Induction of Thermotolerance and Activation of Antioxidant Enzymes in H₂O₂ Pre-applied Leaves of Cucumber and Tomato Seedlings

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The thermotolerance and activation of antioxidant enzymes in the leaves of cucumber and tomato seedlings were induced by foliar application of H₂O₂. The induction of thermotolerance by foliar application of H₂O₂ was detected with decreased electrolyte leakage and malondialdehyde (MDA) content and increased water-soluble protein and chlorophyll content compared with heat-stressed seedlings. The levels of H₂O₂ and superoxide anions in the leaves of heat-stressed seedlings were also reduced by H₂O₂ pre-application before exposure to heat stress. The specific activities of ascorbic peroxidase (APX) and glucose-6-phosphate dehydrogenase (G6PDH) in the leaves of cucumber and tomato seedlings were significantly increased by foliar application of H₂O₂. The gel activities of superoxide dismutase (SOD), hydrogen peroxidase (HPX), and polyphenol oxidase (PPO) isozymes were abruptly increased by foliar application of H₂O₂ and these were maintained for 10 d afterward. The gel activities of guaiacol peroxidase (GPX) in the leaves of cucumber seedlings were significantly increased by foliar application of H₂O₂, but this did not occur in tomato leaves. Although one or two isoforms of SOD, HPX, and PPO isozymes in tomato leaves were suppressed by foliar application of H₂O₂, the total gel activities of the antioxidant enzymes were increased. These results suggest that foliar application of H₂O₂ before a crop is subjected to heat stress may be an effective and method to alleviate heat stress.

Key Words: antioxidant enzymes, cucumber, heat stress, hydrogen peroxide, tomato.

Introduction

The high temperature in protected culture systems, such as in a glasshouse and greenhouse, is a major environmental factor affecting the growth and productivity of horticultural crops during summer. Oxidative stresses are induced by heat stress or high temperature, resulting in harmful changes in normal physiological and biochemical processes, including photosynthetic ability, membrane stability, and respiration levels (Gulen and Eris, 2004; Havaux, 1993). The oxidative stress induced by reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical due to environmental stresses, including high temperature, is the major cause of injury in plants (Almeselmani et al., 2006; Asada, 1999; Bowler et al., 1992). ROS are present in all plants as cytotoxic metabolites and are mediators of the stress response

(Foyer, 1993; Lee and Lee, 2000). Although ROS are generated at various levels in the growth and development process, they are overproduced in response to environmental stresses in all plants (Elstner, 1987). ROS can cause damage to cellular components, such as cell membranes, lipids, proteins, and nucleic acids, disrupting metabolic functions and leading to injury or death under environmental stress (Fridovich, 1991; Hernandez et al., 1995; Zhang and Kirkham, 1994). The levels of ROS in plants are regulated by complex antioxidant systems that include antioxidant enzymes and non-enzymatic agents, such as lipid-soluble antioxidants and water-soluble antioxidants (Foyer, 1993). Complex antioxidant systems, especially antioxidant enzyme systems, are very important as a defense mechanism to protect cellular membranes and organelles against ROS, which are generated by environmental stress in plants (Davies, 1995). A major scavenger in antioxidant enzymes is superoxide dismutase (SOD, EC. 1.15.1.1), which converts superoxide anion radicals to H₂O₂ and oxygen by disproportionation (Fridovich,

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1986). Toxic H_2O_2 produced by dismutation of SOD is reduced to H_2O by peroxidase (POD, EC. 1.11.1.7) or catalase (EC. 1.11.1.6), which are the key enzymes involved in H_2O_2 scavenging systems (Salin, 1991; Anderson et al., 1995).

In recent years, many studies have been devoted to endogenously generated H_2O_2 and exogenously applied H_2O_2 because of its critical role in inducing defense systems against various stresses (de Azevedo Neto et al., 2005; Pellinen et al., 2002; Uchida et al., 2002). Although H_2O_2 has been known as a cytotoxic metabolite in high concentrations (Monk et al., 1989; Zhang and Kirkham, 1994), it is now regarded as a signaling molecule to activate the defense systems against abiotic or biotic stress in plant cells (Borden and Higgins, 2002; Neill et al., 2002). Bowler and Fluhr (2000) suggested that the accumulation of H_2O_2 at appropriate concentrations in plant tissues seems to benefit plants by inducing acclimation or tolerance to environmental stresses. Previous reports have described H_2O_2 playing a critical role as messenger molecule to mediate acclimation (Prasad et al., 1994), triggering tolerance by gene expression (Lamb and Dixon, 1997; Vandenabeele et al., 2003) and hypersensitive reaction (Levine et al., 1994).

High temperature stress is a serious problem affecting the growth and productivity of fruit vegetable crops, such as cucumber and tomato, in protected cultures in the summer season in Korea. The optimum temperature for cucumber and tomato growth is 20–25°C in the daytime; however, the temperature approaches 40°C or higher inside a greenhouse or glasshouse during summer, even with open ventilation. Thus, some growers often give a foliar application of H_2O_2 in the morning to achieve earlier activation of antioxidant enzymes before the crops are exposed to high temperature during the day, and then detoxify the ROS generated by high temperature. The objectives of this study were to determine the effect of alleviating high temperature stress and to better understand the responses of antioxidant enzymes by foliar application of H_2O_2 in cucumber and tomato seedlings.

Materials and Methods

Plant materials and application of H_2O_2 and heat stress

Cucumber (*Cucumis sativus* L. 'Heukjinju') and tomato (*Solanum lycopersicum*, 'Seogeon', Asamizu and Ezura, 2009) seeds were sown in a plastic tray (45 cm $\times$ 35 cm $\times$ 8 cm) containing commercial soil (Shinan Grow, Chinju, Korea) and transplanted into plastic pots (11 cm $\times$ 11 cm) containing the same commercial soil at the one-leaf stage. Seedlings were grown with irrigation once every day to field capacity in a controlled environment growth chamber at 23/20°C (day/night), 12 h photoperiod, and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with RH 80 $\pm$ 5%. When seedlings had three expanded leaves, the seedlings were uniformly sprayed with

15 mM H_2O_2 containing 0.01% (v/v) Tween 20 as surfactant. Foliar application was made to seedlings incubated at 15°C for 6 h to facilitate better absorption of H_2O_2 by the leaves. Distilled water containing 0.01% (v/v) Tween 20 was applied to control seedlings. Samples were collected periodically over a 10 d period and washed in distilled water before enzyme analysis. Seedlings were subjected to heat stress in a growth chamber at 40/28°C (day/night) with 12 h photoperiod for 3 days. Control seedlings were treated at 23/20°C (day/night) with 12 h photoperiod.

Measurement of electrolyte leakage

Leaf samples were collected after 3 days of treatment and washed in distilled water before measuring electrolyte leakage. Leaf discs were punched with holes 1 cm in diameter and placed in test tubes with 30 mL distilled water. Samples were filtered under vacuum with 53.37 kPa pressure for 3 min to allow diffusion of electrolytes and placed on a shaker at 250 rpm for 4 h at room temperature. After incubation, electrical conductivity was measured using a conductivity meter (TOA Electronics Ltd., Japan). After measuring initial electrolyte leakage, samples were autoclaved at 121°C for 15 min, and total electrolyte leakage was measured at room temperature. Electrolyte leakage was calculated using the equation: percentage electrolyte leakage = (conductivity of initial electrolyte leakage/conductivity of total electrolyte leakage) $\times$ 100.

Estimation of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA). The concentration of MDA was determined using the thiobarbituric acid reaction (Heath and Packer, 1968). One gram of leaf samples was homogenized with polytron (PT-3100; Kinematica, Switzerland) in 5 mL of 0.1% trichloroacetic acid (TCA, w/v). The homogenate was centrifuged at 10000 $\times$ g for 10 min, and the supernatant was collected. The supernatant was mixed with 20% TCA containing 0.5% thiobarbituric acid (w/v) as a rate of four volumes. The mixtures were incubated in boiling water of 95°C for 30 min, and then quickly cooled in running water. The cooled mixtures were centrifuged at 10000 $\times$ g for 10 min, and the supernatants collected. Absorbance was measured at 532 nm and 600 nm using a spectrophotometer (DU-650; Beckman, USA). The concentration of MDA was calculated by subtracting 600 nm from 532 nm (extinction coefficient of 155 $\text{mM}^{-1}\cdot\text{cm}$) and expressed as μmol per g fresh weight.

Determination of water-soluble protein and chlorophyll contents

Water-soluble leaf proteins were extracted with 100 mM Tris-HCl (pH 8.0). Samples (1.0 g) were homogenized in a chilled mortar and pestle in 3 mL extraction buffer. The homogenate was centrifuged at

20000 × g at 5°C for 60 min, and then the supernatant was collected to measure total soluble protein contents. Protein contents were measured according to the method of Bradford (1976) with bovine serum albumin as a standard. Fresh leaf samples were cleaned with distilled water to remove surface contamination prior to chlorophyll extraction. Samples were prepared as 10 leaf discs (diameter 10 mm) from whole leaves, and weighed. The samples were incubated with 50 mL 80% acetone in the dark at room temperature for 72 h. After incubation, samples were centrifuged at 1500 × g for 5 min, and then the supernatant was collected. Absorbance was measured with a spectrophotometer (Du-650; Beckman, USA) at 663 and 645 nm. Chlorophyll contents were calculated using the equation proposed by Strain and Svec (1966).

Chl a (mg·mL⁻¹) = 11.64 × (A₆₆₃) – 2.16 × (A₆₄₅)

Chl b (mg·mL⁻¹) = 20.97 × (A₆₄₅) – 3.94 × (A₆₆₃)

Histochemical detection of H₂O₂ and superoxide anions

Hydrogen peroxide was detected using 3,3'-diaminobenzidine (DAB) as a substrate following the method of Schraudner et al. (1998). The leaves were vacuum-infiltrated with 0.1% (w/v) DAB in 10 mM 2-(N-morpholino) ethanesulphonic acid (MES, pH 6.5). Superoxide anions were detected using nitroblue tetrazolium (NBT) staining according to Jabs et al. (1996). The leaves were vacuum-infiltrated with 0.1% (w/v) NBT in 10 mM sodium azide and 50 mM potassium phosphate (pH 6.4). Infiltration was carried out by building up a vacuum with 93.36 kPa pressure for 5 min, and releasing it two or three times until the leaves were completely infiltrated. After infiltration, catalase (100 units/mL) or SOD (100 units/mL) were added to the infiltration buffer prior to DAB or NBT staining, respectively. Incubation was for 60 min under light for DAB or in the dark for NBT; leaves were destained with 90% (v/v) EtOH under boiling at 80°C and mounted in lactic acid, phenol, and distilled water (1 : 1 : 1, v/v/v).

Total leaf protein extraction

Total leaf proteins were extracted with 0.5 M sodium acetate (pH 5.2) containing 15 mM 2-mercaptoethanol. Samples were homogenized with polytron (PT-3100; Kinematica) in extraction buffer. The homogenate was centrifuged to remove insoluble materials at 20000 × g at 5°C for 60 min, and the supernatant was collected. The supernatant was precipitated from crude extracts by incubating in four volumes of cold acetone at –20°C overnight. The precipitate was collected by centrifugation at 15000 × g for 15 min, washed at least twice with cold 80% acetone, and dried in a speed vac. The protein pellets were dissolved in 30 mM sodium acetate (pH 5.2). The suspensions were centrifuged at 15000 × g for 15 min, and the supernatant collected. The crude extractants were passed through a PD-10 column

(Pharmacia-LKB, Sweden), which was previously equilibrated with 30 mM sodium acetate (pH 5.2). A total of 3.5 mL was collected from the column, and this protein fraction was used for enzyme activity and isozyme analysis. Protein concentrations were measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Activity of ascorbic peroxidase and glucose-6-phosphate dehydrogenase

Ascorbic peroxidase (APX, EC 1.11.1.11) specific activities were measured spectrophotometrically by monitoring the decrease in absorbance of the substrate at 290 nm as ascorbate (extinction coefficient = 2.8 mM⁻¹/cm) was oxidized (Chen and Asada, 1989). The reaction mixtures contained 100 mM potassium phosphate (pH 7.5), 0.5 mM ascorbate, 0.2 mM H₂O₂, and enzyme extracts. The reaction was initiated by adding 0.2 mM H₂O₂, and the absorbance decrease was recorded for 1 min at 25°C. Activities were expressed as μmol ascorbate oxidized per min per mg protein. Glucose-6-phosphate dehydrogenase (G6PDH, EC. 1.1.1.49) activity was measured spectrophotometrically by monitoring the formation of NADPH from the oxidation of glucose-6-phosphate (G6P) at 340 nm for 1 min at 25°C (Udvardy et al., 1986). The reaction mixtures consisted of 50 mM Tris-HCl (pH 7.8), 2.0 mM NADP⁺, 1.0 mM G6P, and enzyme extracts. The reaction was initiated by adding G6P, and absorbance was recorded for 1 min at 25°C. Activity was calculated using an extinction coefficient of 6.22 × 10³ M⁻¹·cm⁻¹ at 340 nm. Activities are defined as μmol NADPH oxidized per min per mg protein.

Isozyme analysis of antioxidant enzymes

Isozyme bands were separated electrophoretically in 10% polyacrylamide slab gel under native conditions. Samples were applied with 20 μg total proteins and run using Tris-glycine electrode buffer (pH 8.3) at 4°C for 5 h with a constant of 30 mA. After electrophoresis, the gels were stained for enzyme activities.

SOD isoforms were stained by equilibrating with 50 mM potassium phosphate (pH 7.8) containing 2.5 mM NBT in darkness at 30°C for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 30 mM riboflavin and 2.5 mM NBT in darkness for 30 min. Gels were then exposed to light with shaking until achromatic bands appeared. Hydrogen peroxidase (HPX) isoforms were stained by incubating the gels in 0.1 M sodium citrate (pH 5.0) containing 9.0 mM p-phenylenediamine, and 4.0 mM H₂O₂ in darkness at 30°C for 15 min. Guaiacol peroxidase (GPX) was stained by incubating the gels in 0.1 M potassium phosphate (pH 6.4) containing 20 mM guaiacol and 5.5 mM H₂O₂ in the dark at 30°C until blue bands appeared. Polyphenol oxidase (PPO) isoforms were stained by equilibrating with 100 mM sodium phosphate (pH 7.0) for 5 min, and

then incubating in 100 mM sodium phosphate (pH 7.0) containing 10 mM DL-3,4-dihydroxyphenylalanine on a shaker until dark bands appeared. G6PDH isoforms were stained by incubating the gels in 100 mM Tris-HCl (pH 7.5) containing 1 M $MgCl_2$, 20 mM $NADP^+$, 25 mM NBT, 13 mM PMS, and 140 mM G6P in darkness at 30°C until achromatic bands appeared.

Results

Electrolyte leakage was abruptly increased by heat stress in cucumber and tomato leaves (Fig. 1), however, these increasing patterns were decreased by H_2O_2 pre-treatment in both seedlings before exposure to heat stress. Electrolyte leakage was increased to 51.1% and 40.2% by heat stress compared with 16.9% and 14.6% in the control cucumber and tomato leaves, respectively. Although electrolyte leakage was higher 32.2% and 28.9% in the H_2O_2 pre-treated cucumber and tomato

leaves compared with control, it was reduced to 18.9% and 11.3% by H_2O_2 pre-treatment before exposure to heat stress compared with heat-stressed leaves. The concentration of MDA in cucumber and tomato leaves was increased 2.3 and 2.6 times by heat stress compared with 1.09 and 1.51 μmol per g fresh weight of control leaves, respectively; however, MDA levels in the leaves of H_2O_2 pre-treated cucumber and tomato seedlings were reduced to 30.2% and 23.5% of heat-stressed seedlings, respectively.

The contents of water-soluble protein and total chlorophyll were significantly decreased by heat stress in cucumber and tomato leaves (Fig. 2). The levels of water-soluble protein were decreased to 31.0% and 40.2% by heat stress compared with 7.28 mg and 4.98 mg per g fresh weight in control cucumber and tomato leaves, respectively. The levels of total chlorophyll were also decreased to 20.7% and 22.0% by heat stress

Fig. 1. Percentage of electrolyte leakage and concentration of malondialdehyde (MDA) in the leaves of cucumber and tomato seedlings. Seedlings were grown in a growth chamber set at 23/20°C (day/night), 12 h photoperiod, and light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and RH 80 $\pm$ 5%. When seedlings had three expanded leaves, they were exposed to 23/20°C (day/night, control) and 40/28°C (day/night, heat) for 3 days. A third batch of seedlings was pretreated with 15 mM H_2O_2 before exposure to heat stress, and then exposed to 40/28°C (day/night, H_2O_2 + heat) for 3 days. Data are the means of six replicates; bars indicate standard error of the means. Means in each bar followed by different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD test.

Fig. 2. Content of water-soluble protein and total chlorophyll in the leaves of cucumber and tomato seedlings. Seedlings were grown in a growth chamber set at 23/20°C (day/night), 12 h photoperiod, and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and RH 80 $\pm$ 5%. When seedlings had three expanded leaves, they were exposed to 23/20°C (day/night, control) and 40/28°C (day/night, heat) for 3 days. A third batch of seedlings was pretreated with 15 mM H_2O_2 before exposure to heat stress, and then exposed to 40/28°C (day/night, H_2O_2 + heat) for 3 days. Data are the means of six replicates; bars indicate standard error of the means. Means in each bar followed by different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD test.

compared with 0.769 mg and 1.220 mg per g fresh weight in the control cucumber and tomato leaves, respectively; however, these decreasing levels in water-soluble protein and total chlorophyll were reduced by H_2O_2 pre-treatment in both seedlings before exposure to heat stress (Fig. 2). The levels of water-soluble protein were 20.9% and 31.5% higher with H_2O_2 pre-treatment before exposure to heat stress compared with 5.03 mg and 2.98 mg per g fresh weight in heat-stressed cucumber and tomato leaves, respectively. The levels of total chlorophyll were also 6.4% and 13.8% higher with H_2O_2 pre-treatment before exposure to heat stress compared with heat-stressed cucumber and tomato leaves, respectively.

The levels of H_2O_2 and superoxide anion in the leaves of cucumber and tomato seedlings were detected by histochemical methods using DAB and NBT staining systems (Fig. 3). The generation of H_2O_2 and superoxide anion, expressed by dark brown and blue colors, was abruptly increased by heat stress compared with control cucumber and tomato seedlings. The levels of H_2O_2 and superoxide anion generated by heat stress were reduced by H_2O_2 pre-treatment before exposure to heat stress.

The specific activities of APX and G6PDH in the leaves of cucumber and tomato seedlings were significantly increased by heat stress (Fig. 4). APX activities in the leaves of cucumber and tomato seedlings were increased to 142% and 130% by heat stress compared with 8.14 and 4.48 μmol ascorbate oxidized

per min per mg protein in the leaves of control seedlings, respectively. Although APX activities in the leaves of H_2O_2 -treated cucumber and tomato seedlings were lower than those of heat-stressed seedlings, its activities were also significantly increased to 72% and 108% by foliar application of H_2O_2 compared with control seedlings. The specific activities of G6PDH were increased to 1.7 and 1.8 times by heat stress compared with 0.227 and 0.146 μmol NADPH oxidized per min per mg protein of control cucumber and tomato seedlings, respectively. The specific activities of G6PDH were also increased to 0.337 and 0.276 μmol NADPH oxidized per min per mg protein by foliar application of H_2O_2 . The levels of G6PDH in the leaves of H_2O_2 -treated tomato seedlings were higher than those of heat-stressed seedlings.

The isozyme banding patterns of antioxidant enzymes in the leaves of cucumber and tomato seedlings are shown in Figure 5. When the gels were stained for SOD isoforms, the isozyme banding patterns of SOD detected the presence of five and four isoforms in the leaves of cucumber and tomato seedlings, respectively. The densities of four SOD (SOD-1, SOD-2, SOD-3, and SOD-4) isoforms in cucumber leaves and three SOD (SOD-1, SOD-3, and SOD-4) isoforms in tomato leaves were abruptly increased by foliar application of H_2O_2 , and the activities persisted until 10 days after application; however, specific responses in tomato leaves detected the suppression of SOD-2 bands by foliar application of H_2O_2 compared with control seedlings. The isozyme

Fig. 3. Histochemical detection of H_2O_2 and superoxide anions in the leaves of cucumber and tomato seedlings. Seedlings were exposed to 23/20°C (day/night, control) and 40/28°C (day/night, heat) for 3 days. A third batch of seedlings was pretreated with 15 mM H_2O_2 before exposure to heat stress, and then exposed to 40/28°C (day/night, H_2O_2 + heat) for 3 days. Leaves were vacuum infiltrated with 0.1% (w/v) DAB in 10 mM MES (pH 6.5) or 0.1% (w/v) NBT in 0.1% (w/v) 10 mM sodium azide and 50 mM potassium phosphate (pH 6.4). After infiltration, catalase or SOD was added to the infiltration buffer prior to DAB or NBT staining, and then incubated for 60 min under light for DAB or in the dark for NBT. To visualize the accumulation of H_2O_2 and superoxide anions, leaves were destained with 90% (v/v) EtOH under boiling at 80°C, and mounted in lactic acid, phenol, and distilled water (1 : 1 : 1, v/v/v).

Fig. 4. Specific activities of ascorbic peroxidase (APX) and glucose-6-phosphate dehydrogenase (G6PDH) in the leaves of cucumber and tomato seedlings. Proteins were extracted from leaves exposed to 23/20°C (day/night) without H₂O₂ pre-treatment (control) or with H₂O₂ pre-treatment (H₂O₂) and 40/28°C (day/night, heat) for 3 days. APX activities are defined as μmol ascorbate oxidized per min per mg protein. G6PDH activities are defined as μmol NADPH oxidized per min per mg protein. Data are the means of six replicates; bars indicate standard error of the means. Means in each bar followed by different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD test.

Fig. 5. Changes in gel activities of superoxide dismutase (SOD), hydrogen peroxidase (HPX), guaiacol peroxidase (GPX), and polyphenol oxidase (PPO) in the leaves of cucumber and tomato seedlings. Proteins were extracted from leaves sprayed with distilled water as a control and 15 mM H₂O₂ containing 0.01% Tween 20 as a surfactant. Electrophoresis was conducted using a 10% polyacrylamide slab gel under native conditions.

banding patterns of HPX detected the presence of four and eight isoforms in cucumber and tomato leaves, respectively. In cucumber leaves with foliar application of H₂O₂, three isozyme bands (HPX-1, HPX-2, and HPX-3) markedly increased their activities and these

were maintained until 10 d after application. Although four isozyme bands (HPX-3, HPX-6, HPX-7, and HPX-8) in tomato leaves also had increased activities with foliar application of H₂O₂, two isozyme bands (HPX-4 and HPX-5) were suppressed in comparison with control

seedlings. Differential responses to foliar application of H_2O_2 in guaiacol peroxidase (GPX) were observed between cucumber and tomato leaves. In cucumber leaves, the isozyme banding patterns of GPX detected three isoforms, and their densities abruptly increased with foliar application of H_2O_2 compared with control seedlings; however, the gel activities of GPX in control tomato leaves and those sprayed with H_2O_2 did not differ. The isozyme banding patterns of PPO detected homologous isoforms as HPX with four and eight isoforms in the leaves of cucumber and tomato seedlings, respectively. The gel activities of PPO isozymes, by band density, abruptly increased after foliar application of H_2O_2 and were maintained until 10 days in cucumber and tomato leaves. In tomato leaves, two isozyme bands were also suppressed by foliar application of H_2O_2 .

Discussion

Our results show that H_2O_2 pre-application is an effective method to lessen the impact of heat stress. High temperature is a major environmental stress affecting the growth and productivity of fruit vegetable crops grown in protected systems in summer seasons. It induces oxidative stress, which can lead to the inhibition of photosynthesis (Karim et al., 1999), disruption of cell membranes (Marcum, 1998; Gulen and Eris, 2004), hormonal changes (Maestri et al., 2002), and reduced water availability (Simoes-Araujo et al., 2003). The oxidative stress induced by ROS due to environmental stresses, including high temperature, is the major cause of injury in plants (Asada, 1999; Bowler et al., 1992). ROS, such as hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen, are present in all plants as cytotoxic compounds and are mediators of the stress response (Foyer, 1993; Lee and Lee, 2000). Our study showed a significant increase in electrolyte leakage and MDA content in cucumber and tomato leaves following heat stress (Fig. 1). The contents of water-soluble protein and total chlorophyll were significantly decreased by heat stress in both seedlings (Fig. 2). We also determined that oxidative bursts such as those of H_2O_2 and superoxide anion are significantly generated by heat stress (Fig. 3). These results mean that cell membranes are disrupted by oxidative bursts generated by heat stress. One of the physiological changes under heat stress is lipid peroxidation, which disrupts cellular membranes and breaks down cell compartmentalization (Blum and Ebercon, 1981; Liu and Huang, 2000). When lipid peroxidation occurs in a plant cell, electrolytes leak because of increased membrane permeability. Levels of MDA, a decomposition product of unsaturated fatty acids in phospholipids, were significantly increased during lipid peroxidation (Jiang and Huang, 2001). The levels of electrolyte leakage and MDA content are effective signs that reflect cell membrane thermostability and are often used as an index of oxidative damage to cell membranes under environmental stress (Lester, 1985;

Xu et al., 2006). In our study, increasing levels of electrolyte leakage and MDA content in the leaves of heat-stressed cucumber and tomato seedlings were reduced by H_2O_2 pre-application before heat stress (Fig. 1). On the other hand, decreasing levels of water-soluble protein and total chlorophyll content by heat stress were reduced by H_2O_2 pre-treatment before exposure to heat stress in both seedlings (Fig. 2). The accumulation of ROS was also reduced by H_2O_2 pre-application before exposure to heat stress (Fig. 3). These results suggest that exogenously applied H_2O_2 plays a critical role in minimizing injury brought about by heat stress. Several studies have reported that exogenously applied H_2O_2 can induce acclimation to environmental stresses such as chilling (Prasad et al., 1994), salt (Uchida et al., 2002) and high temperature (Lopez-Delgado et al., 1998). They concluded that H_2O_2 pre-application achieves earlier activation of antioxidant enzymes to alleviate toxic ROS generated by environmental stresses.

To understand the role of exogenously applied H_2O_2 in inducing thermotolerance in terms of antioxidant enzymes, we measured the specific and gel activities of antioxidant enzymes. It has been well documented that ROS, such as hydrogen peroxide, superoxide, and hydroxyl radicals are generated in plant tissues when plants are subjected to environmental stresses, including high temperature (Liu and Huang, 2000; Zhang and Kirkham, 1994). ROS overgenerated by environmental stresses can cause damage to cellular components such as cell membranes, protein, nucleic acids, disrupting metabolic functions and leading to injury or death (Fridovich, 1991; Hernandez et al., 1995); however, fortunately, plants have complex antioxidant systems consisting of enzymatic and non-enzymatic scavenging systems to alleviate toxic ROS-induced injury. The toxic cascades induced by ROS are efficiently prevented by detoxification of antioxidant enzymes (Davies, 1995). We observed that the activities of APX and G6PDH were significantly increased by heat stress in cucumber and tomato leaves (Fig. 4). Increasing patterns of APX and G6PDH enzymes were also detected with foliar application of H_2O_2 in both crops. APX is a specific peroxidase that catalyzes the detoxification of the toxic product H_2O_2 using ascorbate as the electron donor (Asada, 1992). Hausladen and Alscher (1993) reported that ROS in plants is detoxified by the ascorbate-glutathione cycle, which requires reduced coenzyme NADPH. Kuo and Tang (1998) suggested that the reduced coenzyme NADPH is required for ROS detoxification. The cellular source of NADPH is a dehydrogenase in the pentose phosphate pathway, such as G6PDH and 6PGDH. G6PDH is a rate-limiting enzyme that catalyzes the first reaction of the pentose phosphate pathway, which provides precursors for phenolic secondary metabolite synthesis (Kletzien et al., 1994; Leng and Qi, 2003). Our results suggest that activation of G6PDH by foliar application of H_2O_2 could

contribute by providing more NADPH, and could then participate in the activation of antioxidant enzymes against ROS.

We also determined the gel activities of antioxidant enzymes and the duration of activities by foliar application of H_2O_2 . Our results showed clear differences in gel activities of antioxidant enzymes between leaves sprayed with H_2O_2 and those given distilled water (Fig. 5). In cucumber leaves, the density of major isoforms in SOD, HPX, GPX, and PPO isozymes was abruptly increased by foliar application of H_2O_2 , and was maintained until 10 days after application. The density of major isoforms in SOD, HPX, and PPO isozymes in tomato leaves was likewise increased and maintained for 10 days; however, the responses of GPX to exogenously applied H_2O_2 were shown to have different patterns in cucumber and tomato leaves; activities were abruptly increased in cucumber leaves sprayed with H_2O_2 , but this was not observed in the counterpart tomato seedlings. On the other hand, a differential response to exogenously applied H_2O_2 in cucumber and tomato seedlings was detected through the expression patterns of specific isoforms. In tomato leaves, one or two isoforms of SOD, HPX, and PPO isozymes were suppressed by foliar application of H_2O_2 . SODs are enzymes containing metals that convert superoxide anion into H_2O_2 and oxygen by dismutation. Bowler et al. (1992) demonstrated that SOD plays a critical role in protecting cellular molecules from damage caused by ROS in plants. Toxic H_2O_2 produced by dismutation of SOD is reduced to H_2O by peroxidase or catalase, which is the key enzyme involved in hydrogen peroxide scavenging (Anderson et al., 1995; Salin, 1991). Peroxidases are heme-containing oxidoreductases that participate in a number of metabolic processes, such as regulation of cell elongation (Andrews et al., 2002), lignification (Whetten et al., 1998), cross-linking of cell wall structural proteins (Brisson et al., 1994; Fry, 1986) and phenolic oxidation (Chittoor et al., 1999). Peroxidases are classified into HPX, GPX, and APX on the basis of the electron donor (Asada, 1992). Our results revealed that the gel activity of HPX was abruptly increased by foliar application of H_2O_2 in cucumber and tomato leaves; however, the gel activity of GPX showed differential activation. Prasad et al. (1994) reported that exogenously applied H_2O_2 can induce chilling tolerance by applying before exposure to stress. This was partly due to enhanced antioxidant enzymes by exogenously applied H_2O_2 as cofactors. Brisson et al. (1994) reported that H_2O_2 is independently involved in the modification of cell walls by catalyzing cross-linking polymers by peroxidase. PPOs are widely distributed in plants and are involved in the oxidation of polyphenols into quinones using molecular oxygen as an electron acceptor and lignification of plant cells during microbial infection (Chittoor et al., 1999). They play an important role in oxygen scavenging and

providing defense against environmental stress (Shivishankar, 1988; Thipyapong et al., 2004). Our results showed PPO gel activity abruptly increasing following foliar application of H_2O_2 and multiple isoforms of PPO in cucumber and tomato leaves (Fig. 5), as in other plant species (Sommer et al., 1994). Although we cannot determine the role of PPO in thermotolerance, its earlier activation by foliar application of H_2O_2 may enhance thermotolerance when seedlings are subjected to heat stress. In our study, exogenously applied H_2O_2 seems to act as a signal factor for activating antioxidant enzymes and reduces the deleterious effects of ROS to a minimum when seedlings are exposed to heat stress.

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