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ORIGINAL ARTICLE

Apple AP2/EREBP transcription factor MdSHINE2 confers drought resistance by regulating wax biosynthesis

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Abstract

Main conclusion **This study showed that AP2/EREBP transcription factor MdSHINE2 functioned in mediating cuticular permeability, sensitivity to abscisic acid (ABA), and drought resistance by regulating wax biosynthesis.**

Plant cuticular wax plays crucial roles in protecting plants from environmental stresses, particularly drought stress. Many enzymes and transcription factors involved in wax biosynthesis have been identified in plant species. In this study, we identified an AP2/EREBP transcription factor, MdSHINE2 from apple, which is a homolog of AtSHINE2 in Arabidopsis. *MdSHINE2* was constitutively expressed at different levels in various apple tissues, and the transcription level of *MdSHINE2* was induced substantially by abiotic stress and hormone treatments. *MdSHINE2*-overexpressing Arabidopsis exhibited great change in cuticular wax crystal numbers and morphology and wax composition of leaves and stems. Moreover, *MdSHINE2* heavily influenced cuticular permeability, sensitivity to abscisic acid, and drought resistance.

Keywords Apple · AP2/EREBP · ABA sensitivity · Cuticular permeability · Crystal morphology · Drought resistance · Wax load

Abbreviations

AP2/ERF	APETALA2/ETHYLENE-RESPONSIVE FACTOR
CER	*ECERIFERUM*
KCS	*KETOACYLCOA SYNTHASE*
MDA	Malondialdehyde
SHN1	*WAX INDUCER1* (*WIN1*)*/SHINE1*

Introduction

The aerial surface of plants is covered with a cuticle, the major structural component of which is cutin. Cutin is embedded and over layered by intracuticular and epicuticular waxes, complex mixtures of hydrophobic material containing very long-chain fatty acids and their derivatives (Jeffree 1996; Nawrath 2002; Beisson et al. 2012). An important mechanism by which plants adapt to fluctuations in the external environment is by secreting wax substances onto the surface. Wax acts as an initial barrier for plants to regulate epidermal permeability and nonstomatal water loss in addition to protecting plants against insects, pathogens, UV light, and frost (Sieber et al. 2000; Zhang et al.2007). Cuticular wax is produced through multiple biochemical pathways (Zheng et al. 2005; Kunst and Samuels 2009). The cuticular wax synthesis process mainly includes two pathways, alcohol-forming and alkane-forming, with total wax contents of 17–18% and 80%, respectively (Bernard and Joubès 2013). Plants synthesize cuticular wax via epidermal cells, secretion of wax onto the surface of the plant, forming a barrier between the plant and the outside world for

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protection from outside organisms and abiotic stress (Blee et al. 1993; Millar et al. 1999).

Wax synthesis is influenced by many factors, both internal and environmental. Environmental factors such as temperature, light, and humidity exert great influence on plant wax accumulation (Weng et al. 2010). Light and osmotic stresses were shown to up-regulate *ECERIFERUM6 (CER6)* expression (Hooker et al. 2002), whereas several *KETOACYLCOA SYNTHASEs (KCSs)* as well as *CER10* and *KCR1* transcripts were shown to accumulate under NaCl, dehydration, and mannitol treatments while decreasing under low temperature and darkness conditions (Raffaele et al. 2008). To date, numerous wax biosynthesis genes have been identified, most of which encode enzymes or structural genes in the very-long-chain fatty acids (VLCFAs) biosynthesis and derivatization pathways (Millar and Kunst 1997; Bernard and Joubès 2013). For example, *CER2* plays biological functions in two-carbon elongation of VLCFAs, from C28 to C30 (Haslam et al. 2015). The *WAX2* gene was identified in Arabidopsis as the first gene affecting the epidermal wax layer and keratinous layer. The *WAX2* might control conversion from acyl-CoA to aldehydes and other wax components (Gao et al. 2010). Mutations in these genes result in markedly reduced cuticular wax contents. Wax synthesis is also regulated at the transcriptional level, and several transcription factors have been reported to control wax biosynthesis by regulating expression of downstream wax biosynthesis genes. The expression of genes involved in cuticular wax biosynthesis is up-regulated in leaves of transgenic Arabidopsis plants overexpressing *MYB94*. MYB94 activates the expression of wax biosynthetic genes such as *WSD1, KCS2/DAISY, CER2, FAR3*, and *ECR* via direct binding to their promoters (Lee and Suh 2014).

With increasing understanding of wax synthesis mechanisms, recent reports have investigated epigenetics involved in wax biosynthesis. Recently, *DROUGHT HYPERSENSITIVE* (*DHS*), a RING E3 ubiquitin ligase, was identified as a critical regulator of wax biosynthesis in rice. *DHS* negatively regulates cuticular wax biosynthesis via ubiquitinating transcription factor ROC4 (Wang et al. 2018a). Mutations of the Arabidopsis histone acetyltransferase *GENERAL CONTROL NON-REPRESSED PROTEIN5* (*GCN5*) impaired the accumulation of stem cuticular wax. *GCN5* is involved in stem cuticular wax accumulation by modulating *CER3* expression via H3K9/14 acetylation (Wang et al. 2018b). Therefore, the wax synthesis process is affected by structural genes, transcription factors, and post-translational modification proteins (Lam et al. 2012; Borisjuk et al. 2014).

APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF) transcription factor plays an important role in plant growth and development (Shi et al. 2011). Sakuma et al. (2002) divided the AP2/ERF transcription factor family into five branches, namely DREB, ERF, AP2, RAV, and other classes. AP2 transcription factors that coordinate the expression of downstream target genes associated with wax biosynthesis have been shown to alter the leaf surface wax load (Zhang et al. 2005; Seo et al. 2011; Borisjuk et al. 2014; Park et al. 2016). For example, AP2/ERF family members, including *WAX INDUCER1* (*WIN1)/SHINE1* (*SHN1*) in Arabidopsis, positively regulate wax biosynthesis by directly promoting expression of wax and cutin biosynthesis genes (Broun et al. 2004). The Arabidopsis shine gain-of-function mutant and plants overexpressing *WIN1/SHN1, SHN2,* or *SHN3* have increased accumulation of epidermal wax and expression of genes involved in wax biosynthesis, including *ECERIFERUM1* (*CER1*) (Aarts et al. 1995), *3-KETOACYLCOA SYNTHASE1* (*KCS1*), and *ECERIFERUM1 (CER2*) (Aharoni et al. 2004; Broun et al. 2004; Rowland et al. 2006, 2007). These findings indicate that plant AP2 transcription factors are involved in wax synthesis.

The apple is one of the most commonly cultivated fruit trees and an important woody plant (Li et al. 2016; Xu et al. 2016). Cuticular wax on the apple surface protects fruits by enhancing resistance to wind, high temperature, low temperature, light, and other adverse environmental factors (Yang et al. 2017). Cuticular wax also improves surface gloss and post-harvest storage capacity (Bernard and Joubès 2013). Most studies on apple wax have focused on wax synthesis and metabolism; however, few studies have investigated molecular regulation of wax synthesis in apples. In this paper, we isolated an AP2/EREBP transcription factor MdSHINE2 from apple and generated transgenic Arabidopsis plants constitutively expressing *MdSHINE2*. Furthermore, we found *MdSHINE2* overexpression to increase leaf and stem cuticular wax load, reduce water loss, and improve drought resistance. Our study reveals a new molecular regulating mechanism of cuticular wax synthesis in apples, providing a candidate gene to regulate wax biosynthesis in apple and improve apple gloss quality.

Materials and methods

Plant materials and growth conditions

Various apple tissues, tissue-cultured apple seedlings (Gala), 'Orin' apple calli, and WT Arabidopsis seedlings (Col-0) were used as plant materials in this study. Roots of rootstock, spring shoot, young and mature leaves, floral bud, flower, petal, sepals, ovary, stamen, sprout, axillary bud, fruit, pulp, fruit skin, and seed were obtained from 6-year-old 'Royal Gala' apple trees in the experimental station of Shandong Agricultural University in 2018 and then immediately frozen in liquid nitrogen and stored at –80 °C for subsequent analyses.

Tissue-cultured apple seedlings (Gala) were grown on a Murashige and Skoog (MS) medium supplemented with 0.8% agar, 0.5 mmol L^{-1} 6-benzylaminopurine (6-BA), and 0.1 mmol L^{-1} indole-3-acetic acid (IAA) under 16-h light/8-h dark conditions at 25 °C and 60% humidity. After 20 days of growth, tissue-cultured apple seedlings with consistent growth status were selected. Then, these seedlings were treated with 10% polyethylene glycol (PEG 6000), 100 mmol L^{-1} NaCl, 100 µmol L^{-1} ABA, and 150 µmol L^{-1} GA, respectively. Seedlings were sampled at 0, 0.5,1, 3, 6, 12, 24, and 48 h after treatment, immediately frozen in liquid nitrogen, and stored at – 80 °C for subsequent analyses (Zhang et al. 2018). *Arabidopsis thaliana* (ecotype 'Columbia') was grown at 22 °C under a 16-h light/8-h dark photoperiod and used for genetic transformation.

'Orin' apple calli were grown on an MS medium supplemented with 0.8% agar, 0.5 mmol L^{-1} 6-BA, and 0.5 mmol L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) at 26 °C in the dark. 'Gala3' cultures were grown on an MS medium containing 0.2 mg L^{-1} NAA and 0.6 mg L^{-1} 6-BA at a normal temperature under long-day conditions (25 °C, 16 h light/8 h dark). Tobacco (*Nicotiana benthamiana)* was cultivated in a growth room at 25 °C under natural light with a daylight extension of 14 h until at least six leaves were available for infiltration with Agrobacterium (Wang et al. 2018c).

Multiple sequence alignments and phylogenetic analysis

SHINE2 protein sequences of 26 plant species were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). Protein sequences of of AtSHINEs were obtained from the Arabidopsis information resource (http://www.arabidopsis.org/). DNAMAN software was used to construct multiple sequence alignments. Phylogenetic analysis was conducted with MEGA version 7.0 using the neighbor-joining method with 1000 bootstrap replications (Altschul et al. 1990).

Construction of *MdSHINE2* overexpression vector and genetic transformation of *MdSHINE2* into Arabidopsis and apple calli

We inserted the ORF of *MdSHINE2* into the pCXSN-MYC vector to obtain the *MdSHINE2* OE overexpression vector. Transgenic apple calli were obtained by Agrobacterium-mediated genetic transformation. Briefly, 15-day-old WT apple calli were incubated with Agrobacterium strains carrying empty vectors or the *MdSHINE2* OE vector at room temperature for 20 min. The transformed apple calli were plated on a selective medium containing antibiotics. Transgenic apple calli were identified using qRT-PCR and used for the following assays as described by An et al. (2016). Transgenic Arabidopsis seedlings were generated using the floral dip Agrobacterium strain method as described previously (Clough and Bent 1998; Zhang et al. 2006, 2007).

RNA extraction and analysis of real-time quantitative reverse transcription (qRT)-PCR

Total RNAs of plant materials, including apple tissues, apple calli, and Arabidopsis seedlings, were isolated using RNA Plant Plus (Tiangen, Beijing, China) according to the manufacturer's instructions. Reverse transcription was conducted for single-stranded DNA synthesis using the PrimScript™ RT Reagent Kit (Perfect Real Time, TaKaRa, Dalian, China) per the manufacturer's protocol. Specific primers are listed in Supplementary Table S1. qRT-PCR was followed as described in Zhou et al. (2017).

Subcellular localization

Positive clones with correct sequencing were obtained, and the plasmid was extracted. The primers *MdSHINE2*-PRI-F (5′-GTCGACATGGTACAAACAAGGAAGTTCAG-3′) and *MdSHINE2*-PRI-R (5′-GGATCCGTAATGTTC ACGAAGGAAATAATATT-3′) were designed with two restriction endonuclease sites of SalI and BamHI, taking the extracted plasmid as a template with PCR amplification of the *MdSHINE2* fragment. The PCR product and plant high-expression vector PRI-GFP were digested with SalI and BamHI endonucleases, respectively, and the two fragments were ligated with T_4 ligase to construct the fusion expression vector 35S::*MdSHINE2*-GFP. Positive clones were identified by PCR amplification in Bacillus LBA4404. The Agrobacteria with constructed expression vector was injected into tobacco leaves using the Agrobacterium infection method, and the expression of green fluorescent protein in cells was observed via laser confocal microscopy (Zeiss 510 META, Jena, Germany).

Germination assay

Harvested seeds were sterilized with 75% ethanol for 3 min, 2.6% NaClO (Kaitong, Tianjin, China) for 10 min, and washed with double-distilled water five times after sterilization. After 3 days of vernalization, seeds were plated onto a common MS solid medium and MS solid medium containing 0.1 µmol L^{-1} ABA, 0.3 µmol L^{-1} ABA, and 0.5 µmol L^{-1} ABA and placed in a light incubator (light for 16 h, dark for 8 h, 22 °C). Photographs were taken after 7 days. Three biological repeats were applied.

PEG treatment of apple calli and Arabidopsis seedlings treated with ABA and PEG

Apple calli were grown on an MS medium containing 30 g L^{-1} sucrose, 1.0 mg L^{-1} 6-BA, and 1.0 mg L^{-1} 2, 4 days. The calli were treated with 4% PEG and 6% PEG, respectively. Fresh weight (FW) and malondialdehyde (MDA) content were measured after 17 days. The MDA content in apple calli was determined using the thiobarbituric acid method as described in Zhao et al. (1994).

Seeds of WT and transgenic Arabidopsis were sown on an MS medium. After 4 days, seedlings were transferred to MS, MS + 10 µmol L^{-1} ABA, MS + 20 µmol L^{-1} ABA, MS + 30 µmol L^{-1} ABA, MS + 4% PEG, and MS + 6% PEG, respectively, and then placed in a 25 °C light incubator (16 h light/8 h dark photoperiod). Seedling root length was measured after 14 days of ABA and PEG treatment. These experiments were repeated three times.

Wax extraction and GC–MS analysis

Cuticular wax was extracted exhaustively by dipping intact leaves, Arabidopsis rosette leaves, and stems into chloroform. For each Arabidopsis line, three groups of plant tissues were applied. Each group was extracted by 15 mL chloroform for 30 s. Then, the three extracts were combined into one sample. *N*-tetracosane and *n*-heptadecane (C17, C24; Sigma-Aldrich, St. Louis, MO, USA) were added as the internal standard, the extracts were filtered, and the solvent was removed with a gentle steam of N_2 while heating the solution to 50 °C. Then, all samples were treated with BSTFA for derivatization. Analysis was performed, and wax compounds were treated as described in Aharoni et al. (2004).

Scanning electron microscopy (SEM)

The 6-week-old Arabidopsis leaves and stems were vacuum-dried at – 80 °C in a 110 Pa vacuum for 24 h (FDU-1110, 50/60 Hz, 1.7kVA). Next, samples were examined using a scanning electron microscope (JSM-6610LV, JEOL) at an accelerating voltage of either 1 or 2 kV.

Chlorophyll leaching assay

Roots and inflorescence stems of 6-week-old Arabidopsis were cutoff, and the remaining rosettes were cleaned, weighed, and placed into tubes containing 30 mL of 80% ethanol at room temperature with gentle agitation in the dark. Extract was removed from each sample every 10 min during the first hour and up to 100 min. Absorbance was measured at wavelengths of 664 and 647, and the following formula was used to calculate the micromolar concentration of total chlorophyll per gram of fresh weight of tissue: total micromoles chlorophyll = 7.93 (A_{664}) + 19.53 (A_{647}). Detailed method as described in Lolle et al. (1997).

Toluidine blue staining

Arabidopsis rosette leaves, which were healthy for 4 weeks, were cut and immersed completely in 0.05% toluidine blue solution for 2 h. The material was then removed and rinsed 2–3 times with deionized water.

Water loss measurements

4-week-old Arabidopsis was grown under normal conditions. Leaves (root system and inflorescence stem detached) and inflorescence stems (root system and rosette explants detached) were treated in darkness for 12 h to ensure stomatal closure before being dipped into water in dark conditions for 1 h. The leaves were then used for water loss measurements. Leaves and inflorescence stems were weighed during the time intervals shown. Results were derived from three independent experiments and depicted with the standard error of the mean for each time point.

Drought tolerance experiment

For drought tolerance experiments, the soil mixture contained nutrient soil and vermiculite (a mixture of 50% nutrient soil and 50% vermiculite). After 2 weeks of germination, plants were transplanted into trays with the soil mixture and grown at 22 °C under a 16-h light/8-h dark photoperiod. Before drought treatment, the plants were watered every 3 days and photographed when watering stopped. Approximately 20 days after watering stopped, the plant material began to exhibit yellowing of the rosette leaves. Due to sufficient watering before the drought treatment, residual water remained in the soil mixture during the first 2 weeks of watering cessation, and actual drought stress was produced in the third week.

Statistical analysis

All experiments were performed in triplicate. Error bars show the standard deviation (SD) of three replicates. Data were analyzed for significance using the DPS software. Bars with different letters are significantly different at $P < 0.05$ according to Tukey's single factor test. Significant differences were detected by *t* tests using the GraphPad Prism 6.02 software (*$P < 0.05$; **$P < 0.01$).

Results

Gene cloning and relationship analysis of *MdSHINE2*

To determine the *SHINE2* gene in the apple gene genome, the Arabidopsis *SHINE2* gene (*At5G11190*) was used as bait to identify similar sequences in apple by mining the NCBI database with the BLAST program. Using cDNA from the 'Royal Gala' leaf as a template, *MdSHINE2* specific primers (Supplementary Table S1) were used for PCR amplification. After amplification, a 642-bp open-reading frame (ORF) was obtained and labeled *MdSHINE2* (MDP0000178263). The *MdSHINE2* gene contained one intron and two exons (Fig. 1a). *MdSHINE2* encoded a protein of 214 amino acids with a calculated mass of 2.39 kDa and an isoelectric point (pI) of 6.12, predicted by DNAMAN software.

To analyze phylogenetic relationships between MdSHINE2 and SHINE2s from other plants, a neighbor-joining phylogenetic tree was constructed according to the amino acid sequences of MdSHINE2 and homologs from 25 other plant species via MEGA 7 software (Fig. 1b). Results showed that apple MdSHINE2 exhibited the highest similarity to *Prunus persica* SHINE2 (XP_007224520.1); these two sequences formed one clade, both of which were from the Rosaceae family. By contrast, MdSHINE2 showed a relatively further distance to the SHINE2 proteins of monocotyledonous species, including PdSHINE2 (XP_008792041.1), ZmSHINE2 (KMZ68634.1), AcSHINE2 (OAY72031.1), EgSHINE2 (XP_010925213.1), SbSHINE2 (XP_002451740.2), DoSHINE2 (OEL25616.1), and TaSHINE2 (ANY98960.1). MdSHINE2 also has the farthest evolutionary relationship with moss and fern in lower plants. This phylogenetic tree revealed an apparent boundary between monocots and dicots along with an evolutionary relationship from lower plants to higher plants.

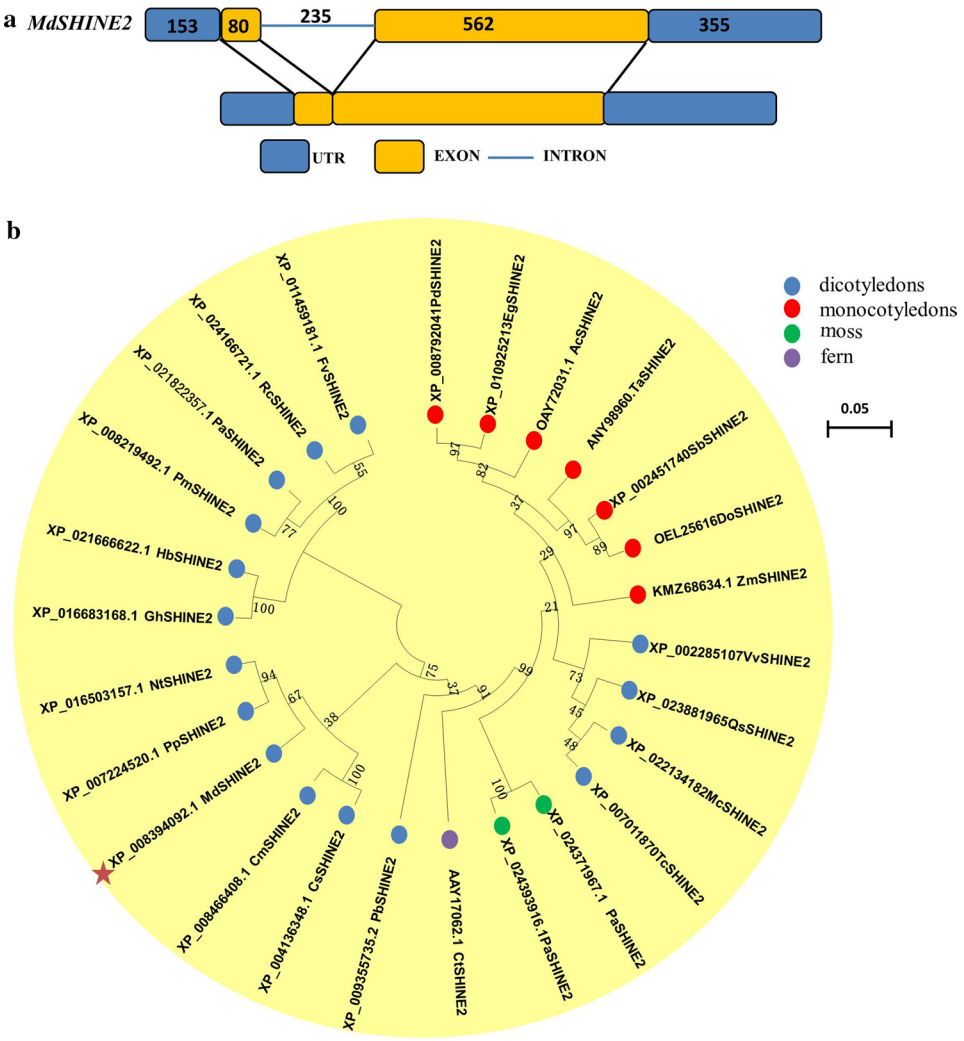

Fig. 1 Schematic diagram of the *MdSHINE2* sequence and phylogenetic relationship analysis of plant SHINE2 proteins. **a** Schematic diagram of *MdSHINE2* genomic and cDNA sequences. UTR means untranslated region. **b** Phylogenetic relationship analysis of plant SHINE2 proteins. MdSHINE2 is denoted by the red asterisk

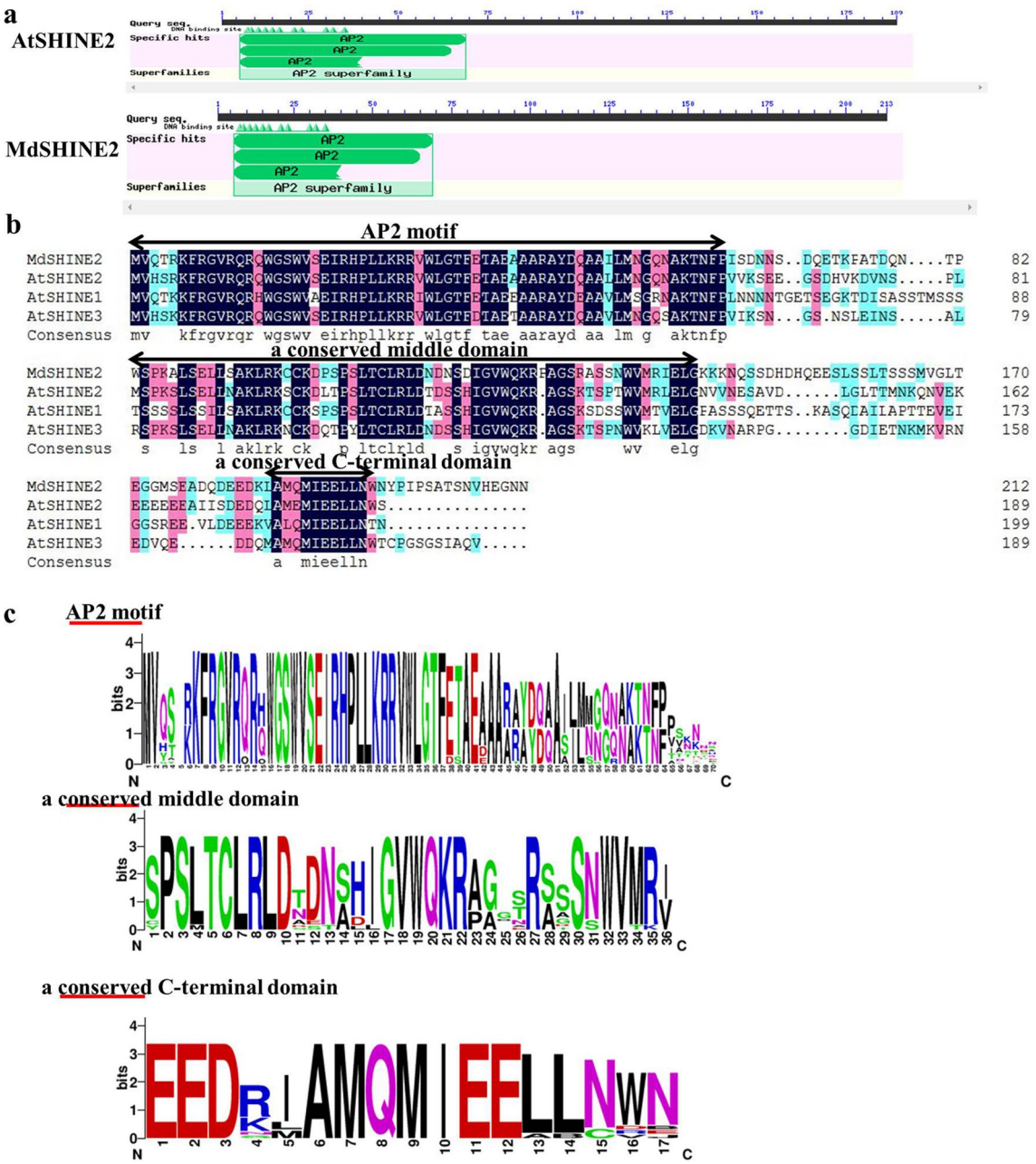

Fig. 2 Multiple sequence alignment and conserved motif analyses of MdSHINE2 and AtSHINE2 proteins. **a** Conserved AP2 domain in MdSHINE2 and AtSHINE2. **b** Alignment of amino acid sequences of MdSHINE2 and AtSHINEs proteins; three conserved motifs are labeled with black lines. **c** Conservation of residues across MdSHINE2 and AtSHINE2 proteins by the height of each letter. Bit scores indicate information for each conserved motif in the sequence. To interpret the colors in this legend, please refer to the Web version of this article

Analysis of MdSHINE2 amino acid sequence

Several common characteristics of the SHINE2 clade suggest related functions (Shen et al. 2008). The feature shows close similarity between MdSHINE2 and AtSHINE2 proteins in terms of sequence; both are members of the plant superfamily of AP2/EREBP transcription factors, which contain a conserved AP2 domain (Fig. 2a). Via amino acid sequence comparison with other AtSHINEs using DNAMAN software, we found that SHINE2 proteins contained a 70-amino acid motif named the AP2/EREBP domain. Besides the AP2 domain, all proteins contained a 36-amino acid sequence (a conserved-middle domain) and a 17-amino

acid sequence (a conserved C-terminal domain). All three domains are conserved in MdSHINE2 and Arabidopsis SHINE proteins, including several invariant amino acid residues (Fig. 2b, c). The SHINE2 proteins might function through these conserved domains.

Tissue-specific expression patterns and subcellular location of *MdSHINE2*

To examine tissue-specific expression of *MdSHINE2*, its transcript levels were analyzed in the root, spring shoot, young and mature leaves, floral bud, flower, petal, sepal, ovary, stamen, sprout, axillary bud, fruit, pulp, fruit skin, and seed using a quantitative RT-PCR assay. Findings indicated that *MdSHINE2* was expressed at different levels in all tested tissues. As demonstrated in Fig. 3a, *MdSHINE2* showed the highest expression in young leaves and fruit skin. Higher expression was also demonstrated in roots and petals. Moreover, *MdSHINE2* was expressed at high levels in young leaves but substantially lower in mature leaves, indicating different functions across developmental stages of the same organ.

To investigate the intracellular location of MdSHINE2 protein, a full-length (642 bp) *MdSHINE2* without termination codon (TAG) was fused to the GFP protein. The generated construct was introduced into tobacco leaf epidermal cells via Agrobacterium-mediated infiltration (Sparkes et al. 2006). Figure 3b shows that the fluorescent signals from the MdSHINE2:GFP construct were observed in the nucleus of tobacco leaf epidermal cells and merged with the fluorescent signals from 40,6-diamidino-2-phenylindole (DAPI) staining, indicating that fluorescent *MdSHINE2* was localized in the nucleus.

cis-element analysis in promoter regions of *MdSHINE2* and expression analysis of *MdSHINE2* under different stresses

Studies have shown that many SHINE genes play important roles in response to various abiotic stresses (Aharoni et al. 2004; Shi et al. 2011). To predict the putative functions of *MdSHINE2* genes in response to abiotic stresses, we analyzed 2-kb upstream of the *MdSHINE2* gene. Potential *cis*-elements in the promoter regions of *MdSHINE2* were determined using PlantCARE software. Several regulatory sequences were found in the promoter sequence of *MdSHINE2*, such as stress responsive *cis*-elements (e.g., fungal elicitor and drought inducibility) and plant-hormone-related *cis*-elements (e.g., auxin, methyl jasmonate, and GA). In addition to stress and hormone-responsive elements, the *MdSHINE2* gene promoter regions also contained many light-responsive elements (e.g., ACE, AE-box, ATC-motif, Box4, G-Box, and TCCC-motif) (Table 1), suggesting that *MdSHINE2* may play important roles in the aerial parts of plants that are exposed to sunlight.

To explore the function of the *MdSHINE2* gene in responding to abiotic stresses and plant hormones in apples, we first studied transcriptional changes of the *MdSHINE2* gene treated with drought PEG, salt (NaCl), abscised acid

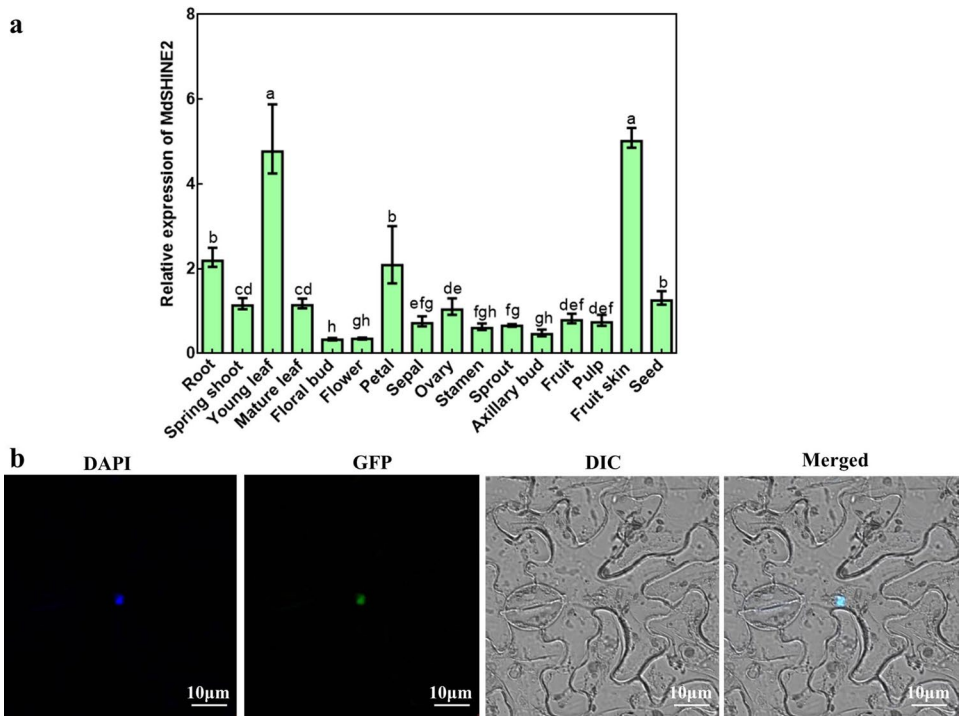

Fig. 3 Expression patterns and subcellular localization of *MdSHINE2* gene. **a** Expression profile of *MdSHINE2* in various apple tissues. Apple actin expression was used as a control. Data are the mean ± SD of three independent replicates. Lowercase letters indicate significant differences at $P < 0.05$. **b** Subcellular localization of *MdSHINE2*. Scale bar = 10 μm

Table 1 Promoter *cis*-acting element analysis of *MdSHINE2*

Regulatory sequence	Sequence	Function of site	Location
ACE	ACGTGGA	*cis*-acting element involved in light	−836
AE-box	AGAAACTT	Part of a module for light response	−480
ATC-motif	GCCAATCC	Part of a conserved DNA module involved in light responsiveness	+876
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	−1045
Box-W1	TTGACC	Fungal elicitor responsive element	−788
CGTCA-motif	CAACTG	*cis*-acting regulatory element involved in the MeJA-responsiveness	−1439
EIRE	TTCGACC	Elicitor-responsive element	−746
G-Box	CACACATGGAA	*cis*-acting regulatory element involved in light responsiveness	+689
GARE-motif	TCTGTTG	*cis*-acting element involved in gibberellin-responsiveness	+1374
GCN4_motif	TGAGTCA	*cis*-regulatory element involved in endosperm expression	+847
MBS	CAACTG	MYB binding site involved indrought-inducibility	−1461
TCCC-motif	TCTCCCT	Part of a light responsive element	−1419
TGACG-motif	TGACG	Auxin-responsive element	+926

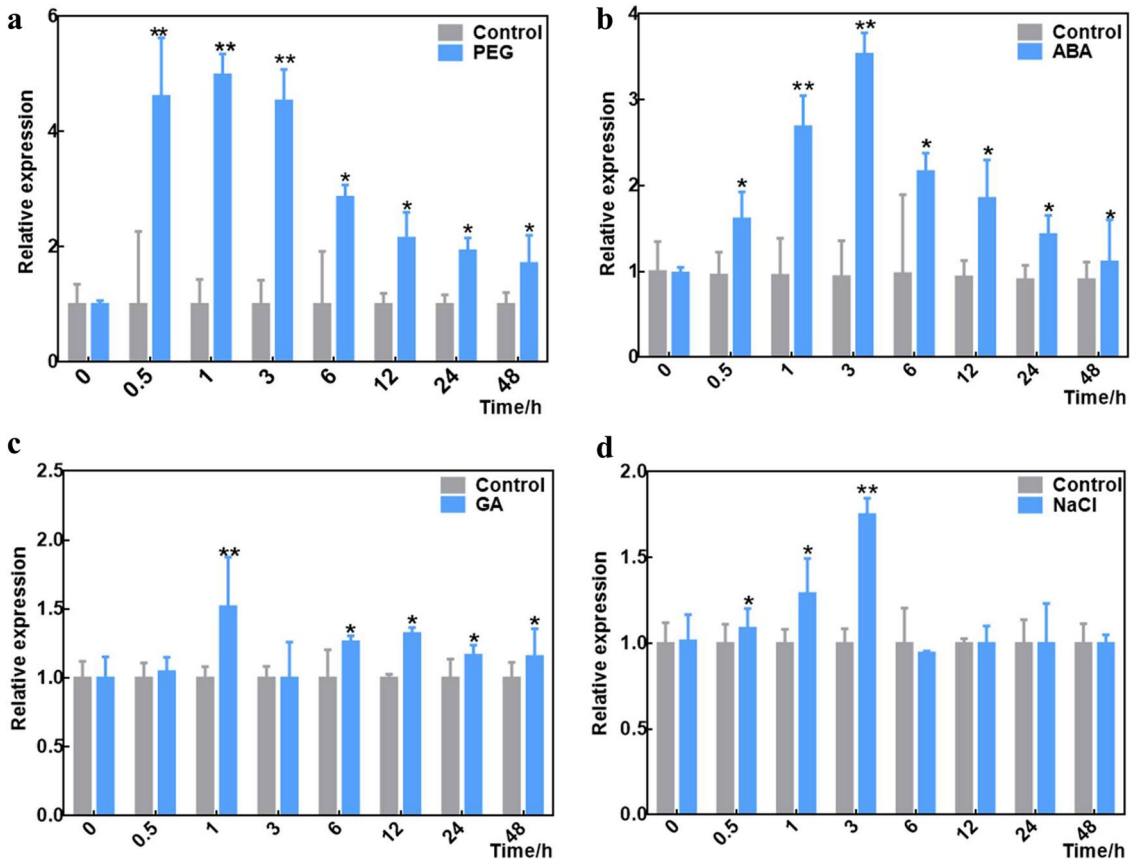

Fig. 4 Expression analysis of *MdSHINE2* in response to abiotic stresses and hormones. Expression level of *MdSHINE2* in response to PEG (**a**), ABA (**b**), GA (**c**), and NaCl (**d**). Data are the mean ± SD of three independent replicates. Asterisks denote significant differences from control (*$P < 0.05$, **$P < 0.01$). Apple actin expression was used as a control

(ABA), and GA, respectively (Fig. 4a–d). As expected, *MdSHINE2* responded to all stress treatments. For example, expression patterns of *MdSHINE2* were significantly up-regulated during early stages by treatments of PEG, ABA, GA, and NaCl and reached their maximum after 1-h or 3-h treatments, respectively, followed by a decline (Fig. 4a–d).

MdSHINE2 altered epicuticular wax load, wax crystal numbers, and crystal morphology

To investigate the function of *MdSHINE2* in plants in detail, the full-length cDNA of *MdSHINE2* was cloned into the expression vector under the control of the 35S promoter and then transformed into the wild-type (WT, Columbia) Arabidopsis. We identified five independent lines after PCR screening (Supplementary Fig. S1). Then, the expression level of *MdSHINE2* in the five lines was determined using qRT-PCR; *MdSHINE2* OE-1, 2, and 3 with higher *MdSHINE2* expression level were used for further analysis (Supplementary Fig. S2).

SHINE2 proteins from several plant species were found to be associated with wax synthesis. To examine the function of *MdSHINE2* in wax accumulation, we tested the expression of Arabidopsis wax-related genes in the WT and *MdSHINE2* OE plants, including *AtMYB30*, *AtMYB96*, *AtLACS2*, *AtCER1*, *AtCER3*, *AtCER6*, *AtKCS1, AtWIN1, AtDEWAX*, and *AtSHINE3* (Supplementary Table S2). Among these, most genes were up-regulated in transgenic plants compared with WT. *AtCER3* and *AtSHINE3* transcripts were significantly up-regulated. *AtMYB30*, *AtMYB96*, *AtLACS2*, *AtCER1*, *AtCER6*, *AtKCS1*, and *AtWIN1* transcripts were also up-regulated; however, *AtDEWAX*, which is a negative regulator for wax synthesis in Arabidopsis, did not exhibit an obvious change (Fig. 5a). To determine the function of *MdSHINE2* in cuticular wax accumulation, total wax was extracted using chloroform. A substantial difference was observed in the total wax load between *MdSHINE2* transgenic and WT plants. The total wax load was 1.4- to 1.5-fold higher in *MdSHINE2 OE* lines compared to WT plants whether on the leaves or stem (Fig. 5b; Supplementary Table S3). Furthermore, wax components in stem samples were analyzed using gas chromatography–mass spectrometry (GC–MS). The epidermis wax composition of *MdSHIE2 OE* and WT plants varied greatly: alkanes were 1.3-fold higher (56.1%) in *MdSHINE2* OE than WT plants (41.7%), alcohols (32.3%) were greatly increased at 1.5-fold more than in WT plants (21.2%), respectively. However, the contents of aldehydes, fatty acids, and others decreased in transgenic plants (Fig. 5c; Supplementary Table S4). In stems of transgenic and WT Arabidopsis, no obvious difference was found in epicuticular wax crystal morphology via scanning electron microscopy, but the number of epicuticular wax crystals in transgenic plants increased notably compared to WT (Fig. 5d). We also examined the wax crystal in leaves. Transgenic Arabidopsis exhibited a slight increase in wax crystals compared to WT, but not as obvious as the stem; however, the wax crystal morphology changed. The wax crystal of WT leaves displayed an irregular shape, whereas that of MdSHINE2 OE exhibited regular ellipses (Fig. 5e). The crystal morphology was clearer when observed at 2500 times magnification (Fig. 5f). Thus, expression of *MdSHINE2* changed the patterns of epicuticular wax crystal numbers and morphology on stems and leaf surfaces.

Alterations to leaf surface properties and cuticle permeability in *MdSHINE2*-overexpressing Arabidopsis

Wax content is often thought to be related to cuticular permeability. Figure 6a displays that *MdSHINE2* OE Arabidopsis exhibited great differences on the leaf surface compared with the WT. Rosette and cauline leaves of the *MdSHINE2* OE plants had a more brilliant, shiny green color compared with WT plants and often had curved-down edges (Fig. 6a). To investigate whether *MdSHINE2* OE plant cuticular membrane properties were altered, we conducted a chlorophyll leaching experiment. The rosette leaves of *MdSHINE2* transgenic and WT plants were submerged in 80% ethanol for different periods, and the chlorophyll concentration in the solution was determined. Results showed that chlorophyll was extracted much more slowly from *MdSHINE2* OE plant leaves than from WT (Fig. 6c, d). Substantially higher quantities of chlorophyll could be extracted from WT (i.e., greener extract) compared to transgenic lines (Fig. 6c), suggesting higher cuticular resistance for chlorophyll leaching in transgenic lines. The TB staining assay was carried out to find whether wax permeability changed, revealing that the three *MdSHINE2* OE Arabidopsis were not easily stained compared to WT (Fig. 6b). These results indicate that the *MdSHINE2* notably reduced the permeability of the Arabidopsis rosette leaf epidermis.

To examine whether enhanced accumulation of cuticular waxes in *MdSHINE2* OE lines affected water loss, we evaluated the water loss rate in *MdSHINE2* OE and WT plants. As shown in Fig. 6e, water transpiration occurred more slowly in *MdSHINE2* OE leaves and more rapidly in WT leaves. We obtained the same results when using stems of WT and *MdSHINE2* OE plants as materials as found in leaves (Fig. 6f), indicating that *MdSHINE2* greatly influenced permeability and water loss, which are important cues for plant drought resistance.

Ectopic expression of *MdSHINE2* in Arabidopsis resulted in increased sensitivity to ABA

We tested the seed germination of *MdSHINE2*-overexpressing lines and WT in response to ABA. When seeds were planted in an MS medium, the seeds of transgenic lines germinated, and these seedlings grew as well as the WT seedlings at 3–7 days. Conversely, when seeds were planted in an MS medium supplemented with different concentrations of ABA treatment, seeds of the overexpressing lines germinated later than WT seeds. Even though transgenic seeds germinated eventually, ABA

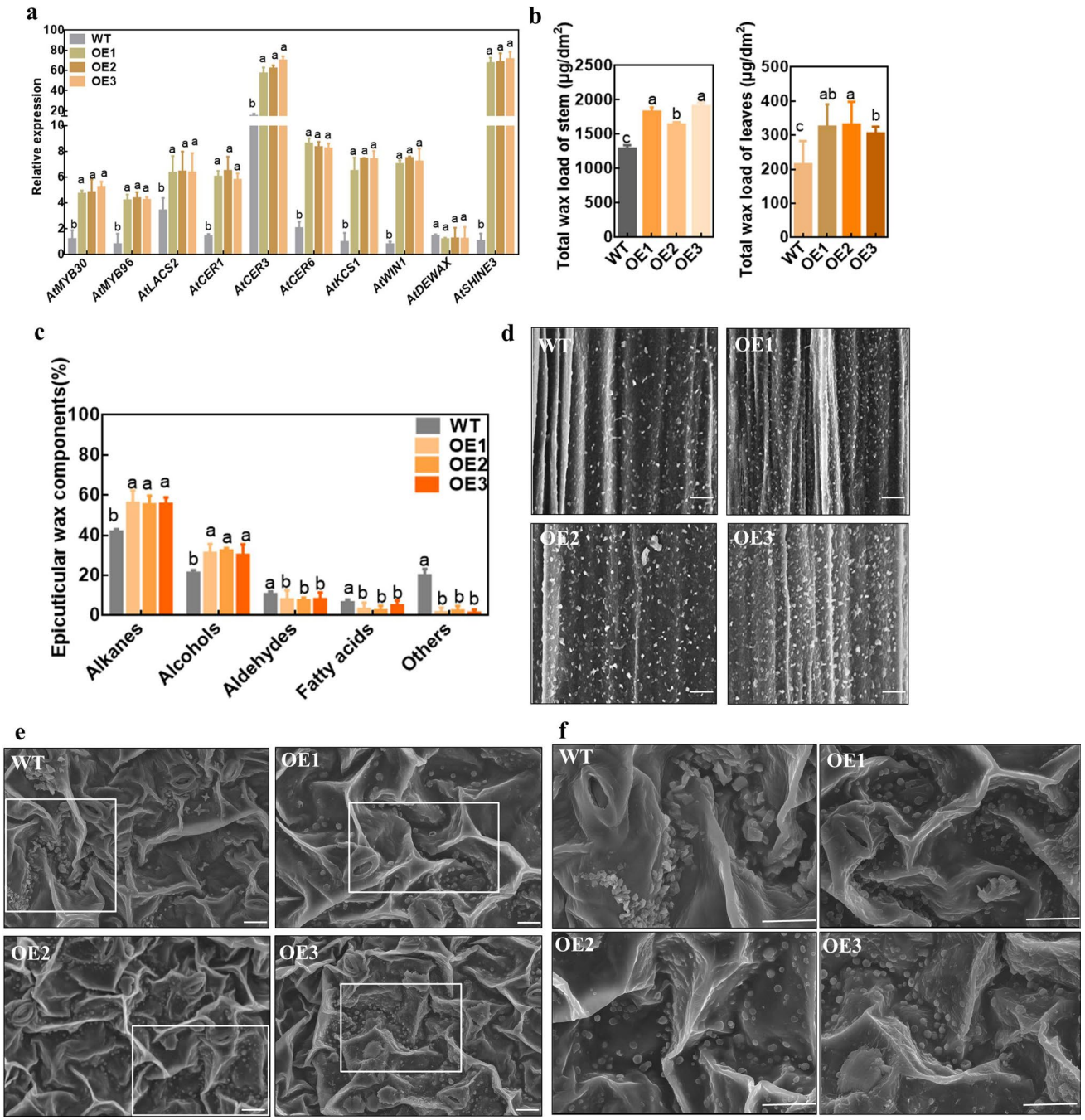

Fig. 5 Changes in wax load and cuticle wax crystal morphology of WT plants and *MdSHINE2* OE plants detected by scanning electron microscopy. **a** *MdSHINE2* affects expression of Arabidopsis wax-synthesis-related genes. **b** Total cuticular wax content of stems and leaves, calculated per unit area of 6-week-old Arabidopsis from WT and *MdSHINE2* OE lines. **c** Cuticular wax composition of 6-week-old Arabidopsis stems for WT and *MdSHINE2* OE lines. Data (**a**–**c**) are the mean ± SD of three independent replicates. Lowercase letters indicate significant differences at $P < 0.05$. **d** Wax crystal morphology of 6-week-old Arabidopsis stems from WT and *MdSHINE2* OE lines; wax was monitored at × 1000 magnification, scale bars = 10 μm. **e, f** Wax crystal morphology of 6-week-old Arabidopsis adaxial side of leaves from WT and *MdSHINE2* OE lines. Wax was monitored at × 1000 (**e**) × 2500 (**f**) magnification, scale bars = 10 μm

treatment delayed their growth and development compared with WT (Fig. 7a); ABA dose–response curves are presented in Fig. 7b. In our quantification of seed germination, only seedlings with white roots were considered germinated. These results suggest that ectopic expression of *MdSHINE2* in Arabidopsis leads to ABA hypersensitivity in germination.

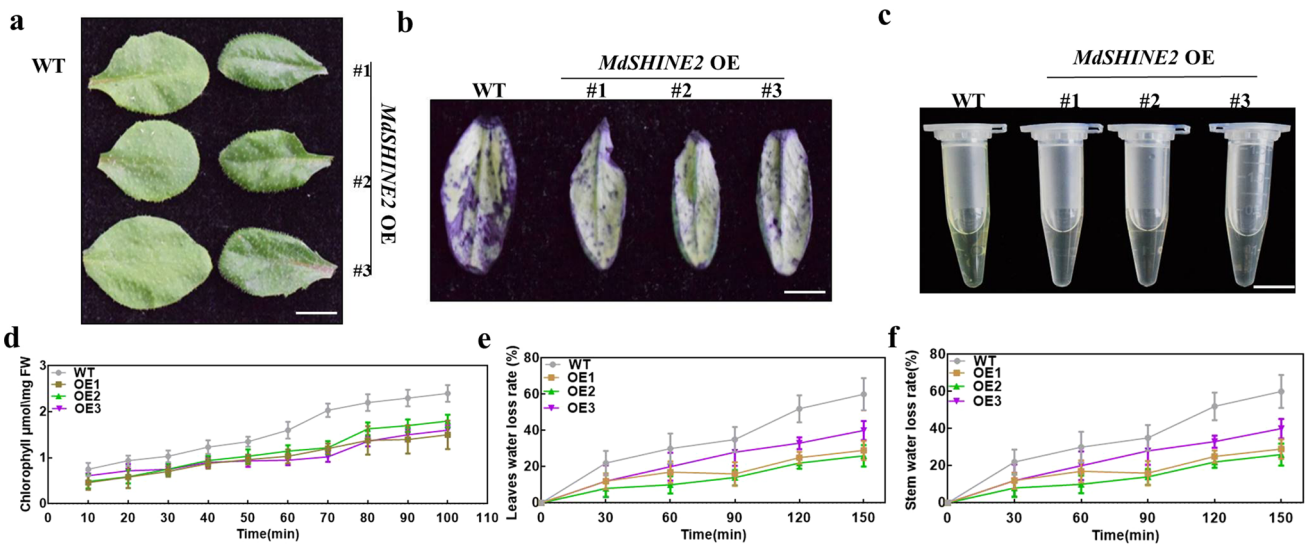

Fig. 6 Alterations to leaf surface properties and cuticle permeability in *MdSHINE2*-overexpressing Arabidopsis. **a** Mature rosette leaves of WT (left) and *MdSHINE2* OE (right) plants. **b** Toluidine blue staining of WT and *MdSHINE2* OE Arabidopsis leaves. **c** Chlorophyll comparison of mature rosette leaves of WT (left containers) and *MdSHINE2 OE* plants (three right containers). Scale bars = 1 cm (**a**–**c**). **d** Chlorophyll leaching rates in mature rosette leaves of WT and *MdSHINE2* OE. Arabidopsis chlorphyll μmol/mg FW (FW = fresh weight). **e, f** Water loss from leaves (**e**) and stems (**f**) in WT and *MdSHINE2* OE plants. Data are the mean ± SD of three independent replicates

To further verify the sensitivity of ectopic expression of *MdSHINE2* in Arabidopsis to ABA, we examined seedling phenotypes of the three transgenic Arabidopsis as well as WT after 14-day growth and found no obvious differences between plants growing in the common MS medium. However, the growth of seedlings changed substantially when growing in an MS medium containing 10, 20, and 30 μM of ABA, respectively. The roots of *MdSHINE2*-overexpressing Arabidopsis became much shorter, and leaves were more yellow compared with the WT (Fig. 7c, d). Germination and the seedling experiments both indicated that ectopic expression of *MdSHINE2* in Arabidopsis resulted in increased sensitivity to ABA, suggesting that *MdSHINE2* acted as a positive regulator in the ABA signaling pathway.

MdSHNE2 conferred to osmotic and drought resistance

MdSHINE2 OE and WT Arabidopsis were grown under normal conditions for 15 days. After 15-day growth, no water was given until leaves began to yellow. The phenotype was observed. After moderate water shortage treatment, the *MdSHINE2* transgenic Arabidopsis exhibited a small number of yellowing leaves but continued to grow normally; by contrast, the WT showed obvious yellowing, although the growth state of WT and transgenic Arabidopsis was consistent before drought treatment (Fig. 8a). Next, we examined chlorophyll content and found that of *MdSHINE2* OE plants to be much greener than in WT after drought treatment (Fig. 8d). This result indicates that *MdSHINE2* can enhance drought resistance of plants. To confirm the function of *MdSHINE2* on drought tolerance, we applied PEG in the cultured medium to simulate drought conditions for tissue-cultured materials. No obvious differences were observed between *MdSHINE2* OE and WT Arabidopsis seedlings in the normal MS medium. However, seedling growth was affected on the MS medium containing 4% and 6% PEG. The roots of *MdSHINE2* OE plants became longer (Fig. 8e), and the number of lateral roots increased compared with those of WT under PEG treatment (Fig. 8b).

To further investigate the function of *MdSHINE2* in apples, we transformed *35S::MdSHINE2* to apple calli and obtained three *MdSHINE2*-overexpressing lines OE1, OE2, and OE3 (Supplementary Fig. S3). Apple calli were treated with the MS medium or that containing 4% and 6% PEG, respectively. We did not identify any differences between the empty vector (EV) and *MdSHINE2* OE calli under normal conditions; however, transgenic apple calli grew much better with PEG treatment compared with WT (Fig. 8c). Then, the fresh weight and malondialdehyde (MDA) content were measured, revealing that the transgenic apple calli exhibited more fresh weight and lower MDA content than the EV (Fig. 8f, g). These results suggested that *MdSHINE2* can greatly increase plant osmotic and drought resistance.

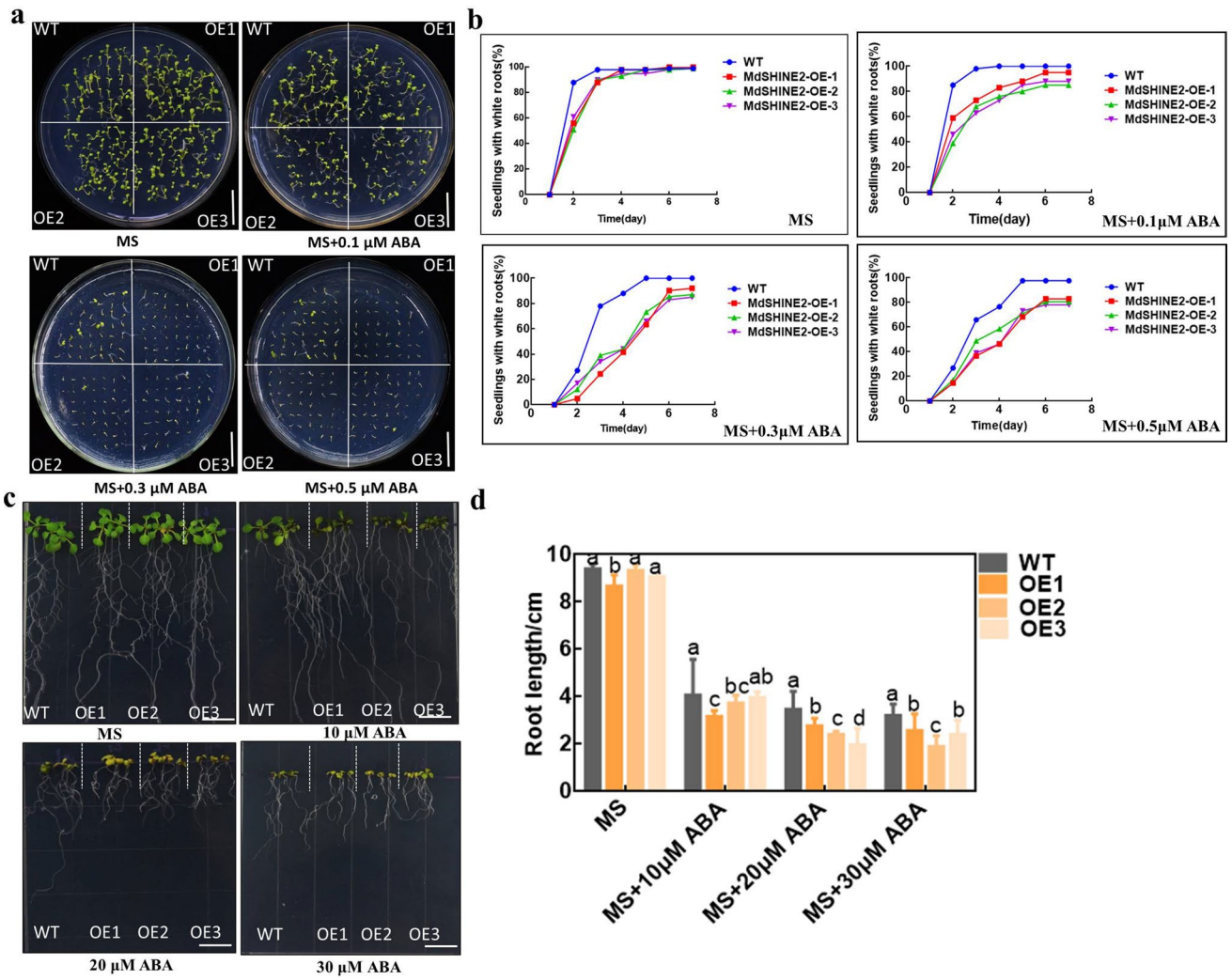

Fig. 7 Ectopic expression of *MdSHINE2* in Arabidopsis increased ABA sensitivity. **a** Seed germination of WT and *MdSHINE2* OE Arabidopsis under ABA treatment, scale bar = 1 cm. **b** Germination frequencies of WT and *MdSHINE2* OE Arabidopsis after 7-day growth. **c** Phenotype of WT and *MdSHINE2* OE Arabidopsis under ABA treatment after 14-day growth, scale bar = 1 cm. **d** Root length in Arabidopsis seedlings shown in **c**. Data are the mean ± SD of three independent replicates. Lowercase letters indicate significant differences at $P < 0.05$

Discussion

Studies have shown the SHINE proteins are members of the plant superfamily of AP2/EREBP transcription factors that function in wax synthesis programs (Singh et al. 2002; Zhang et al. 2005). For example, the regulatory cascade of MIXTA-like proteins and *WIN1/SHINE1* collectively regulate cutin biosynthesis and wax accumulation (Oshima et al. 2013). However, wax-related genes in apples have been rarely studied. In this work, we identified an *SHINE2* gene, named *MdSHINE2* from apple, produced by homologous comparison to the *AtSHINE2* gene.

The resulting parsimony phylogenetic trees showed that apple MdSHINE2 exhibited the highest similarity to dicotyledons and the farthest evolutionary relationship with fern in lower plants, indicating that evolution among SHINEs proceeded from lower plants to higher plants and was consistent with taxonomic lineages similar to other wax-related genes (Aharoni et al. 2004; Qi et al. 2018; Zhang et al. 2018). The entire AP2/EREBP family demonstrated that SHN Clade (SHN1/WIN1, SHN2, and SHN3) contain the highly conserved AP2 domain and share two other conserved motifs in their central portion and C termini; only SHN Clade has two complete motifs outside the AP2 domain, which might associate various SHN proteins with a similar functional role in Arabidopsis (Aharoni et al. 2004). Our results are consistent with previous studies and demonstrate that the main functional domains of AP2 proteins are conserved during the complex evolutionary process and important for their function.

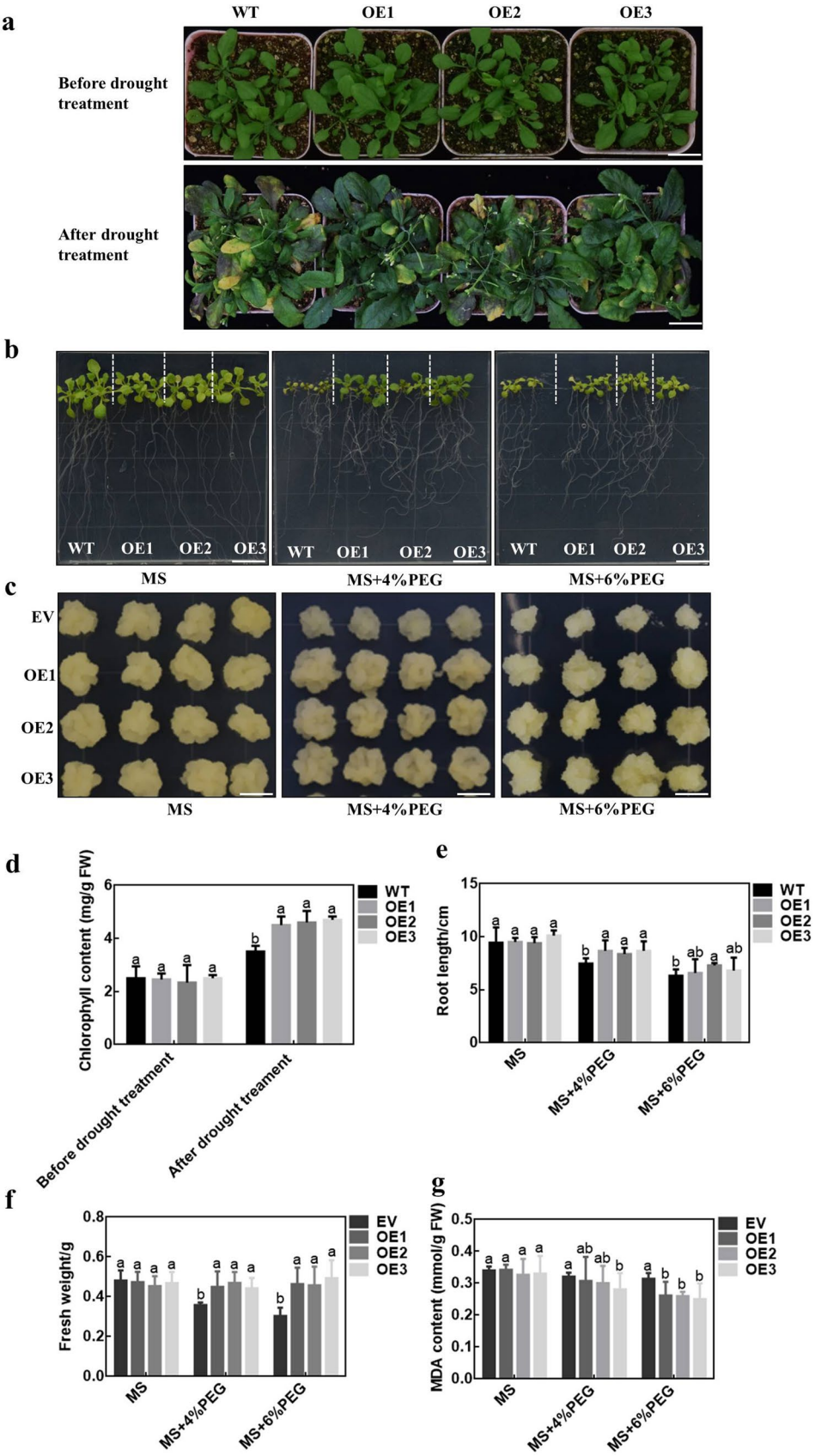

Fig. 8 Drought tolerance of WT and *MdSHINE2* OE Arabidopsis. **a** Phenotype of WT and *MdSHINE2* OE Arabidopsis under drought treatment (five seeds sown per pot). Arabidopsis were exposed to moderate water shortage; photographs were taken before drought treatment and after drought treatment, respectively. **b** Phenotype of WT and *MdSHINE2* OE Arabidopsis under treatment of 4% or 6%PEG. **c** Growth status of WT and *MdSHINE2* OE 'Orin' apple calli treated with or without 4% or 6% PEG (EV = empty vector). Scale bar = 1 cm (**a**–**c**). **d** Chlorophyll content (mg/g FW) in Arabidopsis shown in **a**. **e** Root length of plants shown in **b**. **f** Fresh weight of 'Orin' apple calli shown in **c**. **g** MDA content in mmol/g FW of 'Orin' apple calli shown in **c**. Data are the mean ± SD of three independent replicates. Different lowercase letters indicate significant differences at $P < 0.05$

Among wax-related genes, *MdCER1* is primarily expressed in apple leaves with lower expression in roots (Zhang et al. 2005; Qi et al. 2018). Arabidopsis *CER9* is strongly expressed in stems, leaves, rosette leaves, inflorescences, and siliques (Lü et al. 2009). *WSL4* is mainly found in cortex cells, sheaths, vascular bundles of leaves, and stems in rice. We also performed a spatiotemporal expression analysis to study the expression pattern of *MdSHINE2*. Different from the expression pattern in Arabidopsis, *MdSHINE2* was primarily expressed in the fruit skin and young leaf (Fig. 3a). This is mainly due to the involvement of *MdSHINE2* in the formation of cuticular wax in apples and can be beneficial to apple fruit gloss, water retention, and disease resistance. Most transcription factors have been reported to have nuclear localization signals required for targeting into the nucleus (Lee et al. 2014).

Decrease Wax Biosynthesis (*DEWAX*) encodes an AP2/ERF-type transcription factor in the nucleus. DEWAX negatively regulates the expression of wax synthesis genes and long-chain acyl-CoA synthetase, resulting in lower total wax loads in leaves and stems compared with WT (Go et al. 2014). Similar to other AP2 transcription factors, *MdSHINE2* was localized in the nucleus, demonstrating that *MdSHINE2* can target genes involved in wax synthesis.

From *cis*-elements analysis in the promoter regions of the *MdSHINE2* genes in apples, we found that *MdSHINE2* contains several *cis*-acting elements associated with stresses (Table 1). Furthermore, we tested the response to PEG, ABA, GA, and NaCl and found that *MdSHINE2* showed the fastest response to PEG, reaching the highest point 1 h after treatment; it exhibited the strongest response to ABA, reaching a peak at 3 h after treatment, approximately 3.5-fold that of the control. Therefore, we chose PEG and ABA treatments for further study. Among *cis*-elements in the promoter regions of *MdSHINE2,* many light-responsive elements were identified, implying that *MdSHINE2* could play a more important role in tissues or organs directly exposed to sunshine, similar to our finding that *MdSHINE2* was mainly expressed in fruits and young leaves with a potential function of preventing water loss and maintaining plant surface gloss.

Overexpression of *AtSHINE1*, a transcription factor associated with epicuticular wax biosynthesis to increase the leaf surface wax load in mulberry, is mainly due to increased content of alkanes in wax components (Sajeevan et al. 2017). Compositions of cuticular wax of the *gcn5-2* mutant and WT were quantified by GC–MS analysis in Arabidopsis. The total cuticular wax content in the *gcn5-2* mutant was approximately 63% that of the control, attributable to notable decreases in the major wax constituents in the mutant stem, including alkanes (C29) (Lee et al. 2009; Wang et al. 2018d). In this study, overexpression of *MdSHINE2* increased transcription of some wax-related genes, including structural genes *AtCER1, AtCER3, AtCER6, AtKCS1,* and *AtLACS2*. We further analyzed the promoter sequence of these genes and examined whether *MdSHINE2* could interact directly with promoters to regulate their transcription. In Arabidopsis, SHN2 and SHN3 show the highest sequence identity among the three SHN proteins (71%), whereas SHN1 and SHN2 show the lowest homology among clade members (55%) (Aharoni et al. 2004). Overexpression of the three SHN genes in Arabidopsis indicates a similar phenotype. Therefore, we can assume that the function of SHNE2 and SHNE3 in plants might be redundant, leading to a significantly induced transcription factor AtSHINE3 in MdSHINE2 OE Arabidopsis in our study. Consequently, the observed phenotype might be the cumulative effect of MdSHINE2 and AtSHN3 overexpression. GC–MS results revealed that the wax composition in transgenic plants was characterized by higher proportions of alkanes, alcohols, and others but lower aldehyde and fatty acid contents. The contribution of alkane was most important to an increase in total wax. Research has reported that wax-synthesis-related genes alter the waxy crystal structure on the plant surface. The deposition of epicuticular wax crystals was detected on leaves of *MYB96* OE lines but not on the leaves of WT (Lee et al. 2014). *atx4*, *atxr4*, *ashr2*, and *hda18* mutant variations were found for cuticular wax crystal morphology and crystallization patterns in WT. *atx4*, *atxr4*, and *ashr2* showed more cuticular wax crystals than the WT (Col-0), whereas less abundant wax was observed in *hda18* (Wang et al. 2018e). Scanning electron microscopy (SEM) revealed increased wax crystals on *MdSHINE2* transgenic plants. Our conclusions are consistent with previous studies, suggesting that the main wax components (alkanes) from different species might be conserved, whereas other wax components (alcohols, aldehydes, and fatty acids) differ. *AtSHN1*-overexpressing plants exhibited altered epidermal properties, resulting in tolerance to dehydration stress (Aharoni et al. 2004; Kannangara et al. 2007). Changes in cuticular wax often lead to changes in permeability of the epidermis (Lü et al. 2009). Here, we used the toluidine blue staining assay and water loss rate experiment to find that epidermal permeability may be reduced due to increased waxiness. This result is mostly consistent with previous research, which indicated that an increase of waxiness might enhance water retention of plants, but no clear correlation was found between wax content and composition and water transport.

ABA plays an important role throughout the life cycle of the plant, including during seed development and dormancy, seed germination, early seedling development, flowering, and in response to abiotic and biotic stress. Many studies have shown that overexpression of wax-related transcription factors in plants can increase sensitivity to ABA and improve drought resistance (Zheng et al. 2012). MYB30, an R2R3-type transcription factor related to wax synthesis, is

involved in regulation of ABA signaling. Overexpression of *MYB30* in WT results in an ABA-insensitive phenotype in Arabidopsis (Zheng et al. 2012). MYB96 is also involved in the ABA signaling pathway in Arabidopsis, which regulates plant resistance to drought stress (Pil et al. 2011). Many studies have identified that plants with more ABA sensitivity often have greater drought resistance. Plants lose water primarily by gaseous exchange through stomata on their leaves (Mishra 1997). ABA induces stomatal closure to conserve water, resulting in improved plant drought resistance (Lim et al. 2015). Overexpression of *ZmXerico1* and *ZmXerico2* in Arabidopsis and maize confers ABA hypersensitivity and improved water use efficiency, leading to enhanced maize yield in a controlled drought-stress environment (Brugière et al. 2017). In this study, we found ectopic expression of *MdSHINE2* in Arabidopsis to result in increased sensitivity to ABA and increased plant resistance to drought. Results showed that the more sensitive the plant was to ABA, the stronger the drought resistance, but no direct relationship was identified between ABA sensitivity and drought resistance.

Cuticular wax plays an important role in protecting plants from environmental stress (e.g., increasing the cuticular wax content reduced nonstomatal water loss in plants, thereby improving drought tolerance) (Zhang et al. 2007). The regulation of cuticular wax biosynthesis is important for optimal plant growth and normal plant development. In this study, we identified a novel apple SHINE2 transcription factor that directly or indirectly activates expression of wax-related genes to increase the cuticular wax load and wax deposition, causing greater cuticular wax impermeability and changing the surface pattern to enhance plant ABA sensitivity and drought tolerance. These findings provide fundamental insights into *MdSHINE2* transcription factors, with prospects for utilization in generations of transgenic crops with enhanced stress tolerance and improved apple fruit quality (e.g., gloss and storage period).

Author contribution statement YYL and YJH initiated and designed the research. YLZ, CLZ, GLW, YXW, and CHQ performed the experiments. YLZ analyzed the data. YYL contributed reagents/materials/analysis tools. YLZ wrote the manuscript. YYL and YLZ revised the manuscript.

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