discovery was performed using MEME-Chip suite 5.0.5 (Machanic *et al.*, 2011).

#### Luciferase reporter assay

The LRA was conducted according to previously described procedures (Hellens *et al.*, 2005). Briefly, *PeWRKY1* cDNAs were cloned into the effector plasmid pGreenII 62-SK, and the *PeHA1* promoter (*PeHA1pro*, 1266 bp) sequence was cloned into the reporter plasmid pGreenII 0800-LUC. The negative controls were recombined without the *PeWRKY1* or *PeHA1* promoter. *Agrobacterium tumefaciens* GV3101 (pSoup-p19) was used for transformation of recombinant plasmids. *Agrobacterium tumefaciens* was inoculated into Luria-Bertani (LB) medium containing 25 µg ml<sup>-1</sup> rifampicin and corresponding antibiotics (50 µg ml<sup>-1</sup> kanamycin). The *A. tumefaciens* cultures were incubated at 28 °C in a shaker (250 rpm) until the optical density (OD<sub>600</sub>) reached 1.0. The bacterial solution was centrifuged at 4000 rpm for 5 min at room temperature, resuspended by tobacco injection buffer (10 mM MES pH 5.6, 10 mM MgCl<sub>2</sub>, 150 µM acetosyringone), and the OD<sub>600</sub> was adjusted to 0.6. Equal concentrations and volumes of *A. tumefaciens* constructs, *PeHA1pro*-LUC, pGreenII 0800-LUC, *PeWRKY1*-62-SK, and pGreenII 62-SK were co-transformed into fully developed *Nicotiana benthamiana* leaves (6 weeks) using needle-free syringes, and incubated for 48–60 h (Chen *et al.*, 2008). D-Fluorescein (1 mM) was sprayed onto the tobacco leaves and then photographed using an LB983 Night Owl II fluorescence imaging system (Berthold Technologies, Bad Wildbad, Germany). The relative luminescence intensity was calculated using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). The LRA experiments were repeated at least three times.

#### Virus-induced gene silencing of *PeWRKY1* in *P. euphratica*

VIGS assays were performed according to our previous reports (Shen *et al.*, 2015a, b). Briefly, a 250 bp fragment of *PeWRKY1* was cloned via PCR using the specific primers forward, 5'-CACAACTTCATCAGCCTT-3' and reverse, 5'-AGACTGATACTGTGGTGGTTG-3'. Empty pTRV2,

constructed pTRV2-*PeWRKY1*, and the pTRV1 vector were transformed into *A. tumefaciens* strain GV3101. *Agrobacterium* strains carrying pTRV2-*PeWRKY1* and pTRV1, or empty pTRV2 and pTRV1 at a ratio of 1:1, infiltrated the mature leaves of *P. euphratica*. *Agrobacterium*-infiltrated seedlings were placed in a growth room in a greenhouse at Beijing Forestry University. A temperature of 28 °C was maintained during the light period and 18 °C during the dark period, with a 16 h photoperiod (07.00 h to 23.00 h). PAR was 150–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Relative humidity was maintained at 60–70%. After 45 d of *Tobacco rattle virus* (TRV) infection, seedlings were subjected to NaCl stress by top watering with 2 liters of 200 mM NaCl in full-strength Hoagland's nutrient solution. RNA was extracted from upper mature leaves (leaf index numbers 4–20 from the shoot apex) after 6 h of salt treatment. The transcriptional levels of *PeWRKY1* and *PeHA1* were examined by quantitative RT-PCR as previously described. The housekeeping gene *PeACT7* was used as an internal reference, and three biological replicates were used for each RT-PCR.

#### Yeast one-hybrid assay

Using Gold Matchmaker™ (Clontech Laboratories, Inc., CA, USA), Y1H assays were used to verify the interaction between *PeWRKY1* and the W-box motif of the *PeHA1* promoter. The *PeHA1* promoter was isolated via genomic walking with a series of PCR amplifications. Supplementary Table S2 shows the primer sequences used for *PeHA1* promoter isolation. The promoter of *PeHA1* (1266 bp) containing the W-box was cloned using specific primers and ligated into the pAbAi vector in front of the reporter gene *AUR1-C* [Aureobasidin A (AbA) resistance]. *PeWRKY1* was cloned and expressed by fusion with the pGADT7 vector. The linearized *PeHA1-pro-pAbAi* plasmid was transferred into Y1H yeast, and the positive strain was identified by testing the AbA inhibition. The *PeHA1-pro-pAbAi* strain was plated on synthetic/–uracil (SD/–Ura) medium supplemented with 0, 700, and 800 ng ml<sup>-1</sup> AbA. The Y1H strain was completely inhibited at 800 ng ml<sup>-1</sup> AbA. Both positive yeast and Y1H blank strains were transferred to *PeWRKY1*-pGADT7, with an empty vector as the control, and cultured in SD/–leucine (–Leu) medium supplemented with 800 ng ml<sup>-1</sup> AbA.

To further determine whether *PeWRKY1* binds to the W-box or other regions of the promoter, the sequence of the W-box in the promoter of *PeHA1* was mutated from TTGACC to TTGAAC. The Y1H assays were performed with the mutated W-box, as previously mentioned. Y1H assays were repeated three times, and representative results are shown.

#### EMSA

To construct the *PeWRKY1*-pGEX-4T-1 vector, the full-length coding sequence of *PeWRKY1* was cloned with a glutathione *S*-transferase (GST) tag at the C-terminus of the pGEX-4T-1 vector. An empty pGEX-4T-1 vector was used as a negative control. The construct was transferred to *Escherichia coli* (BL21) for recombinant protein production. Briefly, the bacteria were cultured in LB medium at 37 °C until an OD<sub>600</sub> of 0.5–0.6, and then cultured at 37 °C for 10 h after induction with 0.5 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG). The bacterial solution was collected by centrifugation, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), and centrifuged after sonicating for 15 min. The supernatant of the cell lysate was collected and incubated with glutathione–Sepharose 4B beads (GE Healthcare, Buckinghamshire, UK) for 2 h at 4 °C, followed by washing five times with PBS. The beads were washed four times with 1 ml of GST elution buffer (50 mM Tris–HCl pH 7.3, 10 mM reduced glutathione). The purified protein was collected in a 1.5 ml centrifuge tube. In parallel, 40 bp DNA fragments from the *PeHA1* promoter (5'-CCCTATCGG GCAAGATTTGACCCGCCCGAGTTGATCCCTC-3' and 5'-GAG GGATCAACTCGGGCGGGTCAAATCTTGCCCGATAGGG-3', harboring the W-box motif) were biotin labeled at the 3' end using an EMSA Probe Biotin Labeling Kit (Beyotime, Nantong, China). Unlabeled probes were subjected to cold competition experiments. Mutant W-box (TTGACC→TTAGCC) probes that were not labeled with biotin were used to confirm the binding specificity of the W-box with *PeWRKY1*. The cold probe concentrations were 5 $\times$  and 10 $\times$ , and the mutant probe

was 5 $\times$ . EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Beyotime) according to the manufacturer's instructions. The GST protein was used as a negative control. The biotin signals were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The EMSAs were repeated three times, and representative results are shown.

#### Generation of *PeWRKY1* transgenic tobacco

To construct the *35S::PeWRKY1::pCABIA2300* overexpression vector, a 1788 bp fragment containing the *PeWRKY1* ORF was amplified from *P. euphratica* cDNA using specific primers (forward, 5'-ATGGCTGCTTCTTCAGGGAG-3'; reverse, 5'-CTACCAAAAA CTCTCTACTTCC-3') and then cloned into the pMD18-T vector. Next, the *PeWRKY1* ORF was cloned into the *EcoRI* and *BamHI* sites of the pCABIA2300 vector driven by the 35S promoter. The *35S::PeWRKY1::pCABIA2300* construct was introduced into *A. tumefaciens* strain GV3101, and then transformed into WT *N. tabacum* using an *Agrobacterium*-mediated method (Shen et al., 2015a). The blank pCABIA2300 vector was introduced into WT tobacco plants as a control. Putative transgenic plants were selected on Murashige and Skoog (MS) medium containing 100 mg l<sup>-1</sup> kanamycin and verified by semi-quantitative RT-PCR using *PeWRKY1*-specific primers (Supplementary Table S1; Supplementary Fig. S2; Shen et al., 2013). WT and transgenic tobacco plants were hydroponically cultured in a 1 liter container and placed in a growth chamber at 25 $\pm$ 1 °C with a light intensity of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and a photoperiod of 16 h light and 8 h dark. The relative humidity was maintained at 50–60%. Among the 15 independent transgenic lines, two independent transgenic lines (L-9 and L-12) exhibited higher abundance of *PeWRKY1* transcript and were used for salt experiments. Supplementary Table S2 lists the specific primers used to clone the promoters of *NiHA2* and *NiHA4*.

#### Phenotype tests of transgenic plants

Four-week-old seedlings of the WT, vector control (VC), and transgenic lines L-9 and L-12 were exposed to 0 or 200 mM NaCl for 3 d before measuring leaf fresh weight and malondialdehyde (MDA) content. Three individual plants per treatment were used for MDA and membrane potential measurements.

#### MDA content

Oxidative damage to lipids was estimated by measuring the amount of MDA in plants according to Wang et al. (2007), with modifications. The leaf samples from control and salt-stressed plants were mixed with 10% trichloroacetic acid (TCA) containing 0.6% 2-thiobarbituric acid (TBA) and cooled to room temperature after boiling for 15 min. The absorbance at 450, 532, and 600 nm was examined to calculate the MDA content (Wang et al., 2007).

#### Membrane potential measurements

WT, VC, and transgenic lines L-9 and L-12 were subjected to 0 or 200 mM NaCl for 24 h. The membrane potential at the apical region (300  $\mu\text{m}$  or 400  $\mu\text{m}$  from the root tip) was measured using silver/silver chloride microelectrodes [XY-CGQ03; Xuyue (Beijing) Sci. & Tech Co. Ltd, Beijing, China] as described previously (Sa et al., 2019).

#### Steady-state ion flux measurements

Four-week old seedlings of WT, VC, and *PeWRKY1*-transgenic L-9 and L-12 plants were treated with 0 or 200 mM NaCl for 24 h. The Na<sup>+</sup> flux was recorded using a non-invasive microtest system (NMT-YG-100, Younger USA LLC, Amherst, MA, USA) with the ion flow rate measurement software iFluxes 1.0 (Younger USA) and signal processing software ASET 2.0 (Sciencewares, Falmouth, MA, USA), as previously described (Sun et al., 2009a, b, 2010a). Five to six individual plants of control and salinized plants were used, respectively, to examine the Na<sup>+</sup> flux.

### Detection of Na<sup>+</sup> content in tobacco roots

Four-week old seedlings of WT, VC, L-9, and L-12 were transferred to MS liquid solution containing 0 or 200 mM NaCl for 24 h. The Na<sup>+</sup> content was detected with a specific fluorescent probe (Zhang *et al.*, 2017). The tobacco root was exposed to CoroNa-Green AM (20 μM) for 2 h in 5 mM MES-KCl loading buffer (pH 5.7) in the dark and then rinsed 4–5 times with MS solution. The fluorescent intensity was detected using a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) at an excitation wavelength of 488 nm and emission wavelength of 510–530 nm. The relative fluorescence intensity was calculated using Image-Pro Plus 6.0 (Media Cybernetics). Three individual plants for each treatment were used for Na<sup>+</sup> content measurements.

### Purification of plasma membrane vesicles and H<sup>+</sup>-ATPase activity assays

Tobacco plants (WT, VC, L-9, and L-12) were homogenized in cold extraction lysis buffer [0.33 M sucrose, 0.2% (w/v) BSA, 5 mM EDTA, 5 mM DTT, 5 mM ascorbate, 1% (w/v) polyvinyl pyrrolidone (PVP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 mM HEPES-KOH, pH 7.5]. The homogenate was filtered and centrifuged at 13 000 g for 10 min, then centrifuged for 1 h at 100 000 g. The resulting microsomal pellet was resuspended in buffer (0.33 M sucrose, 1 mM PMSF, and 5 mM potassium phosphate, pH 7.8) and added to a phase mixture of 6.3% (w/w) Dextran T-500 and 6.3% (w/w) polyethylene glycol 3350 in 5 mM potassium phosphate (pH 7.8), 0.33 M sucrose, and 3 mM KCl. The upper phase was collected, diluted with buffer containing 0.33 M sucrose, 1 mM PMSF, 2 mM DTT, and 10 mM MOPS-KOH (pH 7.5), and then centrifuged at 110 000 g for 30 min. The pellet was collected and used to measure H<sup>+</sup>-ATPase activity.

The H<sup>+</sup>-ATPase hydrolysis activity was measured according to protocols described by Wang *et al.* (2013). The reaction mixture (200 μl) contained 10 mM MOPS-KOH (pH 6.5), 50 mM KNO<sub>3</sub>, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM NaN<sub>3</sub>, 0.5 mM Na<sub>2</sub>MoO<sub>4</sub>, and 20 mg of protein. The substrate, ATP (1 μl, 200 mM), was added to activate the reaction in the presence or absence of 400 μM Na<sub>3</sub>VO<sub>4</sub>. After 30 min of incubation at 37 °C, the reaction was stopped by adding 10% (w/v) TCA. H<sup>+</sup>-ATPase hydrolysis activity was measured by determining the Pi released from ATP.

The transport activity of PM H<sup>+</sup>-ATPase was measured by fluorescence quenching of acridine orange at 495 nm (Palmgren, 1990). The PM protein (50 mg ml<sup>-1</sup>) was mixed with reaction buffer containing 20 mM MES-KOH (pH 7.0), 140 mM KCl, 3 mM ATPNa<sub>2</sub>, 10 μM acridine orange, and 0.0125% (v/v) Triton X-100, and then incubated for 10 min at room temperature. The reaction was initiated using 3 mM MgSO<sub>4</sub>. The measurements for hydrolysis and transport activities of H<sup>+</sup>-ATPase were repeated three times, and mean values are given.

### Construction of *PeHA1-pro::GUS* and transformation to tobacco

*PeHA1-pro*, the promoter of *PeHA1*, was cloned into the PMD18-T vector (Takara) and then transferred to a PBI121 vector. The promoter of *PeHA1* was merged with the *GUS* gene and transformed into *A. tumefaciens* GV3101 (Shen *et al.*, 2015a). PBI121-*PeHA1-pro::GUS* was finally transferred into WT tobacco using the leaf disc method (Shen *et al.*, 2015a). We selected the putative transgenic plants on MS medium containing 200 mg l<sup>-1</sup> kanamycin. Tobacco plantlets were incubated in a growth chamber at 25±1 °C with a relative humidity of 50–60%, a photoperiod of 16 h light/8 h dark, and a light intensity of 50 μmol m<sup>-2</sup> s<sup>-1</sup>. WT and *PeHA1-pro::GUS*-transgenic tobacco (T<sub>1</sub>) seeds were allowed to germinate in MS medium. After culturing for 4 weeks, seedlings were treated with 0 or 200 mM NaCl for 24 h. Root, leaf, and stem tissues were sampled for GUS staining. Three individual plants for each treatment were used for GUS assays.

### In situ localization of GUS

Root, leaf, and stem samples from *PeHA1-pro::GUS* transgenic tobacco and WT plants were soaked in X-Gluc solution {50 mM sodium phosphate buffer pH 7.0, 10 mM Na<sub>2</sub>EDTA, 0.5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>]·3H<sub>2</sub>O; 0.5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 0.1% Triton X-100, and 1 mg ml<sup>-1</sup> X-Gluc} at 37 °C for 12 h (Shen *et al.*, 2015a). The chlorophyll in the samples was removed with increasing concentrations of ethanol (70%→100%) at room temperature. The samples were fixed with FAA fixative (1:18:1 mix of formalin:alcohol:acetic acid), followed by gradient dehydration with increasing ethanol solutions (70%→85%→95%→100%→100%, 1–2 h for each ethanol concentration).

The protocols for subsequent infiltration were as follows: an equal volume mixture of dimethylbenzene and ethanol for 1 h→pure dimethylbenzene for 1 h→pure dimethylbenzene for 1 h. The samples were embedded with paraffin solution, and the wax blocks were trimmed to a regular trapezoid shape using a razor blade. Sections with a thickness of 25 μm were cut using an automatic vibration slicer (Leica VT1000 S, Leica Instruments, Nussloch, Germany). The sections were stuck on a glass slide and baked at 40 °C. Dewaxing was performed as follows (5–10 min for each chemical treatment): dimethylbenzene→1/2 dimethylbenzene+1/2 pure ethanol→100% ethanol→95% ethanol (rapid)→100% ethanol→1/2 dimethylbenzene+1/2 pure ethanol→dimethylbenzene. An appropriate amount (1–2 drops) of neutral gum was added to the slide, which was covered with a clean coverslip. The sections were incubated at 42 °C, dried overnight, and detected using an Olympus BX43 light microscope (Olympus Corporation, Tokyo, Japan). We used a stereomicroscope (Leica M205 C, Leica, Wetzlar, Germany) to observe the samples and acquire images. Representative images are shown.

### Protein extraction and fluorometric GUS assays

Based on Jefferson's protocols (Jefferson, 1987), GUS activity in *PeHA1-pro* transgenic tobacco leaves (with main veins), stems, and roots was determined using 4-methyl-umbelliferyl glucuronide (Sigma-Aldrich) as a substrate for fluorescence assays. The protein concentration of the extract was determined using BSA as a standard protein in a microplate fluorescence spectrophotometer (M200, Tecan Group Ltd, Männedorf, Switzerland). The experimentally detected excitation wavelength was 365 nm, and the emission wavelength was 455 nm.

### Statistical analysis

The data were subjected to ANOVA. Significant differences between means were determined by Duncan's multiple range test. Unless otherwise stated, *P*<0.05 was considered significant.

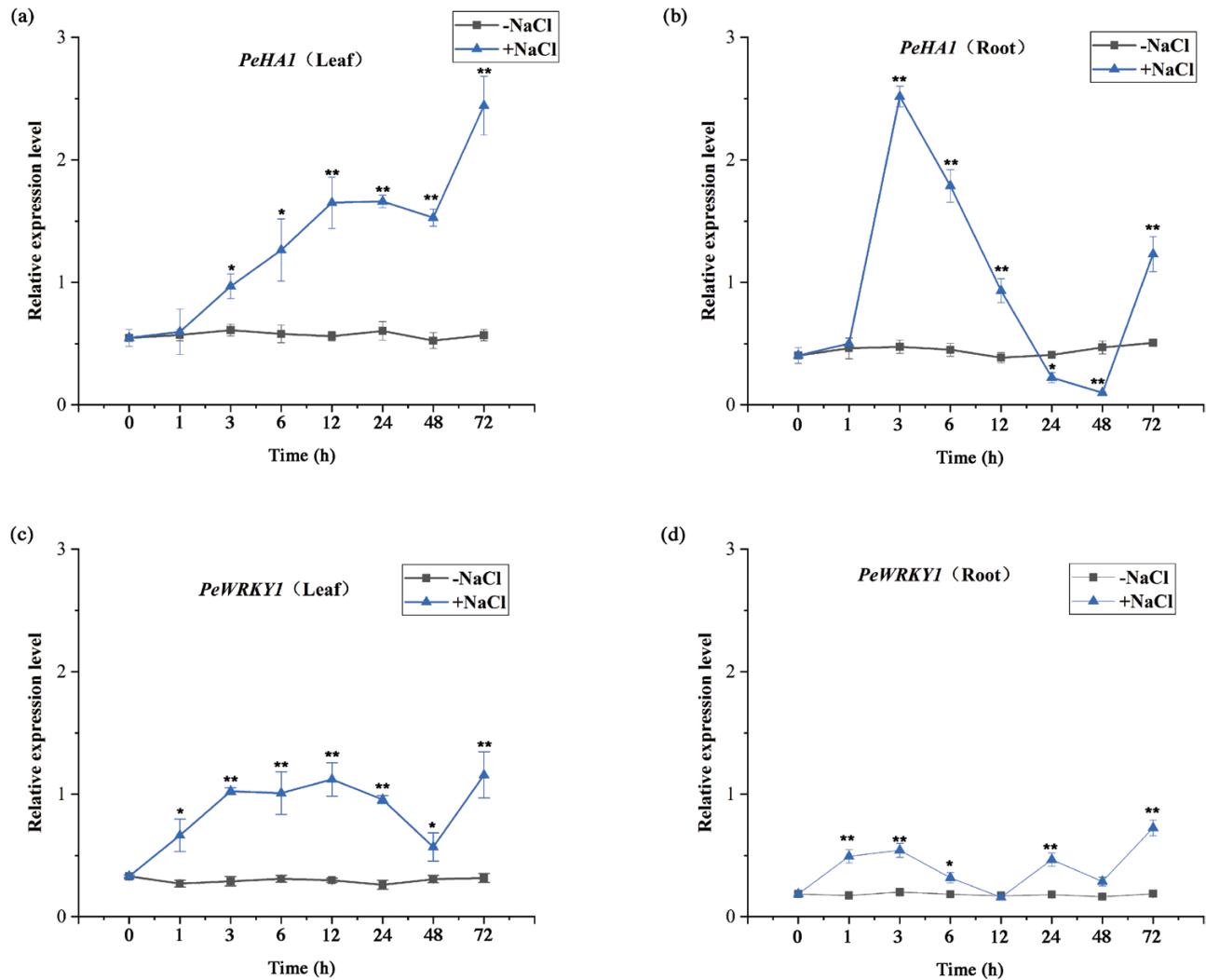
## Results

### Salt-induced transcription of *PeHA1* in *P. euphratica*

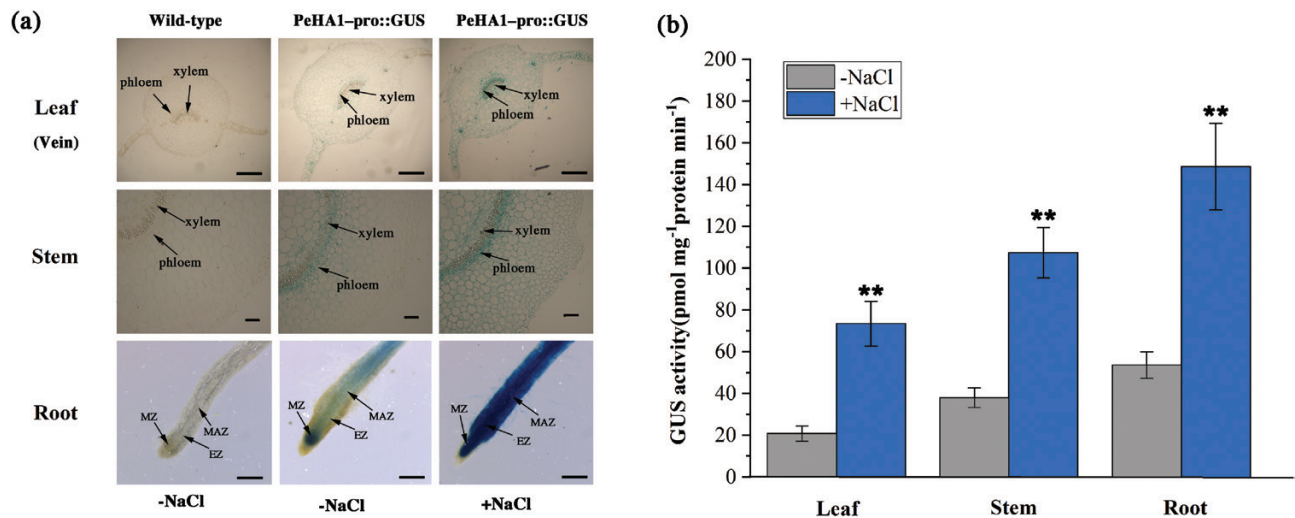
NaCl markedly increased the expression of *HA1* in *P. euphratica*, though the pattern differed between roots and leaves (Fig. 1). In leaves, *PeHA1* steadily increased after the onset of salt exposure, reaching a maximum level at 72 h, although a decline was observed at 24–48 h (Fig. 1a). In roots, an earlier and abrupt increase occurred after 3 h of salt treatment, followed by a significant decline at 24 h and 48 h, then another increase at 72 h (Fig. 1b).

### *PeHA1* promoter analysis

To determine the importance of the upstream regulatory region for salt-induced *PeHA1* expression, a 2022 bp promoter fragment upstream of *PeHA1* was sequenced and



**Fig. 1.** Expression pattern of *PeWRKY1* and *PeHA1* in the roots and leaves of *Populus euphratica* treated with NaCl. *P. euphratica* seedlings were subjected to 200 mM NaCl for 72 h. (a) *PeHA1* in leaves. (b) *PeHA1* in roots. (c) *PeWRKY1* in leaves. (d) *PeWRKY1* in roots. Expression levels of *PeWRKY1* and *PeHA1* were normalized to the *P. euphratica* housekeeping gene *PeACT7* as an internal reference. Primers designed to target *PeWRKY1*, *PeHA1*, and *PeACT7* are shown in [Supplementary Table S1](#). Each point corresponds to the mean of three independent replicates, and bars represent the SEM. \* $P < 0.05$ , \*\* $P < 0.01$ . (This figure is available in color at *JXB* online.)



**Fig. 2.** Anatomical and histochemical analysis of GUS activity in *PeHA1-pro::GUS*-transgenic plants. Four-week-old seedlings of *PeHA1-pro::GUS*-transgenic plants were treated with 0 (-NaCl) or 200 mM NaCl (+NaCl) for 24 h. The wild-type (WT) controls were not treated with NaCl. Root, leaf, and stem samples were taken from control and salinized plants and used for anatomic analysis. (a) GUS staining. Scale bar=500  $\mu\text{m}$ . (b) GUS activity was detectable in *PeHA1-pro::GUS*-transgenic plants regardless of the presence of salt stress, but was not detectable in WT plants. Each column corresponds to the mean of three independent replicates, and bars represent the SEM. \*\* $P < 0.01$ . (This figure is available in color at *JXB* online.)

analyzed using the PLACE and Plant-CARE databases (Supplementary Fig. S1). The promoter sequence contained various *cis*-acting elements, including MRE (MYB-binding site involved in light responsiveness), MYC (*cis*-acting element responsive to drought and abscisic acid), P-box (gibberellin-responsive element), LTR (*cis*-acting element involved in low-temperature responsiveness), CGTCA-motif (*cis*-acting regulatory element involved in methyl jasmonate responsiveness), ARE (*cis*-acting regulatory element essential for anaerobic induction), GC-motif (enhancer-like element involved in anoxic-specific inducibility), MYB (gene element involved in response to drought and ABA signals), G-box (*cis*-acting regulatory element involved in light responsiveness), and ABRE (transcription factors involved in abscisic acid responsiveness). Interestingly, a *cis*-acting element W-box (T)TGAC(C), the binding site for the WRKY family of transcription factors, was present in the promoter region of *PeHA1* (Supplementary Fig. S1).

#### *NaCl* activated the *PeHA1* promoter in root and shoot tissues

The *PeHA1-pro::GUS* fusion was constructed and transferred to tobacco leaves. GUS activity was observed in the root tips, leaf mesophyll and veins, and vascular tissues of the stem (Fig. 2a). NaCl treatment (200 mM, 24 h) significantly increased GUS activity in the root, leaf, and stem tissues (Fig. 2b). Therefore, the salt-enhanced expression of *PeHA1* in *P. euphratica* was shown to be due to promoter activity.

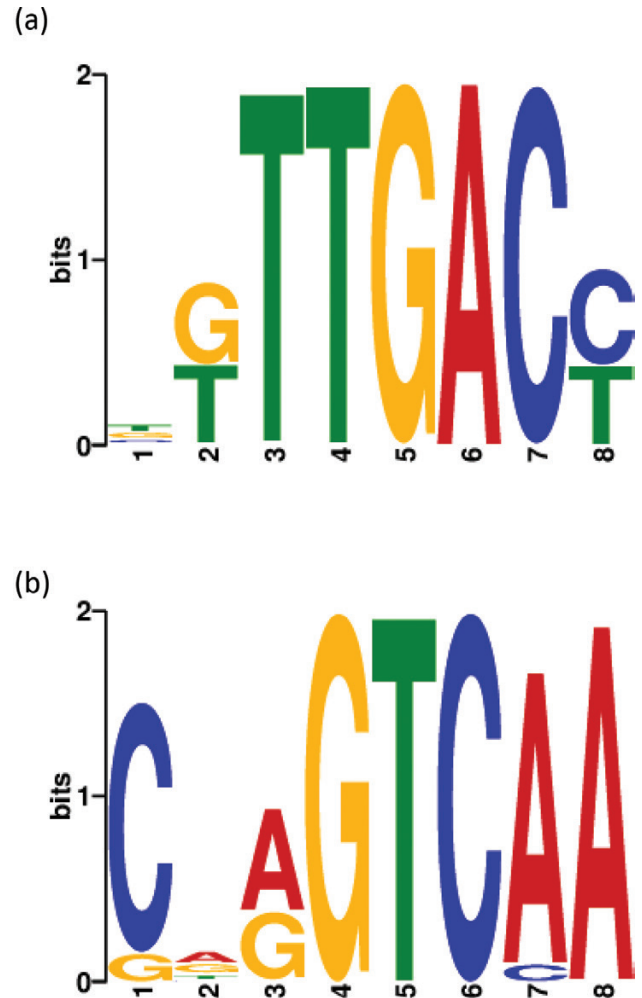
#### Salt-induced transcription of *PeWRKY1* and DAP-seq

NaCl up-regulated *PeWRKY1* transcription in both the roots and leaves (Fig. 1c, d). This result is similar to previous findings by Shen *et al.* (2015a), who found that the *PeWRKY1* transcript level increased during a short-term salt treatment (12 h); however, *PeWRKY1* expression returned to the control level after a long-term salt treatment (7 d). Notably, salt-elicited *PeWRKY1* showed a trend similar to *PeHA1*, but with an earlier increase at 1 h in both roots and leaves (Fig. 1).

DAP-seq was performed to screen direct target genes of the transcription factor, PeWRKY1. The data showed that the top motifs for PeWRKY1-binding were 'TTGAC' and 'GTCAA', respectively (Fig. 3a, b). By screening the binding targets of the transcription factor, *P. euphratica* PM ATPase 4 (LOC105140044), which was named *PeHA1* in our previous study (Wang *et al.*, 2013) and in this work, was the target gene of PeWRKY1 protein (Supplementary Table S3).

#### *PeWRKY1* binds to the W-box of the *PeHA1* promoter

To determine whether *PeWRKY1* interacts with *PeHA1*, Y1H assay, EMSA, LRA, and VIGS were performed to confirm the binding of PeWRKY1 to the W-box in the promoter region of *PeHA1*.



**Fig. 3.** *Populus euphratica* DNA motifs bound by PeWRKY1 protein in DNA affinity purification sequencing (DAP-seq). The two top logos for PeWRKY1 binding are shown in (a) and (b). *P. euphratica* leaves were sampled from each of three individual plants and subjected to DAP-seq. E-value (the statistical significance of the motif)  $1.9\text{e-}108$  or  $1.9 \times 10^{-108}$  (a). E value  $5.2\text{e-}021$  or  $5.2 \times 10^{-21}$  (b). The PeWRKY1 DAP-seq peaks located upstream of the transcription start site (TSS) of the *PeHA1* gene are shown in Supplementary Table S3. (This figure is available in color at JXB online.)

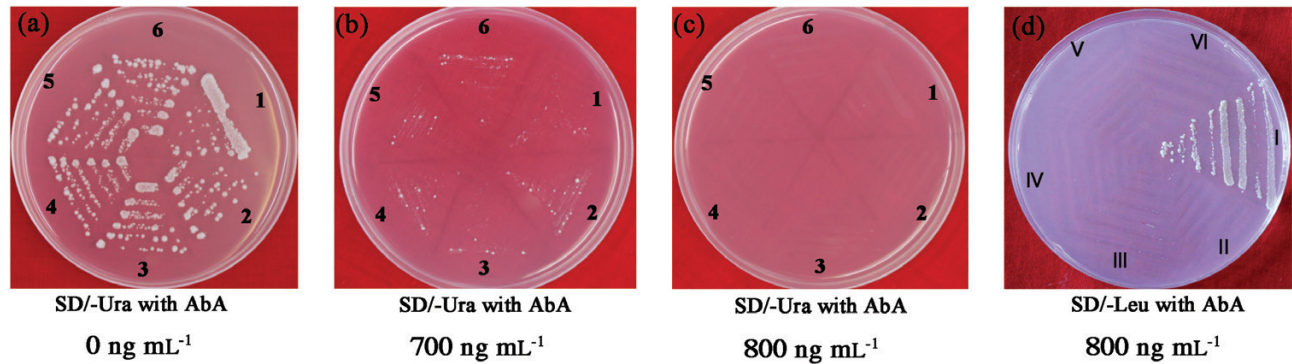
#### Y1H assay

A 1266 bp promoter sequence of *PeHA1* was fused to the yeast vector (pAbAi) with the reporter gene *AUR1-C* (Shen *et al.*, 2015a). pAbAi-*PeHA1pro* grew on SD/-Ura medium containing 0–700 ng ml<sup>-1</sup> AbA, but was inhibited at 800 ng ml<sup>-1</sup> AbA (Fig. 4a–c). However, the Y1H yeast strain grew on SD/-Leu medium (800 ng ml<sup>-1</sup> AbA) with the plasmid carrying *PeWRKY1* (Fig. 4d). The Y1H test demonstrated that PeWRKY1 can interact with *PeHA1-pro*.

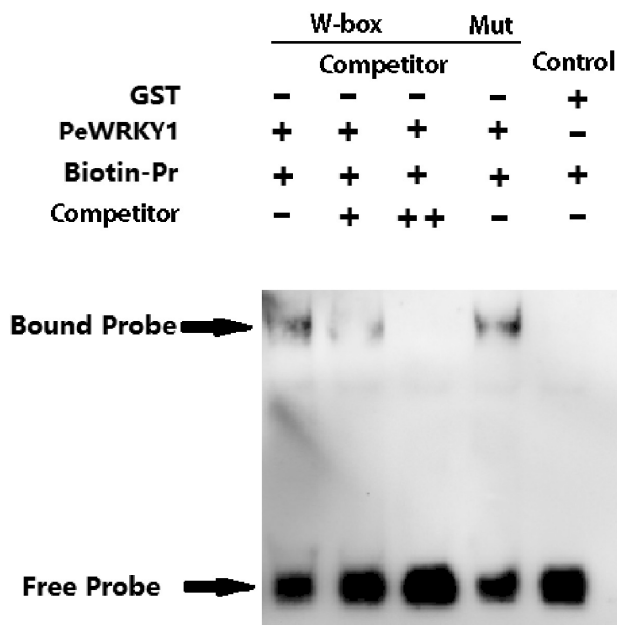
W-box deletion confirmed that PeWRKY1 bound only the W-box *cis*-acting element. Growth of the pAbAi-W-box mutant strain was severely inhibited by AbA in the presence and absence of *PeWRKY1* plasmids (Fig. 4d). Therefore, PeWRKY1 could bind the W-box in the upstream regulatory region of the *PeHA1* promoter.

#### EMSA

The assay was performed with biotin-labeled W-box probes and unlabeled competitive probes. Biotin labeling showed



**Fig. 4.** Yeast one-hybrid analysis of PeWRKY1 binding to the *PeHA1* promoter. The 1266 bp promoter of *PeHA1* was cloned and fused into the pAbAi vector *AUR1-C* gene (with Aureobasidin A resistance). The synthetic W-box mutant promoter sequence (TTGACC mutated into TTGAAC) of *PeHA1* was used as a control. (a–c) pAbAi (*PeHA1pro*)-Y1H (1, 2, 3, and 4) and control (5 and 6) strains were grown on SD/-Ura medium supplemented with 0, 700, or 800 ng mL<sup>-1</sup> AbA. Yeast strains 1, 2, 3, 4, 5, and 6 could not grow on SD medium lacking Ura in the presence of 800 ng mL<sup>-1</sup> AbA, which was used as the screening concentration in the PeWRKY1–W-box interaction experiment. (d) The pAbAi (*PeHA1pro*)-Y1H strain was transferred to the pGADT7 plasmid with or without *PeWRKY1*, and the PeWRKY1–W-box interaction was detected by growth on SD lacking Leu (800 ng mL<sup>-1</sup> AbA). I, pAbAi (W-box)-Y1H-pGADT7 (*PeWRKY1*); II, Y1H-pGADT7; III, pAbAi-Y1H-pGADT7; IV, pAbAi (W-box)-Y1H-pGADT7; V, pAbAi (mutated W-box)-Y1H-pGADT7; VI, pAbAi-Y1H-pGADT7 (*PeWRKY1*). (This figure is available in color at *JXB* online.)



**Fig. 5.** An EMSA validated the PeWRKY1 interaction with the W-box in the *PeHA1* promoter. The purified PeWRKY1–GST protein obtained by prokaryotic expression was used for the *in vitro* assay, and GST protein was used as a negative control. The purified proteins were combined with biotinylated W-box-containing dsDNA probe (1×), and unlabeled probes (Competitor) were subjected to cold competition experiments. The biotin-unlabeled mutant probes (Mut, TTGACC mutated into TTAGCC) were used to confirm the binding specificity of the W-box to PeWRKY1. ‘+’ and ‘-’ indicate the presence or absence, respectively, of proteins and probes in the loading mixture. The cold probe concentrations are 5× and 10×, and that of the Mut probe is 5×. The concentration of the polyacrylamide gel was 4%.

PeWRKY1 protein binding to the W-box motif in the *PeHA1* promoter, whereas the negative control, GST, could not bind to biotin-labeled W-box probes (Fig. 5, lanes 1 and 5). The increasing fraction of competitive probes interfered with the binding of PeWRKY1 to biotin-labeled W-box (Fig. 5, lanes 1–3). The binding specificity of the W-box for PeWRKY1 was

confirmed with a mutated W-box. The W-box mutation probes were obtained with a double-stranded nucleotide mutation and not labeled with biotin. However, the presence of W-box mutation probes did not inhibit the binding of PeWRKY1 to biotin-labeled W-box (Fig. 5, lane 4).

#### LRA

Tobacco leaves co-transformed with *A. tumefaciens* vector controls, pGreenII 0800-LUC and pGreenII 62-SK, showed very low luciferase luminescence (Fig. 6a, b). Co-transformation of pGreenII 0800-LUC and *PeWRKY1*-62-SK exhibited a low luminescence, similar to vector controls (Fig. 6a, b). Luciferase luminescence was observed when *PeHA1pro*-LUC was transformed into tobacco leaves; and the luminescence was significantly enhanced by the co-transformation of *PeWRKY1* (Fig. 6a, b). The LRA confirmed that PeWRKY1 activated the *PeHA1* promoter in *N. benthamiana* leaves.

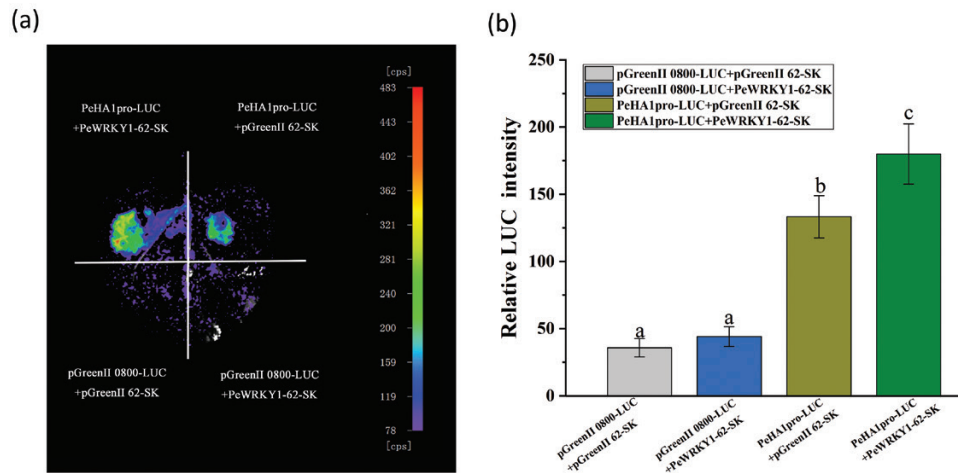
#### VIGS

TRV-based factor pTRV2 was used for VIGS in *P. euphratica* leaves, as described previously by Shen et al. (2015a, b). The expression of *PeWRKY1* in leaves decreased after infiltration of *Agrobacterium* carrying TRV2-*PeWRKY1* (Fig. 7a). Notably, the quantitative RT-PCR assay showed that the expression of *PeHA1* in salinized leaves significantly decreased after 45 d of *Agrobacterium* infiltration (Fig. 7b). This indicates that *PeWRKY1* silencing resulted in the decline of *PeHA1* under saline conditions.

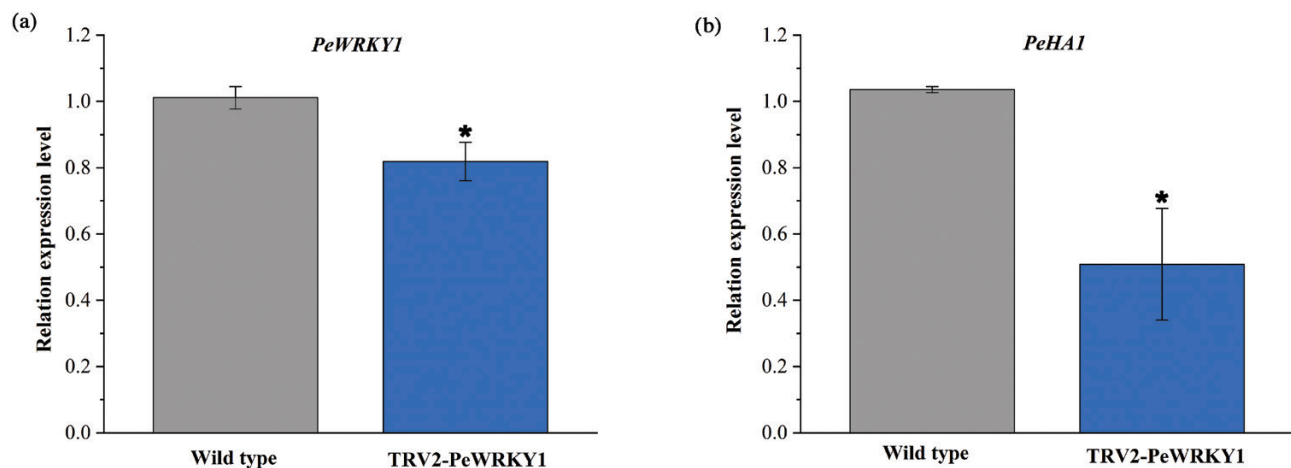
#### Overexpression of PeWRKY1 enhanced salt tolerance in tobacco

In hydroponic culture, *PeWRKY1*-transgenic lines L-9 and L-12 exhibited a greater ability to tolerate salt stress than the WT and VC (Supplementary Fig. S3a). Leaf fresh weight was less suppressed by NaCl and the increase in MDA was lower in transgenic lines than in salt-stressed WT and VC (Supplementary Fig. S3b, c). The salt-elicited oxidative damage





**Fig. 6.** The luciferase reporter assay affirmed that PeWRKY1 activated the *PeHA1* promoter in *Nicotiana benthamiana* leaves. (a) Representative luciferase luminescence image of *N. benthamiana* leaves co-infiltrated with the agrobacterial strains containing *PeHA1pro-Luc* and PeWRKY1-62-SK. Tobacco leaves injected with empty vector controls, pGreenII 0800-LUC and pGreenII 62-SK, were used as a negative control. (b) The luminescence intensity in *Agrobacterium*-injected *N. benthamiana* leaves. Each column corresponds to the mean of three independent replicates, and bars represent the SEM. Columns labeled with different letters denote a significant difference ( $P < 0.05$ ). (This figure is available in color at JXB online.)



**Fig. 7.** Expression of *PeWRKY1* and *PeHA1* in leaves of *PeWRKY1*-silenced *Populus euphratica*. (a) *PeWRKY1* expression. *P. euphratica* leaves were infiltrated with *Agrobacterium* carrying TRV2-*PeWRKY1*, whereas the wild-type controls were transiently injected with a TRV2 empty vector. (b) *PeHA1* expression in *PeWRKY1*-silenced *P. euphratica* seedlings. After 45 d of TRV infection, seedlings were subjected to 200 mM NaCl for 6 h. Expression levels of *PeWRKY1* and *PeHA1* were examined by quantitative RT-PCR. The *P. euphratica* housekeeping gene *PeACT7* was used as an internal reference. Primers designed to target *PeWRKY1*, *PeHA1*, and *PeACT7* are shown in Supplementary Table S1. Each column corresponds to the mean of three independent replicates, and bars represent the SEM. \* $P < 0.05$ . (This figure is available in color at JXB online.)

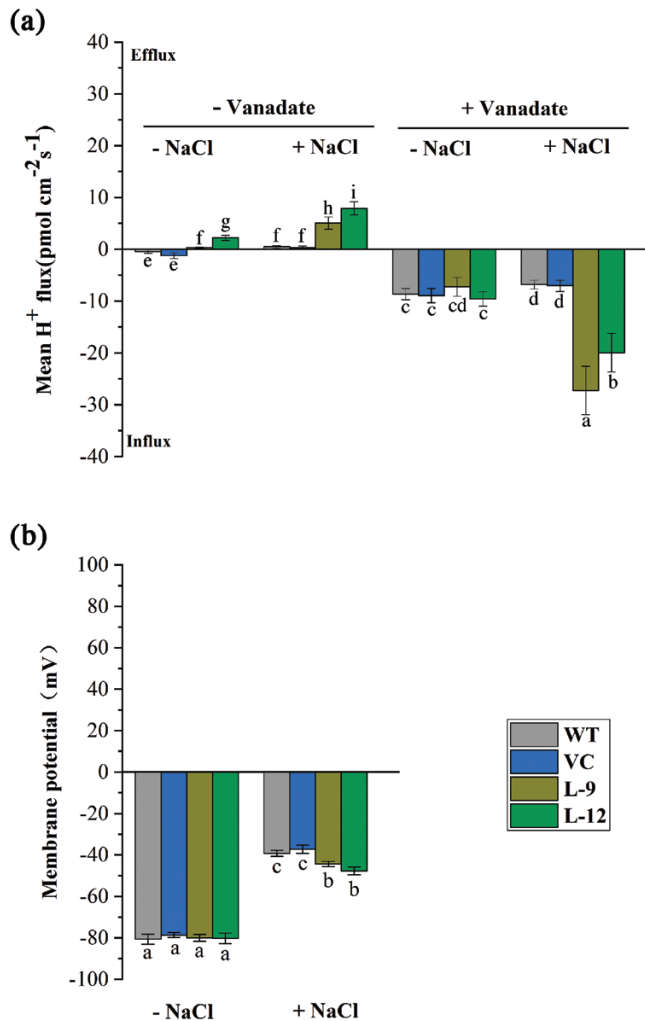
and inhibited growth in WT and VC plants were due to excessive accumulation of  $\text{Na}^+$  ions.  $\text{Na}^+$  concentrations were 36–52% lower in the transgenic lines than in WT and VC plants under salt treatment (Supplementary Fig. S4a). The lower  $\text{Na}^+$  build up was due to the higher  $\text{Na}^+$  efflux in salinized roots of the transgenic lines (Supplementary Fig. S4b).

#### *PeWRKY1* enhanced *NtHA* transcripts and $\text{H}^+$ -ATPase activity

NaCl increased  $\text{H}^+$  efflux in the root tips, and the effect was more pronounced in the transgenic lines (Fig. 8a). However, the salt-elicited  $\text{H}^+$  efflux shifted towards a net influx by vanadate,

a specific inhibitor of  $\text{H}^+$  pumps in the PM (Fig. 8a). Thus, PeWRKY1 promoted proton pumping activity under salinity stress. The membrane potential of root cells was also measured by NMT microelectrodes. The two transgenic lines exhibited a more negative membrane potential than the WT and VC plants, though the membrane hyperpolarization decreased with NaCl treatment (200 mM, 24 h; Fig. 8b). The PM vesicles were isolated from tobacco roots and used for the  $\text{H}^+$ -ATPase activity assay. The hydrolytic and transport activities of  $\text{H}^+$ -ATPase were higher in transgenic lines than in the WT and VC, regardless of whether they were under control or NaCl stress conditions (Fig. 9a, b).

Quantitative RT-PCR showed that NaCl up-regulated the expression of PM  $\text{H}^+$ -ATPase-encoding genes *NtHA2* and *NtHA4* (Fig. 9c, d). Notably, the transcription of *NtHA4* was significantly



**Fig. 8.**  $H^+$  fluxes and membrane potential in roots of wild-type (WT) tobacco and *PeWRKY1*-transgenic plants under salt stress. (a)  $H^+$  fluxes at the root tip. WT, vector control (VC), and transgenic lines (L-9 and L-12) were subjected to 0 (–NaCl) or 200 mM NaCl (+NaCl) for 24 h. The roots were then exposed to sodium orthovanadate (500  $\mu$ M) for 30 min. Steady-state  $H^+$  fluxes were continuously recorded for 10 min at the apical zones (200  $\mu$ m from the root tip) in the absence or presence of the inhibitor. (b) Membrane potential in control and salinized roots was continuously recorded for 10 min at the apical region (200  $\mu$ m from the tip). Each column is the mean of 5–6 individual plants, and error bars represent the SE. Columns labeled with different letters denote a significant difference ( $P < 0.05$ ). (This figure is available in color at JXB online.)

higher in the transgenic lines than in the WT and VC plants in the absence or presence of salt stress (Fig. 9d). We found a W-box in the upstream regulatory region of *NtHA4* (Supplementary Fig. S5) but not in the promoter fragment of *NtHA2* (Supplementary Fig. S6). Therefore, *PeWRKY1* may interact with the W-box and accelerate the transcription of *NtHA4*.

## Discussion

### *NaCl*-elicited *WRKY1* mediates changes in *HA1* expression in *P. euphratica*

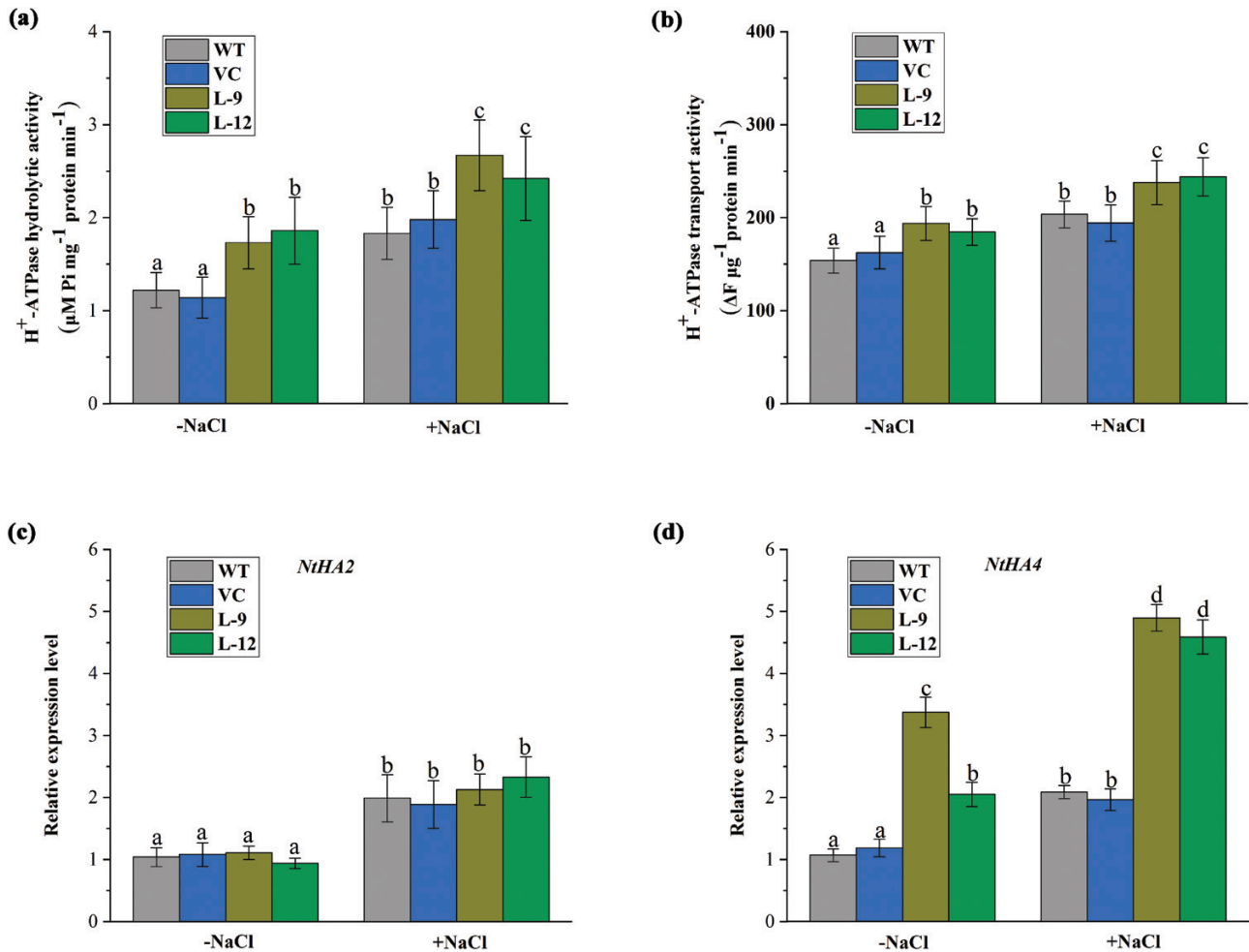
Salt-tolerant *P. euphratica* retain high proton pumping activity to promote  $Na^+$  extrusion under salt stress (Sun *et al.*, 2009a, b,

2010a, b, 2012). Maintaining high expression of ATPase genes is crucial for *P. euphratica* to withstand long-term salt exposure, although regulation of the PM ATPases is primarily post-transcriptional in Arabidopsis (Palmgren *et al.*, 1991; Sennelid *et al.*, 1999; Camoni *et al.*, 2000; Yang *et al.*, 2010). Janz *et al.* (2010) showed that *P. euphratica* exhibits permanent activation of numerous genes responsible for salt exclusion and ion compartmentalization. We have previously shown that control *P. euphratica* plants exhibit a higher transcript abundance of genes related to  $Na^+/H^+$  antiport ( $Na^+/H^+$  antiporters,  $H^+$  pumps) compared with the salt-sensitive poplar, and the expression of these  $H^+$  pump genes did not decrease or was even up-regulated during salt treatment (Ding *et al.*, 2010). Up-regulating the expression of the PM  $H^+$ -ATPase gene increased salt tolerance in Arabidopsis (Gévaudant *et al.*, 2007; Wang *et al.*, 2013) and in *A. littoralis* (Olfatmiri *et al.*, 2014). Here, we showed that NaCl increased the expression of *PeHA1* in *P. euphratica* roots and leaves (Fig. 1). Anatomical and histochemical GUS analyses showed that the *PeHA1* promoter became active in the roots and shoots when the plants were exposed to NaCl (Fig. 2). Therefore, the salt-enhanced expression of *PeHA1* in *P. euphratica* was apparently due to the promoter activity. Notably, *PeHA1* contained a W-box sequence in the promoter (Supplementary Fig. S1), which can be recognized by *PeWRKY1* (Rushton *et al.*, 1996; Eulgem *et al.*, 2000; Rushton *et al.*, 2010). NaCl increased expression of *PeWRKY1* in *P. euphratica* roots and leaves (Fig. 1), which is in agreement with our previous findings in this salt-resistant poplar (Shen *et al.*, 2015a). Similarly, 23 of the 107 WRKY genes can be altered by salt stress in the *P. euphratica* genome (Ma *et al.*, 2015). *PeHA1* induced by salt stress follows a similar trend to *PeWRKY1* in both the roots and leaves (Fig. 1).

DAP-seq revealed *PeHA1* as the target gene of the *PeWRKY1* protein (Fig. 3; Supplementary Table S3). Furthermore, we confirmed that *PeWRKY1* bound the W-box of the *PeHA1* promoter by Y1H assay (Fig. 4), EMSA (Fig. 5), and LRA (Fig. 6). *PeHA1* was down-regulated when *PeWRKY1* was silenced in salinized *P. euphratica* leaves (Fig. 7). Overall, our data revealed that *PeWRKY1* activated *PeHA1* expression by binding to the *cis*-acting W-box of the *PeHA1* promoter in salt-stressed *P. euphratica*. In rice, *WRKY13* physically binds to the promoter region of *SNAC11*, which harbors W or W-like box-type *cis*-regulatory elements for the binding of WRKY transcription factors (Qiu *et al.*, 2009; Tao *et al.*, 2009).

### *PeWRKY1* interacts with *PeHA1*, contributing to increased $H^+$ pumping activity and $Na^+$ exclusion

*PeWRKY1* overexpression enhanced salt tolerance in transgenic plants due to the lower amount of accumulated  $Na^+$  (Supplementary Figs. S3, S4). Similarly, Shen *et al.* (2015a) showed that  $Na^+$  efflux was significantly enhanced in transgenic plants overexpressing *PeWRKY1*.  $Na^+$  exclusion is critical for salt adaption. Excessive accumulation of  $Na^+$  increased lipid peroxidation, as indicated by MDA content (Supplementary Figs. S3, S4; Wang *et al.*, 2007). We have shown that active  $Na^+$  extrusion is promoted by the  $H^+$  pumps in the PM (Wang *et al.*,



**Fig. 9.** H<sup>+</sup>-ATPase activity and gene expression in wild-type (WT) tobacco and *PeWRKY1*-transgenic plants under salt stress. WT, vector control (VC), and transgenic lines (L-9 and L-12) were subjected to 0 (-NaCl) or 200 mM NaCl (+NaCl) for 24 h. Plasma membrane vesicles were isolated from the control and salinized plants and used to measure hydrolytic activity and H<sup>+</sup> transport activity. (a) ATPase hydrolytic activity measured according to the Pi released from ATP. (b) Proton transport activity of ATPase measured by fluorescent quenching of acridine orange at 495 nm. (c and d) Expression of *NtHA2* and *NtHA4*. Expression levels of *NtHA2* (c) and *NtHA4* (d) were examined by quantitative RT-PCR. The tobacco housekeeping gene, *NtEF1α*, was used as an internal reference. Primers designed to target *NtHA2*, *NtHA4*, and *NtEF1α* are shown in [Supplementary Table S1](#). Each column corresponds to the mean of three independent replicates, and bars represent the SEM. Columns labeled with different letters denote a significant difference ( $P < 0.05$ ). (This figure is available in color at *JXB* online.)

2013). *PeWRKY1* overexpression enhanced the activity of PM H<sup>+</sup>-ATPases in both *in vivo* assays with NMT microelectrodes and *in vitro* assays with isolated PM vesicles (Figs 8, 9).

NaCl caused a pronounced H<sup>+</sup> efflux across the PM in the transgenic lines as the result of activated PM H<sup>+</sup>-ATPase, which also caused a negative membrane potential in salinized roots (Fig. 8). *In vitro* H<sup>+</sup>-ATPase assays were consistent with *in vivo* observations (Fig. 9). The activated proton pumps maintained the membrane potential under salt stress and provided the driving force for Na<sup>+</sup> extrusion, thereby avoiding excessive accumulation of Na<sup>+</sup> (Chen *et al.*, 2007; Sun *et al.*, 2009b, 2010a). The high H<sup>+</sup>-pumping activities resulted, at least in part, from increased *HA* transcripts in transgenic tobacco. Our previous studies showed that *PeHA1* overexpression increases the hydrolysis and transport activities of proton pumps in *Arabidopsis* response to salt stress (Wang *et al.*, 2013). Moreover, *PeWRKY1* enhanced Na<sup>+</sup>/H<sup>+</sup> antiport in transgenic plants, which is presumably related to the H<sup>+</sup> pumps (Shen *et al.*, 2015a). *NtHA2* and *NtHA4* were

up-regulated by NaCl in tobacco plants, with *NtHA4* significantly more up-regulated in transgenic plants than in the WT and VC, under both control and saline conditions (Fig. 9). It is likely that *PeWRKY1* bound to the W-box in the promoter of *NtHA4*, enhancing its expression. Consequently, *PeWRKY1* improved the activity of PM H<sup>+</sup>-ATPase, resulting in an increased capacity to tolerate salinity stress. In rice, WRKY13 binds to W-like boxes to regulate gene expression during abiotic and biotic stress (Xiao *et al.*, 2013). In ChIP-quantitative PCR analyses, Hu *et al.* (2013) showed that WRKY8 interacts with one W-box element in the promoter of *RD29A*, directly regulating *RD29A* transcription under salt stress.

Taken together, the results indicate that *PeWRKY1* activated *PeHA1* transcription by binding to the promoter region, conferring H<sup>+</sup> pumping and Na<sup>+</sup> homeostasis control and salt tolerance. Further investigation is needed to determine whether the *PeHA1*-encoded protein was post-transcriptionally regulated in salinized *P. euphratica*.

## Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Promoter sequence analysis of *Populus euphratica* *PeHA1*.

Fig. S2. Semi-quantitative RT-PCR analysis of *PeWRKY1* in wild-type (WT) tobacco and transgenic lines L1–L15.

Fig. S3. Phenotype tests of wild-type (WT) tobacco and *PeWRKY1*-transgenic plants.

Fig. S4. Na<sup>+</sup> concentrations and fluxes in roots of wild-type (WT) tobacco and *PeWRKY1*-transgenic plants.

Fig. S5. Promoter sequence analysis of tobacco *NtHA4*.

Fig. S6. Promoter sequence analysis of tobacco *NtHA2*.

Table S1. Primer sets used for quantitative real-time PCR.

Table S2. Primers used for cloning the promoters of *Populus euphratica* *PeHA1* and *Nicotiana tabacum* L. *NtHA2* and *NtHA4*.

Table S3. *PeWRKY1* DAP-seq peaks located upstream of the transcription start site (TSS) of the *PeHA1* gene.

## Data availability

Physiological data were collected from at least three replicates from wild-type genotype and transgenic plants. Dryad Digital Repository. [https://doi.org/10.5061/dryad.83ng085;Yao \*et al.\* 2020](https://doi.org/10.5061/dryad.83ng085;Yao%20et%20al.%202020).

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## Author contributions

JY, ZS, and SC conceived and designed the experiments; JY, Y-LZ, XW, JW, and GS performed the experiments; JY, JW, RZ, Y-HZ, HZ, CD, JL, SH, YZ, Y-NZ, NZ, and SD analyzed the experimental data; JY, RZ, and SL wrote the article; and SC revised the article.

## Conflict of interest

All authors have no conflict of interest to declare.

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