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...
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 Yima38 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 TCACCGAGCGCAAAAGG and GGCAGCAGCAATTACCAACTGG were designed to isolate the full-length cDNA (namely TaSOD5) from Shi18. 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 µmol m⁻² 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 6000 (chemical pure > 99.5 %), 10 mM H₂O₂ (30 % in water) or 100 µM ABA (m/v, all chemicals Sigma, St. 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.

Measurement of H₂O₂ content and activities of total and Cu/Zn SOD and other ROS scavenging enzymes: The H₂O₂ content was measured with the dimaminobenzidine (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 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 cm³ of homogenization solution on ice. The solution consisted of 50 mM K₂HPO₄, 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 DNase 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 mm³ solution containing 10 mm² 2× SYBR Premix Ex Taq Mix (Takara, Dalian, China), 0.2 µM forward and 0.2 µM reverse primers, 1 mm² 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 calculated using the ΔΔCT 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 ACAAAGCACAACAGGATGTTG and TCCCATGAAACCGACAACCTGC for *TaSOD5*, TCAACAACATGAAAGGTCC and CTAGAAGTTCTTTGTTG for *AtRbohD* (NADPH/respiratory burst oxidase protein D, AT5G47910), CAGCAACCGCATTAAGT and CATCGAACAGTTCCAAATGC for *AtRbohF* (NADPH/ respiratory burst oxidase protein D, AT1G64060), CGAAACCTTCAGTGGCAGCATT and ACCATCACCAGTGAGCAGCAAT for wheat ACTIN gene AB181991, CTCAGAGGTTTCTACGGTA and TCACCTTCTTCTACCGACTT for *Arabidopsis TUBULIN* gene AT1G04820, respectively.
(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 mM-1 cm-1 (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 6.22 103 M-1 cm-1. The unit of CAT was defined as 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 cm3 HCl (1:1 v/v in H2O) was added. After centrifugation at 12 000 g at 25 °C, the supernatant was collected 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 cm3 of a homogenization solution containing 50 mM KH2PO4 and 0.1 mM EDTA. The homogenate was centrifuged at 12 000 g for 15 min, and the supernatant was collected for measuring the activity of NADPH oxidase (NOX) (Grice 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 · 103 M-1 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 (Pa = 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 H2O2, 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 lines of 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 ± SDs, n = 3.
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 H₂O₂, 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 H₂O₂ and the activities of glutathione peroxidase (GPX), ascorbate peroxidase (APX) and catalase (CAT), three ROS removal enzymes catalysing conversion of H₂O₂, the product of SOD, to H₂O. DAB staining indicated that the leaves of the OE lines accumulated less H₂O₂ 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 O₂⁻ to less toxic H₂O₂ (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...
SOD EXPRESSION ENHANCES STRESS TOLERANCE

It should be noted that SOD catalyses the production of H$_2$O$_2$, so the decreased H$_2$O$_2$ content and increased tolerance to H$_2$O$_2$ (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 H$_2$O$_2$ 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 H$_2$O$_2$ removal enzymes reduced H$_2$O$_2$ content and avoided the inhibition of SOD activity by H$_2$O$_2$ accumulation (Allen 1995). The other one is that although H$_2$O$_2$ and O$_2^-$ are two moderately reactive ROS, they can form highly reactive hydroxyl radicals.
difference multiple comparison test. using one-way = 5, different letters indicate significant differences at 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⁺ 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 been found to modulate the mRNA stability of Salt Overly Sensitive 1 (SOS1), the crucial component of SOS pathway (Chung et al., 2008); NOX-catalyzed ROS serve as signal molecules to regulate Na⁺ balance and NOX mutants rbodh and rbodh 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.

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, and 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.

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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 that the DNA methylation of TaSOD5 promoter, and genetic variation may affect the binding capacity of stress-dependent regulation of Na+/K+ homeostasis in Arabidopsis under salt stress. - J. exp. Bot. 63: 305-317, 2012.

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