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Constitutive expression of the wheat *TaSOD5* gene enhances salinity tolerance of *Arabidopsis thaliana*

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Abstract

Superoxide dismutase is a crucial reactive oxygen species (ROS) scavenger and converts the superoxide radical (O_2^-) to H_2O_2, so it is thought to enhance abiotic stress tolerance by reducing ROS accumulation and so avoiding oxidative damage. In this study, we isolated a salt- and oxidative stress-responsive Cu/Zn SOD gene *TaSOD5* from wheat. The ectopic overexpression of *TaSOD5* in *Arabidopsis* increased total and Cu/Zn SOD activities, and offered the plant tolerance to salt stress. *Arabidopsis* ectopically expressing *TaSOD5* possessed a superior resistance to oxidative stress induced H_2O_2. The *TaSOD5* ectopic overexpression elevated the activities of both ROS scavengers and O_2^- producer NADPH oxidase. These findings show that Cu/Zn SOD enhances salt tolerance *via* regulating the machinery of redox homeostasis rather than improving SOD activity alone.

Additional key words: APX, CAT, GPX, NADPH oxidase, redox homeostasis, ROS, superoxide dismutase, *Triticum aestivum*.

Introduction

Soil salinity has a significant limit on crop productivity; it has been estimated that nearly a half of the irrigated areas and about 20 % of the arable land in the world are affected by a varying degree of salinity (Dong *et al*. 2018). Salt stress produces some primary damage to ion balance, water relations, photosynthetic efficiency, and nutrient absorption, and then extends to a secondary damage due to ion toxicity and overproduction of reactive oxygen species (ROS) (Ihsan *et al*. 2016). One source of ROS production is the reduction of O_2, which is excitated to form singlet oxygen (O_2^1) or is transferred with electron(s) to form the superoxide radical (O_2^-) (Mittler 2002). To cope with the damage by ROS, plants have developed efficient ROS scavenging machinery consisting of a set of nonenzymatic compounds and enzymes including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (Mittler 2002). Superoxide dismutase serves as the first defense line to convert O_2^- to hydrogen peroxide (H_2O_2) (Fridovich 1995). The SOD family comprises three classes, Cu/Zn-SOD), Fe-SOD, and Mn-SOD. Of them, Cu/Zn-SOD has been found to enhance tolerance to abiotic stresses in plants (Allen 1995, Gill *et al*. 2015).

On the other hand, a suitable ROS amount is necessary for triggering response to environmental stimuli, and ROS play crucial regulatory performance in numerous biological processes (Mittler *et al*. 2011, Suzuki *et al*. 2012, Sierla *et al*. 2013). Reactive oxygen species amount is governed by homeostasis between ROS production and scavenging pathways (Miller *et al*. 2009). However, only the improvement of ROS scavenging could not ensure the enhancement of tolerance to abiotic stresses (Pitcher *et al*. 1991). These findings suggest the roles of SOD and other ROS scavengers in salt tolerance are performed *via* mechanisms more complicated than we expect, which has not been well addressed so far.

Common wheat (*Triticum aestivum* L.) is one of the most important food crops, but it has only limited resistance to salinity stress (Kumar *et al*. 2018). Salinity hinders leaf growth and tillering by halting the leaf primordia initiation rates and finally lead to reduction of various yield parameters including kernel mass and number (Francois *et al*. 1994). In the past decades, important advances have been made in the understanding of the mechanism of salinity tolerance in wheat (Kumar *et al*. 2018). Wheat plants mitigate the effects of salinity stress by up-regulating various stress-responsive genes,

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; GPX - glutathione peroxidase; NOX - NADPH oxidase; PEG - polyethylene glycol; OE - transgenic *Arabidopsis* lines over-expressing a superoxide dismutase gene *TaSOD5*; ROS - reactive oxygen species; SOD - superoxide dismutase.

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including ion transporters, transcription factors, signaling pathway regulators, and antioxidant enzymes. (Dong *et al*. 2013, Ge *et al*. 2013, Liu *et al*. 2014, Rong *et al*. 2014, Huang *et al*. 2015, Zahid *et al*. 2017). Dynamic expression of these genes authenticates their inevitability and sufficiency in enhancing salt tolerance in wheat. For example, TaHKT1 plays a key role in Na^+/K^+ balance at a high concentration of Na^+, and *Triticum aestivum* Superoxide dismutase 1/2 can crosstalk with other proteins to participate in various signaling pathways (Kumar *et al*. 2018). However, there are few reports on the regulation of salt stress by balancing ROS production and scavenging systems in wheat.

The aim of this research was to identify a stress responsive Cu/Zn SOD gene *TaSOD5* from the salt tolerant wheat and determine if *TaSOD5* ectopic overexpression in *Arabidopsis* can improve the activities of some ROS scavengers and enhance the salinity tolerance *via* altering ROS homeostasis. Discovery and functional identification of this gene might be a potential target for determination of salinity-tolerant and susceptible cultivars for molecular breeding studies in the future.

Materials and methods

Plants and treatments: Two common wheat (*Triticum aestivum*.L.) cultivars, Shi8 with a remarkable salinity tolerance, and Yimai38 which shows salt intolerance were grown in a half strength Hoagland liquid medium (Hoagland and Arnon 1950) in a ceramic pot, with small holes in the lid, under a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, a temperature of 22 °C, and an air humidity of 55 %. For stress treatments, two-week-old seedlings were transferred to media containing 200 mM NaCl, 18 g dm^{-3} polyethylene glycol (PEG) 6000, 10 mM H_2O_2, or 100 μM abscisic acid for 0, 1, 3, 6, 12, and 24 h.

Arabidopsis thaliana L. seeds harvested from *TaSOD5* transgene homozygous plants (OE1 and OE2 lines) and wild type (Col-0) were surface-sterilized and plated on a solidified Murashige and Skoog (1962 agar medium and kept in the dark at 4 °C for 3 d to break dormancy, and then transferred to a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a temperature of 22 °C for 2 d. Then, they were re-plated on half strength Murashige and Skoog agar media supplemented with various concentrations of NaCl for 14 d, or H_2O_2 for 10 d. Each treatment was replicated three times. Plant tissues for RNA extraction were snap-frozen in liquid nitrogen and stored at -80 °C until required.

Isolation of *TaSOD5*: The sequence with annotation as *SOD* for the RNA sequencing dataset was subject to *BLASTN* against the wheat *EST* database in *NCBI*. The matched sequences were assembled using the *CAP3* software (Huang and Madan 1999). According to the assembled sequence, the full-length cDNA (named *TaSOD5*) from the common wheat drought tolerant cultivar Shi8 was isolated. The PCR procedure included a denaturation at 95 °C for 5 min, 35 cycles of 94 °C/30 s, 58 °C/50 s, and 72 °C/60 s, and a final extension at 72 °C for 10 min. The amplified product was inserted into the pMD18-T vector (*Takara*, Dalian, China), and submitted for Sanger sequencing.

Construction of *TaSOD5* transgenic *Arabidopsis* plants: The *TaSOD5* was ligated into the pSTART vector to construct a pSTART-*TaSOD5* vector driven by the CaMV:35S promoter. The pSTART-*TaSOD5* vector was introduced into *Arabidopsis* Col-0 using the floral dip method (Clough and Bent 1998). Homozygous transgenic lines were selected by kanamycin screening. The DNA of the transgenic lines was extracted for PCR to detect the integration of *TaSOD5*. The RNA of the transgenic lines was isolated and reversely transcribed to produce cDNA, and cDNA was used as a template for real-time quantitative PCR to measure the expression of *TaSOD5*.

Total RNA was extracted from samples mentioned above using a *Trizol* reagent (*Takara*), and treated with DNAase I. The RNA was reversely transcribed to synthesize the first cDNA strand using an *M-MLV* reverse transcription system kit (*Takara*). The cDNA was used as a template for real-time quantitative PCR in 0.02 cm^3 of solution containing 10 mm^3 of 2× *SYBR Premix Ex Taq* mix (*Takara*), 0.2 μM forward (5'-GAGGTGAGGGGGAGGGTCTC-3') and 0.2 μM reverse (5'-TCGTTCATCATCCATCGGTG-3') primers, 1 mm^3 of a 1:10 diluted cDNA first strand, and the cycling regime comprised a denaturation step of 95 °C/2 min followed by 45 cycles of 95 °C/10 s, 60 °C/20 s, and 72 °C/20 s. Melting curve analysis was performed over the range of 80 to 95 °C at 0.5 °C intervals. Relative gene expressions were detected using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The wheat *ACTIN* gene (AB181991) or *Arabidopsis TUBULIN* gene (*AT1G04820*) were used as internal references.

Measurement of activities of ROS scavenging enzymes and Na^+/K^+ content: The leaves of three-week-old wheat seedlings and two-week-old *Arabidopsis* seedlings were collected. The leaves (0.5 g) were homogenized in 1 cm^3 of homogenization solution on ice. The solution consisted of 50 mM KH_2PO_4, 0.1 mM Na_2EDTA, and 0.3 % (v/v) *Triton X-100*. The homogenate was centrifuged at 13 000 *g* for 10 min, and a certain volume of the protein extraction solution was sampled to measure enzyme activities. Protein content was determined using the Bradford Coomassie brilliant blue staining assay (Bradford 1976).

Total SOD activity was measured using the nitroblue tetrazolium method with a SOD activity kit; Cu/Zn SOD activities was measured with a Cu/Zn-SOD and Mn-SOD assay kit (*Beyotime*, Shanghai, China). Ascorbate peroxidase activity was measured according to the respective kit instructions (*Jianchen*, Nanjing, China). Catalase activity was measured by detecting a decrease in absorbance at 240 nm for 1 min using a CAT activity measurement kit (*Beyotime*). Glutathione peroxidase (GPX) activity was determined by monitoring a decrease in absorbance at 340 nm of a reaction system using a GPX activity measurement kit (*Beyotime*), and a coefficient of absorbance for NADPH

was assumed as $6.22 \cdot 10^3$ M^{-1} cm^{-1}.

Content of Na^+ and K^+ was determined by an inductively coupled plasma atomic emission spectrometer (*ICP-AES*, *Perkin-Elmer*, Boston, MA, USA) according to Dong *et al* (2012).

Measurement of NADPH oxidase activity: Leaves (0.5 g) were sampled from two-week-old *Arabidopsis* seedlings, and homogenized in 1 cm^3 of homogenization solution containing 50 mM KH_2PO_4 and 0.1 mM Na_2EDTA. The homogenate was centrifugated at 13 000 *g* and 4 °C for 15 min, and the supernatant was collected for measuring the activity of NADPH oxidase (NOX) according to Grace and Logan (1996). A reaction solution contained 100 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA, and 0.2 mM NADPH. The reaction began when NADPH was added. Activity of NOX was calculated by monitoring a decrease in absorbance at 340 nm. The molar coefficient of absorbance for NADPH was $6.22 \cdot 10^3$ M^{-1} cm^{-1}.

Statistics: Values are presented as means of three replicates and standard errors (SDs). Experimental data were analyzed by the *SPSS* software (*IBM*) followed by one-way *ANOVA* to determine significant differences among treatments.

Results

The *TaSOD5* encodes a protein possessing a Cu/Zn SOD catalytic site and shares high identities with Cu/Zn SOD members from other species. To know whether *TaSOD5* is involved in a response to abiotic stresses, the transcriptional profiles of *TaSOD5* under several treatments were detected. After exposure to 200 mM NaCl, *TaSOD5* was gradually induced with a stronger induction in 'Shi8' than in its parent cultivar 'Yimai38' (Fig. 1). When treated with 18 g dm^{-3} PEG 6000 solution to mimic osmotic stress, the expression of *TaSOD5* was also elevated, and peaked at 12 h; during the whole course of treatment, 'Shi8' accumulated more *TaSOD5* transcripts than 'Yimai38' did (Fig. 1). When suffered from oxidative stress stimulated by applying exogenous H_2O_2, the transcription of *TaSOD5* was only slightly higher in 'Shi8' than in 'Yimai38' (Fig. 1). The application of abscisic acid, an abiotic stress associated phytohormone, accelerated the expression of *TaSOD5* especially in 'Shi8' (Fig. 1).

To determine the role of *TaSOD5* in the adaption to abiotic stresses, we constructed two *TaSOD5* ectopic overexpression lines (OE1 and OE2) of *Arabidopsis* ecotype Col-0. The *TaSOD5* was integrated in the genomes of the two transgenic lines, and their transcripts were detected (Fig. 2*A*,*B*). In comparison with Col-0, two OE lines had higher total SOD activities (Fig. 2*C*). Consistently, the Cu/Zn SOD activities of the two OE lines were significantly elevated when compared with Col-0 (Fig. 2*C*). The results show that *TaSOD5* encoded a Cu/Zn SOD, and its ectopic overexpression improved SOD activity in *Arabidopsis*.

The two OE lines had no phenotypic alteration from Col-0 during the whole life course ranging from vegetative to reproductive growth in soil (data not shown) indicating that *TaSOD5* had no effect on development of *Arabidopsis* plants. Similarly, the two OE lines had a comparable growth ability and phenotype to Col-0 when growing on the agar plate (Fig. 3*A*,*B*). On the agar plate containing various concentration of NaCl, the growth of seedlings was restricted, but the restriction was attenuated in the OE lines, which had longer roots and higher shoot masses than Col-0 (Fig. 3*A*,*B*). Salt stress can affect the ionic homeostasis between Na^+ and K^+, and therefore destroy the physiological processes. We found that the content of Na^+ and the content of K^+ were both comparable between Col-0 and the OE lines under the control conditions, and the ratios of K^+ to Na^+ were also similar among all plants (Fig. 3*B*). When subjected to 100 mM NaCl, the content of Na^+ increased (Fig. 3*B*). In comparison with Col-0, the alterations in Na^+ and K^+ content were smaller in the OE lines, so that the OE lines had a lower Na^+ but higher K^+ content than Col-0 did. These data indicate that *TaSOD5* ectopic overexpression enhanced salt tolerance and ionic homeostasis maintenance ability in *Arabidopsis*.

Given that salt and other abiotic stresses often induce over-production of ROS, we further analyzed the role of *TaSOD5* in the response to oxidative stress. After exposure to oxidative stress (by applying H_2O_2), seedling growth was restricted; the restriction was attenuated in the OE lines, which had longer roots and larger shoots than Col-0 (Fig. 4*A,B*). The evidence confirms that *TaSOD5* ectopic overexpression enhanced tolerance to oxidative stress.

Superoxide dismutase is the central effector of the ROS scavenging machinery, in which the close link among

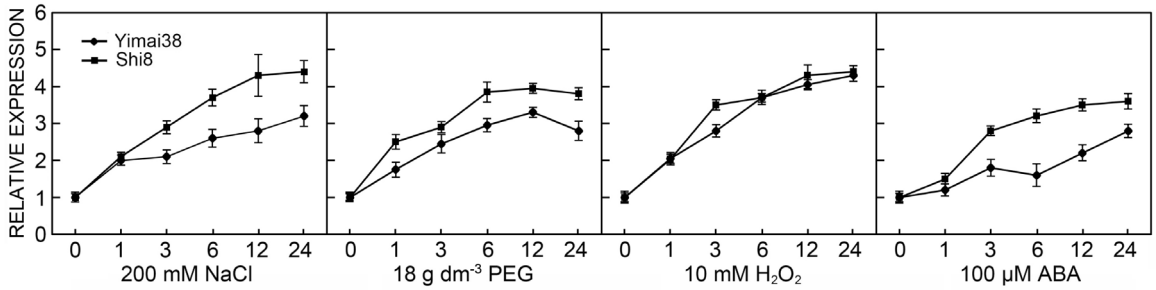

Fig. 1. Expression of a suporoxide dismutase gene *TaSOD5* was responsive to abiotic stresses. Wheat seedlings at three-leaf-stage were subjected to 200 mM NaCl, 18 g dm^{-3} polyethylene glycol (PEG) 6000, 10 mM H_2O_2, or 100 M abscisic acid (ABA) for 0 to 24 h, and *TaSOD5* expressions were detected using real-time quantitative PCR. Means ± SDs, $n = 3$.

components is present. Thus, we detected the activities of GPX, APX, and CAT and in comparison with Col-0, the OE lines had higher APX and GPX activities (Fig. 4*C*). Similarly, the OE lines had also a slightly higher CAT activity than Col-0 although the difference between Col-0 and OE2 was not significant ($P = 0.0542$) (Fig. 4*C*). These data show that *TaSOD5* ectopic overexpression enhanced the activity of ROS scavenging system in addition to SOD activity.

Suitable intracellular ROS production is essential for

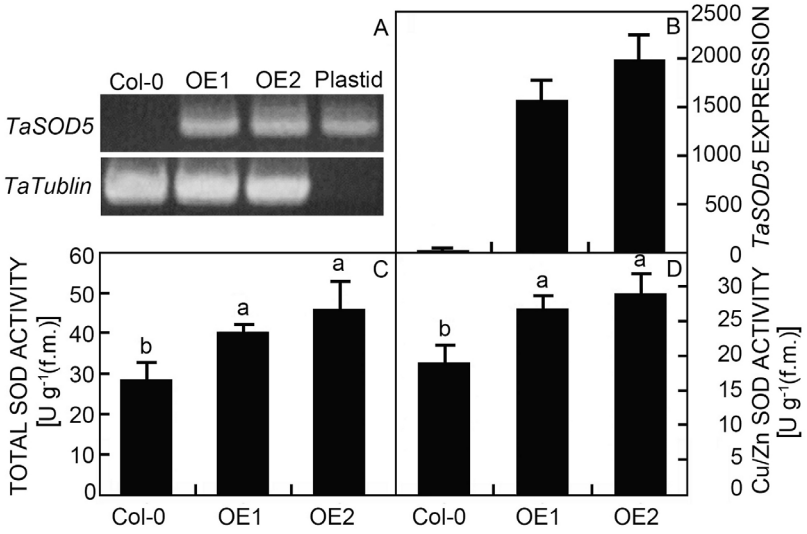

Fig. 2. Superoxide dismutase *TaSOD5* ectopic overexpression improved SOD activity in *Arabidopsis*. *A* - PCR analysis indicates that *TaSOD5* was integrated in the genome of *Arabidopsis*. *B* - Real-time PCR analysis indicates production of transcripts of *TaSOD5* in *Arabidopsis*. *C* - The total SOD and Cu/Zn SOD activities in *Arabidopsis* ecotype Col-0 and the transgenic *Arabidopsis* lines OE1 and OE2 ectopically expressing *TaSOD5*. (Plastid - the pSTART plastid fused with *TaSOD5*; U - the unit of SOD activity which is defined as enzyme activity when the inhibition of the xanthine oxidase is 50 %). Means ± SDs, $n = 3$, different letters indicate significant differences using one-way *ANOVA* ($P < 0.05$).

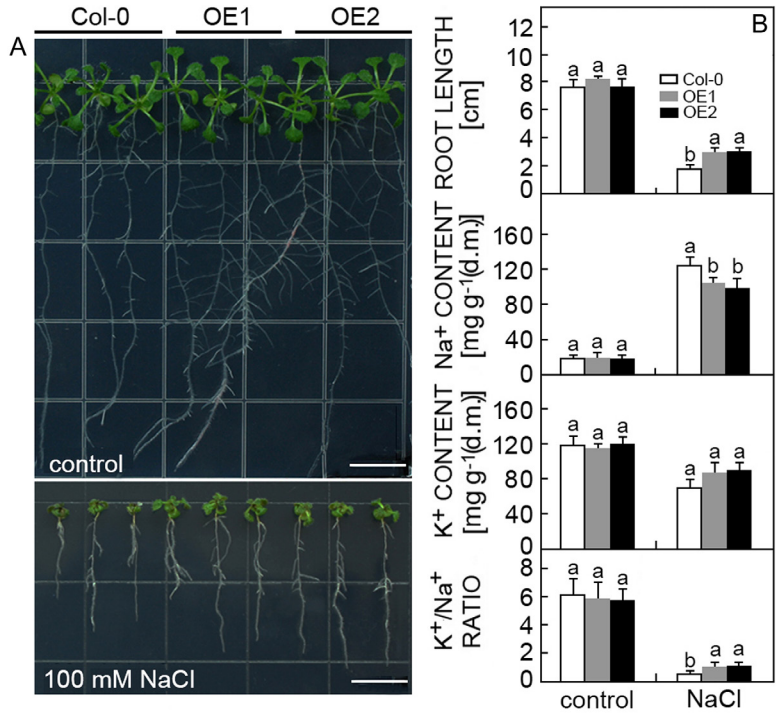

Fig. 3. Superoxide dismutase *TaSOD5* ectopic overexpression offered salt tolerance in OE1 and OE2 transgenic lines in comparison with Col-0. *A* - *Arabidopsis* seedlings growing in plates with none and 100 mM NaCl (the *bar* is 1 cm). *B* - Root length, Na^+ content, K^+ content, K^+/Na^+ ratio as affected by NaCl. Means ± SDs, $n = 3$, different letters indicate significant differences using one-way *ANOVA* analysis ($P < 0.05$).

many physiological processes, so the elevation of activities of ROS scavengers by *TaSOD5* ectopic overexpression may affect ROS production system. Therefore, we also measured the activity of NOX, the major contributor to ROS production. When compared with Col-0, the OE lines had higher NOX activities (Fig. 4*C*).

Discussion

Over-amount of ROS leads to serious oxidative damage. To cope with this, plants have evolved the efficient and complicated ROS scavenging machinery. Within the machinery, SOD appears to play the central role because it converts highly toxic O_2^- to low toxic H_2O_2 (Fridovich 1995). The ectopic overexpression of *TaSOD5* enhanced the activities of total SOD and Cu/Zn SOD in *Arabidopsis* (Fig. 2*C*) indicating that *TaSOD5* expression is connected with a Cu/Zn SOD enzymatic activity.

Salt and other abiotic stresses often induce the production of ROS, so salt tolerance is partially attributed to the capacity of coping with ROS accumulation. Here, *TaSOD5* ectopic overexpression enhanced tolerance to salinity and oxidative stress (Figs. 3, 4). In line with previous studies finding the increase in SOD activity by overexpressing *SOD* genes enhances the tolerance to abiotic stress *via* reducing ROS accumulation (Allen 1995, Gill *et al*. 2015), our work demonstrates that *TaSOD5* enhanced salt tolerance by increasing ROS removal ability.

Note that SOD catalyses the production of H_2O_2, so the increased tolerance to H_2O_2 suggests that downstream ROS scavengers were involved in the enhanced tolerance to salt stress in the *TaSOD5* ectopic overexpression lines. Consistently, *TaSOD5* ectopic overexpression elevated the activities of three H_2O_2 removal enzymes (Fig. 4*C*) indicating that SOD performs roles by comprehensively altering the ROS scavenging system. One possible cause is that the enhanced activities of these H_2O_2 removal enzymes reduce H_2O_2 content and therefore avoid the inhibition of SOD activity because H_2O_2 is an inhibitor of SOD (Allen 1995). The other one is that although H_2O_2 and O_2^- are two moderately reactive ROS, they and can form highly reactive hydroxyl radicals through the Haber-Weiss reaction (Apel and Hirt 2004). The opening question is how *TaSOD* performs the modulation.

Besides as toxic molecules, ROS also serve as signals to

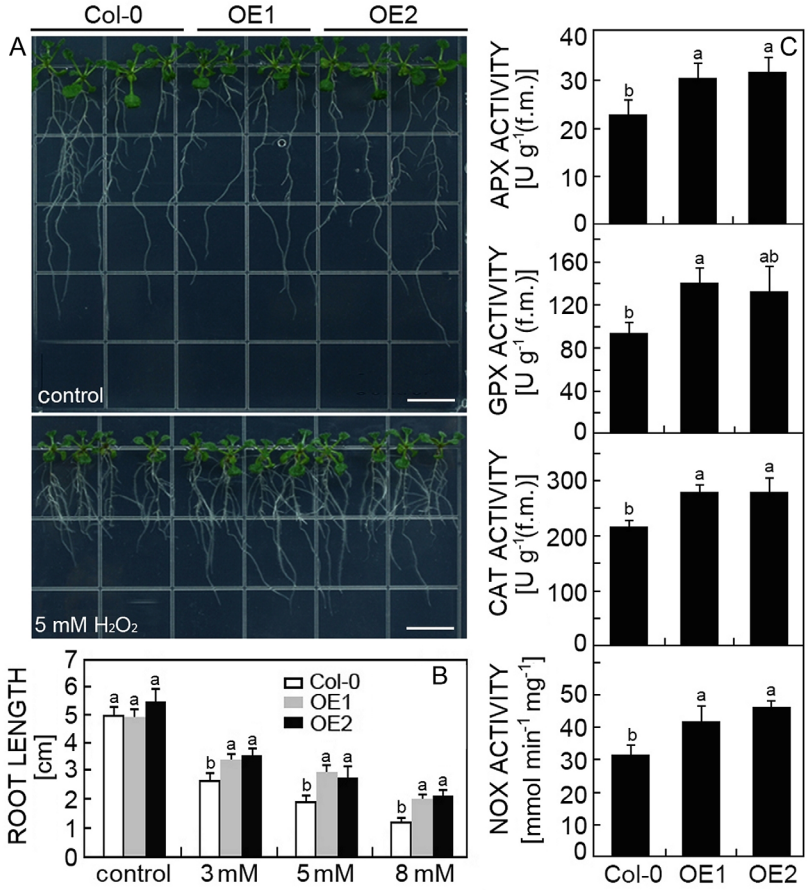

Fig. 4. Superoxide dismutase *TaSOD5* ectopic overexpression enhanced tolerance to H_2O_2 and affected the activities of ROS scavengers ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) and also reactive oxygen species (ROS) producer NADPH oxidase (NOX). *A* - *Arabidopsis* seedlings growing in plates with none and 5 mM H_2O_2. *B* - Root length at different H_2O_2 concentrations. *C* - APX, GPX, CAT, and NOX activities. U - one unit of enzyme activity catalyzes the conversion of 1 μmol of substrate to product in 1 min at 25 °C and pH 7.0; Col-0 - *Arabidopsis* ecotype Col-0; OE1, OE2 - transgenic *Arabidopsis* lines over-expressing *TaSOD5*; the *bar* is 1 cm). Means ± SDs, $n = 3$, different letters indicate significant difference using one-way *ANOVA* ($P < 0.05$).

regulate physiological processes, so suitable ROS amount is necessary for development of plants (Mittler *et al*. 2011, Suzuki *et al*. 2012, Sierla *et al*. 2013,). Control of ROS accumulation is orchestrated by the homeostasis between production and scavenging (Miller *et al*. 2009). The NADPH oxidase is the major ROS production enzyme that produces O_2^- (Torres and Dangl 2005, Suzuki *et al*. 2011), which is dismutated to H_2O_2 spontaneously or catalytically (Lin *et al*. 2009, Wi *et al*. 2012). Here, *TaSOD5* ectopic overexpression increased the activity of NOX (Fig. 4*C*). In consistence with our finding, alteration of ROS scavengers has proved to affect the activity of ROS producers (Suzuki *et al*. 2013). These results indicate that SOD modulates not only the activities of the ROS scavenging system but also the ROS producer, confirming a close association between these two systems (Miller *et al*. 2009). Therefore, *TaSOD5* ectopic overexpression enhanced salt and oxidative tolerance through ROS homeostasis modulation, which was achieved *via* promoting both the ROS scavenging system and the production system.

On the other hand, salt tolerance is comprehensively accomplished by multiple physiological processes including Na^+ exclusion towards the extracellular space or sequestration into the vacuole, better K^+ retention in root and leaf tissues, effective osmotic adjustment, and redox homeostasis (Munns and Tester 2008). Exactly, we found *TaSOD5* ectopic overexpression lowered Na^+ content but improved K^+ content and K^+/Na^+ ratio in *Arabidopsis* under NaCl treatment. This might come from an increased antioxidation ability by which *TaSOD5* ectopic overexpression attenuated the damage by oxidative stress, and therefore maintained a superior physiological status including ionic homeostasis maintenance capacity. Moreover, NOX has proved to modulate the mRNA stability of the Salt overly sensitive 1, the crucial component of the Salt overly sensitive 1/2 pathway (Chung *et al*. 2008); NOX-catalyzed ROS serve as signal molecules to regulate Na^+/K^+ balance, and NOX mutants *rbohd* and *rbohf* are salt-sensitive and have high Na^+/K^+ ratios under salt stress (Ma *et al*. 2011). The accumulated ROS is required for activating downstream signaling transduction pathways (Miller 2009). Thus, a lower Na^+/K^+ ratio and a higher NOX activity by *TaSOD5* ectopic overexpression suggest that *TaSOD5* enhanced salt tolerance *via* mechanisms beyond modulating redox homeostasis alone.

It has been found that apart from positive contribution, SOD has different effects on tolerance to abiotic stresses. For example, in tobacco, the overexpression of a *SOD* gene has no effect on tolerance to oxidative stress (Pitcher *et al*. 1991), but the ectopic overexpression of a pea *SOD* gene increases oxidative stress-induced membrane damage (Gupta *et al*. 1993). These findings suggest the complicated mechanisms governing the performance of SOD in response to abiotic stresses. This brings about a view that the causal link between antioxidant enzymes and overall tolerance to salt and other abiotic stresses is questioned and seriously criticized (Allen *et al*. 1995) because it was argued that salt-tolerance species do not need a higher antioxidation activity as they prevent formation of ROS in the first instance (Bose *et al*. 2014). On the other hand, a rapid burst of NOX-mediated ROS production is required for regulating downstream pathways and acclimation of plants to stress stimuli (Baxter *et al*. 2013). Thus, it could not be excluded that the improved SOD activity may reduce ROS amount to a level lower than a suitable threshold and therefore not enhance tolerance to oxidative stress.

In conclusion, *TaSOD5* may play an important role in responding to salinity and oxidative stresses by enhancing the activities of total SOD and Cu/Zn SOD, keeping a lower Na^+/K^+ ratio and a high NOX activity, as well as promoting both ROS scavenging and the production system. However, the detailed regulatory mechanisms and signaling pathways are still unclear, and further research is needed in the future.

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