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Research Article

MdWRKY30, a group IIa WRKY gene from apple, confers tolerance to salinity and osmotic stresses in transgenic apple callus and *Arabidopsis* seedlings

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ABSTRACT

Abiotic stresses threaten the productivity and quality of economically important perennial fruit crops such as apple (*Malus* × *domestica* Borkh.). WRKY transcription factors play various roles in plant responses to abiotic stress, but little is known regarding WRKY genes in apple. Here, we carried out functional characterization of an apple Group IIa WRKY gene (*MdWRKY30*). qRT-PCR analysis found that *MdWRKY30* expression was induced by salt and drought stress. A subcellular localization assay showed that MdWRKY30 is localized to the nucleus. A transactivation assay found that MdWRKY30 has no transcriptional activation activity. A Y2H assay indicated that MdWRKY26, MdWRKY28, and MdWRKY30 interact with each other to form heterodimers and homodimers. Transgenic analysis revealed that the overexpression of *MdWRKY30* in *Arabidopsis* enhanced salt and osmotic tolerance in the seedling stage, as well as during the seed germination and greening cotyledon stages. *MdWRKY30* overexpression enhanced tolerance to salt and osmotic stresses in transgenic apple callus through transcriptional regulation of stress-related genes. Together, our results demonstrate that MdWRKY30 is an important regulator of salinity and osmotic stress tolerance in apple.

1. Introduction

Environmental stresses, such as high salinity and drought, are primary factors that detrimentally affect growth and development of plants, leading to large reductions in crop yields [1]. Both high salinity and drought cause changes in cellular osmotic pressure, which can squeeze water out from the cytoplasm into the extracellular compartment, and lead to a series of changes at plant physiological and biochemical levels, including accumulation of osmolytes, as well as at molecular and cellular levels, including induced expression of stress-related genes [2–5]. Many key regulators of abiotic stress response have been identified. For example, myeloblastosis (MYB) transcription factors (TFs), NAM (no apical meristem), ATAF1/2and CUC2 (cup-shaped cotyledon) (NAC) TFs, WRKY TFs, Ethylene responsive factor (ERF) TFs, and basic leucine zipper (bZIP) TFs [6–10]. Among these TFs, WRKYs are a large family of regulatory proteins forming an accurate network, that have been demonstrated to play crucial roles in various abiotic and biotic stress responses in several plant species [10–12].

The names of plant-specific WRKY TFs are derived from a highly conserved WRKY protein domain, that consists of approximately 60 amino acid residues at the N-terminal end [13]. The WRKY domain contains an invariant WRKYGQK sequence at the N-terminal and zinc finger motif of the C-terminal, that can specifically bind to W-box (T) TGACC (A/T) in the promoter region of the target gene to regulate their expression [10–12]. Based on the number of WRKY domains and the type of zinc finger motif, WRKY proteins can be classified into three groups (I, II, and III). A typical group I protein comprises two WRKYGQK domains and a C_2H_2 zinc finger motif ($CX_4C_{22-23}HXH$); the group II protein contains one WRKYGQK domain and a C_2H_2zinc finger motif($CX_{4-5}C_{23}HXH$), which can be further divided into five subgroups: IIa, IIb, IIc, IId, and IIe; the group III protein contains one WRKY domain and a zinc finger ($CX_7C_{23}HXC$ or $CX_7C_{23}HXH$) [10–12].

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Further studies have shown that the WRKY gene family is divided into five groups (I, IIa + IIb, IIc, IId + IIe, and III) as based on intron position, conservation domains, and evolutionary relationships of the WRKY domains [14].

Several studies have shown that WRKY proteins play important regulatory roles in plant responses to various biotic stresses [13,15–19], as well as a wide-range of developmental processes, such as seed size, germination, and dormancy [20,21], ovule and pollen development [22,23], root hair growth [24], leaf senescence [25], flowering time [26], and UV-B response [27]. Other researchers have demonstrated that WRKY proteins are involved in plant responses to a broad spectrum of abiotic stresses [28–34]. For example, *GhWRKY34* and *VvWRKY30*, WRKY genes isolated from *Gossypium hirsutum* and *Vitis vinifera*, were showed to be positive transcriptional regulators of salt adaptation in transgenic *Arabidopsis* [29,30]. BnaWGR1, a WRKY-type TF isolated from *Brassica napus*, was showed to be a positive transcriptional regulator of ROS accumulation and leaf senescence in transgenic *Nicotiana benthamiana* and *Arabidopsis* [31]. Constitutive overexpression of wheat *TaWRKY33* resulted in enhancing tolerance to heat stress in transgenic *Arabidopsis* [32]. AtWRKY45 acts as a positive regulator in plant response to Pi starvation by direct up-regulation of *PHT1;1* expression [33]. In rice, *OsWRKY89* participates in plant response to UV-B [27]. In addition, *WRKY46*, *WRKY54*, and *WRKY70* negative are involved in drought responses, and positively regulate BR-mediated plant growth [34].

Apple (*Malus* × *domestica* Borkh.) is a widely cultivated and an economically important perennial fruit crop. However, abiotic stress threatens growth and development of apple, as well as its yield and quality. As such, it is necessary to understand the function and expression of regulatory genes that enhance abiotic stress tolerance [13]. Previous studies have shown that apple WRKY TFs are involved in plant responses to biological stressors. For example, transgenic tobacco plants overexpressing *MdWRKY1* positively regulate resistance to the pathogen *Phytophthora parasitica* [35]. Overexpression of *MdWRKYN1* causes increased resistance to the apple leaf spot fungus *Alternaria alternaria f.* sp. *mali* by up-regulating the expression of some *PR* genes [36]. *MdWRKY15* and *MdWRKY46* enhanced apple resistance to *Botryosphaeria dothidea* by activating the expression of *MdICS1* and *MdPBS3.1*, respectively, in the salicylic acid signaling pathway [19,37]. In addition, the expression of various apple *WRKY* genes was significantly up-regulated under waterlogging and drought stresses, as well as *Alternaria alternata*apple pathotype infection [38,39]. MdWRKY40 was identified as an MdMYB1-interacting protein, and promoted wounding-induced anthocyanin biosynthesis through enhancing binding of MdMYB1 to its target genes in response to wounding [40]. In the present study, we isolated a novel group IIa WRKY TF, *MdWRKY30*, from apple "Golden Delicious", and found that it was up-regulated by drought and salinity stresses. Abiotic stress tolerance assays indicated that overexpression of *MdWRKY30* in transgenic apple callus and *Arabidopsis* seedlings significantly increased tolerance to osmotic and salt stresses. These results provide a basis for exploring the molecular mechanism of MdWRKY30 in response to abiotic stress, and provide a foundation study for engineering stress-tolerant apple crops.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

Tissue cultures of the 'Gala' cultivar in vitro were grown on the subculture medium (MS medium supplemented with 1.5 mg/L 6-BA, 0.2 mg/L IAA) at 24 °C under a 16-h light/8-h dark photoperiod. The roots of 'Gala' tissue-cultured seedlings were treated with water (control), 300 mM Mannitol, or 150 mM NaCl for 0, 2, 4, and 8 h. All of the tissues were immediately frozen in liquid N_2 and stored at −80 °C.

Seedlings of *Arabidopsis thaliana* L. (Heyn), cv. Columbia ("Col"), were used for genetic transformations and assays of NaCl and osmotic tolerance. They were cultured in a growth chamber under a 16-h photoperiod at 23 °C. 'Col' and transgenic seeds were harvested at the same time and stored for 3 months. For seed germination and cotyledon greening analyses, approximately 80 seeds were surface-sterilized and sown on MS agar medium containing 0, 100, or 200 mM of NaCl, and 200 or 300 mM of mannitol. Seeds were vernalized at 4 °C for 3 days before growth at 23 °C under 16 h light/8 h dark conditions. Seedlings with fully emerged radicle tips and green cotyledons were scored for seed germination and cotyledon greening rates, respectively. For the osmotic stress assay, five-day-old seedlings grown on MS agar plates were vertically plated on an MS agar medium supplemented with 0, 100, or 150 mM of NaCl, and 200 or 300 mM of mannitol. Their root lengths, fresh weights, relative electrolyte leakage (REL), and concentrations of chlorophyll, malondialdehyde (MDA), and proline were measured 11 days after that transfer.

2.2. Cloning of MdWRKYs and expression analysis

Total RNA was isolated from *Arabidopsis* leaves using Trizol reagent (Thermo-Fisher Scientific) and from previously frozen apple tissues with a CTAB-based method, before cDNA synthesis was performed with a PrimeScript First-Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) [41]. For cloning *MdWRKY*s, complete open reading frames (ORFs) were obtained via RT-PCR from fully expanded leaves of "Golden Delicious" apple, using specific primers listed in Table S4. For the qRT-PCR assays, reverse-transcription was performed with 1 μg of total RNA from each sample, followed by PCR-amplification of 1 μL of the product. We conducted the qRT-PCR assays in 20-μL reaction mixtures that contained 10 μL of SYBR® Premix Ex Taq™ (TaKaRa), and used a QuantStudio 5 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration of cDNA was diluted to 1–10 ng μL^{-1}, and 1-μL aliquots were used for qRT-PCR. Relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method [42], and the quantification results were normalized by *AtActin2* for transgenic *Arabidopsis* and *MdMDH* for apple tissues [43,44]. At least three independent biological replicates per sample were used, and the primers are listed in Supplementary Table 1.

2.3. Sequence alignments and phylogenetic analysis

Multiple sequence alignments were carried out on 6 full-length WRKY amino acid sequences using DNAMAN 6.0.3.99 with default parameters. The phylogenetic tree of apple WRKY representatives was constructed with MEGA 6.06 software using the neighbor-joining (NJ) method together with amino acid sequences of WRKY proteins from various species. These parameters were used in the NJ method: bootstrap (1000 replicates), complete deletion, and amino: p distance.

2.4. Subcellular localization assays

The full-length cDNAs without the stop codon of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* were introduced into the pCAMBIA2300-GFP vector. The primers are listed in Supplementary Table 1. The fusion vectors were then introduced into *Agrobacterium tumefaciens* strain EHA105 and then infiltrated into tobacco leaves. Those infected tissues were analyzed 72 h after infiltration, under a fluorescence microscope (BX63; Olmypus, Tokyo, Japan).

2.5. Transcriptional activation assays

Transcriptional activation assays were performed according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA, USA). The full-length cDNAs, as well as their various truncations of MdWRKY26 and MdWRKY28 and the full-length cDNA of MdWRKY30, were introduced into the pGBKT7 vector. The fusion vectors were then transformed into the yeast strain 'AH109′ by the lithium acetate method

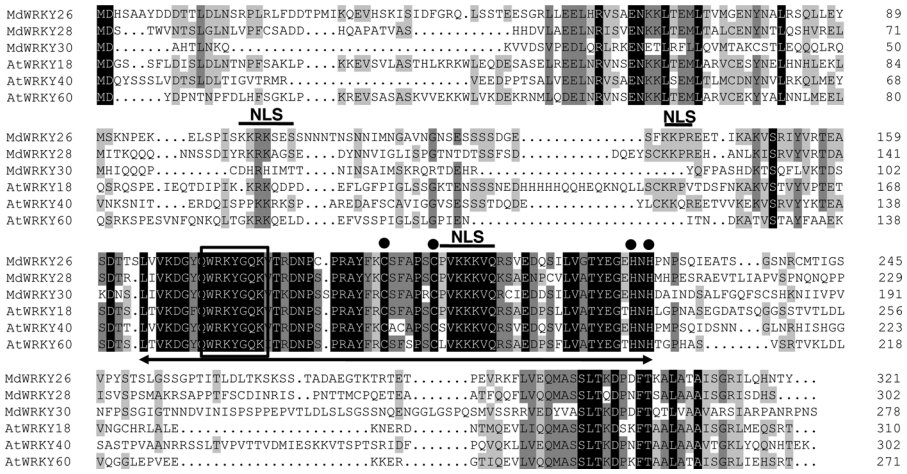

Fig. 1. Alignment of the deduced amino acid sequence of MdWRKY26/28/30 with DNAMAN 6.0 software. Black-highlighted residues are identical, while light gray-highlighted residues are similar in all proteins. The WRKY domain and the C and H residues in the zinc-finger motif are marked by a two-headed arrow and dot, respectively. The WRKYGQK in the WRKY domain is boxed. The putative nuclear localization signals are marked by lines.

and tested for *LacZ* reporter gene expression by assaying for β-galactosidase activity. The cells were plated on yeast synthetic dropout medium lacking Trp (SD/-Trp) and cultured at 30 ˚C for 3 d. To screen for transcriptional activation, we transferred those colonies to yeast synthetic dropout medium that was supplemented with X-α-gal but lacked Trp, His, and Ade (SD/-Trp-His-Ade), and we used empty pGBKT7 vector as the negative control.

2.6. Yeast two-hybrid assays

Yeast (*Saccharomyces cerevisiae*) two-hybrid (Y2H) assays were performed according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA, USA). Because the full-length MdWRKY26 and MdWRKY28 proteins exhibited strong self-activation, we used truncated fragments to test those interactions. The N-terminus deletion versions of MdWRKY26 and MdWRKY28, and the full-length cDNA of MdWRKY 31, were cloned into the Y2H bait vector pGBT9 (GAL4 DNA-binding domain). Meanwhile, the full-length cDNAs of MdWRKY26, MdWRKY28, and MdWRKY30 were recombined into the Y2H prey vector pGAD424 (GAL4 activation domain). The primers are listed in Supplementary Table 1. Different combinations of these plasmids were co-transformed into the yeast strain 'Y2H Gold' by the lithium acetate method and tested for *LacZ* reporter gene expression by assaying for β-galactosidase activity [45]. The cells were plated on yeast synthetic dropout medium lacking Trp and Leu and cultured at 30 ˚C. To screen for interactions, we transferred those colonies to yeast synthetic dropout medium that was supplemented with X-α-gal but lacked Trp, Leu, His, and Ade, and used the empty pGAD424 prey vector and pGBT9 bait vector as negative controls.

2.7. Vector construction and plant transformation

To construct the *MdWRKY* overexpression (OE) vectors, the full-length cDNAs of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* were inserted into pRI 101-AN plant transformation vectors that were driven by the cauliflower mosaic virus (CaMV) 35S promoter. The primers are listed in Supplementary Table 1.

For *Arabidopsis* transformation, the three recombinant plasmids described above were introduced into the 'Col-0′ ecotype via the *Agrobacterium tumefaciens* GV3101-mediated floral dip method [46]. Seeds of the transgenic plants were individually harvested and screened with kanamycin monosulfate. T3 homozygous transgenic lines were used for further phenotypic analysis.

For apple callus transformation, the pRI 101-AN-MdWRKY30 were introduced into the 'Orin' apple callus via the *Agrobacterium tumefaciens* EHA105-mediated method described by Dong et al. [47] and Wang et al. [48]. Resistant callus showing stable growth were used for further investigation.

2.8. Measurements of physiological indices

Relative electrolytic leakage was examined as described by Tan et al. [49]. Chlorophyll concentrations were determined using the protocol of Liang et al. [50], and MDA levels were obtained as described by Dong et al. [44]. Proline concentrations were calculated according to the method described by Dong et al. [48].

2.9. Statistical analysis

All data were examined with the IBM SPSS Statistics v. 20 (https://www.ibm.com/support/knowledgecenter/SSLVMB_20.0.0/com.ibm.spss.statistics_20.kc.doc/pv_welcome.html). One-way ANOVA and Duncan's tests were used to compare the results. Differences between treatments were considered statistically significant at $p < 0.05$.

3. Results

3.1. Isolation and analysis of MdWRKY26, MdWRKY28, and MdWRKY30

In our previous study, we systematically identified members of apple WRKY gene family using the v1.0 apple genome database [51]. There are 8 members in the IIa subgroup, and we cloned three of them, *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* (GeneBank accession numbers: MH150907, MH150909, and MH150910) using fully expanded leaves of "Golden Delicious" apple. The full-length cDNA of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* contained 966, 909, and 837-bp ORFs which encoded 321, 302, and 278-aa polypeptides, respectively. Multiple alignments of the deduced amino acid sequence of MdWRKY26, MdWRKY28, and MdWRKY30 were performed with DNAMAN 6.0.3.99 software. The result indicated that MdWRKY26, MdWRKY28, and MdWRKY30 proteins contained a well-conserved WRKY domain, which had a conserved WRKYGQK core sequence and a C2H2 zinc finger structure (Fig. 1).

To examine the evolutionary relationships of MdWRKY26, MdWRKY28, and MdWRKY30, the full-length amino acid sequences of 71 *Arabidopsis* WRKYs were used, along with various species of Group IIa WRKYs, to generate a phylogenetic tree. Three major Groups were revealed: Group I, Group II, and Group III, of which group II is further classified into five subgroups (IIa, IIb, IIc, IId, and IIe) (Fig. 2). MdWRKY26, MdWRKY28, and MdWRKY30 were classified into Group IIa, along with several other Group IIa WRKY proteins (Fig. 2).

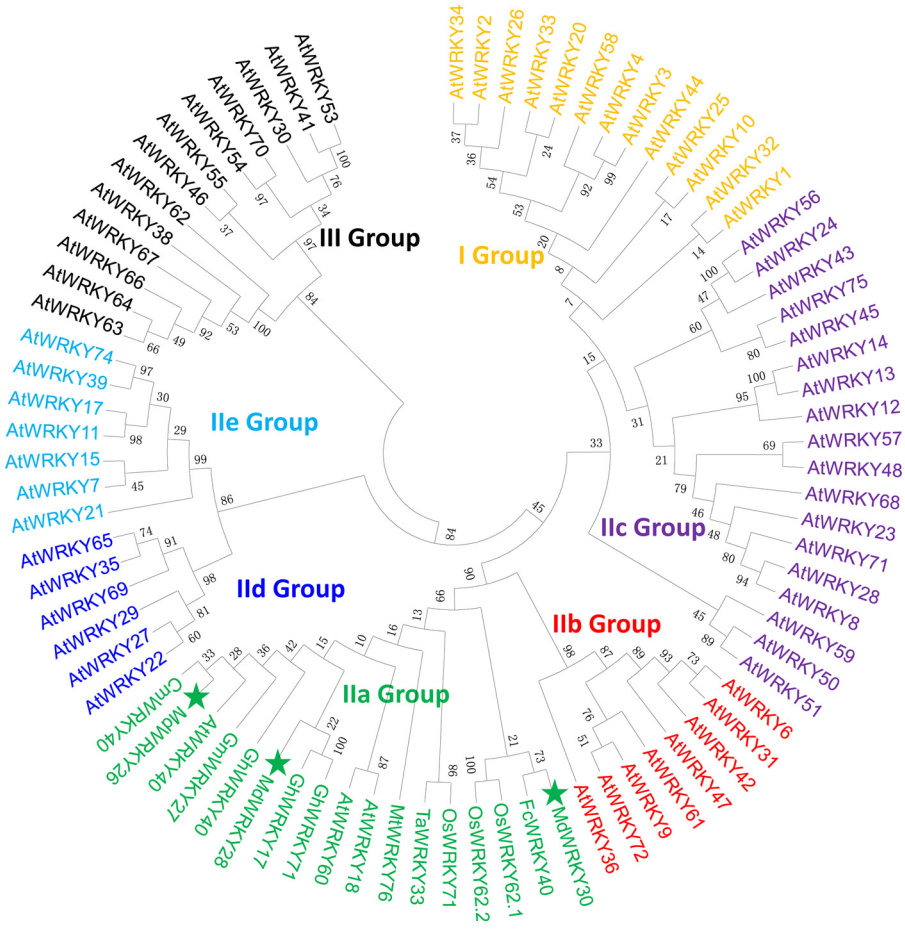

Fig. 2. Phylogenetic relationships and subgroup classification of MdWRKY26/28/30, AtWRKY and other IIa WRKY proteins.
The tree was created using MEGA software (version 6.06). MdWRKY26/28/30 are marked with an asterisk. Accession numbers for other Group IIa WRKY proteins listed are as follows: OsWRKY62.1 (NP_001063185); OsWRKY62.2 (DAA05127); OsWRKY71 (AAT84158); FcWRKY40 (AIZ94612); GmWRKY27 (ABC26917); GhWRKY17 (KF669857); GhWRKY40 (AGX24945); GhWRKY71 (AIE43914); MtWRKY76 (AES59079); CmWRKY40 (AJF11723); TaWRKY33 (ALR88711).

3.2. Expression patterns of MdWRKY26, MdWRKY28, and MdWRKY30 under osmotic and NaCl treatments

The expression patterns of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* under osmotic and NaCl treatments were investigated using qRT-PCR. As shown in Fig. 3A, *MdWRKY26*, *MdWRKY28*, *and MdWRKY30* expression were induced by osmotic stress. The transcript levels of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* reached the highest peaks at 4, 4, and 8 h of treatment, respectively, which were 6.6, 6.9 and 13.9 times of the control. As can be seen in Fig. 3B, *MdWRKY26* expression was increased by 3.4-fold at 8 h after salt treatment, but by less than one-fold at other times. The transcript level of *MdWRKY30* was continuously induced at 4 and 8 h after salt treatment.

3.3. MdWRKY26, MdWRKY28, and MdWRKY30 were localized to the nucleus

Sequence analysis, and online software LocTree3 and WoLF PSORT, showed that MdWRKY26, MdWRKY28, and MdWRKY30 contained the nuclear localization sequence (NLS), and were predicted to be nuclear proteins (Fig. 1), suggesting that they may be nucleus-localized. To confirm their subcellular localizations, the GFP control, MdWRKY26-GFP, MdWRKY28-GFP, and MdWRKY30-GFP constructs were separately transfected into tobacco leaves. Fluorescence microscopy revealed that a green fluorescence signal of the GFP control was ubiquitously distributed throughout the cell, whereas green fluorescence signals of MdWRKY26-GFP, MdWRKY28-GFP, and MdWRKY30-GFP fusion proteins were predominantly detected in the nucleus (Fig. 3C), indicating that *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* encoded proteins that were localized in the nucleus.

3.4. Transcriptional activation activity of MdWRKY26, MdWRKY28 and MdWRKY30

To analyze whether MdWRKY26, MdWRKY28, and MdWRKY30 possess transactivation activity, different truncations of MdWRKY26 and MdWRKY28 and the full-length cDNA of MdWRKY30 were separately fused with the GAL4 DNA-binding domain in the pGBKT7 vector, and then separately transformed into yeast strain AH109. The transformants with each construct grew well on SD/-Trp medium. However, only transformants containing MdWRKY26$^{1-321}$, MdWRKY26$^{1-279}$ MdWRKY28$^{1-302}$, or MdWRKY28$^{1-255}$ grown on SD/-Trp-His-Ade medium (Fig. 3D). Furthermore, they presented strong β-galactosidase activity in the presence of X-α-gal. In contrast, transformants containing pGBKT7, MdWRKY26$^{92-321}$, MdWRKY28$^{72-302}$, or MdWRKY30$^{1-278}$ failed to grow on SD/-Trp-His-Ade medium (Fig. 3D). These results indicated that MdWRKY26 and MdWRKY28 are transcriptional activators and their transactivation domain is in the N terminus, whereas MdWRKY30 has no transcriptional activation activity.

3.5. Self-Interaction of MdWRKY26, MdWRKY28, and MdWRKY30

Previous study has shown that AtWRKY18, AtWRKY40, and AtWRKY60 proteins formed both homocomplexes and heterocomplexes in *Arabidopsis* [16]. To determine whether this was also true for apple, we performed Y2H assays. The full-length CDS of MdWRKY26, MdWRKY28, and MdWRKY30 proteins were introduced into the yeast GAL4 activation domain (AD) in the Y2H prey vector pGAD424. The full-length MdWRKY26 and MdWRKY28 proteins showed strong transcriptional activation activities, whereas the full-length MdWRKY30 protein showed no transcriptional activation activity. So, MdWRKY26 and MdWRKY28 proteins with deleted activation domains and the full-

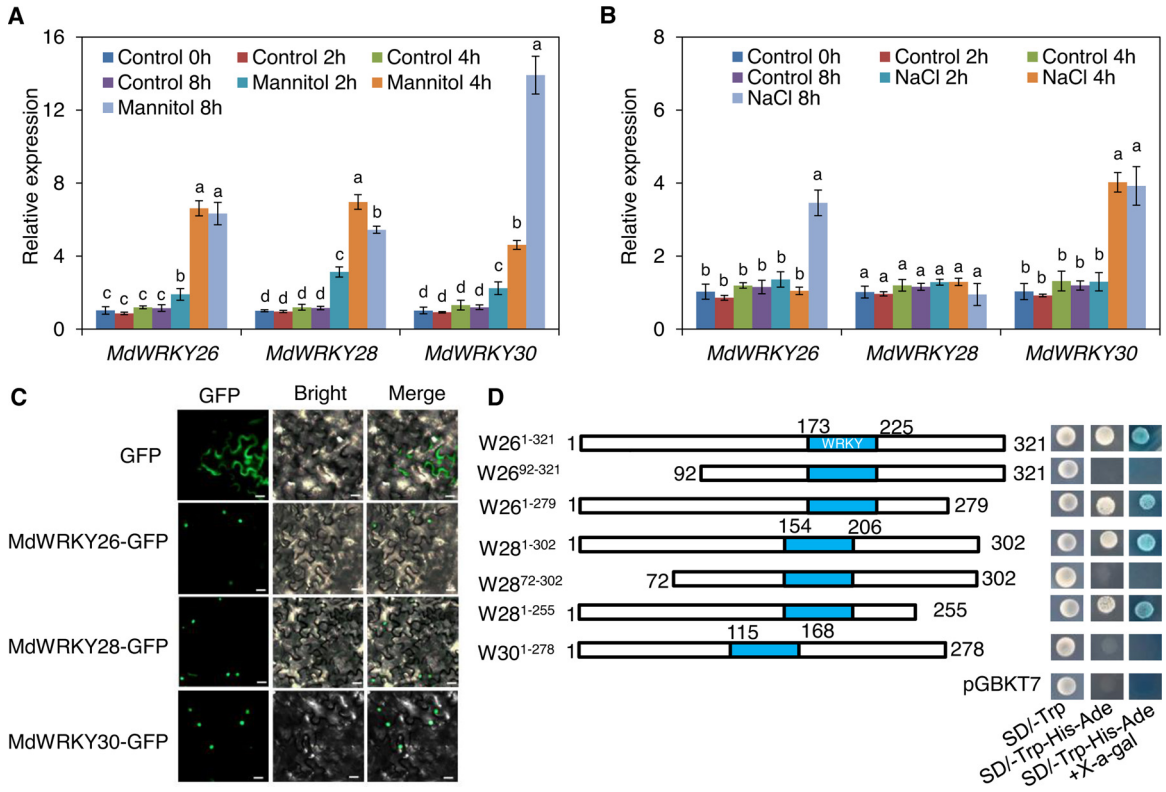

Fig. 3. Expression pattern, subcellular localization, and transcriptional activation analysis of MdWRKY26/28/30.
(A) Relative expression level of *MdWRKY26/28/30* under osmotic stress. (B) Relative expression level of *MdWRKY26/28/30* under NaCl stress. (C) Subcellular localization assay of the MdWRKY26/28/30 proteins. Scale bar: 20 μm. (D) Transcription activation domain identification of MdWRKY26/28/30 proteins in yeast cells. The indicated pGBKT7-MdWRKY fusion bait vectors were transformed into yeast cells. Yeast synthetic dropout medium lacking Trp (SD/-Trp) was used as transformation control, and that lacking Trp, Ade, and His (SD/-Trp-His-Ade) acted as a screen. Empty pGBKT7 bait vector was used as negative control.

length CDS of the MdWRKY30 protein were fused to the GAL4 DNA-binding domain (BD) in the Y2H bait vector pGBT9. The above-described fused pGBT9-MdWRKY and pGAD424-MdWRKY recombinant vectors were then co-transformed into yeast cells and tested for *LacZ* reporter gene expression through assays of β-galactosidase activity. As can be seen in Fig. 4A, MdWRKY26, MdWRKY28, and MdWRKY30 could interact with each other to form a heterodimer, and with themselves to form a homodimer in yeast. For further verify these interactions from the Y2H assays, we performed a BiFC assay. Full-length MdWRKY28 protein was fused to the N-terminal region of YFP (MdWRKY28 + N); MdWRKY30 protein was fused to the C-terminal region of YFP (MdWRKY30 + C). When MdWRKY28 + N was co-infiltrated with MdWRKY30 + C in leaves of tobacco (*Nicotiana benthamiana*), a YFP signal, as revealed by staining with DAPI, was detected in nuclei (Fig. 4B). No fluorescence was observed in all negative controls (YFP-N/MdWRKY30 + C and MdWRKY28 + N/YFP-C) (Fig. 4B). The results showed that MdWRKY28 interacts with MdWRKY30 in the nuclei of plant cells.

3.6. *MdWRKY30* overexpression enhanced osmotic and salt tolerance of *Arabidopsis* seedlings

To determine whether *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* are involved in abiotic stress responses, we generated transgenic *Arabidopsis* plants that constitutively overexpressed *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* genes. The coding sequences of *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* were subcloned behind the cauliflower mosaic virus *35S* promoter in a binary plant transformation vector and transformed into *Arabidopsis* plants. Our PCR-screening of kanamycin-resistant transgenic lines was performed using *Arabidopsis* genomic DNAs as templates, with primers specific to *MdWRKY26*, *MdWRKY28*, and *MdWRKY30* (Fig. S1). Transformants, as identified via qRT-PCR, exhibited elevated levels of transcripts. We chose three transgenic lines to evaluate the potential function in response to abiotic stresses. As shown in Fig. S2 and S3, the growth phenotypes of *MdWRKY26* and *MdWRKY28* overexpressing transgenic seedlings and the wild type (WT) 'Col' were similar on MS agar medium plates and MS agar medium plates supplemented with mannitol or salt, and there was no significant change in primary root lengths and fresh weights. Therefore, subsequent phenotypes of MdWRKY26 and MdWRKY28 under abiotic treatments were not analyzed. As shown in Fig. 5A, the root of 'Col' and *MdWRKY30* overexpressing transgenic seedlings displayed similar growth characteristics on MS agar medium plates; however, growth of 'Col' NaCl treatments at different concentrations, the primary roots and fresh weights of the transgenic seedlings were longer and heavier than those of 'Col' seedlings upon exposure to 200 and 300 mM mannitol or 100 and 150 mM NaCl (Fig. 5B, C).

3.7. *MdWRKY30* overexpressing *Arabidopsis* seedlings have improved physiological traits associated with osmotic and salt tolerance compared with 'Col'

In order to further investigate the *MdWRKY30*-mediated enhanced tolerance to osmotic and salt stresses, we measured electrolyte leakage, as well as chlorophyll, proline, and MDA levels, all of which are important markers of osmotic and salt stress tolerance. As shown in Fig. 6, no obvious diff ;erence in relative electrolyte leakage level was detected under control conditions, but after mannitol treatment at different concentrations, relative electrolyte leakage values were significantly higher in 'Col' than in transgenic seedlings (Fig.6A). In addition, the chlorophyll and proline content of 'Col' and *MdWRKY30* overexpressing transgenic seedlings performed similarly under control conditions. With

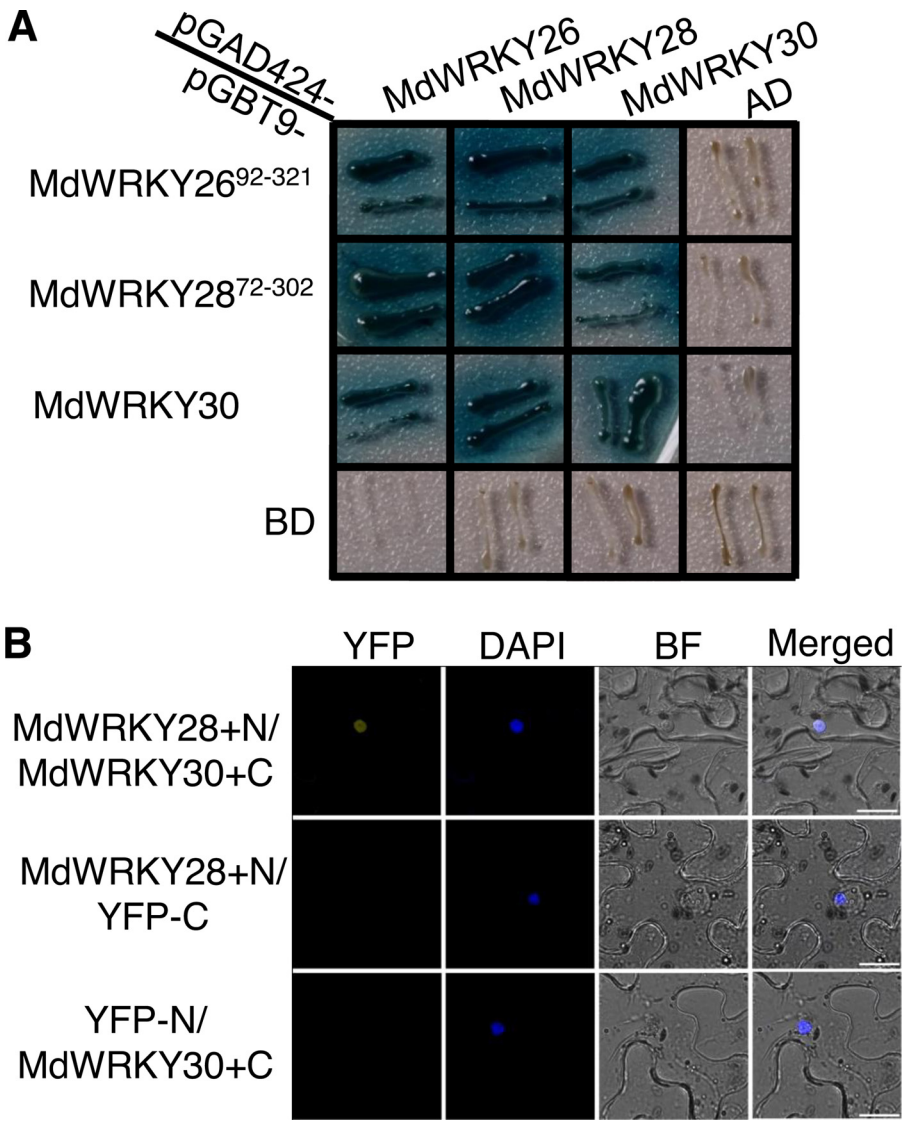

Fig. 4. Physical interactions among MdWRKY proteins.
(A) Yeast two-hybrid assay. pGAD424-MdWRKY fusion prey vectors were co-transformed with pGBT9-MdWRKY fusion bait vectors into yeast cells. Positive interactions were indicated by ability of cells to grow on synthetic dropout medium with additive x-α-gal but lacking Leu, Trp, His, and Ade. Empty AD prey vector and BD bait vectors were used as negative controls. (B) Assay of bimolecular fluorescence complementation (BiFC) assay, showing fluorescence in nuclear compartments of tobacco leaf epidermal cells that resulted from complementation of N-terminus part of YFP fused to MdWRKY28 (MdWRKY28 + N) with C-terminus part of YFP fused to MdWRKY30 (MdWRKY30 + C). No signal was observed from negative controls. Scale bar: 20 μm.

different concentrations of mannitol and NaCl treatments, the transgenic seedlings had higher chlorophyll and proline levels (Fig.6B-C). We also found that the *MdWRKY30* overexpressing transgenic seedlings had lower MDA levels than 'Col' following mannitol treatment, whereas there was no obvious diff ;erence under control conditions (Fig. 6D). These results suggested that over-expression of *MdWRKY30* in *Arabidopsis* seedlings led to enhanced osmotic and salt stress tolerance.

3.8. Analyses of seed germination and cotyledon greening rates under osmotic and NaCl treatments

We examined the germination rates of *MdWRKY30* overexpressing transgenic lines and 'Col' seeds under 100- and 200-mM NaCl or 200- and 300-mM mannitol treatments. When grown on MS agar medium plates for 48 h, the germination rates of *MdWRKY30* overexpressing transgenic lines and 'Col' seeds were close to 100 % (Fig.7); but the germination rates of *MdWRKY30* overexpressing transgenic lines seeds were significantly higher than those of 'Col' under 100- and 200-mM NaCl or 200- and 300-mM mannitol treatments (Fig.7). For example, under the 200-mM NaCl treatment, the germination rate of 'Col' seeds was 24 %, whereas *MdWRKY30* overexpressing transgenic line seeds were 47–49 % (Fig.7B); under the 300-mM mannitol treatment, the transgenic seeds exhibited 16–22 % higher germination rates than those of 'Col' (Fig.7B). We also examined cotyledon greening rates of *MdWRKY30* overexpressing transgenic lines and 'Col' with various concentrations of NaCl or mannitol. After 12 days of culture in MS agar medium plates, the cotyledon greening rates of *MdWRKY30* overexpressing transgenic lines and 'Col' were close to 100 %; but the cotyledon greening rates of 'Col' were significantly lower than those of *MdWRKY30* overexpressing transgenic lines under 100 and 200 mM NaCl or 200 and 300 mM mannitol (Fig. 8). For example, under the 200-mM NaCl treatment, the cotyledon greening of 'Col' was 30 %, whereas *MdWRKY30* overexpressing transgenic lines were 53–61 % (Fig. 8B); under the 300-mM mannitol treatment, transgenic lines exhibited 13–20 % higher cotyledon greening rates than those of 'Col' (Fig. 8B).

3.9. MdWRKY30 overexpression enhanced the osmotic and salt tolerance of apple callus

To further examine the function of *MdWRKY30* under osmotic and salt stresses, we generated a transgenic 'Orin' apple callus that constitutively overexpressed the *MdWRKY30* gene. Two kanamycin-resistant transgenic apple lines over-expressing *MdWRKY30* were confirmed by PCR with genomic DNAs and by qRT-PCR with cDNA (Fig. S4). For further tests, WT and *MdWRKY30* overexpressing transgenic lines were pre-cultured on the MS agar medium plates for 14 d, and then calluses of the same weight were transferred to MS agar medium

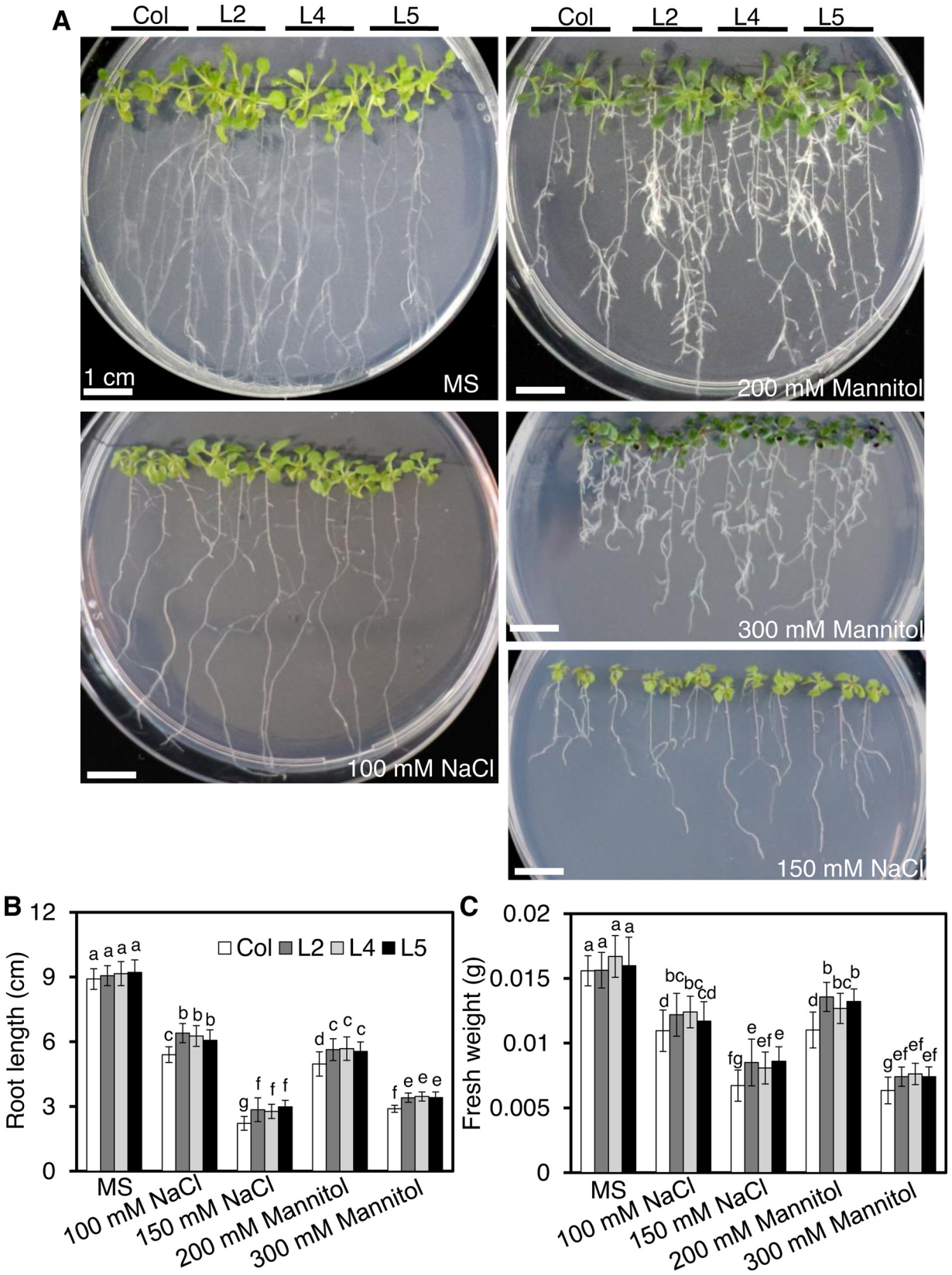

Fig. 5. Phenotypic analysis of transgenic *MdWRKY30*-overexpressing *Arabidopsis* seedlings under NaCl and mannitol treatments.
(A) Representative images of five-day-old 'Col' and transgenic seedlings had been cultivated for 11 d on MS medium supplemented with 0 mM, 100 mM, 150 mM NaCl, 200 mM or 300 mM mannitol. Bars = 1 cm. (B) Primary root lengths; and (C) fresh weights of five-day-old 'Col' and transgenic seedlings measured after plants had been exposed for 11 d to NaCl and osmotic stresses. The T3 homozygous *MdWRKY30* transgenic lines were used for further phenotypic analysis. Error bars represent SD based on 3 independent replicates. For (B) and (C), bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

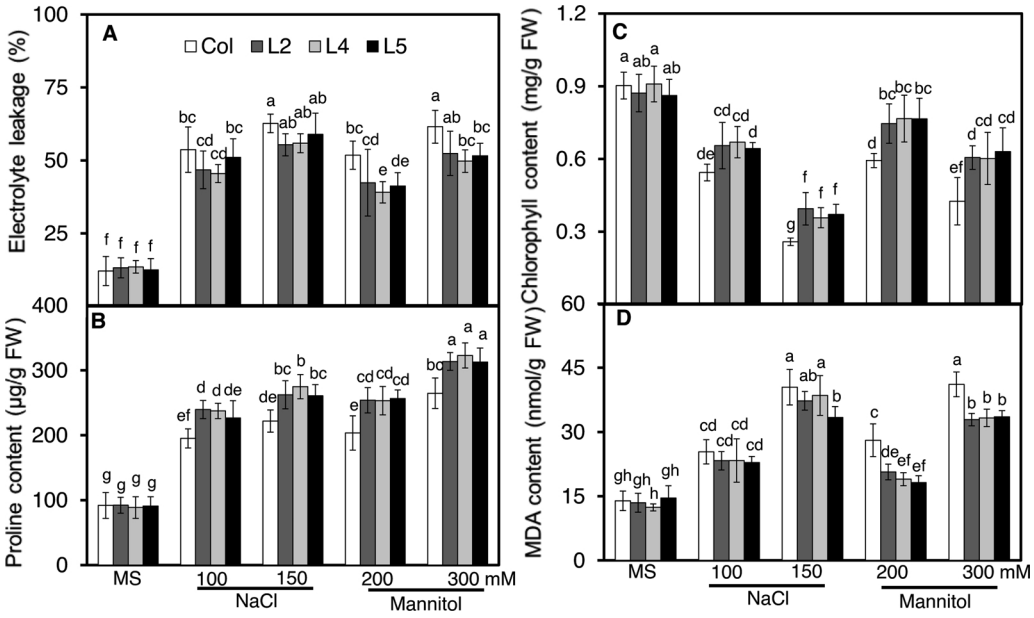

Fig. 6. Physiological changes associated with NaCl and osmotic stressed response in 'Col' and transgenic *Arabidopsis* seedlings.
(A) Relative electrolyte leakage; and levels of proline (B), chlorophyll (C), and MDA (D) of five-day-old 'Col' and transgenic seedlings measured after plants had been exposed for 11 d on MS medium supplemented with 0 mM, 100 mM, 150 mM NaCl, 200 mM or 300 mM mannitol. The T3 homozygous *MdWRKY30* transgenic lines were used for further phenotypic analysis. Error bars represent SD based on 3 independent replicates. For (A), (B), (C), and (D), bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

plates and MS agar medium plates supplemented with mannitol or salt. After 18 d of treatment, the calluses on the MS agar medium were similar in growth status and weight to WT and *MdWRKY30* overexpressing transgenic lines (Fig. 9). On MS agar medium plates supplemented with mannitol or salt, the two transgenic lines had better growth and were significantly heavier than WT (Fig. 9). These results suggested that overexpression of *MdWRKY30* could enhance the osmotic and salt tolerance of apple callus.

The expression of various stress-responsive genes was determined by qRT-PCR in WT and *MdWRKY30* overexpressing transgenic lines after 18 d of mannitol or salt treatment (Fig. 10). The expression of *MdABI1*, *MdABF3*, *MdRD22*, *MdRD29A*, *MdDREB1A*, *MdCAT1*, and *MdSOD1* was found to be similar in WT and *MdWRKY30* overexpressing transgenic lines under control conditions. In contrast, the expression levels of *MdABI1*, *MdABF3*, *MdRD22*, *MdRD29A*, *MdDREB1A*, *MdCAT1*, and *MdSOD1* were significantly greater in *MdWRKY30* overexpressing transgenic lines compared with WT under both osmotic and salt stresses. Interestingly, the expression levels of *MdVHP1*, a multiple-stress gene [47], was significantly higher in *MdWRKY30* overexpressing transgenic lines than in WT, whether they were subjected to a stress treatment or not.

4. Discussion

WRKY TFs have been widely demonstrated to play key roles in plant growth, development, and biotic and abiotic stress tolerance, by binding to target genes containing W-box cis elements and interacting with other proteins [15–29]. Multiple WRKY TFs have been isolated and their biological functions have been identified in *Arabidopsis*, rice, wheat, soybean, cotton, *Fortunella crassifolia*, *Pyrus betulaefolia*, and poplar [20–34,52,53]. However, the WRKY gene family contains many members, and little is known regarding the biological function of most, including non-model plants. In this study, we isolated three WRKY family genes, *MdWRKY26*, *MdWRKY28* and *MdWRKY30*, in apple, which contain a well-conserved WRKY domain and are localized exclusively to the nucleus. Further, the overexpression of *MdWRKY30* in the transgenic *Arabidopsis* and apple callus lines exhibited improved tolerance to osmotic and salt stresses.

In *Arabidopsis*, transcript levels of *AtWRKY18*, *AtWRKY40* and *AtWRKY60* from the subgroup IIa were significantly induced by salt, drought, and ABA [54]. Compared with wild type, single mutant *wrky18* and *wrky60,* and double mutant *wrky18 wrky60*, exhibited different phenotypes under salt and osmotic stress treatments, indicating that they are involved in abiotic stress responses [54]. The expression of *FcWRKY40*, a subgroup IIa gene isolated from *Fortunella crassifolia*, was significantly induced by salt, cold, and ABA. Constitutive overexpression of *FcWRKY40* resulted in enhanced tolerance to oxidative and salt stresses in transgenic tobacco. Overexpression of *FcWRKY40* enhanced tolerance to salt stress in lemon, whereas silencing of *FcWRKY40* increased sensitivity to salt stress [52]. The transcriptional level of *GmWRKY27*, a member of the soybean subgroup IIa, was up-regulated by drought, salt, cold, and ABA. RNAi and over-expression analysis indicated that GmWRKY27 confers salt and drought stress tolerance in transgenic soybean hairy roots [55]. *MtWRKY76* overexpression enhanced salt and drought stress tolerance of transgenic *Medicago truncatula*, which was a subgroup IIa WRKY member, and was rapidly induced by abiotic stresses [56]. In addition, expression of *TaWRKY33* (a member of the wheat subgroup IIa) in transgenic *Arabidopsis* lines enhanced tolerance to osmotic and heat stresses [32]. These studies indicate that the biological function of plant subgroup IIa WRKY members involved in abiotic stress is highly conserved. Based on the highly conserved WRKY domain features and the evolutionary analysis of the *Arabidopsis* WRKY members and subgroup IIa WRKY proteins from various species, MdWRKY26, MdWRKY28, and MdWRKY30 are clearly divided into the subgroup IIa WRKYs. *MdWRKY26*, *MdWRKY28* and *MdWRKY30* expression were rapidly induced by salt and/or osmotic stress. To examine roles of MdWRKY26, MdWRKY28 and MdWRKY30 in osmotic and salt stress tolerance, transgenic *Arabidopsis* plants overexpressing *MdWRKY26*, *MdWRKY28* and *MdWRKY30* were generated and subjected to osmotic and salt stress treatment with mannitol and NaCl, respectively. Using *Arabidopsis* seedlings, we showed that the transgenic lines overexpressing *MdWRKY30* enhanced the tolerance to the stress treatment as compared with 'Col', whereas transgenic lines overexpressing *MdWRKY26* and *MdWRKY28* had no obvious response. Furthermore, *MdWRKY30* overexpression enhanced the osmotic and salt tolerance of apple calluses, suggesting that MdWRKY30 may be an important regulator for mediating tolerance to osmotic and salt stresses, as compared with *MdWRKY26* and *MdWRKY28*, and *MdWRKY30* can be used as a target gene in genetic engineering approaches to improve osmotic and salt tolerance in apple.

In general, when plants are subjected to drought and salt stress, membrane permeability increases and membrane lipid peroxidation occurs, which leads to accumulation of MDA content and changes levels

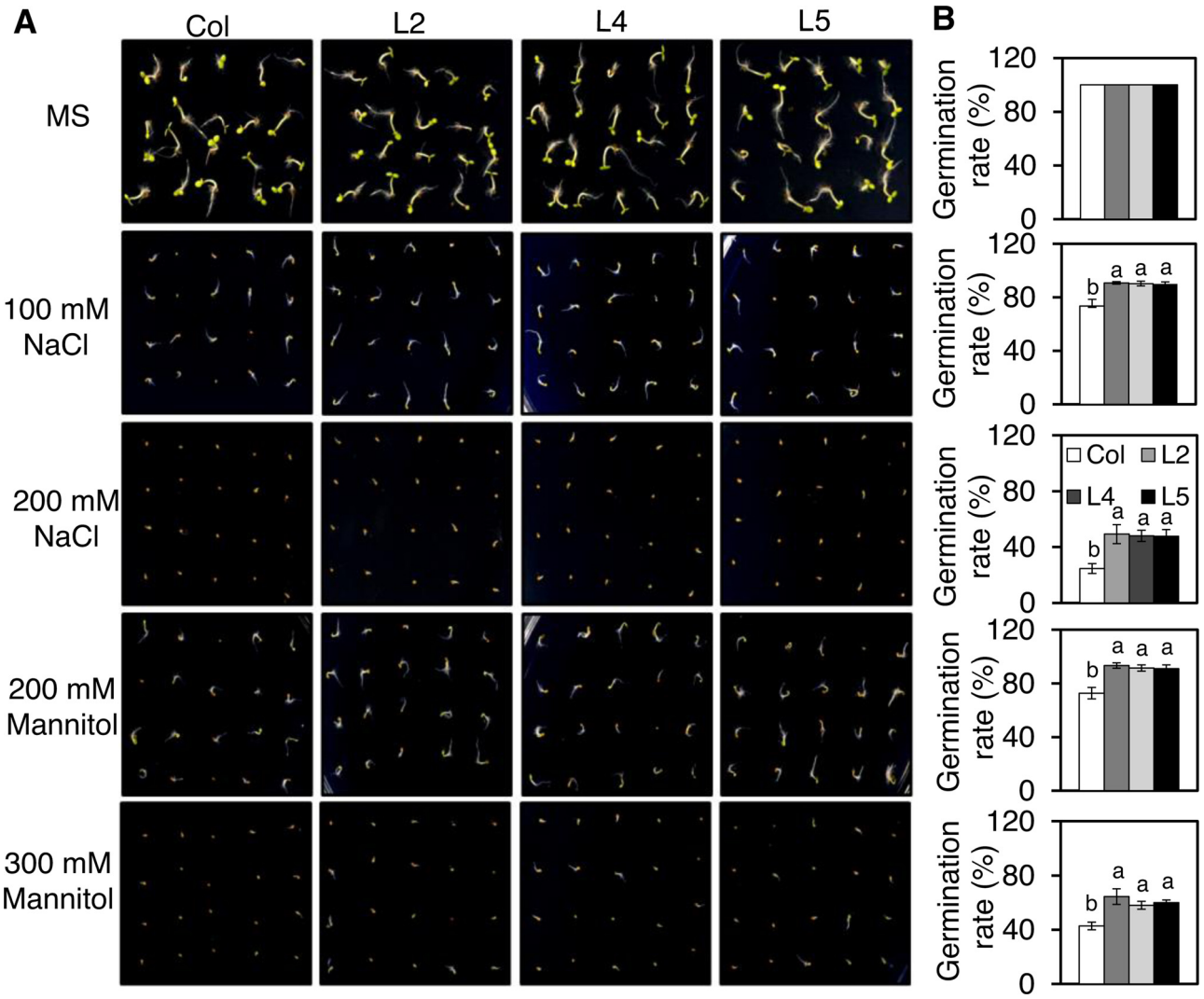

Fig. 7. Phenotypic analysis of transgenic *MdWRKY30*-overexpressing *Arabidopsis* lines at the seed germination stage under NaCl and mannitol treatments. (A) Representative images of seed germination in Col and transgenic lines 48 h after seeds were cultivated on MS medium supplemented with 0 mM, 100 mM, 200 mM NaCl, 200 mM or 300 mM mannitol. (B) Seed germination rates of Col and transgenic lines 48 h after seeds were cultivated on MS medium supplemented with 0 mM, 100 mM, 200 mM NaCl, 200 mM or 300 mM mannitol. The T3 homozygous *MdWRKY30* transgenic lines were used for further phenotypic analysis. For (B), bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

of electrolyte leakage [57]. Therefore, relative electrolyte leakage and MDA levels are important indicators for assessing the degree of abiotic stress resistance in plants [58,59]. In this study, relative electrolyte leakage and MDA levels in 'Col' seedlings were significantly higher than those in *MdWRKY30* transgenic *Arabidopsis* lines under osmotic and salt stresses, suggesting that the degree of membrane damage in 'Col' seedlings was more than that in transgenic lines. Chlorophyll content is also an important indicator for assessing physiological state of plants [60]. Here, our results showed that the chlorophyll content in 'Col' seedlings was significantly lower than in *MdWRKY30* transgenic *Arabidopsis* lines under osmotic and salt stresses. Free proline is one of the most important osmotic protective substances in plants, and plays an important role in maintaining cell membrane structural stability and osmotic balance [61,62]. We observed that, compared with 'Col' seedlings, proline content was higher in *MdWRKY30* transgenic *Arabidopsis* lines under osmotic and salt stresses. Taken together, these results indicated that the over-expression of the *MdWRKY30* gene confers improved tolerance to osmotic and salt stresses in transgenic *Arabidopsis*, and is related to higher free proline content and chlorophyll levels, but lower relative electrolyte leakage and MDA.

WRKY proteins have been reported to function as positive or negative regulators in regulating expression of downstream genes. Most WRKY TFs (e.g., GaWRKY1, NtWRKY12, AtWRKY18 and AtWRKY60) are transcription activators, with some others (e.g., AtWRKY40, OsWRKY51 and OsWRKY71) are repressors [34,63–65]. Interestingly, some WRKY proteins possess both functions. AtWRKY6 has been proved to activate the expression of genes involved in triggering senescence processes and plant defense responses. On the other hand, it was found to act as a repressor of its own promoter activity [66,67]. *Populus trichocarpa* WRKY protein PtrWRKY40 did not possess transcriptional activation ability in yeast [68]. Constitutive expression of PtrWRKY40 in transgenic poplar conferred higher susceptibility to *Dothiorella gregaria* infection by suppressing the expression of SA-associated genes, suggesting that PtrWRKY40 could be a transcription repressor. However, overexpression of PtrWRKY40 in transgenic *Arabidopsis* displayed resistance to necrotrophic fungus *Botrytis cinerea* by activating the expression of JA-defense-related genes [68]. In the present study, though MdWRKY30 showed no transcriptional activation activity in yeast, the significant up-regulation of stress-responsive genes in transgenic plants (Fig. 10) indicated that MdWRKY30 may also function as a positive regulator under stress conditions, similar to PtrWRKY40. This phenomenon may be explained by a hypothesis that the activation activity

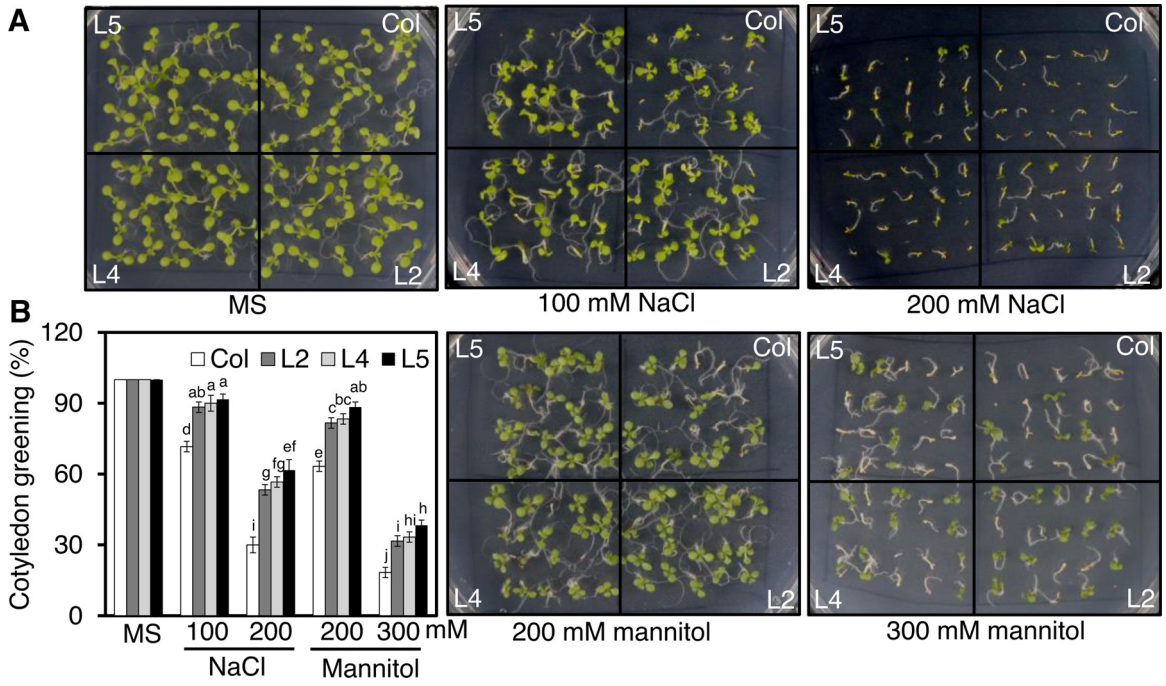

Fig. 8. Phenotypic analysis of transgenic *MdWRKY30*-overexpressing *Arabidopsis* lines at the greening cotyledon stage under NaCl and mannitol treatments. (A) Representative images of greening cotyledons from Col and transgenic lines 12 d after seeds were cultivated on MS medium supplemented with 0 mM, 100 mM, 200 mM NaCl, 200 mM or 300 mM mannitol. (B) Cotyledon greening rates of Col and transgenic lines 12d after seeds were cultivated on MS medium supplemented with 0 mM, 100 mM, 200 mM NaCl, 200 mM or 300 mM mannitol. The T3 homozygous *MdWRKY30* transgenic lines were used for further phenotypic analysis. For (B), bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

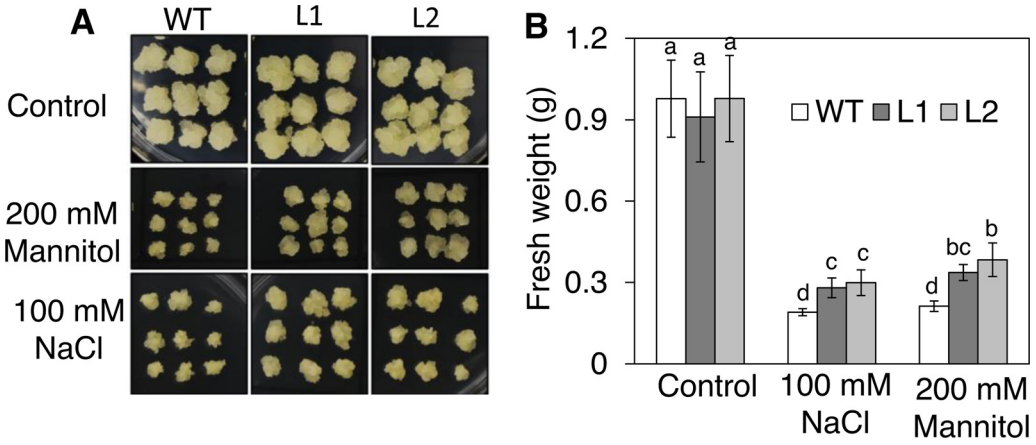

Fig. 9. Phenotypic analysis of transgenic *MdWRKY30*-overexpressing apple callus under NaCl and mannitol treatments. (A) Representative images and (B) fresh weight in WT and transgenic apple callus cultivated on MS medium, or MS medium supplemented with 100 mM NaCl, or 200 mM mannitol for 16 d. For (B), bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

of MdWRKY30 relies on posttranslational modifications or stress-related interaction proteins. Similar findings have been found in other studies of WRKY proteins, such as ZmWRKY17, GmWRKY54, TaWRKY71 − 1, VaWRKY14, and CmWRKY17 [69–74].

Many stress-responsive transcription factors and genes have been identified and validated in multiple species, indicating that plants have evolved sophisticated molecular mechanisms to tolerate abiotic stresses [8,29,75–77]. *TaWRKY33* overexpression enhanced drought and heat tolerance of transgenic *Arabidopsis* plants by regulating the expression of stress-related and ABA-responsive genes (such as *ABA1*, *ABA2*, *ABI1*, *ABI5*, and *RD29A*) [75]. *HaWRKY76* overexpression enhanced drought and heat tolerance of transgenic *Arabidopsis* plants by regulating the expression of *ABI1*, *ABI2*, *ABI3*, *ABI5*, *ADH1*, *RAB18*, *RD29A*, and *RD29B* [76]. *GhWRKY33* overexpression reduced drought tolerance of transgenic *Arabidopsis* plants by down-regulating the expression of *ERD15*, *DREB2A*, and *RD29A* [77]. In the present study, we planned to carry out stress tolerance identification and stress-responsive gene expression analysis of *MdWRKY30* overexpressing transgenic apple plantlets. Unfortunately, *MdWRKY30*-expressing transgenic apple plantlets were not successfully obtained by genetic transformation experiments, but *MdWRKY30*-expressing transgenic apple callus had been obtained. To better understand the mechanisms of salt and osmotic tolerance conferred by *MdWRKY30* overexpression, the expression of several known salt and osmotic tolerance-related genes in WT and *MdWRKY30* overexpressing transgenic apple callus lines was investigated. qRT-PCR analyses suggested that *MdWRKY30* overexpressing transgenic apple callus lines produced high transcript levels in the stress-responsive genes *MdABI1*, *MdABF3*, *MdRD22*, *MdRD29A*, *MdDREB1A*, *MdCAT1*, *MdSOD1*, and *MdVHP1*. Therefore, MdWRKY30 affected salt and osmotic tolerance through regulation of different stress-responsive genes. In addition, ABA-responsive genes ABI, ABF, and RD29A are known to be key regulators of ABA signaling under abiotic stresses [75–77]. MdWRKY may be partly involved in salt and osmotic stresses through the ABA-dependent singling pathway. Moreover, MdWRKY30 interacting with MdWRKY26, MdWRKY28, and itself might act as transcription-regulating complexes involved in the

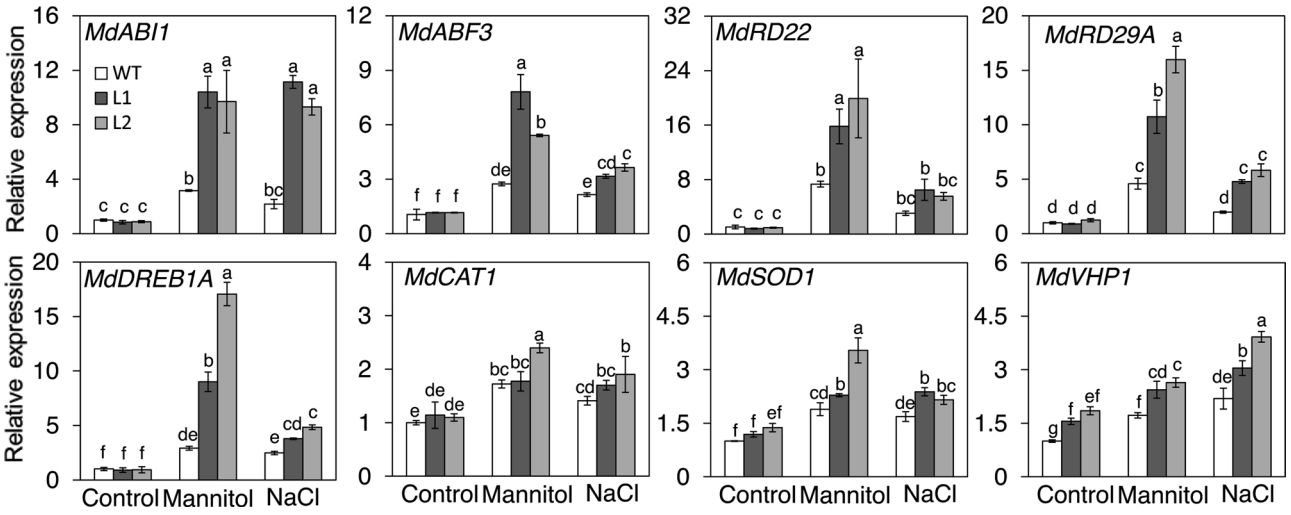

Fig. 10. Expression profiles of stress-marker genes in WT and *MdWRKY30*-expressing transgenic apple callus, analyzed using qRT-PCR. Expression levels were based on total RNA extracted from WT and transgenic apple callus cultivated on MS medium, or MS medium supplemented with 100 mM NaCl, or 200 mM mannitol for 16 d. Data represent mean values ± SD from three independent experiments. Bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

modulation of gene expression regulatory pathways. Thus, further research is needed to elucidate the potential molecular mechanisms of MdWRKY30 in the abiotic stress response.

5. Author contributions

F.M., K.M. and Q.D. conceived and designed the experiments. Q.D., W.Z., D.D., D.H., Q.W., CH.L., C.L., X.G. and K.M. performed the experiments. F.M. and Q.D. analyzed the data and wrote the manuscript. F.M., CY.L. and Q.D. revised the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110611.

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