

Full Length Research Paper

Involvement of calcium and calmodulin signaling in adaptation to heat stress-induced oxidative stress in *Solanum lycopersicum* L. leaves

Hai-Dong Ding¹, Hong-Juan Zhang², Xiao-Hong Zhu¹, Hui Liu¹, Jian-Shen Liang^{1*} and Bing Lu¹

¹Fostering Base for National Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, China.

²College of Horticulture and Plant Protection, Yangzhou University, Yangzhou 225009, China.

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Heat stress is a common form of stress suffered by plants. Therefore, plants have evolved mechanisms to cope with the problems caused by high temperatures. In this study, the involvement of calcium ion and calmodulin (Ca^{2+} -CaM) in the protection against heat stress-induced oxidative damage in tomato (*Solanum lycopersicum* L.) seedlings was investigated. Treatment of tomato leaves with short-term heat stress led to significant increase in the level of malondialdehyde (MDA) and protein oxidation (in terms of carbonyl groups), two important parameters of oxidative stress. However, such oxidative processes were mitigated by pretreatment with Ca^{2+} and aggravated by pretreatment with the Ca^{2+} chelator ethylene glycol-bis(β -amino ethyl ether)-N,N,N',N'-tetraacetid acid (EGTA), the Ca^{2+} channel blockers lanthanum chloride (LaCl_3), or the CaM antagonists chlorpromazine (CPZ), trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesul-fonamide (W7). Besides, Ca^{2+} pretreatment significantly reduced the accumulation of reactive oxygen species (ROS), $\text{O}_2^{\cdot -}$ and H_2O_2 and remarkably induced the total activities of the antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) under heat stress, while EGTA, LaCl_3 , CPZ, TFP and W7 pretreatment reversed them. The results obtained here suggest that Ca^{2+} -CaM signaling pathways are involved in resistance to heat stress-induced oxidative stress by regulation of antioxidant mechanisms in tomato leaves.

Key words: Calcium, calmodulin, heat stress, oxidative stress, *Solanum lycopersicum* L.

INTRODUCTION

Heat stress is a major abiotic factor which disturbs cellular homeostasis and can lead to severe retardation in growth, development, and even death. Plants have evolved a variety of responses to elevated temperatures that minimize damage and ensure protection of cellular homeostasis (Larkindale and Knight, 2002; Kotak et al., 2007). One important mechanism of tolerance to heat stress is the balanced production and elimination of reactive oxygen species (ROS), such as superoxide

radical ($\text{O}_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$) (Mittler, 2002). Oxidative stress occurs as a result of reactive oxygen species (ROS) overproduction, which can cause peroxidation of membrane lipids, denaturation of protein and damage of nucleic acids, ultimately upsetting homeostasis. The generation of ROS can be limited or scavenged by a series of antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POD), glutathione reductase (GR) and catalase (CAT) and antioxidant compounds such as ascorbate and glutathione (Wahid et al., 2007). In view of a number of studies, heat stress tolerance is often correlated well with a more efficient antioxidant system (Suzuki and Mittler, 2006; Kotak et al., 2007; Wahid et al., 2007). The

*Corresponding author. E-mail address: jsliang@126.com. Tel: 0514-87979204. Fax: 0514-8971747.

mechanism of activation of antioxidant system is presently of intense interest.

Accumulated evidences show that different molecular signalings such as abscisic acid (ABA), Ca^{2+} -calmodulin (CaM), hydrogen peroxide (H_2O_2), mitogen-activated protein kinase (MAPK) cascade and nitric oxide (NO) are involved in regulation of plant responses to various environmental stresses including heat stress (Wahid et al., 2007; Saidi et al., 2011). Ca^{2+} , one of the most ubiquitous cellular second messengers, acts as a mediator of stimulus–response coupling in the regulation of plant growth, development, and responses to environmental stresses (Lecourieux et al., 2006; Kudla et al., 2010). Heat stress can transiently increase in the intracellular concentration of free calcium ($[\text{Ca}^{2+}]_i$) in tobacco (Gong et al., 1998), wheat (Liu et al., 2003), suspension-cultured *Arabidopsis* cells (Liu et al., 2006) and Moss plants (Saidi et al., 2009). Increased $[\text{Ca}^{2+}]_i$ was suggested to promote expression of heat shock protein (HSP) genes, synthesis of HSPs and acquisition of HS-induced thermotolerance in plants under heat stress (Liu et al., 2005; Saidi et al., 2011). Ca^{2+} may help protect the photosynthetic system of wheat leaves from oxidative damage induced by the cross stress of heat and high irradiance (Zhao and Tan, 2005). Ca^{2+} signaling pathways were also reported to be involved in the protection against heat stress-induced oxidative damage (Larkindale and Knight, 2002; Bhattacharjee, 2008). However, the signaling pathways including Ca^{2+} signal in tolerance to heat stress-induced oxidative stress are still limited.

Transient $[\text{Ca}^{2+}]_i$ elevations are sensed by several Ca^{2+} sensors such as calmodulin (CaM), calcium-dependent protein kinase (CDPK), and calcineurin B-like protein (CBL). CaM is one of the best characterized intracellular calcium sensors in eukaryotes. It has no catalytic activity of its own but, upon binding Ca^{2+} , activates numerous downstream target proteins (Yang and Poovaiah, 2003; Bouché et al., 2005). In plants, the role of CaM in regulating a variety of calcium-dependent signaling pathways within the cell and in the extracellular matrix has been documented. Gong et al. (1997) observed that CaM was up-regulated during heat stress in maize (*Zea mays*) seedlings. The levels of CaM mRNA and protein increased during heat stress in wheat and *Arabidopsis* were also reported (Liu et al., 2003, 2005; Zhang et al., 2009). More recently, AtCaM3 and CaM-binding protein kinase (CBK3) were identified as an important component in the Ca^{2+} -CaM pathway involved in heat stress signal transduction (Liu et al., 2008; Zhang et al., 2009). Ca^{2+} -CaM signaling has been shown to be involved in activation of antioxidant enzymes in *Zea mays* (Hu et al., 2007) and *Amaranthus lividus* (Bhattacharjee, 2008). However, whether Ca^{2+} -CaM pathways are involved in resistance to heat stress-induced oxidative stress and the activation of antioxidant system remains to be determined.

Tomato, an important greenhouse crop worldwide, is

greatly affected by heat stress, which adversely affects the vegetative and reproductive processes of tomato and ultimately reduces yield and fruit quality (Rivero et al., 2004). In response to heat stress, there are some reports on the physiological metabolism including the antioxidant metabolism, which suggests that tomato grown under high temperature could trigger the defense system (Rainwater et al., 1996; Rivero et al., 2004; Ogweno et al., 2009). Very little is known, however, about the signal transduction pathways of activation of antioxidant system in tomato seedlings against high temperature. In this present study, using biochemical and pharmacological approaches, the role of Ca^{2+} -CaM signaling in the protection against heat stress-induced oxidative damage in tomato (*Solanum lycopersicum* L.) seedlings was investigated.

MATERIALS AND METHODS

Plant material and treatments

Seeds of tomato (*Lycopersicon esculentum* Mill. Puhong 909; from Horticultural Research Institute, Shanghai Academy of Agricultural Sciences, China) warmed at 55°C for 15 min and then soaked in water for 6 h were germinated on moist filter paper in an incubator at 28°C. The germinated seeds were sown in quartz sands in the growth chamber having 14-h days of 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of light at 28°C and 10-h nights at 18°C. Tomato seedlings at the second-true leaf stage were watered with half-strength Hoagland nutrient solution while seedlings at the third-true leaf stage were used for all investigations.

Tomato plants were excised at the base of the stem according to Orozco-Cárdenas and Ryan (2002) and Hu et al. (2006) placed in the beakers wrapped with aluminum foil containing 10^{-3}M potassium phosphate buffer, pH 6.5 for 1 h to eliminate the wound stress, and were used for all experiments. For heat stress treatment, the detached tomato seedlings were pretreated with buffer solutions alone for 2 h at 28°C and then exposed to 42°C for 2, 4, 8, 12, 18 and 24 h in a controlled temperature incubator. To investigate the involvement of Ca^{2+} -CaM signaling in heat stress-induced oxidative stress, the detached seedlings were pretreated with a buffer solution containing 10 mM Ca^{2+} (in the form of CaCl_2) or Ca^{2+} -CaM signaling inhibitors including calcium ion chelator 5 mM EGTA, calcium ion channel blockers 500 μM LaCl_3 , CaM antagonists 200 μM CPZ, 200 μM TFP, 200 μM W7 and 200 μM W5, an inactive structural analog of W7 for 2 h, and then exposed to heat stress for 12 h. The concentrations used in this study were determined from previous studies (Liu et al., 2003, 2005; Hu et al., 2007; Bhattacharjee, 2008) and preliminary experiments in which the chemicals did not have any substantial effect on plants (data not shown). All chemicals were purchased from Sigma-Aldrich. Detached plants treated with a buffer solution under the same conditions for the whole period served as the controls. After treatment of tomato leaves, the second and third leaves were sampled and immediately frozen under liquid N_2 .

Determination of lipids peroxidation

In order to determine whether tomato's tolerance to heat stress-induced oxidative stress was related to the Ca^{2+} -CaM signaling,

various compounds that affected the Ca^{2+} -CaM signaling system were employed to determine oxidative damage under heat stress treatment for 12 h. Oxidative damage to lipids was estimated by measuring the amount of produced MDA by the thiobarbituric acid reaction as described by Jiang and Zhang (2001). The crude extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. The mixture was centrifuged at 3000×g for 10 min and the absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA content was determined by its molar extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

Oxidative damage to proteins was estimated as the content of carbonyl groups (Rezimick and Packer, 1994). Leaf segments (0.5 g) were homogenized with 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM PMSF, 10 mM DTT and $5 \mu\text{g ml}^{-1}$ leupeptin, $5 \mu\text{g ml}^{-1}$ aprotinin and $5 \mu\text{g ml}^{-1}$ antipain. The homogenate was centrifuged with nucleic acids by treatment with streptomycin sulfate, and added an equal volume of 10 mM DNPH in 2 M HCl to solutions containing the oxidized protein. These were allowed to stand in the dark at room temperature for 1 h, with vortex every 10 min. Samples were precipitated with trichloroacetic acid (TCA; 20% final concentration) and centrifuged in a tabletop micro-centrifuge for 5 min. The supernatants were discarded and the protein pellets were washed once more with TCA, and then washed three times with 1 ml portions of ethanol/ethylacetate (1:1) to remove any free DNPH. The protein samples were re-suspended in 1 ml of 6 M guanidine hydrochloride (dissolved in 20 mM phosphate buffer, pH 2.3) at 37°C for 15 min with vortex mixing. Carbonyl contents were determined from the absorbance at 370 nm using a molar absorption coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of and $\text{O}_2^{\cdot-}$ and H_2O_2 content

To evaluate whether heat stress-induced oxidative stress mediated by Ca^{2+} -CaM is associated with ROS production, $\text{O}_2^{\cdot-}$ and H_2O_2 were examined in tomato leaves pretreated with Ca^{2+} , Ca^{2+} inhibitors. $\text{O}_2^{\cdot-}$ was measured as described by Jiang and Zhang (2001) by monitoring the nitrite formation from hydroxylamine in the presence of $\text{O}_2^{\cdot-}$. 1 g of fresh leaves was grounded with 3 ml 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5,000 × g for 10 min. One milliliter of supernatant was mixed with 0.9 ml 65 mM potassium PBS (pH 7.8) and 0.1 ml 10 mM hydroxylamine hydrochloride, and incubated at 25°C for 20 min. After incubation, 17 mM sulfanilamide and 7 mM α -naphthylamine were added to the above mixture, and the mixtures were incubated at 25°C for 20 min. The light absorbance was measured at 530 nm. A standard curve with NO_2^- was used to calculate the production rate of $\text{O}_2^{\cdot-}$ from the chemical reaction of $\text{O}_2^{\cdot-}$ and hydroxylamine.

The content of H_2O_2 was measured by monitoring the A415 of the titanium–peroxide complex following the method described by Jiang and Zhang (2001). Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2 .

Assays of antioxidant enzyme activities

According to the method of Jiang and Zhang (2001), frozen leaf segments (0.5 g) were homogenized in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% soluble polyvinylpyrrolidone (PVP), with the addition of 1 mM ascorbate in the case of APX assay. The homogenate was centrifuged at 15,000g for 20 min at 4°C and

the supernatant was immediately frozen under liquid N_2 and stored at -70°C for the following assays.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μl enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 5000 lx. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT monitored at 560 nm. SOD activity was expressed as U mg^{-1} protein.

APX (EC 1.11.1.11) activity was determined by monitoring the rate of ascorbate oxidation at 290 nm (extinction coefficient $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 , and 200 μl of enzyme extract. Correction was done for the low, non-enzymatic oxidation of AsA by H_2O_2 . The activity of APX was calculated in terms of $\mu\text{mol AsA mg}^{-1} \text{ protein} \cdot \text{min}^{-1}$.

Protein determination

Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Statistical analysis

The results presented were the mean of at least three replicates in the enzyme assays. Means were compared by one-way analysis of variance and Duncan's multiple range test at 5% level of significance.

RESULTS

Heat stress generated oxidative stress in tomato leaves

To examine whether the damage caused by elevated temperature was due to the oxidative stress, we measured the effects of heat stress on oxidation parameters. MDA are the production of lipid peroxidation, which is a common assay for oxidative damage to membranes (Larkindale and Knight, 2002). Carbonylated protein is another good indicator of protein damage due to oxidative stress (Ghezzi and Bonetto, 2003). Incubation of tomato leaves under heat stress resulted in a marked change in the patterns of accumulation of MDA content (Figure 1A) and the level of carbonylated protein (Figure 1B). The MDA and carbonyl groups content increased after 12 h and 4 h treatment, respectively indicating that heat stress brought about lipid peroxidation and protein oxidative damage. After 12 h treatments, the amount of MDA and carbonyl in heat stress-treated leaves increased up to 43.0 and 38.8%, respectively in comparison with the control. And the detached plants under control treatment showed very little oxidative damage. So in subsequent research, tomato seedlings under heat stress for 12 h were used as experimental pattern for assay of Ca^{2+} -CaM signaling pathway.

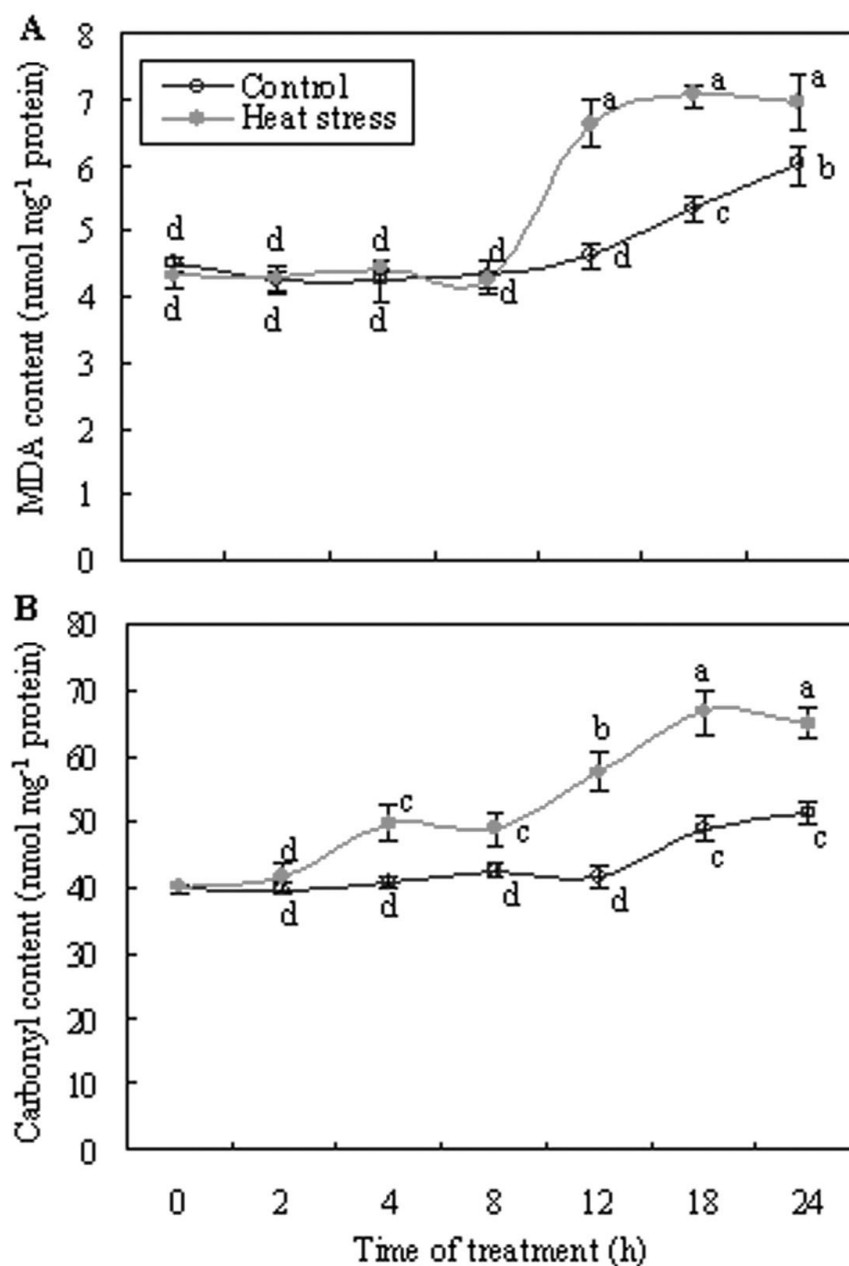


Figure 1. The change in MDA content (A) and carbonyl content (B) in tomato leaves during heat stress treatment. Each date point represents the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

Heat stress-induced oxidative stress was aggravated by inhibition of Ca^{2+} -CaM signaling

To determine whether tomato's tolerance to heat stress-induced oxidative stress was related to the Ca^{2+} -CaM signaling, various compounds such as Ca^{2+} , Ca^{2+} inhibitors EGTA and LaCl_3 and CaM antagonists CPZ, TFP and W7 were employed to determine oxidative

damage under heat stress treatment for 12 h. Ca^{2+} pre-treated plants obviously showed reversal in oxidative damage (reduction in the accumulation of lipid peroxidation and carbonylated protein) caused by heat stress for 12 h (Figures 2A and B). However, the degree of oxidative damage caused by heat stress was significantly aggravated by pretreatment with EGTA, LaCl_3 , CPZ, TFP and W7, compared to the control and heat stress

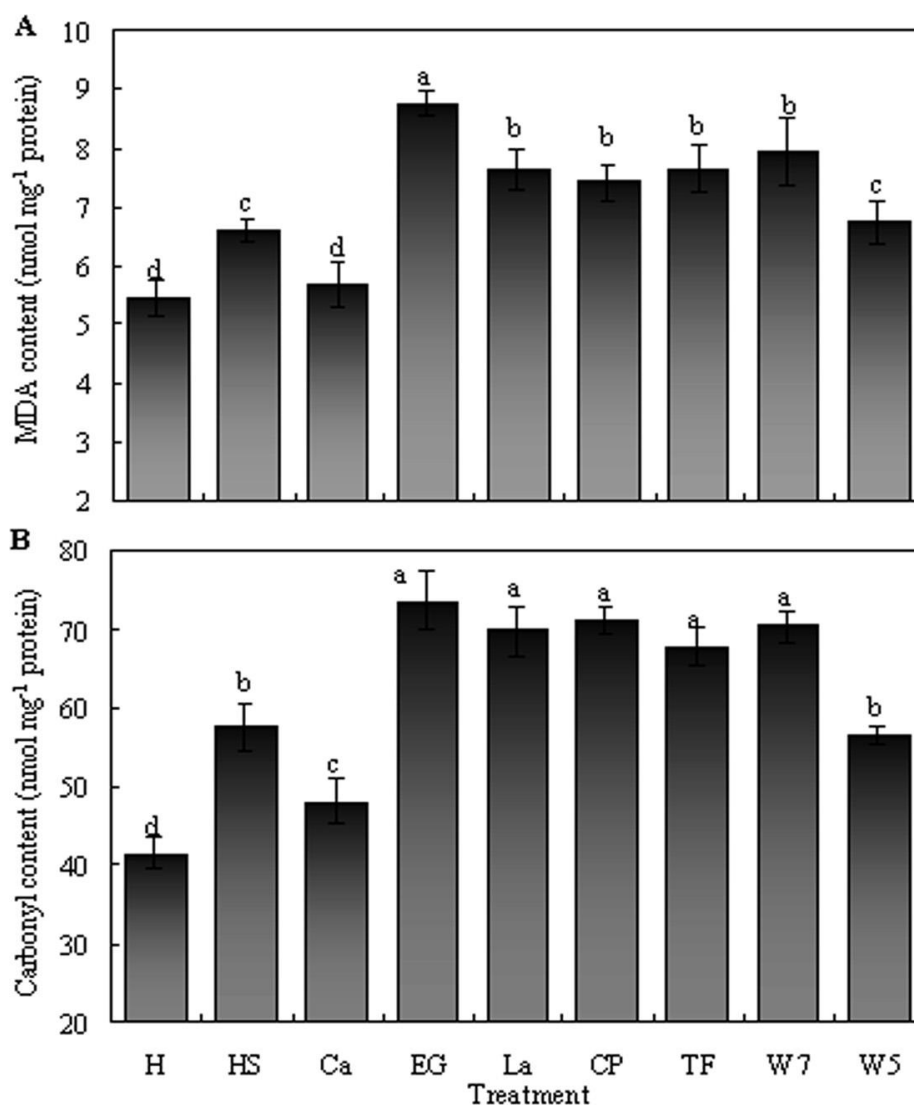


Figure 2. Effects of Ca^{2+} and CaM inhibitors on MDA content (A) and carbonyl content (B) in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM CaCl_2 + HS; EG, 5 mM EGTA + HS; La, 1 mM LaCl_3 + HS; CP, 200 μM CPZ + HS; TF, 200 μM TFP + HS; W7, 200 μM W7 + HS; W5, 200 μM W5 + HS. Each value is the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

treatment (Figure 2A and B). Treatment with W5, an inactive structural analog of W7, did not influence the changes of oxidative partten with respect HS. There was no significant difference in all inhibitors except for EGTA treatment.

Heat stress-induced $\text{O}_2^{\cdot-}$ and H_2O_2 production were mediated by Ca^{2+} -CaM signaling

It is well known that oxidative stress occurs in plants due

to overproduction of ROS such as $\text{O}_2^{\cdot-}$ and H_2O_2 (Mittler, 2002). To evaluate whether heat stress-induced oxidative stress mediated by Ca^{2+} -CaM is associated with ROS production, $\text{O}_2^{\cdot-}$ and H_2O_2 were examined in tomato leaves. Figure 3 shows that heat stress caused significant increase in the content of $\text{O}_2^{\cdot-}$ and H_2O_2 as compared to the control. CaCl_2 pretreatment prior to heat stress obviously reduced the accumulation of both the ROS, whereas all the Ca^{2+} and CaM inhibitors significantly increased the level of ROS production compared with the control and heat stress treatment. W5, an inactive

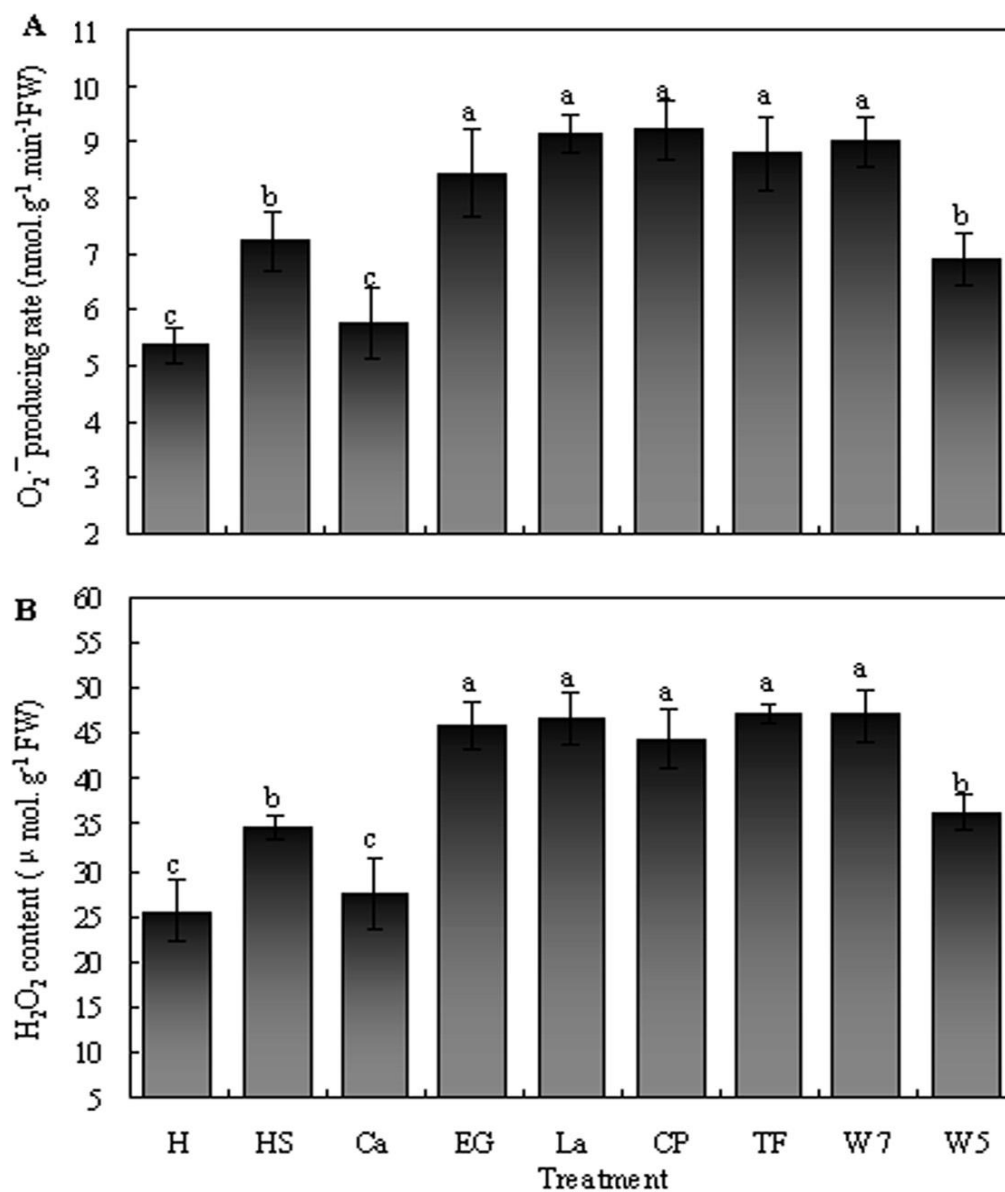


Figure 3. Effects of Ca²⁺ and CaM inhibitors on O₂⁻ content (A) and H₂O₂ content (B) in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM CaCl₂ + HS; EG, 5 mM EGTA +HS; La, 1 mM LaCl₃ +HS; CP, 200 µM CPZ + HS ; TF, 200 µM TFP + HS; W7, 200 µM W7 + HS; W5, 200 µM W7 + HS. Each value is the means of three repeats. Means denoted by the same letter did not significantly differ at P < 0.05 according to Duncan's multiple range test.

structural analog of W7, did not influence the contents of O₂⁻ and H₂O₂ with respect HS.

The antioxidant enzymes SOD and APX were activated in tomato leaves under heat stress

The antioxidant enzymes play vital roles in plant

antioxidant defense system. To further determine the relationship between Ca²⁺-CaM and antioxidant enzymes under heat stress treatment, first of all we studied the change in activities of SOD and APX, two important antioxidant enzymes, in tomato leaves caused by heat stress treatment. As shown in Figure 4, the activities of SOD and APX displayed different pattern, that is, SOD activity increased rapidly and reached highest value within

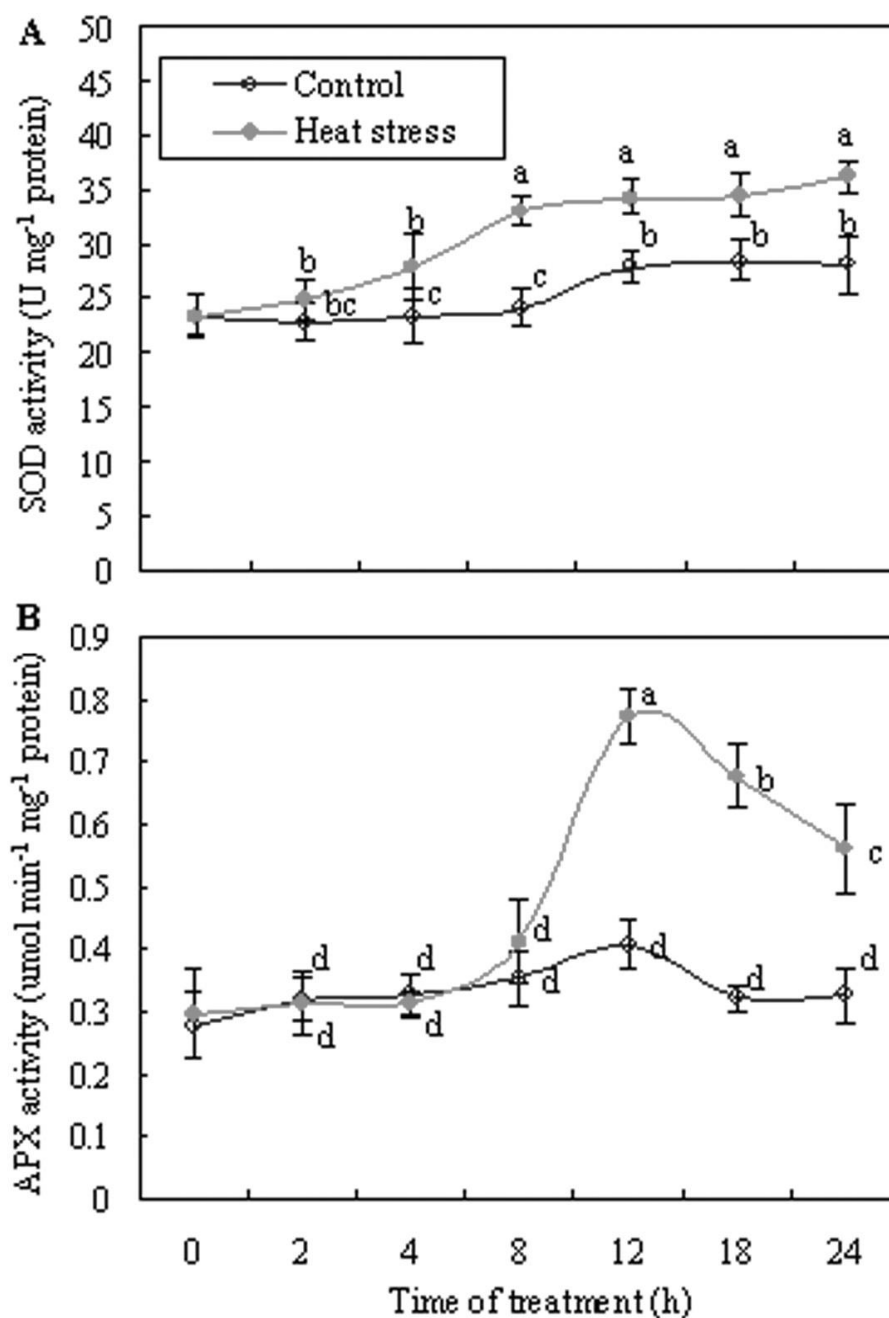


Figure 4. The change in activities of SOD (A) and APX (B) in tomato leaves during heat stress treatment. Each date point represents the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

4 to 8 h, while APX activity reached highest value within 8 to 12 h, indicating that antioxidant enzymes were activated by heat stress. After 12 h treatments, the activities of SOD and APX in heat stress-treated leaves increased up to 22.7 and 90.1%, respectively, compared to the control.

The activation of SOD and APX induced by heat stress were mediated by Ca²⁺-CaM signaling

To explore the relationship between the heat stress-activated SOD and APX and Ca²⁺-CaM signaling, the effect of different inhibitors on the activities of SOD and

APX were determined. Pretreatment of tomato leaves with Ca^{2+} helped to enhanced higher SOD and APX activities compared with the heat treatment. Activities of two enzymes were blocked significantly when tomato seedlings were treated with calcium ion chelator and channel blockers. However, pretreatment with EGTA, LaCl_3 and W7 decreased two enzymes to base-line levels, and CPZ and TFP partly blocked SOD and APX activity and CPZ. W5, an inactive structural analog of W7, showed no significant influence on the SOD and APX activities increased by heat stress.

DISCUSSION

Accumulative evidence have indicated that heat stress, like other abiotic stresses, induced oxidative stress, which resulted in cellular membrane injuries, carbonyl derivatives formation and growth suppression in plants. Survival after heat stress is often associated with the ability to tolerate or repair oxidative damage and it also requires the ability to tolerate or minimize other kinds of heat-induced damage (Wahid et al., 2007). How heat stress signals are perceived and transduced to activate the antioxidant pathways resulting in tolerance to oxidative stress is presently of intense interest. In our work, treatment of tomato leaves with heat stress resulted in increase in levels of MDA and protein carbonylation (Figure 1A and B). Moreover, $\text{O}_2^{\cdot-}$ and H_2O_2 , two main kind of reactive oxygen species (ROS), accumulated significantly under heat stress (Figure 3A and B), which indicated that HS brought about oxidative damage in tomato leaves. Nevertheless, HS-induced oxidative stress was mitigated by pretreatment with Ca^{2+} and was aggravated by pretreatment with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blocker LaCl_3 and the CaM antagonists CPZ, TFP and W7, which have been shown to prevent CaM signaling induced by CaCl_2 treatment (Gong et al., 1997), heat stress (Liu et al., 2003, 2005; Bhattacharjee, 2008) or ABA treatment (Hu et al., 2007).

Using pharmacological approaches, Ca^{2+} -CaM signaling has been shown to involved in regulation of *HSP26* and *HSP70* gene expression in wheat (Liu et al., 2003), *HSP18.2* expression in *Arabidopsis* (Liu et al., 2005), antioxidant enzymes activities and *SOD4*, *cAPX* and *GR1* expression in maize (Hu et al., 2007). In previous studies, by inhibition of *Zea mays* seeds (Gong et al., 1997) or *Amaranthus lividus* seeds (Bhattacharjee, 2008) with CaCl_2 , EGTA, LaCl_3 , CPZ or TFP, Ca^{2+} -CaM signaling was suggested involved in the acquisition of the heat-shock induced thermotolerance. In this present study, by pretreatment of detached tomato leaves with these inhibitors, Ca^{2+} -CaM signaling involved in tolerance of tomato to heat stress-induced oxidative stress was evaluated. The levels of MDA and protein carbonylation caused by heat stress was reduced by exogenous

application of Ca^{2+} , but was aggravated by pretreatment with EGTA, LaCl_3 , CPZ, TFP and W7 (Figure 2A and B). Although the absolute specificities of the inhibitors used in this study can always be question, it has been shown that these inhibitors in the concentration range used in this present study should be suitable for studying the role of Ca^{2+} and CaM signal in plant cells (Liu et al., 2003, 2005; Hu et al., 2007; Bhattacharjee, 2008). Our results clearly suggest that Ca^{2+} and Ca^{2+} dependent pathway acting through CaM in tomato is required to switch on some antioxidant mechanisms by which plants prevent or repair oxidative damage caused by heat stress. Higher CaM level has also been linked to lower level of heat-induced membrane damage in maize (Gong et al., 1997) and in *Arabidopsis* (Liu et al., 2005).

Heat stress can lead to oxidative stress through the increase in ROS, which could potentiate the accumulation of MDA and protein carbonylation, two indicators of oxidative damage to the membranes (Ding et al., 2009). Also, the metabolism balance of ROS can be regulated by a series of antioxidant enzymes (Mittler, 2002). Therefore, we deduced that the vital role of Ca^{2+} -CaM might be related with the elimination of ROS. In this study, the significant reduction in accumulation of $\text{O}_2^{\cdot-}$ and H_2O_2 along with elevated activities of antioxidant enzymes SOD and APX in Ca^{2+} pretreated tomato seedlings further confirm the involvement of Ca^{2+} in mitigating the oxidative stress (Figure 4A and B). Ca^{2+} has also been reported to increase antioxidant enzyme activities and heat resistance in *Festuca arundinacea* and *Poa pratensis* under heat stress (Jiang and Huang, 2001; Larkindale and Huang, 2004; Agarwal et al., 2005). CaM is also involved in the process, as CaM antagonists significantly increased $\text{O}_2^{\cdot-}$ and H_2O_2 production (Figure 3A and B) but decreased SOD and APX activities (Figure 4A and B). SOD is a primary scavenger for $\text{O}_2^{\cdot-}$ generated from exposure to oxidative stress, which catalyzes the $\text{O}_2^{\cdot-}$ to H_2O_2 , which is further utilized by APX. Our results indicate that Ca^{2+} -CaM signaling mediated SOD and APX activation to remove effectively excessive formation of ROS. Furthermore, Ca^{2+} -CaM signal has been proposed to function as a signal downstream of H_2O_2 in the ABA-induced antioxidant enzymes activities (Jiang and Zhang, 2003; Hu et al., 2007). H_2O_2 plays a dual role in plant biology acting on one hand as important signal transduction molecules and on the other as toxic by-products of aerobic metabolism that accumulate in cells during different stress conditions (Mittler et al., 2004, 2008). ROS acting as signal in heat stress signal transduction has also been reported (Wahid et al., 2007). Whether Ca^{2+} -CaM pathways regulate ROS signal in activation of antioxidant system under heat stress remains to be investigated.

Recently, molecular and genetic evidence confirmed that a legible Ca^{2+} -CaM signaling pathways play the key role in heat stress signal transduction in *Arabidopsis* (Liu

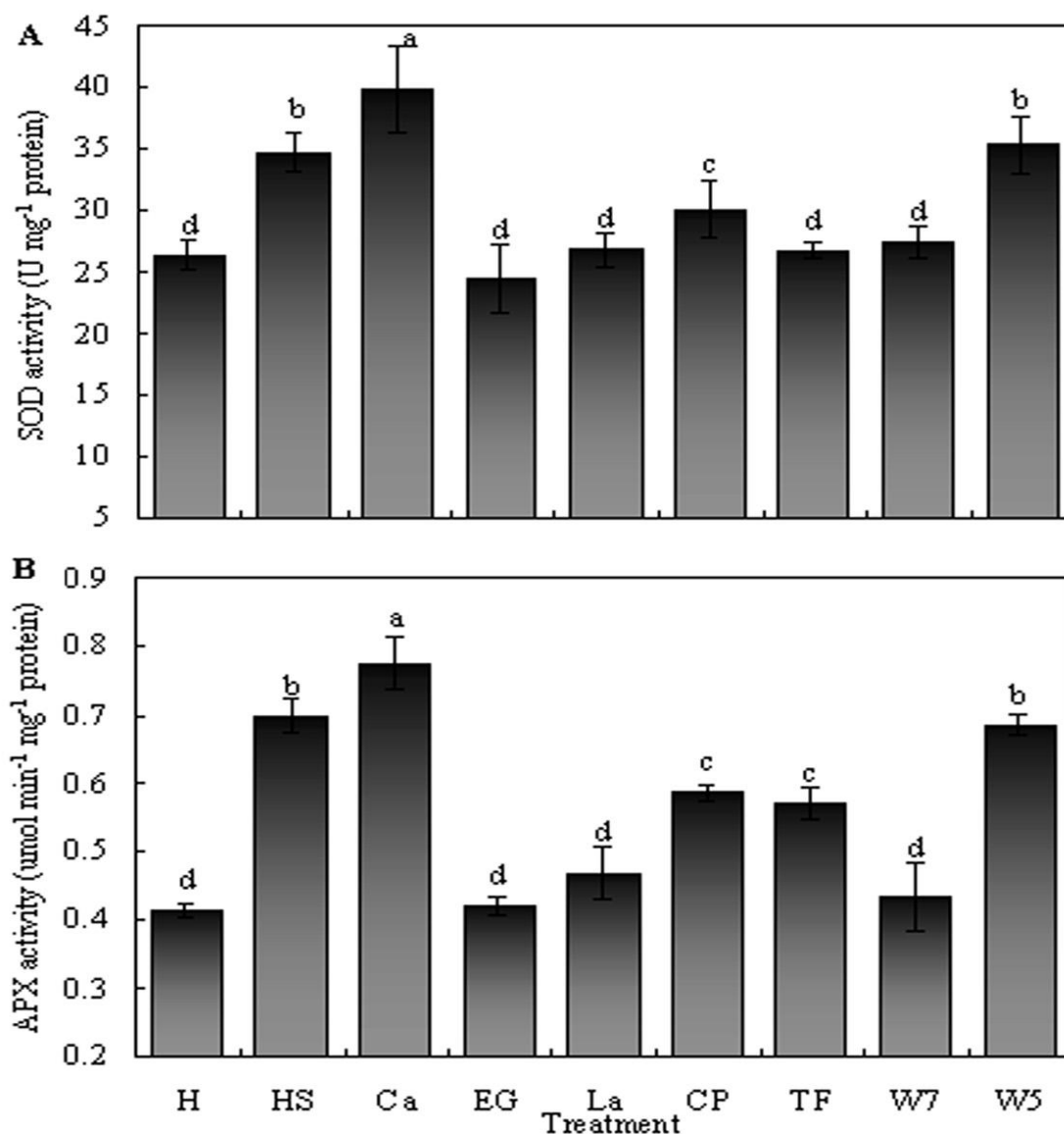


Figure 5. Effects of Ca^{2+} and CaM inhibitors on SOD (A) and APX (B) activities in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM CaCl_2 + HS; EG, 5 mM EGTA +HS; La, 1 mM LaCl_3 +HS; CP, 200 μM CPZ + HS ; TF, 200 μM TFP + HS; W7, 200 μM W7 + HS; W5, 200 μM W7 + HS. Each value is the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

et al., 2008; Zhang et al., 2009). The elevated $[\text{Ca}^{2+}]_i$ directly activates AtCaM3 and, in turn, stimulates AtCBK3, which ultimately regulates the phosphorylation and DNA binding activity of HSFs. By binding to HSEs, HSFs may initiate the transcription of HSP genes as part of the plant's adaptation to environmental heat stress. *AtCaM3* knockout mutant showed decreased thermotolerance and higher MDA level under heat stress, but whether AtCaM3-AtCBK3 pathway are involved in resistance to heat stress-induced oxidative stress and the activation of

antioxidant system are not well-known. The genetic evidences of Ca^{2+} -CaM signaling pathway coupled with HSP in activation of antioxidant defense in other crops under heat stress are still limited, on which the further experiments in tomato seedlings under heat stress are being gone on in our laboratory (Figure 5).

Taken together, the data presented here demonstrate that heat stress-induced ROS production results in oxidative damage. Simultaneously, antioxidant defense enzymes are activated to eliminate over accumulation of

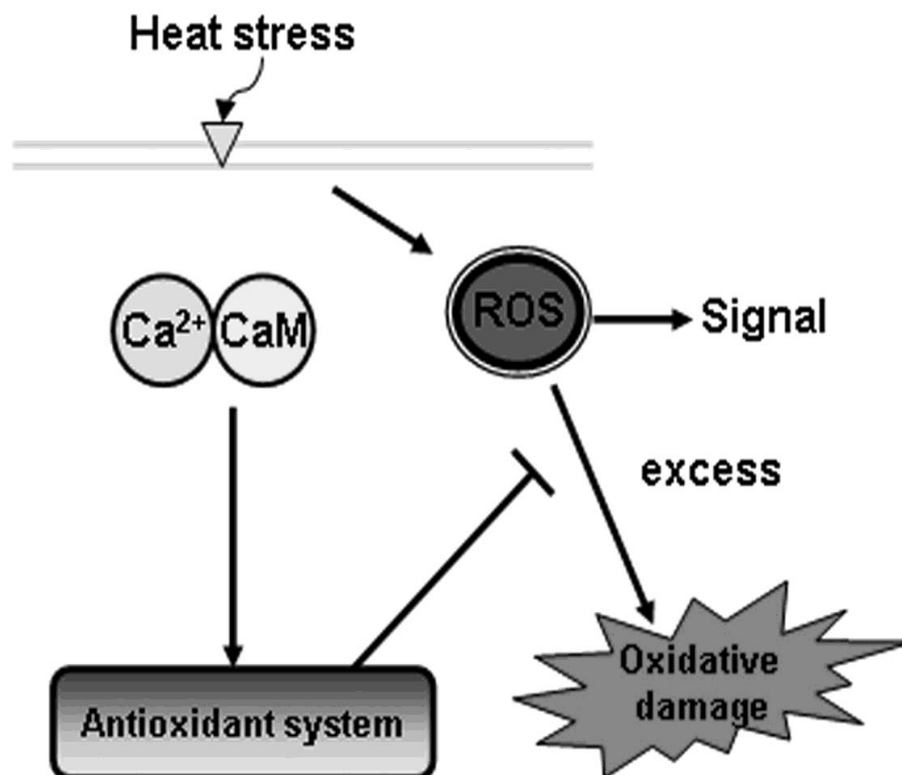


Figure 6. Model summarizing the interaction of Ca^{2+} -CaM, ROS, oxidative damage and antioxidant system in tomato leaves during heat stress. Dashed lines denote indirect or still undescribed pathways.

ROS to reduce oxidative damage in tomato seedlings, which is mediated by Ca^{2+} -CaM signaling (Figure 6). These results can help us to further reveal the signal pathways in resistance to heat stress-induced oxidative stress.

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Abbreviations:

APX, Ascorbate peroxidase; **Ca^{2+} -CaM**, calcium ion and

calmodulin; **CAT**, catalase; **CPZ**, chlorpromazine; **EGTA**, ethylene glycol-bis(β -amino ethyl ether)-**N,N,N',N'**-tetraacetic acid; **MDA**, malondialdehyde; **NBT**, nitrotetrazolium blue chloride; $\text{O}_2^{\bullet-}$, superoxide anion radical; **POD**, peroxidase; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TFP**, trifluoperazine; **W7**, **N**-(6-aminohexyl)-5-chloro-1-naphthalenesulfonyl-**N**-amide.

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