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Function of *Malus prunifolia* WRKY6 transcription factor in response to different stresses

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Abstract

The WRKY transcription factors (TFs) are integral parts of signaling pathways that regulate many processes, such as senescence, seed dormancy, seed germination, and resistance to abiotic and biotic stresses. Stress-related functions of WRKY6 have been characterized in *Arabidopsis* and other plant species, but its role has not been identified in apple. Here, we cloned *WRKY6* genes from *Malus prunifolia*. Two homologues MpWRKY6a and MpWRKY6b found in this species were members of Group II WRKY6 TFs. They were localized to the cell nucleus. MpWRKY6a can bind to W-boxes. Compared with the untransformed wild type plants, *MpWRKY6a*-overexpressing *Arabidopsis* plants were more sensitive to methyl jasmonate (MeJA) and less sensitive to methyl viologen and abscisic acid (ABA), which suggests its role in responses to oxidative stress and MeJA or ABA signaling. The results fill a gap in the WRKY6 function in apple and provide basis for resistance improvement of *Malus*.

Additional key words: abscisic acid, methyl jasmonate, methyl viologen, open reading frame, ubiquitin-like protein.

Introduction

In higher plants, the WRKY transcription factor (TF) family is one of the ten largest TF families. Since first being identified in *Ipomoea batata*, 74 members have been found in *Arabidopsis thaliana*, 103 in *Oryza sativa*, 104 in *Populus trichocarpa*, 136 in *Zea mays*, 47 in *Ricinus communis*, and 81 in *Solanum lycopersicum* (Ishiguro and Nakamura 1994, Eulgem *et al*. 2000, Ross *et al*. 2007, He *et al*. 2012, Huang *et al*. 2012, Li *et al*. 2012, Wei *et al*. 2012). Members contain two key components: the WRKY DNA binding domain (WRKYGQK) in the N-terminal region and an atypical zinc finger motif ($CX_{4–5}CX_{22–23}HXH$ or $CX_7CX_{23}HXC$) in the C-terminal region (Eulgem *et al*. 2000, Rushton *et al*. 2010). The WRKY TFs are divided into three groups according to their numbers of WRKY domains and structure of the zinc finger motif. Group I proteins have two WRKY domains whereas members of Groups II and III have one. The zinc finger structures of Group I and Group II members are C_2H_2 and of Group III are C_2HC (Eulgem *et al*. 2000, Zhang and Wang 2005, Rushton *et al*. 2010). By combining with W-boxes (TTGACC/T) in target gene promoters, WRKY TFs regulate the expression of downstream genes (Eulgem *et al*. 2000).

The WRKYs are integral parts of signaling webs that regulate many plant processes, such as senescence, seed dormancy, seed germination, and resistance to abiotic and biotic stresses (Eulgem and Somssich 2007, Pandey and Somssich 2009, Rushton *et al*. 2010, 2012, Chen *et al*. 2012). In *Arabidopsis*, *WRKY22*, *-53*, *-54*, and *-70* are involved in senescence (Miao and Zentgraf 2010, Zhou *et al*. 2011, Besseau *et al*. 2012); *WRKY28*, *-33*, *-46*, *-53*, and *-70* participate in salicylic acid (SA) signaling (Mao *et al*. 2011, Van Verk *et al*. 2011, Hu *et al*. 2012). Examples from other plant species include *WRKY72* from rice, which interferes with cross-talk between abscisic acid (ABA) signaling and the auxin transport pathway when expressed in transgenic *Arabidopsis* plants (Song *et al*. 2010); *TaWRKY2* and *TaWRKY19*, which improve salt and drought tolerances in *Triticum aestivum* (Niu *et al*. 2012); and *WRKY1* and *-2* in *Vitis pseudoreticulata*, which is induced by *Erysiphe necator* infection or treatment with SA (Li *et al*. 2010).

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Abbreviations: AbA - aureobasidin A; ABA - abscisic acid; MeJA - methyl jasmonate; MV - methyl viologen; ORF - open reading frame; qPCR - quantitative polymerase chain reaction; SA - salicylic acid; TF - transcription factor; WT - wild-type.

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Arabidopsis WRKY6 was firstly reported to have functions in leaf senescence and resistance to pathogen infection (Robatzek and Somssich 2001). It was subsequently found that *Arabidopsis* WRKY6 clearly participates in maintaining phosphorus balance and regulating the expression of target genes in the stress response (Chen *et al*. 2009). Kasajima *et al*. (2010) have also reported that *WRKY6* can be induced by low-boron conditions to assist plants in counteracting the effects of adverse environments. This gene also functions as an arsenate-responsive TF that mediates the expression of arsenate/phosphate transporter genes and restricts the activation of an arsenate-induced transposon (Castrillo *et al*. 2013). Recently, WRKY6 was reported to positively regulate abscisic acid signaling during seed germination and early seedling development (Huang *et al*. 2016). In *Helianthus annuus*, *HaWRKY6* can be induced in response to high-temperature stress through the regulation of miR396 (Giacomelli *et al*. 2012). Both *NaWRKY3* and *NaWRKY6* protect plants of *Nicotiana attenuata* against attack by herbivorous insects (Skibbe *et al*. 2008). In rice, *OsWRKY6* positively regulates the pathogen-defense response by modulating expression of pathogen response gene *OsPR1* (Hwang *et al*. 2011).

Malus × *domestica* Borkh. is one of the most economically important fruits worldwide. In major apple growing regions, apple production can be limited by various stresses, such as heat and/or drought. *Malus prunifolia* (Willd.) Borkh., a wild apple species adapted to the arid region of northern China, offers exceptional promise for use as a rootstock to enhance production in these regions (Wang *et al.* 2011). Potential roles of WRKY6 in stress responses have not been elucidated in *Malus*. In this study, we identified two homologous WRKY6 proteins from *M. prunifolia*, confirmed their basic features as WRKY transcription factors *via* sub-cellular location and yeast-one-hybrid assays and explored their function in stress responses.

Materials and methods

Sequence analysis of genes, cloning genomic genes and promoters: Seeds of *Malus prunifolia* (Willd.) Borkh. were collected from established, open-pollinated individuals at the Horticultural Experimental Station of Northwest A&F University, Yangling, China (34°20'N, 108°24'E). One-year-old seedlings were produced in the greenhouse at the Horticultural Experimental Station. Fully unfolded leaves were used in *MpWRKY6a* and *MpWRKY6b* cloning. Young leaves were also the source of DNA for cloning genomic genes *MpWRKY6a* and *MpWRKY6b* as well as the promoter regions of *MpWRKY6a*, *MpWRKY6b*, and *MpPHO1*.

For the identification of WRKY6 related sequences from *M.* × *domestica*, *Arabidopsis* WRKY6 protein sequence (*TAIR*: AT1G62300.1; http://www.arabidopsis.org) was used as a query to search *M.* × *domestica* open reading frame translations (Genomic Database in *Rosaceae*, *GDR*; http://www.rosaceae.org/), using the program *BLASTP* and expect (E) value limit of 1E-100. Two sequenced were identified and named *MdWRKY6a* (MDP0000301666) and *MdWRKY6b* (MDP0000935652). RNA was extracted from the fully unfolded leaves of *M. prunifolia* by a cetyltrimethylammonium bromide (CTAB) method (Gambino *et al*. 2008). First-strand cDNA was synthesized using the *RevertAid*$^{\text{TM}}$ first strand cDNA synthesis fit (*Fermentas*, *Thermo Fisher Scientific*, Waltham, USA) according to the manufacturer's protocol. Primers for cloning cDNAs from *M. prunifolia* were designed using *Primer Premier 6.0* (Table 1 Suppl.).

The amino acid sequences of WRKY6 proteins from *M. prunifolia* and representative plant species (*A. thaliana*: AT1G62300.1; *Gossypium hirsutum*: AFH01344.1; *Glycine max*: NP_001237313.1; *Triticum aestivum*: ACD80367.1; *Nicotiana attenuate*: AAS13440.1; *Oryza sativa*: XP_015646950.1) were subjected to Bootstrap tests of phylogeny (*MEGA 4.0*) using the neighbor-joining method.

Genomic DNA was isolated from young leaves of *M. prunifolia* by a CTAB method (Porebski *et al*. 1997). *MpWRKY6a* and *MpWRKY6b* genomic clones were obtained by PCR using the same primers as used for cloning *MpWRKY6a* and *MpWRKY6b* in cDNA. *MpWRKY6a* and *MpWRKY6b* promoter regions were cloned by PCR based on the upstream of the *MhWRKY6a* and *MpWRKY6b* genes, which is on contig MDC015642.226 and contig MDC010173.125, respectively. The primers used for cloning those promoters are listed in Table 1 Suppl.

The *MdPHO1* was identified by homology with *Arabidopsis PHO1* (*TAIR*: AT3G23430.1). *MpPHO1* promoter region was cloned by PCR based on the upstream of the *MpPHO1* on contig MDC015642.226. The primers used for cloning MpPHO1 promoter are also listed in Table 1 Suppl.

The PCR-amplified *MpWRKY6* cDNAs, genomic clones, and promoter sequences were sequenced on both strands by *Sanger* technology. The sequences were then compared with those in *GDR*, using the *BLAST* program. Nucleotide/amino acid sequences were analyzed by *DNAMAN*. Finally, the functions for *cis*-acting elements in the *MpWRKY6* promoter were predicted from the *PlantCARE* database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, Rombauts *et al*. 1999, Postel *et al*. 2002).

Protein induction: The reconstructed pET32a plasmids containing *MpWRKY6a* or *MpWRKY6b* were transformed into *Escherichia coli* strain BL21 (DE3) to induce proteins by 1 mM isopropyl -β-D-thiogalactopyranoside at 18 °C for 10 h. Bacterium cells were centrifuged at 8 000 *g* for 10 min and re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 1 mM phenylmethanesulfonyl fluoride). The solution was sonicated (40 % power on for 5 s and off for 10 s, repeated for 30 min) in ice by using an ultrasonic cell disruptor (*FB120220*, *Thermo Fisher Scientific*), and then centrifuged at 12 000 *g* for 30 min. Afterward, the supernatant and precipitate were collected separately and analyzed by SDS-PAGE electrophoresis.

Sub-cellular localization: Onion (*Allium cepa* L.) epidermal cell layers were placed on the MS medium with the interior of the layer facing upwards. pJIT163-*MpWRKY6a-GFP*, pJIT163-*MpWRKY6b-GFP*, and pJIT163-*GFP* (negative control) were transformed into onion epidermal cells *via* gene gun-mediated methods. The conditions of bombardment were vacuum pressure of 94.818 kPa, helium pressure of 7.584 to 8.963 MPa, and 6 cm of target distance using gold microcarriers. After bombardment, tissues were incubated on the agar solidified MS medium at 22 °C for 24 h. Samples were transferred to glass slides and observed under a fluorescence microscope (*Olympus*, Tokyo, Japan). The excitation wavelength was set at 480 nm. Exposure time was adjusted according to the picture status. The light field and corresponding fluorescent field photos were taken under the same view.

Promoter mutation: DNA was subjected to mutagenesis with *Muta-direct*™ site-directed mutagenesis kit (*SBS genetech*; http://www.sbsbio.com/news/ index.php), according to the manufacturer's instructions. Positive clones were sequenced to confirm the intended sequence.

Yeast hybrid assays: Yeast one-hybrid assay used *Matchmaker*™ *Gold* yeast one-hybrid library screening system (*Takara*, Tokyo, Japan) was performed according to the protocol recommended by the manufacturer.

Futher, we collected 20 young fruits of *M. prunifolia* for constructing the yeast two-hybrid cDNA library and yeast two-hybrid assay used *Matchmaker*™ *Gold* yeast two-hybrid library screening system (*Takara*) was performed according to the protocol recommended by the manufacturer.

Tissue specificity and stress response of *MpWRKY6a* and *MpWRKY6b*: For tissue specificity analysis of apple *WRKY6* genes, at least five newly produced shoots, flowers, lateral roots, or fruit were sampled from three 8-year-old trees of *M. prunifolia*. For stress response analysis, the one-year-old *Malus prunifolia* plants were grown outdoors in sand-filled pots for approximately 4 months, and then they were transferred to a chamber for hydroponic culture. For treatment with methyl viologen (*Sigma*, St. Louis, USA), we added MV into the ½-strength Hoagland solution to obtain a final concentration of 50 µM. To represent low-N conditions, we replaced the $Ca(NO_3)_2$ and KNO_3 with $CaCl_2$ and KCl. For low-Pi treatment, the KH_2PO_4 in our ½-strength Hoagland solution was reduced from 0.5 mM to 5 µM, with the difference substituted by KCl. During these stress experiments, we used standard ½-strength Hoagland solution as the control and renewed each solution every 3 d. At least five seedlings were used for each treatment time from which their roots and leaves were sampled for RNA isolations.

Total RNA from snap-frozen *M. prunifolia* tissues was extracted by a CTAB method and reversed using a *PrimeScript*® RT reagent kit with gDNA eraser (*Perfect Real Time*, *Takara*). Real-time quantitative PCR was performed according to protocols for *SYBR*® *Premix Ex Taq*™ II (*Takara*) and the real time PCR system machine (*Bio-Rad iQ*™ *5*; Hercules, USA). Expression levels were monitored for *MpWRKY6a*, *MpWRKY6a*, and *EF* (DQ341381; reference gene). Gene expression was calculated using $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen 2001).

Production and evaluation of transgenic *Arabidopsis* plants over-expressing *MpWRKY6a*: To obtain transgenic plants over-expressing *MpWRKY6a*, the cloned coding sequence of *MpWRKY6a* was inserted into the pWR306 vector under 35S promoter. The 35S: *MpWRKY6a* vector was transferred to *Agrobacterium tumefaciens*, and transformation and selection of transgenic *Arabidopsis* plants were performed per the floral-dip procedure (Zhang *et al*. 2006). Homozygous over-expression lines were identified and expression of *MpWRKY6a* was confirmed by real time qPCR.

For testing the response of *Arabidopsis* to stresses, seeds of the WT (Columbia, or Col-0, ecotype) and selected three homozygous T_3 transgenic lines were surface-sterilized and germinated on a standard MS medium. After 7 d, seedlings of all lines were transferred to either a standard MS medium or one that was supplemented with methyljasmonate (MeJA; 5 µM), MV (0.5 µM), or ABA (5 µM). After 7 d, we recorded the fresh mass and root length of WT and transgenic plants. Data were statistically analyzed with *SPSS 17.0*. Statistical differences between WT and transgenic plants after different treatments were evaluated by independent *t*-tests at $P < 0.05$ and $P < 0.01$.

Results

The coding regions of *MpWRKY6a* and *MpWRKY6b* were 1 821 and 1 836 bp long, respectively. Their sequences were 91.90 % similar. The genomic gene sequences of *MpWRKY6a* and *MpWRKY6b* were 2 567 and 2 502 bp long, respectively. They had six exons and fives introns (Fig. 1*A*).

The amino acid sequences of MpWRKY6a and MpWRKY6b share 88.89 % identify. Amino acid sequences of MpWRKY6a, MpWRKY6b, and AtWRKY6 were also aligned. All of them were Group II members of the WRKY TF family and had a single WRKY domain and a $CX_5CX_{22}HXH$ type zinc-finger motif (Fig. 1*B*, Fig. 1 Suppl.). In a prokaryotic expression system, *MpWRKY6a* and *MpWRKY6b* were translated into approximately 87-kDa proteins when inserted into the pET32a vector. These appeared in both the soluble and insoluble portions of total protein in *Escherichia coli* strain BL21 (not shown).

Protein sequences of MpWRKY6a, MpWRKY6b and homologous sequences in other plants were used to construct a phylogenetic tree (Fig. 2*C*). *Arabidopsis* WRKY6 has the closest evolutionary relationship with *Malus* WRKY6.

To gain insight into potential regulation of these genes, we analyzed their promoter regions for the presence of recognized stress response *cis*-elements. There were many stress-responsive elements in their promoter regions including ARE, MBS, ABRE, W box, and CGTCA -motif (Fig. 1*D*, Tables 2 and 3 Suppl.). They both have abscisic response element (ABRE), anaerobic induction element (ARE), and MeJA response element (CGTCA) motifs.

Our fluorescence observations demonstrated that, when fused with GFP, MpWRKY6a and MpWRKY6b occurred exclusively in the nucleus whereas GFP-alone was detected throughout the cell (Fig. 2). Because MpWRKY6a and MpWRKY6b are highly homologous, we selected only one, MpWRKY6a, for yeast one-hybrid assay. Cells transferred with the *MpPHO1* promoter region and *MpWRKY6a* survived when exposed to 600 ng cm^{-3} aureobasidin A (AbA), whereas only a few transferred with the mutated *MpPHO1* promoter (negative control) and *MpWRKY6a* survived on media containing the same concentration of AbA (Fig. 3). This showed that MpWRKY6a had the ability to bind to W-boxes.

We used a yeast two-hybrid system assay to determine which proteins interact with MpWRKY6a. All yeast cells, whether transferred with the pGBKT7, p-*MpWRKY6a*-GBKT7, or p-*MpWRKY6b*-GBKT7 vectors, were unable to survive on media that lacked Trp, His, and Ade. This demonstrated that MpWRKY6a and

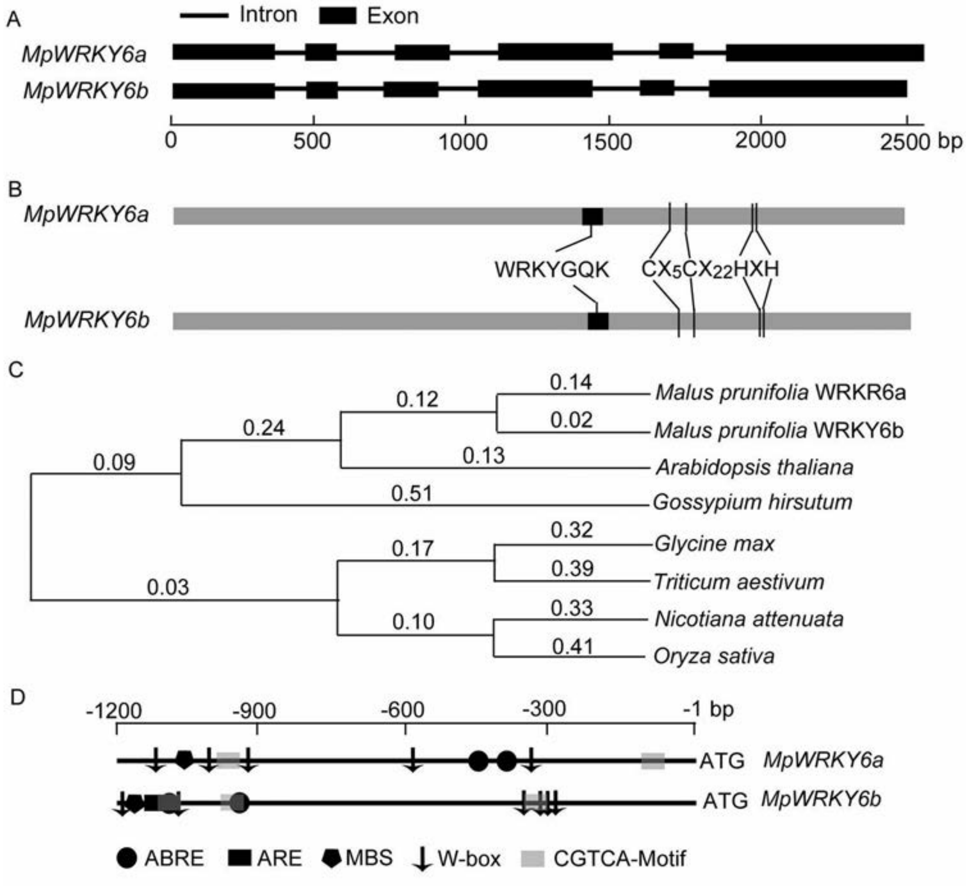

Fig. 1. *A* - Structure analysis of genes *MpWRKY6a* and *MpWRKY6b* showing exons (*black boxes*) and introns (*black lines*). *B* - Schematic diagram of MpWRKY6a and MpWRKY6b, showing the locations of WRKY domain and novel zinc finger structure, $CX_5CX_{23}HXH$. *C* - Phylogenetic analysis of deduced amino acid sequences of WRKR6a, WRKR6b and other WRKYs from 7 plant species. *D* - Main stress-related *cis*-elements in *MpWRKY6a* and *MpWRKY6b* promoter regions.

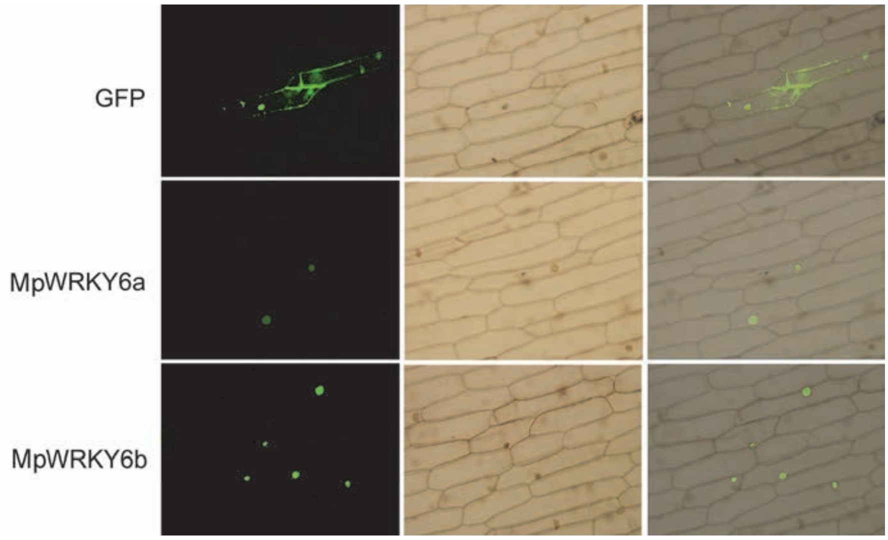

Fig. 2 Sub-cellular localization of MpWRKY6a and MpWRKY6b in onion epidermal cells, based on GFP fluorescence. GFP-alone was localized throughout entire cell. MpWRKY6-GFP was localized exclusively in nucleus. Cells were examined with fluorescence microscope.

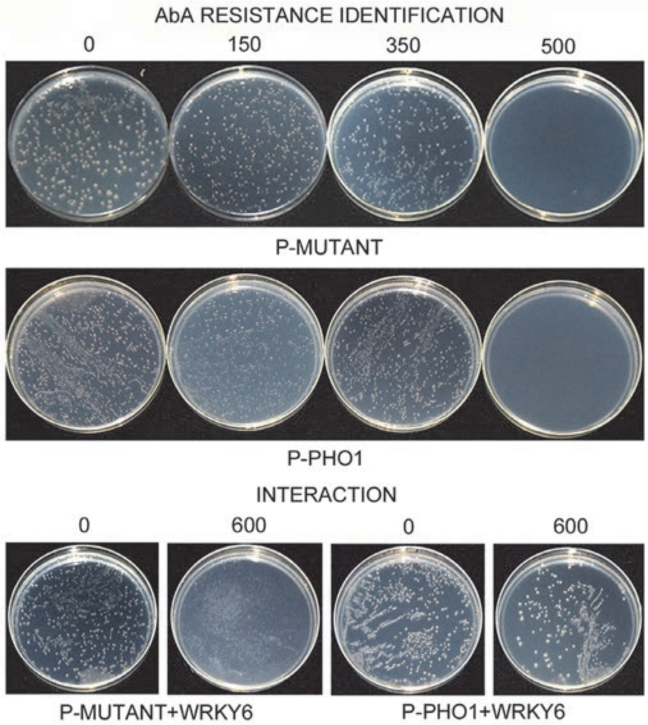

Fig. 3. Yeast one-hybrid assays for W-boxes binding ability of MpWRKY6a. Cells transformed with the *MpPHO1* promoter region and *MpWRKY6a* survived when exposed to 600 ng cm^{-3} aureobasidin (AbA), whereas only a few transformed with the mutated *MpPHO1* promoter (negative control) and *MpWRKY6a* survived on media containing the same concentration of AbA.

MpWRKY6b does not self-activate in yeast and may be transcriptional repressors. After cDNA library screening, only three colonies survived: 6-5, 6-6, and 6-8 (Fig. 3 Suppl.). Among them, only 6-5 and 6-8 turned blue. We then sequenced the cDNA inserted into their plasmids and determined that both contain an ubiquitin-like superfamily protein members (MDP0000204150 in 6-5 and MDP0000169030 in 6-8), which were homologous genes in *Malus*; thereby showing that MpWRKY6a could be modified by ubiquitin-like protein. We also noted here that a member of the Radiation Sensitive23 (RAD23) family (RAD23D; MDP0000291439) could bind with MpWRKY6a, although the interaction between them was not as strong as that found between MpWRKY6a and 6-5 or 6-8 (see colony 6-6 in Fig. 3 Suppl.).

MpWRKY6a and *MpWRKY6b* expressed in all tested plant parts with relatively high levels in leaves, stems, and roots and relatively low levels in flowers and young fruits (Fig. 4*A*). After the plants were treated with MV, both genes showed a transient increase in leaves after 1 h and in roots after 6 h. The fold increase in expression of *WRKY6a* was much greater than of *WRKY6b* in both leaves and roots (Fig. 4*B*). Both genes also showed increased expression in roots in plants transferred to conditions of low N or P (Fig. 4*C* and *D*). But there were no obvious expression changes of *MpWRKY6a* or *MpWRKY6b* in *M. prunifolia* leaves under low-N or P conditions.

To further investigate the potential roles of *MpWRKY6* in abiotic stress responses, we evaluated the phenotype of transgenic plants expressing *MpWRKY6a*. Three homozygous lines representing strong (OE-4), mediate (OE-6), and weak (OE-1) expressions were chosen for the study (Fig. 4 Suppl.). Under non-stress growing conditions, *MpWRKY6* over-expressing lines had longer roots and bigger leaves than WT (Fig. 5). When plants were exposed to MeJA, the fresh mass and root length decreased more in *MpWRKY6a* over-expressing lines than in WT plants, indicating that *MpWRKY6a* over-expression made transgenic plants more sensitive to MeJA (Fig. 5). Different results were found for plants treated with either MV or ABA. Under MV treatment, fresh mass and root lengths were more reduced in WT plants than in transformed lines, indicating that *MpWRKY6a* over-expression made transgenic plants more resistant to MV (Fig. 5). Although there were no significant fresh mass differences between WT and over-expressing lines under ABA treatment, the roots of transgenic lines were longer than those of WT plants, suggesting decreased sensitivity (Fig. 5).

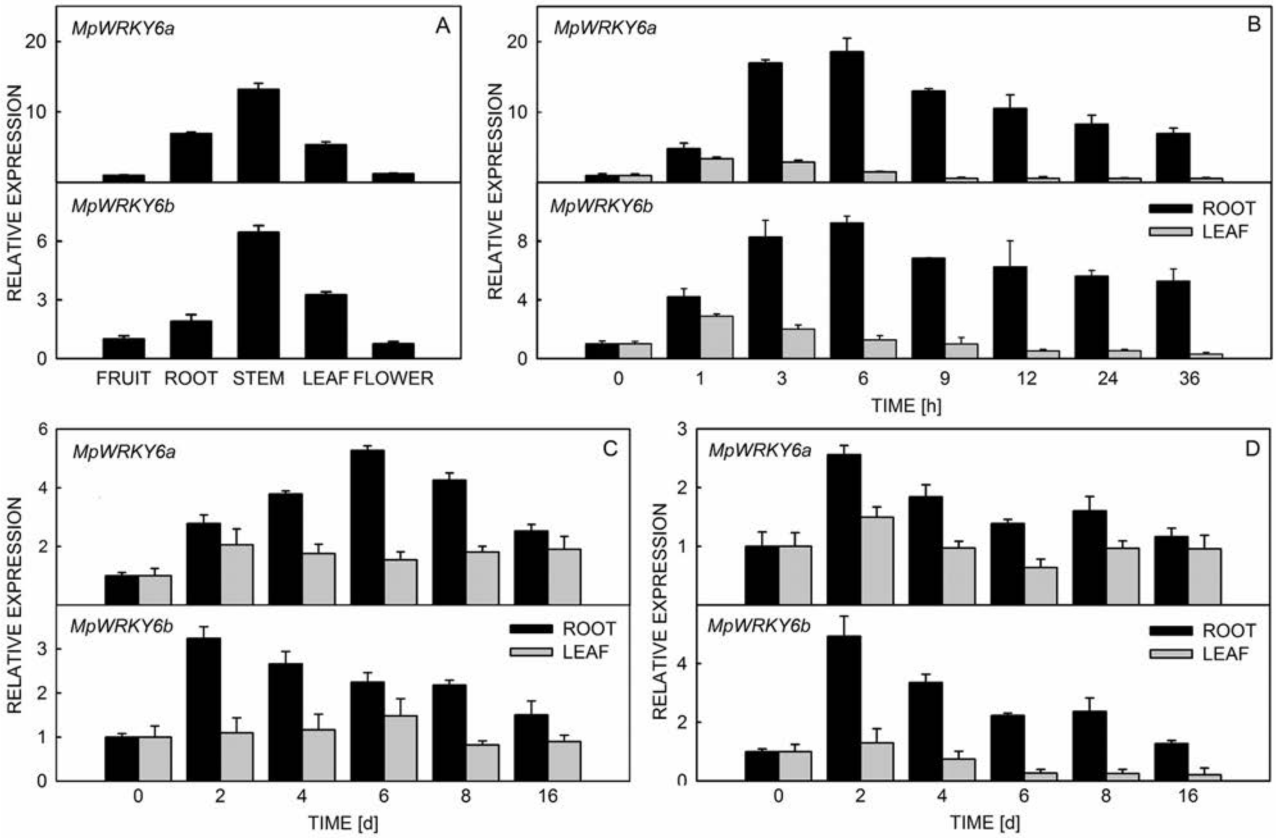

Fig. 4 Relative expression of *MpWRKY6a* and *MpWRKY6b* in different tissues of *M. prunifolia* (*A*) and in roots and leaves from plants exposed to MV (*B*), low N (*C*) and low Pi (*D*). Expressions were calculated relative to expression of *MpEF* mRNA at day 0. Means ± SDs for 5 replicate samples.

Discussion

WRKY6 plays important roles in plant responses to abiotic and biotic stresses (Robatzek and Somssich 2001, Chen *et al*. 2009, Kasajima *et al*. 2010, Castrillo *et al*. 2013, Huang *et al*. 2016). Here, we cloned the homologous *MpWRKY6a* and *MpWRKY6b* from *M. prunifolia.* According to the classification of WRKY transcription family, both MpWRKY6a and MpWRKY6b were Group II members of the WRKY TF family, with one WRKYGQK domain and the $CX_5CX_{22}HXH$ type zinc finger motif (Fig. 1*B*; Fig. 1 Suppl.). As expected for transcription factors, they are localized exclusively in the cell nucleus (Fig. 2). The DNA binding domain of WRKY TFs can recognize *cis*-elements termed W-boxes C/TTGACC/T (Eulgem *et al.* 2000, Ciolkowski *et al.* 2008). Chen *et al.* (2009) have shown that AtWRKY6 binds with W-boxes in the *AtPHO1* promoter region. Our experiments demonstrated that MpWRKY6a can bind with the promoter region of *MpPHO1* in yeast (Fig. 3). Even a single nucleotide base exchange in the W-box sequence can decrease the binding activity of WRKY TFs (Ciolkowski *et al.* 2008). When W-boxes in the *MpPHO1* promoter region had been mutated to TTGATT, MpWRKY6a could no longer bind to that region (Fig. 3, Fig. 2 Suppl.).

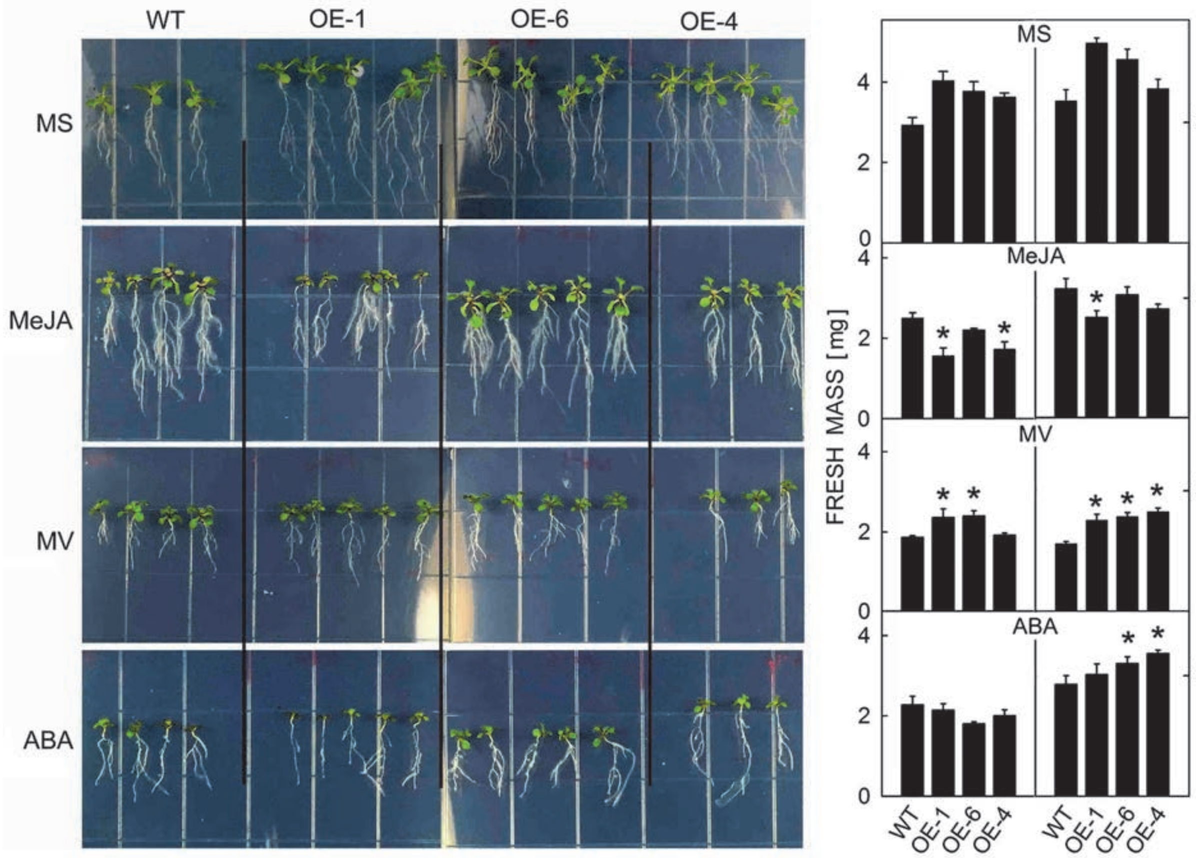

Fig. 5 Phenotype comparisons, fresh mass, and root length of WT and *MpWRKY6a* over-expressing lines grown in MS (control) or MS with MeJA, MV, or ABA. Means ± SDs, $n = 20$, * significant differences between WT and transgenic plants at $P < 0.05$ (*t*-tests).

WRKY TFs can play either transcriptional activator or repressor roles in the stress-response pathways (Hwang et al. 2011, Li *et al.* 2015). Our assays showed that expression of neither *MpWRKY6a* nor *MpWRKY6b* was sufficient to allow yeast to survive. It may suggest that they act as transcriptional repressors in yeast (Fig. 3 Suppl.). If so, this is consistent with the observation that *Arabidopsis* WRKY6 negatively regulates responses to abiotic stresses such as Pi deficiency (Chen *et al.* 2009). Through yeast two-hybrid assay, we were able to obtain only two ubiquitin-like superfamily proteins that interacted with MpWRKY6a. Interestingly, the two proteins are homologous in *Malus* and *Arabidopsis*. This suggested that the WRKY TFs can be modified by ubiquitin-like superfamily members in addition to MAPKs (Mao *et al.* 2011). Other researchers (Chen *et al.* 2009, Miao and Zentgraf 2010) have reported that E3 ubiquitin ligase can interact with and degrade WRKY TFs through 26S-proteosome pathway. We also noted here that a member of the *Radiation sensitive23* (*RAD23*) family (*RAD23D*; MDP0000291439) could bind with MpWRKY6a (see colony 6-6 in Fig. 3 Suppl.). RAD23D can bind with ubiquitin and is an essential connection between the 26S proteasome and ubiquitylated proteins (Farmer *et al.* 2010). However, we did not further investigate here whether MpWRKY6 degradation is achieved *via* the 26S-proteosome pathway with the help of RAD23D.

The observation that *MpWRKY6a* and *MpWRKY6b* were expressed in all selected plant parts is consistent with the expression of *WRKY6* in *Arabidopsis*, which suggests an important and general function of WRKY6 (Fig. 4; Robatzek and Somssich 2001).

AtWRKY6 participates in responses to Pi deficiency and boron starvation (Chen *et al*. 2009, Kasajima *et al*. 2010). *MpWRKY6a* and *MpWRKY6b* are induced by Pi or N deficiencies in *M. prunifolia*, thereby suggesting their possible roles in nutrition-starvation responses (Fig. 4*C* and *D*). Interestingly, Chen *et al*. (2009) hypothesized that the repression of PHO1 by WRKY6 was released under low Pi conditions and the expression of *WRKY6* did not change significantly under Pi deficiency in *Arabidopsis*. Thus, more work should be done to determine if down-regulation of WRKY6 is affected at the transcriptional or posttranscriptional level.

There are many stress associated *cis*-elements in the promoters of *MpWRKY6a* and *MpWRKY6b*, including ARE for anaerobic induction, CGTCA-motif for the MeJA response, and ABRE for the ABA response (Tables 2 and 3 Suppl.), indicating functions of *MpWRKY6a* and *MpWRKY6b* in these stress responses. *MpWRKY6a* and *MpWRKY6b* can be up-regulated by MV treatment and *MpWRKY6a*-overexpressing *Arabidopsis* plants are more resistant to MV treatment illustrating their possible functions in oxidative stress response (Fig. 4*B* and Fig. 5).

MpWRKY6a over-expressing *Arabidopsis* plants are more sensitive than the WT to treatments with MeJA and insensitive to ABA, indicating roles in MeJA and ABA signaling pathways and related stress responses. Phytohormone-related functions of WRKY TFs have been reported previously. In *A. thaliana*, *WRKY18*, *-40*, and *-60* participate in ABA signaling during seed germination and seedling growth (Chen *et al*. 2010). Ramamoorthy *et al*. (2008) have shown that many WRKY TF family members in rice respond to exogenous phytohormones, *e.g*., ABA, auxin, MeJA, SA, and gibberellins (GAs); In *Gossypium hirsutum*, *GhWRKY3* can also be induced by MeJA, ABA, GAs, ethylene, pathogen infection, or wounding (Guo *et al*. 2011). In *Malus*, *MhWRKY1*, similar to *AtWRKY7*, is involved in resistance pathways mediated by SA, MeJA, and ethylene, as well as the defense reaction triggered by *Botryosphaeria berengeriana* (Zhang *et al*. 2011).

In summary, *M. prunifolia* contained two homologous *WRKY6* genes. Both MpWRKY6a and MpWRKY6b were members of Group II WRKY TFs and were localized exclusively to the cell nucleus. MpWRKY6a can bind to W-box *cis*-elements. Expression of *MpWRKY6a* and *MpWRKY6b* was up-regulated in plants exposed to low-N and low-Pi conditions, suggesting their roles in nutrition-starvation responses. Their expression levels were also up-regulated in plants treated with MV and transgenic plants were more resistant to MV treatment, indicating their functions in oxidative stress. Transgenic plants were more sensitive to MeJA and resistant to ABA treatment, providing participations of MpWRKY6 TFs in MeJA or ABA signaling related abiotic and biotic stresses responses.

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