Plant Science 301 (2020) 110654

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Overexpression of *MdMIPS1* enhances salt tolerance by improving osmosis, ion balance, and antioxidant activity in transgenic apple

Lingyu Hu [a,b,1], Kun Zhou [a,1], Yuan Liu [a], Shulin Yang [a], Jingyun Zhang [a], Xiaoqing Gong [a], Fengwang Ma [a,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas/Shaanxi Key Laboratory of Apple, College of Horticulture, Northwest A&F University, Yangling, Shaanxi, 712100, China

[b] Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Key Laboratory of South Subtropical Fruit, Biology and Genetic Resource Utilization, Ministry of Agriculture, Guangdong Key Laboratory of Tropical and Subtropical Fruit Tree Research, Guangzhou, 510640, China

ARTICLE INFO

Keywords:
Apple
MdMIPS1
myo-inositol
Salt tolerance

ABSTRACT

Myo-inositol and its derivatives play vital roles in plant stress tolerance. *Myo*-inositol-1-phosphate synthase (MIPS) is the rate-limiting enzyme of *myo*-inositol biosynthesis. However, the role of apple MIPS-mediated *myo*-inositol biosynthesis in stress tolerance remains elusive. In this study, we found that ectopic expression of *MdMIPS1* from apple increased *myo*-inositol content and enhanced tolerance to salt and osmotic stresses in transgenic *Arabidopsis* lines. In transgenic apple lines over-expressing *MdMIPS1*, the increased *myo*-inositol levels could promote accumulation of other osmoprotectants such as glucose, sucrose, galactose, and fructose, to alleviate salinity-induced osmotic stress. Also, it was shown that overexpression of *MdMIPS1* enhanced salinity tolerance by improving the antioxidant system to scavenge ROS, as well as Na^+ and K^+ homeostasis. Taken together, our results revealed a protective role of *MdMIPS1*-mediated *myo*-inositol biosynthesis in salt tolerance by improving osmotic balance, antioxidant defense system, and ion homeostasis in apple.

1. Introduction

Apple (*Malus domestica)* is one of the most popular fruits worldwide and an indispensable part of a nutrition human diet. Currently, China dominates global apple production and is the greatest exporter, as it has the largest areas of apple cultivation. In China, the Loess Plateau region is the largest and most ideal area for apple production because of its abundant light and wide diurnal temperature variations. However, salinity is increasingly severe and seriously limits apple production in this area.

Salinity is mainly caused by excessive accumulation of Na^+ and Cl^- in the soil, which results in osmotic stress and ion toxicity to plants, in turn, impair their ability to absorb water and nutrients [1]. Under osmotic stress, the water potential of the root surface is reduced, thereby decreasing water uptake and leading to an inhibition of cell division and expansion [2]. Also, Na^+ over-accumulation impairs the absorption of essential element K^+ into plant cells and causes detrimental effects by interfering with K^+-dependent metabolic processes [3]. Plant salinity injuries mainly include impaired photosynthesis, decreased chlorophyll content, reduced stomatal conductance, and biomass reduction [4]. Both osmotic stress and ion toxicity disturb aerobic metabolism and lead to an excessive accumulation of reactive oxygen species (ROS), such as H_2O_2, O_2^-, and O'H [5]. ROS over-accumulation in plant cells can cause oxidation of proteins, lipids, and nucleic acids, leading to cellular structure damage and metabolic dysfunction [6,7].

To cope with salt stress, plants would adjust their own physiological and biochemical processes, such as re-establishment of osmotic and ion equilibrium, alleviation of salt-induced damage, and performance of detoxification reactions [8]. Under salt stress, accumulating osmoprotectants is crucial for reducing plant cell osmotic potential and stabilizing cellular and protein structures [9]. Osmoprotectants mainly fall within the following categories: N-containing substances, such as proline and betaine; cyclic polyols, *myo*-inositol and pinitol; straight-chain polyols, mannitol and sorbitol; and soluble sugars, glucose, sucrose, fructose, and raffinose [10]. Maintenance of cellular ion homeostasis is another important strategy by which plants achieve salt tolerance. An appropriate Na: K ratio can be maintained by reducing Na^+ content and increasing K^+ content in the cytoplasm, thereby alleviating ion toxicity

* Corresponding author.
E-mail addresses: fwm64@sina.com, fwm64@nwsuaf.edu.cn (F. Ma).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plantsci.2020.110654
Received 27 May 2020; Received in revised form 15 August 2020; Accepted 30 August 2020
Available online 1 September 2020

and nutrient deficiency [7,11]. Enzymatic and nonenzymatic systems are activated to scavenge ROS under salt stress, which is key to the reduction of cytotoxicity and oxidative damage. Enzymatic scavengers include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and enzymes involved in the ascorbate-glutathione cycle. Nonenzymatic scavengers include ascorbic acid, glutathione, carotenoids, phenolic compounds, and flavonoids [11].

Myo-inositol (MI) is widely existed in eukaryotes and serves as a precursor for various inositol-containing compounds. In higher plants, MI and its derivatives play broad protective roles in abiotic stress responses, pathogen resistance, hormonal regulation, and programmed cell death [12–15]. As for stress tolerance, MI and its derivatives play dual roles as both key metabolites and signaling molecules [16]. During the biosynthesis of MI, the rate-limiting enzyme *myo*-inositol-1-phosohate synthase (MIPS) mediates the conversion of D-glucose-6-phosphate (G-6-P) to D-*myo*-inositol-1-phosphate (Ins1P). Then, the Ins1P is dephosphorylated to MI by inositol monophosphatase (IMP) [17]. *MIPS* has been widely characterized in plants such as *Arabidopsis*, potato, sweet potato, kiwifruit, and poplar [18–22]. Also, overexpression of *MIPS* has shown that increased MI level confers tolerance to multiple abiotic stresses, including salinity, drought, heavy metals, and chilling, in transgenic plants [15,21–23]. However, the role of apple MIPS-mediated MI biosynthesis under stressed conditions has received little attention.

In a previous study, we found that exogenous application of 50 μM MI could alleviate salt-induced damage to *Malus hupehensis* by improving MI metabolism [24]. In this study, we investigated the role of MdMIPS1-mediated MI biosynthesis in salt tolerance using transgenic *Arabidopsis* and apple lines that overexpressed *MdMIPS1,* a *MIPS* gene from *M. domestica* [25]. MI level and tolerance to salt and osmotic stresses were increased in transgenic *Arabidopsis* lines. Moreover, it was showed that the increased MI levels could improve salt tolerance in transgenic apple lines by improving osmotic balance, antioxidant systems, and ion homeostasis.

2. Materials and methods

2.1. Genetic transformation of Arabidopsis and apple

MdMIPS1 (MDP0000698835) was cloned from mature leaves of 'Royal Gala' and introduced into pCambia2300 vector to create an overexpressing construct as described previously [25]. The sequencing-confirmed plasmid was transformed into *Agrobacterium tumefaciens* strains EHA105 and GV3101. Wild type (WT) *Arabidopsis thaliana* 'Columbia-0' (Col-0) plants were grown in nutrition pots (8 × 8 × 7.5 cm) that contained a mixture of soil and perlite (1:1, v:v) in a phytotron (23 ℃, 16 h light). The *Agrobacterium*-mediated floral-dip method was used for *Arabidopsis* genetic transformation [26]. The collection and screening of T3 homozygous seeds were carried out as previously described [27]. Seeds from WT and two T3 homozygous lines were used in subsequent experiments. The primers used are list in Supplemental Table S1.

GL-3, a line with a high regeneration capacity isolated from 'Royal Gala', was used for apple genetic transformation [28]. *MdMIPS1* overexpression (OE) and silencing apple lines were generated using *Agrobacterium*-mediated transformation in previous [25].

2.2. Subcellular localization of MdMIPS1 in Nicotiana benthamiana leaves

The coding region of MdMIPS1 without stop codon was cloned into the pGWB405-GFP vector (35S: GFP). The fusion construct was transformed into *Agrobacterium tumefaciens* strains GV3101 (pSoup-p19) and infiltrated into 5-week-old leaves of tobacco (*Nicotiana tabacum*). The infiltrated tobacco plants were cultured, and the fluorescence was detected using a confocal microscope (Nikon, Tokyo, Japan) as described previously [29]. The primers used are listed in Supplemental Table S1.

2.3. Plant materials and treatments

Seeds of transgenic and WT *Arabidopsis* were surface-sterilized and sown in Murashige and Skoog (MS) agar medium. After placement in darkness at 4 ℃ for 3 days, the seeds were transferred to a tissue culture room (23 ℃, 14 h light) for 7 days. Plants of uniform size were transferred to medium for treatment. For NaCl treatment, plants were divided into two groups: 1) Control group, 2.22 g L^{-1} MI-depleted MS medium +8 g L^{-1} sucrose +8 g L^{-1} agar; 2) NaCl treatment group, 2.22 g L^{-1} MI-depleted MS medium +8 g L^{-1} sucrose +8 g L^{-1} agar +150 mM NaCl. For mannitol treatment, plants were divided into two groups: 1) Control group, 2.22 g L^{-1} MI-depleted MS medium +8 g L^{-1} sucrose +8 g L^{-1} agar; 2) Mannitol treatment group, 2.22 g L^{-1} MI-depleted MS medium +8 g L^{-1} sucrose +8 g L^{-1} agar +250 mM mannitol. At the end of the treatment, the root length, fresh weight, and bolting rate were measured.

After rooting on MS medium, GL-3 and transgenic apple plantlets were transferred to nutrition pots (8 × 8 cm) that contained a mixture of soil and perlite (1:1, v:v). After 1 month of adaptation in a growth chamber (25 ℃, 14 h light), plants of uniform size were transferred to a hydroponics system as described by Hu et al. [24]. For NaCl treatment, plants were divided into two groups: 1) Control group, ½-strength Hoagland's nutrient solution; 2) Salt stress group, ½-strength Hoagland's nutrient solution supplemented with 100 mM NaCl. The stress period spanned 15 days.

2.4. Physiological measurements

On day 15 after initiation of salt treatment, relative electrolyte leakage was measured with a conductivity meter as described by Thalhammer et al. [30]. Malondialdehyde (MDA) content, H_2O_2 content, SOD activity, POD activity, and anti-O_2^- activity were quantified using detection kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Accumulation of H_2O_2 and O_2^- was also detected by histochemical staining with DAB (3, 3'-diaminobenzidine) and NBT (nitro blue tetrazolium), respectively [31].

During salt treatment, the net photosynthetic rate (Pn) was measured using a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, MA, USA) between 9:00 and 11:00 am. Measurements were performed at 200 μmol photons m^{-2} s^{-1} and a constant airflow rate of 300 μmol s^{-1}. On day 15, chlorophyll content, plant height, total fresh weight, total dry weight, and root architecture were measured [24,32]. Na^+ and K^+ concentrations were quantified by flame spectrometry as described by Liang et al. [33].

Soluble sugars and sugar alcohols were extracted, derivatized, and analyzed as previously described [34]. In brief, the samples were extracted with 75 % methanol and fractionated with chloroform. Then, the extract solutions were vacuum-dried and derivatized with methoxamine hydrochloride and N-methyl-*N*-trimethylsilyl-trifluoroacetamide. The metabolites were analyzed with a GC/MS-ISQ & TRACE ISQ (Thermo Fisher Scientific, United States).

2.5. RNA extraction and RT-qPCR analysis

Total RNA was extracted using a Plant RNA Isolation Kit from Wolact (Wolact, Hong Kong, China), the first-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit with the gDNA Eraser (Takara, Tokyo, Japan). The RT-qPCR analysis was carried out as previously described by Zhou et al. [35]. The primers used are listed in Supplemental Table S1.

2.6. Statistical analysis

Data were presented as means ± SD (standard deviation) and analyzed via one-way ANOVA with SPSS software (version 17.0).

3. Results

3.1. Ectopic expression of MdMIPS1 enhanced salt and osmotic tolerance in Arabidopsis thaliana

To investigate a potential role for MdMIPS1 in salt tolerance, we first generated transgenic *Arabidopsis* lines over-expressing *MdMIPS1*. Two transgenic lines with higher *MdMIPS1* expression and MI levels relative to WT (OE-15 and OE-36) were selected for subsequent experiments (Fig. 1A–C). Under normal growth conditions (control group), there were no significant differences in growth between WT and transgenic *Arabidopsis* lines. After NaCl treatment, the root lengths and fresh weights of transgenic lines were significantly higher than those of WT (Fig. 1D–F). In addition, root lengths and bolting rates of the transgenic lines were significantly higher than those of WT after the treatment of mannitol (Fig. 1G–I). These results suggest that overexpression of *MdMIPS1* enhanced MI biosynthesis and improved salt and osmotic tolerance in transgenic *Arabidopsis* lines.

3.2. Overexpression of MdMIPS1 enhanced salt tolerance in transgenic apple lines

To further investigate the role of MdMIPS1-mediated MI biosynthesis in salt-stressed apple, the transgenic apple lines that overexpressed *MdMIPS1* were used here [25]. Under salinity conditions, *MdMIPS1* was up-regulated in both leaves and roots of transgenic and non-transgenic apple lines (Fig. 2A and B). Previous analysis showed that a higher *MdMIPS1* expression level was identified in leaves and fruits of 'Royal Gala' [25]. Here, RT-qPCR further revealed a higher expression level of *MdMIPS1* in the leaves than roots in GL-3 (Fig. 2C). Thus, apple leaves were chosen for analysis in this study. Moreover, MI level was significantly increased in GL-3 and transgenic apple leaves after salt treatment (Fig. 2D). In addition, subcellular localization showed that both MdMIPS1-GFP and GFP were observed on the whole cell, suggesting an extensive role of MdMIPS1 in apple cells (Fig. 2E).

Under normal growth conditions (control group), no obvious differences in growth were observed between transgenic apple lines and GL-3. Under salt stress, GL-3 displayed obvious browning and necrosis on the leaf margins, but such damage was much less pronounced in transgenic apple lines (Fig. 3A). Meanwhile, the values of relative electrolytic leakage and MDA content were significantly lower in transgenic apple lines than in GL-3 (Fig. 3B and C). Under normal conditions, there were no obvious differences in chlorophyll content and Pn between transgenic apple lines and GL-3 (Fig. 3D and E). Salt stress led to an obvious reduction in chlorophyll *b* content, but the degree of reduction was much smaller in transgenic apple lines (Fig. 3D). Salt stress also lowered Pn. However, on Day 8 and Day 15 of salt treatment, Pn was significantly higher in transgenic apple lines than in GL-3 (Fig. 3E). Ultimately, the values of plant height, total fresh weight, and total dry weight (Fig. 4A), as well as root length, volume and surface area (Fig. 4B) were significantly higher in transgenic apple lines than in GL-3 under salt stress, although these parameters did not differ under control conditions. Therefore, MdMIPS1-mediated MI biosynthesis appeared to confer salt tolerance in apple.

3.3. Overexpression of MdMIPS1 improved accumulation of soluble sugars in transgenic apple lines under salt stress

Salinity causes osmotic stress and disrupts plant water uptake [1]. The accumulation of osmotic adjustment substances such as soluble sugars is essential for reducing cell osmotic potential and stabilizing cellular structure [9]. In addition, MIPS-mediated MI biosynthesis directly affects sugar metabolism in plants. Thus, we examined the level of soluble sugars in transgenic apple lines and GL-3 under both control and salt stress conditions. Compared with the control group, salt stress induced the accumulation of soluble sugars such as glucose, sucrose, galactose, and fructose (Fig. 5). Moreover, the levels of these soluble

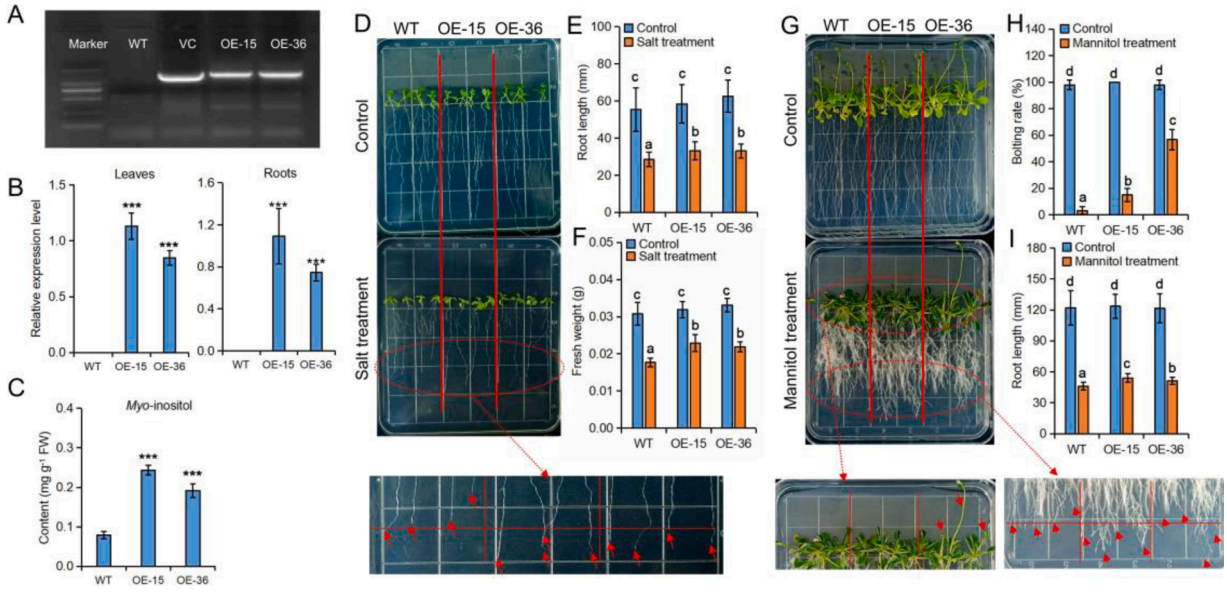

Fig. 1. (A) Genomic-PCR identification of transgenic *Arabidopsis* plants, Marker, DL2000, 'VC' represents vector control; (B) Relative expression levels of *MdMIPS1* in transgenic *Arabidopsis* leaves and roots. *Arabidopsis Actin2* served as the reference gene for normalization. The highest expression level in the transgenic lines (OE-15) was defined as 1.0; (C) Levels of *myo*-inositol in transgenic *Arabidopsis* leaves; (D) Performance, (E) root length, and (F) fresh weight of *MdMIPS1*-overexpressing *Arabidopsis* lines and the WT after salt treatment for 10 days; (G) Performance, (H) bolting rate, and (I) root length of *MdMIPS1*-overexpressing *Arabidopsis* lines and the WT after mannitol treatment for 20 days. Fresh weight indicates the weight of four pooled plants. Data are means ± SD ($n = 3$ for B, C, three biological replicates, each biological replicate contains 12 plants; $n = 5$ for E, F, H, I, five biological replicates, each biological replicate contains 16 plants). In comparison with WT, ***, $P < 0.001$. Values not followed by the same letter are significantly different ($P < 0.05$).

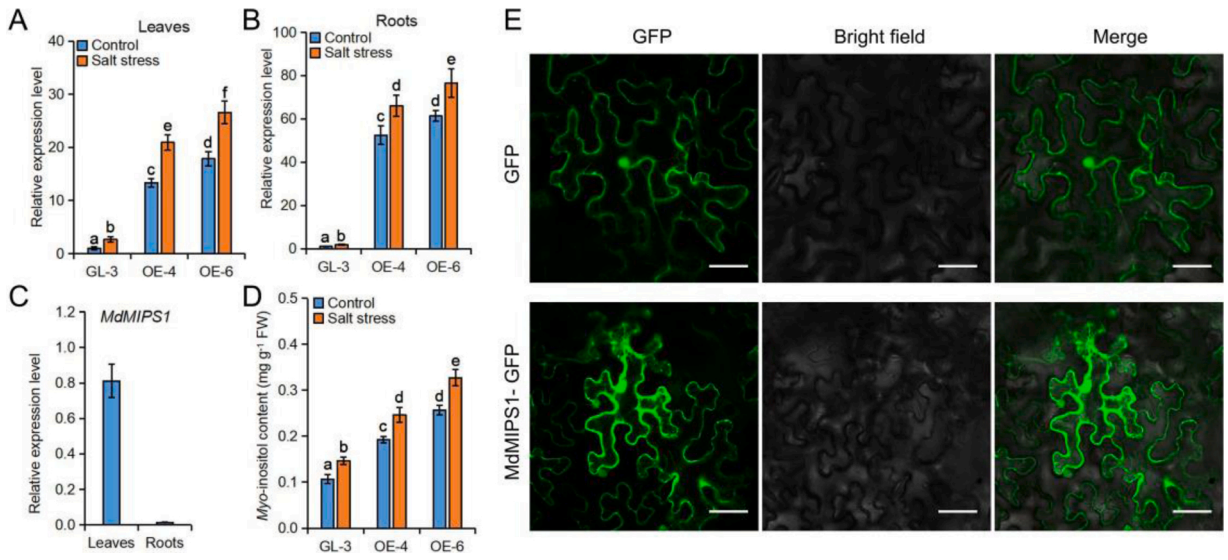

Fig. 2. Identification of MdMIPS1. Relative expression levels of *MdMIPS1* in GL-3 and transgenic apple leaves (A) and roots (B) under control and salt stress conditions; (C) Expression levels of *MdMIPS1* in the leaves and roots of GL-3; (D) Levels of *myo*-inositol in GL-3 and transgenic apple leaves under control and salt stress conditions; (E) Subcellular localization of MdMIPS1 in *Nicotiana benthamiana* leaves. Bars =50 μm. Data are means ± SD ($n = 3$, three biological replicates, each biological replicate contains 8 plants). Values not followed by the same letter are significantly different ($P < 0.05$).

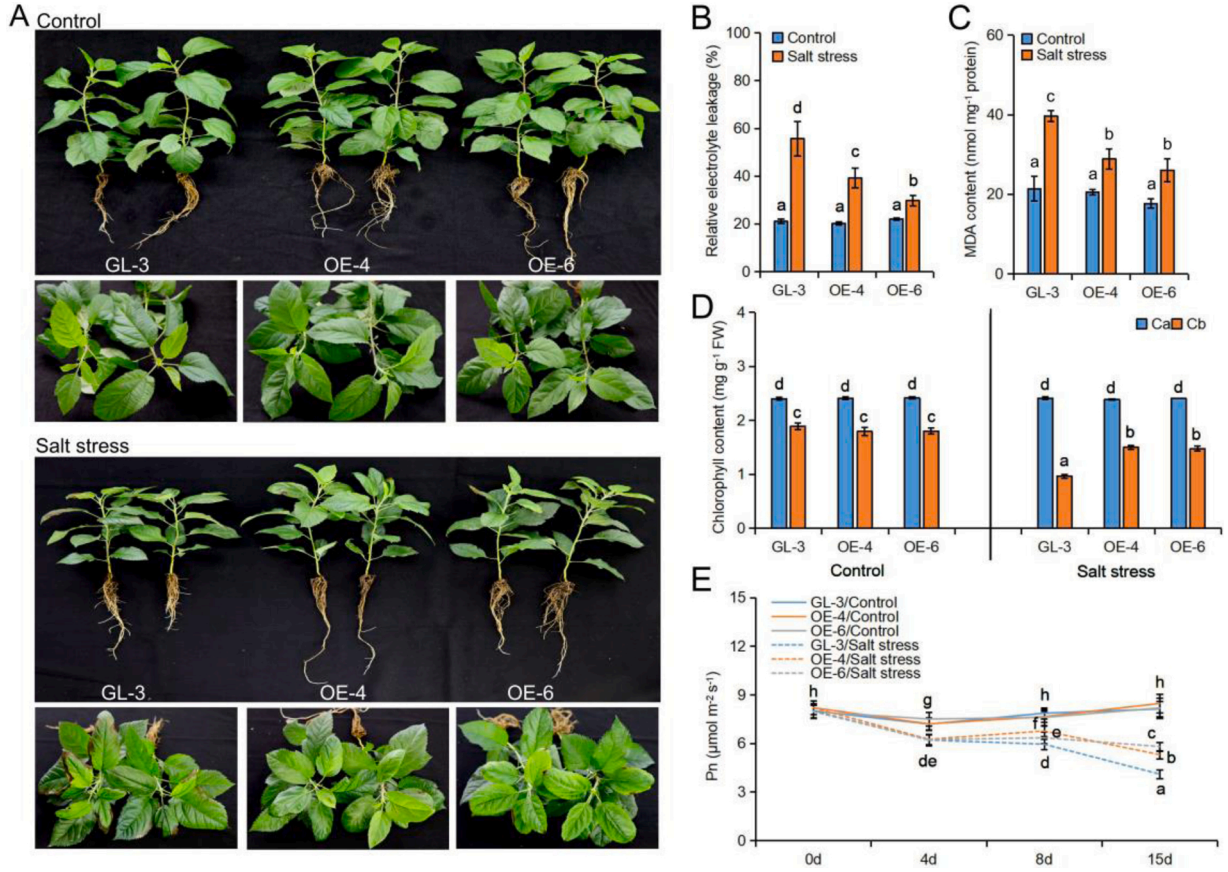

Fig. 3. Plant salt tolerance parameters of *MdMIPS1*-overexpressing apple lines and GL-3. (A) Performance, (B) relative electrolyte leakage, (C) malonaldehyde (MDA) content, (D) chlorophyll content, and (E) net photosynthetic rate in leaves of *MdMIPS1*-overexpressing apple lines and GL-3. Data are means ± SD ($n = 4$ for B, C, and D, four biological replicates, each biological replicate contains 6 plants; $n \geq 8$ for E, at least 8 plants). Values not followed by the same letter are significantly different ($P < 0.05$).

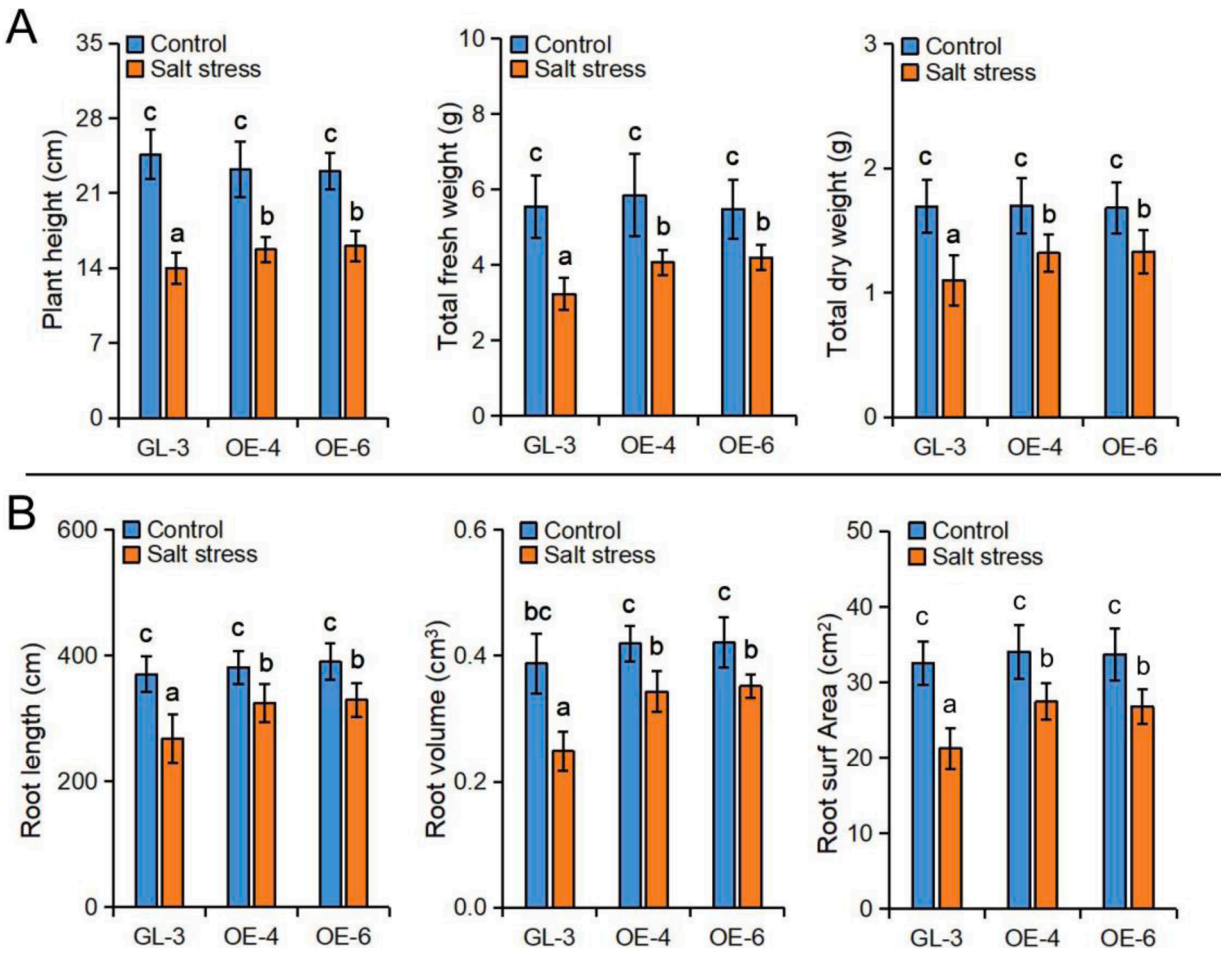

Fig. 4. Growth parameters of *MdMIPS1*-overexpressing apple lines and GL-3 under salt stress. (A) Plant height, total fresh weight, and total dry weight; (B) root length, root volume, and root surface area. Data are means ± SD ($n \geq 8$, at least 8 plants). Values not followed by the same letter are significantly different ($P < 0.05$).

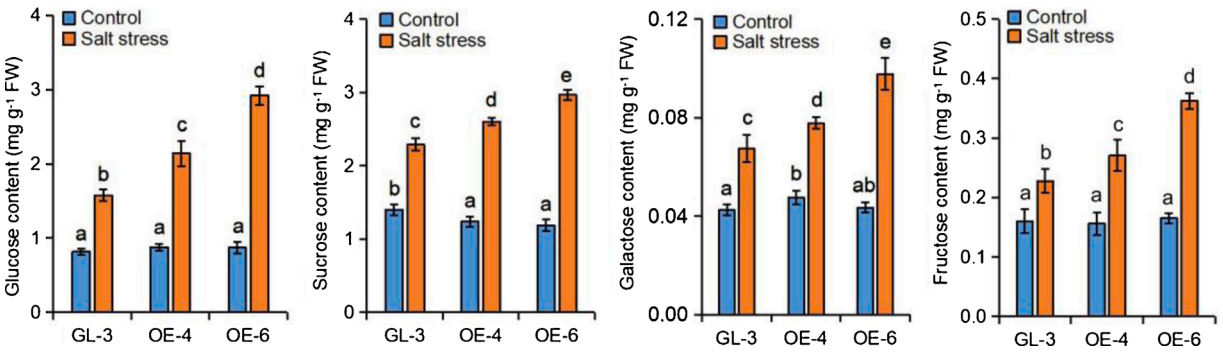

Fig. 5. Accumulation of soluble sugars in leaves of *MdMIPS1*-overexpressing apple lines and GL-3 under salt stress. FW, fresh weight. Data are means ± SD ($n = 3$, three biological replicates, each biological replicate contains 8 plants). Values not followed by the same letter are significantly different ($P < 0.05$).

sugars in transgenic apple lines were significantly higher than in GL-3 under salt stress (Fig. 5). Thus, apple MdMIPS1-mediated MI biosynthesis appeared to alleviate osmotic stress by increasing the accumulation of soluble sugars under salt stress.

3.4. Overexpression of MdMIPS1 alleviated oxidative stress in transgenic apple lines under salt stress

Salt stress causes excessive accumulation of ROS, leading to oxidative stress in plants. Plant enzymatic scavengers such as SOD, POD, and CAT may be activated to scavenge ROS in response to salinity [11]. Staining with NBT and DAB revealed an obvious reduction in H_2O_2 and O_2^- accumulation in transgenic apple leaves relative to GL-3 under salt stress, but there were no significant differences under normal conditions (Fig. 6A). We also quantified the H_2O_2 level of leaves and confirmed a significant reduction in H_2O_2 accumulation in transgenic apple lines compared with GL-3 under salt stress (Fig. 6B). In response to the over-accumulation of ROS, the activities of SOD, POD, and anti-O_2^- were enhanced in both transgenic apple lines and GL-3 under salt stress. Under normal conditions, these parameters did not differ between transgenic and non-transgenic apple lines. However, under salt stress, a significant increase in the activities of SOD, POD, and anti-O_2^- was

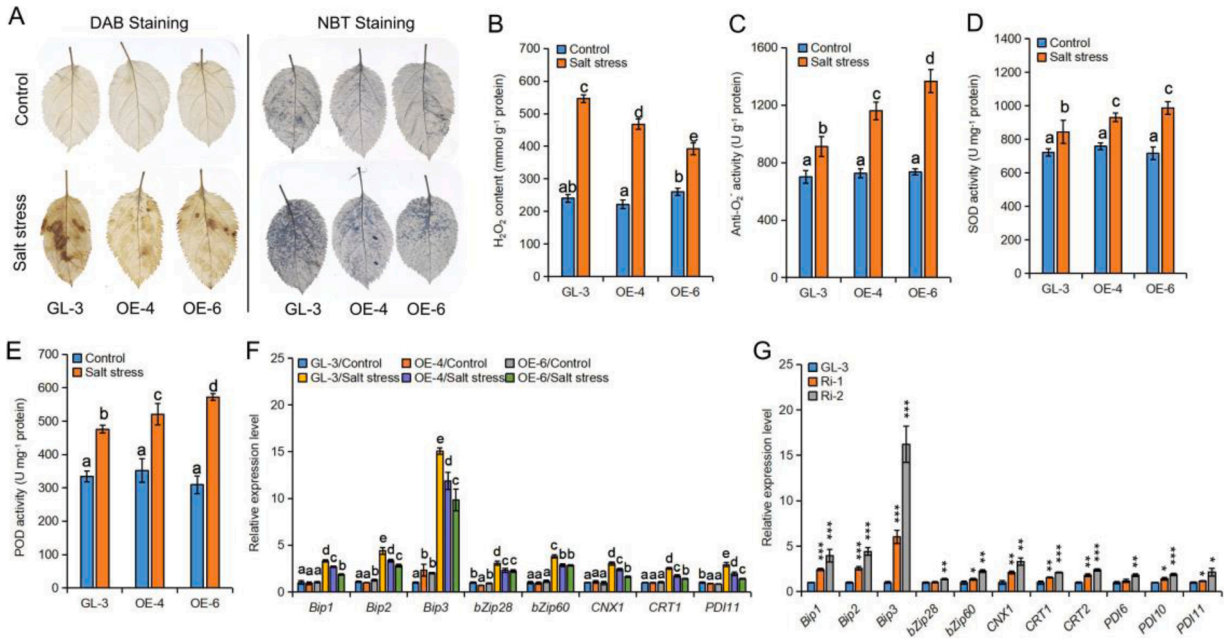

Fig. 6. (A) H_2O_2 and O_2^- staining, (B) H_2O_2 content, (C) anti-O_2^- activity, (D) SOD activity, and (E) POD activity in leaves of *MdMIPS1*-overexpressing apple lines and GL-3 under salt stress; (F) Expression levels of *Bip1*, *Bip2*, *Bip3*, *bZip28*, *bZip60*, *CNX1*, *CRT1* and *PDI11* in the leaves of salt-stress GL-3 and *MdMIPS1*-overexpressing apple lines; (G) Expression levels of *Bip1*, *Bip2*, *Bip3*, *bZip28*, *bZip60*, *CNX1*, *CRT1*, *CRT2*, *PDI6*, *PDI10* and *PDI11* in the leaves of MdMIPS1-RNAi lines and GL-3 under MI-depletion MS conditions. Data are means ± SD ($n = 3$, three biological replicates, each biological replicate contains 8 plants). In comparison with GL-3, ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; and n.s. indicates no significant differences. Values not followed by the same letter are significantly different ($P < 0.05$).

observed in transgenic apple lines compared with GL-3 (Fig. 6C–E).

Salt stress induces unfolded protein response (UPR) in plants, and excessive accumulation of UPR, in turn, causes endoplasmic reticulum (ER) stress [36,37]. Also, ER stress causes oxidative damage as shown by H_2O_2 over-accumulation and oxidation of proteins, lipids [38]. To investigate whether overexpression of *MdMIPS1* could affect the expression level of genes related to ER stress under salt stress, *Bip1* (*Binding Protein 1*), *Bip2*, *Bip3*, *bZip60* (*basic leucine zipper 60*), *bZip28*, *CNX1* (*calnexin 1*), *CRT1* (*calreticulin 1*), *CRT2*, *PDI6* (*protein disulfide isomerase 6*), *PDI10*, and *PDI11* were examined [38,39]. RT-qPCR results showed that these genes were up-regulated in GL-3 and transgenic apple leaves under salt stress. However, the transcript abundance of these detected genes were lower in transgenic apple lines than in GL-3 under salt stress (Fig. 6F). In previous, we found that decreasing MI biosynthesis in apple lines by RNA silencing of *MdMIPS1* led to excessive accumulation of ROS and programmed cell death (PCD) [25]. By contrast, we found these genes related to ER stress were up-regulated in *MdMIPS1* silencing leaves under MI-depletion MS conditions (Fig. 6G). Together, apple MdMIPS1-mediated MI biosynthesis appeared to reduce oxidative and ER stresses by improving the antioxidant system under salt stress.

3.5. Overexpression of MdMIPS1 maintained ion homeostasis in transgenic apple lines under salt stress

Over-accumulation of Na^+ not only results in ion toxicity but also disturbs K^+ absorption and multiple metabolic processes in plants [3]. Under salt stress, plant salt overly sensitive (SOS) pathway is activated, taking charge of Na^+ export from cells and the maintenance of ion homeostasis [40]. Here, we first evaluated the expression of *SOS1*, *SOS2*, *SOS3*, and *NHX1* to investigate whether overexpression of *MdMIPS1* could activate the SOS pathway to protect apple plants from salt stress. As shown in Fig. 7A, these four genes were up-regulated under salt stress, and higher expression levels were found in transgenic apple leaves when compared with GL-3.

We subsequently quantified the Na^+ and K^+ levels in leaves, stems, and roots of GL-3 and transgenic apple lines under both conditions. Under salt stress, K^+ concentration decreased significantly in all tissues, especially roots, of both transgenic apple lines and GL-3. However, there was a higher K^+ level in leaves and roots of transgenic apple than in those of GL-3, although no differences were observed in stems (Fig. 7B). By contrast, Na^+ level and Na: K ratio increased significantly under salt stress conditions (Fig. 7C and D). However, both parameters were significantly lower in all measured tissues of transgenic apple lines than in those of GL-3 (Fig. 7C and D). Thus, apple MdMIPS1-mediated MI biosynthesis appeared to promote ion homeostasis under salt stress.

4. Discussion

Soil salinity is an increasing problem that is mainly attributed to improper fertilization or irrigation and industrial pollution [41]. Salinity affects almost all aspects of plant growth and development, including seed germination and vegetative and reproductive growth, thereby seriously limiting crop yield [42]. MIPS is the rate-limiting enzyme in MI biosynthesis and determines plant MI content [43]. Previous studies showed that overexpression of *MIPS* can increase MI content and enhance salt tolerance in transgenic plants [15,21,22]. However, it is unclear whether apple MIPSs have a similar role. In this study, our results suggested that apple MdMIPS1-mediated MI biosynthesis conferred salt tolerance by improving osmotic balance, ion homeostasis, and the activity of the antioxidant system under salt stress.

Under salt stress, the restoration of osmotic balance is crucial for the enhancement of plant salt tolerance. MI exists extensively in plants and can function as an osmotic adjustment substance to regulate cell osmotic pressure. In this study, increased MI level enhanced the tolerance to osmotic and salt stress in *MdMIPS1*-overexpressing *Arabidopsis* lines (Fig. 1). Also, an enhanced salt tolerance was identified in MdMIPS1-OE apple lines (Fig. 3). Kusuda et al. [44] showed that overexpression of *MIPS* could activate various aspects of basal metabolism, such as glycolysis, inositol metabolism, and the pentose phosphate pathway, thereby enhancing salt tolerance in transgenic rice. Therefore, it seems possible that overexpression of *MdMIPS1* also led to a wide range of

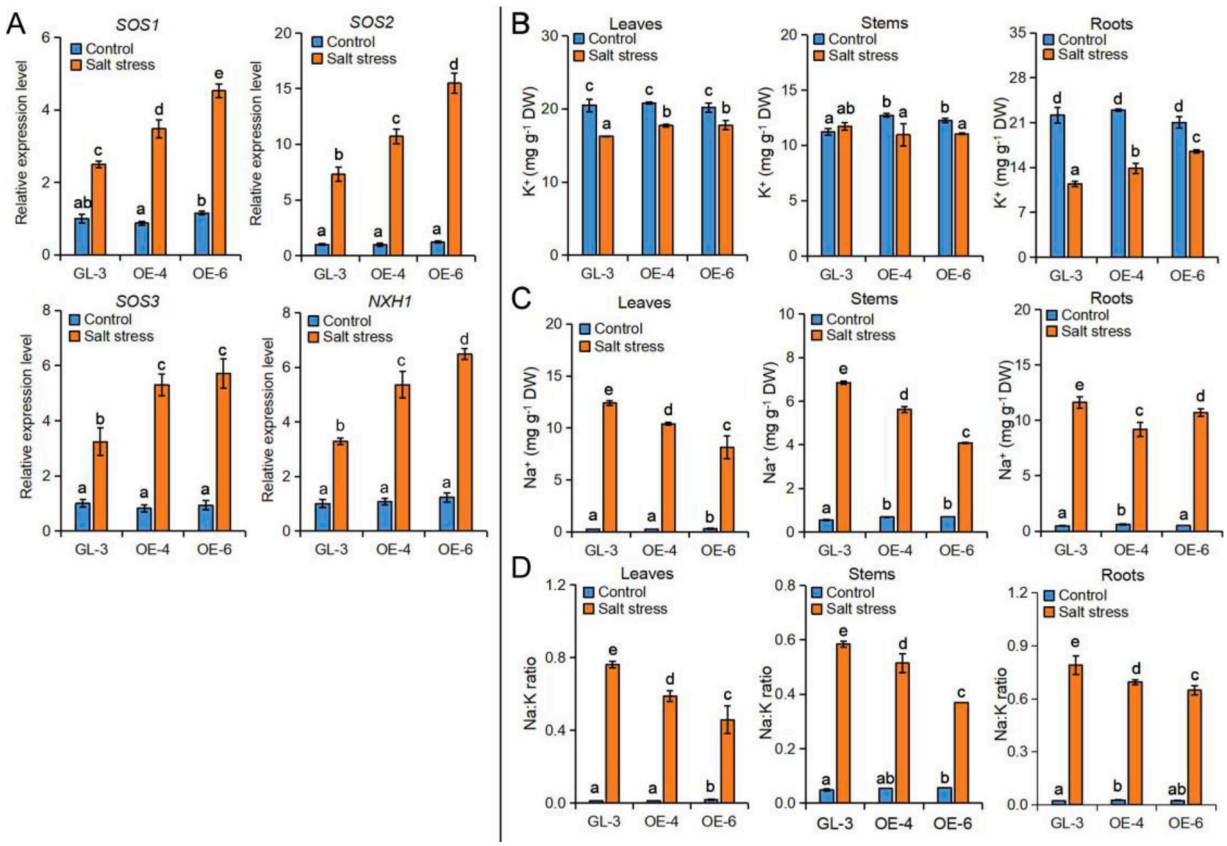

Fig. 7. (A) Expression levels of *SOS1*, *SOS2*, *SOS3*, and *NHX1* in the leaves of *MdMIPS1*-overexpressing apple lines and GL-3 under salt stress; (B) K^+ concentrations, (C) Na^+ concentrations, and (D) Na: K ratio in the leaves, stems, and roots of *MdMIPS1*-overexpressing apple lines and GL-3 under salt stress. DW, dry weight. Data are means ± SD ($n = 3$, three biological replicates, each biological replicate contains 8 plants). Values not followed by the same letter are significantly different ($P < 0.05$).

metabolic changes in transgenic apple lines. Similarly, a higher increase for glucose, sucrose, galactose, and fructose was identified in transgenic apple lines relative to GL-3 under salt stress (Fig. 5). It has been suggested that soluble sugars such as sucrose, glucose, and fructose were accumulated and acted as osmoprotectants to maintain osmotic balance in plants under salt and osmotic stresses [45,46].

Besides, it seems possible that MI-derivatives, such as phosphatidylinositol 5-phosphate (PtdIns5P) and compatible solutes, would be increased in *MdMIPS1*-overexpressing *Arabidopsis* and apple plants [16]. Increasing levels of PtdIns5P have been detected in response to hyperosmotic, salt, and dehydration stresses, suggesting PtdIns5P can function as a signaling molecule in osmotic stress [16]. It was shown that the binding of PtdIns5P to *Arabidopsis* trithorax-like factor (ATX1, a chromatin modifier) linked lipid signaling under osmotic stress to gene transcription [47]. The compatible solutes such as galactinol, raffinose-family oligosaccharides (RFOs), and pinitol could serve as potent osmoprotectants enhancing plant salt tolerance [16]. Also, the phosphatidylinositol 4-phosphate 5-kinase (PIP5K) mediating PtdIns bisophosphates production, has been considered as a partner of cytosolic neutral invertase, regulating invertase activity. Influencing this interaction resulted in alterations of glucose and fructose levels [48]. Moreover, PIP5Ks seem key players in abiotic stress tolerance. *AtPIP5K1* was induced by salinity [49]. However, this speculation needs to be elucidation in the future. Nevertheless, our results suggest that MdMIPS1-mediated MI biosynthesis could maintain osmotic balance and confer salt tolerance in apple.

Salinity leads to ROS over-accumulation, which in turn destroys cellular structures, membrane lipids, enzymes, and DNA [10]. ROS-scavenging ability is closely related to salt tolerance in plants. SOD converts peroxides to H_2O_2, and POD catalyzes the decomposition of H_2O_2 [1,50]. Apart from enzymatic scavengers, MI and its derivatives are considered as key metabolites that scavenge ROS and stabilize the membrane system [12,51]. It has been shown that increased MI level improves SOD activity and reduces the accumulation of H_2O_2 and MDA in *IbMIPS1*-overexpressing sweet potato plants under salt stress [21]. Also, overexpression of *PeMIPS1* enhanced the activities of SOD and CAT and accounted for the decrease in H_2O_2 and MDA contents in transgenic poplar lines under salt stress [22]. Similarly, we found that the activities of SOD, POD, and anti-O_2^- were enhanced, and ROS and MDA levels were decreased in transgenic apple lines under salt stress (Fig. 6A–E, and 3 C). By contrast, silencing *MdMIPS1* compromised the antioxidant defense system and resulted in ROS over-accumulation and severe PCD occurrence in apple [25]. Also, genes related to ER stress were up-regulated, indicating dysfunction of ER in *MdMIPS1* silencing lines (Fig. 6G). Consistent with salinity-induced ROS level, a lower transcript abundance of ER stress-related genes were found in salt-stressed transgenic apple lines than GL-3 (Fig. 6F). Due to the fact that ER stress could cause oxidative damage, it was unclear whether MI directly functions in the regulation of ER stress [38]. At least, it was indicated that MdMIPS1-mediated MI biosynthesis confers salt tolerance by improving the antioxidant system and alleviating oxidative stress in apple.

In addition, one important strategy for achieving salt tolerance under stress conditions is to control the Na^+ concentration in cells [52]. Plants achieve ion homeostasis by precisely regulating the uptake of toxic and essential ions and compartmentalizing toxic ions into vacuoles [53,54]. The SOS pathway is important for the maintenance of ion homeostasis. SOS1 (a plasma membrane Na^+/H^+ antiporter) is responsible for Na^+ efflux from plant cells. Meanwhile, both SOS2 (a serine/threonine protein kinase) and SOS3 (a calcium-binding protein) are involved in SOS1 regulation [40]. The Na^+/H^+ antiporter NHX functions in Na^+

compartmentalization, which is also regulated by the SOS2-SOS3 complex [7]. Previously, overexpression of *MdSOS2* or *MdNHX1* was shown to enhance salt tolerance in apple [55,56]. Our RT-qPCR results showed that the expression levels of *SOS1*, *SOS2*, *SOS3*, and *NXH1* were up-regulated to a greater extent in transgenic apple lines than in GL-3 under salt stress (Fig. 7A). Consistent with the RT-qPCR results, Na^+ accumulation, and the Na: K ratio were significantly reduced in transgenic apple lines under salt stress (Fig. 7B–D). Therefore, apple MdMIPS1-mediated MI biosynthesis also confers salt tolerance by improving ion homeostasis.

5. Conclusions

In summary, our results showed a protective effect of MdMIPS1-mediated MI biosynthesis on salt tolerance in apple. Ectopic expression of *MdMIPS1* in *Arabidopsis* not only increased MI biosynthesis but also enhanced tolerance to salt and osmotic stresses. In MdMIP1-OE apple lines, the increased MI level appeared to alleviate salinity-induced osmotic stress by improving the accumulation of other osmoprotectants. Moreover, it was revealed that overexpression of *MdMIPS1* enhanced apple salt tolerance by improving the ability of the antioxidant system and the homeostasis of Na^+ and K^+. Taken together, our results highlight the potential for improvement of salt tolerance by genetic engineering of MdMIPS1 in apple.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by National Key Research and Development Program of China (2018YFD1000303) and by the earmarked fund for the China Agriculture Research System (CARS-27). We thank Dr. Zhihong Zhang, Shenyang Agricultural University, for providing tissue-cultured GL-3 plants.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110654.

References

[1] A. Ismail, S. Takeda, P. Nick, Life and death under salt stress: same players, different timing? J. Exp. Bot. 65 (2014) 2963–2979.
[2] M. Singh, A. Singh, S.M. Prasad, R.K. Singh, Regulation of plants metabolism in response to salt stress: an omics approach, Acta Physiol. Plant 39 (2017) 48.
[3] J.L. Zhang, H.Z. Shi, Physiological and molecular mechanism of plant salt tolerance, Photosynth. Res. 115 (2013) 1–22.
[4] A. Rahnama, R.A. James, K. Poustini, R. Munns, Stomatal conductance as a screen for osmotic stress tolerance in durum wheat growing in saline soil, Funct. Plant Biol. 37 (2010) 255–263.
[5] D.M. Almeida, M.M. Oliveira, N.J.M. Saibo, Regulation of Na^+ and K^+ homeostasis in plants: towards improved salt stress tolerance in crop plants, Genet. Mol. Biol. 40 (2017) 326–345.
[6] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930.
[7] Y.Q. Yang, Y. Guo, Unraveling salt stress signaling in plants, J. Integr. Plant Biol. 60 (2018) 796–804.
[8] J.K. Zhu, Plant salt tolerance, Trends Plant Sci. 6 (2001) 66–71.
[9] M.P. Apse, E. Blumwald, Engineering salt tolerance in plants, Curr. Opin. Biotech. 13 (2002) 146–150.
[10] P. Parihar, S. Singh, R. Singh, V.P. Singh, S.M. Prasad, Effect of salinity stress on plants and its tolerance strategies: a review, Environ. Sci. Pollut. R. 22 (2015) 4056–4075.
[11] Y.Q. Yang, Y. Guo, Elucidating the molecular mechanisms mediating plant salt-stress responses, New Phytol. 217 (2018) 523–539.
[12] P.H. Meng, C. Raynaud, G. Tcherkez, S. Blanchet, K. Massoud, S. Domenichini, Y. Henry, L. Soubigou-Taconnat, C. Lelarge-Trouverie, P. Saindrenan, J.P. Renou, C. Bergounioux, Crosstalks between *myo*-inositol metabolism, programmed cell death and basal immunity in *Arabidopsis*, PLoS One 4 (2009) e7364.
[13] S. Chaouch, G. Noctor, *Myo*-inositol abolishes salicylic acid-dependent cell death and pathogen defence responses triggered by peroxisomal hydrogen peroxide, New Phytol. 188 (2010) 711–718.
[14] H. Chen, L. Xiong, *Myo*-inositol-1-phosphate synthase is required for polar auxin transport and organ development, J. Biol. Chem. 285 (2010) 24238–24247.
[15] J.L. Tan, C.Y. Wang, B. Xiang, R.H. Han, Z.F. Guo, Hydrogen peroxide and nitric oxide mediated cold- and dehydration-induced *myo*-inositol phosphate synthase that confers multiple resistances to abiotic stresses, Plant Cell Environ. 36 (2013) 288–299.
[16] R. Valluru, W.V.D. Ende, *Myo*-inositol and beyond-emerging networks under stress, Plant Sci. 181 (2011) 387–400.
[17] F.A. Loewus, P.P.N. Murthy, *Myo*-inositol metabolism in plants, Plant Sci. 150 (2000) 1–19.
[18] M.D. Johnson, The *Arabidopsis thaliana myo*-inositol-1-phosphate synthase (EC 5.5.1.4), Plant Physiol. 105 (1994) 1023.
[19] R. Keller, C.A. Brearley, R.N. Trethewey, B. Müller-Röber, Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-*myo*-inositol-3-phosphate synthase, Plant J. 16 (1998) 403–410.
[20] M. Cui, D. Liang, S. Wu, F.W. Ma, Y.S. Lei, Isolation and developmental expression analysis of L-*myo*-inositol-1-phosphate synthase in four *Actinidia* species, Plant Physiol. Biochem. 73 (2013) 351–358.
[21] H. Zhai, F.B. Wang, Z.Z. Si, J.X. Huo, L. Xing, Y.Y. An, S.Z. He, Q.C. Liu, A *myo*-inositol-1-phosphate synthase gene, *IbMIPS1*, enhances salt and drought tolerance and stem nematode resistance in transgenic sweet potato, Plant Biotech. J. 14 (2015) 592–602.
[22] J. Zhang, N. Yang, Y.Y. Li, S.D. Zhu, S.N. Zhang, Y.D. Sun, H.X. Zhang, L. Wang, H. Y. Su, Overexpression of *PeMIPS1* confers tolerance to salt and copper stresses by scavenging reactive oxygen species in transgenic poplar, Tree Physiol. 38 (2018) 1566–1577.
[23] L. Goswami, S. Sengupta, S. Mukherjee, S. Ray, R. Mukherjee, A.L. Majumder, Targeted expression of L-*myo*-inositol-1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka confers multiple stress tolerance in transgenic crop plants, J. Plant Biochem. Biotech. 23 (2014) 316–330.
[24] L.Y. Hu, K. Zhou, Y.T.S. Li, X.F. Chen, B.B. Liu, C.Y. Li, X.Q. Gong, F.W. Ma, Exogenous *myo*-inositol alleviates salinity-induced stress in *Malus hupehensis* Rehd, Plant Physiol. Biochem. 133 (2018) 116–126.
[25] L.Y. Hu, K. Zhou, G.J. Ren, S.L. Yang, Y. Liu, Z.J. Zhang, Y.T.S. Li, X.Q. Gong, F. W. Ma, *Myo*-inositol mediates reactive oxygen species-induced programmed cell death via salicylic acid- and ethylene-dependent pathways in apple, Hortic. Res. (2020), https://doi.org/10.1038/s41438-020-00357-2.
[26] X. Zhang, R. Henriques, S.S. Lin, Q.W. Niu, N.H. Chua, *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method, Nat. Protoc. 1 (2006) 641–646.
[27] Y. Shao, X.L. Zhang, S. van Nocker, X.Q. Gong, F.W. Ma, Overexpression of a protein kinase gene *MpSnRK2.10* from *Malus prunifolia* confers tolerance to drought stress in transgenic *Arabidopsis thaliana* and apple, Gene 692 (2019) 26–34.
[28] H.Y. Dai, W.R. Li, G. Han, Y. Yang, Y. Ma, H. Li, Z. Zhang, Development of a seedling clone with high regeneration capacity and susceptibility to *Agrobacterium* in apple, Sci. Hortic. 164 (2013) 202–208.
[29] L.Y. Hu, K. Zhou, S.L. Yang, Y. Liu, Y.T.S. Li, Z.J. Zhang, J.Y. Zhang, X.Q. Gong, F. W. Ma, MdINT1 enhances apple salinity tolerance by regulating the antioxidant system, homeostasis of ions, and osmosis, Plant Physiol. Biochem. 154 (2020) 689–698.
[30] A. Thalhammer, D.K. Hincha, E. Zuther, Measuring freezing tolerance: electrolyte leakage and chlorophyll fluorescence assays, in plant cold acclimation: methods and protocols, in: D.K. Hincha, E. Zuther (Eds.), Methods in Molecular Biology 1166, Springer Science+ Business Media, New York, 2014, pp. 15–24.
[31] X.S. Huang, W. Wang, Q. Zhang, J.H. Liu, A basic helix-loop-helix transcription factor, PtrbHLH, of *Poncirus trifoliata* confers cold tolerance and modulates peroxidase-mediated scavenging of hydrogen peroxide, Plant Physiol. 162 (2013) 1178–1194.
[32] H.K. Lichtenthaler, A.R. Wellburn, Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents, Biochem. Soc. Trans. 11 (1983) 591–592.
[33] B.W. Liang, C.Y. Li, C.Q. Ma, Z.W. Wei, Q. Wang, D. Huang, Q. Chen, C. Li, F. W. Ma, Dopamine alleviates nutrient deficiency-induced stress in *Malus hupehensis*, Plant Physiol. Biochem. 119 (2017) 346–359.
[34] H.C. Wang, F.F. Ma, L.L. Cheng, Metabolism of organic acids, nitrogen and amino acids in chlorotic leaves of 'Honeycrisp' apple (*Malus domestica* Borkh) with excessive accumulation of carbohydrates, Planta 232 (2010) 511–522.
[35] K. Zhou, L.Y. Hu, P.M. Li, X.Q. Gong, F.W. Ma, Genome-wide identification of glycosyltransferases converting phloretin to phloridzin in *Malus* species, Plant Sci. 265 (2017) 131–145.
[36] T. Yang, P. Zhang, C.Y. Wang, AtHSPR may function in salt-induced cell death and ER stress in *Arabidopsis*, Plant Signal Behav. 11 (2016), e1197462.
[37] L.J. Liu, F. Cui, Q.L. Li, B.J. Yin, H.W. Zhang, B.Y. Lin, Y.R. Wu, R. Xia, S.Y. Tang, Q. Xie, The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance, Cell Res. 21 (2011) 957–969.
[38] R. Ozgur, I. Turkan, B. Uzilday, A.H. Sekmen, Endoplasmic reticulum stress triggers ROS signalling, changes the redox state, and regulates the antioxidant defence of *Arabidopsis thaliana*, J. Exp. Bot. 65 (2014) 1377–1390.
[39] I.M. Martinez, M.J. Chrispeels, Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes, Plant Cell 15 (2003) 561–576.
[40] J.K. Zhu, Regulation of ion homeostasis under salt stress, Curr. Opin. Plant Biol. 6 (2003) 441–445.

[41] C. Ouhibi, H. Attia, F. Rebah, N. Msilini, M. Chebbi, J. Aarrouf, L. Urban, M. Lachaal, Salt stress mitigation by seed priming with UV-C in lettuce plants: growth, antioxidant activity and phenolic compounds, Plant Physiol. Biochem. 83 (2014) 126–133.
[42] S.I. Allakhverdiev, Y. Nishiyama, S. Miyairi, H. Yamamoto, N. Inagaki, Y. Kanesaki, N. Murata, Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*, Plant Physiol. 130 (2002) 1443–1453.
[43] J.L. Donahue, S.R. Alford, J. Torabinejad, R.E. Kerwin, A. Nourbakhsh, W.K. Ray, M. Hernick, X. Huang, B.M. Lyons, P.P. Hein, G.E. Gillaspy, The *Arabidopsis thaliana myo*-inositol-1-phosphate synthase1 gene is required for *myo*-inositol synthesis and suppression of cell death, Plant Cell 22 (2010) 888–903.
[44] H. Kusuda, W. Koga, M. Kusano, A. Oikawa, K. Saito, M.Y. Hirai, K.T. Yoshida, Ectopic expression of *myo*-inositol-3-phosphate synthase induces a wide range of metabolic changes and confers salt tolerance in rice, Plant Sci. 232 (2015) 49–56.
[45] I. Kerepesi, G. Galiba, Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings, Crop Sci. 40 (2000) 482–487.
[46] A.K. Parida, A.B. Das, P. Das, NaCl stress causes changes in photosynthetic pigments, proteins and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*, in hydroponic cultures, J. Plant Biol. 45 (2002) 28–36.
[47] K. Mikami, T. Katagiri, S. Iuchi, K. Yamaguchi-Shinozaki, K. Shinozaki, A gene encoding phosphatidylinositol-4-phosphate 5-kinase is induced by water stress and abscisic acid in *Arabidopsis thaliana*, Plant J. 15 (1998) 563–568.
[48] I. Ndamukong, H. Lapko, R.L. Cerny, Z. Avramova, A cytoplasm-specific activity encoded by the trithorax-like *ATX1* gene, Nucleic Acid Res. 39 (2011) 4709–4718.
[49] Y. Lou, J.Y. Gou, H.W. Xue, PIP5K9, an *Arabidopsis* phosphatidylinositol monophosphate kinase, interacts with a cytosolic invertase to negatively regulate sugar-mediated root growth, Plant Cell 19 (2007) 163–181.
[50] W.J. Liang, X.L. Ma, P. Wan, L.Y. Liu, Plant salt-tolerance mechanism: a review, Biochem. Biophl. Res. Co. 495 (2017) 1–6.
[51] S. Sengupta, S. Mukherjee, P. Basak, A.L. Majumder, Significance of galactinol and raffinose family oligosaccharide synthesis in plants, Front. Plant Sci. 6 (2015) 656.
[52] R. Munns, M. Tester, Mechanisms of salinity tolerance, Annu. Rev. Plant Biol. 59 (2008) 651–681.
[53] M. Tester, R.A. Davenport, Na^+ tolerance and Na^+ transport in higher plants, Ann. Bot. 91 (2003) 503–527.
[54] V. Chinnusamy, J.H. Zhu, J.K. Zhu, Salt stress signaling and mechanisms of plant salt tolerance, Genet. Eng. 27 (2006) 141–177.
[55] D.G. Hu, M. Li, H. Luo, Q.L. Dong, Y.X. Yao, C. You, Y.J. Hao, Molecular cloning and functional characterization of *MdSOS2* reveals its involvement in salt tolerance in apple callus and *Arabidopsis*, Plant Cell Rep. 31 (2012) 713–722.
[56] C. Li, Z.W. Wei, D. Liang, S.S. Zhou, Y.H. Li, C.H. Liu, F.W. Ma, Enhanced salt resistance in apple plants overexpressing a *Malus* vacuolar Na^+/H^+ antiporter gene is associated with differences in stomatal behavior and photosynthesis, Plant Physiol. Biochem. 70 (2013) 164–173.