Ectopic expression of a wheat superoxide dismutase gene *TaSOD5* enhances salt and oxidative stress tolerance in *Arabidopsis*

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Abstract

Superoxide dismutase (SOD) is a crucial reactive oxygen species (ROS) scavenger, which converts superoxide radical to H₂O₂, so it is thought to enhance abiotic stress tolerance by reducing ROS and thus avoiding oxidative damage. In this study, we isolated a salt- and oxidative stress-responsive copper-zinc (Cu/Zn) SOD encoding gene *TaSOD5* from wheat. The ectopic overexpression of *TaSOD5* in *Arabidopsis thaliana* increased total SOD and Cu/Zn SOD activities and enhanced tolerance to salt stress. *Arabidopsis* ectopically expressing *TaSOD5* possessed a superior resistance to oxidative stress stimulated by exogenous H₂O₂. Ectopic overexpression of *TaSOD5* elevated the activities of both ROS scavengers and an O₂^{•−} producer - NADPH oxidase. These findings show that Cu/Zn SOD enhanced salt tolerance *via* regulating the machinery of redox homeostasis rather than improving SOD activity alone.

Keywords: *Arabidopsis thaliana*, H₂O₂, superoxide dismutase, salt stress, redox homeostasis, *Triticum aestivum*.

Introduction

Salt stress induces overproduction of reactive oxygen species (ROS), and accumulated ROS cause secondary oxidative damage. One source of ROS production is the reduction of O₂ to form singlet oxygen (O₂¹) or superoxide radical (O₂^{•−}) (Mittler 2002). To cope with the damage of ROS, plants have developed an efficient ROS scavenging machinery, consisting of a set of nonenzymatic compounds and enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GTR) (Mittler 2002). SOD serves as the first defense line to convert O₂^{•−} to hydrogen peroxide (Fridovich 1995). SOD family constitutes three classes, copper-zinc SOD (Cu/Zn-SOD), iron SOD (Fe-SOD) and manganese SOD (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, suitable amount of ROS is necessary

for the development and response to environmental stimuli, because ROS play crucial regulatory performance in numerous biological processes (Mittler *et al.* 2011, Suzuki *et al.* 2012, Sierla *et al.* 2013). ROS content is governed by ROS production and scavenging pathways and there is a close link between the two systems (Miller *et al.* 2009). Thus, the improvement of ROS scavenging could not ensure the enhancement of tolerance to abiotic stresses. For example, the overexpression of a tobacco SOD gene did not alter tolerance toward oxidative stress (Pitcher *et al.* 1991). These findings suggest SOD function in abiotic stress response *via* the mechanisms more complicated than simply scavenging ROS, which have not been addressed well so far.

Bread wheat (*Triticum aestivum*) is one of the most important crops, but it has only limited resistance to abiotic stresses. We previously bred a wheat cultivar Shi8 with remarkable salt tolerance. Here, we identified an abiotic stress responsive Cu/Zn SOD gene *TaSOD5*, and

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; GPX - glutathione peroxidase; NBT - nitroblue tetrazolium; NOX - NADPH oxidase; OE - overexpression; ROS - reactive oxygen species; SCR - Shanghai Chemical Reagent; SOD - superoxide dismutase.

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we studied its ectopic overexpression in *Arabidopsis* to determine if *TaSOD5* ectopic overexpression can enhance its tolerance to salt and oxidative stresses by improved activities of some ROS scavengers and a ROS producer NADPH oxidase.

Materials and methods

Isolation of *TaSOD5*: The sequences from the RNA sequencing dataset of common wheat (*Triticum aestivum* L.) cultivars Yimai38 with moderate drought tolerance and Shi18 with strong drought tolerance that were bred by our own group were subjected to BLASTN against the wheat EST database in NCBI (EST number: 2506684; Jul 28, 2020). The matched sequences were assembled using the CAP3 software (Huang and Madan 1999). According to the assembled sequence, the primers TGCACCGAGGCGACAAAGG and GGCGCAGCAATACCAACATGG were designed to isolate the full-length cDNA (namely *TaSOD5*) from Shi8. 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 of 72 °C/10 min.

Transgenic *Arabidopsis* plants with *TaSOD5* construct: The *TaSOD5* was ligated into pSTART vector to construct pSTART-*TaSOD5* vector driven by the CaMV 35S promoter. The pSTART-*TaSOD5* vector was introduced into *Arabidopsis thaliana* L. Col-0 (retained in our group) using the floral dip method (Clough and Bent 1998). Homozygous transgenic lines were selected by kanamycin screening. DNA of the transgenic lines was extracted for PCR to detect the integration of *TaSOD5*. RNA of the transgenic lines was isolated for real-time PCR to measure the expression of *TaSOD5* in *Arabidopsis*.

Plant treatments: Wheat was grown in a half strength Hoagland's liquid medium (pH = 7.0) (Peng *et al.* 2009) under a 16-h photoperiod, an irradiance of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a temperature of 22 °C, and a relative humidity of 70 %. Two-week-old wheat seedlings were transferred to the medium containing either 200 mM NaCl (analytically pure > 99.8%), 18 % (m/v) PEG 6 000 (chemical pure > 99.5 %), 10 mM H₂O₂ (30 % in water) or 100 μM ABA (m/v, all chemicals Shanghai Chemical Reagent (SCR), Shanghai, China) for 0, 1, 3, 6, 12, and 24 h. The leaves were sampled for RNA extraction to measure the responsive patterns of *TaSOD5* in wheat. To avoid the influence of circadian rhythm on *TaSOD5* expression, the treatment was started at different time points, and the leaves were sampled at the same time point.

Arabidopsis seeds were sterilized and plated on a half strength Murashige and Skoog agar medium. The seeds were kept in the dark at 4 °C for three days to break dormancy, and then transferred to the 16-h photoperiod an irradiance of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a temperature of 22 °C, and a relative humidity of 70 % for two days. They were re-plated on the half strength Murashige and Skoog agar medium supplemented with 50 and 100 mM NaCl (for

14 d), or 3, 5 and 8 mM H₂O₂ (for 10 d) and grown under the same conditions. The seedlings were used for RNA extraction.

Quantitative real-time PCR analysis: Total RNA was extracted from samples mentioned above using the Trizol reagent (Invitrogen, Carlsbad, USA), and treated with DNAase I (> 2000 units mg^{-1} , Sigma, St. Louis, USA). RNA was reversely transcribed to synthesize the first cDNA strand using the M-MLV reverse transcription system kit (Invitrogen, Carlsbad, USA). The cDNA was used as the template for real-time PCR in 20 μm^3 solution containing 10 μm^3 2 $\times$ SYBR Premix Ex Taq Mix (Takara, Dalian, China), 0.2 μM forward and 0.2 μM reverse primers, 1 μm^3 of a 1:10 dilution of the cDNA first strand, and the cycling regime comprised a denaturation step at 95 °C/2 min, followed by 45 cycles of 95 °C/10 s, 60 °C/20 s, 72 °C/20 s. A melting curve analysis was performed over the range 80 to 95 °C at 0.5 °C intervals. Relative gene expressions were detected using the 2^{- $\Delta\Delta\text{CT}$} method (Livak and Schmittgen 2001). The wheat ACTIN gene (AB181991) or *Arabidopsis* TUBULIN gene (AT1G04820) were used as internal references. The forward and reverse primers were ACAAGCCAACAAGGATGGTG and TCAGCATGAACGACAACCTGC for *TaSOD5*, TCAACAACATGAAAGGTCC and CTAGAAGTTCTCTTTGTGG for *AtRbohD* (NADPH/respiratory burst oxidase protein D, AT5G47910), CAGCAACCGCCATTAATG and CATCGAACAGTTCCAATGC for *AtRbohF* (NADPH/respiratory burst oxidase protein D, AT1G64060), CGAAACCTTCAGTTGCCCCAGCAAT and ACCATCACCAGAGTCGAGCACAAT for wheat ACTIN gene AB181991, CTCAAGAGGTTCTCAGCAGTA and TCACCTTCTTCATCCGCAGTT for *Arabidopsis* TUBULIN gene AT1G04820, respectively.

Measurement of H₂O₂ content and activities of total and Cu/Zn SOD and other ROS scavenging enzymes: The H₂O₂ content was measured with the diaminobenzidine (DAB) staining method. The leaves of four-week-old seedlings were selected, and placed in DAB (Sigma, Saint Louis, USA) solution (1 mg cm^{-3} , pH 4) at room temperature in the dark for 6 h. The leaves were then transferred into 95 % (v/v) ethanol, and subjected to boiling bath to remove chlorophyll.

The leaves of two-week-old *Arabidopsis* seedlings were collected for measuring total SOD and Cu/Zn SOD activities. Total SOD activity was measured using the nitroblue tetrazolium (NBT) method with a SOD activity kit (Beyotime Institute of Biotechnology, Shanghai, China). The Cu/Zn SOD activity was measured with the Cu/Zn-SOD and an Mn-SOD assay kits with WST-8 (Beyotime Institute of Biotechnology). The unit of SOD was defined as the amount of SOD that inhibits 50% photochemical reduction of NBT by 1 mg protein .

For measuring the activity of ROS scavenging enzymes, 0.5 g leaves of two-week-old *Arabidopsis* seedlings were sampled and homogenized in 1 cm^3 of homogenization solution on ice. The solution consisted of 50 mM KH₂PO₄

(99.5 %, SCR, Shanghai, China), 0.1 mM EDTA (99.5 %, SCR), and 0.3 % (m/v) Triton X-100 (Sigma). The homogenate was centrifuged at 1 200 g and 4 °C for 15 min, the pellet was removed and the supernatant was retained. Protein content of the supernatant was determined using the Bradford Coomassie brilliant blue staining assay (Bradford 1976). The protein extraction solution was used to measure the activities of catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). The CAT activity was measured by detecting the decrease in absorbance at 240 nm for 1 min using a CAT activity measurement kit (Beyotime Institute of Biotechnology). APX activity was measured at 290 nm by applying an ascorbic acid (AsA) extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-2}$ (Beyotime Institute of Biotechnology). The GPX activity was determined by monitoring an decrease in absorbance at 340 nm of a reaction system using a GPX activity measurement kit (Beyotime Institute of Biotechnology), and a coefficient of absorbance for NADPH was assumed to be $6.22 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Sebastiani *et al.* 2007). The unit of CAT was defined as the decrease of optical density value by 0.01 per minute by 1 mg protein. The unit of GPX was defined as catalyzing the oxidation of 1 nmol reduced glutathione (GSH) per minute by 1 mg protein. The unit of APX was defined as catalyzing the oxidation of 1 μmol ascorbic acid (AsA) per minute by 1 mg protein.

Measurement of Na^+ and K^+ content: The *Arabidopsis* seedlings grown on the medium plates containing none and 100 mM NaCl were sampled. After clearing with distilled water, the seedlings were dried at 80 °C for 3 d. Dried seedlings (5 mg) were placed in a muffle furnace at 570 °C for 6 h. Then, 2.5 cm^3 HCl (1:1 v/v in H_2O) was added, and mixed well. After centrifugation at 12 000 g at 25 °C, the supernatant was collected, and diluted to a final volume of 25 cm^3 with distilled water. The solution was used for measuring Na^+ and K^+ contents with an atom absorption spectrometry detector (Persee TAS-990, Persee Analytics, Beijing, China).

Measurement of NADPH oxidase activity: Leaves (0.5 g) were sampled from two-week-old *Arabidopsis* seedlings, and homogenized in 1 cm^3 of a homogenization solution containing 50 mM KH_2PO_4 and 0.1 mM EDTA. The homogenate was centrifuged at 12 000 g and 4 °C for 15 min, and the supernatant was collected for measuring the activity of NADPH oxidase (NOX) (Grace and Logan 1996). A reaction solution contained 100 mM Tris-HCl (99 %, SCR, Shanghai, China), pH 8.0, 1 mM EDTA, and 0.2 mM NADPH. The reaction began when NADPH was added. Activity of NOX was calculated by monitoring decrease in absorbance at 340 nm (Shimadzu UV-3600, Kyoto, Japan). The molar coefficient of absorbance for NADPH was $6.22 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The unit of NOX was defined as the oxidation of 1 μmol reduced NADPH per minute by 1 mg protein.

Statistical analysis: The differences of indices among Col-0 and two overexpression (OE) lines were calculated by the one-way ANOVA analysis with post-hoc Tukey's

honest significant difference (HSD) multiple comparison ($P\alpha = 0.05$) using SPSS v. 19 (Chicago, IL, USA).

Results

Our transcriptional analysis by transcriptome sequencing found out that the genes of antioxidant machinery were remarkably enriched in the common wheat salt tolerant cultivar Shi8, among which a fragment annotated with SOD (namely *TaSOD5*) was induced to a stronger extent than the others (data not shown). 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 the response to abiotic stresses, its transcriptional profiles upon several treatments were detected. After exposure to 200 mM NaCl to mimic salt stress, *TaSOD5* was gradually induced, with a stronger induction in Shi8 than in its parent cultivar Yimai38 (Fig. 1A). When treated with 18 % PEG to mimic osmotic stress, the expression of *TaSOD5* was elevated over the whole course of treatment and peaked at 12 h; Shi8 accumulated more *TaSOD5* transcripts than Yimai38 did (Fig. 1B). After exposure to exogenous H_2O_2 , *TaSOD5* expression was promoted, and the expression was similar in Shi8 and in Yimai38 (Fig. 1C). The application of ABA, an abiotic stress associated phytohormone, accelerated the expression of *TaSOD5* especially in Shi8 (Fig. 1D).

To determine the role of *TaSOD5* in the adaption to abiotic stress, we constructed two *TaSOD5* ectopic

Fig. 1. Responses of *Triticum aestivum* superoxide dismutase 5 to abiotic stresses: A - 200 mM NaCl, B - 18 % polyethylene glycol 6000, C - 10 mM H_2O_2 , D - 100 μM ABA. Wheat seedlings at the three-leaf-stage were subjected to these treatments for 0 - 24 h, and the expression was detected using real-time PCR analysis. Means $\pm$ SDs, $n = 3$.

Fig. 2. The effect of *Triticum aestivum* superoxide dismutase 5 (*TaSOD5*) ectopic overexpression on superoxide dismutase (SOD) activity in two *Arabidopsis* transgenic lines OE1 and OE2: A, B - integration of *TaSOD5* in the genome of *Arabidopsis*, *AtTubulin* - *Arabidopsis thaliana tubulin*; C - total SOD activity, D - Cu/Zn SOD activity. Col 0 - wild type of *A. thaliana*. Means $\pm$ SDs, $n = 5$, different letters indicate significant differences at $P < 0.05$ using one-way ANOVA followed by Tukey's honest significant difference multiple comparison test.

overexpression lines (OE1 and OE2) derived from *Arabidopsis* ecotype Col-0. The *TaSOD5* was integrated in the genomes of two transgenic lines (Fig. 2A), and its transcripts were detectable (Fig. 2B). In comparison with Col-0, two OE lines had higher total SOD activities (Fig. 2C). Consistently, the Cu/Zn SOD activities of two OE lines were significantly elevated when compared with Col-0 (Fig. 2D). The results show that *TaSOD5* encodes a Cu/Zn SOD, and its ectopic overexpression improves SOD activity in *Arabidopsis*.

Two OE lines had no phenotypic alteration from Col-0 during the whole life span ranging from vegetative to reproductive phase when growing in soil (data not shown), indicating that *TaSOD5* had no effect on development of *Arabidopsis*. Similarly, two OE lines had comparable growth ability and phenotype to Col-0 when growing on the agar plate (Fig. 3A,C). On the agar plate containing various concentrations of NaCl, the growth of seedlings was restricted, but the restriction was attenuated in the OE lines so that they had longer roots and higher shoot

mass than Col-0 (Fig. 3B,C). Salt stress can affect the ionic homeostasis between Na^+ and K^+ . We found that the content of Na^+ and K^+ were both comparable between Col-0 and the OE lines under the control conditions, and the ratios of K^+ to Na^+ were similar among all plants (Fig. 3D-F). When subjected to 100 mM NaCl, Na^+ content was increased, but K^+ content was declined (Fig. 3D-F). In comparison with Col-0, the alterations of Na^+ and K^+ content were both smaller in the OE lines, leading to higher K^+ to Na^+ ratios. These data indicated that *TaSOD5* ectopic overexpression enhances salt tolerance and ionic homeostasis maintenance ability in *Arabidopsis*.

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

The SOD is the central effector in the ROS scavenging machinery, in which the close link among components is present. Thus, we detected the content of H_2O_2 and the activities of glutathione peroxidase (GPX), ascorbate peroxidase (APX) and catalase (CAT), three ROS removal enzymes catalysing conversion of H_2O_2 , the product of SOD, to H_2O . DAB staining indicated that the leaves of the OE lines accumulated less H_2O_2 than Col-0 leaves did (Fig. 5A). In comparison with Col-0, the OE lines had higher APX and GPX activities (Fig. 5B,C). Similarly, the OE lines also had slightly higher activities of CAT than Col-0, although the difference between Col-0 and OE2 was not significant (Fig. 5D). These data show that *TaSOD5* ectopic overexpression enhanced the activity of ROS scavenging system instead of SOD activity alone.

Suitable intracellular ROS content is essential for physiological processes, so the increased activities of ROS scavengers by *TaSOD5* ectopic overexpression may affect ROS production system. Thus, we measured the activity of NADPH oxidase (NOX), the major contributor of ROS production. When compared with Col-0, the OE lines had higher NOX activities (Fig. 5E). Consistently, the expressions of *RbohD* and *RbohF*, two NOX encoding genes, were higher in the OE lines than in Col-0 (Fig. 5F,G).

Discussion

High content of intracellular ROS leads to serious oxidative damage. To cope with this stress, plants have evolved the efficient and complicated ROS scavenging machinery. Within the machinery, SOD appears to play a central role because it converts highly toxic O_2^- to less toxic H_2O_2 (Fridovich 1995). The ectopic overexpression of *TaSOD5* enhances the activities of total SOD and Cu/Zn SOD in *Arabidopsis* (Fig. 2C,D). Salt and other abiotic stresses often induce the production of ROS, so salt tolerance is partially attributed to the capacity of coping with ROS excess. Here, *TaSOD5* ectopic overexpression

Fig. 3. *Triticum aestivum* superoxide dismutase 5 ectopic overexpression enhanced salt tolerance of two *Arabidopsis* transgenic lines OE1 and OE2. A, B - *Arabidopsis* seedlings grown in plates with none (A) and 100 mM (B) NaCl (scale bar - 1 cm), C - root length (results from panels A and B), D - Na⁺ content, E - K⁺ content, F - K⁺/Na⁺ ratio. Col 0 - wild type of *A. thaliana*. Means $\pm$ SDs, $n = 15$ in (C) and $n = 5$ in (D-F), different letters indicate significant differences at $P < 0.05$ using one-way ANOVA followed by Tukey's honest significant difference multiple comparison test.

Fig. 4. *Triticum aestivum* superoxide dismutase 5 ectopic overexpression enhanced oxidative tolerance of two *Arabidopsis* transgenic lines OE1 and OE2. A, B - *Arabidopsis* seedlings grown in plates with none (A) and 5 mM (B) H₂O₂ (scale bar - 1 cm), C - root length. Col 0 - wild type of *A. thaliana*. Means $\pm$ SDs, $n = 15$, different letters indicate significant differences at $P < 0.05$ using one-way ANOVA followed by Tukey's honest significant difference multiple comparison test.

reduces H₂O₂ content and enhances tolerance to salt and oxidative stresses. It was in line with the previous finding that 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).

It should be noted that SOD catalyses the production of H₂O₂, so the decreased H₂O₂ content and increased tolerance to H₂O₂ (Figs. 4 and 5) suggested that downstream ROS scavengers may contribute to the enhanced salt tolerance of *TaSOD5* ectopic overexpression lines. Consistently,

TaSOD5 ectopic overexpression elevated the activities of three H₂O₂ removal enzymes (CAT, APX, and GPX; Fig. 5B-D), indicating that SOD performed the roles, at least in a large part, by comprehensively altering the ROS scavenging system. One possible cause was that the enhanced activities of these H₂O₂ removal enzymes reduced H₂O₂ content and avoided the inhibition of SOD activity by H₂O₂ accumulation (Allen 1995). The other one is that although H₂O₂ and O₂⁻ are two moderately reactive ROS, they can form highly reactive hydroxyl radicals

Fig. 5. *Triticum aestivum* superoxide dismutase 5 ectopic overexpression reduced H₂O₂ content and increased enzyme activities in two *Arabidopsis* transgenic lines OE1 and OE2. *A* - content of H₂O₂ (scale bar - 1 cm); *B-D* - activities of H₂O₂ scavenging enzymes: ascorbate peroxidase (APX) (*B*), glutathione peroxidase (GPX) (*C*), and catalase (CAT) (*D*); *E* - activity of reactive oxygen species producer - NADPH oxidase (NOX); *F,G* - relative expressions of NOX encoding genes, *AtRbohD* (*F*) and *AtRbohF* (*G*). Col-0 - wild type of *A. thaliana*. Means $\pm$ SDs, $n = 5$, different letters indicate significant differences at $P < 0.05$ using one-way ANOVA followed by Tukey's honest significant difference multiple comparison test.

(HO $\cdot$) through Haber-Weiss reaction (Apel and Hirt 2004), so SOD-catalyzed O₂⁻ removal can avoid the formation of HO $\cdot$.

Besides being toxic chemicals, ROS also serve as signal molecules to regulate physiological processes, so suitable ROS content is necessary for development of plants (Mittler *et al.* 2011, Suzuki *et al.* 2012, Sierla *et al.* 2013). ROS content is orchestrated by the homeostasis between ROS production and scavenging (Miller *et al.* 2009). NADPH oxidase (NOX) is the major ROS production enzyme that produces O₂⁻ (Suzuki *et al.* 2011, Torres and Dangl 2005), which is dismutated to H₂O₂ spontaneously or catalytically *via* SOD (Lin *et al.* 2009,

Wi *et al.* 2012). Here, *TaSOD5* ectopic overexpression increased the activity of NOX and the expressions of NOX encoding genes (Fig. 5E-G). In consistence with our finding, alteration of ROS scavengers has proved to affect the activities of ROS producers (Suzuki *et al.* 2013). These results indicate that SOD modulates not only the activities of ROS scavengers but also ROS producers, confirming a close association between these two systems (Miller *et al.* 2009). Therefore, *TaSOD5* ectopic overexpression enhanced salt and oxidative tolerance through modulating ROS homeostasis, which was achieved *via* promoting both ROS scavenging and production systems. It is possible that to achieve the modulation, ROS are maintained at a low level, so it can activate downstream genes including ROS producers to maintain a suitable level (Miller *et al.* 2009), but not to accumulate them to a toxic level. However, how plants would balance the activities of ROS producers and removers to modulate intracellular ROS content needs to be answered in the future.

On the other hand, salt tolerance is comprehensively accomplished by multiple physiological processes including Na⁺ exclusion towards extracellular spaces or sequestration into the vacuoles, better K⁺ retention in root and leaf tissues, effective osmotic adjustment, and redox homeostasis (Munns and Tester 2008). Exactly, we found that *TaSOD5* ectopic overexpression decreased Na⁺ content but increased K⁺ content and K⁺/Na⁺ ratio in *Arabidopsis* under salt treatment. This result might owe to the possible cause that increased antioxidation ability by *TaSOD5* ectopic overexpression attenuated the damage caused by oxidative stress, and so maintained ionic homeostasis. Moreover, increasing evidence shows that besides acting as important signals, ROS can alter the redox state of some proteins and affect their roles and activities. Thus, it could not be excluded that ROS might affect the activities of Na⁺/K⁺ transporting proteins, and lower ROS content due to *TaSOD5* ectopic overexpression modulated their abilities of Na⁺/K⁺ transporting. Thirdly, NOX has proved to modulate the mRNA stability of *Salt Overley Sensitive 1* (*SOS1*), the crucial component of SOS 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 higher Na⁺/K⁺ ratio under salt stress (Ma *et al.* 2012). The accumulation of ROS is required for activating downstream signaling transduction pathways (Miller *et al.* 2009). Thus, lower Na⁺/K⁺ ratio and higher NOX activity by *TaSOD5* ectopic overexpression suggested that *TaSOD5* enhanced salt tolerance *via* the mechanisms beyond modulating redox homeostasis alone.

It has been found that SOD has different effects on tolerance to abiotic stress. 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 the response to abiotic stress. This brings about a view that the causal link between anti-oxidation enzymes and overall tolerance to

salt and other abiotic stresses is questioned and seriously criticized (Allen 1995), because it is argued that salt-tolerant species do not need higher anti-oxidation activity as they prevent formation of highly toxic ROS species 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.* 2014). Thus, it could not be excluded that the improved SOD activity may reduce ROS accumulation to a level lower than the suitable threshold, and, therefore, does not enhance tolerance to oxidative stress.

The *TaSOD5* expression was induced more in Shi8 than in Yimai38 after exposure to NaCl and other treatments (Fig. 1). This result implied that there might be genetic variation such as single nucleotide polymorphism (SNP) and indels or epigenetic variation such as DNA methylation in the promoter sequences of *TaSOD5* between two cultivars, which contributes to the different responsive profiles of *TaSOD5* expression to abiotic stresses. It should be noted that *TaSOD5* had comparable expression between two cultivars under the control conditions (Fig. 1), indicating the contribution of putative genetic and epigenetic variation to *TaSOD5* expression was achieved in a stress-dependent manner. The possible causes were that the genetic variation may affect the binding capacity of stress-induced transcription factor(s) to *TaSOD5* promoter, and the DNA methylation of *TaSOD5* was differentially altered between two cultivars under the stressful conditions.

In summary, the ectopic expression of *TaSOD5* enhanced the activities of both ROS scavenging and producing enzymes to alter ROS homeostasis machinery, and, therefore, avoided oxidative damage caused by ROS over-accumulation. This alteration consequently helped plants reconstruct ionic homeostasis under salt stress. Thus, the change of a component in ROS homeostasis machinery appeared to enhance stress tolerance *via* comprehensive altering physiological processes.

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