Ectopic expression of Mn-SOD in *Lycopersicon esculentum* leads to enhanced tolerance to salt and oxidative stress


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Abstract

Production of reactive oxygen species (ROS) is associated with a number of physiological disorders in plants. Superoxide dismutase (SOD) catalyzes the breakdown of superoxide (O$_2^-$) into O$_2$ and H$_2$O, and provides the first line of defense against the toxic effects of elevated levels of ROS. The effect of increased expression of Mn superoxide dismutase (Mn-SOD) on salt stress tolerance was studied using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. Northern blots confirmed expression of the heterologous Mn-SOD in transgenic plants. Strong Mn-SOD enzyme activity was detected by native PAGE in transformed plants. Transgenic plants showed resistance to the superoxide-generating herbicide methyl viologen (MV). The total SOD activity was one and one half- to two-fold higher, and APX (ascorbate peroxidase) activity was six to seven fold higher in transgenic, than in wild-type (WT) plant under MV stress. Germination of transgenic tomato seeds at a NaCl concentration of 150 mM was greater than that observed in WT plants. Also, the total APX activity of transgenic plants was 4 to 5 fold higher than that of WT under NaCl (200 mM) stress.

Key words: Superoxide dismutase, oxidative stress, salt stress, transgenic tomato.

Introduction

Salt stress is one of the most common types of abiotic stress that limits the production of agricultural plants around the world (Zhu, 2000). Plants subjected to salt stress also show elevated levels of activated forms of oxygen and free radicals. These elements often are associated with damage to membranes and essential macromolecules such as protein, DNA, and lipids (Fadzilla et al., 1997; Gueta-Dahan et al., 1997; Hernández et al., 1995).

Superoxide dismutase (SOD) is an important enzyme in a plant’s defense against oxidative stress. It catalyzes the conversion of two superoxide anions (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and O$_2$, and alleviates oxidative stress (Bowler et al., 1992). SODs are a group of metal-containing enzymes and are classified into three types according to their metal cofactor requirements: iron SOD (Fe-SOD) is localized in the chloroplast; copper-zinc SOD (Cu/Zn-SOD) is localized in the chloroplast, cytosol, and possibly the extracellular space; and manganese SOD (Mn-SOD) is found mainly in mitochondria and peroxisomes (Alscher et al., 2002). Antioxidant enzyme activity is found in plants responding to various environmental and chemical stresses (Allen, 1995; Baek et al., 2006), such as freezing (Martinez et al., 2001), chilling (Baek and Skinner, 2005; Iannelli et al., 1999), salt (Gueta-Dahan et al., 1997; Hernández et al., 1995; Rajiguru et al., 1999), and methyl viologen (MV) (Bowler et al., 1991; Donahue et al., 1997).

The role of SOD during salt stress has received much attention. Exposure of salt-tolerant pea plants to NaCl resulted in the formation of O$_2^-$ and H$_2$O$_2$, and increased the activity of SOD and other antioxidant enzymes, such as ascorbate peroxides (APX). Transcripts levels for Mn-SOD, Cu/Zn-SOD, and APX were strongly induced in the salt-tolerant variety but not in the salt-sensitive one (Hernández et al., 2000). Reports dealing with rice (Dionisio-Sese and Tobita, 1998) and tomato plant (Shalata et al., 2001), have also reported increased SOD activity in salt-tolerant cultivars when exposed to salt stress. Additionally, Tanaka et al. (1999) confirmed that overexpression of a yeast *Mn-SOD* gene in rice confers tolerance to salt stress.

Enhanced expression of SODs in transgenic plants has demonstrated tolerance to MV (Allen, 1995; Perl et al., 1993), freezing (McKersie et al., 1999), and salt (Tanaka et al., 1999). In order to further understand the role of APX and SOD in response to oxidative stress induced by abiotic stresses, transgenic tomato plants were produced that overexpress either cAPX (Wang et al., 2005 and 2006) or Mn-SOD and their response to several abiotic stresses was evaluated. The purpose of this study was to evaluate the tolerance of transgenic Mn-SOD tomato plants to salt (NaCl) and oxidative (MV) stress.

Materials and methods

Generation and analysis of transgenic tomato plants: Mn-SOD cDNA was synthesized from rubber tissue (*Hevea brasiliensis*) based on primers by Miao and Gaynor (1993). The cDNA was mobilized into the binary vector pDU92.3103 (Tao et al., 1995) between the cauliflower mosaic virus 35S promoter and...
results from the Northern blot analysis of the Mn-SOD specific transcript in WT and T1 transgenic plants. The hybridization was conducted under high stringency conditions.

Application of methyl viologen: Shoots were treated with methyl viologen (MV, Sigma) following the procedure described by Perl et al. (1993). Shoot cuttings (with three to four leaves) from WT and T1 transgenic plants were obtained from containerized mature plants grown in the greenhouse. The cut end of the shoots was placed in 100 mM MV. After 16 h, the cut ends were transferred to tap water for an additional two days. Two leaf discs (1.02 cm in diameter) of the third leaf from the apex were excised after the MV treatment, and MV-induced oxidative damage was evaluated using the leaf electrolyte leakage (Wisniewski et al., 1997) with a conductivity meter (Markson Science, Inc., Del Mar, CA). Means for all values were an average of two subsamples in each plant with three replications. The data were subjected to Duncan’s multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformations were performed before data analysis; nontransformed means are presented. MV treated leaf discs were also sampled and stored at –80°C prior to APX and SOD analyses.

Salt Stress: To evaluate salt stress, seeds from WT and T1 plants were placed in petri dishes (40 seeds per box) on filter paper (Whatman 3MM) saturated with 150 mM NaCl solution. They were germinated at 23/21°C (± 2°C, day/night temperature) under cool white fluorescent lights (100-150 µmol m⁻² sec⁻¹) using a 16-h photoperiod. At the end of two weeks, germination was measured. Germination was considered successful when the radicle protruded through the seed coat. Values are means ± SE (three repeats). The data were subjected to Duncan’s multiple range test.

To evaluate the tolerance of developing roots to salt stress, shoot cuttings from 10-day-old WT and T1 seedlings were grown in sterile, solidified Murashige-Skoog (MS; Murashige and Skoog, 1962) medium (Sigma) amended with either 200 mM or 250 mM NaCl. After 5 weeks at 23/21°C (± 2°C, day/night temperature), the fresh roots were excised, blotted on filter paper, and weighed. Values are means ± SE (six replicates). The data were subjected to Duncan’s multiple range test.

WT and T1 shoot cuttings were grown in rooting medium for 2 weeks. Healthy seedlings were transferred to 5.8 × 5.8 × 8-cm plastic pots with peat moss soil and watered with tomato fertilizer (9N-4.4P-12.5K, Schultz, Inc., Bridgeton, MO) for one week. Stress was imposed by watering plants with tomato fertilizer and NaCl (200 mM or 250 mM) solution every three days. Controls received only fertilizer. The plants were maintained in a greenhouse with natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) for a 16-h photoperiod. At approximately 23/21°C (± 2°C, day/night temperature), height (from the apex to soil) was measured after 10 days (d). The extent of injury was evaluated visually at 10, 20, and 30 d following treatment. The scale was as follows: 0, no injury; 1, slow growth but no obvious damage, ≤20% leaf area exhibited injury; 2, leaves turned yellow, 21 to 40% of the leaf area injured; 3, plants wilted, 41 to 60% leaf area injured; 4, seriously damaged, the plant became soft and could not remain upright, 61 to 80% leaf area injured; 5, 81 to 100% leaf area injured or plant died. Mean values of 5 replicates were calculated and the ranks were subjected to the non-parametric Kruskal-Wallis test. The leaf discs from WT and transgenic plants grown for 10-d under NaCl (200 mM) stress were then frozen in liquid nitrogen and stored at –80°C for further enzyme activity gel analyses.
Results

Overexpression of the Mn-SOD gene: No detectable GUS activity was seen in WT leaves. Transgenic leaves stained intensely blue, indicating high levels of GUS activity. No PCR products could be amplified from WT plants, whereas products of the expected size (~800 bp) were amplified from all transgenic lines (data not shown). Northern-blot analysis was performed to assess the mRNA levels in transgenic plants. All the transgenic plants contained transcripts but levels among individual lines varied. WT plants did not exhibit transcript that hybridized to the probe (Fig. 1). Two transgenic lines, S4 and S20, were selected for further study because they showed higher expression levels.

The selected transgenic lines were screened by gel assay for the presence of Mn-SOD activity. Two isoforms were observed in all plants, and may represent chloroplastic (chl) and cytosolic (cyt) Cu/Zn-SOD (Perl et al., 1993). Transgenic plants displayed an additional lower mobility band corresponding to the Mn-SOD enzyme activity (Fig. 2A). Total APX activity was also measured.

Lines S4 and S20 exhibited 2- to 3-fold higher APX activity than in WT plants (Fig. 2B).

Effects of MV stress: Methyl viologen-induced electrolyte leakage in transgenic plants was significantly less than in WT (P<0.05, Fig. 3). Mean electrolyte leakage in WT leaves was about 47%. In comparison, Mn-SOD-expressing lines (S4, S20) had significantly lower electrolyte leakage (approximately, 30%). After two days under MV treatment, total SOD activity was 1.5- to 2-fold higher in transgenic plants. APX activity of transgenic plants was 6- to 7-fold higher than WT plants following MV treatment (data not shown).

Effects of NaCl stress: The effect of 150 mM NaCl on the percent seed germination at 13 days was 76 and 81% germination for transgenic line S20 and S4, respectively and 39% for WT seeds (Fig. 4). After 5 weeks of treatment, the fresh root weight of transgenic plant (14 g and 8 g for S4; 14 g and 7 g for S20) was significantly (P<0.05) greater than that of WT (8 g and 2 g) tomato plants at 200 mM and 250 mM NaCl, respectively (Fig. 5). Irrigation of transgenic and WT tomato plants with 200mM

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Fig. 1. Northern blot analysis of RNA isolated from the T<sub>1</sub> plants. 30 µg of total RNA was used per lane for each blot. Blots were probed with γ<sup>32</sup>P-labeled Mn-SOD PCR products. WT, wild-type plant; S1 to S20, independent transgenic lines.

Fig. 2. Detection of (A) SOD and (B) APX activity in leaves of wild-type (WT) and transgenic Mn-SOD (Lines S4, S20) tomato plants. About 70 µg protein was loaded on the non-denaturing polyacrylamide gel.

Fig. 3. Effects of methyl viologen (10<sup>-4</sup> M) stress on electrolyte leakage from transgenic Mn-SOD (Lines S4, S20) and wild-type (WT) tomato plants. Bars represent SE for three replicates. Different letters indicate significant differences (P<0.05) between means (Duncan’s multiple range test).

Fig. 4. Germination in T<sub>1</sub> transgenic Mn-SOD (Lines S4, S20) and wild-type tomato seeds treated with NaCl (150 mM) for various periods of time. Values are means ± SE (n=3). Different letters indicate significant differences (P<0.05) between means within each sampling time (Duncan’s multiple range test).
and 250 mM NaCl solution severely inhibited their shoot height growth (Fig. 6). The margins of leaflets in WT plants became necrotic following 10 days of salt treatment. Visible injury to WT was rated as 2.2. In contrast, injury to transgenic plants was significantly lower \( (P<0.05) \), showing only slight leaf area injury (scale 1). The effect of the salt treatment was still apparent but severe after 20 d. WT plants displayed visible necrotic injury (scale 3.6) after 20 d. In contrast, the transgenic seedlings showed less injury (Fig. 7A). At 250 mM NaCl, WT seedlings showed injury scale 5 after 20 d, whereas the transgenic plants exhibited less wilting injury (average scale 2.2 for S4 and 3 for S20). The differences in visible injury between transgenic and WT plants were statistically significant \( (P<0.05, \text{Fig. 7B}) \). The leaf APX activity in transgenic plants was about 4- to 5-fold higher than that in WT plants after 10 d of NaCl (200 mM) treatment (data not shown).

**Discussion**

Numerous studies have indicated that oxidative stress enhances SOD activity (Donahue et al., 1997; El-Saht, 1998). Abiotic stresses, such as chilling, drought, and salt stress have been correlated with increase in SOD activity (Baek et al., 2006; Dionisio-Sese and Tobita, 1998; Fadzilla et al., 1997; Scandalios, 1993).

Our study indicated that transgenic tomato plants expressing the Mn-SOD gene from *H. esculenta* displayed an enhanced tolerance to both MV and salt-induced oxidative stress. Transgenic plants had less electrolyte leakage than WT plants (Fig. 3), suggesting that overexpression of Mn-SOD in the transgenic plants reduced cellular damage caused by ROS (Bowler et al., 1991). After MV treatment, APX and SOD activity was higher in transgenic plants and was related to a plant’s resistance to ROS damage (Allen, 1995; Van Camp et al., 1994).

An increase in ROS scavenging capacity is required to enable rapid removal of ROS produced during early seed imbibition (Gidrol et al., 1994). In this study, transgenic seeds were more tolerant to NaCl than WT seeds. The transgenic plants had higher SOD and APX activity, which could prevent the accumulation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) concentration during the early stages of seed germination.

In our study, we found that shoot cuttings of transgenic tomato plants produced more root biomass than WT plants under salinity stress, indicating that overexpression of Mn-SOD enabled the transgenic plants to better deal with oxidative stress. Also, seedlings of transgenic *Mn-SOD* tomato plants showed less injury (Fig. 7) following NaCl (200 mM and 250 mM) stress than WT plants. This is consistent with the theory that increased antioxidant enzyme activity can prevent NaCl-induced oxidative stress (Fadzilla et al., 1997; Tanaka et al., 1999).

Our study showed that APX activity increased due to NaCl treatment and is consistent with other reports (Hernández et al., 2005).
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1999: Mittova et al., 2002; Sairam and Srivastava, 2002; Wang et al., 2005, 2006). This confirms earlier reports that APX plays an important role in scavenging H$_2$O$_2$ induced by NaCl stress. However, SOD activity decreased after 10 d of NaCl treatment (data not shown). The reason for this decrease in activity is not known but may be related to the long exposure to NaCl.

In this study, the overexpression of Mn-SOD in transgenic tomato plants enhanced seed germination, root development and seedling tolerance to NaCl stress. We conclude that increased antioxidant levels may play an important role in scavenging ROS when plants are exposed to salt stress.

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**References**


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