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Research article

Ectopic expression of apple hexose transporter *MdHT2.2* reduced the salt tolerance of tomato seedlings with decreased ROS-scavenging ability

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ABSTRACT

Salt is one of the main stresses that limit plant growth, especially at the seedling stage, reducing crop production and severely impacting food security. However, the relationship between salt stress and sugar content regulated by sugar transporters remains unknown. Here, we investigated the salt tolerance of transgenic tomato seedlings ectopically expressing *MdHT2.2*, which is a fructose and glucose/H^+ symporter located on the plasma membrane in apple. Although the contents of fructose, glucose and sucrose in the leaves of seedlings ectopically expressing *MdHT2.2* obviously increased compared with those of WT seedlings, the transgenic seedlings were significantly less tolerance to salt stress. Under salt stress, the *SlSOS1/2* and *SlNHX1* genes were highly expressed, and the accumulation of Na^+ was lower in the transgenic seedlings than in WT, however, ROS accumulated to a greater degree in the former, and the ROS-scavenging-related enzyme activities and AsA content were lower in the transgenic seedlings than WT. Taken together, these results indicated that the relatively low salt tolerance of the *MdHT2.2* transgenic seedlings was related with the accumulation of ROS, which was caused by reduced ROS-scavenging ability. Our results offer proof that changes in sugar content caused by sugar transporters are related to salt tolerance, and provide new insight into the regulation of sugar content, quality improvement and stress tolerance.

1. Introduction

Soil salinity is one of the most harmful abiotic stresses, limiting plant growth, especially at the seedlings stage, reducing crop production and severely impacting food security. Currently, inadequate manual irrigation has led to water and soil salinization (Ouhibi et al., 2014; Slama et al., 2015). In turn, high salinity leads to hyperosmotic conditions, which impede the ability of plant to absorb water and nutrients from the soil, and impact seed germination, root and shoot development and even yield (Cuartero and Fernandez-Munoz, 1999; Ismail et al., 2014).

Salt stress induces ionic stress, osmotic stress and secondary stress (especially reactive oxygen species (ROS)) (Qi et al., 2017). To defend against the stress, plants employ a series of mechanisms to survive. The first mechanism involves ROS-scavenging. ROS is most harmful secondary stress to plants, which damage the cellular structure, and it must therefore be scavenged rapidly by several enzymes (Zhu, 2001), such as catalase (CAT), peroxidase (POD), superoxidase (SOD), ascorbate peroxidase (APX), and several antioxidant substances, such as ascorbic acid (AsA) and glutathione. The second mechanism involves the reestablishment of homeostasis, as the ionic and osmotic balance must be reestablished for water and nutrient uptake. For Na^+ homeostasis, decreasing the Na^+ content in the cytoplasm prevents damage to enzyme activities and other processes. In the salt overly sensitive (SOS) signaling pathway, *SOS1* which is a Na^+/H^+ antiporter located on the plasma membrane, exports Na^+ from the cytoplasm to reduce excess the Na^+-induced damage (Yang et al., 2009). *NHX1*, which is a Na^+/H^+ antiporter, located on the vacuolar membrane, transports Na^+ into the tonoplast, which is beneficial for decreasing the osmotic potential compared with that of the extracellular space (Xiong and Zhu, 2002; Chinnusamy et al., 2006). To counteract osmotic stress, as well as to

Abbreviations: HT, hexose transporter; WT, wild type; Suc, sucrose; Fru, fructose; Glc, glucose; ROS, reactive oxygen species; SOS, salt overly sensitive; NHX, Na^+-H^+ exchanger; REC, relative electrical conductivity; RWC, relative water content; MDA, malondialdehyde; CAT, catalase; POD, peroxidase; APX, ascorbate peroxidase; SOD, superoxidase; AsA, ascorbic acid.

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achieve ionic homeostasis, plants accumulate compatible osmolytes (polyols such as mannitol and sorbitol; sugars such as hexoses, sucrose (Suc) and trehalose; ions such as K^+) to decrease the osmotic potential to allow water to be taken up from saline soils (Zhu, 2001; Yang and Guo, 2018).

As important osmolytes, soluble sugars increase under salt stress, which has been observed in rice (Cha-um et al., 2009), poplar (Janz et al., 2010), *Arabidopsis* (Yamada et al., 2011; Gong et al., 2015; Sellami et al., 2019), tomato (Yin et al., 2010) and apple (Yang et al., 2019). While the increasing sugar content is due to the metabolism and transport of sugar, its accumulation in plant cells is highly regulated by sugar transporters (Li et al., 2018), and altering sugar transporter expression/activity can alter the sugar content impacting stress tolerance. Located on the plasma membrane, the *Arabidopsis* sucrose transporter *AtSUC2/4* functions in Suc phloem loading, *atsuc2* and *atsuc4* knockout seedlings present increased Suc content in the shoots but decreased content in the root, and the mutant seedlings were hypersensitive to a variety of stresses, such as NaCl, drought and cold (Gong et al., 2015). These phenomena was also observed for *atsuc9* T-DNA insert mutant seedlings, which presented higher Suc contents in the shoots but lower Suc contents in the root, and reduced salt resistance (Jia et al., 2015). In addition, *Arabidopsis* tonoplast sugar transporters abundance and activity on vacuolar membrane increase to accumulate increased amounts of sugar in the vacuole to defense against stress (Schulze et al., 2012). There are also reports that *AtTST1/2* expression were induced by stress, and *AtTST1* knockout plants had a lower hexose content and were less resistant to cold stress (Wormit et al., 2006). Similar finding have also been reported in rice, after the expression of *OsGMST1*, which encodes a Golgi-localized monosaccharide transporter, was reduced, the transgenic seedlings were hypersensitive to salt (Cao et al., 2011).

On the basis of all the studies above, it is easy to see that the salt stress can facilitate sugar accumulation and improve tolerance to salt stress, similarly, decreasing the expression of sugar transporters could decrease the sugar content as well as the tolerance. However, those reports were based on the knocking-out of sugar transporters (those located on either the plasma membrane or the vacuolar membrane) and involved mostly Suc, the tolerance of plants overexpressing sugar transporters (especially hexose transporters (HTs) has rarely studied, despite HTs possibly increasing the hexose content (McCurdy et al., 2010; Slewinski, 2011; Wang et al., 2020), which would be beneficial to salt tolerance. Thus, we hypothesized that increasing the expression of HTs would increase the hexose content and enhance salt tolerance.

To determine the tolerance of hexose increasing plants that accumulated large amounts of hexose vis HT overexpression, we used transgenic tomato seedlings ectopically expressing *MdHT2.2* (Wang et al., 2020), MdHT2.2 functions as an energy-dependent, low-affinity monosaccharide/H^+ symporter specific for fructose (Fru) and glucose (Glc), and compared with WT seedlings, seedlings ectopically expressing *MdHT2.2* presented a series of beneficial agronomic traits, such as early flowering, dwarf, stature, large fruits, high seed numbers, increased soluble solid content (SSC) and increased hexose contents in the fruit. Despite all the beneficial agronomic traits of the transgenic plants, their ability to resist stress remains unclear. Therefore, we treated tomato plants with 150 mM NaCl at the seedling age to investigate their salt tolerance. Compared with those in the WT seedlings the sugar contents in the transgenic seedlings were greater, as well as the relative electrical conductivity (REC), but relative water content (RWC) and the salt tolerance were lower. Afterwards, we measured the Na^+ and K^+ content, and the results showed lower amount of Na^+ accumulated in the transgenic seedlings than in WT. We then measured the ROS contents and the ROS-scavenging-related enzyme activities, gene expression levels and antioxidant content, and the results revealed that, compared with the WT seedlings, the transgenic seedlings accumulated more ROS and had a lower ROS-scavenging ability. All the results indicated that the sugar content changes caused by *MdHT2.2* ectopic expression related with the ability of the ROS-scavenging system to perceive salt stress. This article offers proof that changes in the sugar content caused by sugar transporters are related with salt tolerance, and provides new insight into the regulation of sugar content, quality improvement and stress tolerance.

2. Materials and methods

2.1. Plant material

Homozygous *MdHT2.2* transgenic tomato seedlings (L5, L11, L12) were used, which were first described in our previous work (Wang et al., 2020). Tomato (*Solanum lycopersicum* cv. Micro-Tom) seedlings were grown in a growth chamber at 70% relative humidity under 16 h of light and 140 μmol photons/m^2/s at 25 °C and under 8 h of darkness at 22 °C. The seedlings were watered regularly and supplied with half-strength Hoagland's nutrient solution once a week to maintain healthy growth. To induce salt stress, 30-day-old tomato plants were irrigated with 150 mM NaCl. Samples of the mature leaves were harvested 0 day before treatment (DBT) and at 3, 5 and 7 days after treatment (DAT), with at least three biological replicates included per experiment.

2.2. Salt stress tolerance and antioxidant analysis

The fresh weight (FW) of the mature leaves was measured, after which they were immersed in distilled water for 24 h to measure the full turgid weight (TW), and then oven dried at 70 °C for 72 h; afterwards, the dry weight (DW) was measured to calculate the relative water content (RWC) (Levine et al., 1994; Sun et al., 2018). The relative electrical conductivity (REC) in the leaves was calculated according to the method of Thalhammer et al. (2014). In addition, malondialdehyde (MDA) level were measured as previously described (Heath and Packer, 1968). The contents of Na^+ and K^+ were measured with a flame photometer after they were ashed in a muffle furnace and digested with H_2SO_4. The H_2O_2 and O_2^- contents were measured according to the method of Chen et al. (2013).

Diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining was preformed to analysis the H_2O_2 and O_2^- contents, respectively. Fresh leaves were immersed in DAB solution (1 mg/ml DAB in 0.02 mol/L PBS) or NBT solution (1 mg/ml NBT in 0.02 mol/L PBS), incubated at 25 °C in darkness for 8 h, destained with 95% ethyl alcohol at 80 °C for 2 h, and then stored in 50% glycerol (Wang et al., 2015).

Tomato mature leaves (0.1 g) were ground with 1.2 ml of ice-cold buffer consisting of 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA-Na_2, 0.3% Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 13,000 g for 20 min at 4 °C, after which the supernatant was used for the assays as Rangani et al. (2016) described. The activity of CAT was analyzed with the reaction mixture (50 mM potassium phosphate (pH 7.0) and 10.5 mM H_2O_2) by measuring the initial linear rate of the decrease in absorbance at 240 nm due to the disappearance of H_2O_2, one unit of CAT activity was defined as 1 mg protein catalytic decomposition 1 μM H_2O_2 in 1min. The activity of POD was analyzed with the reaction mixture (50 mM potassium phosphate (pH 7.0), 9 mM guaiacol, and 19 mM H_2O_2) by measuring the formation of tetraguaiacol at 470 nm, one unit of POD activity was defined as 1 mg protein catalytic produce 1 μM tetraguaiacol in 1min. SOD activity was measured by its ability to inhibit photoreduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM KPO4 (pH 7.8), 9.9 mM L-methionine, 58 μM NBT, 0.025% Triton X-100, 2.4 μM riboflavin. The increase in absorbance due to formazan formation was read at 560 nm. Reaction mixture with no enzyme developed maximum color because of the maximum rate of reduction of NBT. One unit of SOD activity was defined as the amount of enzyme that inhibits 50% NBT photoreduction. The activity of APX was analyzed with the reaction mixture (50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM H_2O_2) by measuring the initial linear rate of the decrease in absorbance at 290 nm due to the

disappearance of AsA, one unit of APX activity was defined as 1 mg protein catalytic oxidate 1 μM AsA in 1min.

The content of AsA was extracted by 6% TCA, and measured with the reaction mixture (0.2 M phosphate buffer (pH 7.4), 10% TCA, 42% H_3PO_4, 4% 2, 2′-dipyridyl dissolved in 70% ethanol, 3% $FeCl_3$) and measuring absorbance at 550 nm after incubating at 42 °C for 40min (Kampfenkel et al., 1995).

2.3. Sugar content measurements

As previously described (Li et al., 2018), soluble sugars and hexose phosphates were extracted in 75% methanol to which ribitol was added as an internal standard and then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide. After derivatization, the metabolites were analyzed via a Shimadzu GCMS-2010SE (Shimadzu Corporation, Tokyo, Japan) with a DB-5MS capillary column (20 m × 0.18 mm × 0.18 μm) and a 5-m Duraguard column (Agilent Technology, California, USA).

2.4. RNA extraction and qRT-PCR

Total RNA was isolated from frozen samples, and cDNA was synthesized via PrimeScript^{TM}II Reverse Transcriptase (Takara, Dalian, China). Gene-specific primers sequence were retrieved from the NCBI database and examined via qRT-PCR and melting curve analysis. The PCR products were quantified by a LightCycler® 96 real-time PCR detection system (Roche, Basel, Switzerland) in conjunction with Light Cycler Ultra SYBR Mixture (CWBIO, Beijing, China). *SlActin* was used for the normalization of target gene transcripts via the $2^{-\triangle\triangle Ct}$ method. The primers used are listed in Supplementary Table 1.

2.5. Statistical analysis

All the data were analyzed by IBM SPSS Statistics 21 (IBM, California, USA) and graphed with Sigma Plot 12.0 (Systat software, California, USA) software. The data were analyzed by independent t-tests at a significance level of $p \leqq 0.05$. The values are presented as the means ± standard errors (SEs) of at least three biological replicates per measurement.

3. Results

3.1. Transgenic tomato seedlings displayed decreased salt stress tolerance

Our previous work showed that MdHT2.2 is a typical HT that is an energy-dependent, monosaccharide/H^+ symporter specific for Fru and Glu, and that MdHT2.2 is located on the plasma membrane and is highly expressed in mature apple fruits (Wang et al., 2020). *MdHT2.2* was highly expressing in mature fruits, but the *SlHT1/2/3* were highly expressed in roots, leaves, and young fruits in tomato plants (Fig. S1), and they share about 54.42%, 56.68% and 60.83% sequence identity to *MdHT2.2*, respectively. To determine the salt tolerance of HT overexpression plants, we used homozygous tomato plants ectopically expressing *MdHT2.2* (L5, L11, L12, Fig. S1), whose fruits presented increased soluble solid contents and increased hexoses contents. We irrigated all the plants with 150 mM NaCl, and the plant growth status was similar for all plant types, expect the dwarf transgenic seedlings before treatment, but at 7 DAT, the leaves of the transgenic seedlings were severely wilted (Fig. 1A). Afterwards, we measured the REC, RWC and MDA content in the mature leaves, which are typical indicators used to evaluate salt damage. Under normal conditions, these indexes did not significantly differ between the WT seedlings and the transgenic seedlings. However, after treatment with NaCl, the REC of the transgenic seedlings was approximately 5%, 16% and 14% greater than that of the

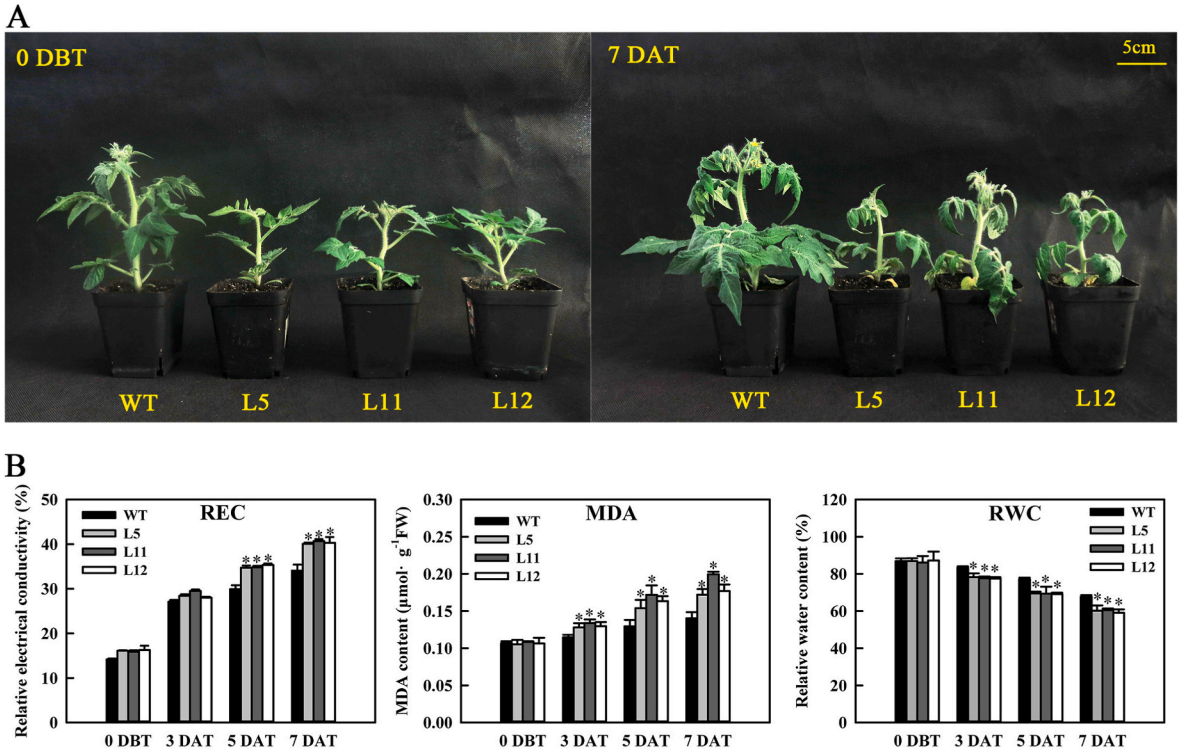

Fig. 1. Salt stress tolerance evaluation. All the plants (transgenic seedlings: L5, L11, L12; wild type: WT) were treated with 150 mM NaCl. Sample were collected at 0 day before treatment (DBT) and 3, 5, 7 day after treatment (DAT). (**A**) Phenotype of all plant types at day 0 and day 7, yellow bar = 5 cm. (**B**) Evaluation of the relative electrical conductivity (REC), malondialdehyde content (MDA) and relative water content (RWC) under salt stress. The different colors indicate different seedlings. The bars represent the mean values±SEs ($n \geqq 3$). The asterisk indicates a significant difference compare with WT on that day, $p \leqq 0.05$.

WT seedlings at 3, 5, 7 DAT, respectively. The MDA content also increased in all plant types after treatment, but the increase in the transgenic seedlings was much greater than that in the WT seedlings. Additionally, the RWC of the leaves of the transgenic seedlings was lower than that of the WT seedlings under salt stress (Fig. 1B). Taken together, these results revealed that the salt tolerance of the transgenic seedlings was lower than that of WT.

3.2. Changes in sugar content and metabolism of tomato plants under salt stress

As our previous work indicated, the hexose content of the fruits of seedlings ectopically expressing *MdHT2.2* increased. In the leaves of the transgenic seedlings, the Glc and Fru contents were approximately 20% greater than those in the WT seedlings under normal conditions (Fig. 2), and the expression levels of the genes encode the plasma membrane sugar transporters *SlHT* and *SlSUT*, which take up hexoses/Suc from the apoplast into cells, were lower in the transgenic seedlings than in the WT seedlings, while the expression of the vacuolar membrane sugar transporter *SlTST1/2* were greater. With respect to Suc invertase enzymes, there were differences in the various of cellular compartments. The expression of cell wall invertase (*SlLIN6*), which is located on the cell wall and cleaves Suc into hexoses, was greater in the transgenic seedlings than in the WT seedlings, but the expression of neutral invertase (*SlNINV*) and tonoplast invertase (*SlTIV*), which are located in the plasma membrane and vacuolar membrane, respectively, was lower in the transgenic seedlings. With respect to sugar metabolism, the expression of Suc phosphate synthase (*SlSPS*), which synthesizes Suc, was greater in the transgenic seedlings than in the WT seedlings, but the expression of Suc synthase (*SlSUSY1*), which cleaves Suc, was lower in the transgenic seedlings. The expression of sugar signaling-related sugar metabolism kinases *SlHK* and *SlFK* was lower in WT seedlings, but that of *SlSnRK* was the opposite (Fig. 3).

After treatment, the sugar content of all the seedlings increased, and the sugar content in the WT seedlings reached that of the transgenic seedlings and gradually continued to increase. At 7 DAT, there were no significant differences between any of the seedlings (Fig. 2). In addition, the transcript levels of sugar metabolism- and transport-related genes continued to increase after treatment, and the expression of the sugar transporter genes *SlHT* and *SlSUT* in the transgenic seedlings reached that in the WT seedlings after being significantly lower, the results of which were opposite those of *SlTST1/2*. While the expression of the Suc cleavage gene of *SlLIN6* in the cell wall of the WT seedlings presented a greater rate of increase than did that of the transgenic seedlings, at 7 DAT, there were no significant differences between the plant types, but the expression of the other cleavage-related genes, *SlNINV* and *SlSUSY1* increased faster in the transgenic seedlings. Interestingly, the expression of *SlTIV* in the transgenic seedlings was lower at 0 DBT, but greater at 3 DAT and remained greater than that in WT. With respect to the sugar metabolism-related kinases, all the expression levels increased and remain greater in the transgenic seedlings compared with the WT seedlings (Fig. 3). These results indicated that the damage caused by osmotic stress to the transgenic lines was less than that of WT.

3.3. The Na^+ and K^+ contents in transgenic tomato plants under salt stress

While the osmotic stress caused less damage to the transgenic seedling than to the WT, to detect whether ion stress led to a decrease in salt tolerance in the transgenic seedlings, we next measured the Na^+ and K^+ contents and the expression of the Na^+ transport-related genes *SlSOS1/2* and *SlNHX1*. The results showed that the Na^+ and K^+ contents were not significantly different between the plants at 0 DBT. After treatment, the Na^+ content and Na^+/K^+ ratio were lower in the transgenic seedlings than in WT, while the K^+ content was greater, although they continued to increase like in the WT seedlings after treatment (Fig. 4). This was due to the increased expression of *SlSOS1/2* and *SlNHX1*, which encode transporters that reduce the cytoplasmic Na^+ content to alleviate cell damage (Ma et al., 2012).

3.4. Increased ROS content in transgenic seedlings under salt stress

Both the osmotic stress and ionic stress seemed less harmful to the transgenic seedlings than to WT, and the decreased salt tolerance was hypothesized to be, because ROS severely injured the transgenic seedlings. The H_2O_2 and O_2^- contents accumulated to much greater levels in

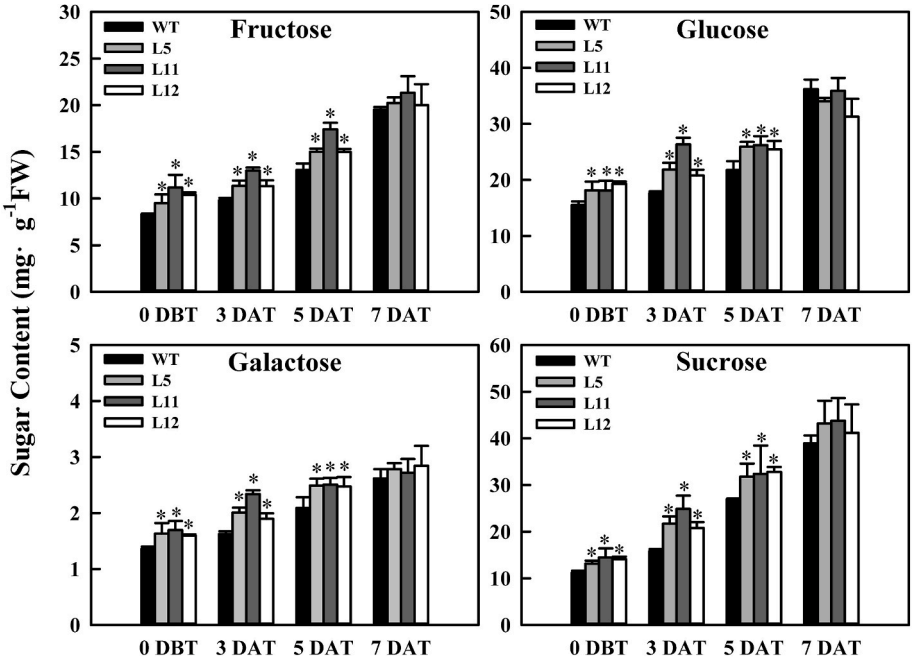

Fig. 2. Changes in sugar content under salt treatment. The different colors indicate different seedlings. The bars represent the mean values±SEs (n ≧ 3). The asterisk indicates a significant difference compare with WT on that day, p ≦ 0.05.

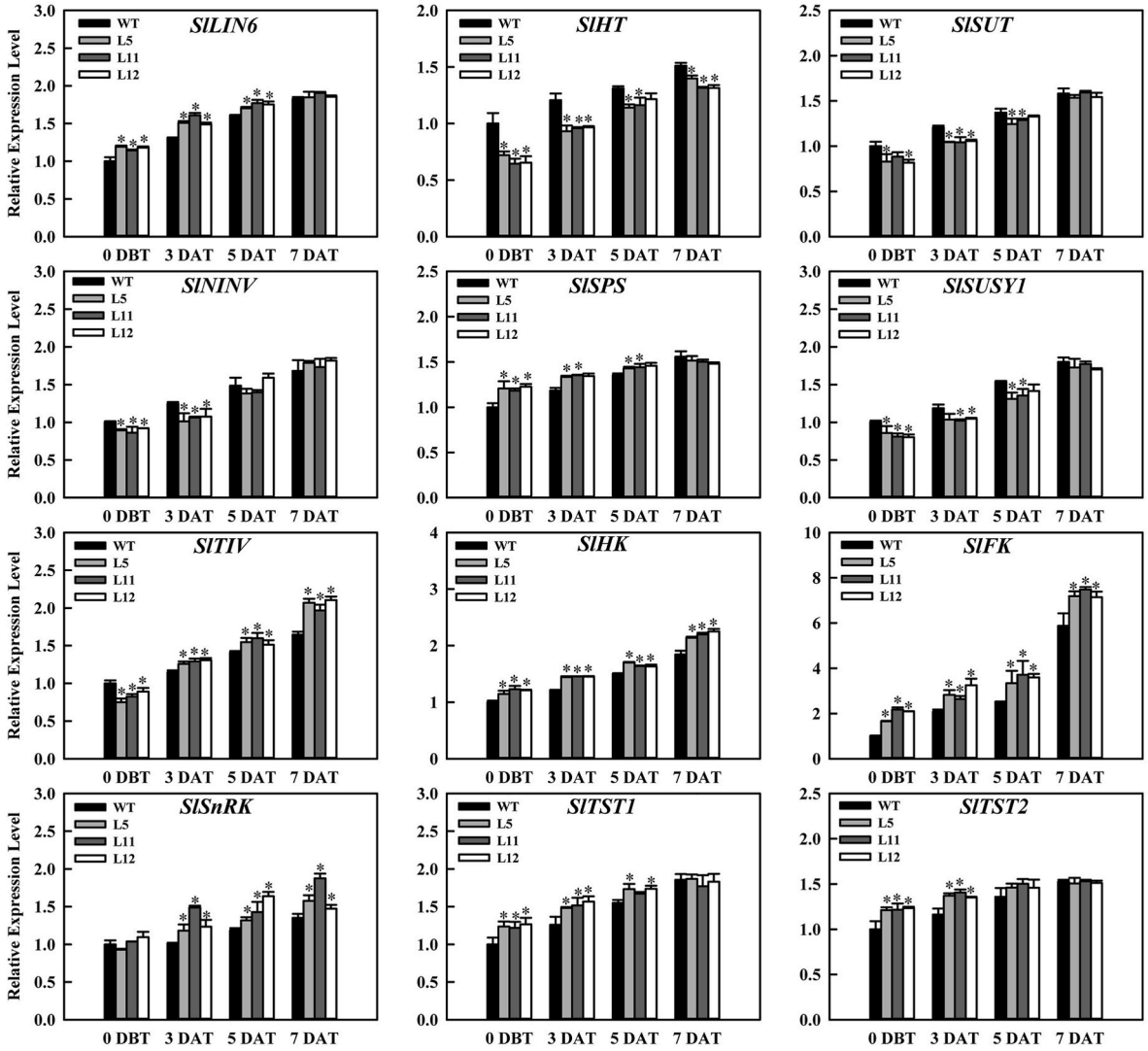

Fig. 3. Gene expression of sugar metabolism- and transporter-related genes. The gene expression levels was calculated relative to that of *SlActin*, and the expression in the WT seedlings in 0 DBT was set to 1. The different colors indicate different seedlings. The bars represent the mean values±SEs ($n \geqq 3$). The asterisk indicates a significant difference compare with WT on that day, $p \leqq 0.05$.

the transgenic seedlings than in WT after treatment and continuously increased with increasing stress, the values were approximately 11%–14% and 33%–50% greater in transgenic seedlings than in WT seedlings, respectively, after treatment (Fig. 5A). DAB and NBT histochemical staining, which were used to determine the H_2O_2 and O_2^- contents, respectively (Fig. 5B and C), was consistent with the ROS contents.

3.5. Decreased ROS-scavenging system in transgenic seedlings

To investigate why the ROS accumulated to a greater degree in the transgenic seedlings than in WT, we measured ROS-scavenging-related enzyme activities and gene expression and the antioxidant-AsA content (Figs. 6 and 7). The enzyme activities, gene expression and AsA content were not significantly different between either plant types at 0 DBT. After salt treatment, although the enzyme activities, gene expression and AsA content in the transgenic seedlings continued to increase, they remained obviously lower than that those in the WT seedlings, and decreases by approximately 20%–25%. This indicates that the ROS accumulation was greater in the transgenic seedlings than in WT, while the ROS-scavenging ability was lower.

Taken together, all of the results above indicate that compared with the WT seedlings, the relative low salt tolerance of the transgenic seedlings was related to the higher ROS accumulating and lower ROS-scavenging ability.

4. Discussion

4.1. Ectopic expression of apple MdHT2.2 in tomato seedlings alters the ROS-scavenging ability under salt stress

Salt stress is complex, in that it induces ionic stress, osmotic stress and secondary stresses (especially ROS). Osmotic stress mainly increased the osmotic potential, which reduces water and nutrient uptake and photosynthesis because of stomatal closure and reduced turgor for expansion and growth (Flowers and Colmer, 2008; Flowers et al., 2015). In addition to osmotic stress, excess ions also cause decreases in enzyme activities and water loss (Hanin et al., 2016). Both ionic stress and osmotic stress can induce ROS (Zhu, 2001), and weak and temporary ROS signaling in turn induce plant responses to stress, however strong and continuous ROS severely damage macromolecular substances such as lipids, proteins and nucleic acids, leading to changes in lipid fluidity and membrane transport ability, impeding protein synthesis and enzyme activities, and ultimately causing cell death (Dangol et al., 2019).

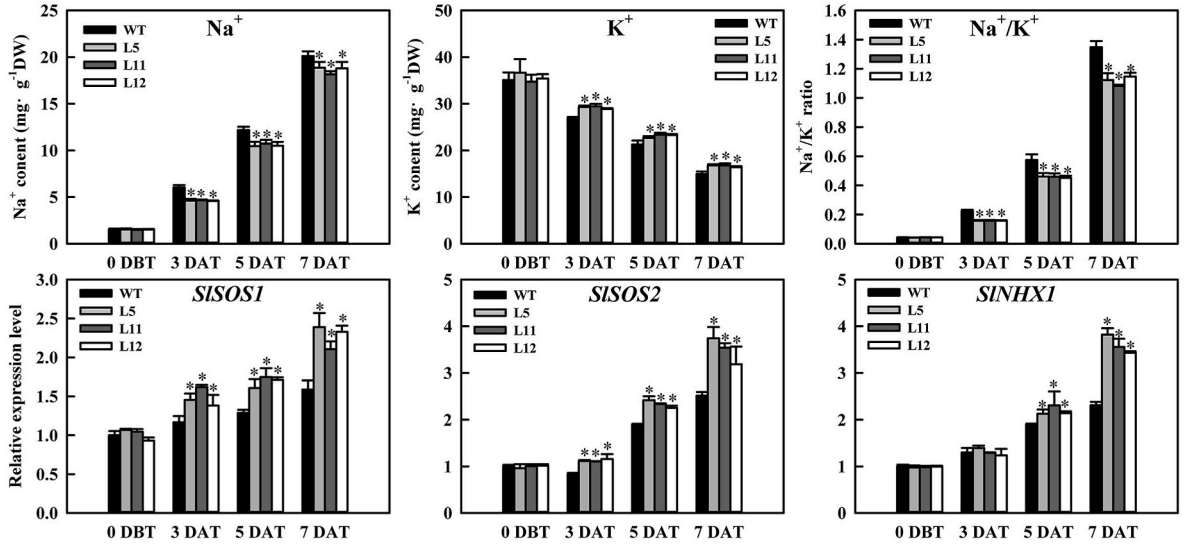

Fig. 4. Ionic stress response under salt treatment. Na^+ and K^+ contents were measured by a flame photometer, the gene expression was calculated relative to that of *SlActin*, and the expression in the WT seedlings in 0 DBT was set to 1. The different colors indicate different seedlings. The bars represent the mean values±SEs (n ≧ 3). The asterisk indicates a significant difference compare with WT on that day, p ≦ 0.05.

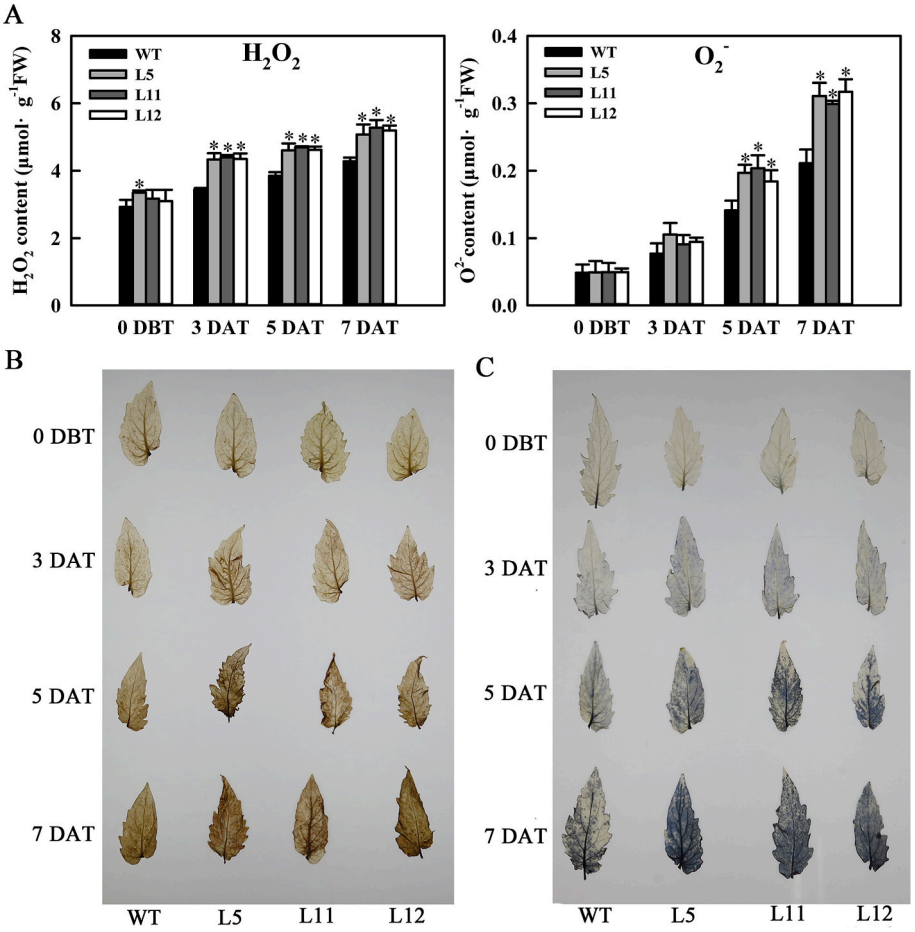

Fig. 5. Changes in ROS content under salt treatment. (**A**) Content of H_2O_2 and O_2^-. The different colors indicate different seedlings. The bars represent the mean values±SEs (n ≧ 3). The asterisk indicates a significant difference compare with WT on that day, p ≦ 0.05. (**B**) H_2O_2 staining. (**C**) O_2^- staining.

After we evaluated the salt tolerance by measuring the REC, RWC and MDA content, the results revealed that the transgenic seedlings were less salt tolerance than the WT seedlings (Fig. 1), and the sugar content in the transgenic seedlings was greater than that in the WT seedlings (Fig. 2). These results were opposite those from previous reports that declared that increasing the expression of sugar transporters could increase the sugar content, which was beneficial for the salt tolerance (Yamada et al., 2011; Gong et al., 2015). At 0 DBT, the sugar

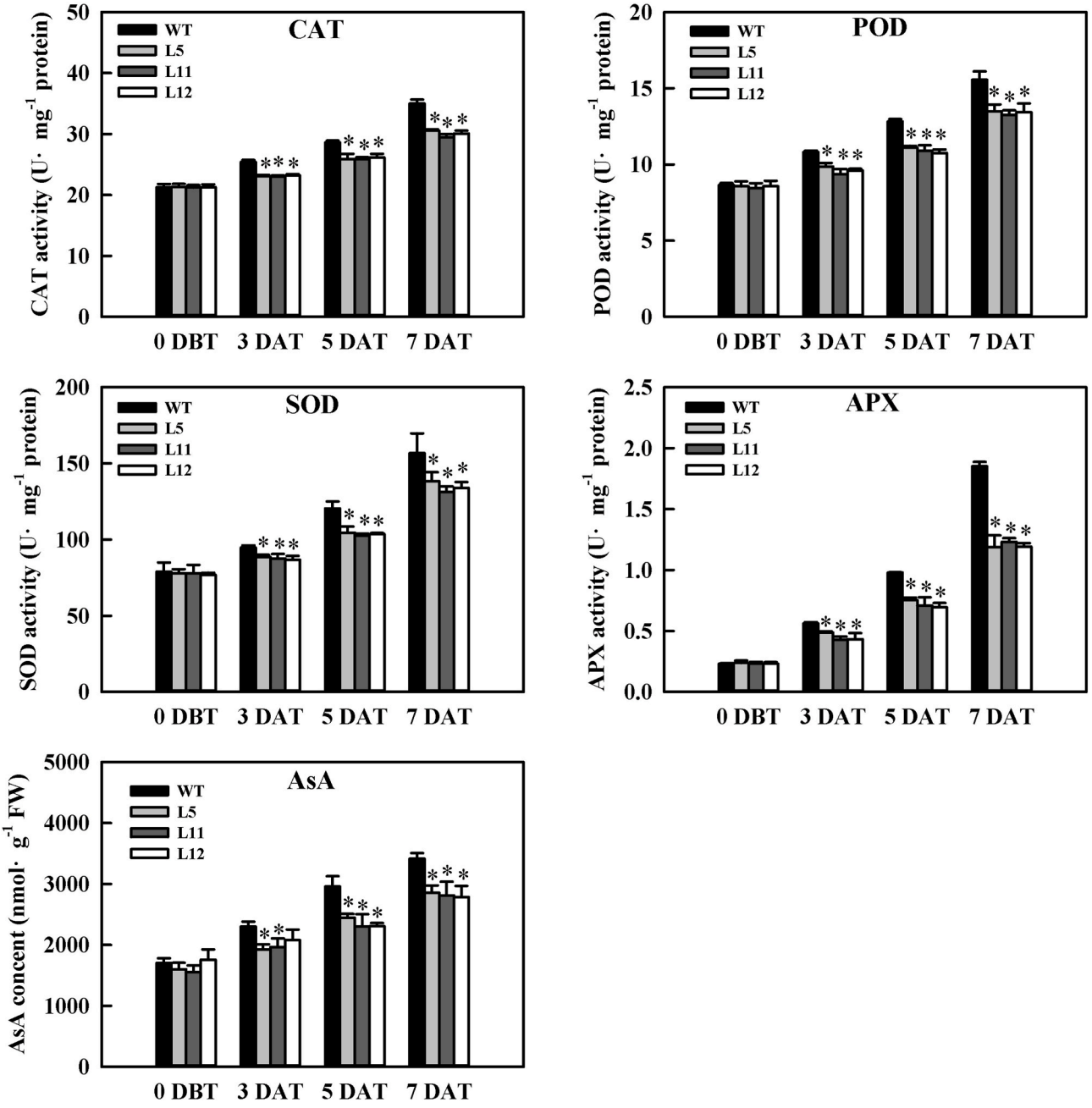

Fig. 6. Enzyme activities of the ROS-scavenging system. The activities of CAT (catalase), POD (peroxidase), APX (ascorbate peroxidase), SOD (superoxidase) and the content of AsA (ascorbic acid). One unit of CAT activity was defined as 1 mg protein catalytic decomposition 1 μM H_2O_2 in 1min. One unit of POD activity was defined as 1 mg protein catalytic produce 1 μM tetraguaiacol in 1min. One unit of SOD activity was defined as the amount of enzyme that inhibits 50% NBT photoreduction. One unit of APX activity was defined as 1 mg protein catalytic oxidate 1 μM AsA in 1min. The different colors indicate different seedlings. The bars represent the mean values±SEs (n ≧ 3). The asterisk indicates a significant difference compare with WT on that day, p ≦ 0.05.

concentration of WT was lower than transgenic lines, but at 7 DAT, there were no significantly difference between all the plant types, that might because the salt stress makes the leaves wilting, which inhibited the photosynthetic capacity in transgenic lines, lead to the reduction of the sugar accumulation.

We then measured the ion content to estimate the damage to cells, and the results showed that the damage caused by Na^+ was less extensive in the transgenic seedlings than that in WT (Fig. 4). Afterwards, we measured the H_2O_2 and O_2^- contents, which are typical ROS and hinder cell growth. The ROS content was much greater in the transgenic seedlings after treatment compared with that in normal conditions (Fig. 5). Taken together, all of the above results revealed that the reason for the decrease in tolerance was related to the increase in ROS content. To determine why this happened, we measured activities of the enzyme and expression levels of the genes involved in the ROS-scavenging system. The results showed that all the enzyme activities and gene expression levels significantly decreased in the transgenic seedlings compared with the WT seedlings (Figs. 6 and 7), which means that compared with the WT seedlings, the relative low salt tolerance of the transgenic seedlings was related to the higher ROS accumulating and lower ROS-scavenging ability.

Although seedlings ectopically expressing *MdHT2.2* presented greater hexose contents than did the WT seedlings, the former were less salt tolerance. Interestingly, with the salt treatment, the expression level

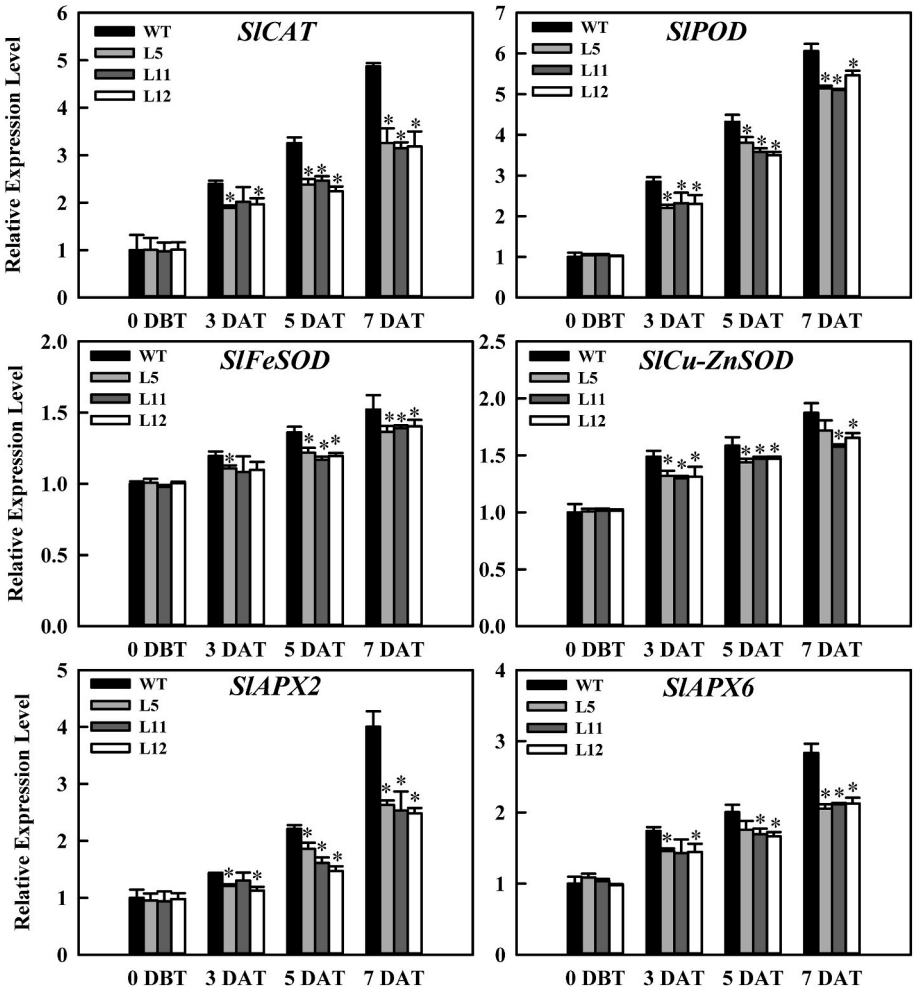

Fig. 7. Expression levels of genes encoding ROS-scavenging enzymes. The expression of *SlCAT*, *SlPOD*, *SlAPX* and *SlSOD* was calculated relative to that of *SlActin,* and the expression in the WT seedlings in 0 DBT was set to 1. The different colors indicate different seedlings. The bars represent the mean values±SEs (n ≧ 3). The asterisk indicates a significant difference compare with WT on that day, p ≦ 0.05.

of *MdHT2.2* keeps increasing to accumulate more sugar in apple seedlings (Fig. S1C), it might be a kind of stress response to reduce damage caused by osmotic stress (Zhu, 2001). Similarly, the *SlHT* expression level in ectopic lines was increasing as WT, despite the transcript level of *SlHT* in the transgenic lines keeping lower than WT. However, the *MdHT2.2* ectopic expression seedlings showed lower salt stress resistance, that might be related with the fact that the CaMV35S driven-MdHT2.2 constitutively expressed in whole plant. This altering sugar homeostasis might cause intracellular/extracellular sugar signaling change (Couee et al., 2006; Xiang et al., 2011; Liu et al., 2013), and lead to decreased ROS-scavenging ability in the transgenic lines.

4.2. Altered sugar contents result from changes in the expression of sugar metabolism-related genes under salt stress

The content of sugar can be determined by its metabolism and transport (Ruan, 2014; Li et al., 2018). Here, we analyzed sugar metabolism- and transporter-related gene expression to investigate the regulation of sugar content. The findings of the indicator *SlTST1*, whose expression is induced by Glc, and *SlTST2*, whose transport product mainly Suc, were consistent with the changes in sugar content (Fig. 3).

Under normal conditions, ectopically expressed *MdHT2.2* was homologous to *SlHTs*, which functions in the transport of hexoses from the apoplast to cells, and the high concentration of hexoses taken up by MdHT2.2 suppresses the expression of *SlHT* by a feedback mechanism (Fig. 3). The expression of *MdHT2.2* can upregulate the expression of *SlLIN* as reported in our previous research (Wang et al., 2020). The increased Suc content in the transgenic seedlings was due to excess hexose synthesis, which can be indicated by the increased expression of *SlSPS*, which is a gene involved in Suc synthesis, and the decreased expression of *SlNINV*, *SlSUSY1* and *SlTIV*, all of which are genes involved in Suc cleavage (Fig. 3), those results mean that Suc synthesis increased but that Suc hydrolysis decreased and that the downregulation of *SlSUT* expression was suppressed by a high Suc content.

As the treatment duration increased, the sugar content increased, which occurred as part of a stress defence mechanism to reduce the osmotic potential. However, at 7 DAT, there were no significant differences between either plant types (Fig. 2). With respect to the Suc content, under the conditions of similar expression of *SlSUT*, *SlSPS*, *SlNINV* and *SlSUSY1*, the main cause was the upregulated expression of *SlTIV*, whose product cleaves excess Suc. For similar hexose contents, the increased *SlHK* and *SlFK* expression may explain the phosphorylation of hexoses to maintain hexose homeostasis (Fig. 3).

4.3. Changes in the sugar content of leaves of tomato plants between different growth stages

Our previous work showed that the hexose content in mature fruits of transgenic tomato plants was greater than that of WT plants, while the Suc content was lower in transgenic seedlings. However, the sugar

content was different in the leaves of the plants at the mature stage, which was opposite that which occurred in the fruits, and we inferred that there maybe different regulatory pathways between them (Wang et al., 2020). Interestingly, during the seedling stage under normal conditions, the sugar content in the transgenic seedlings was greater than that in the WT seedlings (Fig. 2). The greater hexose content was mostly due to the greater expression of *MdHT2.2* and *SlLIN6*, which is induced by the former, after which TST1 transports the hexose into the vacuole. The greater Suc content was mainly due to the excess hexose synthesized via SPS and Suc transport into the vacuole by TST2. In contrast to the leaves of transgenic plants at the seedlings stage, the leaves at the mature stage contain lower amount of hexose but more Suc than do the leaves of WT, and the hexose content decreased mainly because of the low expression of *SlLIN* and *SlHT*, both of which transport hexose from the apoplastic into cells, moreover the low *SlTST1* expression means a relatively low amount of hexose transported into the vacuole. The increasing Suc content was due to the reduced expression of *SlNINV*, *SlSUSY1/4* and *SlTIV*, which encode Suc hydrolase. However, the relationship between sugar content and developmental stage remains unclear.

There are reports about the sugar signaling of HKs, FKs and SnRKS are components of the nutrient signaling network that links sugar availability to growth (Smeekens et al., 2010; Lastdrager et al., 2014; Wingler, 2018), and sugar regulates the plant development via hormones such as abscisic acid, auxin and ethylene (Eveland and Jackson, 2012). Previous researchers have also reported that in grape and tomato (Ruan and Patrick, 1995; Zhang et al., 2006), the Suc unloading pathway shifts from a symplastic pathway to an apoplastic pathway during fruit development. On the basis of the above information, we believe that the sugar content and metabolism are regulated according to plant development stage, which caused the sugar content to change at different developmental stages. However, in the regulatory pathways, hormone, sugar signaling and others components involved require further research.

5. Conclusion

In the transgenic seedlings under normal conditions, the higher content of hexose was due to the ectopic expression of *MdHT2.2*, and the excess sucrose was due to the decreasing cleavage and increasing synthesis from hexose (Figs. 2 and 3). While after treatment, sugar content keep increasing, until day 7, there were no significant difference of sugar content between all plant types, this was mainly because the sucrose cleavage increased and hexose phosphorylation to maintain sugar homeostasis (Figs. 2 and 3). Although the osmotic and ionic stress were alleviated by the increasing sugar content and decreasing Na^+/K^+ ratio (Figs. 2 and 4), but the salt resistance were still low in transgenic seedlings (Fig. 1), which were mainly caused by the increasing ROS content and reducing ROS scavenge ability (Figs. 5–7). Furthermore, how the sugar content changing caused by hexose transporter overexpression influenced the ROS system sensing NaCl under salt stress need to be fully researched.

Author contributions

Li, M.J., Ma, B.Q. and Ma, F.W. conceived and supervised this study; Li, M.J. and Wang, Z.Y. designed the experiments; Wang, Z.Y., Liang, Y. H., Jin, Y.R., Tong, X.L. and Wei, X.Y. performed the experiments; Wang, Z.Y. and Li, M.J. wrote the original draft. All authors reviewed and edited the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.10.001.

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