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Changes in Antioxidant Enzyme Activities in Detached Leaves of Cucumber Exposed to Chilling

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We studied changes in biochemical and physiological status, level of oxidative damage, and antioxidant enzyme activities in detached leaves of cucumber plants (*Cucumis sativus* L. cv. Pyunggangnaebyungsamchuk) that were exposed to a low temperature (4°C). Chlorophyll fluorescence (F_v/F_m) declined during the chilling treatment, but was slowly restored after the tissues were returned to 25°C. Likewise, the fluorescence quenching coefficient and relative water content decreased during the stress period, but then increased during recovery. In contrast, we detected a significant rise in protein and hydrogen peroxide contents in the chilled leaves, as well as higher activities for superoxide dismutase, ascorbate peroxidase, peroxidase, and glutathione reductase. However, the level of catalase decreased not only during chilling but also after 24 h of recovery. These results indicate that exposure to low temperatures acts as an oxidative stress. Moreover, we propose that a regulating mechanism exists in the detached cucumber leaves and contains an antioxidant defense system that induces active oxygen species, thereby alleviating the effects of chilling stress within 12 h.

Keywords: antioxidant enzyme, chilling stress, chlorophyll fluorescence, *Cucumis sativus*, H_2O_2

To enhance their growth and metabolism, plants must maintain a balance between generating free radical oxygen species and utilizing their scavenging mechanisms. Increased their understanding of those reactive oxygen free radicals would help researchers define how cucumber plants adapt to chilling. At lower temperatures, oxidative stress is thought to be mediated by active oxygen species composed of superoxide (O_2^-), hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Wise and Naylor, 1987). These may act as both cytotoxic compounds and mediators to induce stress tolerance. Complex antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. Various tolerance mechanisms have been suggested, based on the biochemical and physiological changes related to chilling injury (Elstner, 1991; Aroca et al., 2001).

SOD (superoxide dismutase) is a group of metalloenzymes that catalyze the disproportionation of superoxide to H_2O_2 and O_2, thereby playing an important role in protecting against superoxide-derived oxidative stress in plant cells (Asada and Kiso, 1973; Fridovich, 1986). Cellular H_2O_2 is detoxified through the action of the Asada-Halliwell scavenging cycle, which is found in the chloroplast and cytosol. There, ascorbate and glutathione are oxidized and reduced via the activation of enzymes such as APX (ascorbate peroxidase) and GR (glutathione reductase) (Foyer et al., 1994). The former catalyzes the reaction of ascorbic acid with H_2O_2, while the latter is involved in the regeneration of ascorbic acid (Jiang and Zhang, 2001). Catalase (CAT) can also reduce H_2O_2 to water, but it has a very low affinity for H_2O_2 compared with APX (Graham and Patterson, 1982). SOD and APX isoforms are specific to the chloroplast and cytosol (Scandalios, 1993; Asada, 1994), whereas those of GR are localized to the chloroplast, cytosol, and mitochondria (Madamanchi et al., 1992).

Chlorophyll fluorescence is an excellent tool for studying *in vivo* changes to the photosynthetic apparatus when plants are exposed to various environmental stresses (Lee et al., 1990). This approach is frequently used for determining the influence of such factors as temperature, salinity, and light because it is easy to monitor the function of the photosynthetic apparatus, including changes in the carbon reduction cycle (Schreiber et al., 1986).

The objective in this study was to clarify the protec-

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tive mechanism of antioxidant enzymes against chilling stress in cucumber. Specifically, we investigated changes in H_2O_2 content, quantum yields in chlorophyll fluorescence, and fluctuations in the activation of various antioxidant enzymes.

MATERIALS AND METHODS

Plant Materials and Experimental Conditions

Seeds of cucumber (*C. sativus* L. cv. Pyunggangnaebyungsamchuk) were germinated in the dark at 25°C for 5 d in a Petri dish containing Whatman No. 2 filter paper and distilled water. The germinants were then planted in a pot filled with commercial soil, and reared in a growth chamber for 20 d. Environmental conditions were 70% humidity, 25°C, and a light intensity of 200 μmol m^{-2} sec^{-1}, with a 14-h photoperiod. For the chilling treatment, 25-d-old plants were transferred to a cold chamber at 4°C under 12 h of continuous illumination (light intensity of 50 μmol m^{-2} sec^{-1}). Afterward, those plants were returned to the previous growth chamber and held at 25°C for 24 h. Secondary leaves subjected to chilling or the post-stress period were used as the experimental materials. The controls were secondary leaves of 25-d-old plants that had not undergone these chilling and recovery treatments. All experiments were independently repeated at least three times.

Calculation of Chlorophyll Fluorescence

Emission of chlorophyll *a* fluorescence from the upper leaf surface was routinely monitored under light, using a Plant Efficiency Analyzer (Hansatech, UK) and a PAM Chlorophyll Fluorometer (Walz; Effeltrich, Germany). The initial level (F_o) of fluorescence was elicited by a weak red light (655 nm, 1 μmol m^{-2} s^{-1}, modulated at 1.6 KHz), and measured with a photodiode at a wavelength >700 nm. Maximal fluorescence (F_m) was induced by a 1-s pulse of white light (4000 μmol m^{-2} s^{-1}). The maximum variable fluorescence (F_v) was calculated as the difference between F_m and F_o at a specific time. Quenching parameters were calculated according to the equations of Oxborough and Horton (1988):

$$qQ = [(Fv)s - Fv]/(Fv)s$$
$$qNP = [(Fv)m - (Fv)s]/(Fv)m$$
$$qE = [(Fv)E - (Fv)s]/(Fv)E$$
$$qR = [(Fv)m - (Fv)E]/(Fv)m$$

Measurement of Relative Water Content

Leaf relative water content (RWC) was estimated by first recording the turgid weight of 1 g of fresh leaf samples that were hashed from the second leaves. These tissues were kept in water for 24 h, then oven-dried until a constant weight was achieved for 48 h. RWC was calculated according to the following: RWC (%) = (fresh weight – dry weight)×100/(saturated weight dry weight) (Whetherley, 1950).

Measurement of H_2O_2 Content

For the assay of H_2O_2 content, 1 g of leaves was homogenized in 3 mL of 100 mM sodium phosphate buffer (pH 6.8). To remove cellular debris, the homogenate was filtered through four layers of cheesecloth, then centrifuged at 18,000*g* for 20 min at 4°C. After the supernatant was collected, H_2O_2 content was measured according to the modified method of Bernt and Bergmeyer (1974), using peroxidase enzyme. To initiate the enzyme reaction, an aliquot of 0.5 mL of supernatant was mixed with 2.5 mL of peroxide reagent that consisted of 83 mM sodium phosphate (pH 7.0), 0.005% (w/v) *o*-dianisidine, and 40 μg peroxidase mL^{-1}, and was incubated for 10 min at 30°C in a water bath. The reaction was stopped by adding 0.5 mL of 1 N perchloric acid, followed by centrifuging at 5000*g* for 5 min. The resultant supernatant was read at 436 nm; its absorbance was compared to the extinction of an H_2O_2 standard.

Preparation of Enzyme Extracts and Protein Content Determination

To determine the activities of the antioxidant enzymes, 1 g leaf samples were homogenized in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP), and 0.5% (v/v) Triton X-100 at 4°C. An exception was made for APX, in which case the leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. Homogenates were filtered through four layers of cheesecloth and centrifuged at 18,000*g* for 20 min at 4°C. The resultant supernatants were then collected and stored at -80°C. Protein content was measured according to the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

Enzyme Assay

Catalase activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient: 39.4 mM cm^{-1}) at 240 nm, as per the method of Aebi (1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and plant extract in a 3-mL volume. This reaction was initiated by adding 10 mM H_2O_2. One unit of catalase was defined as the amount of enzyme that liberated half the peroxide oxygen from a 10 mM H_2O_2 solution in 100 s at 25°C. Peroxidase activity was measured by monitoring the formation of the guaiacol dehydrogenation product (extinction coefficient: 6.39 mM cm^{-1}) at 436 nm, per the method of Pütter (1974). The reaction mixture (3.18 mL) comprised 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol, and plant extract; the reaction was initiated by adding 0.1 mM H_2O_2. One unit of peroxidase specific to guaiacol was defined as the oxidation of 1.0 mmol of guaiacol from 0.3 mM guaiacol and 0.1 mM H_2O_2 per min at 25°C at pH 7.0.

SOD activity was determined by the method of Beyer and Fridovich (1987). Here, the 30.25 mL reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 µM nitroblue tetrazolium (NBT), and the appropriate volume of plant extract. The reaction was initiated by illumination. One unit of SOD was defined as the amount of enzyme that caused a 50% decline in SOD-inhibitable NBT reduction. APX activity was measured by following the decrease in absorbance at 290 nm (extinction coefficient: 2.8 mM cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2, and a suitable volume of the enzyme extract (Chen and Asada, 1989). Finally, GR activity was determined according to the oxidation of NADPH at 340 nm (extinction coefficient: 6.2 mM cm^{-1}), as described by Rao et al. (1996). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG), and the appropriate volume of enzyme extract in a 1 mL volume. This reaction was initiated by the addition of NADPH at 25°C.

RESULTS

Photosynthetic Efficiency Measured with Quantum Yields in Chlorophyll Fluorescence

Leaves of cucumber plants were exposed to 4 °C to determine their photosynthetic responses to chilling stress as well as their ability to recover after refreshing. F_v/F_m (variable yield of fluorescence / maximum yield of fluorescence) is generally used as an indicator of the photochemical efficiency of PSII. In our study, the level of F_v/F_m was dramatically lower after 12 h of chilling treatment (Fig. 1), indicating that this stress induced significant inhibition of photosynthetic efficiency. That response may have resulted from a physical dissociation of the light-harvesting complex from the PSII core. After 24 h of post-stress recovery, F_v/F_m was restored to almost the same value as for the control plants. The oxidation state of Q_A (i.e., the primary quinone acceptor of PSII), as monitored in terms of qQ (photochemical quenching), markedly declined in leaves chilled for 12 h (Fig. 2). This demonstrated that both qNP (non-photochemical quenching) and qE (energy-dependent quenching) were nearly lost. Nevertheless, PSII capacity regained its potential quantum yield during the 24-h recovery period.

Changes in Relative Water Content and Protein and H_2O_2 Contents

RWC initially decreased during 12 h of chilling, then rose after 24 h of post-stress recovery. In contrast, protein content significantly increased during the stress treatment (Fig. 3). After 24 h of recovery, however, protein content returned to values similar to those measured in the control plants. This chilling-induced increase may be partially explained by the decrease in RWC in the stressed plants (Fig. 3). Chill-

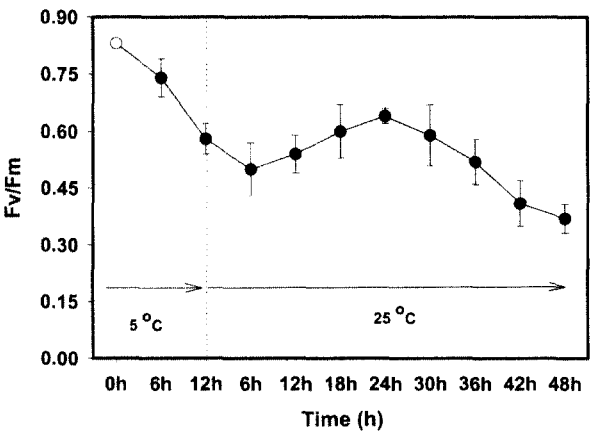

Figure 1. Changes in F_v/F_m of detached cucumber leaves. Samples were exposed to 4°C in the light (50 µmol·m^{-2}·s^{-1}), then pre-treated in the dark for 20 min at 25°C before chlorophyll fluorescence was induced. Circles represent mean ± SE of three leaves.

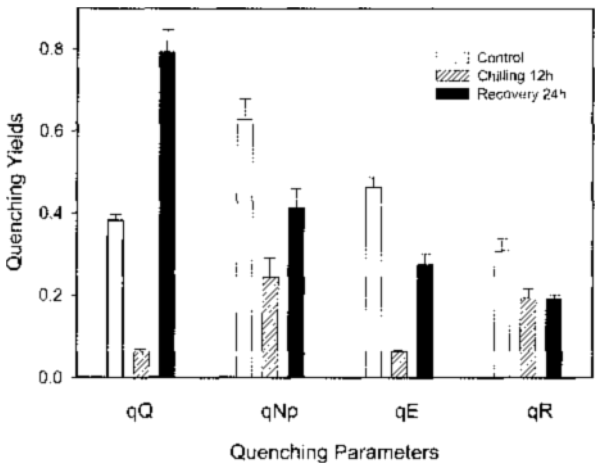

Figure 2. Changes in chlorophyll fluorescence quenching parameters qQ, qNP, qE, and qR in detached cucumber leaves. Samples were exposed to 4°C in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$), then pre-treated in the dark for 20 min at 25°C before chlorophyll fluorescence was induced. Data represent averages of at least three independent experiments.

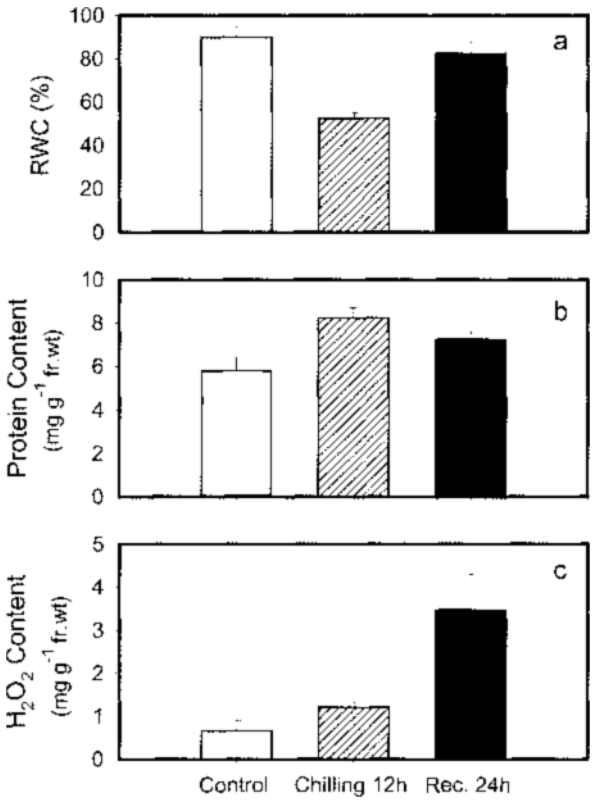

Figure 3. Changes in RWC (**a**), protein content (**b**), and H_2O_2 content (**c**) for detached leaves of cucumber exposed to 4°C or 25°C in the light. Untreated tissues (control), leaves chilled for 12 h (Chilling 12h), and leaves after 24-h recovery following chilling (Rec. 24h).

ing also caused a marked rise in the amount of H_2O_2, a level that continued to rise during the recovery period. This post-stress increase in H_2O_2 content may have been associated with visible injury symptoms, such as leaf yellowing and senescence, which began after 24 h of post-stress restoration.

Changes in Activation of Antioxidant Enzymes

Activities of catalase and peroxidase (POD) were monitored during the 12-h chilling treatment as well as for 24 h following the stress (Fig. 4). The CAT level in chilled tissue was lower than for the control; its activity remained depressed after 24 h of recovery. Peroxidases are known to utilize different substrates to metabolize H_2O_2. For example, when guaiacol was used here, peroxidase activity was enhanced in the chilling-stressed plants compared with the controls. After 24 h of recovery, the level of catalase activity was lower than the level at chilling stress. In contrast, peroxidases that comprise multiple isozymes require H_2O_2 as an essential substrate. When we used guaiacol as a substrate, POD activity was markedly increased, not only during chilling stress but also after 24 h of

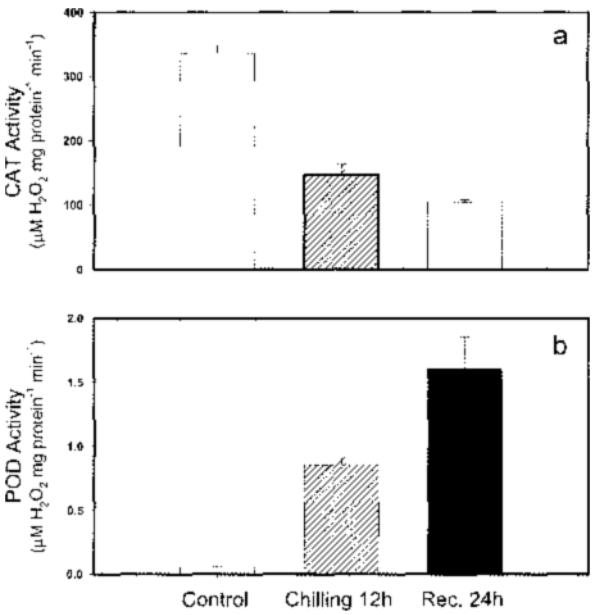

Figure 4. Activities of CAT (**a**) and POD (**b**) specific to guaiacol in detached leaves of cucumber exposed to chilling. Soluble proteins were isolated from untreated tissues (control), leaves chilled for 12 h (Chilling 12h), and leaves after 24-h recovery following chilling (Rec. 24h). CAT activity was determined by monitoring decomposition of H_2O_2 at 240 nm; POD activity, by monitoring formation of guaiacol dehydrogenation product at 436 nm.

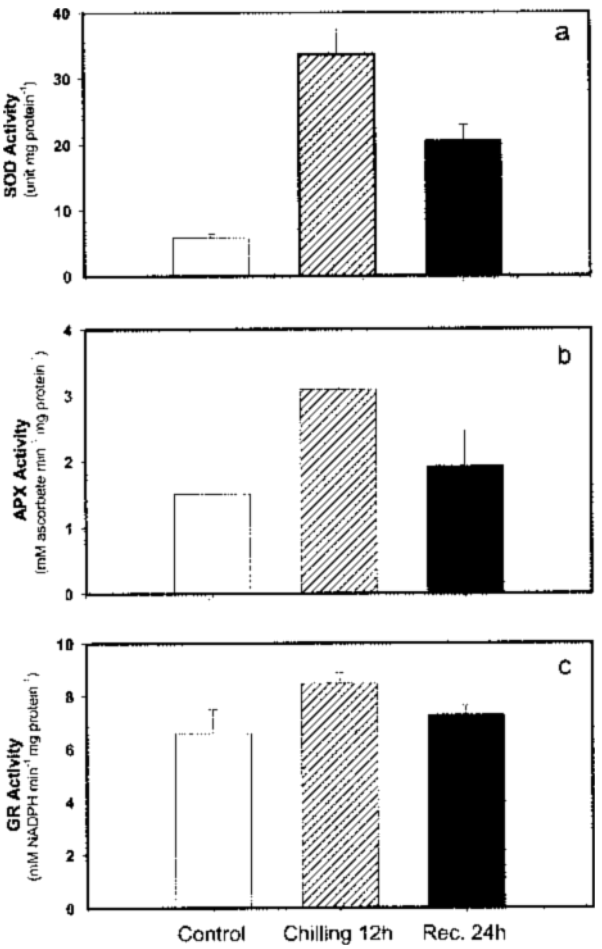

Figure 5. SOD (**a**), APX (**b**), and GR (**c**) activities in detached leaves of cucumber exposed to chilling. Soluble proteins were isolated from untreated tissues (control), leaves chilled for 12 h (Chilling 12h), and leaves after 24-h recovery following chilling (Rec. 24h). One unit of SOD was defined as amount of enzyme causing a 50% decrease in SOD-inhibitable NBT reduction. APX activity was determined by monitoring oxidation of 1 μM ascorbate at 290 nm; GR activity, by monitoring oxidation of NADPH at 340 nm.

recovery (Fig. 5). Compared with the control, chilling induced a significant increase in total SOD activity, whereas after 24 h of post-stress restoration, its activity was slightly decreased (Fig. 5a). This pattern of change in SOD activity during recovery differed from that seen with H_2O_2 content (Fig. 3).

Few reports have been made on the metabolic role of APX and other antioxidant enzymes in H_2O_2 scavenging. Thus, we examined the chilling-stressed cucumber leaves and found that APX activity was enhanced over that measured in our control plants (Fig. 5b). After 24 h of post-stress recovery, this level was even higher than during the chilling period. Although APX plays an important role in converting H_2O_2 to water, GR is also an essential catalase in this conversion in order to maintain the redox state of ascorbate and glutathione (Foyer et al., 1994). Because the potential for APX to metabolize H_2O_2 depends on the redox state of such compounds, we also monitored GR activity and found that its level was increased by chilling stress compared with performance by the control (Fig. 5c). However, following 24 h of post-stress recovery, GR activity did not change significantly.

DISCUSSION

The decreases in F_v/F_m (Fig. 1) measured here reflect a depression in the potential quantum yield of photosystem II, which results from an increase in F_o and/or a decrease in F_v. Higher calculations for F_o may possibly be the result of a decrease in the probability of energy transfer from the antenna chlorophyll to the reaction center (Chun et al., 1996). In contrast, measured decreases in F_v seem to be attributable either to the limitation on electron donation to PSII (which is induced by the accumulation of stably reduced Q_A by the PSII reaction centers), or to a partial dissociation of the antennae from the centers (Laasch, 1987; Vass et al., 1992).

The severe decline in qQ (Fig. 2) during 12 h of chilling resulted in unstable Q_A. Moreover, this stress induced a significant decrease in the coefficients of non-photochemical quenching (qNP) (Fig. 2). The coefficients for energy-dependent quenching seemed to occupy most portions of qNP, changing in a parallel fashion with those of qNP following the chilling treatment. These results indicate that, for qNP formation, qE is dominant among factors such as state transitions and photoinhibition (Krause and Weis, 1991). As an important component of energy dissipation at PSII, qE is triggered by the enhancement of proton concentrations in the lumen of the thylakoid. It has also been proposed that qE is critical in protecting the photosynthetic apparatus that is exposed to excessive light (Krause and Behrend, 1986). Therefore, the rapid decline in qE and qNP observed in our stressed cucumber leaves may have resulted from a collapse in the buildup of protons because of either limited electron transport or the chilling stress-stimulated disassembly of the thylakoid membrane components. Demmig-Adams et al. (1989) have suggested that qE depends on the conversion of violaxanthin into zeaxanthin in the xanthophyll cycle. Our findings here also indicate that cucumber leaves are sensitive to

low temperature as it pertains to the weakening of photosynthetic functions.

Dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell. The discrepancy between H_2O_2 content and total SOD activity after 24 h of post-stress recovery indicates that overproduction of H_2O_2 could be due to a reduction of superoxide by SOD, ascorbate, thiols, ferredoxin, and Mn ions, or even by self-dismutation (Graham and Patterson, 1982; Aroca et al., 2001). The enhancement of H_2O_2 levels by chilling would, therefore, be alleviated through the combined activity of catalase and APX. Otter and Polle (1994) have suggested that anionic peroxidases, which are known to utilize phenolic compounds as substrate, play a central role in synthesizing secondary metabolites such as lignin. Therefore, further studies are necessary to clarify the role of peroxidase specific to coniferyl alcohol on the lignification of chilled plants. GR activity was also enhanced in the stressed plants, and increased again after the 24-h recovery period (Fig. 5). Edwards et al. (1994) have also suggested that the rise in total GR activity in cold-stressed peas appears to be due to changes in the isoform population.

In summary, both F_v/F_m and the quenching coefficient for cucumber leaves are decreased by 12 h of exposure to low temperatures, but then increase during recovery at 25°C. The accumulation of H_2O_2 during such chilling periods leads to increased activity by several enzymes, e.g., peroxidase, APX, and GR, for catalase deactivation. However, levels of CAT activity decline not only during chilling stress but also in the 24-h post-stress period, while POD activity is markedly increased. The metabolism of active oxygen species such as H_2O_2 depends upon various functionally interrelated antioxidant enzymes, such as CAT, POD, SOD, APX, and GR. Our results indicate that chilling acts as an oxidative stress. Therefore, we believe a regulating mechanism exists within detached cucumber leaves, which alleviates this stress within 12 h by operating an antioxidant defense system against those active oxygen species. However, it is unclear whether the responses of the antioxidant enzyme system against stress-induced, excessive H_2O_2 levels may offer a tolerance or cytotoxicity.

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