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Increased autophagic activity in roots caused by overexpression of the autophagy-related gene *MdATG10* in apple enhances salt tolerance

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ABSTRACT

Autophagy is a conserved pathway to degrade and recycle damaged proteins and organelles, which has generally been reported to play an important role in plant adaption to various abiotic stressors. Here, we isolated a new apple autophagy-related gene, *MdATG10*, from *Malus domestica*. Expression of *MdATG10* was induced by salt stress, particularly in roots. To investigate the effects of increased autophagic activity on salt tolerance of apple, we generated three *MdATG10*-overexpressing apple lines and exposed them to salt stress. The transgenic apple plants exhibited enhanced salt tolerance, accompanied by slightly damaged photosynthetic ability and a milder growth limitation under the salt treatment. In addition, damage to growth and vitality of the root system caused by the salt treatment was alleviated by overexpressing *MdATG10*. Furthermore, reduced accumulation of Na^+ and a lower Na^+: K^+ ratio was detected in the *MdATG10*-overexpressing apple lines under salt stress. The salt treatment induced expression of genes involved in ion homeostasis in transgenic apple roots. These results demonstrate a promoting role of autophagy in ion transport when plants encounter salty conditions.

1. Introduction

Soil salinity is a major environmental constraint that detrimentally affects the growth and development of plants, limiting agricultural productivity worldwide. Almost all crop plants are highly salt sensitive, including apple trees, so they cannot tolerate high soil salt concentrations [1]. Sodium chloride (NaCl) is the most widespread soil salinity factor [2]. Under NaCl stress, plants encounter hyperosmotic stress and subsequent hyper-ionic imbalance, leading to disrupted plant physiological processes and limitations to plant growth and productivity [2,3].

Salt stress initially causes an imbalance in key physiological processes, such as seed germination, root growth, and leaf gas exchange [4–6]. In addition, the proteins and nucleic acids of plant cells become severely damaged when challenged with salinity stress [7]. Consequently, a decrease in biomass and yield are commonly observed in plants under salt stress [8]. Salt tolerance of crop plants includes the ability to alleviate inhibited growth, and reestablishment of homeostasis [9]. Plants have developed several strategies to protect themselves from salt stress. First, with the decrease in the chlorophyll concentration, salt stress can affect the photosynthesis rate of plants, suggesting that the ability of plants to maintain appropriate photosynthetic performance could be an important factor in plant salt tolerance [10]. Moreover, to avoid Na^+ overload inside of cells, plants have evolved adaptive molecular mechanisms in response to salt stress, including reduced absorption of NaCl by roots and its exclusion and compartmentalization in tissues and cells [11].

Salt treatment activates various ion transporters, which mediate ionic homeostasis and Na^+ tolerance in plants [12]. Among them, the salt overly sensitive (SOS) pathway has been well characterized and is a key determinant of Na^+ homeostasis [13]. Na^+ efflux from the cytoplasm to the apoplast is mediated by the plasma membrane Na^+/H^+ antiporter SOS1 [14]. The serine/threonine protein kinase SOS2 phosphorylates and activates SOS1 and interacts with the calcium-binding protein SOS3 [13]. Overexpression of *AtSOS1* in *Arabidopsis* improves salt tolerance by limiting Na^+ accumulation in cells [15]. In addition, vacuolar partitioning of Na^+ from the cytoplasm is mediated by the tonoplast Na^+/H^+ exchanger NHX, and overexpressing *MdNHX1* in apple rootstock M.26 enhances salt resistance [16]. HKT1 is considered to be a key salt tolerance determinant that coordinates with SOS1 in the response to salt stress [17]. In *Arabidopsis*, HKT1 recirculates Na^+ from the shoot to the roots and decreases Na^+ accumulation in shoot tissues [18]. Moreover, the K^+ transporter AKT1 has been reported to modulate K^+ homeostasis in *Arabidopsis* by mediating K^+ influx into plant cells and displaying more selectivity for K^+ than Na^+ [19].

Autophagy is an important pathway for plant cells to recycle damaged proteins and organelles, and it is positively involved in plant salt

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stress adaptation [20]. Several lines of evidence have demonstrated that autophagy is quickly induced by salt stress. For example, salt treatment induces autophagic activity in *Arabidopsis*, concomitant with upregulation of *AtATG18a* expression [21]. In addition, the effect of salinity on autophagy in roots is also important for salt resistance in plants. Exposing roots to NaCl results in its accumulation in the autophagosome of pepper [22], *Arabidopsis* [21], tobacco [23], and rice [24], accompanied by activation of the core *ATG* genes. Autophagy-deficient plants exhibit hypersensitive to salinity stress. For example, seed germination and growth of RNAi-*AtATG18a* plants is more sensitive to salt conditions [21]. The autophagy-deficient mutants *atg5* and *atg7* exhibit sensitivity to salt treatment [25]. In addition, suppressing the *ATI1* and *ATI2* genes, which bind to the ATG8 protein, renders *Arabidopsis* plants more sensitive to salt stress [26].

Two ubiquitin-like conjugation systems were originally identified in yeast and are largely conserved in all eukaryotes to properly form autophagic vesicles [27]. The *ATG10* gene has been demonstrated to function as an E2-like enzyme, which is essential for ATG12-ATG5 conjugation in *Arabidopsis* [28]. In addition, two rice *ATG10* genes have been characterized and rice *atg10b* mutants are sensitive to high salt treatment, accompanied by increased amounts of oxidized proteins in salt-treated *atg10b* mutant seedlings [24]. Here, we isolated a homologous sequence of *Arabidopsis AtATG10* in apple and named it *MdATG10*. We obtained three *MdATG10*-overexpressing (OE) apple lines and using them to investigate the effects of increased autophagic activity on salt tolerance in apple. The results showed that the transgenic apple plants exhibited enhanced salt tolerance, accompanied by less damaged photosynthetic ability and milder growth limitation under the salt treatment than the control condition. In addition, Na^+ accumulation and K^+ deficiency under salt stress was also alleviated in transgenic plants, particularly in the roots. As the salt treatment induced expression of genes involved in ion homeostasis in transgenic apple roots, we believe that ion homeostasis of apple roots was improved by overexpressing *MdATG10*, thereby improving salt tolerance in apple.

2. Materials and methods

2.1. Plant materials and salt treatments

Mature leaves were collected from three 2-year-old apple (*Malus domestica* Borkh. “Golden Delicious”) plants growing at the Horticultural Experimental Station of Northwest A&F University (Yangling, Shaanxi, China) to clone *MdATG10* and its promoter.

Seedlings of wild-type tobacco (*Nicotiana benthamiana*) and two homozygous T_3 transgenic lines were used. Seeds were sterilized and sown on Murashige and Skoog (MS) medium. Five-day old seedlings of uniform size grown on MS agar plates were transferred vertically into unmodified media or MS media supplied with 100 mM NaCl for 5 d. Seedlings were grown at 25 °C under a 14 h light/10 h dark cycle.

GL-3 is a derivative line isolated from the apple cultivar ‘Royal Gala’, which has a high regeneration capacity and is susceptible to *Agrobacterium* [33]. Tissue-cultured GL-3 plants were cultured as described previously [29], and they were sub-cultured every 4 weeks. After 30 d on rooting media, transgenic and wild-type (WT) apple plantlets were transferred to a growth chamber and cultivated using a method described previously [29]. After 40 d of acclimation in the growth chamber, transgenic and WT apple plants of similar size were transferred to a hydroponics system, as described previously [30]. After a 2-week preincubation, plants of uniform size were selected for treatment in half-strength Hoagland’s nutrient solution supplemented with 120 mM NaCl. Plants cultured in normal half-strength Hoagland’s nutrient solution were used as the control. Control and treated GL-3 plants were divided into roots, stems, and leaves 24 h after applying the salt for the *MdATG10* expression analysis. After 12 d of this experiment, the fourth through sixth leaves from the apex of the stem (fully mature leaves) were sampled from six plants per strain for damage analyses, and the roots were sampled from nine plants per strain.

2.2. Sequence analysis, construction of plasmids, and subcellular localization

Homologous sequences and putative conserved domains were predicted from the NCBI BLASTp program. The sequences were aligned with homologues sequences from other species using DNAMAN 6.0 with default parameters. Phylogenetic trees were constructed using MEGA 10.0 software and the neighbor-joining method was performed with 1,000 replications for the bootstrap analysis. Putative cis-regulatory elements in the *MdATG10* promoter region were examined with the PlantCARE program.

To construct vectors for subcellular localization and for the transgenic lines, the *MdATG10* coding region was introduced into the pGWB405-GFP and pCambia2300 vectors, both driven by the CaMV 35S promoter and carried by the kanamycin (Kan) selectable marker in plants. To generate the *ProMdATG10*: GUS construct, a 1,981-bp genomic promoter sequence upstream of the *MdATG10* coding region was amplified separately and inserted into the pGWB433 vector. After being confirmed by PCR and sequencing analysis, the resulting plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The primers used for constructing the vector are listed in Supplemental Table S2.

Leaves of 5-week-old tobacco (*N. benthamiana*) were transiently transformed to determine subcellular localization, as described previously [31]. Three days later, green fluorescent protein (GFP) signals in transformed tobacco leaves were observed using confocal microscopy, and the images were processed with FV10-ASW software.

2.3. Transformation of tobacco and apple plants

Wild-type tobacco (*N. benthamiana*) seedlings were transformed using the *A. tumefaciens* EHA105-mediated leaf dip method [32]. The polymerase chain reaction (PCR)-positive plantlets were transplanted into soil in the growth chamber. After the transgenic plants were harvested individually, the seeds were screened on MS medium supplemented with 50 mg L^{-1} Kan. T_2 homozygous lines were obtained after selecting transgenic lines at a 3:1 segregation ratio, and the T_3 seeds were collected.

GL-3 apple plants were used as the genetic background and *Agrobacterium*-mediated leaf fragments were transformed as described previously [33]. Regenerated Kan-resistant buds were sub cultured every 4 weeks on MS medium containing 25 mg L^{-1} Kan as a selectable marker. Lines showing normal growth were evaluated by PCR analysis. Overexpression of *MdATG10* was confirmed by quantitative real-time PCR (qRT-PCR). Untransformed GL-3 plants were cultured in the same way without selection pressure and served as the control.

2.4. RNA extraction, DNA isolation, and qRT-PCR analysis

Total RNA was extracted using a Wolact® plant RNA isolation kit (Wolact, Hong Kong, China). 1 μg of total RNA was used to synthesize first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Genomic DNA was isolated with the Wolact® Plant Genomic DNA purification kit. The qRT-PCR analysis was carried out as described previously [34]. Three biological replicates were conducted in each assay, and values were calculated using *MdMDH* as the endogenous control [35]. The relative expression level of each gene was obtained according to the $2^{-\Delta\Delta CT}$ method [36], and the specificity of each gene was determined by a dissociation curve analysis. The gene-specific primers are shown in Supplemental Table S2.

2.5. Physiological analysis and GUS histochemical staining

Relative electrolyte leakage (REL) and total chlorophyll concentrations in leaves were determined according to methods described previously [29,37]. Na^+ and K^+ concentrations were determined by flame spectrometry (M410; Sherwood Scientific, Cambridge, UK) as described previously [38].

On day 6 of the hydroponics experiment, the net photosynthesis rate (Pn), stomatal conductance (Gs) and intercellular CO_2 concentration (Ci) were monitored between 9:00 and 11:00 a.m. as described previously [38]. Data were collected from fully expanded, fully light-exposed leaves from eight plants.

The GUS staining of transgenic tobacco was performed using 5-bromo-4-chloro-3-indolyl-β-glucuronide as the substrate, as described previously [39].

2.6. Growth measurements and observation of autophagosomes

Nine plants per strain were collected after the hydroponic salt treatment to evaluate differences in growth among the different genotypes. Plant height was measured from the stem base to the terminal bud of the main stem. Whole plants were divided into root, stem, and leaf portions. The fresh and dry weights were measured as described previously [38]. Briefly, after the fresh weight of each sample plant was recorded, the dry weight was obtained after the plants were fixed at 105 °C for 15 min and oven-dried at 75 °C for at least 72 h to constant weight. Relative growth was calculated as the weight of the salt-treated plant divided by the weight of the control plant.

The autophagosomes were observed under a JEOL-1230 transmission electron microscope (TEM, Hitachi, Tokyo, Japan) as described previously [29].

2.7. Measurements of root activity and root hydraulic conductance

To analyze root activity, fresh and white root samples were collected at the end of the stress treatment. The triphenyl tetrazolium chloride (TTC) method was applied to monitor root activity, which was defined as the capacity for deoxidization (mg g^{-1} FW h^{-1}) [34].

Root hydraulic conductance was measured using a pressure chamber (Model 3500) according to a method described previously [40].

2.8. Statistical analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Three independent replicates were used for each determination. Experimental data are presented as mean ± standard error. The statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple range test. A P-value < 0.05 was considered significant.

3. Results

3.1. Molecular cloning, sequence analysis, and subcellular localization of MdATG10

Using *Arabidopsis AtATG10* (AT3G07525) to blast against the apple genome, we identified a homologous sequence in *M. domestica* and named it *MdATG10*. The 690-bp open reading frame (ORF) encoded a 229 amino acid-deduced protein. Protein alignment revealed high homology between the MdAT10 protein and other ATG10 proteins from *A. thaliana* (44.78 %), *Glycine max* (47.39 %), *Nicotiana tabacum* (40.30 %), *Populus euphratica* (51.49 %), and *Solanum lycopersicum* (39.93 %) (Fig. 1a). There was a conserved HPC motif among all aligned sequences, and the cysteine residue within the HPC motif is the putative active-site residue unique to autophagic E2 enzymes for recognizing the ATG5 subunit of the autophagosome complex [41]. The phylogenetic tree analysis indicated that the MdATG10 protein formed a close cluster with AtATG10 and PeATG10 (Fig. 1b).

To investigate subcellular localization of MdATG10, we fused its ORF with the GFP at the N-terminus under control of the CaMV35S promoter. For transient expression in tobacco epidermal cells, the fluorescence observations indicated the presence of the MdATG10–GFP fusion protein in the cytoplasm and nucleus of the cells (Fig. 1c). In addition, we cloned the *MdATG10* promoter region and analyzed it *in silico* using the PlantCARE online program, revealing the presence of several recognized stress-responsive *cis*-elements (Fig. S1, Table S1).

3.2. Salt-responsive expression patterns of MdATG10 in different tissues

We used GL-3 apple plants cultured in a hydroponic system to determine whether expression of *MdATG10* is induced by salt stress. Under normal hydroponic conditions, the *MdATG10* transcription level was similar in roots and stems, and it was almost two-fold higher in leaves. After a 12 h 120 mM NaCl treatment, *MdATG10* expression increased in all three tissues of apple plants, and it increased most in roots. The transcripts were upregulated 5.3-fold in roots, 3.8-fold in stems, and 1.6-fold in leaves in response to the salt treatment (Fig. 2a).

In addition, using *pro-MdATG10*-GUS transferred *Nicotiana* plants (Fig. S2), we detected strong staining in the roots of two transgenic lines P-10 and P-17 in response to salt treatment, while staining was relatively lighter under the control condition (Fig. 2b). These results suggest that *MdATG10* is induced by salt stress, particularly in roots.

3.3. Overexpression of MdATG10 enhances salt tolerance in apple

To further valid the roles of *MdATG10* under salt stress in apple, we constructed an overexpression vector with its ORF and transformed it into GL-3 apple plants. We obtained three overexpressing (OE) lines in which *MdATG10* expression levels were noticeably elevated compared with the WT levels. The transcripts were constitutively increased by 49.5-, 146.8-, and 35.1-fold in the roots of lines OE-1, OE-4, and OE-5, respectively, when compared with the levels of the untransformed WT control (Fig. 3a, b).

We used a hydroponic system to investigate performance of the apple plants in a 120 mM NaCl treatment. Under normal conditions, the phenotypes did not differ between the OE lines and WT (Fig. 3c). Although a 12-d salt stress treatment led to conspicuous damage in all apple plants, leaves of WT plants showed more brown stains compared with those of the OE lines (Fig. 3c). Due to the salt damage, significantly increased REL was detected in all genotypes, but it was still much lower in the transgenic lines than in the WT (Fig. 3d). The salt treatment led to degradation of total chlorophyll concentrations in plants, but they were still higher in the OE lines than in the WT (Fig. 3e). After the salt treatment, plant height decreased among the genotypes, but the reduction was much smaller in OE plants than in the WT (Fig. 3f). These results show that overexpressing *MdATG10* conferred higher tolerance to salt stress in apple.

3.4. Apple lines over-expressing MdATG10 maintain a higher photosynthetic capacity and growth under salt stress

To examine damage to the photosynthetic capacity of different apple lines under salt stress, we measured their gas exchange parameters 6 d after the salt treatment. Under normal conditions, no significant difference was found among the genotypes in terms of photosynthesis rate (Pn), stomatal conductance (Gs), or intercellular CO_2 level (Ci). Salt stress significantly decreased Pn among the genotypes, but it was approximately 1.4 times higher in the OE lines than that in WT plants (Fig. 4a). Gs and Ci followed a similar trend (Fig. 4b, c). These data suggest that the photosynthetic ability of plants over-expressing *MdATG10* was less damaged under the salt treatment.

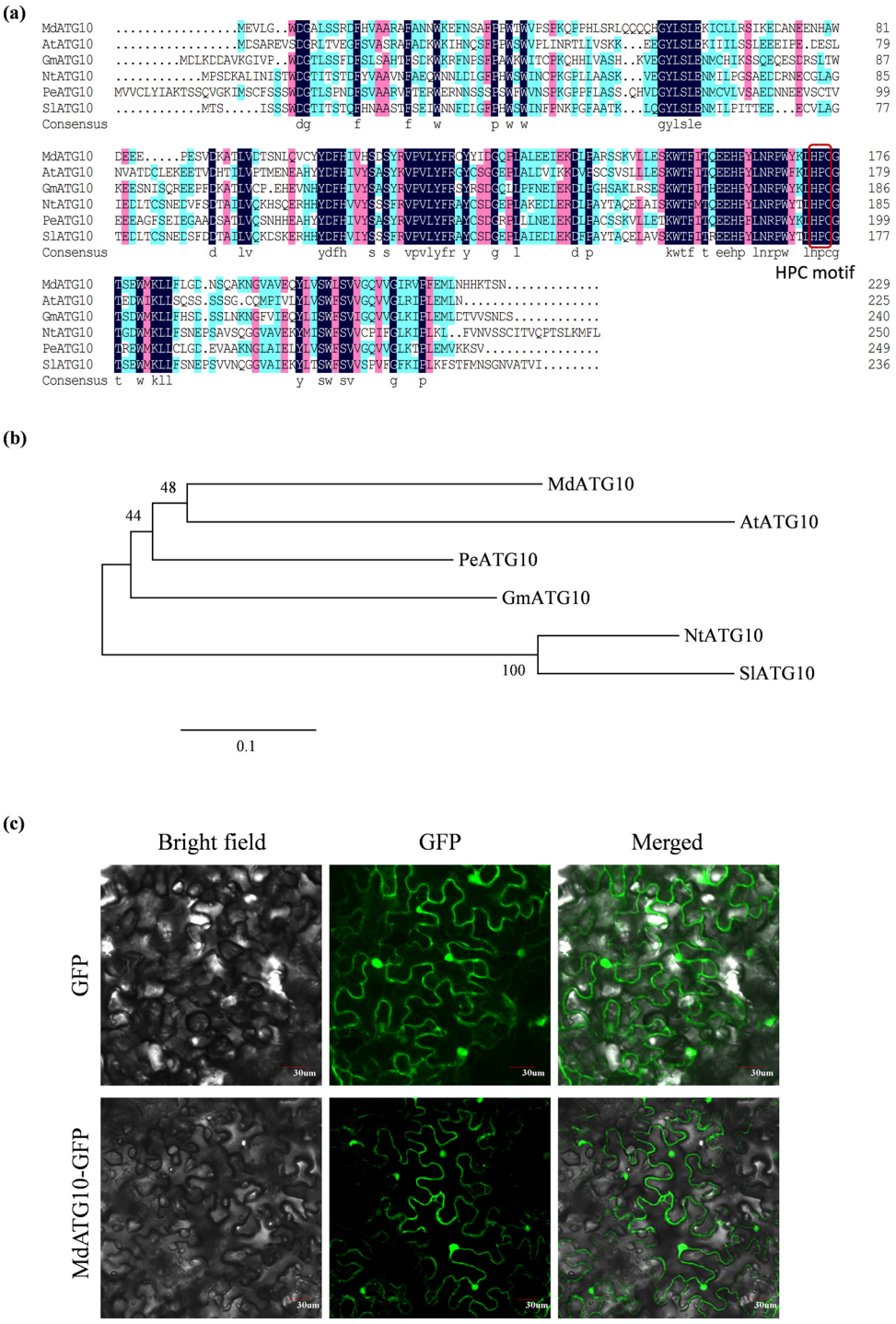

Fig. 1. Sequence analysis and cellular localization of MdATG10. (a) Alignment of deduced ATG10 amino acid sequence from *Malus domestica* (*Md*), *Arabidopsis thaliana* (*At*), *Populus euphratica* (*Pe*), *Glycine max* (*Gm*), *Nicotiana tabacum* (*Nt*), and *Solanum lycopersicum* (*Sl*). (b) Phylogenetic analysis of MdATG10 protein with ATG10 proteins from other species. (c) Subcellular localization analysis of MdATG10-GFP fusion protein in tobacco epidermal cells.

In addition, the decrease in growth rates was retarded in OE plants during the salt treatment period. When compared with plants cultured under normal conditions, the total fresh weight of the WT decreased to 55.6 % of the control after the salt treatment, while those of the OE lines decreased to 81.7 % in OE-1, 74.0 % in OE-4, and 76.2 % in OE-5 (Table 1). The fresh weights of the roots, stems, and leaves also showed less of a decrease in the OE lines. Dry weights revealed the same trend (Table 2). These results suggest that the adverse effect of salt stress on biomass production of the apple lines was effectively eased by over-expressing *MdATG10*.

3.5. Apple lines over-expressing MdATG10 maintain better root activity under salt stress

In addition to the salt damage to leaves, we also observed differences in root growth among the genotypes. Root growth was inhibited under salt stress, but the inhibition was partially alleviated in the OE lines, as represented by more vibrant white roots (Fig. 5a). TTC staining is commonly used as an H^+ receptor and we observed the reduction ability of root cells was induced among all genotypes under salt treatment, which was increased higher in OE lines (Fig. 5b). Additionally,

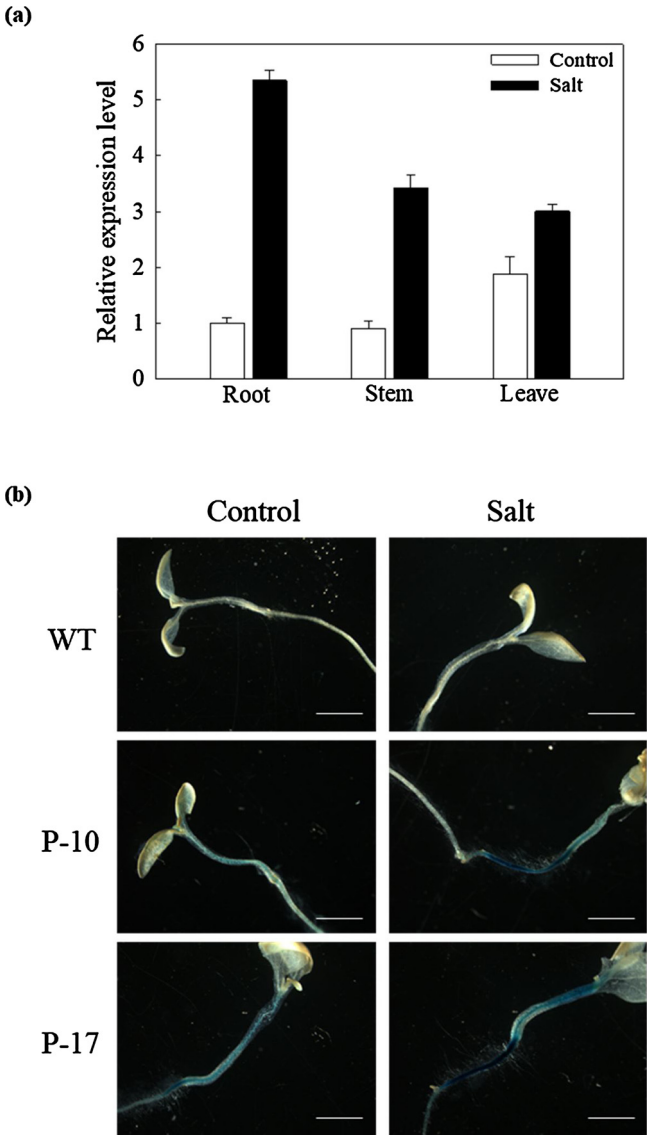

Fig. 2. The expression of *MdATG10* is induced by salt treatment. (a) Changes in the expression of *MdATG10* in different tissues of GL-3 apple plants after treating with 120 mM NaCl for 12 h. (b) Gus staining of *pro-MdATG10*-GUS transferred *Nicotiana* plants without or with 100 mM NaCl for 5 d. P-10 and -17, *pro-MdATG10*-GUS transferred *Nicotiana* plants. Bars: 2 mm.

root hydraulic conductivity decreased significantly under salt stress, but was much higher in the OE lines than in the WT plants (Fig. 5c). While no significant difference was found in root hydraulic conductivity among the genotypes under the control condition, the root hydraulic conductivity of the OE lines was almost two times that of the WT after the salt treatment, suggesting a better ability of roots to take up water under salt stress.

3.6. Overexpression of MdATG10 reduces the Na^+/K^+ ratio in apple under salt stress

As a balanced Na^+/K^+ ratio is an important characteristic for salt tolerance, we measured the levels of Na^+ and K^+ in the roots, stems, and leaves at the end of the experiment. The salt treatment greatly improved the accumulation of Na^+ in different tissues, but the increase was still lower in the OE lines compared with the WT. In addition, the difference was most significant in roots (Fig. 6a–c). The K^+ concentrations in different tissues decreased after the salt treatment, but the reduction was alleviated in transgenic plants. Although the difference was not always significant in terms of different tissues, the K^+ concentrations were still higher in all transgenic lines than in the WT (Fig. 6d–f). As a result, the Na^+/K^+ ratios in the different tissues were markedly lower in the OE lines than the WT, particularly in roots (Fig. 6g–i).

Due to the significant difference in the Na^+/K^+ ratios of roots, we examined the expression of genes involved in the maintenance of ion homeostasis in the roots of all genotypes. The transcript levels of three genes in the salt overly sensitive (SOS) pathway, i.e., *MdSOS1*, *MdSOS2*, and *MdSOS3*, increased after the salt treatment, particularly *MdSOS1*, and they were all expressed higher in the transgenic lines than in the WT (Fig. 7a–c). The expression pattern of *MdNHX1* (Na^+/H^+ antiporter 1) showed the same trend (Fig. 7d). In addition, expression of *MdAKT1* (K^+ transporter 1) was downregulated by salt stress in WT plants, but it was upregulated almost two-fold in transgenic plants (Fig. 7e). The *MdHKT1* transcripts (high-affinity K^+ transporter 1) were induced by salinity treatment and were expressed at higher levels in WT plants (Fig. 7f). These findings indicate that tolerance of the transgenic apple plants to salt stress was correlated with balance of the Na^+/K^+ ratio.

3.7. Salt stress-induced autophagy in apple roots is intensified in the transgenic lines overexpressing MdATG10

Autophagy is activated in plants under stress. To assess the change in autophagic activity in apple roots in response to salt stress, we examined the expression of several autophagy-related genes in the roots of all genotypes after salt treatment, including *MdATG3a*, *MdATG3b*, *MdATG5*, *MdATG7a*, *MdATG7b*, *MdATG8c*, *MdATG8f*, *MdATG8i*, and *MdATG18a*. Apparently, the transcript levels of most detected *MdATG*s were induced by salt stress in the roots, and they were all expressed at higher levels in the OE lines than in the WT. Transmission electron microscopy revealed higher autophagic activity in the roots of the transgenic lines under salt stress than in the WT. A few autophagosomes were observed in the roots of all genotypes under normal conditions. After the salt treatment, an increase in the number of autophagosomes was detected in root tissues, and the number of autophagosomes accumulated in the OE lines was twice as many as in the WT plants. These results indicate that autophagy is triggered in apple roots by salt stress and was intensified in the OE lines.

4. Discussion

Autophagy is a bulk degradation pathway in which cellular components are enveloped by autophagosomes and transported into vacuoles to maintain cellular homeostasis [21,42]. The autophagosomes are induced under stress conditions, such as salt or drought stress, and the important roles of autophagy in plant resistance have been demonstrated in model plants [21,25]. Here, we charaterized an important autophagy-related gene, *MdATG10* in apple, and used *MdATG10*-OE apple plants to investigate the effects of increased autophagic activity on salt tolerance in apple.

The MdATG10 protein had a conserved HPC motif and presented high homology with other ATG10 s from various plant species (Fig. 1a, b). The subcellular localization analysis showed that the MdATG10–GFP fusion protein occurred in the cytoplasm and nucleus of tobacco epidermal cells (Fig. 1c). In addition, we isolated the *MdATG10* promoter region and obtained *pro-MdATG10*-GUS transferred *Nicotiana* plants (Figs. S1, S2). A previous study in *Arabidopsis* reported that autophagic flux is induced rapidly by NaCl treatment in *Arabidopsis* root cortex cells [43]. After analyzing the changes in expression of *MdATG10* in the roots, stems, and leaves of GL-3 apple plants after a 12 h salt treatment, we found that it was also induced by salt stress in all three tissues, particularly in roots (Fig. 2a). Consistent with this observation, higher GUS activity was detected in the roots of *pro-MdATG10*-GUS transferred *Nicotiana* plants under the salt treatment

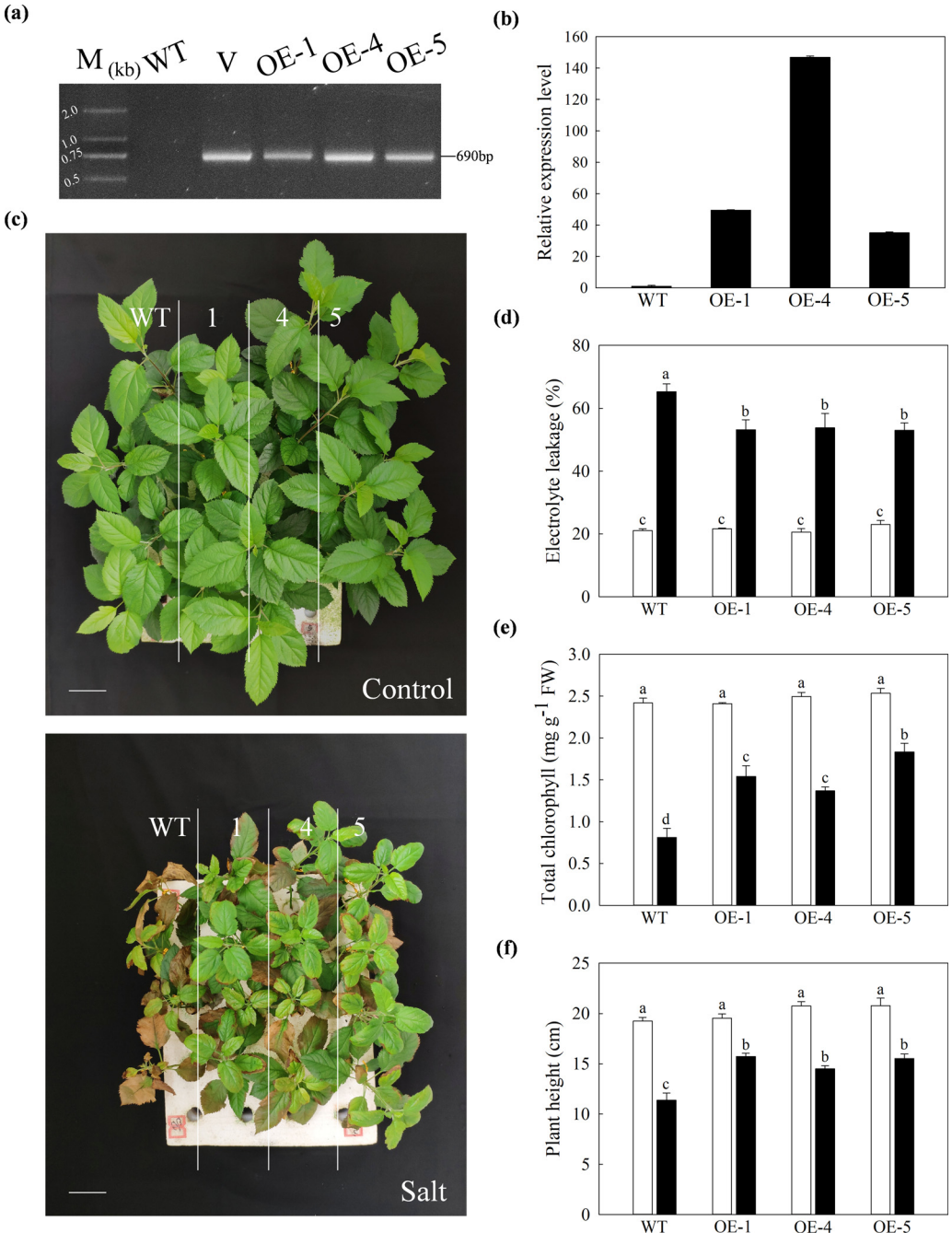

Fig. 3. Overexpression of *MdATG10* confers enhanced salt tolerance in apple. (a) PCR confirmation of transgenic apple plants. Lanes: M, molecular marker DL2000; V, positive vector containing pCambia2300-*MdATG10* plasmid; WT, non-transformed wild-type; OE-1, -4 and -5, *MdATG10*-transgenic apple lines. (b) qRT-PCR analysis of *MdATG10* transcripts in the roots of Lines OE-1, OE-4 and OE-5. (c) Phenotypes of WT and transgenic apple plants under normal hydroponic conditions and after 12 d of treatment with 120 mM NaCl. Bars: 5 cm. (d) Electrolyte leakage, (e) total chlorophyll concentrations and (f) plant height of WT and transgenic plants treated with and without salt. Data are the means of six replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

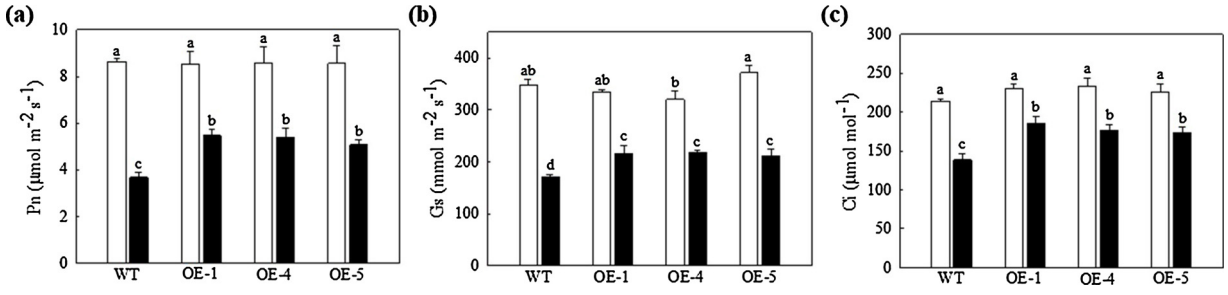

Fig. 4. Overexpression of *MdATG10* in apple leads to higher photosynthetic capacity under salt stress. Changes in (a) net photosynthesis rate (Pn), (b) stomatal conductance (Gs) and (c) intercellular CO_2 concentration (Ci) were measured at 6 d after salt treatment. Data are the means of three replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

Table 1
Tissue fresh weights (FW, g) measured from WT and *MdATG10*-OE plants after 12 d of salt treatment. All data are means ± SE of 9 seedlings. The percentages in parentheses after the SE denote the data in OE versus WT lines. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

	Root (M ± SD)			Stem (M ± SD)			Leaf (M ± SD)			Total (M ± SD)		
	Control	Salt	Relative growth	Control	Salt	Relative growth	Control	Salt	Relative growth	Control	Salt	Relative growth
WT	1.5975 ± 0.1315a (100 %)	0.8098 ± 0.0629d (100 %)	50.7 %	1.0907 ± 0.0453a (100 %)	0.6216 ± 0.0494d (100 %)	57.0 %	3.8523 ± 0.1651a (100 %)	2.2082 ± 0.0689c (100 %)	57.3 %	6.5405 (100 %)	3.6396 (100 %)	55.6 %
OE-1	1.5200 ± 0.1319a (95.1 %)	1.1289 ± 0.0413c (139.4 %)	74.3 %	1.1348 ± 0.0342a (104 %)	0.8942 ± 0.0291b (143.8 %)	78.8 %	3.5940 ± 0.1362a (93.3 %)	3.0861 ± 0.0956b (139.8 %)	85.9 %	6.2488 (95.5 %)	5.1092 (140.4 %)	81.7 %
OE-4	1.4170 ± 0.0618ab (88.7 %)	1.0781 ± 0.0420c (133.1 %)	76.1 %	1.0681 ± 0.0484a (97.9 %)	0.7838 ± 0.0272c (126.1 %)	73.4 %	3.8726 ± 0.2053a (100.5 %)	2.8459 ± 0.0874b (128.9 %)	73.5 %	6.3577 (97.2 %)	4.7078 (129.3 %)	74.0 %
OE-5	1.6262 ± 0.1251a (101.8 %)	1.1752 ± 0.0325bc (145.1 %)	72.3 %	1.0646 ± 0.0294a (97.6 %)	0.7744 ± 0.0178c (124.6 %)	72.7 %	3.8059 ± 0.1647a (98.8 %)	3.0013 ± 0.0817b (135.9 %)	78.9 %	6.4967 (99.3 %)	4.9509 (136.0 %)	76.2 %

Table 2
Tissue dry weights (DW, g) measured from WT and *MdATG10*-OE plants after 12 d of salt treatment. All data are means ± SE of 9 seedlings. The percentages in parentheses after the SE denote the data in OE versus WT lines. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

	Root (M ± SD)			Stem (M ± SD)			Leaf (M ± SD)			Total (M ± SD)		
	Control	Control	Relative growth	Control	Salt	Relative growth	Control	Salt	Relative growth	Control	Salt	Relative growth
WT	0.2110 ± 0.0076a (100 %)	0.1271 ± 0.0129b (100 %)	60.2 %	0.2623 ± 0.0184a (100 %)	0.1544 ± 0.0032b (100 %)	59.2 %	1.1385 ± 0.0629a (100 %)	0.6802 ± 0.0292d (100 %)	59.7 %	1.6118 (100 %)	0.9627 (100 %)	59.7 %
OE-1	0.2033 ± 0.0097a (96.4 %)	0.1532 ± 0.0243b (120.5 %)	75.3 %	0.2745 ± 0.0219a (104.7 %)	0.1983 ± 0.0104b (127.6 %)	72.2 %	1.1528 ± 0.0635a (101.3 %)	0.9328 ± 0.0722c (137.1 %)	80.9 %	1.6306 (101.2 %)	1.2843 (133.4 %)	78.8 %
OE-4	0.2015 ± 0.0139a (95.5 %)	0.1498 ± 0.0188b (117.9 %)	74.3 %	0.2589 ± 0.0051a (98.7 %)	0.1882 ± 0.0065b (121.1 %)	72.7 %	1.0762 ± 0.0687ab (94.5 %)	0.8965 ± 0.0553bc (131.8 %)	83.3 %	1.5366 (95.3 %)	1.2345 (128.2 %)	80.3 %
OE-5	0.2046 ± 0.0174a (97.0 %)	0.1577 ± 0.0188b (124.1 %)	77.1 %	0.2588 ± 0.0218a (98.6 %)	0.1908 ± 0.0046b (122.8 %)	73.7 %	1.0946 ± 0.0581ab (96.1 %)	0.9095 ± 0.0670bc (133.7 %)	83.1 %	1.5580 (96.7 %)	1.2580 (130.7 %)	80.7 %

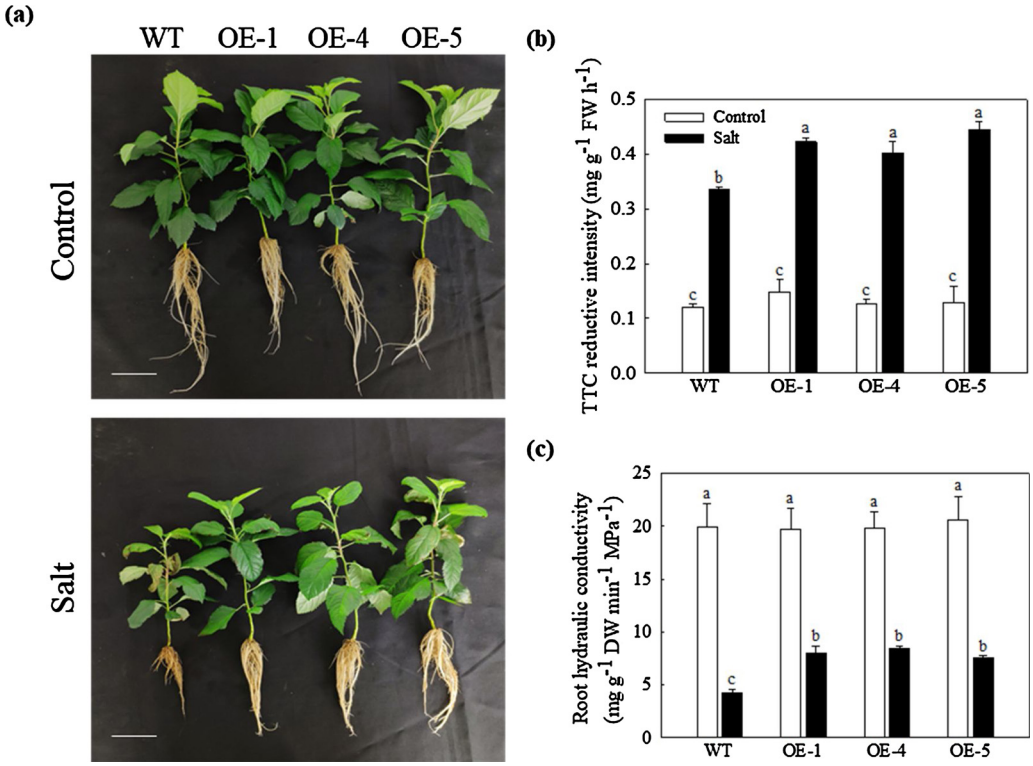

Fig. 5. Overexpression of *MdATG10* in apple leads to better root growth under salt stress. (a) Phenotypes of the root of WT and transgenic apple plants with or without 12 d of treatment with 120 mM NaCl. Bars: 5 cm. (b) Root triphenyl tetrazolium chloride (TTC) reductive intensity and (c) root hydraulic conductivity of WT and transgenic plants treated with and without salt. Data are the means of three replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

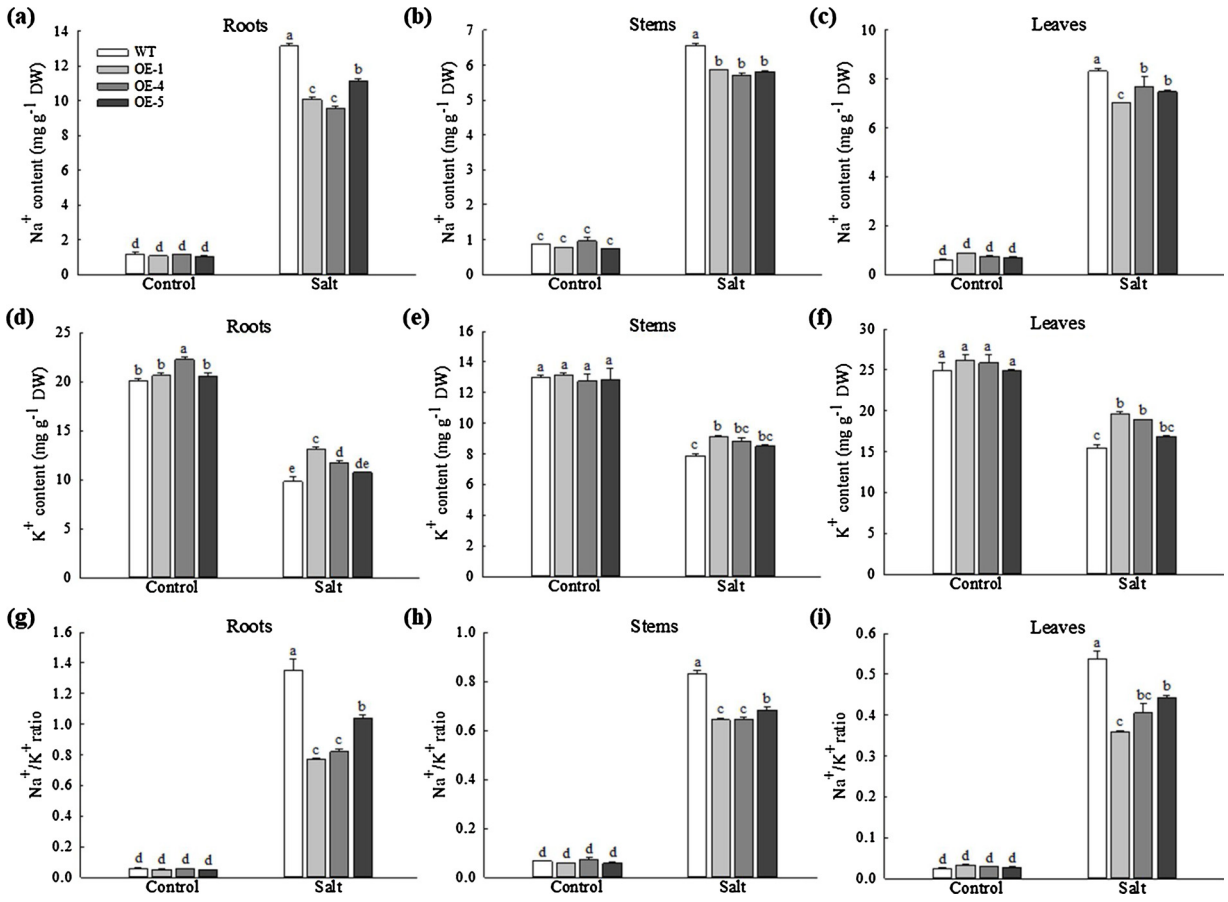

Fig. 6. Overexpression of *MdATG10* in apple reduces the accumulation of Na^+ and the deficiency of K^+ under salt stress. Changes in concentrations of Na^+ (a-c), K^+ (d-f) and Na^+: K^+ ratios (g-i) in the roots, stems, and leaves of WT and transgenic apple plants. Data are the means of three replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

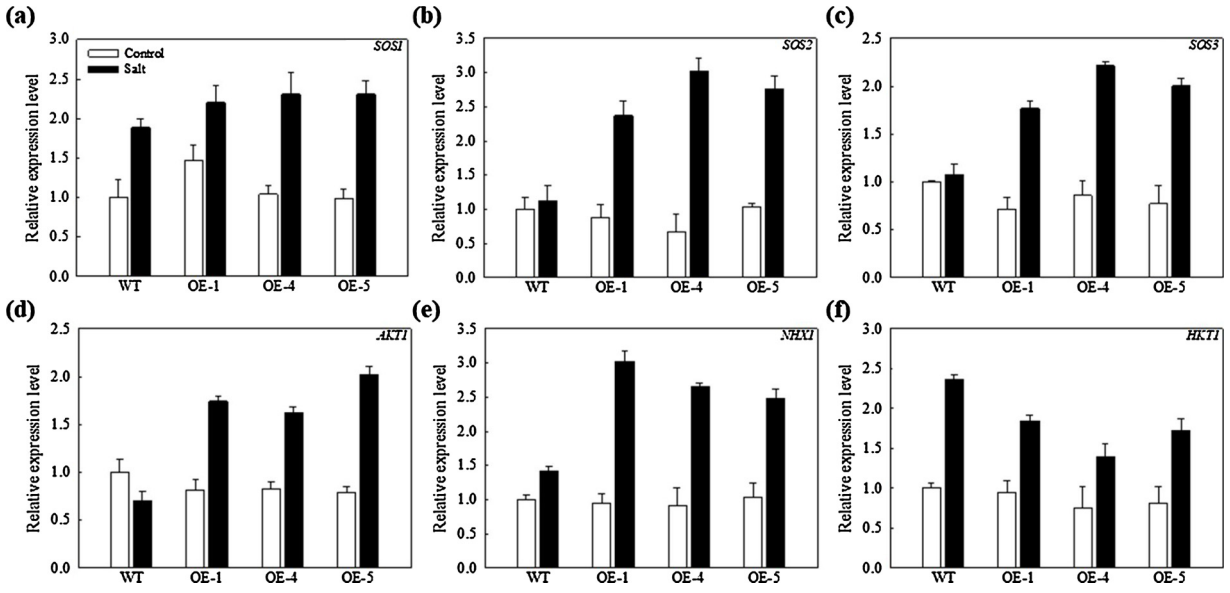

Fig. 7. Changes in transcript levels of genes involved in ion homeostasis in the apple roots following salt treatment. Changes in expression of (a) *MdSOS1*, (b) *MdSOS2*, (c) *MdSOS3*, (d) *MdAKT1*, (b) *MdNHX1*, and (d) *MdHKT1* under salt treatment. Data are means of three replicates with SE.

compared with under the control condition (Fig. 2b).

We observed higher salt tolerance of the OE lines by implementing a hydroponic NaCl treatment in the WT and transgenic apple plants (Fig. 3). Saline conditions affect the photosynthetic processes of plants due to stomatal closure and the loss of photosynthetic pigments, leading to a limitation in growth [44]. The ability of plants to maintain higher photosynthetic ability could be an important factor in plant salt tolerance [45]. In this experiment, the photosynthetic ability of transgenic plants was less damaged during the salt treatment compared with the WT (Fig. 4), and the total chlorophyll concentrations in the OE lines were higher than those in WT plants (Fig. 3e). Combined with the better biomass production of the transgenic lines under salt stress, we believe that *MdATG10*-overexpression could enhance salt tolerance in the plants by decreasing damage to their photosynthetic systems.

Salt stress is first perceived by the root system, and root growth is significantly inhibited under salt treatment [7]. The decrease in the root hydraulic conductivity caused by salt stress reduces water transport from the soil to the plant, affecting leaf water status and therefore growth and other physiological responses of the plant [46]. Furthermore, the weak root system under the salt treatment inhibits uptake of essential nutrients from the roots, leading to nutrient imbalances and a significant loss in productivity [47,48]. Here, we found that the inhibited root growth caused by salt stress was apparently alleviated in the OE lines, and root hydraulic conductivity was much higher in the OE lines than WT plants after the salt treatment (Fig. 5). These results indicate that *MdATG10*-overexpression could protect the growth and vitality of the root system under salt stress, thereby reducing salt damage to the apple plants.

Ionic toxicity is one of the main threats imposed by salt stress on plants, which is associated with excessive Na^+ uptake, leading to K^+ deficiency and to other nutrient imbalances [2,49]. The accumulation of toxic ions in the root zone also leads to a decrease in the root osmotic potential, causing irreversible damage to roots [50]. The capacity to maintain a suitable Na^+: K^+ ratio has been proposed to be a major adaptive trait of salt-tolerant plants, as it is indispensable for sustaining normal functions of plant organelles in response to salt stress [4,51]. Herein, we also found that the enhanced salt tolerance of the OE lines was consistent with reduced accumulation of Na^+ and a lower Na^+: K^+ ratio, and this difference was most pronounced in the roots of OE and WT plants (Fig. 6). As the expression of many genes changed in response to salt stress [52,53], we measured the expression of genes involved in the maintenance of ion homeostasis in the roots of all genotypes to investigate whether autophagy affected these genes in roots.

As results, the salt treatment induced expression of the three genes involved in the SOS pathway in apple roots, which is a highly conserved pathway in plants to export Na^+ ions out of cells [53]. The increase in *MdSOS1*, *MdSOS2*, and *MdSOS3* expression was higher in the transgenic plants (Fig. 7a–c), suggesting a greater ability of the roots of OE plants to increase Na^+ efflux under the salt treatment. In addition, the same tendency was also found in the expression of *MdNHX1* (Fig. 7d), suggesting that the transgenic lines might stimulate a more active mechanism to separate Na^+ into vacuoles to reduce cytoplasmic ion toxicity in the plants under salt treatment. The *AKT1* gene expressed in epidermal and cortex cells of *Arabidopsis* roots has been identified as an important K^+ channel that mediates K^+ uptake from soil into roots [19]. Here, we found that the *MdAKT1* transcript level was downregulated by salt stress in WT plants but upregulated in the transgenic lines (Fig. 7e), which is consistent with the higher K^+ concentrations in the roots of OE lines compared with the WT after the salt treatment. The *MdHKT1* transcript was also induced by the salt treatment but it was expressed at a higher level in WT plants (Fig. 7f), which might be the reason why the difference in the Na^+: K^+ ratio among genotypes was not as significant in leaves as in roots. In conclusion, the increased autophagic activity affected ion homeostasis in apple roots, thereby maintaining an optimal cytoplasmic Na^+: K^+ ratio, which was conducive to root development and growth while plants were growing under a relatively high salt condition.

We also detected stronger autophagic activity in the roots of transgenic plants, which was supported by the fact that other *MdATG* genes were upregulated higher and autophagosomes formed more frequently under the salt treatment (Fig. 8). The conserved function of autophagy to degrade and recycle damaged proteins and organelles plays an important role when plants encounter abiotic stress [20,54]. *HsfA1a* has been demonstrated to play a critical role in tomato plant drought tolerance by activating the expression of *ATG10* and *ATG18f* and inducing the formation of autophagosomes [54]. In this study, we also demonstrated that increased autophagic activity in roots caused by overexpression of *MdATG10* in apple enhanced their salt tolerance. Damage to the growth and vitality of the root system caused by the salt treatment was alleviated by *MdATG10*-overexpression, which promoted the absorption of water and nutrients by apple plants under the salt

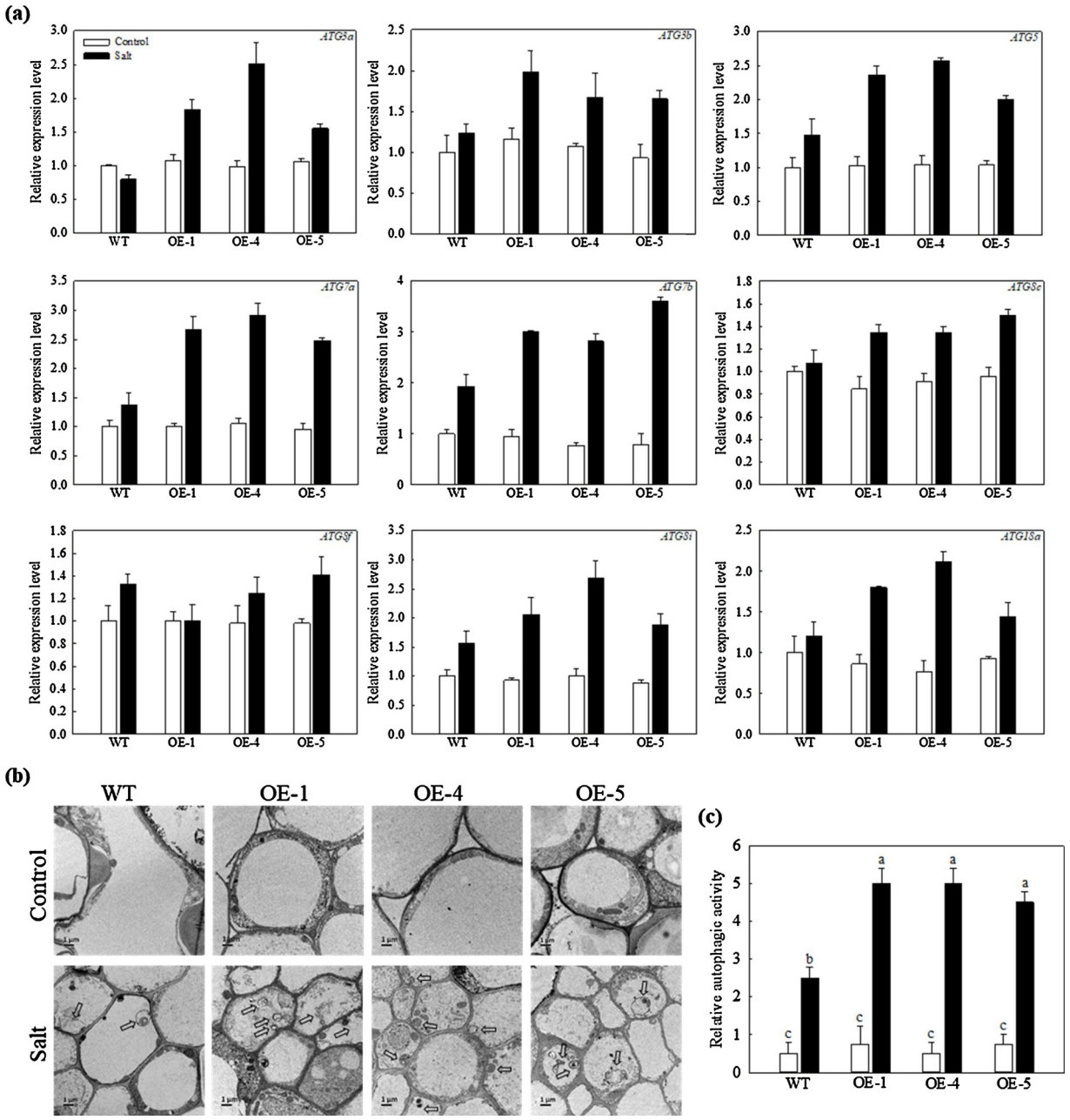

Fig. 8. Expression of other *MdATGs* and formation of autophagosomes in apple roots under salt stress. (a) Changes in expression of other *MdATGs* in the roots of WT and *MdATG10*-OE plants following salt stress. (b) Representative TEM images of autophagic structures in mesophyll cells from the roots of WT and *MdATG10*-OE plants. Autophagic bodies are indicated by arrows. Bars: 1 μm. (c) Relative autophagic activity normalized to the activity of WT or *MdATG10*-OE plants shown in (b). More than 10 cells were used to quantify structures. Data are the means of six replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

condition. In addition, we revealed that ion homeostasis of roots under salt treatment was improved in apple plants overexpressing *MdATG10*, suggesting a possible linkage between autophagy and ion transport in response to salt stress.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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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.2020.110444.

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