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RESEARCH PAPERS

Heterologous Expression of a Halophilic Archaeon Manganese Superoxide Dismutase Enhances Salt Tolerance in Transgenic Rice[1]

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Abstract—In order to investigate new gene resource for enhancing rice tolerance to salt stress, manganese superoxide dismutase gene from halophilic archaeon (*Natrinema altunense* sp.) (*NaMnSOD*) was isolated and introduced into *Oryza sativa* L. cv. Nipponbare by *Agrobacterium*-mediated transformation. The transformants (L1 and L2) showed some *NaMnSOD* expression and increased total SOD and CAT activity, which contributed to higher efficiency of ROS elimination under salt stress. The levels of superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) were significantly decreased. In addition, they exhibited higher levels of photosynthesis, whereas lower relative ion leakage and MDA content compared to wild-type plants. Therefore, transgenic seedlings were phenotypically healthier, and heterologous expression of *NaMnSOD* could improve rice salt tolerance.

Keywords: Natrinema altunense sp., *Oryza sativa*, superoxide dismutase, salt stress, transgenic rice

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INTRODUCTION

There are many extremely harsh environments, such as hot springs, salt lakes, and submarine volcanic habitats with abundant resources of “extremophiles”. The abnormal temperature, low nutrient levels, abundant sunlight, and remote geographical location of these regions make them relatively special ecosystems [1, 2]. However, isolation and applications of stress genes in extremophiles still remains quite limited. With the excess consumption of resources and the deterioration of the environment, characterization of gene information and protein properties of extremophiles, as well as expression in other organisms promise to be beneficial for tolerance.

Salinity is a global problem that threatens crop yield and quality [3, 4]. Exposure to severe salt stress can lead to oxidative damages, ion toxicity, and nutritious imbalance [5]. ROS play an important role in these toxic effects [6]. In the antioxidative process, superoxide dismutase (SOD), as the first defense line, converts superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). H_2O_2 can be rapidly decomposed into nontoxic components O_2 and H_2O by peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), or in the ascorbate–glutathione cycle [7]. Overexpression of SOD was in positive correlation with stress tolerance of many transgenic plants [8–10].

SOD can be classified into four groups: copper-zinc SOD (Cu/ZnSOD), iron SOD (FeSOD), manganese SOD (MnSOD), and nickel SOD (NiSOD). MnSOD could be found in prokaryotic organisms as well as in eukaryotes. NaSOD, putative MnSOD from halophilic archaeon (*Natrinema altunense* sp.) was isolated in our previous work [11]. By the experimental verification, the NaSOD was expressed in colibacillus, and the tolerance of the colibacillus to a high-salt environment enhanced. Up to now, overexpression of archaeon SOD in plants has not been reported. Rice (*Oryza sativa*) is a tremendously important food crop and is particularly sensitive to salt stress. In this study, *NaSOD* was introduced into rice by *Agrobacterium*-mediated transformation and proved to be a MnSOD coding gene. Phenotypic changes and physiological parameters were measured, and the results showed the transgenic rice plants were more tolerant to salt stress than wild-type (WT) plants.

[1] This text was submitted by the authors in English.

Abbreviations: CaMV—cauliflower mosaic virus; CAT—catalase; Chl—chlorophyll; F_0—the minimum fluorescence yield; F_m—the maximum fluorescence yield; F_s—stable fluorescence yield; GUS—β-glucuronidase; L1/L2—transgenic lines; NBT—nitroblue tetrazolium chloride; $O_2^{\bullet-}$—superoxide anion radical; PCR—polymerase chain reaction; RT-PCR—reverse transcription–polymerase chain reaction; SOD—superoxide dismutase; WT—wild type.

MATERIALS AND METHODS

Construction of vector and rice transformation. An extremely halophilic archaeon strain *Natrinema altunense* sp., AJ2 was isolated from Ayakekum salt lake in Altun Moutain in Xinjiang, China [1]. For protein screening, AJ2 was cultured with 1.7 M or 3.0 M NaCl and harvested in the logarithmic growth phase. Differential proteins were analyzed and purified with SDS-PAGE and HPLC. A DNA fragment of *NaSOD* was cloned after amino acid sequencing and PCR-amplification. Homologous analysis of sequences and amino acid similarity indicated that the NaSOD was a MnSOD.

For construction of the plant express vector, the primers *NaMnSOD*-F, 5'-CGG GGT ACC ATG ACT GAT CAC GAA CTT CCAC-3', and *NaMnSOD*-R, 5'-AAA CTG CAG TTA CTC GAA GTG GTC GAG GCAG-3' were used for fidelity PCR. Then subcloned into reconstructive vector pCAMBIA1301 under cauliflower mosaic virus 35S (CaMV 35S) promoter. The p1301-*35S::NaMnSOD* was ready for transforming rice after introducing into *Agrobacterium tumefaciens* strain EHA105.

Transformation of rice (*Oryza sativa* L., cv. Nipponbare) was achieved by co-cultivation of rice calli derived from mature seed scutella with *A. tumefaciens* containing p1301-*35S::NaMnSOD* according to a previously reported protocol [12].

Screening of transgenic plants. All the putative T_0 transgenic plants were screened using PCR analysis and GUS staining [13]. Plants of T_1 generation were firstly screened by 50 mg/L hygromycin selection. The lines with 3 : 1 segregation ratio were chosen and propagated.

SOD activity staining was performed according to the method of Luo and Wang [14]. The total proteins (50 μg per sample) were separated by a nondenaturing 10% PAGE with a 2.5% stacking gel and stained by riboflavin-nitroblue tetrazolium method for SOD activity. Incubation of the gels with 5 mM H_2O_2 inhibited FeSOD and Cu/ZnSOD to identify MnSOD isoforms in the transgenic and untransformed plants [15].

Growth condition and stress treatment. Seeds of homozygous lines, named as L1 and L2, and wild-type (WT) rice were sterilized with 10% NaClO (v/v) for 20 min, rinsed three times with sterilized water, and soaked for 1–2 days at 37°C in the dark. Then germinated seeds were hydroponics with the Yoshida's culture solution [16] refreshed every 5 days. The seedlings were grown at a 13-h photoperiod (200 μmol/(m^2 s)), 26/22°C (day/night) temperature, and 80% relative humidity.

Uniform 35-day-old seedlings were transferred to culture solution containing 100 mM NaCl, while others without NaCl served as controls. After exposure to salt stress for 3 and 6 days, the roots and the shoots were collected separately for further assays.

Reverse transcription–polymerase chain reaction (RT-PCR). For the analysis of *NaMnSOD* expression level, 0.1 g leaves from WT and transgenic plants treated with 100 mM NaCl were collected. Total RNA was isolated using TRIzol reagent (Invitrogen). RT-PCR was performed using a Prime Script™ 1st Strand PCR Kit (TaKaRa Biotech.). Reverse transcription products (2 μL) were subjected to PCR with *NaMnSOD* primers. *Actin* gene was used as an internal control [17].

Determination of ion leakage and lipid peroxidation. Leaf relative electrolyte leakage was detected with the methods described by Arora et al. [18] with some modifications. Leaf samples of 0.1 g from WT and transgenic plants were vacuum infiltrated in 20 mL of distilled water for 10 min and then left overnight at 25°C. After overnight incubation, the initial electric conductivity (C_1) was measured using a conductivity meter (HORIBA). Thereafter, samples were boiled for 10 min and total electric conductivity (C_2) was read at 25°C. Relative electrolyte leakage was expressed as a percentage of the total conductivity: $C_1/C_2 \times 100\%$.

MDA was extracted from the leaves with 10% (w/v) trichloroacetic acid and determined according to Huang et al. [19]. MDA was expressed as nmol/g initial fresh weight.

Assays of superoxide anion radical and hydrogen peroxide. $O_2^{\bullet -}$ was estimated by the method of monitoring the nitrite formation from hydroxylamine in the presence of superoxide radical [20]. H_2O_2 level was measured according to the previously described method [21] and calculated using the extinction coefficient of 0.28/(μM cm).

Enzyme assays. Enzymes were extracted according to the reported method [19]. Leaf tissue (0.1 g) was ground into powder in liquid nitrogen and homogenized in 2 mL of precooled (4°C) extraction buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 3 mM DTT, and 5% PVP-40). The homogenate was centrifuged at 15000 *g* for 20 min at 4°C, and the resulting supernatant was collected for further antioxidant enzyme assays.

Total SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm. The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 67 μM NBT, 13 mM L-methionine, 1.3 μM riboflavin, and suitable aliquot of the enzyme extract. Reaction was carried out at 30°C, under light intensity of about 150 μmol/(m^2 s) through 10 min.

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm (extinction coefficient 39.4 /mM cm). The reaction mixture (3 mL) contained 50 mM PBS (pH 7.0), 0.1 mM EDTA, 2 mM H_2O_2, and the enzyme extract.

Measurements of photosynthetic gas exchange and chlorophyll fluorescence. Gas exchange of the second

fully developed leaf was measured using an LI-6400 portable photosynthesis system (LI-COR, United States) during 8:30 to 11:00 a.m. after 0, 3, and 6 days of treatment according to Huang et al. [19].

Chlorophyll (Chl) fluorescence was measured with an integrating fluorescence fluorometer (LI-6400-40 leaf chamber fluorometer, Li-Cor). Before each measurement, plants were dark-adapted for 30 min. F_0 (the minimum fluorescence yield) and F_m (the maximum fluorescence yield) were measured before and after a 0.8-s saturation pulse of 8000 μmol/(m^2 s). Immediately, the leaf was continuously irradiated with an actinic light (1500 μmol/(m^2 s)) and equilibrated for 30 min. After the stable fluorescence yield during actinic illumination (F_s) was recorded, another 0.8-s saturation pulse was applied to determine maximal fluorescence in the light-adapted state (F'_m). After the flash, a far-red light was given, and the minimum fluorescence yield (F'_0) was determined.

Statistical analysis. All data points were based on a mean of three replications. Data were analyzed by Student's *t*-test at 95% confidence limits. For these analyses SPSS 10.0 software (SPSS, Chicago, United States) was used.

RESULTS AND DISCUSSION

Construction and Transformation of Plant Expression Vector

The differential protein of halophilic archaeon (*Natrinema altunense* sp., AJ2) was screened under different salt treatment [11]. Then the amino acid sequence was determined and a DNA fragment was cloned (Fig. 1b) and presumed to be a *MnSOD*. To overexpress *NaMnSOD* in rice, the plant expression vector p1301-35S::*NaMnSOD*, under the control of the cauliflower mosaic virus 35S promoter and NOS terminator, was constructed (Fig. 1a). The vector was then transformed into rice using *Agrobacterium*-mediated transformation.

Screening of the Transgenic Plants

Twenty-two lines were regenerated from hygromycin-resistant calli. PCR analysis and GUS staining confirmed the integration of T-DNA region in the genomic DNA of transgenic plants (Fig. 2a). Total RNA from leaves was isolated, and cDNA was synthesized. PCR reaction was performed firstly with *Actin* primers for the uniform concentration of template. Then RT-PCR proved the transcription of *NaMnSOD* just in transgenic plants (Fig. 2b). There were no apparent differences between the phenotypes of transgenic and WT plants. All T_1 seeds were firstly screened by 50 mg/L hygromycin selection. Two of them, named as L1 and L2, showed 3 : 1 segregation ratio and high *NaMnSOD* expression level (Fig. 2c). The segregation pattern of T_2 progeny was 2 : 1, which indicates that the integration of *NaMnSOD* gene occurred just at a single locus.

As shown in Fig. 2c (left), six endogenous isoform bands of rice SOD were observed in the control. When treated with H_2O_2, FeSOD and Cu/ZnSOD were inhibited, and MnSOD isoforms are shown in Fig. 2c (right). WT exhibited 2 bands, while the transformants exhibited an additional band, which was resistant to H_2O_2. Therefore, the additional enzymatic band was identified as MnSOD from *N. altunense*.

Effect of Salt Stress on Rice Growth

The 20-day-old seedlings were treated with 100 mM NaCl. The WT plants started wilting within 3 days after salt treatment, while the transgenic plants did not show signs of wilting until the 5th day. By the end of the 8th days, the transgenic plants withstood the stress better in comparison with the untransformed control plants, which wilted almost completely.

Effect of Salt Stress on Damaging the Cell Membrane

In addition to visible damage, disruption of the membrane integrity by stress is usually quantified by relative ion leakage, and the content of MDA reflects membrane lipid peroxidation state. When subjected to salt stress, ion leakage and MDA content in plants increase [7]. In our study, the relative ion leakage and MDA content of the WT rice increased dramatically, while transgenic plants showed a slight increase when treated with 100 mM NaCl. So the transformants kept significantly lower ion leakage ($P \le 0.01$ at the 3rd day and $P \le 0.05$ at the 5th day) (Fig. 3a). The content of MDA showed the same tendency with relative ion leakage (Fig. 3b). These data revealed that the membranes of WT plants were damaged severer than those of transgenic plants, indicating that transgenic plants overexpressing *NaMnSOD* had the higher ROS-scavenging activity since membrane damage was mainly caused by ROS under salt stress.

Effect of Salt Stress on Level of ROS

As shown in Fig. 4a, when treated with 100 mM NaCl for 6 days, the $O_2^{\cdot -}$ level of WT plants increased by 49.9%, while transgenic plants – by 15.4 and 16.2%, respectively, which was remarkably less than that of WT. Meanwhile, after 3-day salt treatment, H_2O_2 content in leaves of WT plants increased acutely by 62%, while in transgenic plants – by 24 and 19%, respectively (Fig. 4b). During extended treatment time, H_2O_2 content declined slightly in all plants. However, the content in transgenic plants was still significantly lower than that in the WT plants.

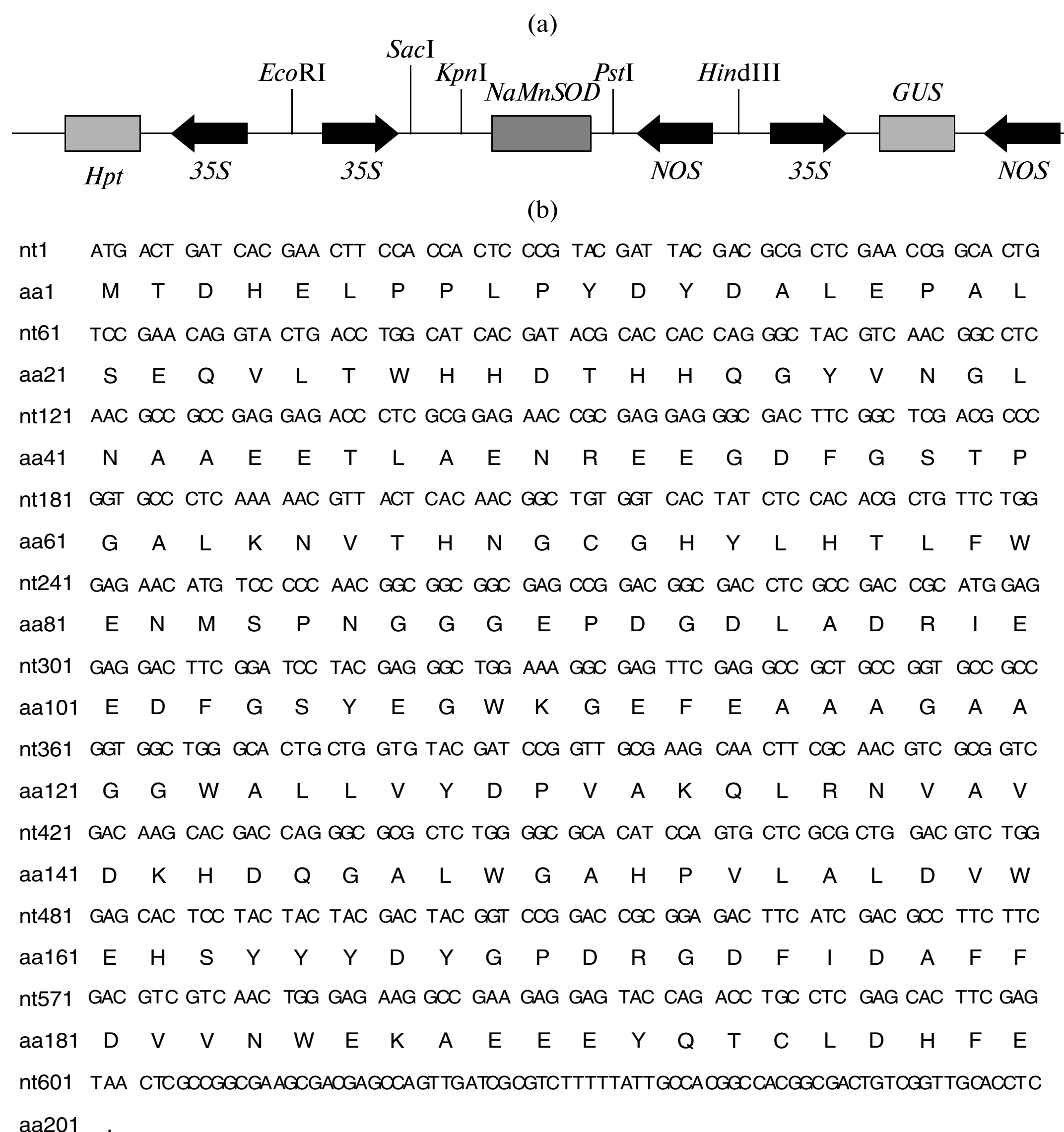

Fig. 1. (a) Plant express vector of p1301-*35S::NaMnSOD*. *35S* represents CaMV 35S promoter; *NOS* represents nopaline synthase terminator; *NaMnSOD* represents the gene of SOD from *Natrinema altunense* sp., AJ2. *GUS* represents the gene of β-glucuronidase; *Hpt* represents the gene of hygromycin phosphotransferase. (b) DNA and amino acid sequences of NaMnSOD.

Effect of Salt Stress on Activities of SOD and CAT

In plant cell, SOD dismutate $O_2^{\bullet -}$ into H_2O_2 and O_2 to defend plants against oxidative stress. As reported in previous work, when treated with NaCl, the content of H_2O_2 in plants was elevated but the activities of Cu/ZnSOD and CAT decreased [22]. However, Parida et al. [23] found that the expression of MnSOD was preferentially activated by salt stress. This may be due to the fact that H_2O_2 can inactivate Cu/ZnSOD and FeSOD but not MnSOD. Furthermore, H_2O_2 can react with superoxide anions to form hydroxyl radical ($^{\bullet}$OH), the most toxic ROS, unless it is quickly scavenged by CAT or other H_2O_2-scavenging systems. Thus, the moderate increase in the $O_2^{\bullet -}$ and H_2O_2 decomposing systems could protect the plant from oxidative damage. Therefore, we detected the total SOD and CAT activities to evaluate the properties of transformants. The results showed that, under normal conditions, the total SOD activities in transgenic plants were significantly higher (by 14.3 or 14.5%) than that of WT (Fig. 5a). When subjected to salt stress, all plants had enhanced total SOD activity at the 3rd day. But the total SOD activity in WT declined almost to the level of the control after 6 days of salt treatment. In contrast, the total SOD activity of transgenic plants was kept increased. It may be suggested that transgenic plants with *NaMnSOD* increase

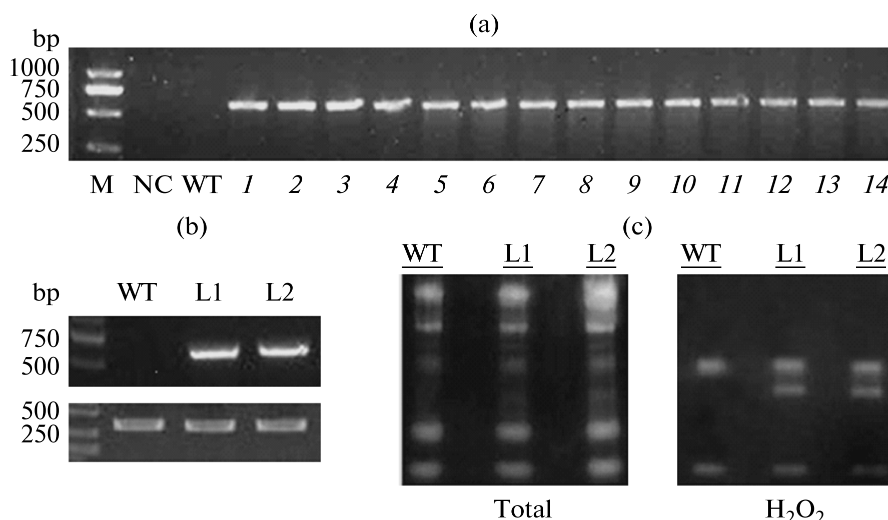

Fig. 2. Generation of *NaMnSOD* overexpressing plants.

(a) PCR analysis of T_0 transgenic rice plants. The expected size of the product was 621 bp. M—DNA marker; NC—negative control (water); WT—wild-type plant; *1–14*—transgenic plants. (b) RT-PCR analysis of the transcriptional level of foreign *NaMnSOD* expression in leaves from WT and transgenic rice plants (L1 and L2). *Actin* was used as an internal control. (c) Total SOD isozyme analysis (left) and MnSOD isozyme analysis, which is resistant to H_2O_2 (right).

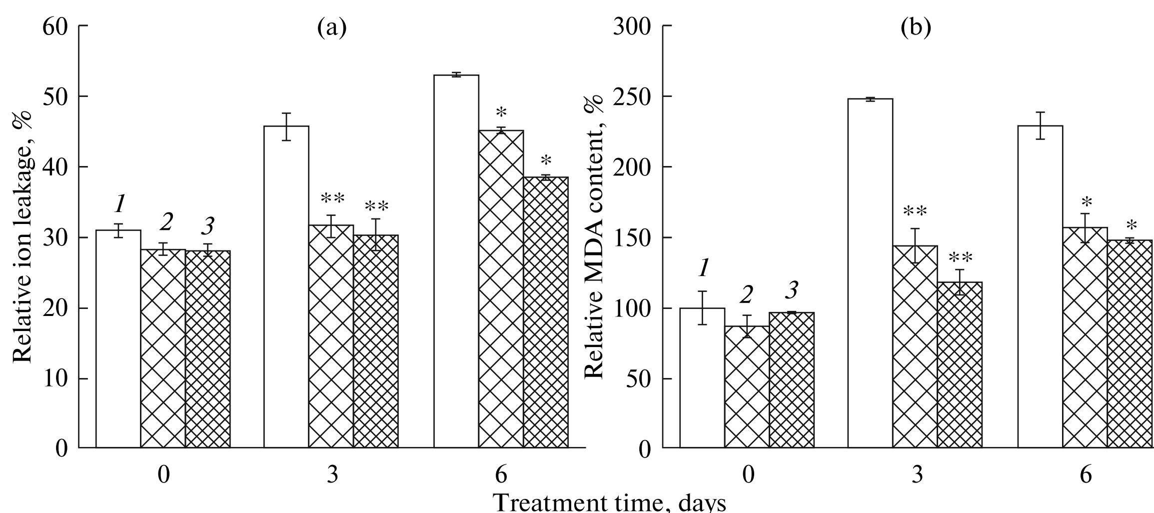

Fig. 3. Membrane damage determination after salt stress for 3 and 6 days.

(a) Effect of NaCl on the relative ion leakage from WT and transgenic plants. (b) Effect of NaCl on MDA content in WT and transgenic plants. Each value in this figure is expressed as the percentage of the control. L1 and L2 are transgenic rice lines; WT—wild-type rice plants. Means of three replicates ± SE are presented. Values significantly different from the WT in the same group according to *t*-test are indicated by asterisks (* $P \leq 0.05$; ** $P \leq 0.01$).

(*1*) WT; (*2*) L1 line; (*3*) L2 line.

the total SOD activity and contribute to the lower ROS levels. In addition, the activity of CAT was continuously depressed as salt stress continued (Fig. 5b). However, a decrease in the CAT activity was much less in transgenic plants, compared to WT. So, the increased activity of SOD as ROS scavenger cleaned up the increasing $O_2^{\cdot -}$ under salt stress in time and prevented the cell from oxidative damage, resulting in the enhancement of salt tolerance.

Effects of Salt Stress on Net Photosynthetic and Fluorescence Parameters

One of the most important reasons for crop production decrease under salt stress is ascribed to its severe inhibition of photosynthesis, e.g., the depression in PSII activity and electron transport [24]. Yang et al. [25] reported that the reduction of photosynthetic activity under salt stress was attributed to the ROS-induced oxidative damage. In this study, trans-

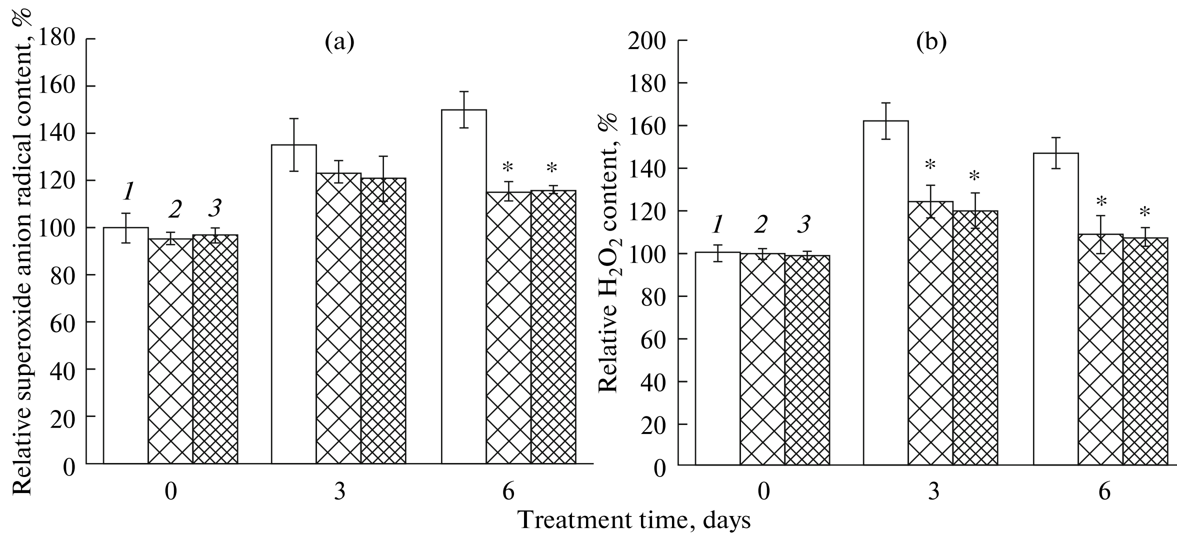

Fig. 4. Effects of salt stress on superoxide radical ($O_2^{\bullet -}$) production rate (a) and H_2O_2 content (b) of wild type rice (WT) and transgenic rice lines (L1 and L2). Asterisks—see Fig. 3.
(*1*) WT; (*2*) L1 line; (*3*) L2 line.

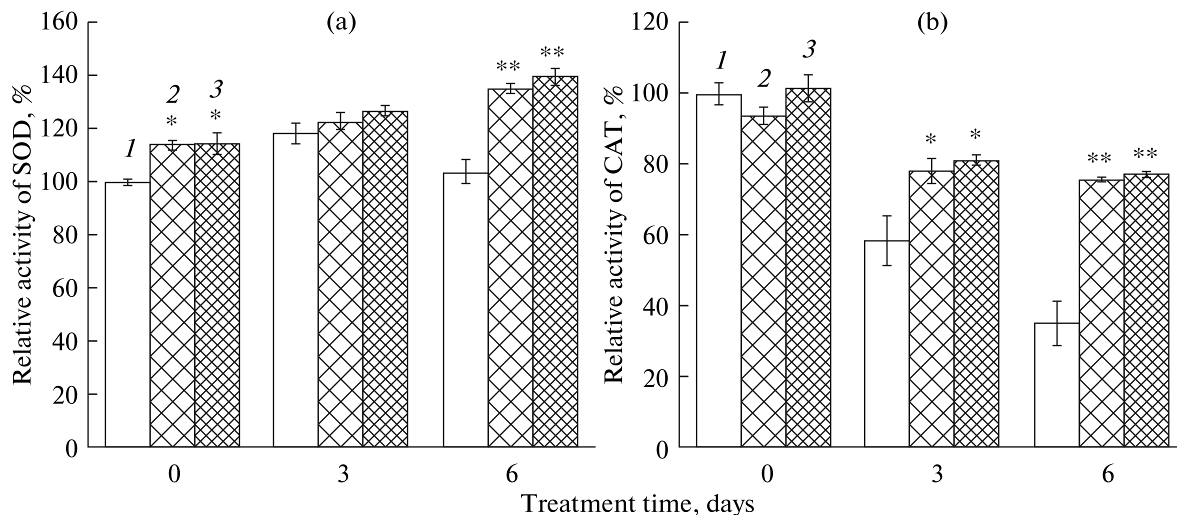

Fig. 5. Effects of salt stress on activities of antioxidant enzymes in the leaves of transgenic and WT rice plants during the 6-day salt treatment.
(a) Relative activity of SOD; (b) relative activity of CAT. Asterisks—see Fig. 3.
(*1*) WT; (*2*) L1 line; (*3*) L2 line.

genic plants exhibited better photosynthetic performance than WT plants under salt stress. As shown in Fig. 6a, net photosynthesis (P_n) was continuously decreased in all plants when salt stress lasted. However, a decline in the transformants was slower. After 6 days of saline treatment, P_n of transgenic plants was more than twice in comparison with WT. The changes of maximal photochemical efficiency (F_v/F_m), quantum yield of open PSII (F'_v/F'_m), and electron transport rate (ETR) exhibited the similar tendency. Over the 6-day-long period of treatment, the values of them in transgenic plants were significantly higher than those in WT, although the values decreased in both plants as the 100 mM NaCl treatment progressed (Figs. 6b–6d). F_v/F_m data showed that overproduced NaMnSOD in transgenic plants protected the PSII reaction center against oxidative damage caused by salt stress. Probably, the excess $O_2^{\bullet -}$, which could not be eliminated only by endogenous detoxicant enzymes during the salt stress, were dismutated into H_2O_2 by

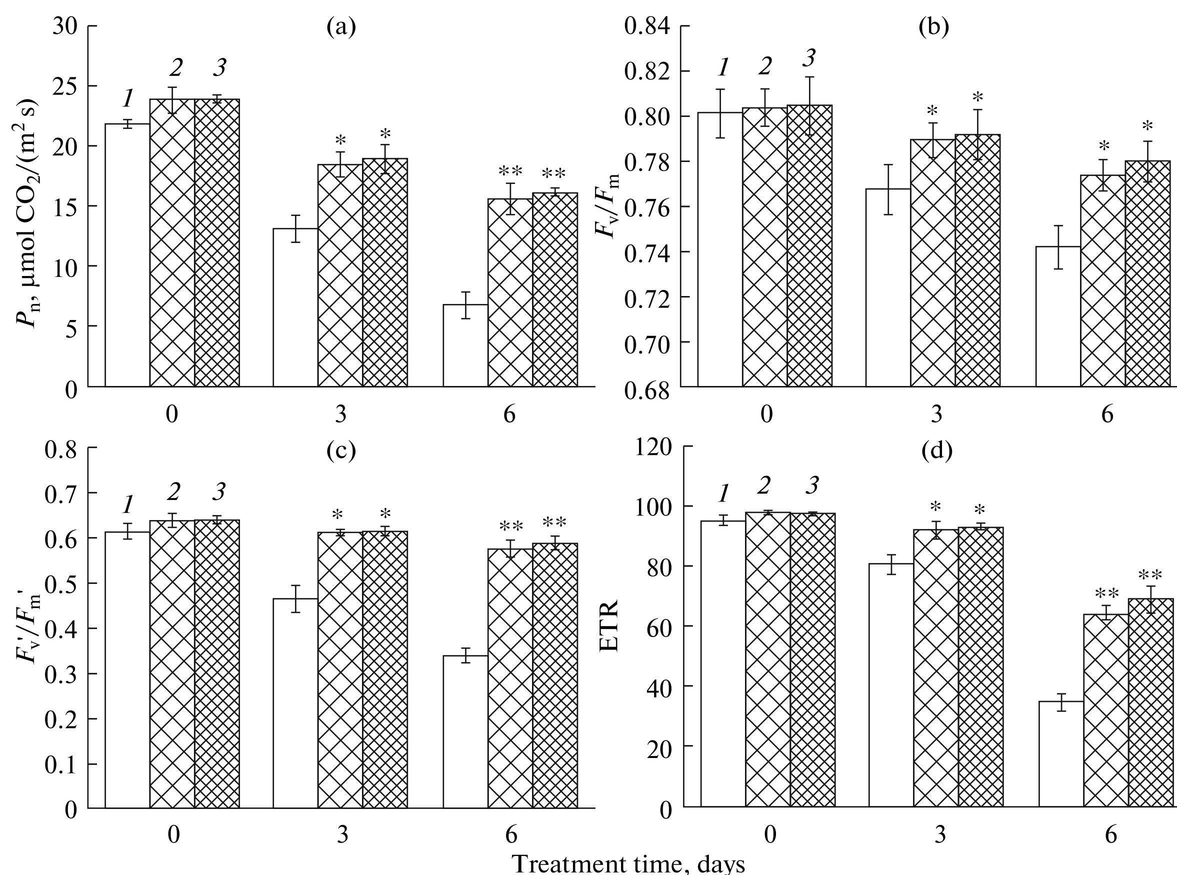

Fig. 6. Effects of salt stress on photosynthetic characteristic of WT rice and transgenic rice lines (L1 and L2).
(a) Net photosynthetic rate (P_n); (b) maximal photochemical efficiency (F_v/F_m); (c) quantum yield of open PSII (F_v'/F_m'); (d) electron transport rate (ETR). Asterisks—see Fig. 3.
(*1*) WT; (*2*) L1 line; (*3*) L2 line.

the overproduct of *NaMnSOD*, prior to interaction of $O_2^{\cdot -}$ with target molecules. In the end, the higher SOD activity in transgenic plants kept the superoxide radical at the lower level, leading to reduced oxidative damage.

In conclusion, we developed transgenic rice plants overexpressing *NaMnSOD*, which encodes the MnSOD from halophilic archaeon (*N. altunense* sp.). The transformants exhibited an increased total SOD activity and enhanced tolerance to salt stress with the higher levels of photosynthesis, whereas less membrane damage when treated with 100 mM NaCl, compared to WT plants. Moreover, the higher CAT activity in transgenic plants was observed under salt stress than that in WT plants. So, transgenic rice had the higher efficiency of ROS elimination during salt-induced oxidative stress. These results suggested that SOD plays an essential role in the plant adaptive responses under salt stress and the overexpression of *NaMnSOD* could improve salt tolerance in rice.

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