



Contribution of methylation regulation of *MpDREB2A* promoter to drought resistance of *Malus prunifolia*

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Abstract

Background and aims *Malus prunifolia* (Chinese name: Fu Ping Qiu Zi), a wild relative of cultivated apple (*Malus x domestica* Borkh), is extremely resistant to drought compared with domesticated

cultivars, such as ‘Golden Delicious’. However, the molecular mechanisms underlying drought resistance of *M. prunifolia* have not been characterized. This study investigates a new regulatory mechanism to improve apple drought resistance.

Xuewei Li and Yinpeng Xie contributed equally to this work.

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Methods *M. prunifolia* and ‘Golden Delicious’ were each grafted on *M. hupehensis* for gene expression analysis. The methylation level of the *DREB2A* promoter was determined by bisulfite sequencing and ChIP-qPCR. Chromatin immunoprecipitation sequencing (ChIP-seq) was used to identify target genes of MpDREB2A in apple.

Results The exposure to drought stress stimulated the expression level of *DREB2A* gene more than 100-fold in *M. prunifolia*, but only 16-fold in ‘Golden Delicious’. This difference in gene expression could not be explained in terms of difference in leaf relative water content. Correspondingly, the methylation level of *M. prunifolia* *DREB2A* (MpDREB2A) promoter region was significantly reduced. Additionally, MpDREB2A conferred enhanced drought resistance when ectopically expressed in *Arabidopsis*. Over 2800 potential downstream target genes of MpDREB2A were identified by ChIP-seq and these downstream genes have diverse potential functions related to stress resistance.

Conclusions Methylation regulation in promoter of MpDREB2A may contribute to the drought resistance of *M. prunifolia*.

Keywords DREB2A · *Malus* · Drought resistance · DNA methylation · Gene expression · ChIP-seq

Introduction

One of the most important ways by which plants adapt to the environmental alterations can be displayed by modulating expression of genes that influence physiology, growth, and development. The Dehydration Responsive Element Binding proteins (DREB), which is a member of AP2/ERF class of transcription factor, are induced in response to multiple abiotic stresses. Several recent studies have characterized *DREB* genes in various species and discussed their potential roles in resistance to high salt, heat, cold, and water stress (Liao et al. 2016; Zhao et al. 2012, 2013; Century et al. 2008; Chen et al. 2007; Qin et al. 2007; Sakuma et al. 2006a, b; Dubouzet et al. 2003; Liu et al. 1998; Yamaguchi-Shinozaki and Shinozaki 1994). DREB factors recognize the dehydration responsive *cis* element (DRE)/CRT (C-repeat sequence) (Century et al. 2008; Yamaguchi-Shinozaki and Shinozaki 1994) in the promoter region of downstream genes. In *Arabidopsis thaliana*, AtDREB2A enhances drought resistance by regulating a diverse set of genes including

COR15A/B, *RD17/29A* and *KINI* (Sakuma et al. 2006a, b). *DREB2A* levels are regulated post-transcriptionally through a suite of factors including DRIP1 (DREB2A-INTERACTING PROTEIN1), DRIP2, and BPM (BTB/POZ AND MATH DOMAIN) proteins (Morimoto et al. 2017; Blomberg et al. 2012; Elfving et al. 2011; Qin et al. 2008). In addition, *DREB2A* is regulated at the transcriptional level by stress-response transcription factors (Kim et al. 2012).

DNA methylation plays an important role in gene activity and establishment of developmental programs (Tan 2010; Slotkin et al. 2009). DNA methylation and demethylation have been shown to be associated with response to abiotic and biotic stress through impacts on gene expression patterns (Dowen et al. 2012). For example, loss of flavor in chilled tomatoes is associated with transient and fast changes in DNA methylation of volatile synthesis genes (Zhang et al. 2016). It was reported that genome-wide demethylation occurred in root tissues of maize seedlings in response to cold stress (Steward et al. 2002). In addition, drought and salt stress can induce DNA hypermethylation in pea and wheat (Kumar et al. 2017). DNA methylation has also been associated with maintenance of stress memory (Chinnusamy and Zhu 2009). So far, few researches have been focused on the relationship between DNA methylation and abiotic stress resistance in perennial woody plants, such as apple.

Malus prunifolia (Chinese name: Fu Ping Qiu Zi), a wild relative of domesticated apple, is native to arid regions of eastern Asia. Compared with cultivated apple varieties, such as ‘Golden Delicious’, *M. prunifolia* shows extreme resistance to drought (Wang et al. 2012). Previously, it was investigated that the *M. prunifolia* *DREB* gene MpDREB2A was dramatically induced by drought, cold stress, heat stress, and abscisic acid (ABA) treatment (Zhao et al. 2012). However, the molecular mechanisms underlying drought resistance of *M. prunifolia* have not been characterized. This study investigates a new regulatory mechanism of *DREB2A* to improve apple drought resistance.

Materials and methods

Plant materials

Seedlings of *M. prunifolia* were used for MpDREB2A cloning. Three-month-old *M. prunifolia* and ‘Golden

Delicious' plants grafted on *M. hupehensis* rootstock were used for gene expression and DNA methylation analysis. 'Golden Delicious' plants grafted on *M. hupehensis* were also used for *MdDREB2A* cloning. All apple plants were grown in a greenhouse at Northwest Agriculture and Forest University, Yangling (34°20'N, 108°24'E), Shaanxi Province, China.

Arabidopsis accession Col-0 was used for ectopic expression of *MpDREB2A*. Seeds were sterilized with 70% ethanol for 1 min, 5% sodium hypochlorite for 10 min, and then washed with sterile water 5 times. After stratification at 4 °C for two days, seeds were distributed onto Murashige and Skoog (MS) medium (4.43 g/L, #M519, Phyto Technology Laboratories, USA) containing 20 g/L sugar and solidified with 6 g/L agar. Seedlings were grown at 22 °C

under 16 h light/8 h dark photoperiods. After 14 d, plants were transplanted to soil and grown in a growth chamber at 22 °C under 16 h light/8 h dark photoperiods and 60% relative humidity.

Stress treatment

Three-month-old *M. prunifolia* and 'Golden Delicious' were each grafted on *M. hupehensis* rootstock and grown in a greenhouse under natural light and temperature conditions in July, 2016. The plants were planted in plastic pots (15 cm × 20 cm, ~1.3 L) filled with a mixture of garden soil and substrate (PINDSTRUP, Denmark) (1:1, v/v). Before treatment, soil field capacity (FC) was measured using the following formula:

$$FC (\%) = [(Saturated\ mixed\ soil\ weight - dry\ mixed\ soil\ weight) / dry\ weight] * 100$$

To impose soil drying, plants were withheld with water for up to 8 d, and soil relative water content (SRWC) was calculated daily at 18:00 pm by a weighing method (Shao et al. 2006; Yildirim and Kaya 2017). SRWC (as a percentage of field capacity) was 85% (day 0), 70% (day 2), 55% (day 4), 40% (day 6) and 30% (day 8) when leaf samples were collected for further analysis. Surface evaporation was minimized by covering the pots with a 3 cm-layer of sieved sand (2 mm in diameter). The external of the pots was packaged with reflective film to

prevent the soil from heating up too much. To avoid edge effects, all pots were rotated daily.

Leaf relative water content (LRWC) was determined by Wang et al. (2012). Plant leaf water status was measured on day 0 and day 8 after drought. Ten leaves were collected randomly from *M. prunifolia* and 'Golden Delicious', weighed quickly and then transferred to deionized water for 12 h to measure turgid weight. Leaves were then dried in an oven at 80 °C for 72 h and weighed to measure dry weight. LRWC was measured by the following formula:

$$LRWC (\%) = 100 * [(fresh\ weight - dry\ weight) / (turgid\ weight - dry\ weight)]$$

For gene expression analyses, mature leaves were collected after 0, 2, 4, 6, 8 d of drought. Leaves harvested at day 0 and 8 were also used to determine the methylation level of the *DREB2A* promoter. For cold stress, three-month-old plants were subjected to 4 °C for 0, 4, and 12 h. For ABA treatment, plants were sprayed with abscisic acid (100 µM, Sigma, USA) and leaves were collected after 0, 2, 4, and 8 h. For salt stress, soil-grown plants were treated with 200 mM NaCl and leaves were collected after 0, 12, and 24 h.

To investigate drought resistance of *Arabidopsis* plants, water was withheld for 10 d from four-week-old plants grown in soil. The *Arabidopsis* were planted in plastic pots

(4 cm × 4 cm × 5 cm) filled with substrate (PINDSTRUP, Denmark) in a plant growth chamber (20,000 Lx, 25 °C, 40% humidity). Substrate relative water content was calculated daily at 18:00 by the same method. The SRWC was 70% (day 0) and 35% (day 10). Survival rate was measured after 5 d of rewatering. *Arabidopsis* LRWC was determined by the method described above.

Dehydration assay

Two-week-old transgenic *Arabidopsis* and Col-0 grown on MS medium were transferred to filters exposed to the environment (25 °C, 35% humidity).

Plants were quickly weighed after 0, 20, 40, 60, 120, and 180 min.

Sequence alignment, phylogenetic analysis and structure model of *MpDREB2A* and *MdDREB2A*

The conserved AP2 domains were identified using Pfam (<http://pfam.sanger.ac.uk/>). Sequences were aligned with CLC Combined Workbench version 7. Full-length gene sequences were obtained from NCBI (the National Center for Biotechnology Information) and the phylogenetic tree was constructed by neighbor-joining (NJ) and bootstrap (Tamura et al. 2011).

RNA extraction

Extraction of total RNA from apple leaves was performed by a CTAB method. Total RNA of *Arabidopsis* was extracted with TRIzol Reagent (Invitrogen, USA). Residual genomic DNA was removed by RNase-free DNase I (Fermentas, USA).

Quantitative real-time PCR

qRT-PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad, USA) using GoTaq® qPCR Master Mix Kit (Promega, USA) (Guan et al. 2013). The apple *MDH* (malate dehydrogenases) gene was used as the endogenous control. Expression data were presented with apple *MDH* gene as the internal control, per the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Primers used were shown in Table S1.

Vector construction and genetic transformation

The coding region of *MpDREB2A* was cloned into the vector pDONR222 using the BP reaction, and then into pGWB411 by the LR reaction (Invitrogen). The resulting plasmid 35S::*MpDREB2A* was introduced into *Agrobacterium* strain *EHA105*. Four-week-old *Arabidopsis* Col-0 was used for transformation with the floral dip method (Clough and Bent 1998).

Plasmid pMDC164 was used to examine GUS expression. Promoter of DREB2A from ‘Golden Delicious’ or *M. prunifolia* was cloned into pMDC164. The resulting plasmids *DREB2A::GUS* were introduced into *Agrobacterium* strain *C58C1*. Four-week-old

Nicotiana benthamiana was used for transient transformation (Sparkes et al. 2006).

Primers used were shown in Table S1.

Subcellular localization of MpDREB2A

The coding region of *MpDREB2A* was cloned into vector pGWB406 by BP and LR reactions (Invitrogen). The resulting plasmid, 35S::*MpDREB2A-GFP*, was introduced into *Agrobacterium* strain *C58C1*. Four-week-old *Nicotiana benthamiana* were infected and after three days, leaves were observed for GFP signal and imaged using a Nikon A1R/A1 confocal microscope (Nikon, Tokyo, Japan).

MpDREB2A transcriptional activity assay

The entire coding region and five 3'-truncated fragments of MpDREB2A (encoding 1-137aa, 1-163aa, 1-268aa, 1-296aa and 1-354aa, respectively) were amplified and cloned into vector pGBKT7 (Clontech, USA). The resulting plasmids were transformed into yeast strain AH109 grown on synthetic dropout (SD) medium lacking Tryptophan (Trp). Positive clones were further observed for X- α -gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside) activity on SD medium containing 4 mg/L X- α -gal but lacking Trp, Histidine (His), and Adenine (Ade).

Histochemical and fluorometric assays for GUS activity

Histochemical staining of transiently transformed *Nicotiana benthamiana* was performed as previously described by Yu et al. (2013b). GUS activity was measured with a Multimode Microplate Reader (Infinite M200 PRO, TECAN), and the total protein extracted from the tested samples was detected using the BSA method (Bradford 1976). GUS activity was calculated as nM of 4-methylumbelliferon (4-MU, Sigma-Aldrich) generated per min per mg of total protein. This experiment was repeated three times.

DNA methylation analysis

Genomic DNA was extracted using a Wolact® Plant Genomic DNA Purification Kit (Vicband life sciences company (HK) limited, NP11003.50). Bisulfite conversion was conducted with an EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA).

Briefly, the input DNA was converted by bisulfite first and then the bisulfite-converted DNA was used as a template to clone the DREB2A promoters. Colonies carrying DREB2A promoters were used for sequencing. For each treatment, at least 20 clones were chosen randomly and sequenced. The sequences were aligned and analyzed using Kismeth (<http://katahdin.mssm.edu/kismeth/revpage.pl>) (Gruntman et al. 2008, Jian and Chattopadhyay, 2010).

For the ChIP-qPCR analysis, methylated-DNA IP Kit (Zymo Research, Orange, CA, USA) was used to capture methylated DNA from genome. Briefly, the input DNA was firstly fragmented to 200–500 bp by dsDNA Shearase Plus (Zymo Research, Cat # E2018) and then denatured. The DNA was pulled down with mouse 5-Methylcytosine monoclonal antibody to capture the methylated DNA and then followed by qPCR (Zymo Research, Cat # D5101). This experiment was repeated three times and three biological replicates were used each time.

Primers used for bisulfite sequencing are listed in Table S1.

Root morphology analysis

Five-day-old *Arabidopsis* wild type and transgenic plants grown on MS agar medium were transferred to MS agar medium supplemented with -0.7 Mpa PEG8000 for 7 d (Verslues et al. 2006). Plants transferred to MS agar medium were used as controls. Lateral roots were observed using a stereo microscope (Leica S8 APO). Root length was measured by ImageJ software.

To make MS agar plates containing -0.7 Mpa PEG8000, MS agar medium was poured into a petri dish (100 mm \times 100 mm) after autoclaving. 400 g PEG8000 (Sigma, Catalog number P-2139) was dissolved in 600 ml sterile water. After the MS agar medium congealed, 30 ml PEG solution was poured onto the surface of MS agar medium. The plates were placed 15 h inside bench with lid open for the PEG to diffuse into the agar medium (Verslues et al. 2006).

Chromatin immunoprecipitation followed by Illumina sequencing (ChIP-seq)

Leaves of drought-treated *M. prunifolia* were used for ChIP-seq assay which was performed as described previously (Xie et al. 2018).

Results

Isolation and sequence analysis of DREB2A protein in *Malus*

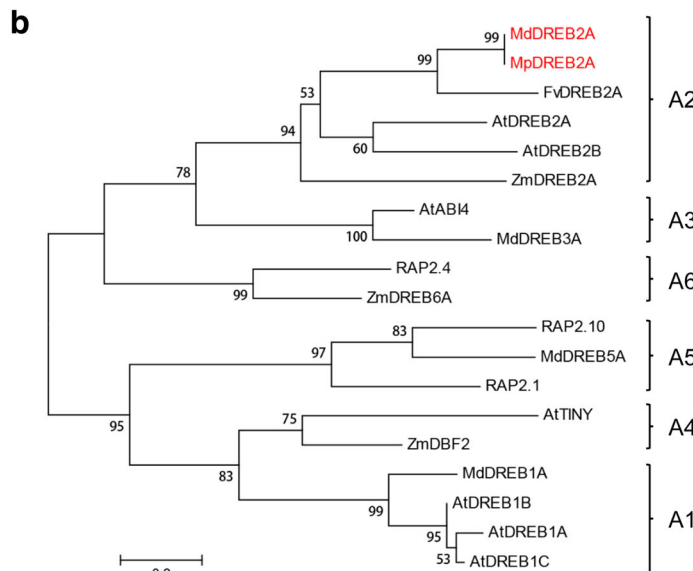
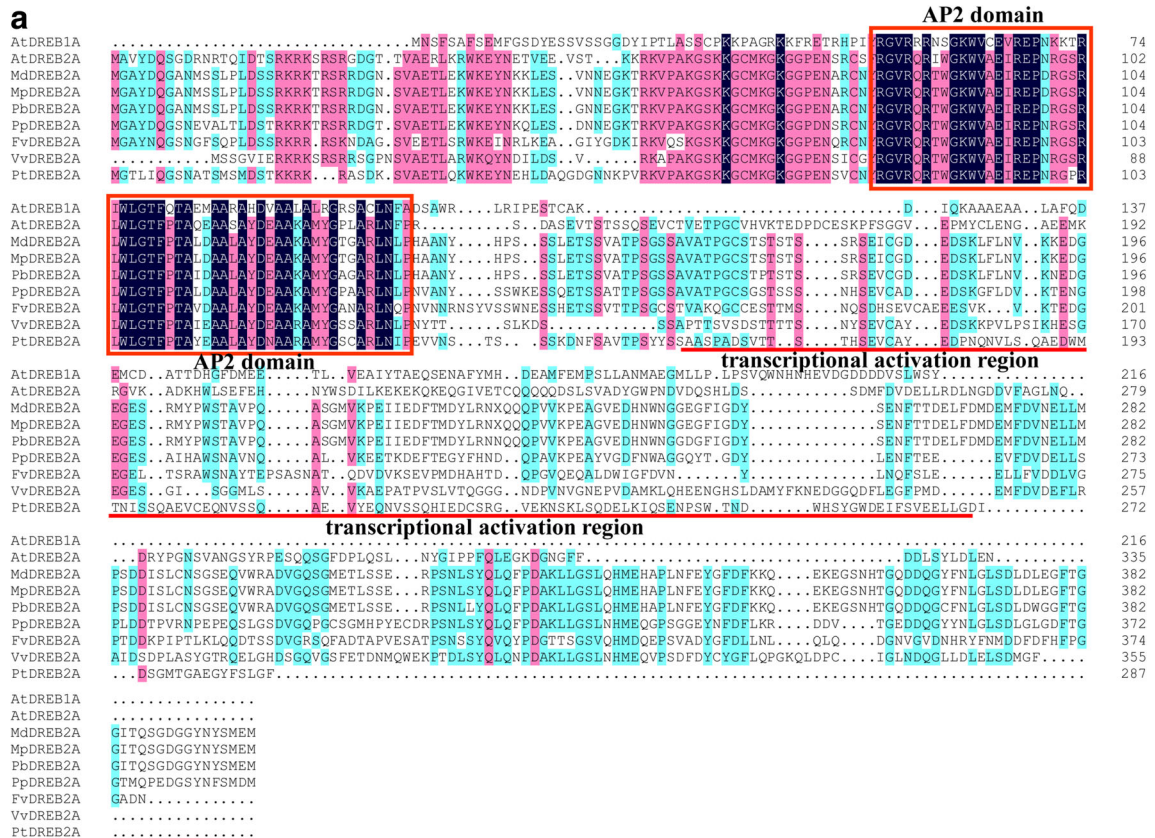
As an initial step to characterize *MpDREB2A*, we cloned the entire open reading frame from *M. prunifolia* cDNA and sequenced the clone in its entirety. The putative protein encoded by *MpDREB2A* is identical with that of *MdDREB2A* from ‘Golden Delicious’, comprising 398 amino acids and with a predicted molecular mass of 43.8 kDa (Fig. S1). Like other DREB2A proteins from various plants, *MpDREB2A*/*MdDREB2A* contain conserved AP2 domain at N-terminus and an acidic C-terminal region that might act as an activation domain for transcription (Fig. 1a). In addition, *AtDREB2A* has less similarity with other DREB2A proteins from rosaceous plants (*MdDREB2A*, *MpDREB2A*, *PbDREB2A*, *FvDREB2A*) (Fig. 1a). Phylogenetic analysis showed that *MdDREB2A*/*MpDREB2A* belong to the A-2 subgroup of the DREB subfamily, with the highest homology (46.6%) to *FvDREB2A* (Fig. 1b).

Transcriptional activity of *MpDREB2A*

To evaluate the transcriptional activation potential of *MpDREB2A*, we carried out transactivation activity assay using full length and 3'-truncated fragments of *MpDREB2A* (encoding 1–137aa, 1–163aa, 1–268aa, 1–296aa and 1–354aa) as translational fusions to the GAL4 DNA binding domain. When expressed in yeast cells, full length *MpDREB2A*, as well as amino-terminal *MpDREB2A* fragments encoding 1–268 aa, 1–296 aa, and 1–354 aa conferred transcriptional activity. In contrast, shorter amino-terminal *MpDREB2A* fragments (1–137 aa and 1–163 aa) were ineffective (Fig. 2a). These results indicate that *MpDREB2A* has a transcriptional activation domain at positions of aa 164 to aa 268. The results of the present investigation showed that full-length *MpDREB2A* sequence was sufficient to direct a GFP fusion protein to the nucleus (Fig. 2b), consistent with a presumed role as a transcription factor.

Ectopic expression of *MpDREB2A* in *Arabidopsis* suggested drought resistance

In order to investigate the potential role of *MpDREB2A* in drought resistance, we ectopically expressed



MpDREB2A in *Arabidopsis* (Fig. S2). When treated with -0.7 Mpa polyethylene glycol (PEG) for 7 d, leaves of the transgenic plants stayed green while Col-0 turned yellow (Fig. S3a). Under both control

conditions and PEG treatment, transgenic plants had more lateral roots than Col-0 (Fig. 3, Fig. S3b). As shown in Fig. S3b, under control conditions, transgenic plants had longer roots than Col-0 plants.

Fig. 1 Sequence alignment, and phylogenetic tree analysis of **DREB2A**. **a** Alignment of DREB2A amino acid sequences from different species. The amino acids in red frame indicate AP2 domain, and amino acids underlined were transcriptional activation region. The accession numbers for the aligned proteins are: AtDREB1A (ABD42992.1), AtDREB2A (AED90871.1), MdDREB2A (XP_008354169.1), MpDREB2A (MG488272), FvDREB2A (XP_004307690.1), PbDREB2A (XP_009345422.1), PpDREB2A (XP_020413813.1), VvDREB2A (XP_002273838.1), PtDREB2A (XP_002312185.2). **b** Phylogenetic tree of MpDREB2A and their close homologs in different species. A-1 to A-6 indicate subgroups proposed in *Arabidopsis*. MpDREB2A and MdDREB2A in the A-2 subgroup were marked as red font. The accession numbers for the DREB related proteins are: AtDREB1B (AEE85066.1), AtDREB1C (ABI93900.1), AtDREB2B (AEE74994.1), AtAB14 (ABE65896.1), RAP2.4 (AAN41307.1), RAP2.1 (ABD57516.1), RAP2.10 (ABF83647.1), AtTINY (AAT44909.1), ZmDREB2A (BAE96012.1), ZmDBF2 (AF493799), ZmDREB6A (AAM80486.1), MdDREB5A (NP_001288046), MdDREB3A (XP_008362743.1), MdDREB1A (ART8558.1)

We also measured drought resistance of soil-grown Col-0 and transgenic plants. When water was withheld for 10 d, and resupplied for 5 d, 80% of transgenic plants recovered, while only 20% of Col-0 plants recovered (Fig. 4a, b). Moreover, in a controlled dehydration experiment, transgenic plants lost less water than Col-0 (Fig. 4c). These results indicate that *MpDREB2A* is a positive regulator for plant drought resistance.

Expression of *MpDREB2A* in various tissues and in response to various stresses

To explore the function of *MpDREB2A* at the whole plant level, the expression of *MpDREB2A* in various tissues and in response to various stresses was assessed. We detected transcripts of *MpDREB2A* in roots, stems, leaves, flowers, and fruits of *M. prunifolia*, with dominant expression levels in

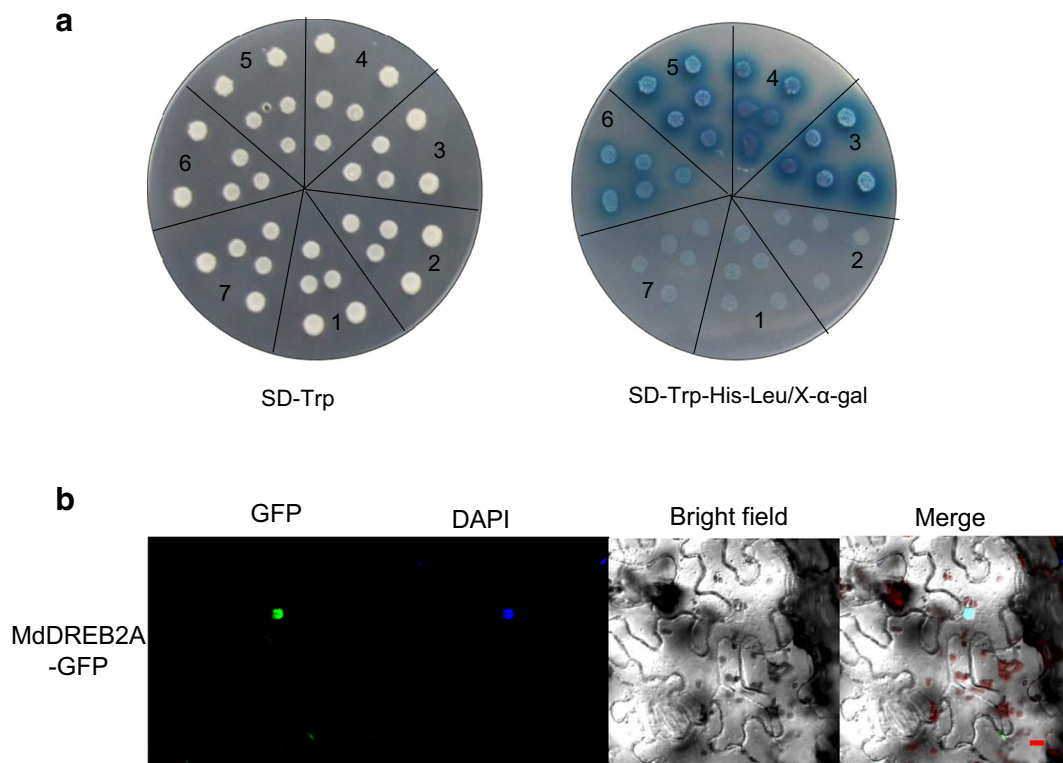


Fig. 2 *MpDREB2A* functions as a self transcriptional activator. **a** Transcriptional activation assay of *MpDREB2A*. The yeast AH109 was transformed with pGBKT7 containing DNA fragment encoding following proteins, 1: *MpDREB2A* 1–137 aa, 2: *MpDREB2A* 1–163 aa, 3: *MpDREB2A* 1–268 aa, 4: *MpDREB2A* 1–296 aa, 5: *MpDREB2A* 1–354 aa, 6:

MpDREB2A full length and 7: pGBKT7 empty vector. Left panel, yeast growth on SD/-Trp medium. Right panel, yeast growth on SD/-Trp-His-Leu medium supplemented with x-α-gal. **b** Subcellular localization analysis of *MpDREB2A* in tobacco leaf epidermal cells. Bars = 20 μm

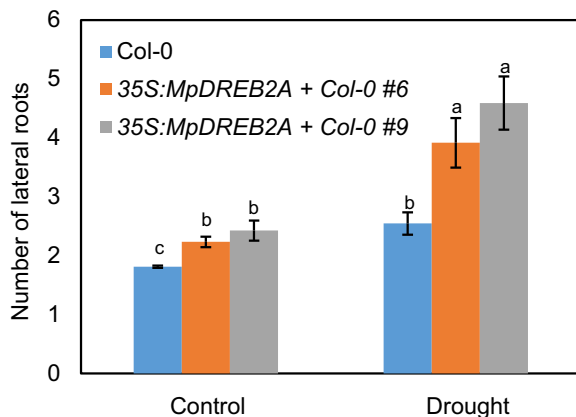


Fig. 3 Number of lateral roots in Col-0 and transgenic plants in response to PEG treatment. Error bars indicate standard deviation ($n = 30$). Seven-day-old seedlings were transferred to MS or MS medium supplemented with PEG for 7 days. Two-way ANOVA was performed, and statistically significant differences were indicated by different letters (Tukey test), $P < 0.05$

roots and flowers (Fig. 5). As shown in Fig. S4a, *MpDREB2A* was up-regulated by 350-fold when plants were exposed to 45 °C for 2 h. However, transcripts of *MpDREB2A* increased 9-fold over the control after 12 h treatment in response to 200 mM NaCl (Fig. S4b). When ABA or cold treatment was applied, the mRNA level of *MpDREB2A* increased, and eventually reached a maximum of 25- or 5- fold

enhancement, respectively (Fig. S4c-d). When water was withheld for 8 d, *DREB2A* was induced in both *M. prunifolia* trees and ‘Golden delicious’ trees grafted on *M. hupehensis*. However, *MpDREB2A* expression level was dramatically enhanced about 160-fold in *M. prunifolia*, whereas *MdDREB2A* expression was increased only 16-fold in ‘Golden Delicious’ (Fig. 6). We also examined leaf relative water content (LRWC) of *M. prunifolia* and ‘Golden Delicious’ grafted on *M. hupehensis* under control and drought stress. A similar LRWC (90%) was observed in both genotypes under control conditions, whereas a LRWC of 65% in *M. prunifolia* and 47% in ‘Golden Delicious’ were observed after 8 d of drought (Fig. S5, Table S2), indicating a drought avoidance strategy in *M. prunifolia*.

Sequence and GUS activity analysis of *DREB2A* promoter

Variation in expression of a given gene between species may be conditioned at the transcriptional level through genetic or epigenetic differences in regulatory elements (Zhang et al. 2016). To reveal the molecular basis for the different expression levels of *DREB2A* between ‘Golden Delicious’ and

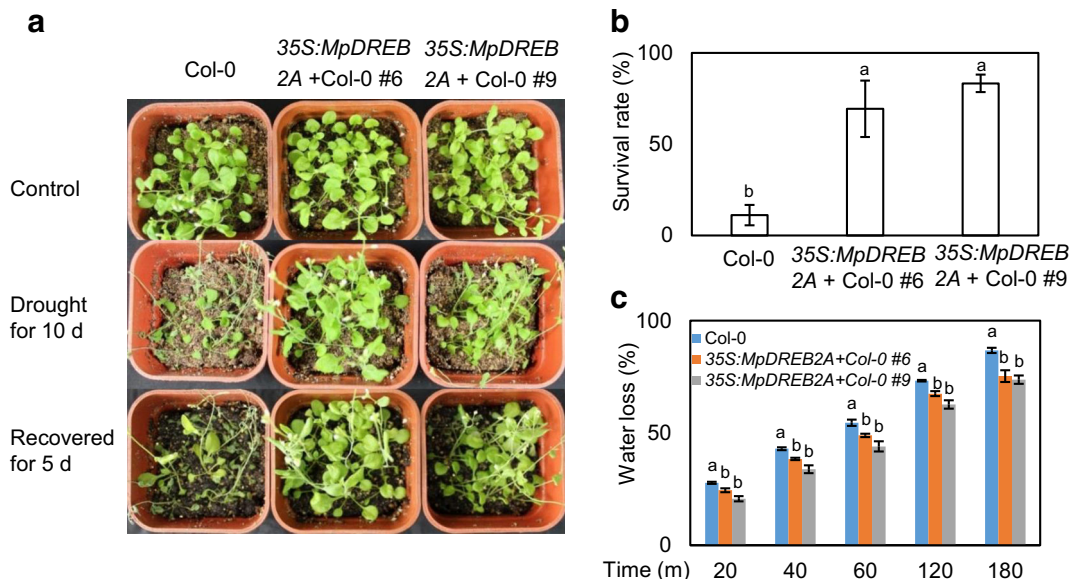


Fig. 4 *MpDREB2A* enhanced drought tolerance of transgenic *Arabidopsis*. **a** Phenotypes of Col-0 and transgenic plants before and after drought treatment. **b** Survival rate of Col-0 and transgenic plants after drought treatment. One-way ANOVA was performed for data in (b) ($n = 36$) (Tukey), and statistically significant

differences were indicated by different letters, $P < 0.05$. **c** Water loss of Col-0 and transgenic plants. Error bars indicate standard deviation ($n = 5$). One-way ANOVA was performed and different letters indicate statistically significant differences ($P < 0.05$) among each time point (Tukey test)

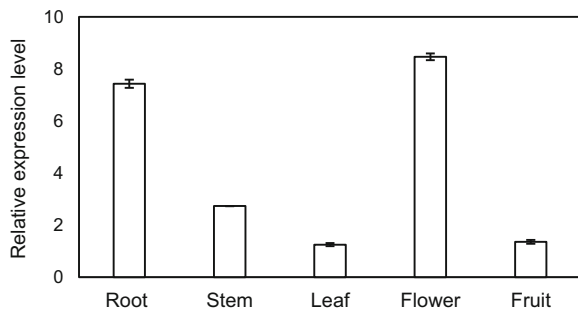


Fig. 5 Expression level of *MpDREB2A* in different tissues of *M. prunifolia* seedlings. Error bars indicate standard deviation (n = 3)

M. prunifolia under drought conditions, the promoter regions for *DREB2A* (GenBank accession number of *MpDREB2A*: MG712295; *MdDREB2A*: MG712294) in both genotypes were cloned and sequenced. The nucleotide sequence of the *DREB2A* promoter in the two genotypes show some insertions/deletions (In/Dels) and SNPs (Fig. S6). To examine if the In/Dels and SNPs may contribute to the incongruent expression of *DREB2A* in the two genotypes, two promoters from both genotypes were fused to the β -glucuronidase (GUS) coding region. Our results showed that there was no difference in GUS activity across both genotypes under normal condition. After dehydration for 0.5 h, GUS was induced rapidly at comparable levels in ‘Golden Delicious’ and *M. prunifolia* (Fig. S6b-c, Table S2).

A search of plant *cis*-regulatory elements in the PlantCARE database (Lescot et al. 2002)

(<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) revealed that there are numerous *cis*-elements in the *DREB2A* promoter. In general, the promoter region of *DREB2A* contains typical CAAT and TATA boxes, as well as light-responsive elements, including G-box, GA-motif, I-Box and TCCC-motif. Important *cis*-regulatory elements in response to hormones were also identified, such as ABRE which is involved in the abscisic acid response, the CGTCA-motif and TGACG-motif which are associated with MeJA-response, TCA-element (core of salicylic acid-responsive element), and TGA-element (core of auxin-responsive element). The Skn-1 motif required for endosperm expression, a *cis*-element related to zein metabolism regulation, and heat stress responsive element (HSE) were also identified in *DREB2A* promoter (Table 1). In addition, promoter alignment analysis suggests that *DREB2A* promoter in ‘Golden Delicious’ also contains the CG-motif and ERE-motif (core of ethylene-responsive element) which were absent in *M. prunifolia* due to two SNPs (Fig. S6a).

Drought stress induces DNA demethylation in the *MpDREB2A*

Since different expression levels of *DREB2A* resulting from drought between ‘Golden Delicious’ and *M. prunifolia* were not due to SNPs of promoter, we speculated that different expression levels could possibly relate to DNA modification. As DNA methylation is

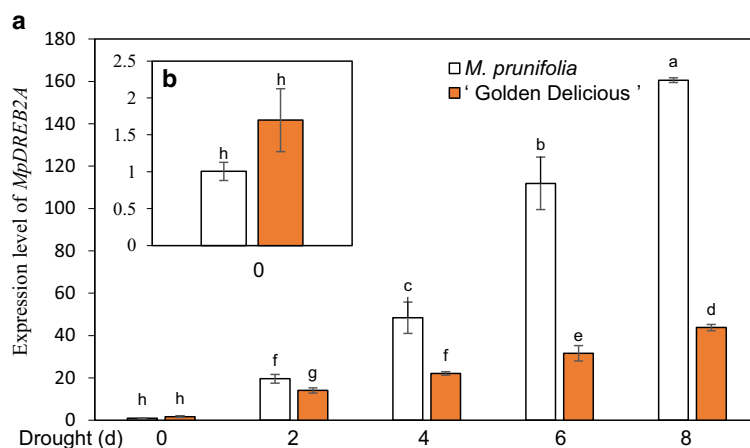


Fig. 6 mRNA level of *DREB2A* in leaves of *M. prunifolia* or ‘Golden Delicious’ grafted on *M. hupehensis* under drought stress. **a** mRNA level of *DREB2A* in leaves of *M. prunifolia* or ‘Golden Delicious’ grafted on *M. hupehensis* under drought stress.

(b) The magnifying figure of (a) in day 0. Error bars indicate standard deviation (n = 3). Two-way ANOVA was performed for data in (a) and (b), and statistically significant differences were indicated by different letters (Tukey test), $P < 0.05$

Table 1 Predicted cis-acting elements in *MpDREB2A* promoter region

Cis-acting elements Name	Cis-elements Sequence	Cis-element Position	Function
ABRE	ACGTGGT	+1374	abscisic acid responsiveness
	CACGTG	−1349	
CG-motif	CCATGGGG	−1234	light responsive element
CGTCA-motif	CGTCA	−727	MeJA-responsiveness
		−1514	
ERE	ATTTCAAA	−1037	ethylene-responsive element
G-Box	CACGTT	−1379	light responsiveness
	CACGTT	+1164	
	CACGTG	−1355	
	CACGAC	−1469, −751	
GA-motif	AAAGATGA	−1063	light responsiveness
HSE	AAAAAATTTC	−1132	heat stress responsiveness
I-box	AAGATAAGA	−835	light responsive element
O2-site	CATGACATGA	−1411	zein metabolism regulation
	GATGACGTGG	+726	
Skn-1_motif	GTCAT	+1574, −728	endosperm expression
TC-rich repeats	ATTTTCGTCA	−1568	defense and stress responsiveness
TCA-elements	GAGAAGAATT	−1222	salicylic acid responsiveness
TCCC-motif	TCTCCCT	−1285	light responsive element
TGA-box	TGACGTGGC	−730	auxin-responsive element
TGACG-motif	TGACG	+726, −1573, +1514	MeJA-responsiveness
CAAT-box	CAAT/CCAAT/CAAT		common cis-acting element in promoter and enhancer regions
TATA-box	TATA		core element around -30 of transcription start

Note: Location of first nucleotide 5' upstream of start codon is designated as −1 position. The complementary strand designated as + position

one of the most important epigenetic factors, we explored the DNA methylation status of the *DREB2A* promoter. Since most of the *cis*-elements were enriched in the region from −1266 to −1642 bp in *DREB2A* promoter (Table 1), this region was selected to examine DNA methylation levels in both genotypes under control and drought conditions by performing bisulfite sequencing 20 clones (Fig. 7a). The present results showed that DNA methylation levels of this region decreased under drought stress in both genotypes, which was consistent with the induced expression of *DREB2A* in both genotypes under drought (Fig. 7b). Total methylation levels of this region in both genotypes were similar under normal conditions (Fig. 7b, Fig. S7). However, under drought stress total methylation levels of this region decreased from 18.52% to 14.31% in *M. prunifolia*, and from 18.28% to 17.57% in 'Golden Delicious' (Fig. 7b, Fig. S7). For further confirmation of the present results, ChIP was analyzed by using anti-5-methylcytosine monoclonal antibody to

capture the methylated DNA from genome. The following qPCR results showed that the fold enrichment was lower in *M. prunifolia* than in 'Golden Delicious' under drought stress conditions, consistent with bisulfite sequencing results (Fig. 7c, Table S2). Given that decreased DNA methylation levels in promoters usually correlates with increased gene expression, our results suggested that the higher expression of *MpDREB2A* induced by drought stress could be, at least in part, a result of decreased cytosine methylation in the *MpDREB2A* promoter.

Among all the motifs in the region of −1266 to −1642 bp in *DREB2A* promoter, only one motif (CGTCA motif) showed a methylation difference between both genotypes under drought conditions (Fig. S7). Our analysis showed that the methylation level of cytosine at −1518 bp was reduced from 60% to 25% by drought stress in *M. prunifolia*, while the cytosine methylation of 'Golden Delicious' was only reduced from 45% to 40% (Fig. S7).

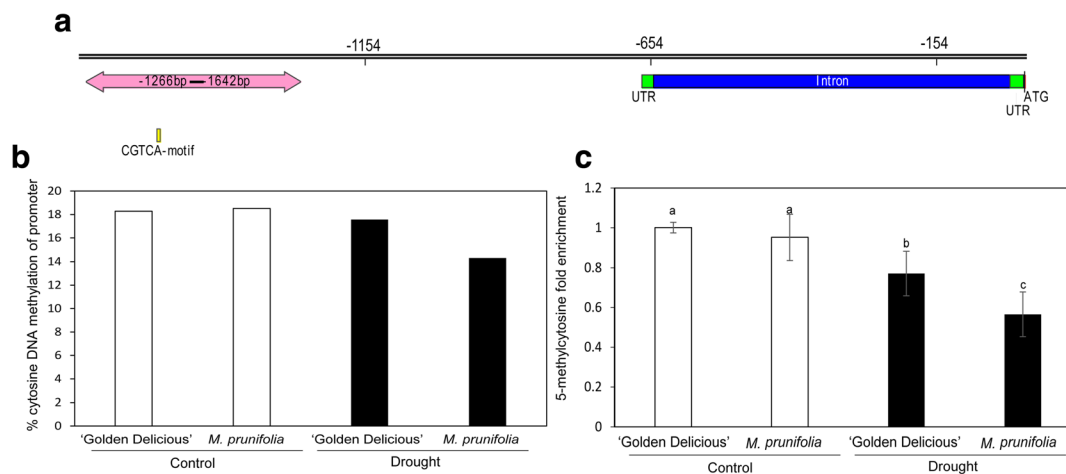


Fig. 7 Effects of drought stress on DNA methylation of *DREB2A* promoter in 'Golden Delicious' and *M. prunifolia*. **a** Schematic structure of the *DREB2A* promoter. Pink region represents the region for bisulfite sequencing. Green regions represent the UTR region and blue region represents an intron. **b** DNA methylation level of *DREB2A* promoter under control or

drought stress. **c** DNA methylation status detected by ChIP–qPCR assay using an anti-5-methylcytosine monoclonal antibody. Two-way ANOVA was performed for data in (c), and statistically significant differences were indicated by different letters (Tukey test), $P < 0.05$

MpDREB2A downstream target genes were responsive to drought stress

To identify potential regulatory targets of MpDREB2A in apple, we generated a polyclonal antibody of MpDREB2A and performed chromatin-immunoprecipitation coupled with Illumina sequencing (ChIP-seq) using drought treated leaves of *M. prunifolia*. Through this approach, we identified 2848 potential target genes (Table S3). To identify the binding motif of MpDREB2A, we performed MEME-ChIP analysis on the peaks located in the promoter region and found only one distinct consensual *cis*-element sequence for MpDREB2A: CCGAC (Fig. 8a). Go Ontology (GO) analysis suggested that most target genes contributed to catalytic activity, cellular process, binding, organelle, metabolic process, and cell part (Fig. 8b). Eighteen biological processes were enriched significantly through this experiment, such as development process, multicellular organismal process, regulation of biological process, immune system process and others. While binding, transporter activity, transporter regulator activity and catalytic activity were abundant in molecular function category, and the most significant cellular components were macromolecular complex, cell part and organelle part (Fig. 8b).

Among these potential target genes, some were responsive to drought stress, including homologs of early-responsive to dehydration stress (*ERD*) family protein,

and late embryogenesis abundant protein (*LEA*) family protein in *Arabidopsis* (Table S3). In addition, some potential target genes were related to root development, including homologs of *Arabidopsis thaliana* *MYOSIN XI B* (*MYOSIN XI B*), *ARABIDILLO1*, *ARF16* and *WRKY70*. (Table S3, 4 and Fig. S8). qRT-PCR analysis showed that these four genes were induced by drought stress in both *Malus* species. Compared with 'Golden Delicious', expression level of these four genes was higher in *M. prunifolia* under control or drought conditions (Fig. S8). GO enrichment analysis of developmental processes (GO: 0032502) and multicellular organisms (GO: 0032501) showed that root development-related processes (GO: 0048364) and post-embryonic morphogenesis (GO: 0009886) were enriched significantly (Fig. 8c), which is consistent with the root phenotype of transgenic *Arabidopsis* plants (Fig. 3, Fig. S3, Table S2).

Discussion

DREB2A is reported to be an important transcription factor in response to various abiotic stresses in different species (Lata and Prasad 2011). It activates expression of downstream genes by binding to DRE/CRT elements in their promoter regions (Sakuma et al. 2002; Liu et al. 1998). Because of the vital regulatory function of DREBs under abiotic stress, it has long been the interest

of researchers to identify them from the entire genomes in different species. Previous researches have reported that overexpression of *DREB* homologous genes could enhance resistance of transgenic plants to abiotic stress in *Arabidopsis* (Sakuma et al. 2006a), rice (*Oryza sativa*) (Dubouzet et al. 2003), maize (*Zea mays*) (Qin et al. 2004) soybean (*Glycine max*) (Chen et al. 2007), and apple (*M. sieversii*) (Liao et al. 2016). Though numerous downstream target genes of DREB proteins have been well studied, only a few regulators of *DREB* genes were identified, including GRF7, DRIP1, DRIP2, and BPM proteins (Morimoto et al. 2017; Sadhukhan et al. 2014; Kim et al. 2012; Qin et al. 2008). Moreover, epigenetic regulation of *DREB2A* is largely unknown. Our current data displayed that *MpDREB2A* was up-regulated much more by drought in *M. prunifolia* grafted on *M. hupehensis* than in *M. domestica*. Further study revealed that the highly induced *MpDREB2A* expression is associated with DNA cytosine demethylation in its promoter.

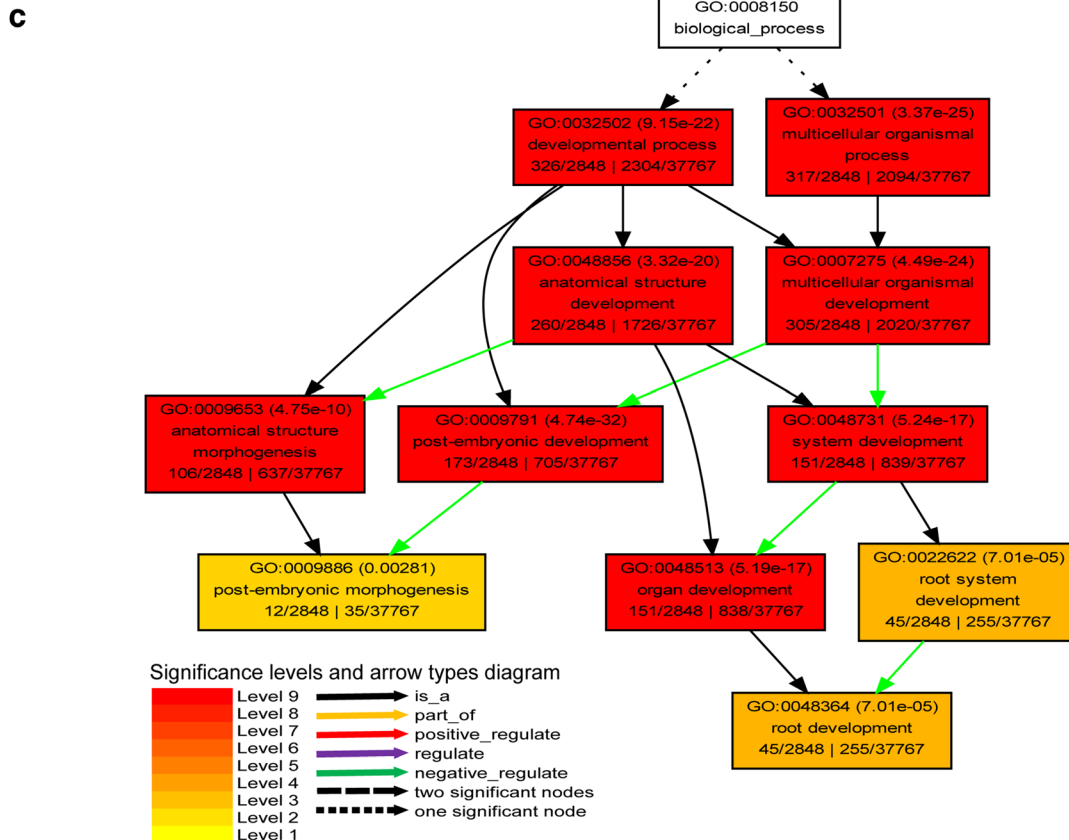
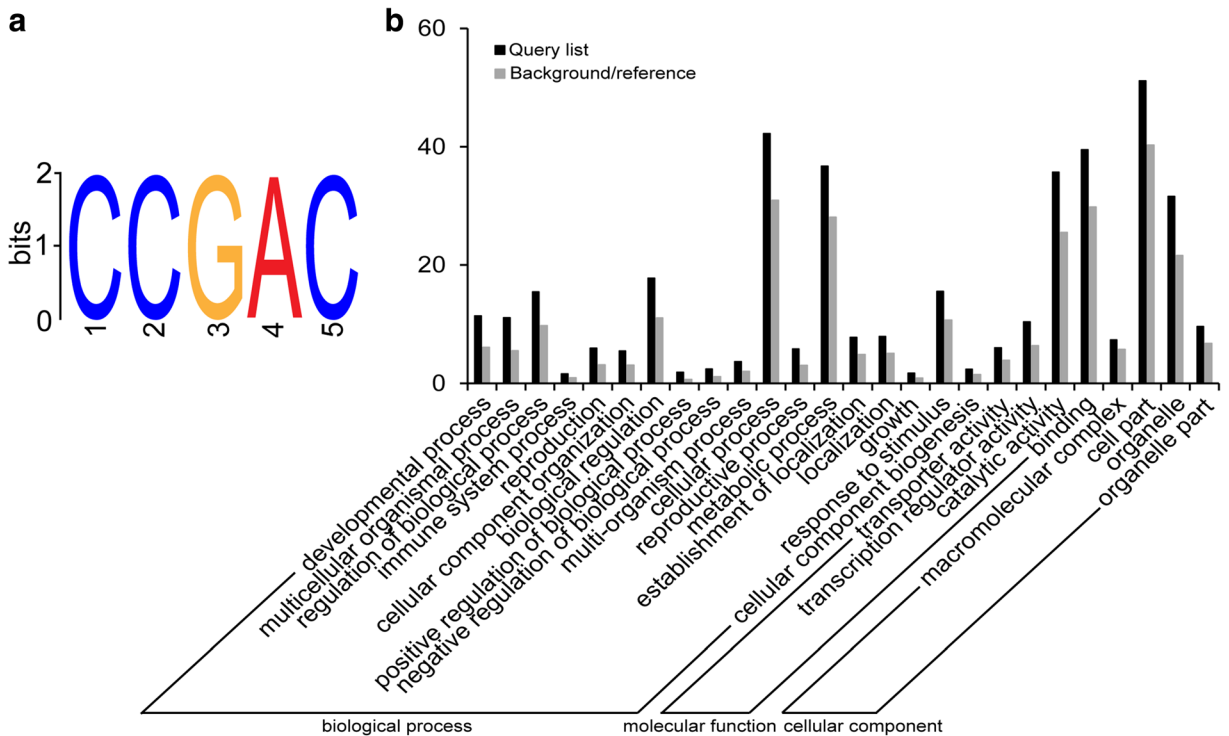
Recently, there have been frequent reports of epigenetic factors contributing to transcriptional regulation of gene expression for stress responses (Xu et al. 2017 and 2018; Mirouze and Paszkowski 2011). Cytosine methylation in gene promoters is tightly associated with repression of transcription. Previous studies have shown that heat stress-induced methylation levels in tobacco contribute to gene expression and growth alteration as a response to environmental changes (Centomani et al. 2015). Drought and salt stress are also related to DNA methylation-induced gene transcript changes in plants (Kumar et al. 2017). A reduction in the overall levels of *DREB2A* DNA methylation was observed in rice under drought stress at the tillering stage (Wang et al. 2011) and *Quercus ilex* (Rico et al. 2014). Our analysis showed that *DREB2A* methylation levels in *M. prunifolia* decreased much more after drought than did *DREB2A* methylation levels in ‘Golden Delicious’ (Fig. 7b). This change is consistent with the higher expression levels of *DREB2A* in *M. prunifolia* and lower transcript levels in ‘Golden Delicious’ after drought (Fig. 6, Table S2). Hence, a notable result emerging from this study is that the alteration in the expression of *DREB2A* in drought-stressed ‘Golden Delicious’ and *M. prunifolia*, appears to be associated with the changes in the methylation status of its promoter.

Previous studies showed that DNA methylation change is required for the induction of the gene expression. When exposed to aluminum, *NtGPDH* transcripts

Fig. 8 Sequence alignment, and phylogenetic tree analysis of *DREB2A*. **a** Alignment of *DREB2A* amino acid sequences from different species. The amino acids in red frame indicate AP2 domain, and amino acids underlined were transcriptional activation region. The accession numbers for the aligned proteins are: AtDREB1A (ABD42992.1), AtDREB2A (AED90871.1), MdDREB2A (XP_008354169.1), MpDREB2A (MG488272), FvDREB2A (XP_004307690.1), PbDREB2A (XP_009345422.1), PpDREB2A (XP_020413813.1), VvDREB2A (XP_002273838.1), PtDREB2A (XP_002312185.2). **b** Phylogenetic tree of MpDREB2A and their close homologs in different species. A-1 to A-6 indicate subgroups proposed in *Arabidopsis*. MpDREB2A and MdDREB2A in the A-2 subgroup were marked as red font. The accession numbers for the DREB related proteins are: AtDREB1B (AEE85066.1), AtDREB1C (ABI93900.1), AtDREB2B (AEE74994.1), AtAB14 (ABE65896.1), RAP2.4 (AAN41307.1), RAP2.1 (ABD57516.1), RAP2.10 (ABF83647.1), AtTINY (AAT44909.1), ZmDREB2A (BAE96012.1), ZmDBF2 (AF493799), ZmDREB6A (AAM80486.1), MdDREB5A (NP_001288046), MdDREB3A (XP_008362743.1), MdDREB1A (ART8558.1)

of wild type tobacco plants are induced within 6 h, and the CCGG sites of corresponding genomic loci are demethylated within 1 h (Choi and Sano 2007). In *Arabidopsis*, some transposable elements are demethylated and the transcription levels increase rapidly after bacterial infection (Yu et al. 2013a). Moreover, decitabine (5-aza-2'-Deoxycytidine), a DNA methylation inhibitor, can reactivate the expression of genes silenced by hypermethylation (Bai et al. 2015; Nie et al. 2014). The CGTCA motif (−1514 bp to −1518 bp) is required for inducing gene expression of methyl jasmonate (MeJA), a compound which is responsive to various abiotic stresses (Su et al. 2011). Further analysis also showed that drought stress-related *cis*-element CGTCA motif displayed much lower methylation levels in *M. prunifolia* than in ‘Golden Delicious’ under drought stress (Fig. S7). Since this *cis*-element is involved in abiotic stress (Zhu et al. 2015; Raikwar et al. 2015; Zhu et al. 2014), we hypothesized that lower methylation levels in the *MpDREB2A* promoter in *M. prunifolia* might induce more stress-related transcription factors to bind to the *cis*-element, which in turn promotes the higher expression level of *MpDREB2A* under drought stress.

DREB is a typical stress-induced transcription factor in most plant species, and its expression level can increase more than 100-fold in apple (Zhao et al. 2013; Zhao et al. 2012; Yang et al. 2011). Though numerous downstream target genes of DREB proteins have been well studied, only a few upstream regulators of *DREB* genes have been identified. In the promoter region of



DREB2A in apple, we also identified several *cis* elements which might be recognized by certain transcription factors. Thus it is possible that some drought-inducible transcription factors might bind to these elements and therefore contribute to the induction of *DREB2A* under drought. Moreover, epigenetic regulation of *DREB2A* is largely unknown. Therefore, we conclude that DNA methylation change is required for the induction of the *DREB2A* gene; however, we cannot rule out the possibility of *DREB2A* activation by its upstream transcription factors under drought conditions.

Roots play essential roles in plant growth by regulating water and nutrient absorption and transportation. When subjected to drought stress, plant roots form a large surface area for efficient absorption of water. Enhanced lateral root formation promoted the development of poplar (*Populus*) under drought stress (Dash et al. 2017). Previous study has isolated the *MsDREB2C* gene from *M. sieversii* (Zhao et al. 2013). *MsDREB2C* transgenic plants had longer primary roots than the non-transgenic plants exposed to drought conditions (Zhao et al. 2013). In our study, we found that the transgenic plants carrying *35S::MpDREB2A* had more lateral roots under drought (Fig. 3), which might be one of the contributors of the transgenic plants to drought avoidance.

We examined expression level of *AtDREB2A* downstream targets in response to drought stress in transgenic *Arabidopsis thaliana*. Although less similarities between two genomes of *A. thaliana* and *M. prunifolia*, the *cis* element recognized by *DREB2A* is identical. We selected three known *AtDREB2A* targets, *AtRD29A*, *AtCOR15A*, and *AtLEA7*. Our results displayed that these three target genes were highly induced in *35S::MpDREB2A* transgenic plants under dehydration conditions (Fig. S9a–c, Table S2). *AtRD29A*, a hydrophilic protein, endows plants with drought resistance by both ABA-independent and dependent pathways (Datta et al. 2012). *AtCOR15A* has been reported to play positive roles in drought stress resistance in *Arabidopsis* by regulating stomatal movement through ABA-signaling way (Meng et al. 2015). *AtLEA7* functions in drought stress resistance by stabilizing membrane and protecting enzymes (Popova et al. 2015). In our study, increased drought resistance of *Arabidopsis* carrying *35S::MpDREB2A* (Figs. 3 and 4) might be due to the positive modulation of such target genes by *MpDREB2A* under drought stress.

We identified 2848 potential target genes of *MpDREB2A* by ChIP-seq analysis. Among the

potential targets of *MpDREB2A*, some play essential roles in drought resistance (Popova et al. 2015; van den Burg et al. 2010; Mao et al. 2015). We analyzed expression level of *MpDREB2A* targets, *LEAs*, in *M. prunifolia* and ‘Golden Delicious’ in response to drought (Fig. S9d–f, Table S2), and found that expression of *LEAs* was induced more in *M. prunifolia* than that in ‘Golden Delicious’. *LEA* protein was shown to enhance drought resistance in sweet orange (Pedrosa et al. 2015), cotton (Magwanga et al. 2018), wheat (Kosová et al. 2014), and *Kosteletzkya virginica* (Tang et al. 2016). Hence, it is possible that drought-induced *LEAs* by demethylated *MpDREB2A* contributes to drought of *M. prunifolia*. In addition, homologs of root development-related genes *MYOSIN XI B*, *ARABIDILLO1*, *ARF16* and *WRKY70* were induced by drought stress in both *Malus* species (Fig. S8, Table S2). Compared with ‘Golden Delicious’, expression level of these four genes was higher in *M. prunifolia* under drought conditions (Fig. S8, Table S2). *MYOSIN XI B* accumulates at root hair tips and promotes root hair growth in *Arabidopsis* (Ojangu et al. 2007; Park and Nebenführ 2013). *ARABIDILLO1*, a F-box protein, promotes lateral root development by depressing the GA3 function (Mu et al. 2010). *ARF16* is a target of microRNA160 which is a negative regulator for root development (Bustos-Sanmamed et al. 2013; Wang et al. 2005). Increased *WRKY70* could reduce the JA which inhibits root growth (Chen et al. 2017). Considering the important roles of root development under drought stress, we speculate that the higher expression of *MYOSIN XI B*, *ARABIDILLO1*, *ARF16*, and *WRKY70* in *M. prunifolia* may contribute to its drought resistance by promoting its root system under drought stress (Fig. S8, Table S2). Transgenic *Arabidopsis* plants overexpressing *DREB2A CA* showed 483 genes with the average of expression ratios greater than two times in compared to vector control plants under water and heat stress (Sakuma et al. 2006a, b). The apple genome is highly heterozygous and recently duplicated into a haploid consensus sequence (Daccord et al. 2017; Li et al. 2016; Velasco et al. 2010), thus the genome size of apple is larger than that of *Arabidopsis*. There are ~50,000 genes in ‘Golden Delicious’ genome but ~27,000 in *Arabidopsis* genome. In addition, the similarity between *AtDREB2A* and *MpDREB2A* is 35.6%. Hence, it is possible that *MpDREB2A* function might be more diverse and it may target more downstream genes.

In the present study, the lower overlapping of targets genes of AtDREB2A and MpDREB2A might be due to the lesser similarity between these two plant genomes. For example, *RD29A* is the first identified target gene of DREB2A in *Arabidopsis*, but we did not find the homolog of *RD29A* in the apple genome. GO analysis suggested that most target genes participate in drought stress, cold/oxidative stress, heat stress, cell cycle, DNA binding, cell division, growth, root development, auxin signaling, RNA splicing and ABA signaling (Table S4). This indicates that the function of MpDREB2A could be more diverse in apple. We also identified one consensus binding sequence of MpDREB2A, CCGAC, which is identical to the DRE/CRT elements in *Arabidopsis* (Yamaguchi-shinozaki and Shinozaki 1994). Hence it is possible that MpDREB2A plays a similar role to AtDREB2A, regulating expression of downstream genes by binding to the same DRE/CRT element.

For woody plants which need grafting in agricultural production, rootstocks were used to balance growth vigor, enhance biotic and abiotic stress resistance, improve yield and fruit quality of scions (Sun et al. 2013; Mudge et al. 2009). Our previous research found that an apple cultivar (*M. x domestica* Borkh. cv. ‘Qinguan’) was more drought-tolerant than another (*M. x domestica* Borkh. cv. ‘Naganofuji No. 2’) irrespective of whether these scions were grafted on *M. hupehensis* (Zhou et al. 2015) or M9-T337 (Sun et al. 2013) rootstocks. In addition, previous researches also observed higher *DREB* expression levels in the drought tolerant cultivars than in the sensitive ones, including wheat (*Triticum aestivum* L.) (Qin et al. 2016; Hu et al. 2018), chickpea (*Cicer arietinum*) (Agarwal et al. 2016) and rice (*Oryza sativa* L.) (Khattab et al. 2014). In our unpublished study, *M. prunifolia* seedlings were more drought resistant than ‘Golden Delicious’ seedlings. Therefore, it is reasonable that *DREB2A* expression is higher in *M. prunifolia* than in ‘Golden Delicious’, no matter what the rootstocks are used.

Plants have developed multiple strategies to adapt to drought environment, including drought escape, drought avoidance and drought tolerance (Levitt 1980). A drought resistant plant usually has physiological properties of both drought avoidance and drought tolerance, such as higher leaf water content and osmotic adjustment solutes under drought conditions in black poplar (*Populus nigra* L.) (Yildirim and Kaya 2017), fennel (*Foeniculum vulgare* Mill.) (Khodadadi et al. 2017), rice (Zhou et al. 2016), snap bean (*Phaseolus*

vulgaris) (Omae et al. 2005), and apple (Wang et al. 2012). In our study, higher LRWC in *M. prunifolia* after exposure to drought for 8 d is consistent with its better performance under drought. This result suggests that the drought resistance of *M. prunifolia* should be partially due to the drought avoidance of leaf, which maintains a high turgor pressure by preventing water loss during drought. Previous data suggest that the improved performance of *M. prunifolia* under drought is also contributed by osmotic adjustment, cellular elasticity and protoplasmic resistance (Wang et al. 2012). Similarly, LRWC of transgenic *Arabidopsis* is higher than that of Col-0 under drought stress (Fig. S10c, Table S2), resulting in the drought resistance phenotype of transgenic *Arabidopsis* (Fig. S10).

In summary, we have identified a *DREB2A* gene, *MpDREB2A*, in *M. prunifolia* that is more strongly induced by drought stress than its counterpart in ‘Golden Delicious’. Ectopic expression of *MpDREB2A* in *Arabidopsis* improved drought tolerance by promoting root system development. The present investigation showed that DNA demethylation in the *DREB2A* promoter potentially contributes to *M. prunifolia*’s enhanced ability to tolerate drought, as *DREB2A* expression in *M. prunifolia* was over 10 times greater than that of ‘Golden Delicious’ after drought. As a whole, this study sheds light on the relationship between the *DREB2A* gene expression level and the methylation status of its promoters in *M. prunifolia* and ‘Golden Delicious’ and provides a novel point for studying epigenetic regulation and stress.

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