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Molecular cloning and functional characterization of the Aluminum-activated malate transporter gene *MdALMT14*

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ABSTRACT

The salinity of soil severely restricts the rate of land use and crop productivity. Plants have developed complex mechanisms, including osmotic adjustment, to resist salt stress. Here, we report that the *MdALMT14* gene in the apple is a homolog of the *Arabidopsis* malate acid transporter gene *AtALMT14*. The expression of *MdALMT14* was induced by salt treatment. The ectopic expression of *MdALMT14* in *Arabidopsis* decreased the malondialdehyde (MDA) content and the relative electronic conductivity under salt conditions. In addition, our results showed that *MdALMT14* impacted salt tolerance by promoting the accumulation of malic acid in *Arabidopsis*. The overexpression of *MdALMT14* increased salt tolerance in apple calli. Taken together, these findings provide evidence that the apple Aluminum-activated Malate Transporter MdALMT14 is involved in salt stress tolerance and the regulation of malic acid content. These data provide new ideas for further attempts to improve stress tolerance and fruit quality through cultivation and breeding methods.

1. Introduction

Much of the irrigation soil (approximately 20% of land worldwide) is affected by salinity, and this trend is getting worse (Zhao et al., 2014; Zhang et al., 2016). There is evidence that more than 50% of the land will be salinized by 2050 (Wang et al., 2003). The salinity of soil severely restricts the rate of land-use and crop productivity (Wang et al., 2016). Therefore, understanding the molecular regulating mechanism of salt stress is important.

It is well known that salt causes soil salinity, and high salinity causes ion toxicity and osmotic stress (Zhu, 2002). Salt stress can also inhibit protein synthesis, reduce the rate of photosynthesis and respiration, and promote the loss of cellular integrity (Munns, 2002). During the course of evolution, plants have developed complex mechanisms to resist salt stress, including reducing their growth rate or adjusting their osmotic balance (Munns and Gilliham, 2015). Plants have adopted two strategies: one is the exclusion of Na^+ and/or Cl^- depending on the organic solutes available for balancing osmotic pressure, while another strategy is storing Na^+ in vacuoles to prevent salt entry or to minimize its concentration in the cytoplasm (Shabala, 2013). Indeed, osmotic regulation can raise osmotic pressure in the cytoplasm thus maintaining water absorption and cell turgor pressure (Blum et al., 1983; Tiwari et al., 2010).

Previous studies have found osmotic regulators mainly include proline, free amino acids, soluble proteins, sugars or organic acids (Rahnama and Ebrahimzadeh, 2004; Hajlaoui et al., 2010). The amount of organic acid is high in plants and plays an important role in many kinds of metabolic processes as well as the regulation of ionic balance and pH. Among these organic acids, malic acid plays an important role in different plants and plays an important role in maintaining cell turgor and pH balance in the cytoplasm (Kovermann et al., 2007; Wu and Chen, 2016). In addition, organic acid can not only participate in photosynthesis but can also be involved in mechanisms such as regulating osmotic pressure, nutrient stress and metal stress (Lo´pez-Bucio et al., 2000). Meanwhile, the main organic acids, including malic acid, oxalic acid, citric acid and succinic acid, especially malic acid also play a vital role in the tolerance of salt stress (Guo et al., 2010). For example, results have shown that large amounts of malic acid can accumulate under salt stress to adapt to a high-salt environment in *Mesembryanthemum crystallinum* (Cushman et al., 1989). In *Leymus chinensis*, the content of malic acid changes significantly in both leaves and roots under saline-alkali stress (Liu et al., 2014). Moreover, the content of malic acid in *Belamcanda chinensis* first increased and then decreased in leaves, while the malic acid concentration decreased in roots in a high salt environment (Duan, 2014). How plants regulate the accumulation of malic acid under salt stress is not very clear.

Al-activated malate transporters (ALMTs) play an important role in malate accumulation and transport. ALMTs localized to the plasma

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membrane have been discovered in wheat, *Arabidopsis* and rape (Sasaki et al., 2004; Hoekenga et al., 2006; Ligaba et al., 2006). They can also participate in metal toxicity avoidance, mineral nutrition, ion homeostasis, turgor regulation, fruit quality, and guard cell function. A recent study found that *SlALMT9* in tomato regulates malate accumulation in both fruit and root tissue, thus improving both fruit quality and Al tolerance (Ye et al., 2017). In grapes, the overexpression of *VvALMT9* can up-regulate the content of malate and tartrate in the vacuole of grape berries, thereby affecting berry development and wine quality as well as production (Conde et al., 2007; De Angeli et al., 2013a). Vacuolar malate channels were observed in *Arabidopsis* such as the gene *AtALMT9* (Kovermann et al., 2007). Regarding anion homeostasis, studies have found that the gene *ZmALMT1* in maize encodes a protein localized to the plasma membrane and mediates anion influx and efflux rather than participating in Al-activated organic acid exudation. *ZmALMT2*, however, encodes a plasma membrane-localized protein that also plays an important role in ion homeostasis (Piñeros et al., 2008a; Ligaba et al., 2012).

Additionally, ALMTs are involved in abiotic stress. In *Arabidopsis*, the *AtALMT* family has 14 members, most of which are relevant to Al tolerance. For instance, *AtALMT1* has been described to be involved in Al-tolerance (Hoekenga et al., 2006), as has *MsALMT1* from *Medicago sativa* and *HlALMT1* from the grass *Holcus lanatus* (Chen et al., 2013a,b). It was also reported that the AtALMT6 channel can be activated by cytosolic calcium and is regulated by vacuolar pH and cytosolic malate, which obviously demonstrates that *AtALMT6* plays an important role in stomatal opening and closure (Meyer et al., 2011). There was also study showed that AtALMT9 localized to the vacuole membrane of guard cells is a major vacuolar chloride channel and was related to the opening of stomata (De Angeli et al., 2013b). AtALMT12, different from AtALMT6 and AtALMT9, is targeted in the guard cell plasma membrane and is crucial for the closure of stomata (Meyer et al., 2010). It has been found that knockout mutants of *almt4* show impaired stomatal closure and increased water loss in response to ABA and are more sensitive to drought (Eisenach et al., 2017). The overexpression of *HvALMT1* shows a disruption stomatal closure phenotype and a reduced growth rate in comparison to WT plants (Gruber et al., 2011). *OsALMT1* in rice was reported to be related to salt and osmotic stress (Liu, 2016). In *Arabidopsis*, Mora-Macías et al found that *AtALMT1* responds to low Pi, thus affecting the accumulation of Fe in root (Mora-Macías et al., 2017). Salt and drought are main abiotic stresses that affect the growth of fruit trees in the natural environment, and there are researches indicate that *AlMT* genes such as *AtALMT4*, *AtALMT9* as well as *OsALMT1* can participate in regulating of salt and drought (De Angeli et al., 2013b; Liu, 2016; Eisenach et al., 2017), whether *MdALMT* genes in apple could regulate salt stress is unclear.

The apple tree is one of the most important fruit trees in temperate regions. Variable environmental factors severely impact apple fruit quality. Plants have developed a series of mechanisms to adapt to environmental changes, such as monitoring malate levels to alter the distribution and balance of malate. Here, the *MdALMT14* gene was cloned from the apple as a homolog of the *Arabidopsis* Aluminum-activated malate transporter gene *AtALMT14*. Its function in salt stress response was characterized in both *Arabidopsis* and apple calli.

2. Materials and methods

2.1. Identification and bioinformatics analysis of the ALMT genes in apples

The *AtALMT* genes from the *Arabidopsis* genome database (https://www.ncbi.nlm.nih.gov/) were obtained and then used as bait to search and download *MdALMT* genes from the Genome Database for Rosaceae (GDR) (http://www.rosaceae.org/). To analyze the genetic relationship of *ALMT* genes in the apple that were homologous with *Arabidopsis ALMT* genes, the DNA sequences of these genes were analyzed using the NJ method of the MEGA5 program to construct an unrooted phylogenetic tree, and the gene was named according to the phylogenetic tree.

The protein structure was analyzed using the EXPASY (https://web.expasy.org/protscale/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Subcellular localization of ALMT proteins were analyzed using PredictProtein (https://open.predictprotein.org/).

2.2. Plant material and treatments

For the gene expression analysis, tissue cultures of Malus × domestic cv. 'Royal Gala' apples were used as the wild type. The cultures were grown on MS medium supplemented with 1.0 mg/l naphthyl acetate (NAA) and 0.5 mg/l 6-benzylaminopurine (6-BA) at 25 °C under long-day conditions (16 h light/8 h dark). They were subcultured at 30-day intervals. For salt treatment, apple shoot cultures were cultivated on MS medium supplemented with 1.0 mg/l naphthyl acetate (NAA), 0.5 mg/l 6-benzylaminopurine (6-BA) and 200 mM NaCl for 0 h, 1 h,3h, 6 h respectively. The samples (both leaves and shoots) were then stored at −80 °C for future investigation. All of the samples were tested in three biological replicates. To investigate the salt-tolerance of transgenic *Arabidopsis*, NaCl treatment was implemented. Wild-type and transgenic *Arabidopsis* seeds were seeded into MS culture medium and 4 days later, the seedlings were transferred to MS, MS + 100 mM NaCl media. Ten days later, the phenotypes were observed. To further study the salt tolerance of *MdALMT14*, after germination under normal conditions for 20 days, then plants were flooded for with NaCl solution every 3 days for 25 days, incrementally increasing with each successive watering from 100 to 150, 200, and 250 to a final concentration of 300 mM NaCl. The resulting calli from WT and transgenic lines were grown for 15 days and were then treated with MS, MS + 50 mM NaCl, MS + 100 mM NaCl, MS + 150 mM NaCl media. Fifteen days later, we measured the relevant indices, such as the fresh weight and the contents of both MDA and malic acid.

2.3. Extraction of RNA, qRT-PCR and RT-PCR

The total RNA was extracted using the RNA plant plus Reagent kit (TianGen, Beijing, China) according to the manufacturer's instructions. The reverse transcription products, which were derived from the PrimeScript® RT reagent kit (Perfect Real Time, TaKaRa), were used for qRT-PCR and RT-PCR.

For the semi-quantitative RT-PCR, the reactions were conducted according to the method described in Ma et al. (2017). The cycling parameters were as follows: pre-incubation at 95 °C for 5 min, followed by 30 cycles of 95 °C (20 s), 58 °C (20 s), and 72 °C (20 s), with a final extension at 72 °C for 10 min. The primers used in these reactions are listed in Table S1.

For the real-time qRT-PCR analysis, we applied the iQ SYBR Green Supermix in an iCycler iQ5 system (Bio-Rad, Hercules, CA, USA) to the reactions for qRT-PCR, following the manufacturer's instructions. The specific mRNA levels were analyzed by relative quantification using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table S1.

2.4. Length of roots, fresh weight, and content of MDA

To measure the length of taproot, the software package MBF from ImageJ was applied, and each measurement obtained had three biological replicates and three mechanical replicates.

To detect the concentration of malondialdehyde (MDA), the leaf tissue (0.1 g for seedlings and 0.5 g for plants and calli) was ground in 5 ml 0.2 M phosphate buffered saline and then centrifuged at 12,000*g* for 5 min. The supernatant was transferred to a new centrifuge tube, 5 ml 0.5% thiobarbituric acid (TBA, in 10% TCA) was added, and then it was moved to a boiling water bath for 10 min, followed by cooling the