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TGase regulates salt stress tolerance through enhancing bound polyamines-mediated antioxidant enzymes activity in tomato

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ABSTRACT

The accumulation of free polyamines (PAs) plays critical role in plants under abiotic stress conditions, while protective traits of bound PAs receiving much less attention, our understanding of the regulation of free PAs converted to bound PAs is limited. In this study, we demonstrated that the PA mediator transglutaminase (TGase) play an important role in improving tomato tolerance to salinity stress, in part because its critical role in inducing accumulation of bound PAs under salt stress. *TGase* expression was induced upon salt stress. Exogenous phenanthroline (O-phen, a TGase and bound PAs synthesis inhibitor) abolished the TGase-mediated salt tolerance with terminating bound PAs accumulation in *TGase*OE plants. O-phen also had a negatively altered on Na^+/K^+ uptake or homeostasis under salt stress in leaves of *TGase*OE plants. Instead, the overexpression of *TGase* effectively alleviated salt-induced oxidative damage and reduced reactive oxygen species (ROS) overproduction, but it was significantly aggravated by O-phen in the *TGase*OE plants. Although the ROS generating enzymes (PA oxidase, diamine oxidase and NADPH oxidase) was not affected by application of O-phen, the antioxidant enzyme (superoxide dismutase, catalase, ascorbate peroxidase and monodehydroascorbate reductase) activity was significantly decreased by exogenous O-phen in plants. Furthermore, further analysis showed that the antioxidant enzyme activity positively and closely correlated with the level of bound PAs in *TGase*OE plant. Together, these results uncovered a probably function mechanism of TGase-mediated antioxidant enzyme activity to reduced salt-induced oxidative damage, which is partially depended on bound PAs accumulation.

1. Introduction

Salinity stress is one of the most important environmental stresses adversely affects worldwide agricultural productivity (Munns and Tester, 2008; Shabala, 2013). It subsequently causes ionic, osmotic, and oxidative stress to plant. Excess levels of Na^+ was absorbed in the cytosol hinders water uptake and leads to adverse physiological response, including disorders of ion homeostasis, stomatal closure, photosynthesis inhibition and biomass loss, thereby resulting in ion toxicity and osmotic damage (Zhu, 2002). Additionally, salinity stress leads to oxidative stress through an over-accumulation of reactive oxygen species (ROS) (Zhang et al., 2011). High levels of ROS that cause a loss of crop productivity and negatively affect cellular functions and damage to DNA, protein or lipids (Schieber and Chandel, 2014). Fortunately, plant have evolved a fine and complex enzymatic antioxidant system to repair damage initiated by ROS, such as superoxide dismutase (SOD), ascorbate catalase (CAT), peroxidase (APX) and monodehydroascorbate reductase (MDAR) (Mittler et al., 2004; Nahar et al., 2016). For instance, application of exogenous phytohormones and phytoprotectants induced the gene expression of antioxidant enzyme and increased their activities, which mitigated the detrimental effects of salt stress (Qiu et al., 2014; Rizwan et al., 2015; Wang et al., 2015). In other cases, manipulation of SOD, CAT and APX biosynthesis have all been demonstrated to enhance salt tolerance and neutralize salt-induced oxidative damage (Badawi et al., 2004; Luo et al., 2013). However, the regulatory mechanisms involved in these antioxidant enzymes are still not fully understood.

Polyamines (PAs) are present in free forms, or are often conjugated forms and interact with several important macromolecules (bound forms) (Groppa and Benavides, 2008; Michael, 2016). Free PAs, one of

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the original and important PA species, are widely distributed and response to various abiotic stress through increasing antioxidant enzyme activity (Alcázar et al., 2010; Gupta et al., 2013). Additionally, it has been demonstrated that conjugated or bound PAs exhibit higher ability to stability or activate antioxidant enzyme activity than free PAs through their cross-linked with antioxidant enzyme (Shevyakova et al., 2006; Saha et al., 2015). Although the crucial roles of conjugated and bound PAs have been demonstrated in a series of different biological processes, including salt stress response, our understanding about the regulatory mechanism of conjugated and bound PAs is extremely limited. Most studies on the regulation mechanism of conjugated and bound PAs has focused on the transglutaminase (TGase) (mediator of PA) (Del Duca et al., 1997, 2000). TGases catalyze the protein-bound glutamine residues incorporated into the primary amino groups of PAs, resulting in covalent posttranslational modification of proteins (Lilley et al., 1998). Endogenous bound Put increment is accompanied by an increase TGase activity was observed in plants under natural conditions, indicating that TGase is a positive mediator of bound PAs in plants (Pintó-Marijuan et al., 2007). Additionally, PAs were conjugated to light-harvesting complex of photosystem II (LHCII) to protect the structural stability by TGase (Della Mea et al., 2004). Thus, TGase may mediate the levels of conjugated and bound PAs in plants. Recently, we found that overexpression of *TGase* enhance salt tolerance and induced the accumulation of PAs in chloroplast, suggesting the possible involvement of TGase in bound PAs accumulation (Zhong et al., 2019b). However, little information is available concerning the functions and regulation of mechanisms between TGase and bound PAs under salt stress conditions.

To examine the potential function of *TGase* by genetic manipulation, we generated TGase-overexpressing tomato plant and compared their salt tolerance, endogenous conjugated and bound PAs content, Na^+/K^+ homeostasis, antioxidant enzyme activity with wild type (WT) plants. To our knowledge, this is the first study by genetic manipulation to demonstrate that the PA mediator TGase through maintaining bound PAs-mediated activity of antioxidant enzyme, and decreasing ROS overproduction, thereby alleviating oxidative damage, which play a critical role in increasing plant tolerance to salt stress. Additionally, our results indicate that the regulation of free PAs to bound PAs by TGase might be a valid strategy for the improvement of plant' abiotic stress tolerance.

2. Materials and methods

2.1. Plant materials and treatments

Seeds of *Solanum lycopersicum* cv. 'Ailsa Craig' from WT and transgenic plants were germinated in quartz sand and cultured in a greenhouse at 28 ± 2℃/20 ± 2℃ (day/night) under a maximum photosynthetic photon flux density (PPFD) of approximately 1200 μmol m^{-2} s^{-1} with a relative humidity of 70–80 %. In Experiment I, abiotic stresses were induced in WT seedling at the 30-day-old, RNA samples were collected from leaves under the control condition and under different stress conditions over 6 h. These stresses were salt (125 mM NaCl), drought (20% PEG6000), heat (42℃) and cold (4℃). In Experiment II, fifteen-day-old seedlings were transplanted into 20 L black plastic containers containing aerated full Hoagland's nutrient solution. After 15 days of preculture, two types of salt treatments were performed. (1) the seedlings were treated at 125 mM NaCl for 144 h, expression induction by salt of interesting genes were determined in shoot apex, young leaf (not fully developed leaf), upper adult leaf, stem, and root of plants after 3-day salt treatments (3 DST). Additionally, gene expression was also determined in upper leaf after 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 h of salt stress; (2) The experimental design consisted of a 'Control' (no added NaCl) and 'Salt stress' (125 mM NaCl). After 0 and 3 days of treatment, the second and third true leaves of each seedling were taken for further assay. In Experiment III, the WT and two independent homozygous transgenic plants overexpressing *TGase* (*TGase*OE-1# and *TGase*OE-2#) were used for experiment as described by Zhong et al. (2019a). After a 15 d' preculture, seedlings of WT and *TGase*OE plants were transformed to an aerated full Hoagland's nutrient solution with or without 125-mM NaCl for 1 d. For experiments with the TGase inhibitor treatment, seedlings were grown in a normal solution containing 125 mM NaCl and sprayed with 10 μM phenanthroline (O-phen, a TGase and bound PAs synthesis inhibitor) (Icekson et al., 1987; Liu et al., 2005).

2.2. Bound and conjugated PAs analysis

The levels of endogenous PAs in leaves were determined by high-performance liquid chromatography (HPLC) as previously described with small modifications (Shu et al., 2015; Zhong et al., 2019b). Leaves samples were homogenized in 5 % (v/v) cold perchloric acid (PCA), and incubated on ice for 1 h. After centrifugation for 20 min at 12,000 × *g*, the supernatant I was hydrolyzed and used to determine conjugated PAs, the pellet was used to measure bound PAs. The remaining residue was resuspended in 2 mL of 5 % PCA to obtain supernatant II and used to measure the conjugated PAs. The level of conjugated PAs was calculated by removing the content of free PAs determined in supernatant I from supernatant II. The pellet content of bound PAs was measured. PAs were measured using an HPLC 1200 series system (Agilent Technologies, Santa Clara, CA) with a C18 reversed-phase column. Samples were eluted from the column with 64 % (v/v) methanol at a flow rate of 0.8 mL min^{-1}. Put, Spd, and Spm (Sigma, St. Louis, MO 63178, USA) were used as standard samples and treated in a similar manner.

2.3. Chlorophyll fluorescence and transpiration rate measurements

The maximum quantum efficiency of photosystem II (PSII), expressed as the Fv/Fm, was measured in the third leaves after 30 min of dark adaptation using Dual-PAM-100 system (Walz, Germany), as described elsewhere (Zhong et al., 2019b). Transpiration rate was determined on the third leaves using a portable photosynthesis system (LI-6800; LI-COR, Inc., Lincoln, NE, USA) as described by Yuan et al. (2014).

2.4. Ion content analysis

Measurement of ion concentration in tomato tissue were performed as describe previously (Suzuki et al., 2016). In brief, plant material dried for 1 week for 65 ℃, milled to powder, and digested with ultrapure HNO_3 for one day and then boiled at 95℃ for 10 min followed by 3 cycles. Na^+ and K^+ were determined by an inductively coupled plasma spectrometry (ICP-MS Agilent 7700 series, USA).

2.5. Analysis of relative electrolyte leakage, malondialdehyde, H_2O_2 and $O_2^{\cdot -}$ content

The relative electrolyte leakage (REL, %) was measured following protocols described previously (Zhou et al., 2014). The membrane lipid peroxidation was estimated by measuring the content of MDA in leaves as described by Hodges et al. (1999). Leaf H_2O_2 content was measured according to Doulis et al. (1997) with some modifications. The $O_2^{\cdot -}$ germination rate was determined by the method of (Elstner, 1976).

2.6. Enzymes activities assay

Polyamine oxidase (PAO) and diamine oxidase (DAO) activities were determined according to the methods by Sun et al. (2005) and Fan et al. (2013). The NADPH oxidase activity was determined by plant NADPH oxidase ELISA assay kit (MEIMIAN, China) according to the manufacturer's instruction.

2.7. Antioxidant enzyme activity assays

Leaf samples were snap frozen in liquid N2 and extracted using ice-cold 50 mM phosphate buffer (2% (w/v) PVPP, and 0.2 mM EDTA, pH 7.8). The supernatant obtained post centrifugation for determination of enzyme activity. The activities of SOD, CAT, APX and MDAR were measured as previously described (Nakano and Asada, 1981). The protein content was determined using a BCA Protein Assay Kit (Pierce). All spectrophotometric analyses were performed using a Shimadzu UV-2410PC spectrophotometer (Shimadzu Co., Japan).

2.8. Total RNA extraction and gene expression analysis

Total RNA was isolated from total tissues using RNAsimple Total RNA extraction Kit (Tiangen, DP419; Beijing, China) according to the manufacturer's instruction. Total RNA (1 μg) was used to reverse transcribed for cDNA template using the HiScript™ Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China). The qPCR assays were performed using the ABI ViiA7 real-time PCR system (Applied Biosystems, USA). The *Actin* gene was used as a reference gene, Gene-specific primers designed according to cDNA sequences as shown in supplemental Table S1. Relative gene expression was calculated as Livak and Schmittgen (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data from experiments with three or more mean values were statistically analysed using SAS 13.0 software (SAS Institute, Inc, Cary, NC, USA) by Duncan's multiple range. The difference was considered to be statistically significant at $P < 0.05$.

3. Results

3.1. TGase expression was induced by abiotic stresses

Given that gene transcription levels under abiotic stresses may be related to gene functions, the *TGase* responded to a wide range of abiotic stresses was associated using WT seedling at 30-day. Samples were collected under the control and stress conditions for 6 h; stress consisted of NaCl, drought, heat and cold. Significantly increased *TGase* expression was observed under abiotic treatments compared with the expression in plants under normal conditions, with the exception of cold treatment. As shown the Fig. 1, the greatest fold change in the *TGase* expression was observed for 125-mM NaCl and heat treatments compared with controls condition, indicating that TGase may response to a series of abiotic stresses, especially salinity and heat stress.

3.2. TGase was differentially expressed in tomato tissue and was induced by salt stress

To further analyze the expression pattern of *TGase* in salt stress. Changes mRNA transcripts of *TGase* induced by salinity were measured in 30-day WT plants grown in a hydroponic system. The *TGase* expression was induced by salt stress for 3 days in all tissue analyzed (Fig. 2A). In aerial part, the highest expression of *TGase* was detected in upper adult leaves (9-fold increase), followed by young leaves (6-fold increase). The expression of *TGase* in the roots was lower than that in leaves, although a significant increase was also detected in salt-treated plants (Fig. 2B).

The temporal effect of salinity on *TGase* expression was detected in the first developed leaf of WT plants according to the same experiment mentioned above (Fig. 2C). The *TGase* expression was significantly induced after 12 h under salt treatment, and reaching the maximum levels between 48 and 60 h of treatment. A further increase transcripts levels of *TGase* was observed at 6 day of salt treatment (6 DST), indicating that two induction phases may exist during exposure to salt treatment.

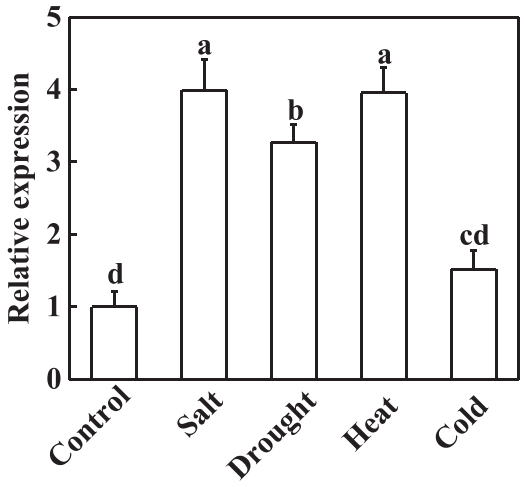

Fig. 1. *TGase* expression pattern under various stresses. The seedlings were subjected to Salt (125 mM NaCl), Drought (20% PEG6000), Heat (42 °C) and Cold (4 °C) stress treatments. Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

3.3. Overexpression of TGase enhanced salt tolerance and greatly influenced bound PAs accumulation

To further directly analyze the role of TGase in salt tolerance, we compared WT and F2 progeny of *TGase*-overexpressing (*TGase*OE) plants from 2 independent lines (#1# and #2#) expressing high protein levels of TGase (Fig. S1). After treating with salt stress for 3 days, the *TGase*OE seedlings showed significantly reduced salinity sensitivity (Fig. S2A) and exhibited a delay in leaf senescence with chlorophyll content obviously higher than that of WT plants (Fig. S2B). Accordingly, their root vigor and dry weight was significantly different in WT plants after 3 days of salt treatment (Fig. S2C and D). Interestingly, significant differences in profiles of bound PAs were detected in the leaves of WT and *TGase*OE plants after the treatment of 24 h with 125 mM NaCl (Fig. 3). Among the bound PAs, the levels of Spm was too low to be detectable in the leaves. As shown in Fig. 3A and C, the levels of conjugated/bound Put and Spd was similar in all the plants under normal conditions, the levels of conjugated Put and Spd was increased, but no significant difference in WT and *TGase*OE plants upon 24 h of salt stress. However, the bound Spd and Put content continuously accumulated upon 24 h of NaCl stress in *TGase*OE plants, with salt stress resulting in an approximately 1.5-fold and 2-fold increase, respectively (Fig. 3B and D). These results indicate that endogenous bound PAs likely to account for TGase-regulated salt tolerance in tomato.

3.4. TGase-induced bound PAs accumulation was abolished in O-phen treatment

To directly analyze whether *TGase* was involved in bound PAs accumulation under salt stress, O-phen, an inhibitor of TGase and bound PAs synthesis, was used to analyze the role of TGase in bound PAs-mediated salt tolerance (Liu et al., 2004; Serafini-Fracassini and Del Duca, 2008). The exogenous application of O-phen significantly reduced bound PAs accumulation (Fig. 4A) and aggravated NaCl-induced chlorophyll degradation (Fig. 4B), Fv/Fm and transpiration inhibition (Fig. 4C and D) in *TGase*OE plants upon 3 days of salt stress. Accordingly, the mRNA transcripts of senescence-related genes *NAP2*, *SAG12*, *SAG113* and *NYC1* was reduced in *TGase*OE plants but enhanced in O-phen treatment compared to the WT plants following 3 days of salt stress (Fig. S3), strongly supporting the role of TGase for protecting

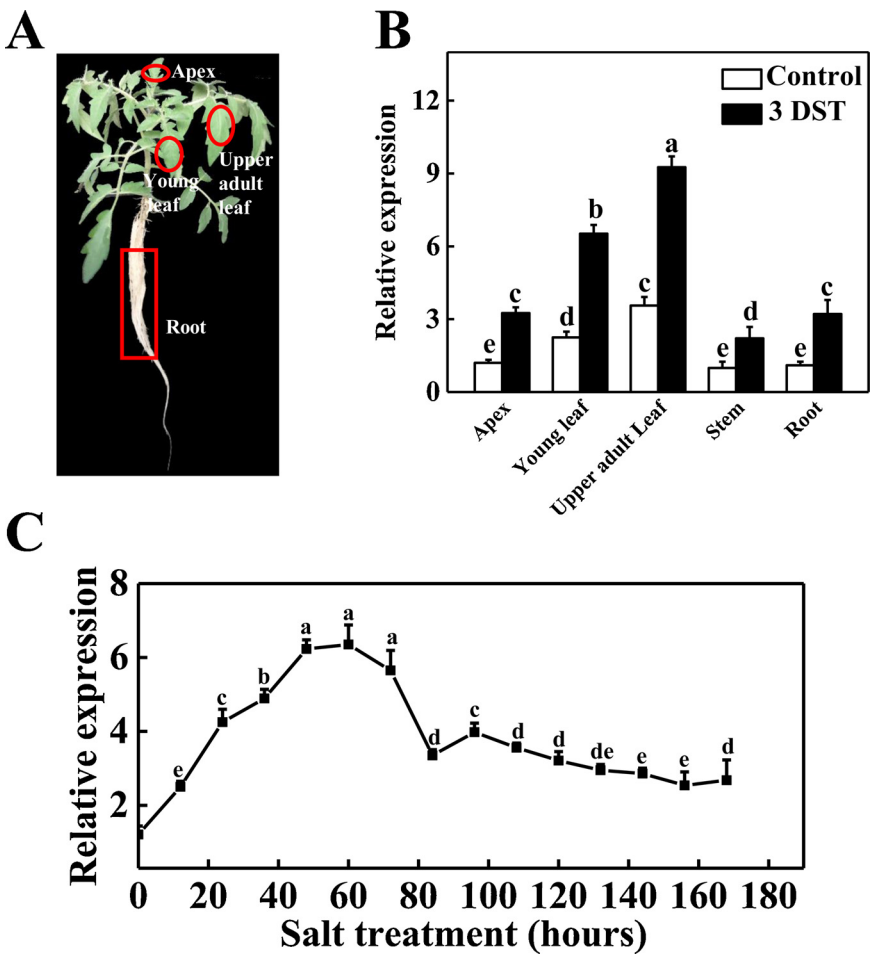

Fig. 2. Expression pattern of *TGase* gene in wild-type (WT) plants under salt stress conditions. The plants grown in a hydroponic system and 125 mM NaCl treatment (**A**). Levels of *TGase* transcripts were quantified by qPCR in apex, young leaf, upper adult leaf (first fully developed leaf), and root of WT plants developed in absence of salt (0 day) and after 3 day of salt treatment (3 DST) (**B**). Time course analysis of *TGase* expression during 6 day of salt treatment (6 DST) (**C**). Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

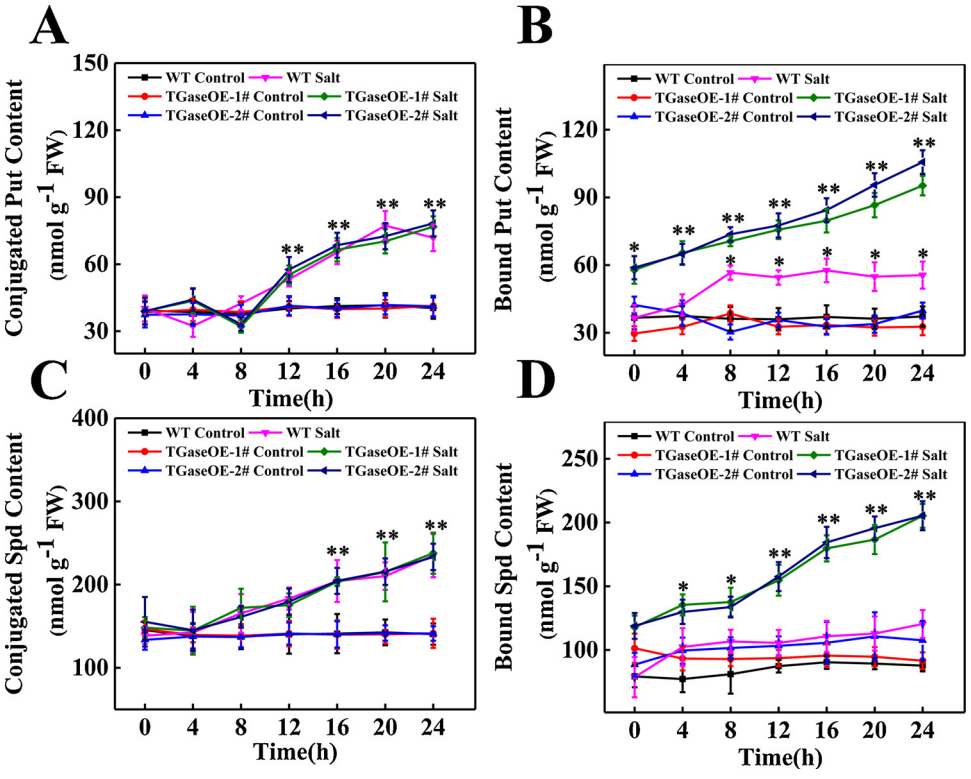

Fig. 3. Response of endogenous conjugated/bound polyamines content to NaCl stress in WT and *TGase*OE plants. Each histogram represents a mean ± SE of three independent experiments (n = 3). Thirty-d-old seedlings exposed to 125 mM NaCl for 24 h. * and ** indicate significant differences between control and salt stress treatments at $P < 0.05$ and $P < 0.01$, respectively.

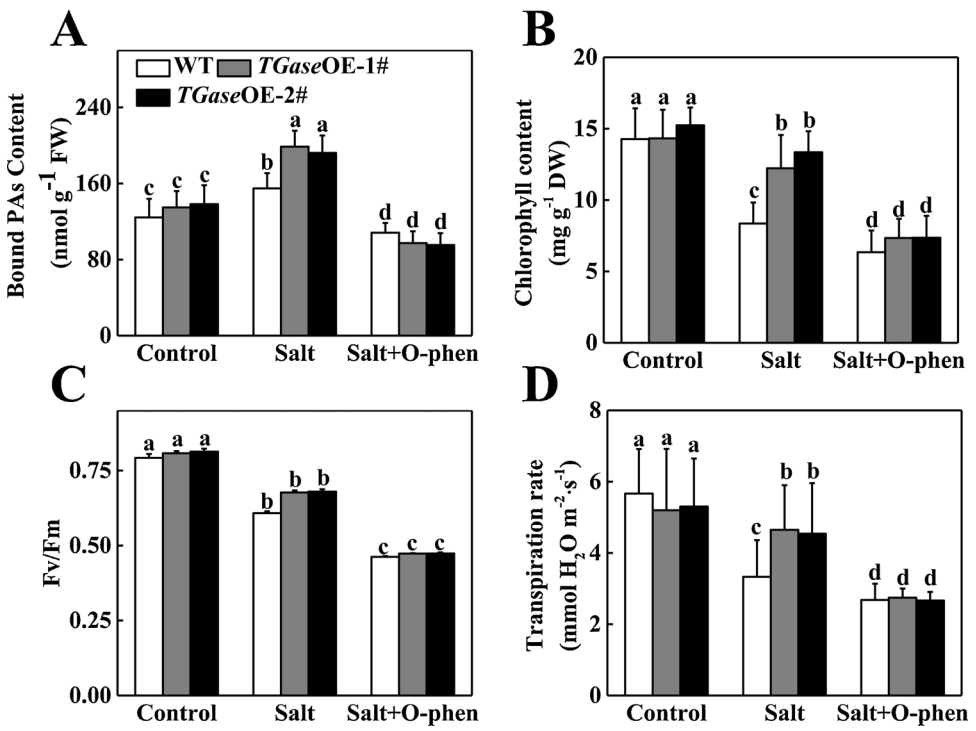

Fig. 4. Effects of phenanthroline (O-phen) on bound PAs (**A**) and chlorophyll (**B**) content, maximum quantum yield of PSII (Fv/Fm) (**C**), and transpiration rate (**D**) under NaCl stress in WT and *TGase*OE plants. Thirty-d-old seedlings exposed to 125 mM NaCl was treated with or without 10 μM O-phen for 3 day. Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

against the deleterious effects of salinity stress.

3.5. Effect of O-phen on TGase-induced Na^+/K^+ homeostasis

Salt is known to induce excessive accumulation of Na^+ and inhibited K^+ uptake, which causes to ion toxicity and negatively affects plant growth. To test if TGase was involved in Na^+/K^+ homeostasis. Here, we performed the Na^+ and K^+ contents in shoots and roots of 30-day-old WT and *TGase*OE plants exposed to salinity stress, 125 mM NaCl (with or without O-phen), for 3 days (no NaCl and O-phen as control). Compared with the control, it was dramatically decreased the Na^+ contents and increased the K^+ accumulation in shoots and roots of transgenic plants. Consequently, the Na^+/K^+ ratio under salinity stress was significantly decreased in *TGase*OE plants (Figs. 5 and S4). As expected, treatment with O-phen further caused to higher content of Na^+ and decreased K^+ levels in shoots of the WT and *TGase*OE plants. Moreover, in shoots of *TGase*OE plants, O-phen applied to NaCl treatment almost increased Na^+ content and decreased K^+ content to control level of WT plants in NaCl treatment (Fig. 5). However, in roots, no considerable difference in the Na^+ and K^+ content was observed between the WT and *TGase*OE plants after O-phen application. Similarly, the Na^+/K^+ of ratio was also no significant difference (Fig. S4).

To further investigate the mechanism underlying of TGase in the altered accumulation of Na^+ of leaves, we performed a series of key genes in shoots (fifth leaf) of WT and *TGase*OE plants, including the Na^+/H^+ antiporter (*SOS1*), the vacuole membrane antiporter (*NHX4*), the xylem parenchyma localized Na^+ transporters (*HKT1;1* and *HKT1;2*). Basically, salt treatment led significant increase in the expression of these genes in leaves of WT and *TGase*OE plants (Fig. S5). In detail, *SOS1* and *NHX4* expression was significantly higher in *TGase*OE than WT leaves. Expression of *HKT1;1* and *HKT1;2* was significantly higher in leaves of *TGase*OE plants than WT, whereas the addition of O-phen significantly diminished TGase-induced increase in transcript levels of *SOS1*, *NHX4*, *HKT1;1* and *HKT1;2* in leaves of *TGase*OE plants to levels close to that of WT (Fig. S5). These results are consistent with the observed decreased Na^+ content and Na^+/K^+ ratios in shoots of *TGase*OE plants.

3.6. Effect of O-phen on TGase-reduced oxidative damage and ROS accumulation

Reactive oxygen species (e.g. H_2O_2 and $O_2^{\cdot -}$) are responsible for damage to cellular membranes and other cellular components, which are important parameters used for assessing tolerance to salt in plants (Miller et al., 2010; Schmidt et al., 2013). H_2O_2 and $O_2^{\cdot -}$ levels were extremely low in WT and *TGase*OE plant before salt treatment (Fig. 6A and B). However, with 125 mM NaCl, evaluation of H_2O_2 and $O_2^{\cdot -}$ content indicated that salt stress caused more seriously oxidative damage in leaves of WT than that of *TGase*OE plants. O-phen further increased H_2O_2 and $O_2^{\cdot -}$ content, respectively, more than the NaCl treatment alone in the leaves of WT and *TGase*OE plants. Importantly, O-phen eliminated that overexpression of *TGase* suppressed the salt-induced oxidative stress (Fig. 6A and B). Additionally, the content of MDA increased dramatically with NaCl treatment, these was less in *TGase*OE plants compared with that in WT plants. Application of O-phen further increased the MDA content in leaves of WT and *TGase*OE plants under salinity stress, which resulted in no significant difference in MDA content in leaves of WT and *TGase*OE plants (Fig. 6C).

3.7. Effect of O-phen on TGase-enhanced PAO, DAO and NADPH oxidase

To test if O-phen overshadowed TGase-induced salt tolerance may be due to an alteration of ROS generation, PAO, DAO and NADPH oxidase activity were measured. The results showed that overexpression of *TGase* conspicuously increased the PAO, DAO and NADPH oxidase activity compared with that in leaves of WT plants upon salinity stress, and O-phen treatment had no significantly change on the enzyme activity compared with salt treatment (Fig. 6D, E and F). These results indicate that O-phen is not involved in exacerbating ROS generation during salinity stress

3.8. Effect of O-phen on TGase-enhanced antioxidant enzyme activities

It is well documented that ROS-scavenging enzymes are major players in salt stress tolerance (Hazman et al., 2015; You and Chan, 2015). As shown in Fig. 7, the activities of SOD, CAT, APX and MDAR

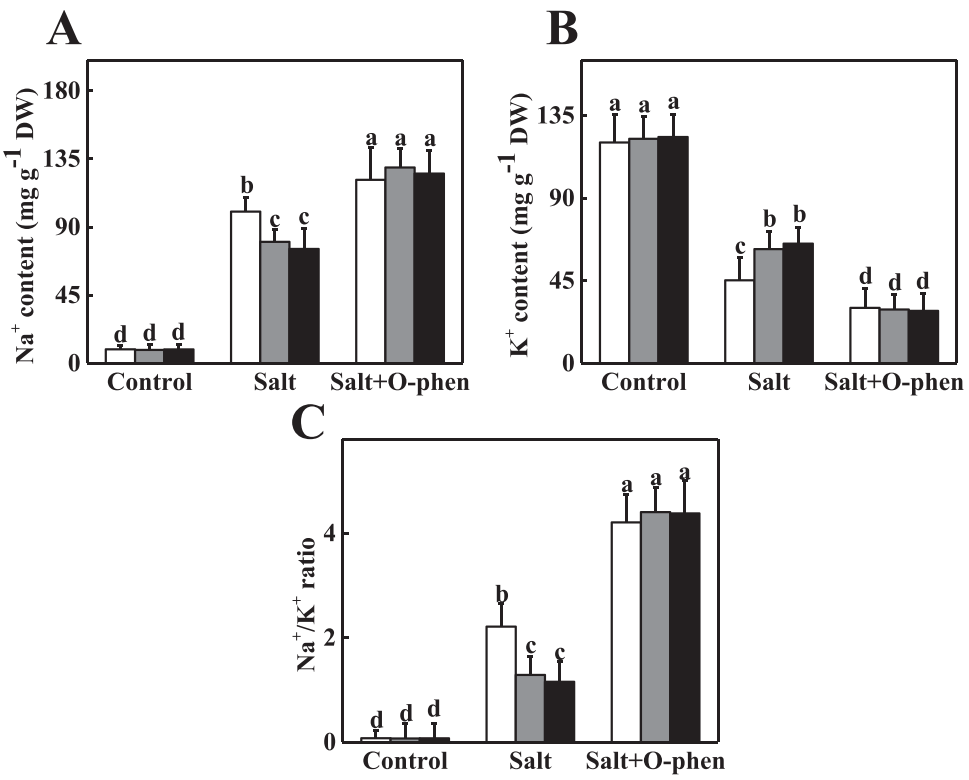

Fig. 5. Effects of NaCl treatments on accumulation of Na^+ (**A**) and K^+ (**B**) and the Na^+/K^+ ratio (**C**) in leaves of WT and *TGase*OE plants. Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

were induced by salt stress. In addition, the expression of genes encoding the ROS-scavenging enzymes were induced by salinity stress (Fig. S6). Moreover, the results showed more significant increases in the antioxidant enzymes activity in leaves of *TGase*OE plants than WT during salt stress (Figs. 7 and S6). The induction of ROS-scavenging enzymes in response to salt stress had little effect in WT plants under O-phen treatment, whereas it abolished the ROS-scavenging capacity in *TGase*OE plants, the activities of antioxidant enzyme in *TGase*OE plant to levels close leaves that of WT (Fig. 7). Surprisingly, Correlation analyses showed that bound PAs content was positively correlated to SOD, CAT, APX and MDAR activity in *TGase*OE plants (Fig. 8). Together, these results indicate that slighter oxidative damage in *TGase*OE plants might be due to overexpression of *TGase* increases the bound PAs content that maintain antioxidant enzyme activity.

4. Discussion

Results reported here indicate that TGase was induced by abiotic stresses, and expression analysis shown that *TGase* was induced in WT plants under salt stress, the highest expression level being examined in upper adult leaves. These results indicate that TGase involved in adaptive response to salinity through protecting tissue from physiological caused by salt stress (Figs. 1 and 2B). Comparable results were reported for TGase activity strongly increased to protect the tissue and photosynthetic organs in cucumber leaves following NaCl treatment (Shu et al., 2019). Additionally, the present study proved that salt stress induced the expression of *TGase* (Fig. 2C). Herein, in our present study, changes in chlorophyll content, root vigor and dry weight, the important indicators of plant growth status, are consistent with the physiological detected in the *TGase*OE plants and support the role of TGase in protecting tissues from salinity conditions (Fig. S2).

Numerous studies have revealed the endogenously altered and implicated of PAs balance in plant tolerance to salinity stress (Gill and Tuteja, 2010; Li et al., 2016). In our previous study, the positive role of free PAs alteration, such as free PAs content and (Spd + Spm)/Put value, in plant to salinity tolerance has been widely demonstrated (Duan et al., 2008; Fan et al., 2013). Additionally, conjugated/bound PAs are also positively linked with salinity tolerance (Quinet et al., 2010). Our studies have shown that the levels of conjugated/bound PAs increased could stabilized the molecules and resisted salinity tolerance of plants (Yuan et al., 2016). Furthermore, TGase catalyze proteins by establishing ε-(γ-glutamyl) links among proteins, and PAs act as a tether and acceptor molecules in this process, this leads to transformation of free PAs to bound PAs (Serafini-Fracassini et al., 1988; Serafini-Fracassini and Del Duca, 2008; Aloisi et al., 2016). In addition, O-phen, as an inhibitor of TGase, able to inhibit TGase-mediated modification of PAs or analyze the biological functions of bound PAs (Liu et al., 2005). Thus, gathered evidence indicates that conjugated/bound PAs is mediated by TGase/O-phen and plays an important role under salinity stress. In this study, *TGase*OE plants showed increased tolerance to salinity, with a corresponding accumulate in the levels of bound PAs under short-term salt treatment (Fig. 3B and D). Furthermore, bound PAs content continuously increased with prolonged salt treatment in the *TGase*OE plants, but barely varied in the WT plants (Fig. 4A). Importantly, our previous studies indicated that salt stress inhibited chlorophyll synthesis, photosynthetic and transpiration rate (Shu et al., 2015; Yuan et al., 2019). Here, the *TGase*OE plants displayed enhanced salt tolerance, as indicated by higher levels of chlorophyll content, Fv/Fm and transpiration rate, but mostly abolished by O-phen treatment in the *TGase*OE plants, and the application of O-phen aggravated salinity toxicity accompanied by a decrease in bound PA content (Fig. 4B–D). These results indicated that the function of TGase in salinity tolerance might be associated with the accumulation of bound PAs.

Salt stress lead to disturb ionic homeostasis and ion toxicity in plants. Plants have evolved inherent mechanisms to exclude or sequester Na^+ ion during salt stress (Zhu, 2002; Carden et al., 2003). Similarly, when exposed to NaCl, we observed a lower accumulation of Na^+ in leaves and roots of *TGase*OE plant than WT plants. As expected, the exogenous application of O-phen to salt-stressed tomato seedlings significantly increased Na^+ content in the leaves of WT and *TGase*OE plants. However, O-phen barely increased the accumulation of Na^+ in WT and *TGase*OE roots (Figs. 5 and S4). This result indicate that TGase

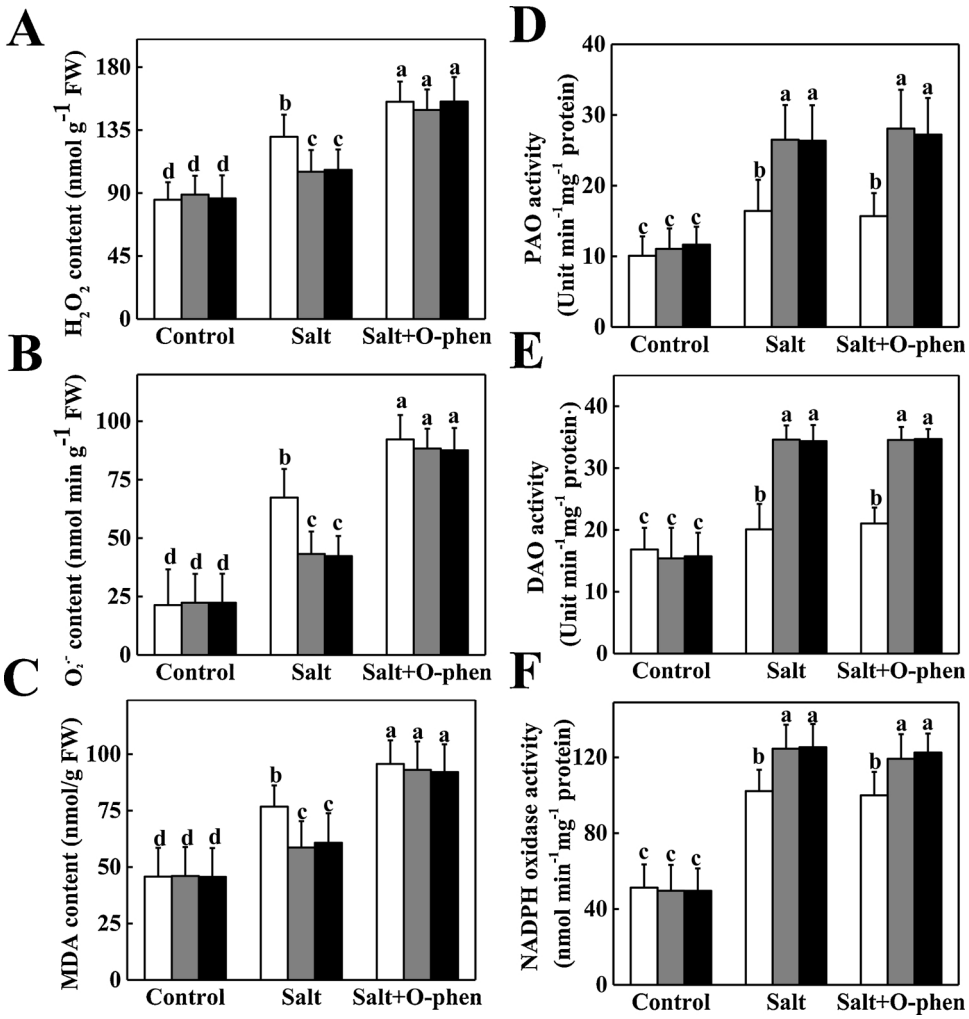

Fig. 6. Effects of phenanthroline (O-phen) on H_2O_2 (**A**), $O_2^{\cdot-}$ (**B**) MDA (**C**) content, cell wall bound polyamine oxidase (PAO) (**D**), diamine oxidase (DAO) (**E**) and NADPH oxidase (**F**) activity under NaCl stress in WT and *TGase*OE plants. Thirty-d-old seedlings exposed to 125 mM NaCl was treated with or without 10 μM O-phen for 3 day. Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

improve salt tolerance by contributing to the lower accumulation of Na^+ in plants. Our results also suggest that the differences in bound PAs content of WT and *TGase*OE plants might be associated with the variation in salt tolerance between them.

Additionally, results from expression analysis showed that the increased capacity of *TGase*OE plants to restrict Na^+ in leaves was accompanied with a significant higher salt induced expression of gene involved in prevent its transport, such as *SOS2* and *NHX4* Na^+/H^+ antiporters genes, that are located on plasma membrane and vacuole membrane, respectively. The *SOS2* and *NHX4* meditate the toxic accumulation of Na^+ in the cytosol through compartmentalizing Na^+ into vacuole or exporting Na^+ back to apoplast (Liu et al., 2000; Bassil and Blumwald, 2014). Moreover, an increase of *HKT1;1* and *HKT1;2* involved in Na^+ transporter gene expression was also observed in the leaves of *TGase*OE plants, the two genes are indicating main function for retrieving Na^+ from the xylem into phloem sieves, these are expressed in xylem parenchyma, phloem cells and loading Na^+ into phloem sieves (Møller et al., 2009; Asins et al., 2013). However, induction of these genes expression was compromised in O-phen treatment under salt stress (Fig. S5). Taken together, the above data supports the hypothesis that TGase is needed for regulating Na^+/K^+ homeostasis through managing the retrieval of Na^+ from the xylem into phloem sieves through mediating the accumulation of bound PAs under saline conditions.

Recently, previous studies strongly suggested that salinity stress rapidly induced a large of ROS production, which triggering membrane lipid peroxidation, oxidative stress and physiological metabolic disorders (Asada, 2006; Anjum et al., 2015; Nahar et al., 2016). To adapt to salt stress and redox balance, plant usually control ROS homeostasis by regulating ROS scavenging systems and generating. Our experimental results showed that uncontrolled increment of H_2O_2, $O_2^{\cdot-}$ and MDA content after 3 day of salt treatment induced oxidative stress. Meanwhile, we observed that the *TGase*OE plants had lower ROS accumulation relative to the WT plants. Furthermore, the application of O-phen resulted in hypersensitive to salt stress and decreased TGase-mediated plant salt tolerance in *TGase*OE plants (Fig. 6), supporting that the TGase-mediated bound PAs were involved in TGase-mediated ROS scavenging systems and generating, it has been shown to play a critical role in plant cell by the precise equilibriums between its generation and clear (Mittler, 2002; Miller et al., 2010). The cross-talk of PAs and ROS is to be sophisticated because PAs not only act as ROS scavenger and enhance the mechanism of antioxidant to enhance plant salt tolerance but also as ROS producers by PAs oxidase (Pottosin et al., 2014; Gupta et al., 2016). To acquire further insight into the mechanism of bound PAs in ROS homeostasis, we further tested the effects of O-phen on both ROS generating and scavenging systems. On the one hand, we tested the activities of DAO, PAO and NADPH oxidase activity, that play role in ROS generating. Interestingly, O-phen treatment

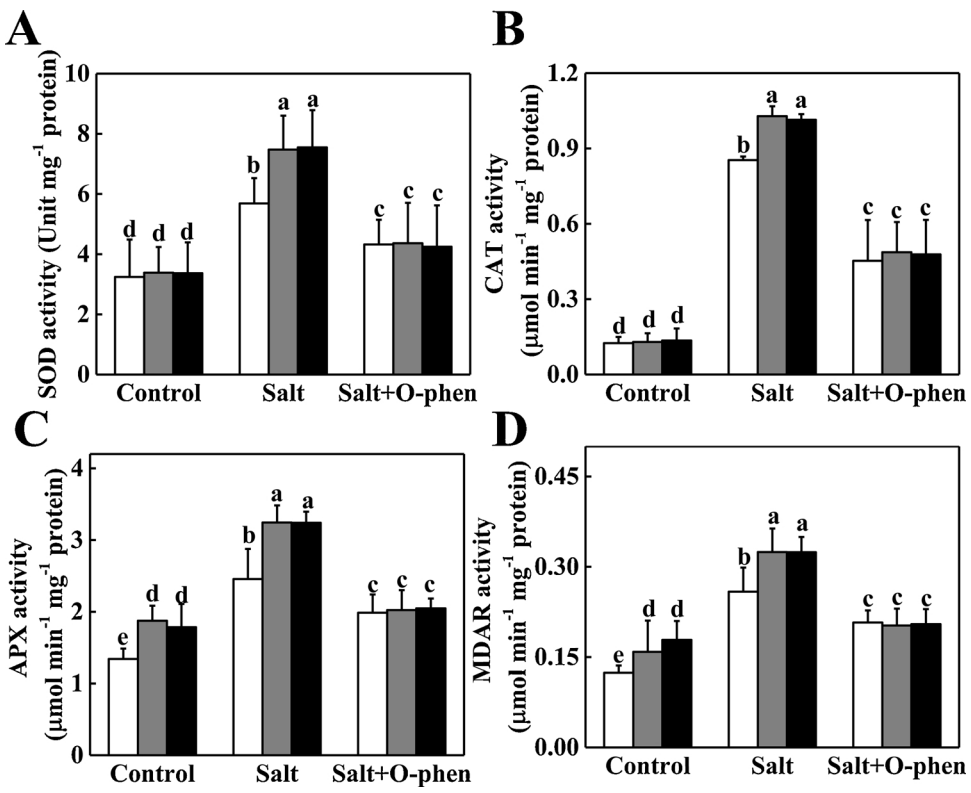

Fig. 7. Effects of phenanthroline (O-phen) on superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**), ascorbate peroxidase (APX) (**C**) and monodehydroascorbate reductase (MDAR) (**D**) activity under NaCl stress in WT and *TGase*OE plants. Thirty-d-old seedlings exposed to 125 mM NaCl was treated with or without 10 μM O-phen for 3 day. Each histogram represents a mean ± SE of three independent experiments (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's multiple range test.

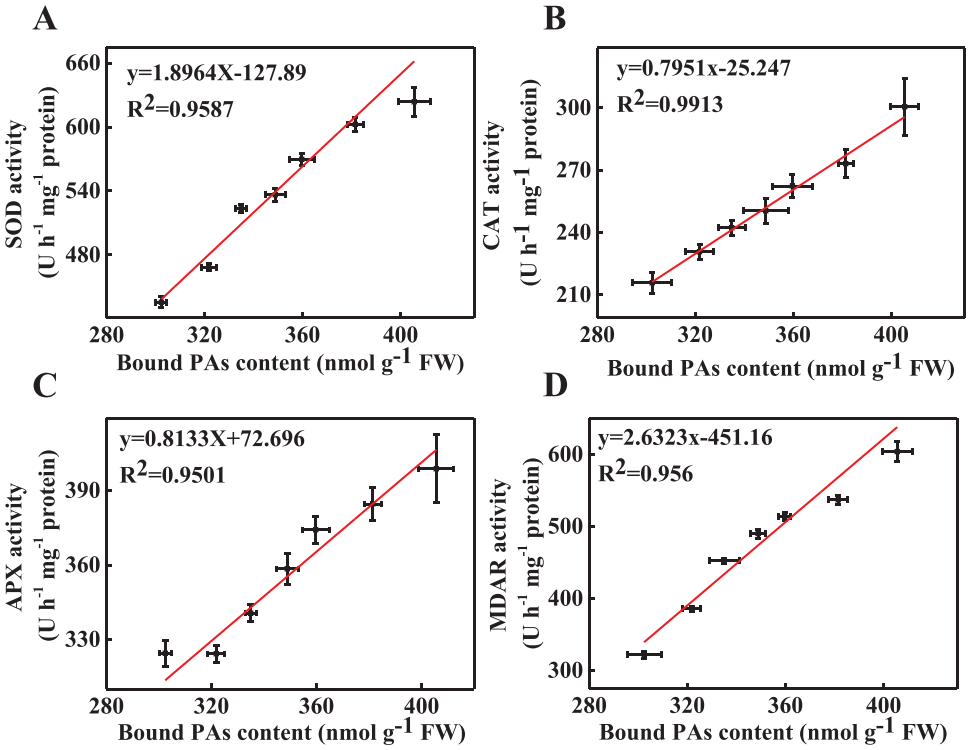

Fig. 8. The relationship between bound PAs content and antioxidant enzyme activities in *TGase*OE plants. Thirty-d-old seedlings were treated with and without 125 mM NaCl for 0, 3, 6, 9, 12, 18, and 24 h. Correlation between content of bound PAs and activities of superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**), ascorbate peroxidase (APX) (**C**) and monodehydroascorbate reductase (MDAR) (**D**) activity in thirty-d-old seedlings of *TGase*OE plants were determined.

had no significant inhibit these enzyme activities, suggesting that TGase-mediated bound PAs may have no effect on the generation of ROS (Fig. 6). On the other hand, NaCl treatment significantly reinforced the activities of antioxidant enzymes (including SOD, CAT, APX, MDAR), and the *TGase*OE plants showed stronger ROS-scavenging capacity than the WT plants, but the application O-phen significantly reduced the activity of these enzymes in *TGase*OE plants, indicating that TGase-mediated bound PAs may play a critical role in activating the antioxidant enzyme activity (Fig. 7). Furthermore, the hypothesis could be supported as the antioxidant enzyme activity positively correlates with the levels of bound PAs in *TGase*OE plants (Fig. 8). Thus, in this study, our results suggest that TGase-mediated bound PAs accumulation may conduce to salt tolerance by maintaining the activity of antioxidant system, mediating redox homeostasis. Similarly, in our previously

study, the regulation of antioxidant enzyme by TGase has been demonstrated and attributed to maintain the cellular redox homeostasis in tomato (Zhong et al., 2019a).

In summary, in this study, we provide a comprehensive genetic and analysis of TGase and bound PAs in response to salinity stress in tomato plants. Salt stress induces the expression of *TGase* which acts as a positive mediator of the transpiration rate, K^+/Na^+ ratio, and photosynthetic system damage. Furthermore, bound PAs increment promotes the activity of antioxidant enzymes leading to mediating ROS over-accumulation rather than regulating the activity of DAO, PAO and NADPH oxidase activity to influence the production of ROS. Further studies are needed to provide more molecular and genetic evidence of the involvement of TGase-meditated the conversion of free PAs to bound forms, and to elucidate the molecular mechanisms of between TGase and PA metabolism.

Author statement

Neither the entire paper nor any part of its content has been published or accepted elsewhere. It is not being submitted to any other journal. All of the authors have read and approved the final version of the manuscript and approved its submission to *Environmental and Experimental Botany*. The authors further declare no competing interests.

Author contributions

SG designed the research and supervised this study. MZ performed the experiments and wrote the original draft. YW revised the manuscript. SS and JS improved the manuscript. All authors reviewed and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict 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.envexpbot.2020.104191.

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