African Journal of Biotechnology Vol. 11(14), pp. 3259-3269, 16 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2784
ISSN 1684–5315 © 2012 Academic Journals

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 Ding[1], Hong-Juan Zhang [2], Xiao-Hong Zhu[1], Hui Liu[1], Jian-Shen Liang[1]* and Bing Lu[1]

[1]Fostering Base for National Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, China.
[2]College of Horticulture and Plant Protection, Yangzhou University, Yangzhou 225009, China.

Accepted 1 February, 2012

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), $O_2^{\cdot-}$ 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 ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (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: jssliang@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 transient increase in the intracellular concentration of free calcium ($[Ca^{2+}]i$) 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 $[Ca^{2+}]i$ 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 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 $[Ca^{2+}]i$ 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 sensor 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 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 (*Lycopersicom 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 μmol $m^{-2}s^{-1}$ 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) tricholoroacetic 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 mM^{-1} 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 µg ml^{-1} leupeptin, 5 µg ml^{-1} aprotintin and 5 µg 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 $mM^{-1}cm^{-1}$.

Determination of and $O_2•^-$ and H_2O_2 content

To evaluate whether heat stress-induced oxidative stress mediated by Ca^{2+}-CaM is associated with ROS production, $O_2^{\cdot-}$ and H_2O_2 were examined in tomato leaves pretreated with Ca^{2+}, Ca^{2+} inhibitors. $O_2•^-$ was measured as described by Jiang and Zhang (2001) by monitoring the nitrite formation from hydroxylamine in the presence of $O_2•^-$. 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 $O_2•^-$ from the chemical reaction of $O_2•^-$ 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 ethylene-diaminetetraacetic 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 $mM^{-1}cm^{-1}$). 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 umol AsA mg^{-1}protein.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.

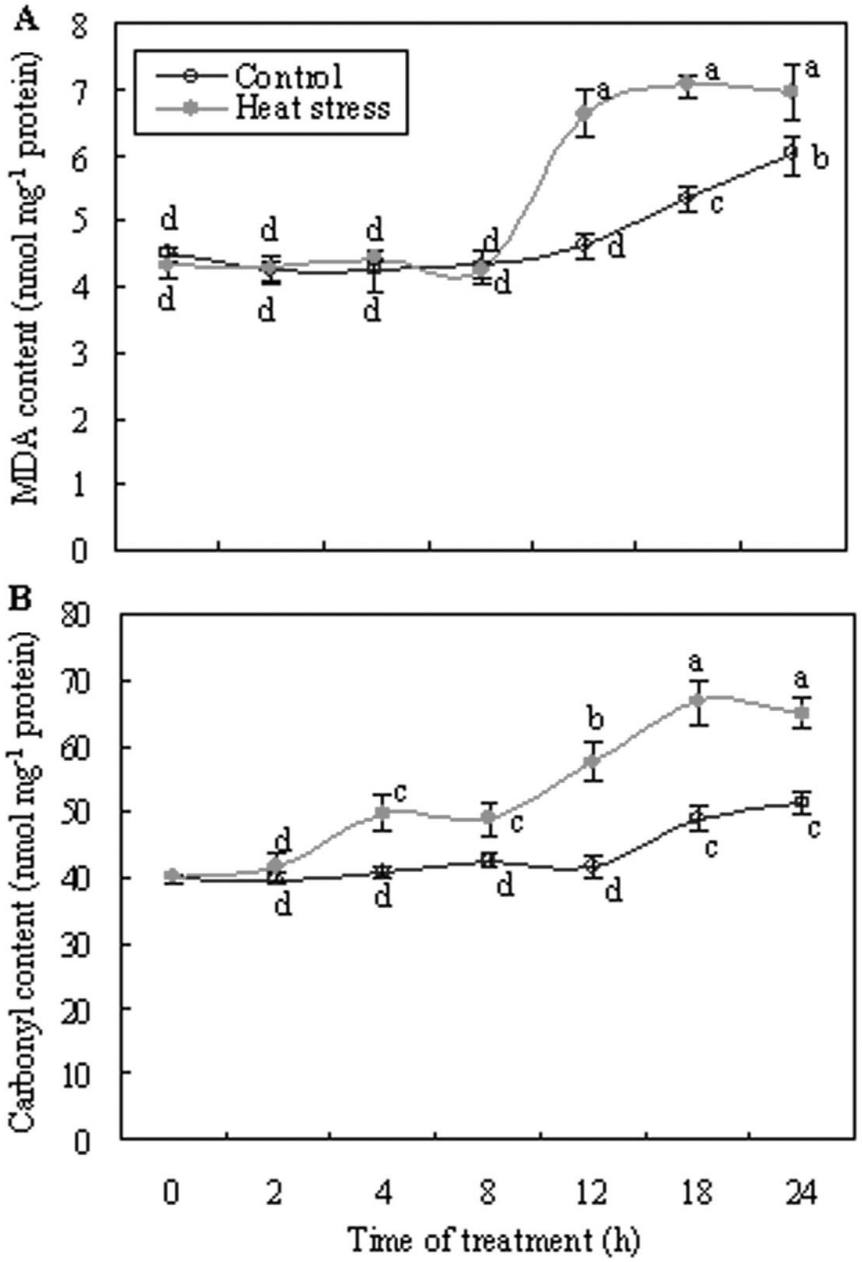

Figure 1. The change in MDA content (A) and carbonyl content (B) in tomato leaves during heat stress treatment. Each date point represents the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

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

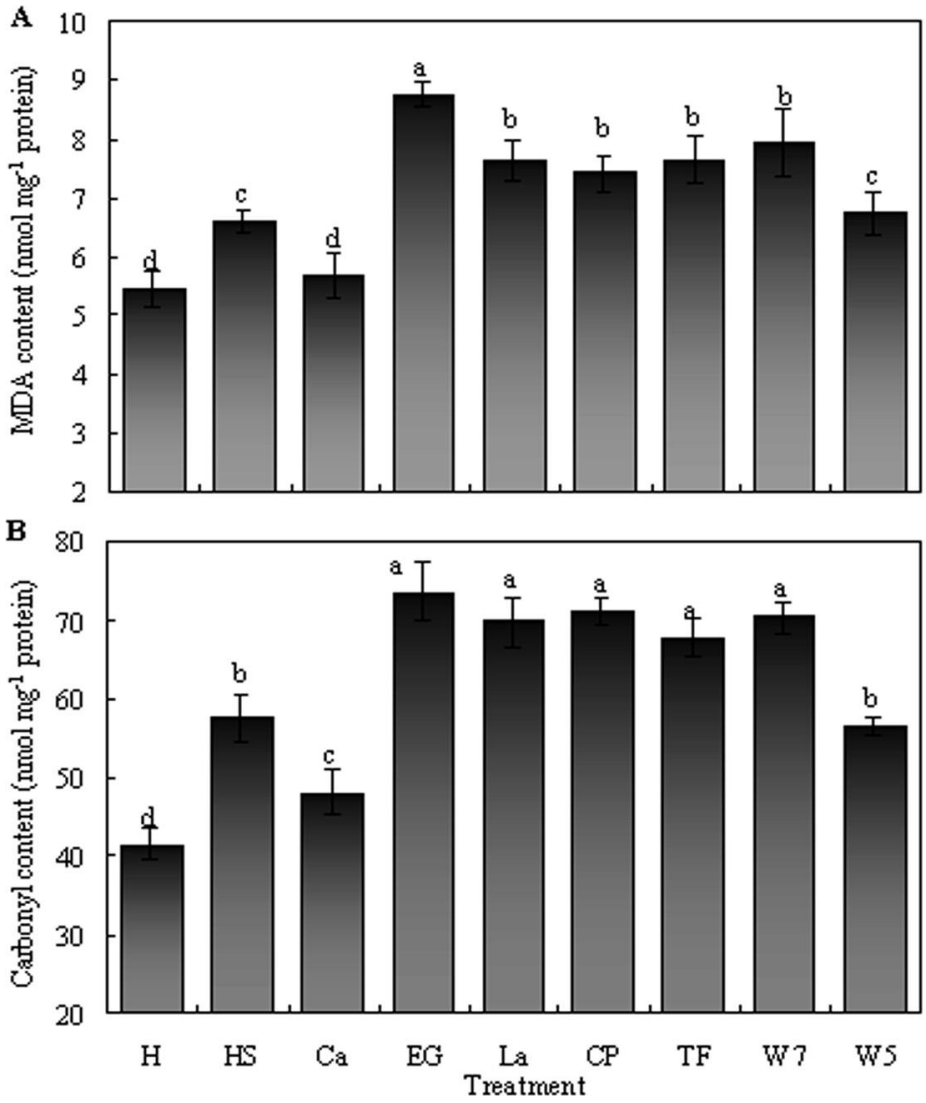

Figure 2. Effects of Ca^{2+} and CaM inhibitors on MDA content (A) and carbonyl content (B) in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM $CaCl_2$ + HS; EG, 5 mM EGTA +HS; La, 1 mM $LaCl_3$ +HS; CP, 200 μM CPZ + HS ; TF, 200 μM TFP + HS; W7, 200 μM W7 + HS; W5, 200 μM 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.

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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ and H_2O_2 (Mittler, 2002). To evaluate whether heat stress-induced oxidative stress mediated by Ca^{2+}-CaM is associated with ROS production, $O_2^{\cdot-}$ and H_2O_2 were examined in tomato leaves. Figure 3 shows that heat stress caused significant increase in the content of $O_2^{\cdot-}$ 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

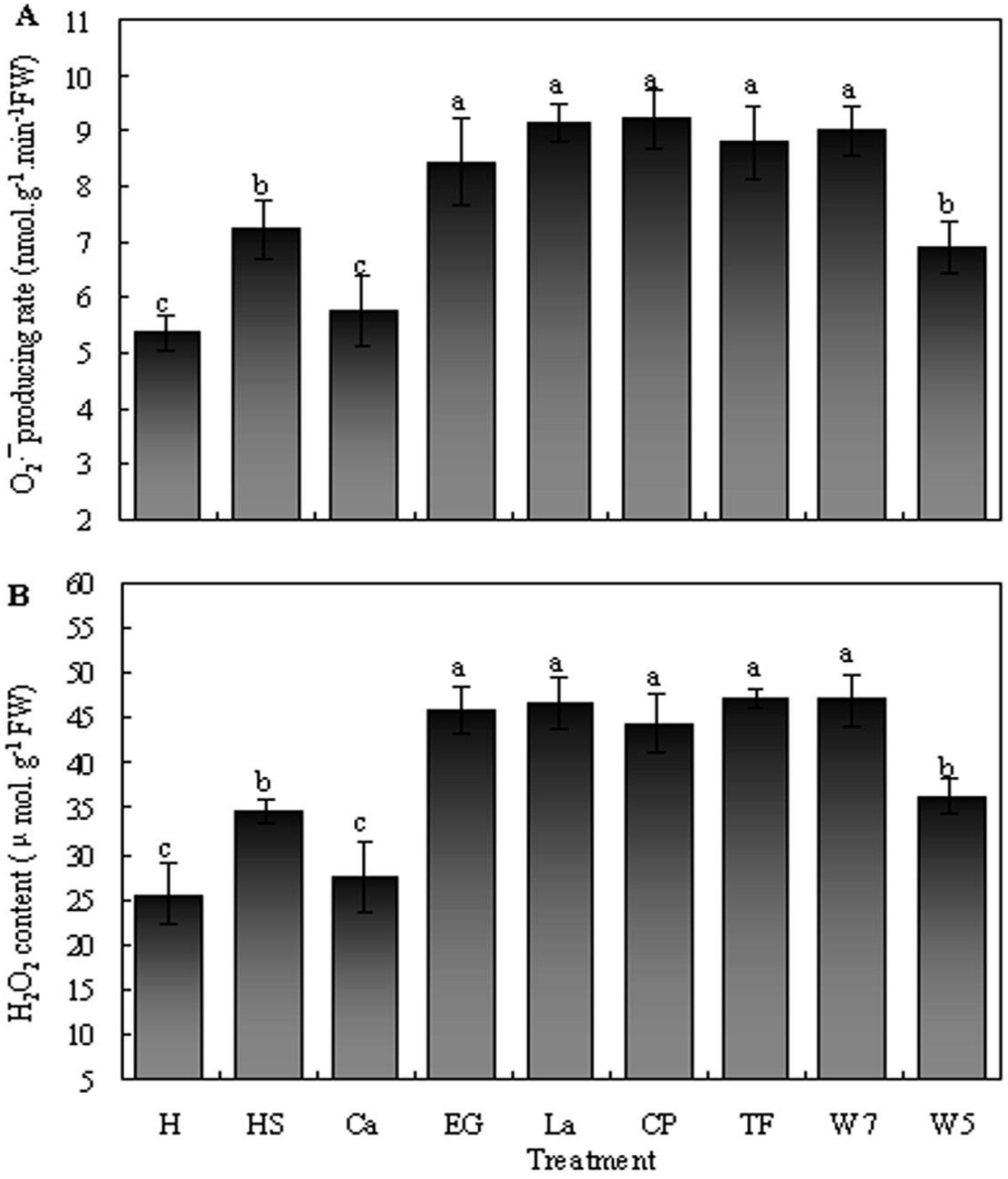

Figure 3. Effects of Ca^{2+} and CaM inhibitors on $O_2^{\cdot-}$ content (A) and H_2O_2 content (B) in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM $CaCl_2$ + HS; EG, 5 mM EGTA +HS; La, 1 mM $LaCl_3$ +HS; CP, 200 μM CPZ + HS ; TF, 200 μM TFP + HS; W7, 200 μM W7 + HS; W5, 200 μM 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 $O_2^{\cdot-}$ and H_2O_2 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 Ca^{2+}-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

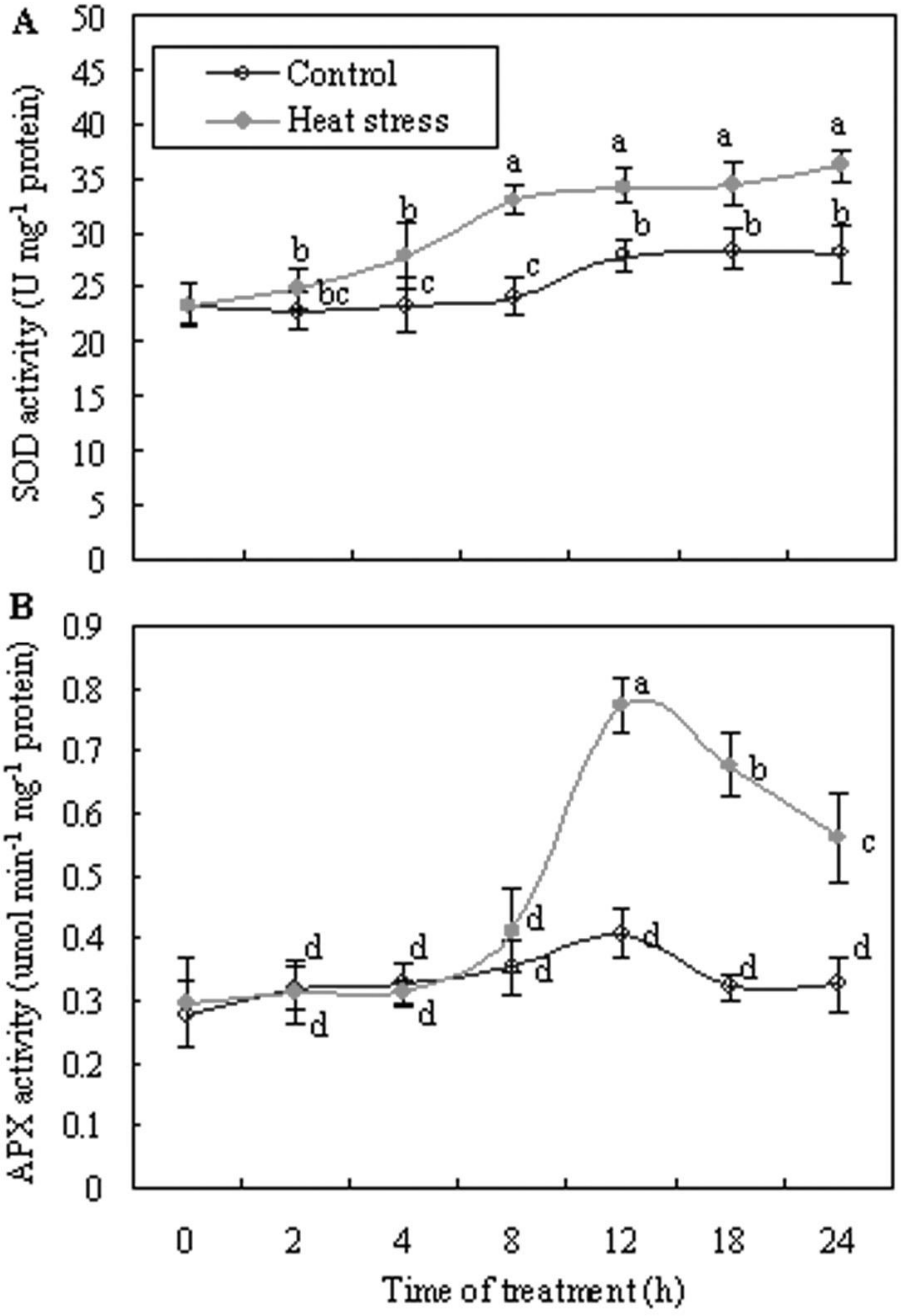

Figure 4. The change in activities of SOD (A) and APX (B) in tomato leaves during heat stress treatment. Each date point represents the means of three repeats. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

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 Ca^{2+}-CaM signaling

To explore the relationship between the heat stress-activated SOD and APX and Ca^{2+}-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, $O_2^{\cdot-}$ 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 may* 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 $O_2\bullet^-$ generated from exposure to oxidative stress, which catalyzes the $O_2\bullet^-$ 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

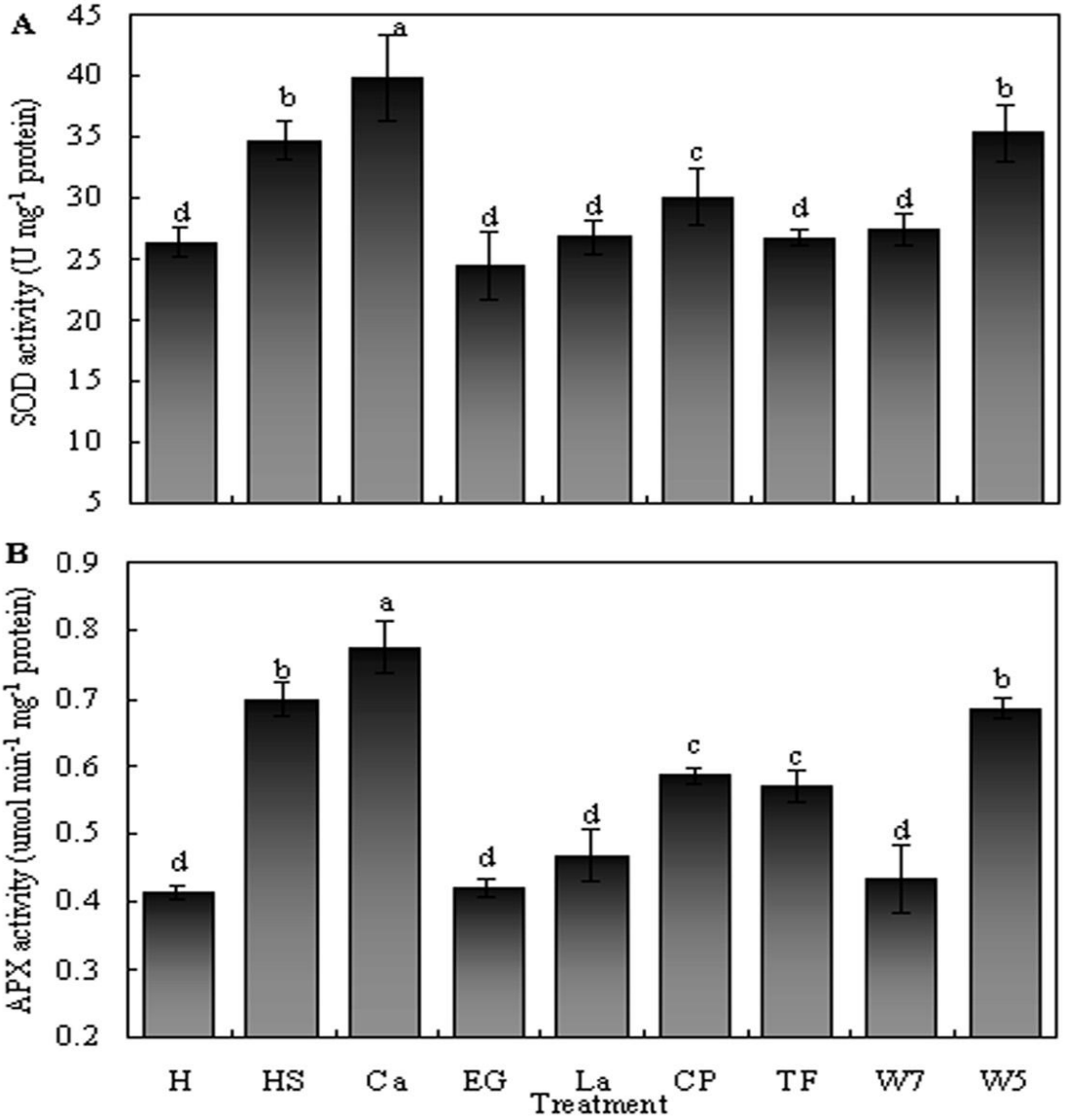

Figure 5. Effects of Ca^{2+} and CaM inhibitors on SOD (A) and APX (B) activities in tomato leaves exposed to heat stress treatment. The tomato seedlings were treated as follows: H, PBS (control); HS, heat stress; Ca, 10mM $CaCl_2$ + HS; EG, 5 mM EGTA +HS; La, 1 mM $LaCl_3$ +HS; CP, 200 μM CPZ + HS ; 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 $[Ca^{2+}]i$ 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

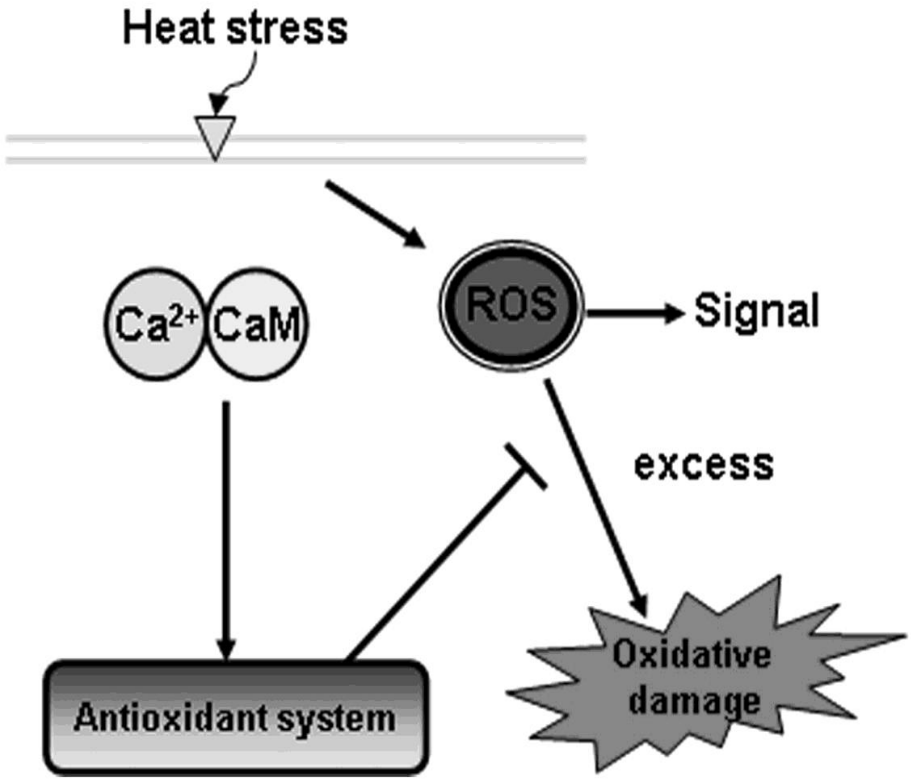

Figure 6. Model summarizing the interaction of Ca^{2+}-CaM, ROS, oxidative damage and antioxidant system in tomato leaves during heat stress. Dashed lines denote indrect or still undescribed pathways.

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.

ACKNOWLEDGEMENTS

We are grateful for the funding supported by the Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (Grant No. 10KJB180010), the National Natural Science Foundation of China (Grant No. 31101092 to H.D., Grant No. 90917005 to J.L.), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions and the Natural Science Foundation of Jiangsu Province, China (Grant No. BK2009185).

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'**-tetraacetid acid; **MDA,** malondialdehyde; **NBT,** nitrotetrazolium blue chloride; $O_2^{\bullet-}$, superoxide anion radical; **POD,** peroxidase; ROS, reactive oxygen species; **SOD,** superoxide dismutase; **TFP,** trifluoperazine; **W7, N-**(6-aminohexyl)-5-chloro-1-naphthalenesul –fonamide.

REFERENCES

Agarwal S, Sairam RK, Srivastava GC, Tyagi A, Meena RC (2005). Role of ABA, salicylic acid, calcium and hydrogen peroxide on antioxidant enzymes induction in wheat seedlings. Plant Sci. 169: 559-570.

Bhattacharjee S (2008). Calcium-dependent signaling pathway in the heat-induced oxidative injury in *Amaranthus lividus*. Biol. Plant, 52: 137-140.

Bouché N, Yellin A, Snedden WA, Fromm H (2005). Plant-specific calmodulin-binding proteins. Annu. Rev. Plant Biol. 56: 435-466.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248-254.

Ding HD, Zhang XH, Xu SC, Sun LL, Jiang MY, Zhang AY, Jin YG. (2009). Induction of protection against paraquat-induced oxidative damage by abscisic acid in maize leaves is mediated through mitogen-activated protein kinase. J. Integr. Plant Biol. 51: 961-972.

Ghezzi P, Bonetto V (2003). Redox proteomics: Identification of

oxidatively modified proteins. Proteomics 3: 1145-1153.
Gong M, Li YJ, Dai X, Tian M, Li ZG (1997). Involvement of calcium and calmodulin in the acquisition of heat-shock induced thermotolerance in maize. J. Plant Physiol. 150: 615-621.
Gong M, Van der Luit AH, Knight MR, Trewavas AJ (1998). Heat-shock-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. Plant Physiol. 116: 429-437.
Hu X, Jiang M, Zhang J, Zhang A, Lin F, Tan M (2007). Calcium-calmodulin is required for abscisic acid-induced antioxidant defense and functions both upstream and downstream of H_2O_2 production in leaves of maize (*Zea mays*) plants. New Phytol. 173: 27-38.
Jiang M, Zhang J (2001). Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. Plant Cell Physiol. 42: 1265-1273.
Jiang M, Zhang J (2003). Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defense in leaves of maize seedlings. Plant Cell Environ. 26: 929-939.
Jiang Y, Huang B (2001). Effects of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. J. Exp. Bot. 52: 341-349.
Kotak S, Larkindale J, Lee U, Von Koskull-Döring P, Vierling E, Scharf KD (2007). Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. 10: 310-316.
Kudla J, Batistič O, Hashimoto K (2010). Calcium Signals: The lead currency of plant information processing. Plant Cell. 22: 541-563.
Larkindale J, Knight MR (2002). Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol. 128: 682-695.
Larkindale J, Huang B (2004). Thermotolerance and antioxidant systems in *Agrostis stolonifera* : Involvement of salicylic acid , abscisic acid , calcium, hydrogen peroxide , and ethylene. J. Plant Physiol. 161: 405-413.
Lecourieux D, Ranjeva R, Pugin A (2006). Calcium in plant defence-signalling pathways. New Phytol. 171: 249-269.
Liu HT, Gao F, Cui SJ, Han JL, Sun DY, Zhou RG (2006). Primary evidence for involvement of IP3 in heat-shock signal transduction in *Arabidopsis*. Cell Res. 16: 394-400.
Liu HT, Gao F, Han JL, Li GL, Liu DL, Sun DY, Zhou RG (2008). The calmodulin-binding protein kinase 3 is part of heat shock signal transduction in *Arabidopsis thaliana*. Plant J. 5: 760-773.
Liu HT, Li B, Shang ZL, Li XZ, Mu RL, Sun DY, Zhou RG (2003). Calmodulin is involved in heat shock signal transduction in wheat. Plant Physiol. 132: 1186-1195.
Liu HT, Sun DY, Zhou RG (2005). Ca^{2+} and AtCaM3 are involved in the expression of heat shock protein gene in *Arabidopsis*. Plant Cell Environ. 28: 1276-1284.
Mittler R, Miller G, Shulaev V (2008). Reactive oxygen signaling and abiotic stress. Physiol. Planta.133: 481-489.
Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410.
Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004). Reactive oxygen gene network of plants. Trends Plant Sci. 9: 490-498.
Ogweno JO, Song XS, Hua WH, Shi K, Zhou YH, Yu JQ (2009). Detached leaves of tomato differ in their photosynthetic physiological response to moderate high and low temperature stress. Sci. Hortic. 123: 17-22.
Orozco-Cárdenas ML, Ryan CA (2002). Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol. 130: 487-493.
Rainwater DT, Gossett DR, Millhollon EP, Hanna HY, Banks SW, Lucas MC (1996). The relationship between yield and the antioxidant defense system in tomatoes grown under heat stress. Free Rad. Res. 25: 421-435.
Rezimick AZ, Packer L (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Method Enzymol. 233: 357-363.
Rivero RM, Ruiz JM, Romero L (2004). Oxidative metabolism in tomato plants subjected to heat stress. J. Hort. Sci. Biotechnol. 79: 560-564.
Saidi Y, Finka A, Goloubinoff P (2011) Heat perception and signalling in plants: a tortuous path to thermotolerance. New Phytol. 190: 556-565.
Saidi Y, Finka A, Muriset M, Bromberg Z, Weiss YG, Maathuis FJM, Goloubinoff P (2009). The heat shock response in moss plants is regulated by specific calcium permeable channels in the plasma membrane. Plant Cell. 21: 2829-2843.
Suzuki N, Mittler R (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol. Plant. 126: 45-51.
Wahid A, Gelani S, Ashraf M, Foolad MR (2007). Heat tolerance in plants: An overview. Environ. Exp. Bot. 61: 199-223.
Yang T, Poovaiah BW (2003). Calcium/calmodulin-mediated signal network in plants. Trends Plant Sci. 8: 505-512.
Zhang W, Zhou RG, Gao YJ, Zheng SZ, Xu P, Zhang SQ, Sun DY (2009). Molecular and genetic evidence for the key role of AtCaM3 in heat-shock signal transduction in *Arabidopsis*. Plant Physiol. 149: 1773-178.
Zhao HJ, Tan JF (2005). Role of calcium ion in protection against heat and high irradiance stress-induced oxidative damage to photosynthesis of wheat leaves. Photosynthetica, 43: 473-476.