Front. Agr. Sci. Eng. 2021, 8(2): 247–261 https://doi.org/10.15302/J-FASE-2021388

RESEARCH ARTICLE

APPLE SUMO E3 LIGASE MDSIZ1 NEGATIVELY REGULATES DROUGHT TOLERANCE

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KEYWORDS

apple, drought tolerance, gene expression, *MdSIZ1*

HIGHLIGHTS

- *MdSIZ1* RNAi transgenic apple trees are drought tolerance than wild type—GL-3.
- *MdSIZ1* RNAi plants get enhanced ability to keep water and scavenge ROS under drought conditions.
- MdSIZ1 may participate in apple drought tolerance by affecting ABA biosynthesis.

Received November 1, 2020;
Accepted February 1, 2021.

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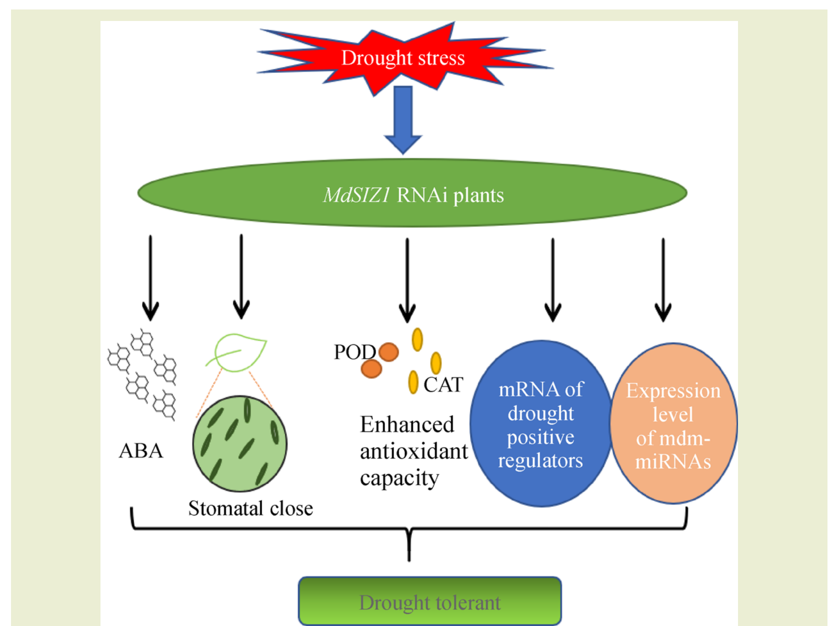
GRAPHICAL ABSTRACT



ABSTRACT

Drought stress typically causes heavy losses in apple production and uncovering the mechanisms by which apple tolerates drought stress is important in apple breeding. MdSIZ1 is a SUMO (small ubiquitin-like modifier) E3 ligase that promotes SUMO binding to substrate proteins. Here, we demonstrate that *MdSIZ1* in apple has a negative relationship with drought tolerance. *MdSIZ1* RNAi transgenic apple trees had a higher survival rate after drought stress. During drought stress they had higher leaf water potential, reduced ion leakage, lower H_2O_2 and malondialdehyde contents, and higher catalase activity. In addition, *MdSIZ1* RNAi transgenic plants had a higher net photosynthetic rate during the latter period of drought stress. Finally, the transgenic apple trees also altered expression levels of some microRNAs in response to drought stress. Taken together, these results indicate that apple MdSIZ1 negatively regulates drought stress by enhancing leaf water-holding capacity and antioxidant enzyme activity.

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1 INTRODUCTION

Water has a range of functions in plant physiological and metabolic processes including seed germination, seedling growth, flower bud differentiation, root growth, leaf transpiration, plant respiration, stomatal opening and antioxidant enzyme activity[1]. Water deficiency is therefore an important global agricultural challenge inhibiting the yield and quality of crops and fruit trees[2]. Elucidating the mechanisms by which plants respond to drought stress is a useful approach in breeding stress-resistant crops, thereby contributing to worldwide food security[3,4]. Apple is a widely grown fruit with high nutritional value and excellent flavor. Water deficiency is the most important challenge in the main Chinese apple producing area on the loess plateau. Hence, it is important to understand the function of key drought-responsive genes and investigate the physiological mechanisms by which apple trees respond to drought[5,6]. Plants may respond to drought stress by controlling stomatal movement[7], hormone biosynthesis[8], the ability to scavenge reactive oxygen species[9], accumulation of metabolic products[10], root development[3], transcriptional regulation[10] and microRNA biogenesis[11].

SUMOylation (small ubiquitin-like modifier, SUMO) is a very important posttranslational modification in eukaryotes and has been implicated in the growth and development of animals, plants and fungi[12,13]. During the SUMOylation process, SUMO is combined with a substrate protein through a multi-enzyme cascade reaction mediated by E1 (SUMO activating enzyme), E2 (SUMO conjugating enzyme) and E3 (SUMO ligase)[14,15]. Compared with ubiquitination, SUMOylation is a reversible, cyclic process that functions both in degrading conjugated proteins and in increasing protein stability[16–18]. Two types of SUMO E3 ligase have been identified in *Arabidopsis*: SIZ1 (SAP and Miz 1) and MMS21/HPY2 (methyl methanesulfonate-sensitivity protein 21/HIGH PLOIDY 2)[18–21]. The basic structure of SIZ1 comprises five domains, namely the Sp-RING domain, the PHD domain, the SXS motif, the SAP domain, and the PINIT motif, each of which performs a different function[22]. The basic function of SUMO E3 ligase is to interact with E2 to promote the transfer of SUMO from E2 to the substrate protein[23,24]. Although E3 ligase is not needed for SUMOylation of every protein, it is usually involved to accurately target substrate molecules. In other words, the specificity of SUMOylation is determined by the substrate specificity of the E3 ligase[9,25]. Studies have therefore focused increasingly on the importance of SIZ1-mediated processes in growth, development, abiotic stress response and immunity[9,25,26].

SIZ1 affects various physiological processes. Its mutation causes dwarfing, early flowering, abnormal seed development, low germination rate and inhibition of primary root growth[23,27,28]. SIZ1 also influences plant growth and development by regulating the signaling pathways of phytohormones such as abscisic acid (ABA), gibberellin (GA) and salicylic acid (SA). However, SIZ1 appears to have different functional characteristics in different species in response to drought stress. In *Arabidopsis*, AtSIZ1 can independently regulate drought stress response through the ABA non-dependent pathway and can also regulate ABA signal transduction and ABA-related gene expression[29–31]. Overexpression of tomato *SIZ1* in transgenic tobacco confers drought tolerance to the transgenic plants, as does overexpression of rice *OsSIZ1* in cotton[32–34]. However, the *Arabidopsis siz1* mutant results in a drought-sensitive phenotype compared with the wild type[28]. MdSIZ1-mediated SUMO conjugation of target proteins plays an important role in regulating the adaptation of apple plants under different abiotic stresses[35]. In addition, MdSIZ1 influences iron homeostasis in apple[17], while AtSIZ1 is involved in excess copper[26]. Previous studies report that SIZ1 is involved in apple cold, phosphate and iron deficiency stresses but its role in apple drought stress remains unclear.

It is necessary to further understand how SUMO E3 ligase responds to drought stress when regulating the ABA accumulation under drought conditions and changing ROS scavenging ability, subsequently leading to a change in physiological index. Here, we demonstrate that apple MdSIZ1 is a negative regulator of drought tolerance. *MdSIZ1* RNAi transgenic plants had a higher survival rate and greater leaf photosynthetic capacity under drought stress conditions. We provide evidence that MdSIZ1 participated in the drought resistance of apple by negatively regulating the response of ABA to drought stress. The stomatal sensitivity to ABA and ROS scavenging ability increased in transgenic RNAi lines. In addition, MdSIZ1 altered the expression of some drought-related genes and microRNAs in response to drought.

2 MATERIALS AND METHODS

2.1 Generation of transgenic apple trees

We lowered the expression level of *MdSIZ1* by the RNA interference (RNAi) method in order to obtain apple *MdSIZ1* RNAi plants. A fragment of 118 bp of *MdSIZ1* was first introduced into pDONR222 with the gateway system and then into pK7WIWG2D, resulting in *MdSIZ1*-pK7WIWG2D. The

plasmid was introduced to *Agrobacterium* strain EHA105. *Agrobacterium*-mediated transformation of apple was conducted using GL-3 as genetic background. Four-week-old tissue cultured GL-3 was used for gene transformation according to Xie et al.[36]. The plants were grown on Murashige and Skoog (MS) medium (4.43 $g\cdot L^{-1}$ MS salts, 30 $g\cdot L^{-1}$ sucrose, and 7 $g\cdot L^{-1}$ agar, pH 5.8) supplemented with 0.2 $mg\cdot L^{-1}$ 6-benzylamino-purine(6-BA) and 0.2 $mg\cdot L^{-1}$ indoleacetic acid under long-day conditions (14:10 h L:D photoperiod) at 25°C. The primers used are listed in Table 1.

2.2 Plant materials and stress treatment

The transgenic plants and GL-3 were sub-cultured and rooted (2.22 $g\cdot L^{-1}$ MS salts, 20 $g\cdot L^{-1}$ sucrose, 7.5 $g\cdot L^{-1}$ agar, 0.5 $mg\cdot L^{-1}$ IAA, 0.5 $mg\cdot L^{-1}$ indolebutyric acid, pH 5.8), and transplanted into pots (15 cm × 13 cm) containing a matrix (Pindstrup Mosebrug, Pindstrup, Denmark), perlite and vermiculite (5:1:1 by volume) two months later. The plants grew in a growth chamber with an air temperature of 25–28°C, a light intensity of 3000–4000 lx and an air humidity of 45%–50% (14:10 h L:D photoperiod). Three months later, *MdSIZ1* RNAi transgenic plants and GL-3 were subjected to drought stress for 21 d and then reirrigated for a further 7 d. Soil water content was determined with a FieldScout TDR 350 (Spectrum Technologies, Inc, Aurora, IL). The soil water content (VWC, volume water content) changed from 35% to 40% (control) or 5% to10% (drought), and reduced to 0% on day 21.

Table 1 Primers used in this study

Primer name	Primer sequence (5' to 3')	Purpose
MdSIZ1 RNAi F	AAGCAGGGGAAGAAGCAG	*MdSIZ1* RNAi plants
MdSIZ1 RNAi R	CAAGTTCCGCCACCTGT	*MdSIZ1* RNAi plants
MdSIZ1 qPCR F	GACGACTGGATCTCTCTTCGAC	qRT-PCR analysis
MdSIZ1 qPCR R	CTTTGTTAGACCTAACTTCAT-CAGTTCC	qRT-PCR analysis
MdSIZ1-like qPCR F	GACGACTGGATCTCTCTTCGAC	qRT-PCR analysis
MdSIZ1-like qPCR R	CTTTGTTAGACCTAACTTCAT-CAGTTCC	qRT-PCR analysis
MdDREB2A qPCR F	GAGTCTGCCGCACATGGA	qRT-PCR analysis
MdDREB2A qPCR R	GTTGTAACCTACGTCTCCGGAT	qRT-PCR analysis
MdPIP1;3 qPCR F	CCAAGAGGAATGCCAGAGAC	qRT-PCR analysis
MdPIP1;3 qPCR R	GCAAGCATCATTGTTTCCCC	qRT-PCR analysis
MdNCED3 qPCR F	CAGCTACAGGTACACGGGTT	qRT-PCR analysis
MdNCED3 qPCR R	TGTGGCACTGGCTTTGAAGA	qRT-PCR analysis
mdm-miR168 qPCR F	TCGCTTGGTGCAGGTCG	qRT-PCR analysis
mdm-miR172 qPCR F	GAGAATCTTGATGATGCTGCAT	qRT-PCR analysis
mdm-miR393 qPCR F	TCCAAAGGGATCGCATTGATCC	qRT-PCR analysis
mdm-miR408 qPCR F	GCGATGCACTGCCTCTTC	qRT-PCR analysis
stem-loop Universal R	GTGCAGGGTCCGAGGT	qRT-PCR analysis
MdMDH qPCR F	CGTGATTGGGTACTTGGAAC	qRT-PCR analysis
MdMDH qPCR R	TGGCAAGTGACTGGGAATGA	qRT-PCR analysis

2.3 DNA and RNA extraction and qRT-PCR analysis

A modification of the CTAB method was used to extract DNA and total RNA according to Xie et al.[36]. qRT-PCR analysis was conducted as described by Xie et al.[36]. The primers used for qRT-PCR analysis are arranged and summarized in Table 1.

2.4 Leaf relative water content and leaf water potential

Leaves from *MdSIZ1* RNAi transgenic plants and GL-3 were collected when the soil VWC was 35%–40% (control) or 5%–10% (drought). Three replicates comprising 10 plants were used. Leaf relative water content (LRWC, Eq. (1)) was calculated as follows. Leaves were cut from the plants and weighed immediately to determine fresh weight and then immersed in distilled water for 24 h to give the turgid weight. Finally, the leaves were oven-dried at 65°C for 3 d.

$$\text{LRWC}\ (\%) = 100 \times [(\text{fresh weight} - \text{dry weight})/(\text{turgid weight} - \text{dry weight})] \quad (1)$$

Leaf water potential measurement (Ψleaf) was determined with a Model 600 pressure chamber (PMS Instrument Company, Albany, OR) according to the manufacturer's instructions.

2.5 Leaf ion leakage

Leaves were collected to determine the ion leakage when soil VWC was 35%–40% (control) and 5%–10% (drought). Three replicates comprising 10 plants were used. Leaf discs were placed in 7 mL deionized water in a clean tube for 12 h and the conductivity was determined using a Five Easy Plus conductivity meter (Mettler Toledo, Columbus, OH). Finally, the tubes were boiled at 100°C for 30 min and cooled to room temperature to

measure the conductivity. Leaf relative conductivity (%) was calculated as the ratio of these two conductivities.

2.6 Hydrogen peroxide, O_2^- and malondialdehyde

The contents of H_2O_2, O_2^- and malondialdehyde (MDA) were determined using a peroxide assay kit (Suzhou Comin Biotechnology Institute, Suzhou, China), a superoxide anion assay kit (Suzhou Comin Biotechnology Institute, Suzhou, China), and a BC0020 malondialdehyde assay kit (Solarbio, Beijing, China), respectively. Three replicates comprising 10 plants were used. Histological staining of H_2O_2 and the content measurement were conducted as described by Thordal-Christensen et al.[37].

2.7 Peroxidase and catalase activities

Peroxidase (POD) and catalase (CAT) activities were determined according to Wang et al.[38] with minor modifications. Total proteins were extracted with enzyme extracting buffer (100 $mmol \cdot L^{-1}$ phosphate buffer, 1 $mmol \cdot L^{-1}$ EDTA, 0.1% Triton-X-100 and 1% PVP). Frozen samples (50 mg) were weighed into 2-mL centrifuge tubes and 800 µL extracting buffer were added. The samples were mixed on ice and then centrifuged at 13,000 $r \cdot min^{-1}$ for 20 min at 4°C. Each supernatant was transferred into a new 1.5-mL centrifuge tube for subsequent detection of enzyme activity. The CAT activity was determined with a spectrophotometer at 240 nm wavelength, with 3.12 mL of 50 $mmol \cdot L^{-1}$phosphate buffered saline (PBS, pH 7.8), 800 µL of 50 $mmol \cdot L^{-1}$ H_2O_2, and 80 µL protein extracting buffer. The POD activity was detected with a Victor Nivo Multiskan Spectrum (PerkinElmer, Waltham, MA) (795 µL of 50 $mmol \cdot L^{-1}$ PBS at pH 7.0, 100 µL of 100 $mmol \cdot L^{-1}$ H_2O_2, 100 µL of 100 $mmol \cdot L^{-1}$ guaiacol, and 5 µL protein extracting buffer).

2.8 Photosynthetic rate

The photosynthetic parameters were measured with an LI-COR6400 portable photosynthetic system (LI-COR Biosciences, Lincoln, NE) between 9 and 10 a.m. The mature leaves of each plant (leaves 5 and 7 from the top of the plant) were measured. The light source comprised LEDs (red and blue) with a light intensity of 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, an airflow of 500 $\mu mol \cdot s^{-1}$ and an environmental CO_2 concentration of 400±5 $\mu mol \cdot mol^{-1}$. The LI-COR6400 instruction manual was followed in detail.

2.9 Stem-loop qRT-PCR

Total RNA was extracted from leaves using CTAB[36]. cDNA was synthesized with a HiScript II 1st Strand cDNA Synthesis Kit (with gDNA wiper) (Vazyme R212-02, Nanjing, China) according to the manufacturer's instructions. The specific steps were as follows: (1) miRNA168-P5, miRNA393 g-P5, miRNA408a, and miRNA172-P3 premature primers and MDH reversed primer were mixed into one tube (final primer concentration 1 $\mu mol \cdot \mu L^{-1}$). (2) Mixed primers were denaturalized at 65°C for 5 min. These mixed primers were used as the primer for the subsequent reverse transcription. (3) cDNA of *MdSIZ1* RNAi 2, *MdSIZ1* RNAi 4 and GL-3 before and after drought treatment were used as the template for fluorescent quantitative PCR. ChamQ SYBR qPCR Master Mix (Vazyme Q311-02/03) and a Bio-Rad CFX96 (Bio-Rad, Hercules, CA) fluorescent quantitative PCR instrument were used to analyze the relative expression of miRNAs. Specific primer sequences are shown in Table 1.

2.10 Stomatal aperture

Two-month-old tissue cultured transgenic and GL-3 plants were used in a stomata opening and closing experiment. Mature leaves in the middle of each plant were collected and placed directly in a buffer (30 $mmol \cdot L^{-1}$ KCl, 0.1 $mmol \cdot L^{-1}$ $CaCl_2$, 10 $mmol \cdot L^{-1}$ MES-KOH, pH 6.15). The leaves were incubated at 22°C for 2 h under illumination (120 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in order to induce the stomata opening. After 2 h, 5 $\mu mol \cdot L^{-1}$ ABA was added to the buffer for an additional hour. Finally, the leaves were cut into slices and placed to observe stomatal movement (microscope, 200× magnification). The width and length of the stomata were measured by Image J software (University of Wisconsin, Madison, WI) and the stomatal aperture was calculated as the width/length ratio. Ten leaves were used and at least 40 stomatal apertures were measured in each treatment.

2.11 ABA content

The ABA extraction method has been described previously[39] and is summarized as follows. About 50 mg frozen sample was ground into a powder in liquid nitrogen and then extracted with 1 mL of cold extraction buffer (A, 0.5 g $C_6H_8O_7 \cdot H_2O$ dissolved in 200 mL ddH_2O_2; B, 0.2 g BHT dissolved in 400 mL methyl alcohol. Solutions A and B were mixed and then diluted with methyl alcohol to 1 L. The samples were mixed with extracting buffer by vortex for 5 min at 4°C. The supernatant was collected after centrifugation (4°C, 12,000 $r \cdot min^{-1}$ for 10 min) and the pellets were re-extracted with 600 µL of cold extraction buffer. The extraction process was repeated three times and a constant amount of internal standard was added. Finally, the combined supernatant was filtered through a 0.22-µm PTFE filter (Shimadzu, Kyoto, Japan). Eight standard ABA (Sigma-Aldrich,

Steinheim, Germany) solutions were prepared ranging from 0.5 to 100 ng·mL^{-1}. Samples were then analyzed using a Sciex 5500 Qtrap LCMS/MS (Danaher Corporation, Washington, DC). Gradient elution was conducted with solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid) at the following solvent rates: 0–2 min with 20% B, 2–6 min increasing to 90% B, 6–11 min with 90% B, 11–12 min decreasing to 20% B, and 12–15 min with 20% B. Experiments were conducted in negative ionization mode. The capillary voltage was −4.5 kV and the temperature was 60°C. The parameter of declustering potential was −60 V and collision energies were −14 V and −27 V. Five independent replicates of each treatment were used. Data were analyzed and processed using MultiQuant Software (Danaher Corporation, Washington, DC).

3 RESULTS

3.1 Identification of the apple SUMO E3 ligase gene *MdSIZ1*

We used *Arabidopsis AtSIZ1* as a query and identified four homologs in the apple genome (GDDH13 v1.1). Given their ancient hybridization and whole genome duplication, apple *MdSIZ1* homologs are located on four chromosomes, namely 1, 3, 7 and 11. MD03G1112700 and MD11G1131700 are located on chromosomes 3 and 11 and share high similarity (Fig. 1(a)), MD01G1111200 and MD07G1177400 are located on chromosomes 1 and 7 and show high similarity in their protein sequences (Fig. 1(b)). We designated MD11G1131700 and MD03G1112700 *MdSIZ1*, and MD01G1111200 and MD07G1177400 *MdSIZ1-like*. Both MdSIZ1 and MdSIZ1-like contained conserved domains similar to those of AtSIZ1, including an SAP domain (which functions in DNA binding in yeast, although its function in plants is not yet clear), a PHD domain (which participates in ligase catalytic substrate connection) and an Sp-RING finger domain (which endows AtSIZ1 ligase with its catalytic function) (Fig. 1).

3.2 Expression of the *MdSIZ1* genes in different tissues and in response to abiotic stresses

We previously conducted an RNA-seq analysis using three-month-old *Malus prunifolia* seedlings and found that *MdSIZ1* genes (MD11G1131700 and MD03G1112700) were abundantly expressed in all tissues but an *MdSIZ1-like* gene (MD07G1177400) was expressed only at trace levels in all tissues (Fig. 2(a)). Expression of MD01G1111200 was not detected in the RNA-seq data. We postulated that *MdSIZ1* had the main function in apple rather than *MdSIZ1-like*. Given its higher expression in leaves and roots, we speculate that *MdSIZ1* might be involved in stress response. Indeed, it was significantly induced by chilling stress (4°C), drought (drought lasting 6 d) and heat (45°C). In particular, drought stress upregulated *MdSIZ1* expression 2.2-fold (Fig. 2(b)). These results suggest that MdSIZ1 was involved in the apple drought stress response. By contrast, the expression of *MdSIZ1-like* was low in response to cold, drought and heat stresses (Fig. 2(b)).

3.3 MdSIZ1 is a negative regulator of drought tolerance in apple

We generated *MdSIZ1* RNAi transgenic apple trees to investigate the biological function of MdSIZ1 in the apple drought response. Given the high similarity between *MdSIZ1* and *MdSIZ1-like*, we reduced the expression level of both genes simultaneously (Fig. 3). We identified the transgenic plants at both the DNA and mRNA levels and examined the drought tolerance of three-month-old *MdSIZ1* RNAi and non-transgenic GL-3 plants. After withholding water for 21 d and reirrigating for 7 d, 80%–90% of the *MdSIZ1* RNAi plants survived but only 40% of the control plants survived (Fig. 4). These results indicate that MdSIZ1 is a negative regulator of apple drought tolerance.

3.4 MdSIZ1 regulated leaf water potential in response to drought stress

Compared with GL-3 plants under 21 d of drought, the leaves of *MdSIZ1* RNAi plants had higher relative water content and higher water potential (Fig. 5). According to the LRWC and leaf, we found a higher water holding capacity of *MdSIZ1* RNAi transgenic plants compared to GL-3. Given that drought stress often damages plant cell membranes, we measured electrolyte leakage and found that it was lower in *MdSIZ1* RNAi plants than in GL-3 plants under drought stress (Fig. 6(a)). This indicates better integrity of the cell membrane in *MdSIZ1* RNAi plants. In addition, *MdSIZ1* RNAi plants had lower MDA content than GL-3, suggesting that they sustained less oxidative damage under drought stress (Fig. 6(b)). In addition, the higher status of *MdSIZ1* RNAi plant leaves increased the photosynthetic rate and water use efficiency under drought conditions (Fig. 7).

3.5 MdSIZ1 regulated antioxidant enzyme activity in response to drought stress

Plants accumulate reactive oxygen species (ROS) when faced with stress conditions. Here, staining with 3,3'-diaminobenzidine tetrahydrochloride and the less brown polymer in *MdSIZ1* RNAi plants suggest that *MdSIZ1* RNAi plants contained less H_2O_2 under drought stress (Fig. 8(a)). Further analysis confirms

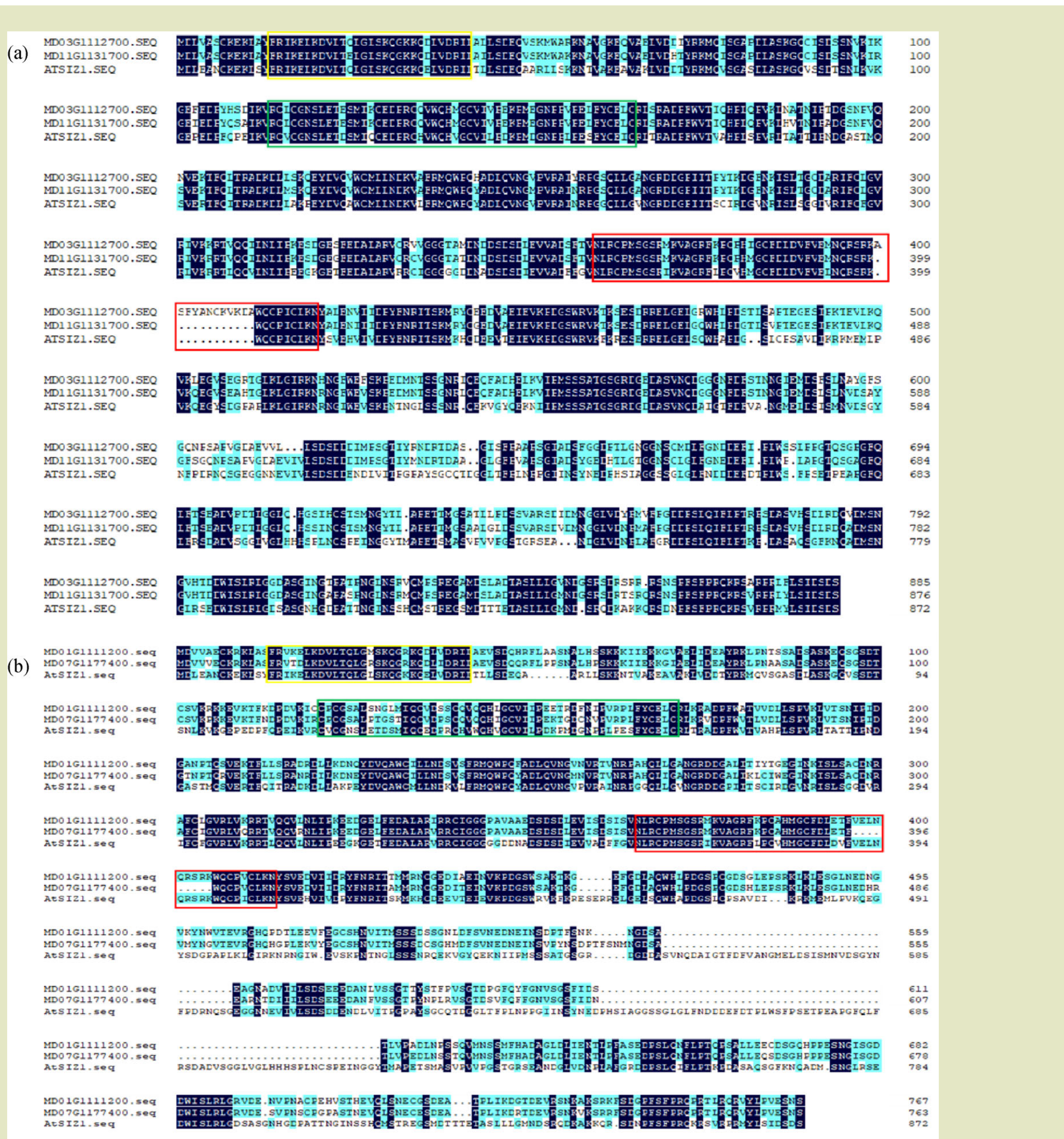

Fig. 1 Protein alignment of MdSIZ1, MdSIZ1-like and AtSIZ1. (a) Protein alignment of MdSIZ1 and AtSIZ1. (b) Protein alignment of MdSIZ1-like and AtSIZ1. Sequences of MdSIZ1 and MdSIZ1-like were obtained from the *Malus × domestica* (GDDH13 v1.1 genome) and AtSIZ1 (accession NM_125434) from NCBI. Yellow, green and red boxes indicate the SAP, PHD, and SP-RING finger domains, respectively.

this result (Fig. 8(b)). We also measured the activity of the main H_2O_2 scavenging enzymes, CAT and POD. After drought stress, *MdSIZ1* RNAi plants exhibited increased CAT and POD activities (Fig. 8(c,d)). In addition, the lighter nitro blue tetrazolium staining results indicate that there was a lower content of superoxide (O_2^-) in *MdSIZ1* RNAi plants, suggesting a stronger ability of the transgenic plants to scavenge hydrogen peroxide and O_2^- under drought stress conditions.

3.6 MdSIZ1 regulates drought resistance by modulating stomatal movement induced by ABA

To further explore the mechanism by which MdSIZ1 regulates apple drought resistance we detected ABA content in *MdSIZ1* RNAi plants and GL-3. ABA plays a key role in plant drought response through its role in regulating stomatal movement[7].

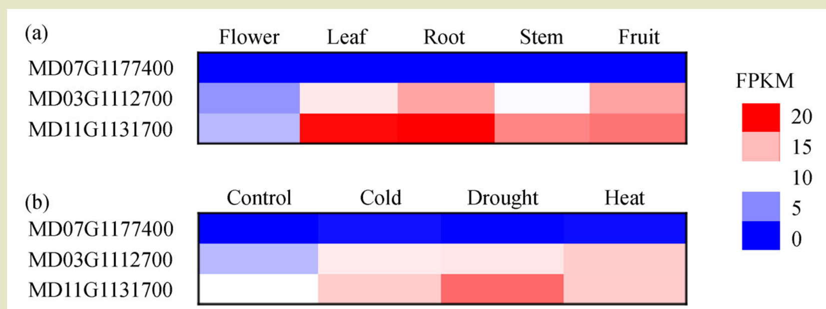

Fig. 2 Heat map of *MdSIZ1* and *MdSIZ1-like* expression (FPKM) in different tissues and under abiotic stress. Higher transcript levels are shown in red and lower transcript levels in blue. (a) Three-month-old *Malus prunifolia* seedlings were used for RNA-seq measurement of gene expression in different tissues and under abiotic stresses. (b) For cold stress, the seedlings were exposed to 4°C for 3 h in a growth chamber before the leaves were harvested. For heat stress, the seedlings were exposed to 45°C for 30 min in a growth chamber, and for drought stress, water was withheld from the seedlings for 6 d.

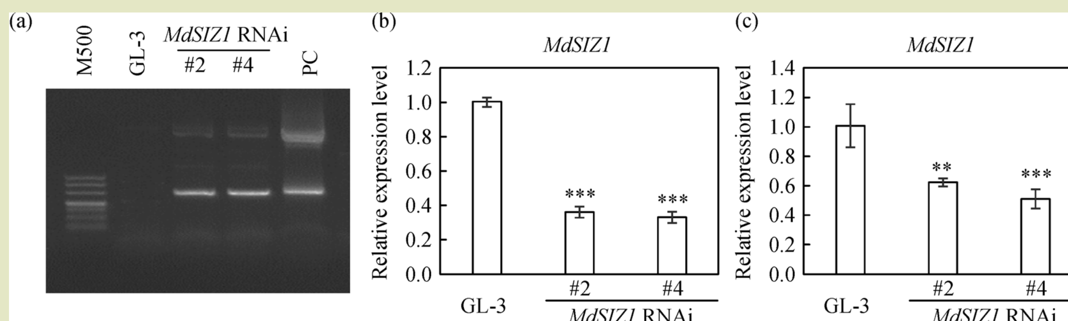

Fig. 3 Identification of *MdSIZ1* RNAi transgenic plants. (a) Detection of *MdSIZ1* RNAi plants at the DNA level. M500, DNA Marker 500; PC, positive control with MdSIZ1-pK7GWIWG2D as a template. A sequence from the CaMV 35S promoter was used as the forward primer and a sequence of *MdSIZ1* RNAi was used as the reverse primer. Transcript levels of *MdSIZ1* (b) and *MdSIZ1-like* (c) in *MdSIZ1* RNAi plants. Data are means±SD (n = 3). Student's t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

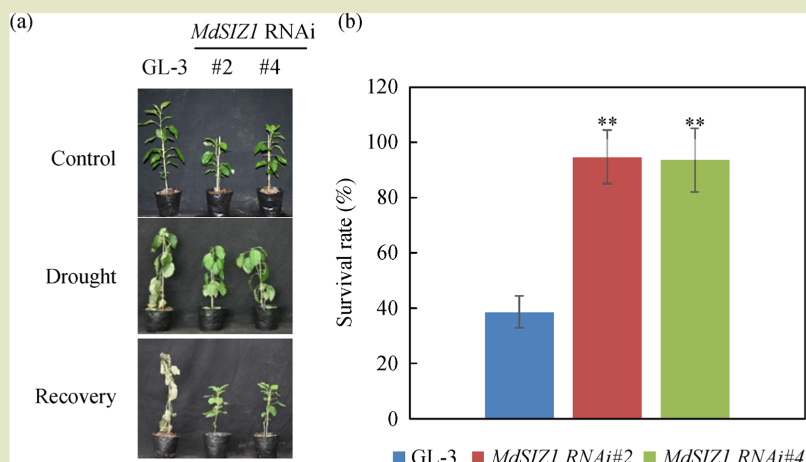

Fig. 4 Drought tolerance of *MdSIZ1* transgenic apple plants under drought stress conditions. (a) Morphological characteristics of GL-3 and *MdSIZ1* RNAi transgenic apple plants in response to drought stress. Three-month-old plants were exposed to drought for 21 d, then reirrigated for 7 d. (b) Survival rate of plants shown in (a). Thirty plants per line were used to analyze survival rate. Data are means±SD (n = 30). Student's t-test: *, $P<0.05$; **, $P<0.01$.

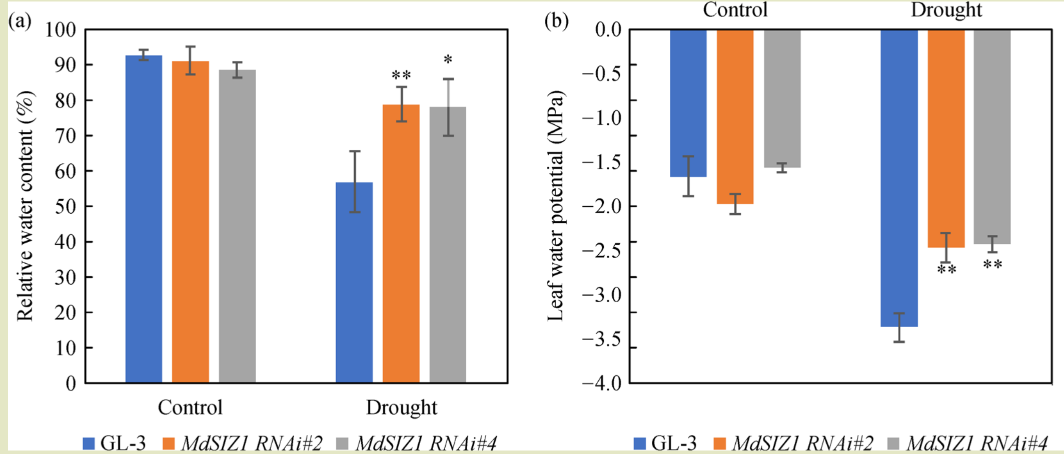

Fig. 5 Water deficit levels of *MdSIZ1* transgenic plants in response to drought stress. (a) Leaf relative water content. (b) Leaf water potential. Data are means±SD (n = 10). Student's t-test: *, $P<0.05$; **, $P<0.01$.

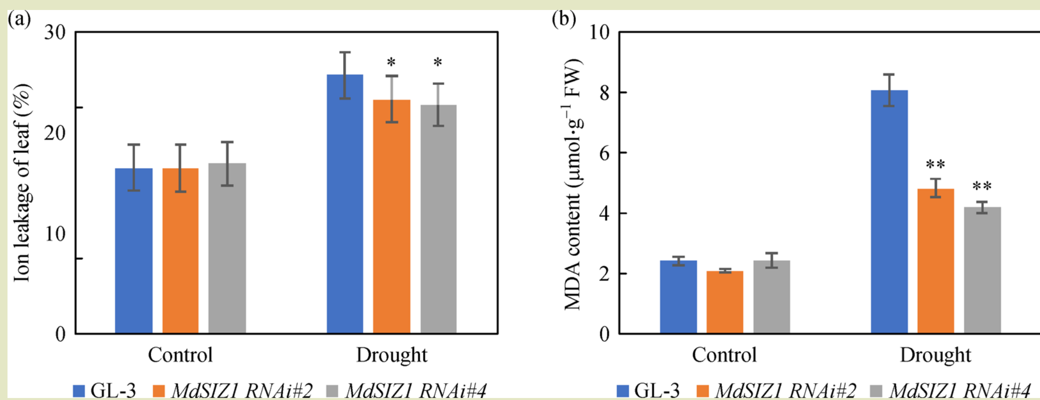

Fig. 6 The membrane integrity of *MdSIZ1* transgenic plants in response to drought stress. (a) Leaf ion leakage and (b) MDA content in leaves. Data are means±SD (n = 10). Student's t-test: *, $P<0.05$; **, $P<0.01$.

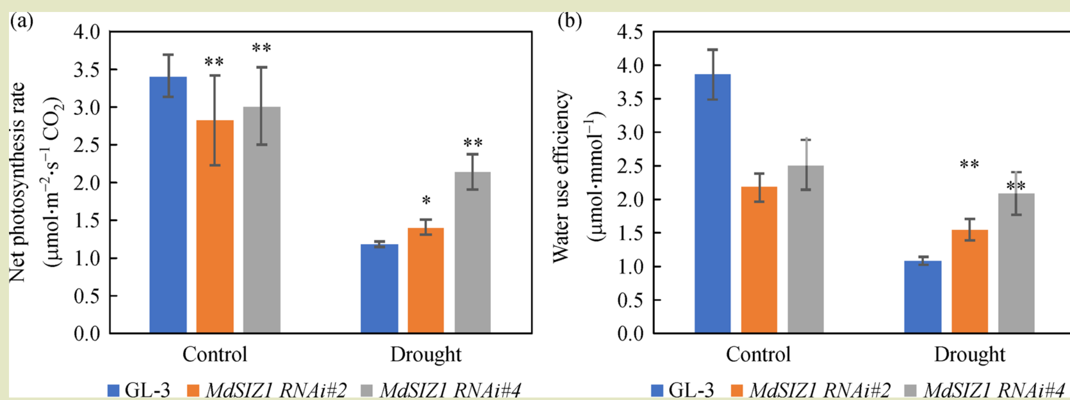

Fig. 7 Photosynthetic capacity and water use efficiency of GL-3 and *MdSIZ1* RNAi plants after drought stress. (a) Net photosynthetic rate and (b) water use efficiency. Data are means±SD (n = 10). Student's t-test: *, $P<0.05$; **, $P<0.01$.

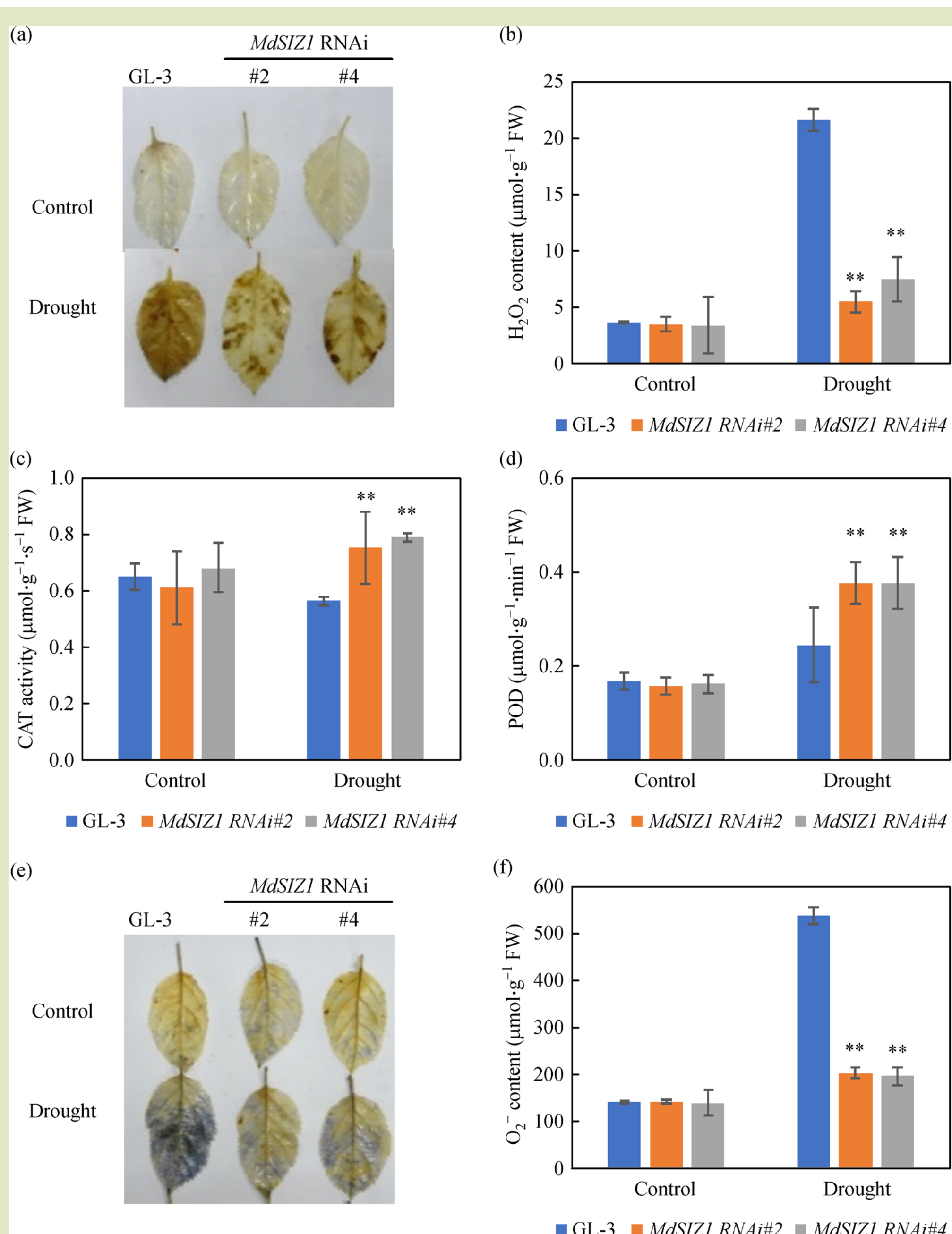

Fig. 8 *MdSIZ1* RNAi plants have higher scavenging ability for hydrogen peroxide (H_2O_2) and superoxide (O_2^-) under drought stress conditions. (a) Results from staining to detect H_2O_2 and O_2^- in leaves of *MdSIZ1* RNAi and GL-3 plants exposed to drought treatment for 21 d. (b) H_2O_2 content in leaves. (c) Catalase activity (CAT). (d) Peroxidase activity (POD). (e) Staining of O_2^- in *MdSIZ1* RNAi and GL-3 plants under drought stress. (f) O_2^- content in leaves. H_2O_2 and O_2^- were stained with DAB and NBT, respectively. Data are means±SD (n = 10). Student's t-test: *, $P < 0.05$; **, $P < 0.01$.

Our results indicate that ABA accumulated significantly in both *MdSIZ1* RNAi transgenic plants and GL-3 under induced drought stress conditions. Also, *MdSIZ1* RNAi transgenic plants contained significantly more ABA content than GL-3 (Fig. 9(a)). In contrast to ABA content, we found that *MdSIZ1* RNAi plants were hypersensitive to ABA-induced stomatal closure compared to GL-3 (Fig. 9(b,c).

3.7 MdSIZ1 regulates the expression of drought-responsive genes and miRNAs under drought

Previous studies report that drought stress tolerance is mediated by changes in gene expression[40–42]. We therefore conducted qRT-PCR for three drought-responsive genes to determine whether MdSIZ1 influenced their expression.

Dehydration-responsive element binding factor (*MdDREB2A*), aquaporin (*MdPIP1;3*) and 9-cis-epoxycarotenoid dioxygenase 3 (*MdNCED3*) genes were upregulated to a greater extent in *MdSIZ1* RNAi than in GL-3 under drought conditions (Fig. 10). These genes are positive regulators of drought response[6,41,43] and MdSIZ1 appears to negatively regulate their expression.

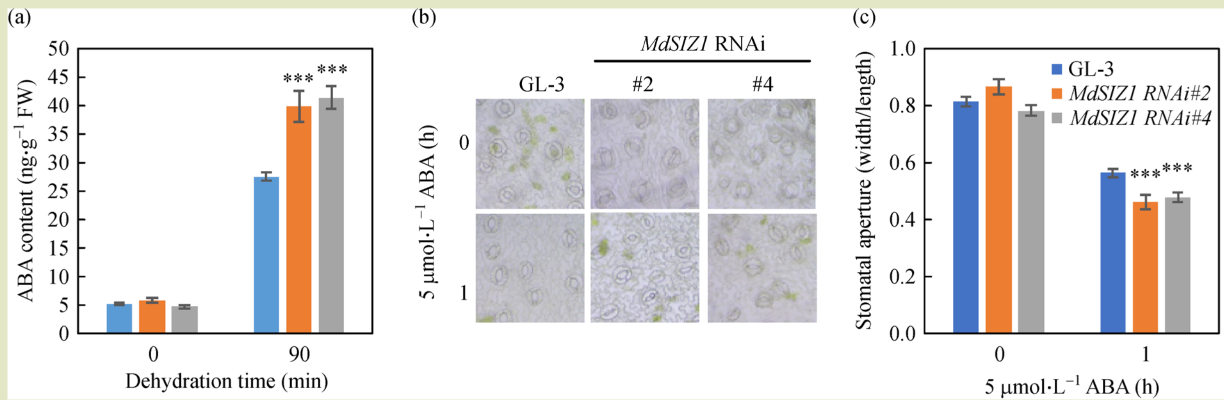

Fig. 9 ABA response and content in *MdSIZ1* RNAi transgenic plants and GL-3. (a) ABA content in GL-3, *MdSIZ1* RNAi plants under control and dehydration conditions. Error bars indicate standard deviation (n = 8). (b) Representative images of stomata of GL-3 and *MdSIZ1* transgenic plants in response to ABA treatment. (c) Stomatal aperture of GL-3 and *MdSIZ1* transgenic plants under ABA treatment. 10 leaves were used, and at least 40 stomatal apertures were measured for each treatment. Student's *t*-test: *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

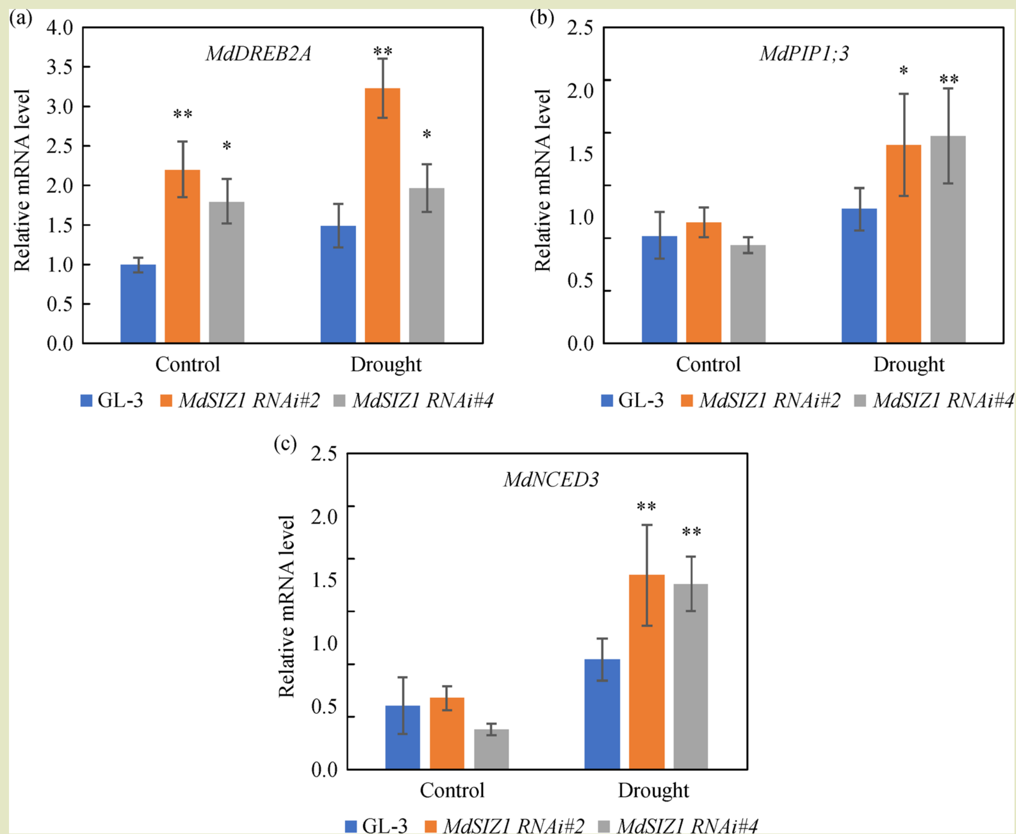

Fig. 10 Transcript levels of drought-responsive genes in GL-3 and *MdSIZ1* RNAi plants under drought stress. (a) *MdDREB2A*. (b) *MdPIP1;3*. (c) *MdNCED3*. Data are mean±SD (n = 3). Student's *t*-test: *, $P<0.05$; **, $P<0.01$.

We also found that *MdSIZ1* RNAi plants had a dwarf phenotype. Given that many microRNA (miRNA) mutants show developmental deficiencies, we speculate that MdSIZ1 might influence miRNA biogenesis. We used stem-loop qRT-PCR to analyze the expression of multiple drought-responsive miRNAs: mdm-miR168, mdm-miR172, mdm-miR393 and mdm-miR408. Mdm-miR168 and mdm-miR172 have a positive relationship in the *Arabidopsis* drought response[44,45] but mdm-miR393 and mdm-miR408 are negative drought regulators in rice and *Arabidopsis*[46,47]. The expression levels of mdm-miR168 and mdm-miR172 increased in *MdSIZ1* RNAi plants but those of mdm-miR393 and mdm-miR408 decreased (Fig. 11), consistent with the drought-tolerant phenotype of *MdSIZ1* RNAi plants. These data indicate that MdSIZ1 may negatively modulate drought stress through the regulation of drought-related genes and miRNAs.

4 DISCUSSION

SUMOylation carries out important plant response functions to abiotic stresses, including extreme temperatures, water availability, salinity, oxidative stress and nutrient imbalance[12,16,48–51]. SIZ1 is an E3 SUMO ligase and determines the specificity of substrate protein SUMOylation[17,19,34]. Although SIZ1 functions in various plant growth and developmental processes, its function in apple is still not clearly understood. We therefore characterized an apple SUMO E3 ligase, MdSIZ1, that has a negative relationship in apple drought tolerance by altering the activity of CAT and POD and modulating the expression of drought-responsive genes and miRNAs.

In *Arabidopsis*, two types of SUMO E3 ligase have been identified, SIZ1 and MMS21 (also designated HPY2). They participate in both abiotic and biotic stress responses and also in root development, DNA damage response and meiosis[21,52]. The reported drought tolerance of *siz1* differs among studies. Catala showed that *siz1* was sensitive to drought stress and mediated anthocyanin synthesis and jasmonate response genes involved in the drought response[27]. However, Miura found that *siz1* reduced stomatal aperture and enhanced drought tolerance by controlling salicylic acid signaling[9]. Kim et al. also found that the *siz1* mutant had a drought-tolerant phenotype[28]. Recent studies also found that apple MdSIZ1 was involved in other abiotic stresses including phosphate-deficiency[53], Fe-deficiency[17] and low-temperature stress[54]. Apple MdSIZ1

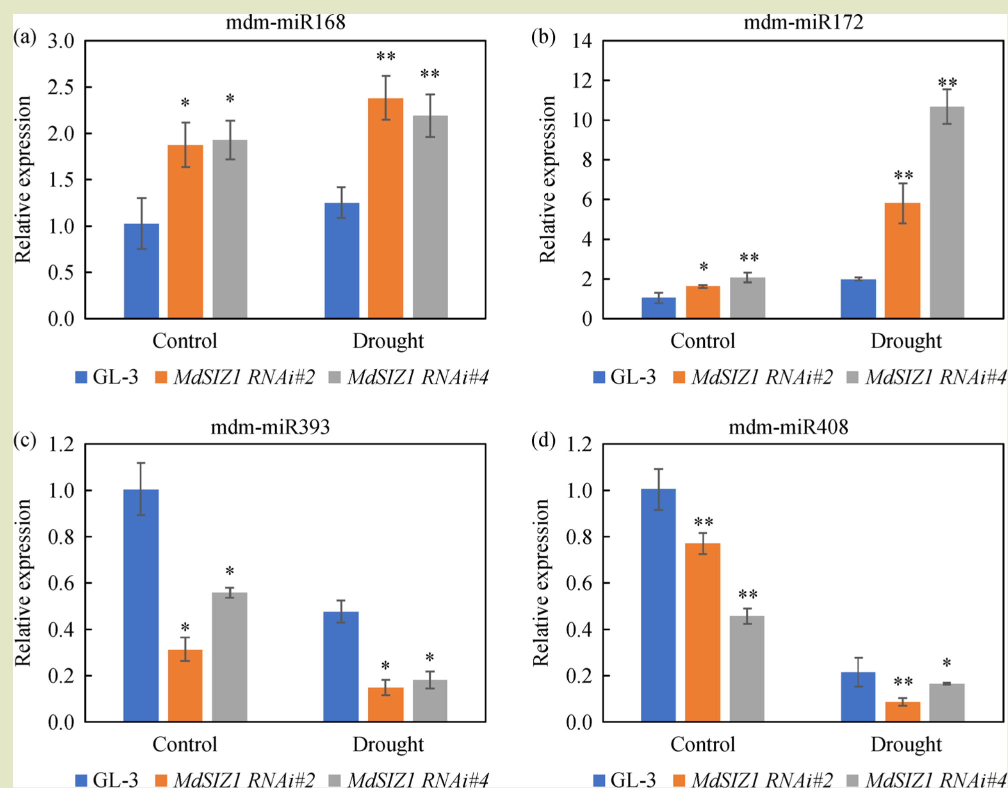

Fig. 11 Transcript levels of drought-responsive microRNAs in GL-3 and *MdSIZ1* RNAi plants under drought stress. (a) mdm-miR168. (b) mdm-miR172. (c) mdm-miR393. (d) mdm-miR408. Data are means±SD (n = 10). Student's t-test: *, $P<0.05$; **, $P<0.01$.

responded to cold stress by regulating anthocyanin biosynthesis, and AtSIZ1 recognized trimethylated histone H3K4 and finally controlled transcriptional suppression under cold stress conditions[55]. These results suggest different functions of SIZ1 in apple and *Arabidopsis* in response to cold stress. However, due to the lack of research, how apple MdSIZ1 responds to drought stress and whether SIZ1 has similar functions in apple and *Arabidopsis* during drought stress condition remain unclear.

Due to the complexity and heterozygosity of the apple genome, we identified four apple *MdSIZ1* genes compared with the single ortholog in the *Arabidopsis* genome (Fig. 1). However, the two *MdSIZ1*s located on chromosomes 1 and 7 were minimally expressed in different tissues and under low-temperature, drought or high-temperature stress (Fig. 2). The *MdSIZ1*s located on chromosomes 3 and 11 had expression patterns similar to those reported in a previous study[27,35]. We therefore speculate that only the *MdSIZ1*s on chromosomes 3 and 11 functioned as important SUMO E3 ligases in apple. We found high similarity between MdSIZ1 and AtSIZ1 in the conserved domains SAP, PHD and SP-RING finger domains. The full length of MdSIZ1 and AtSIZ1 protein sequences shared were compared in the protein alignment of MdSIZ1 with AtSIZ1 and it was found that the similarity of the total length sequences had up to 66.9% similarity. This suggests some conserved functions of SIZ1 in apple and *Arabidopsis*, but some unique functions also occurred in the apple genome. Our results are consistent with the previously reported induction of *SIZ1* by heat, cold and drought stress[9,27,35], and our *MdSIZ1* RNAi transgenic plants exhibited the same dwarf phenotype as the *siz1* mutant[9,28] (Fig. 4). Although apple *MdSIZ1* was upregulated upon drought stress, we found that *MdSIZ1* RNAi transgenic plants were drought tolerant (Fig. 2, Figs. 4–9). In consideration of various post-translation modifications of SIZ1, we speculate that the fine-tuning regulation functions of SIZ1 in response to drought stress and the detailed molecular mechanism still require further exploration.

We found that *MdSIZ1* RNAi transgenic plants were more tolerant to drought stress conditions than GL-3 plants (Fig. 4). Given that LRWC usually serves as an early indicator of plant response to drought stress[38,56], we measured the LRWC of *MdSIZ1* RNAi transgenic plants. LRWC remained higher in transgenic plants than in GL-3 under drought stress, as did leaf water potential (Fig. 5). We therefore propose that *MdSIZ1* RNAi transgenic plants maintain a greater capacity for water retention under drought stress[4,57].

Leaf ion leakage and MDA content are two important indicators of cell membrane integrity and oxidative damage under stress conditions. Here, leaf ion leakage and MDA content were significantly lower in *MdSIZ1* RNAi transgenic plants than in GL-3, indicating that cell damage was alleviated by *MdSIZ1* knockdown (Fig. 6). Previous studies found that drought-stress-tolerant apple maintained a higher photosynthetic capacity[54,58]. Likewise, *MdSIZ1* RNAi transgenic plants maintained higher net photosynthetic rates and higher water use efficiency than GL-3 under drought stress conditions (Fig. 7).

ROS accumulate in response to abiotic stress and cause oxidative damage to cellular components[59,60]. Specifically, ROS-mediated peroxidation of membrane lipids impairs the normal functions of plant organs. Plants with increased ability to scavenge ROS are therefore better able to maintain normal growth processes under stress conditions[56,61]. The *MdSIZ1* RNAi transgenic plants accumulated less H_2O_2 and O_2^- after drought stress treatment and showed enhanced CAT and POD activity. We speculate that the enhanced antioxidant system of *MdSIZ1* RNAi transgenic plants caused greater ROS scavenging in the leaves, thereby maintaining membrane integrity to avoid water loss under drought stress. The above physiological indexes all pointed to *MdSIZ1* RNAi transgenic plants having a greater capacity to maintain leaf water potential and membrane integrity. In addition, accumulated ABA content, enhanced antioxidant activity and more sensitive stomatal response of *MdSIZ1* RNAi transgenic plants conferred greater drought tolerance.

In addition, the expression of drought-responsive genes and miRNAs changed in both GL-3 and *MdSIZ1* RNAi plants under drought stress. The positive regulators of drought stress, *MdDREB2A*, *MdPIP1;3* and *MdNCED3*[6,41,43], were induced to a greater extent in *MdSIZ1* RNAi transgenic plants than in GL-3 (Fig. 9). These genes respond to drought stress by participating in the regulation of stomatal movement, ABA biogenesis, water transport, gene expression and cellular metabolism[43,54,62,63]. We speculate that their upregulation may have contributed to the increased drought tolerance of *MdSIZ1* RNAi plants. In addition, previous studies have found that the dwarf phenotype may be related to plant miRNA level[6,64]. Our stem-loop qRT-PCR analysis demonstrates that the drought positive regulators mdm-miR168 and mdm-miR172 were upregulated in transgenic lines under normal and drought conditions[44,45]. In contrast, the drought negative regulators mdm-miR393 and mdm-miR408 were downregulated in transgenic plants[46]. Changes in gene and miRNA expression may therefore contribute to the drought tolerance of *MdSIZ1* RNAi plants.

5 CONCLUSIONS

In conclusion, we have functionally characterized *MdSIZ1* by reducing its expression level in apple. The explanations for *MdSIZ1* RNAi transgenic apple trees exhibiting enhanced drought tolerance are as follows. Firstly, the accumulated ABA content and enhanced stomatal sensitivity to ABA led to the enhanced leaf water holding capacity of *MdSIZ1* RNAi transgenic apple trees. Secondly, the lower leaf ion leakage, lower ROS, MDA content and greater photosynthetic capacity under drought stress conditions may have been due to the increased antioxidant capacity of *MdSIZ1* RNAi transgenic apple trees. Finally, the upregulation of genes and miRNAs known to positively influence plant drought response may have increased growth and reduced oxidative damage in *MdSIZ1* RNAi plants under drought. Overall, our findings indicate that MdSIZ1 has a negative effect on apple drought tolerance and may represent a candidate gene for drought improvement through molecular breeding.

Acknowledgements

We thank Dr. Zhihong Zhang of Shenyang Agricultural University for providing tissue-cultured GL-3 plants. This work was funded by the National Natural Science Foundation of China (31872080) and the Start-up Funds of Northwest A&F university to Xuewei Li (2452020216).

Compliance with ethics guidelines

Baohua Chu, Jia Sun, Huan Dang, Ziqing Ma, Shuang Zhao, Qingmei Guan, and Xuewei Li declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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