Impact of Salicylic Acid on the Antioxidant Enzyme System and Hydrogen Peroxide Production in *Cucumis sativus* under Chilling Stress

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Salicylic acid (SA) is a naturally produced compound and has been implicated to play important roles in defense of plants against diverse biotic and abiotic stresses. To understand how SA functions in the tolerance of cucumber (*Cucumis sativus*) to chilling stress, endogenous SA levels in two different cultivars with opposite chilling responsiveness were quantified. Membrane integrity, including malondialdehyde (MDA) content and leakage of electrolyte, was also examined in SA-pretreated cucumber plants under chilling conditions. In addition, activities of the two antioxidant enzymes peroxidase (POD) and catalase (CAT) were quantified, and hydrogen peroxide (H_2O_2) production was investigated histochemically in SA-treated leaves under chilling temperature. Chilling stress resulted in greater induction of SA levels in the chilling-tolerant cultivar Changchun mici in both leaves and seeds compared to the chilling-sensitive one Beijing jietou, while the former one contained higher levels of SA than the latter one in the seeds under normal conditions. Pretreatment with SA diminished the increased electrolyte leakage and MDA content caused by chilling in the leaves of both cultivars, while much less MDA and electrolyte leakage were produced in Changchun mici compared to Beijing jietou. Moreover, exogenous application of SA increased significantly the POD and CAT activities and soluble protein content. Most importantly, exogenous SA treatment could eliminate the accumulation of H_2O_2 in leaves and cotyledons of both cultivars caused by chilling stress. The data clearly demonstrated that the chilling-tolerant cultivar displays a higher SA level than the chilling-sensitive one, and that exogenous SA can enhance the chilling tolerance ability, which might be achieved through modulating the antioxidant system in cucumber.

Key words: Chilling Stress, *Cucumis sativus*, Salicylic Acid

Introduction

Plants constantly encounter a plethora of abiotic and biotic stresses in the natural environment, including drought, salinity, cold, freezing, heavy metals, mechanical wounding, as well as insects and pathogen attack (Fujita *et al.*, 2006). Such stresses usually cause severe damage to plants hence result in considerable loss of yield. To adapt to changes of the environmental factors, plants integrate a multitude of diverse environmental signals to respond properly under certain environmental conditions, achieved by many efficient defense mechanisms, including changes of a series of physiological features, defense gene expression, and modulation of phytohormone levels.

One of the well-known phytohormones, salicylic acid (SA), is a phenolic compound that has been shown to influence many plant physiological processes, including flower induction, sexual differentiation, garlic bulb expension (Raskin, 1992). Moreover, SA is widely demonstrated to play a role in responses to biotic stresses (Janda *et al.*, 2007), as a large body of evidence over the past decade has shown that SA is an essential molecule involved in plant defense response against diverse pathogens by inducing pathogenesis-related (PR)

gene expression and/or systemic acquired resistance (SAR) (Malamy *et al.*, 1990; Shah, 2003). Meanwhile, the accumulating evidences support the essential role of SA in modulating the plant response to diverse abiotic stresses as well. For example, application of exogenous SA could improve the thermotolerance and heat acclimatization in mustard seedlings (Dat *et al.*, 1998). SA was also found to be required for the tolerance to ozone (O$_3$) (Sharma *et al.*, 1996), cadmium (Cd) (Metwally *et al.*, 2003), and salt stresses (Borsani *et al.*, 2001; Cao *et al.*, 2009). In addition, SA is capable of promoting the tolerance of maize to chilling stress (Janda *et al.*, 1999), most likely accompanied by increasing antioxidant enzyme activity.

Chilling stress is probably one of the most severe abiotic stress factors, restricting plant growth and productivity and thereby resulting in considerable yield loss worldwide. Generally, chilling constrains plant growth by causing severe injuries in chilling-sensitive crop species, such as cucumber (*Cucumis sativus* L.). The injuries include immediate mechanical constraints, activity changes of macromolecules, reduced osmotic potential in the cellular milieu (Xiong *et al.*, 2002), and a series of physiological, biochemical, and molecular modifications, such as the photoinhibition of photosystem I (PS I) (Kudoh and Sonoike, 2002) and increased hydrogen peroxide (H$_2$O$_2$) accumulation in chilled leaves (Zhou *et al.*, 2006). In addition, the levels of endogenous phytohormones, such as abscisic acid (ABA) (Anderson *et al.*, 1994) as well as polyamines (PAs) (Shen *et al.*, 2000; Zhang *et al.*, 2009), are modulated to enable enhanced adaptation of plants to chilling stress. In northern China chilling and low-light conditions during winter and early spring usually cause significant damage on the tissues of cucumber at the early development stages, such as germinating seeds, cotyledons, and young leaves; therefore, cucumber can only be grown in sunlight-heated greenhouses. Although a few previous studies have suggested that SA might be associated with the tolerance of cucumber to chilling stress (Yang *et al.*, 2004; Kang and Saltveit, 2002), it is still unclear how SA impacts the response of cucumber plants to such stress. To address this question, we used two cucumber cultivars possessing different chilling responses to investigate the effects of exogenous SA on protection against chilling stress as well as the changes of physiological features

and the fluctuation of free SA content during early response of cucumber plants exposed to chilling stress.

Material and Methods

Plant materials and growth conditions

Two cucumber (*Cucumis sativus* L.) cultivars, Changchun mici and Beijing jietou, were used in this study; cv. Changchun mici is chilling-tolerant, whereas cv. Beijing jietou is chilling-sensitive, assessed at a temperature of 15 °C (Lu *et al.*, 2005). The seeds were surface-sterilized with 0.1% hypochlorite, followed by rinsing with distilled H$_2$O for five times, and then germinated in a Petri dish with two layers of wet filter paper at 28 °C in the dark. The geminated seeds were subsequently transferred to a plastic tray filled with autoclaved peat moss soil. The seedlings were grown in a growth chamber equipped with lamps (Philips TLD18W/54; Shanghai, China) of an irradiance of approximately 200 μmol m^{-2} s^{-1}, kept at 28 °C (day)/18 °C (night) under a 10 h (day)/14 h (night) dark photoperiod. The relative aerial humidity fluctuated between 60% and 75%. About 2- to 3-week-old plants with two fully expanded leaves were used as experimental materials for treatments as described below.

Chilling and SA treatments

Seedlings were subjected to exogenous spray with 1 mм SA (pH 7.0) to the whole leaves of plants for 12 h prior to chilling treatment. The control plant received the approximately same amount of H$_2$O. To perform the chilling treatment, the seedlings were transferred to a growth chamber with the same conditions as above except temperature set at 15 °C (day)/8 °C (night). As much lower temperature (4 °C) causes severe injury and subsequent death of cucumber plants exposed to such chilling condition for 2~3 d, the experiments were not conducted at such low temperature conditions. The SA-treated or control plants were then kept in the same chamber at 15 °C/8 °C for 3 d and one additional day at 28 °C/18 °C for rewarming. For rewarming, following chilling treatments with or without SA pretreatment, the plants were transferred to the initial growth chamber for another 24 h. Fresh Hoagland buffer was supplemented at every other day to maintain the nutrition. The first leaves

were harvested at 0, 1, 2, 3 d after chilling initiation, and at the end of the subsequent warming period (4 d after chilling initiation), respectively. Three independent experiments were performed with three replicates of each treatment containing three uniform seedlings.

Determination of MDA content and electrolyte leakage

The malondialdehyde (MDA) content was measured by the thiobarbituric acid reaction method (Dhindsa *et al.*, 1981) with some modifications. Leaf samples (0.2 g) were homogenized in 1.8 ml of 5% trichloroacetic acid (TCA) and centrifuged at 10,600 x *g* for 20 min. The supernatant was mixed with 1.5 ml of 0.67% thiobarbituric acid (TBA), heated at 100 °C for 30 min, and then immediately cooled down on ice. After centrifugation at 660 x *g* for 10 min, absorbance of the supernatant was measured at 450, 532, and 600 nm, respectively. To measure electrolyte leakage, 10 leaf discs of the same size (0.25 cm^2) per treatment were excised from SA- or H_2O-treated plants upon chilling stress. The electrolyte leakage was measured by the electrical conductivity method (Gong *et al.*, 1998).

Assays of antioxidant enzyme activity

To extract antioxidant enzymes, 0.2 g of fresh leaves was homogenized using 1.6 ml of 50 mM ice-cold phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10,600 x *g* for 20 min at 4 °C. The supernatant was subsequently used for antioxidant enzyme activity assays.

Peroxidase (POD, EC1.11.1.7) activity was assayed according to Kochba *et al.* (1977). The POD reaction solution (3 ml) contained 20 mM phosphate buffer (pH 6.0), 20 mM guaiacol, 40 mM H_2O_2, and 40 μl enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined every 40 s. One unit of activity was defined as the amount of enzyme required to increase the optical density at 470 nm within 1 min by 1 absorbance unit.

Catalase (CAT, EC1.11.1.6) activity was assayed according to Dhindsa *et al*. (1981). The CAT reaction solution (3 ml) contained 15 mM phosphate buffer (pH 7.0), 15 mM H_2O_2, and 0.1 ml enzyme extract. The reaction was initiated by adding enzyme extract. Changes in absorbance of the reac-

tion solution at 240 nm were read every 40 s. One unit of activity was defined as the amount of enzyme required to decrease the optical density at 240 nm within 1 min by 0.1 absorbance unit.

The activity of each enzyme was expressed on a protein basis. The protein content was determined according to Bradford (1976), using bovine serum albumin as standard.

Quantification of endogenous SA content in germinating seeds and young leaves

Uniform seeds were selected and put on three layers of filter paper to allow germination in the dark at 28 °C in a Petri dish after surface-sterilization. The germinating seeds and 2- to 3-week-old plants were subjected to 15 °C for 24 h. Entire germinating seeds or young leaves were subsequently harvested and frozen immediately in liquid nitrogen and stored at –70 °C until use. The germinating seed radicles with approximately 5 mm in length, and young leaves without chilling treatment were used as control at 0 h. The SA content was quantified using GC-MS. Briefly, 0.5 – 1 g tissue was ground in liquid nitrogen and extracted with 3 ml 90% methanol followed by extraction with 2 ml 100% methanol. The extracts were then resuspended in 2.5 ml 5% (v/v) TCA, followed by two extractions with an equal volume of ethyl acetate/cyclopentane/isopropanol [100:99:1 (v,v,v)]. The extracts were dried and then resuspended in 20% (v,v) methanol. SA was identified and quantified by spiking a noninduced sample with a known amount of an authentic standard on a 5-μm C-18 column (4.6 × 250 mm) using reverse-phase HPLC; detection was with a Waters 474 scanning fluorescence detector (Milford, MA, USA) (excitation wavelength, 295 nm; emission wavelength, 400 nm). Three replicates were used with 20 seeds or young leaves from two plants per replicate per time point.

Histochemical detection of H_2O_2 production in leaves and cotyledons

H_2O_2 production was visually detected using the 3,3'-diaminobenzidine (DAB) (Sigma, Shanghai, China) staining method as described by Orozco-Cardenas and Ryan (1999) with minor modifications. Briefly, the two cultivars of cucumber were grown under the conditions described above. Plants with two fully expanded leaves were subjected to different combinations of treat-

ments with chilling at 15 °C or chilling plus spray with 1 mM SA. To investigate whether ROS production is influenced by SA at low temperature, cotyledons from seedlings chilled at 8 °C were collected. Following treatment, the first fully expanded true leaf and both cotyledons were excised at the base with a razor blade and immersed immediately in 1 mg ml^{-1} solution of DAB (pH 3.8, dissolved in H_2O with 0.01% Triton-X-100). The leaves and cotyledons were infiltrated under reduced pressure for 30 min, followed by incubation overnight at room temperature. After incubation with DAB, leaves were fixed and cleared in alcoholic lacto-phenol [95% ethanol/lactic acid/phenol (2:1:1) (v,v,v)] at 65 °C for 30 min, rinsed with 50% ethanol once, and with water twice. This treatment decolourized the leaves and facilitated the visualization of a dark brown polymerization product upon reaction of DAB with H_2O_2.

Statistical analysis

The data were analysed for significance of difference by analysis of variance (ANOVA) using the Statistical Analysis System program (SAS Institute, Cary, NC, USA). Two independent experiments were performed with three replicates each and consistent results were obtained. The data were expressed as mean ± SE ($n = 3$) of samples. Differences among means within the same treatment were separated by the least significance difference (LSD) test at 0.05 probability level.

Results

Effect of chilling stress on endogenous SA levels in cucumber leaves and geminating seeds

Endogenous SA levels in the two cultivars with different chilling responsiveness were determined in the germinating seeds and young leaves under chilling stress. As shown in Fig. 1A, the geminating seeds of chilling-tolerant cultivar Changchun mici were found to contain higher levels of SA constitutively, compared to the chilling-sensitive one, Beijing jietou. SA levels increased remarkably in the germinating seeds of Changchun mici upon chilling stress for 24 h, whereas no obvious changes of SA levels were observed in those of Beijing jietou. Chilling stress for 24 h also caused an increase of SA levels in young leaves of both cultivars, with significantly higher levels of SA in the former than in the latter one (Fig. 1B), despite

of that the non stressed leaves of Changchun mici displayed no significantly higher level of SA compared to Beijing jietou. In addition, endogenous SA levels in young leaves were much higher than those in the geminating seeds of both cultivars, while SA levels were significantly induced in both cultivars 24 h post chilling stress (Fig. 1B).

Effect of exogenous SA on the membrane integrity of cucumber under chilling stress

It is known that chilling stress causes reduction of the membrane integrity (Alonso *et al.*, 1997). To investigate the role of SA in the protection of the cucumber membrane integrity under chilling stress, MDA content and electrolyte leakage in leaves were determined in the two cultivars subjected to chilling stress or chilling plus SA treat-
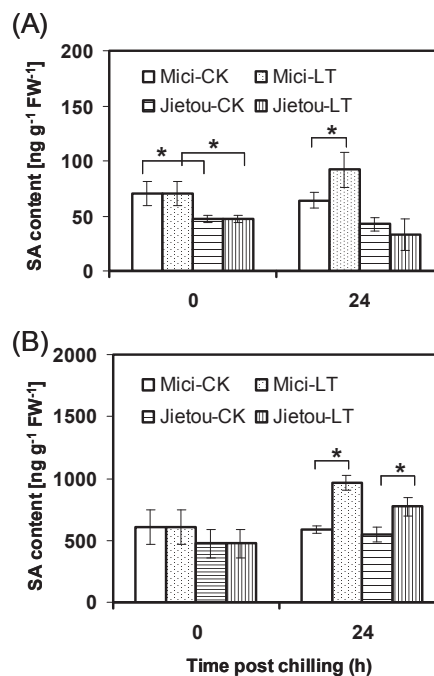

Fig. 1. Effect of chilling stress on endogenous SA levels in cucumber (*Cucumis sativus* L.) (A) seeds and (B) leaves. Two independent experiments were conducted and consistent results were obtained. Data are the mean ± SE ($n = 3$), and asterisks denote a significant difference ($P < 0.05$, ANOVA) between control and chilling temperature treatments within the same cultivar at the same time point. CK, control; LT, low temperature at 15 °C/8 °C. Mici, chilling-resistant cultivar Changchun mici; Jietou, chilling-sensitive cultivar Beijing jietou.
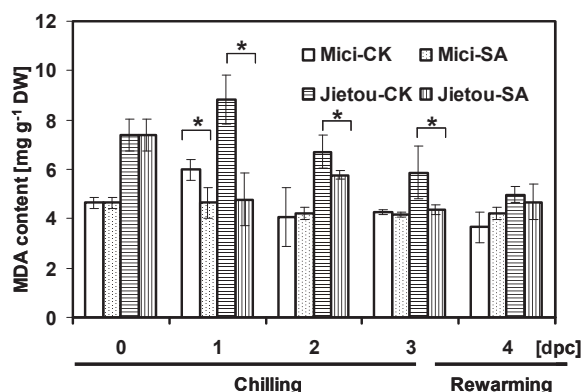
Fig. 2. Effect of exogenous application of SA on MDA contents in cucumber (*Cucumis sativus* L.) seedlings under chilling stress at 15 °C/8 °C. Two independent experiments were conducted and consistent results were obtained. Data are the mean ± SE ($n = 3$), and asterisks denote a significant difference ($P < 0.05$, ANOVA) between control and SA treatments within the same cultivar at the same time point. CK, control without SA treatment; SA, salicylic acid; Mici, chilling-resistant cultivar Changchun mici; Jietou, chilling-sensitive cultivar Beijing jietou; dpc, days post chilling.

ment. As a marker, the content of the end product of membrane lipid peroxidation, MDA, usually increases when the plants encounter environmental stress. As demonstrated in Fig. 2, the chilling-tolerant cultivar Changchun mici contained a significantly lower level of MDA compared to Beijing jietou. Moreover, chilling stress caused a significant elevation of the MDA content in seedlings of both cultivars on the first day post chilling (dpc) stress, especially in Beijing jietou, followed by a decline to the level prior to chilling. However, exogenous application of SA reduced the higher levels of MDA caused by chilling stress, in both cultivars. Rewarming brought down the MDA content to a level similar to that without chilling treatment. Significantly, the MDA levels in Changchun mici were much lower, through day 0 to day 4, compared to those in Beijing jietou (Fig. 2).

In addition, as shown in Fig. 3, chilling stress caused an increase in electrolyte leakage in the leaves of both Changchun mici and Beijing jietou one dpc, with significantly higher electrolyte leakage in Beijing jietou at 3 dpc. However, SA reduced the electrolyte leakage caused by chilling stress in the leaves of both cultivars, with the notable reduction at 1 dpc and 2 dpc. Moreover, the electrolyte leakage in the leaves of Changchun mici was always lower than that in Beijing jietou during the period of chilling stress. Rewarming at 4 dpc brought down the electrolyte leakage to a level similar to that without chilling stress (Fig. 3).

Effect of exogenous SA on the content of soluble protein in cucumber leaves under chilling stress

Chilling stress significantly alleviated the soluble protein content in Beijing jietou, but not in Changchun mici, with the lowest level at 3 dpc (Fig. 4). However, an exogenous spray with SA attenuated the reduction of soluble protein caused
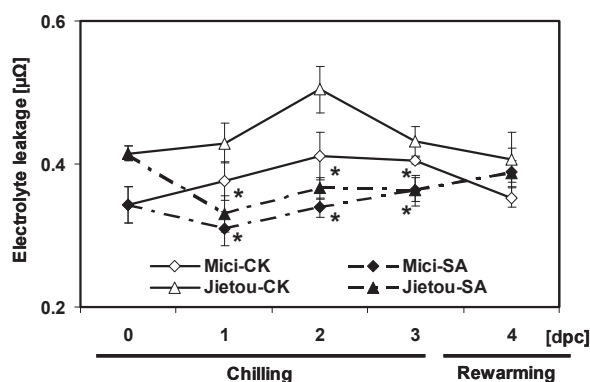

Fig. 3. Effect of exogenous application of SA on electrolyte leakage in cucumber (*Cucumis sativus* L.) seedlings under chilling stress at 15 °C/8 °C. Two independent experiments were conducted and consistent results were obtained. Data are the mean ± SE ($n = 3$), and asterisks denote a significant difference ($P < 0.05$, ANOVA) between control and SA treatments within the same cultivar at the same time point. CK, control without SA treatment; SA, salicylic acid; Mici, chilling-resistant cultivar Changchun mici; Jietou, chilling-sensitive cultivar Beijing jietou; dpc, days post chilling.

by chilling, starting from 1 dpc, with a significantly sharp increase in Changchun mici. The soluble protein content declined subsequently at 2 dpc and dropped to the level prior to chilling. Notably, the soluble protein content in Changchun mici was markedly higher than that in Beijing jietou through the entire chilling period. Rewarming maintained the normal level of soluble protein in both cultivars (Fig. 4).

Effect of exogenous application of SA on antioxidant enzyme activity in cucumber seedlings under chilling stress

As shown in Fig. 5A, chilling stress at 15 °C caused rapid reduction of the POD activity at 3 dpc in Beijing jietou. Similarly, chilling resulted in a reduction of the CAT activity in Beijing jietou at 1 dpc, whereas an increase of the CAT activity was observed in Beijing jietou starting on 1 dpc (Fig. 5B). While an exogenous spray of SA elevated the POD and CAT activities in both cultivars, with the induction maintained through the entire chilling period in Changchun mici, the greatest induction of both POD and CAT activities in Beijing jietou was only observed at 3 dpc. The POD activity enhanced by SA was maintained in both

cultivars after the seedlings were recovered from chilling stress, whereas rewarming brought the CAT activity to the level similar to that prior to chilling (Fig. 5A and B).

Effect of SA on H_2O_2 accumulation caused by chilling stress in cucumber

The induction of the antioxidant enzyme activity by SA prompted us to investigate whether exogenous application of SA could impact the production of reactive oxygen species (ROS), which might be associated with increased injury

Fig. 5. Effect of exogenous application of SA on (A) peroxidase (POD) activity and (B) catalase (CAT) activity in cucumber (*Cucumis sativus* L.) seedlings under chilling stress at 15 °C/8 °C. Two independent experiments were conducted and consistent results were obtained. Data are the mean ± SE (*n* = 3), and asterisks denote a significant difference (*P* < 0.05, ANOVA) between control and SA treatments within the same cultivar at the same time point. CK, control without SA treatment; SA, salicylic acid; Mici, chilling-resistant cultivar Changchun mici; Jietou, chilling-sensitive cultivar Beijing jietou; dpc, days post chilling.
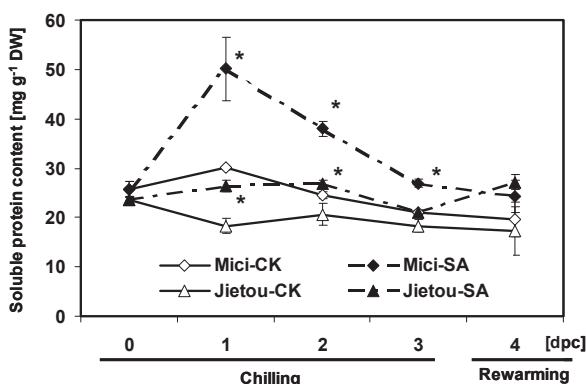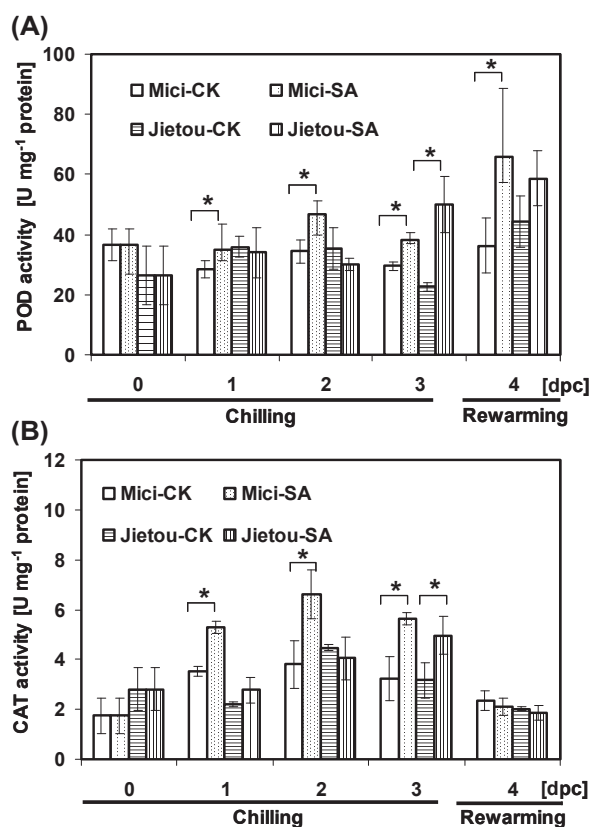

Fig. 4. Effect of exogenous application of SA on soluble protein content in cucumber (*Cucumis sativus* L.) seedlings under chilling stress at 15 °C/8 °C. Two independent experiments were conducted and consistent results were obtained. Data are the mean ± SE (*n* = 3), and asterisks denote a significant difference (*P* < 0.05, ANOVA) between control and SA treatments within the same cultivar at the same time point. CK, control without SA treatment; SA, salicylic acid; Mici, chilling-resistant cultivar Changchun mici; Jietou, chilling-sensitive cultivar Beijing jietou; dpc, days post chilling.

caused to plant cells. We examined histologically the production of H_2O_2 in young leaves and the cotyledon of cucumber under chilling stress with or without SA pretreatment. As shown in Fig. 6, H_2O_2 accumulated in both young leaves (Fig. 6A) and cotyledons (Fig. 6B) in both cultivars. Compared to Changchun mici, the chilling-sensitive cultivar Beijing jietou accumulated more H_2O_2, upon chilling stress for 24 h. However, a spray with SA eliminated the H_2O_2 production caused by chilling in both cultivars (Figs. 6A and B).

When the cotyledons were subjected to stress under lower temperature (8 °C), much greater H_2O_2 accumulation was observed compared to that at 15 °C. As evidenced in Fig. 6C, H_2O_2 accumulated remarkably in the cotyledons of Beijing jietou starting at 4 h post chilling stress, whereas it started to accumulate in Changchun mici only at 24 h post chilling stress. Obviously, pretreatment with SA abolished the H_2O_2 accumulation in both cultivars, with the greater effect in Changchun mici (Fig. 6C).
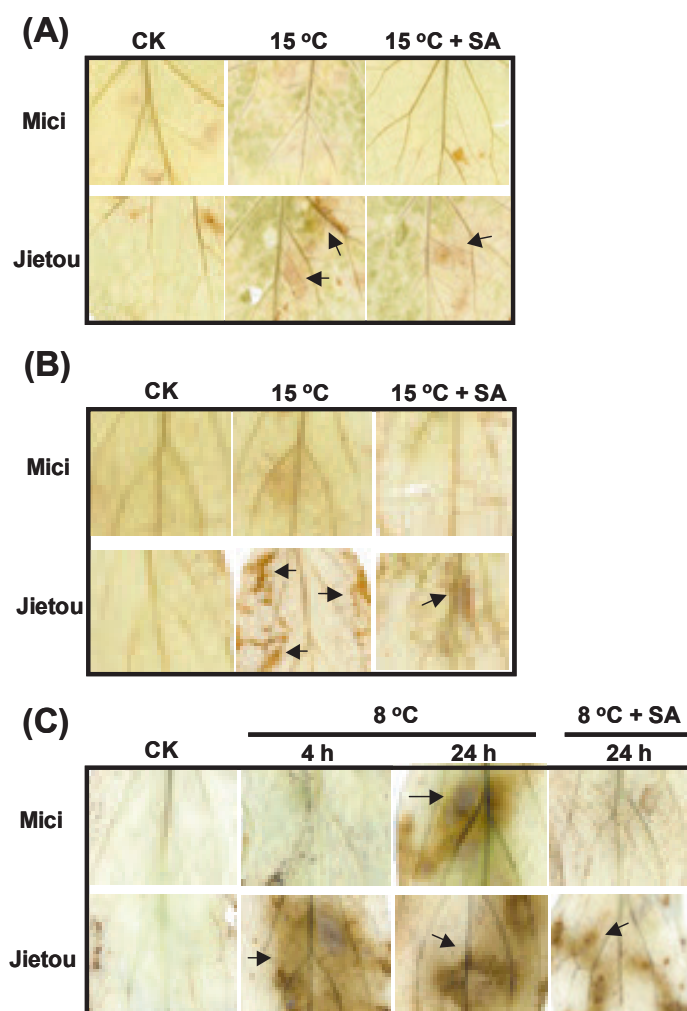

Fig. 6. Effect of exogenous application of SA on hydrogen peroxide produced by chilling stress in cucumber (*Cucumis sativus* L.) (A) young leaves and (B and C) cotyledons with or without pretreatment with SA. CK refers the normal temperature at 28 °C/18 °C and chilling stress at (A and B) 15 °C or (C) 8 °C. The arrows indicate production of hydrogen peroxide visible as dark deposits stained by DAB.

Discussion

Higher endogenous SA levels are associated with enhanced tolerance of cucumber to chilling stress

Although several previous studies have shown that exogenously applied SA could effectively improve the cold tolerance in economically important crops, including maize (Janda *et al.*, 1999), banana (Kang *et al.*, 2003a), cucumber, and rice (Kang and Saltveit 2002), very little is known about the association of the endogenous SA content with the tolerance of plants to chilling stress. *Arabidopsis NahG* (expressing a bacterial salicylate hydroxylase) transgenic plants, in which SA is degraded into catechol or SA perception/ production is defective, become more susceptible to abiotic stress (Rao and Davis, 1999; Clarke *et al.*, 2004), suggesting a positive correlation of SA to chilling tolerance. In supporting this hypothesis, we found that the chilling-resistant cultivar Changchun mici contains higher levels of endogenous SA in the geminating seeds and young leaves, compared to those in the chilling-sensitive cultivar Beijing jietou. The difference of endogenous SA levels in the two cultivars suggested that SA is not only associated with tolerance to chilling stress, but can also be used as a physiological marker for screening chilling tolerance traits.

In addition, much higher levels of SA were found in young leaves compared to those in germinating seeds, which is consistent with a previous finding in *Arabidopsis* (Abreu and Munné-Bosch, 2009). The difference of endogenous SA levels in various organs might be due to the differential evolutionary response of different organs to severe stress conditions (Abreu and Munné-Bosch, 2009), or to the inhibitory effect of a high SA content on seed germination (Xie *et al.*, 2007).

Exogenous application of SA enhances chilling tolerance of cucumber through protection of cell membrane from damage by chilling stress

It has been reported previously that SA might be involved in the tolerance response of cucumber to chilling stress (Kang and Saltveit, 2002). However, how SA enhances cucumber tolerance to chilling is still obscure. In the present study, we used two different cucumber cultivars that are either tolerant or sensitive to chilling (Lu *et al.*, 2005) to monitor the effect of SA on several important indicators used for several physiologi-

cal and biochemical parameters of plants upon environmental stresses, whose changes might be due to the decrease of membrane integrity and lipid peroxidation (Moore and Roberts, 1998; Bajji *et al.*, 2002). We found that while chilling stress caused a significant reduction of the soluble protein content in both cultivars, especially in Beijing jietou, exogenous SA could attenuate effectively this reduction. On the other hand, exogenous SA could diminish the higher level of MDA caused by chilling stress in both cultivars, indicating that SA might enhance cucumber chilling tolerance by impacting cellular components, including elimination of cell membrane oxidation and reinforcement of cell walls.

Exogenous SA improves chilling tolerance of cucumber through impacting antioxidant enzyme activities and ROS production

It has been well documented that chilling causes not only changes of the membrane structure of plants (Xu *et al.*, 2008), but also decreases activities of oxidant enzymes that constitute the cellular defense against oxidative stress (Lee and Lee, 2000) and, therefore, function importantly in protection of plants from stress-induced damage (Zhou *et al.*, 2006; Rider *et al.*, 2007). Previous studies have shown that pretreatment of banana seedlings with 0.5 mM SA could enhance the chilling tolerance to 5 °C chilling temperature, and this treatment altered the activities of SOD, CAT, and ascorbate peroxidase (APX) (Kang et al., 2003a), which is thought to be associated with a reduction of the H$_2$O$_2$ level in SA-pretreated but chilling-stressed banana seedlings (Kang *et al.*, 2003b). There have been few studies so far on the effect of SA on the antioxidant enzymes in cucumber upon chilling stress (Kang and Saltveit, 2002), in which pretreatment with SA increased significantly the activities of glutathione reductase (GR) and glutathione peroxides (GPX), but not CAT and APX, to levels higher than those in untreated plants under chilling condition. Despite of these findings, previous studies did not provide direct evidence by showing the difference in the responsiveness of two different cultivars of cucumber in the tolerance to chilling stress, regarding both the antioxidant enzyme activity and ROS production. This study investigated more comprehensively the impact of SA on the antioxidative system during induction of cucumber tolerance to chilling stress, demonstrating that the enhanced

tolerance in cucumber to chilling stress is likely associated with higher antioxidant enzyme activity, thereby leading to lower ROS production and, probably, alteration of gene expression.

Indeed, while Changchun mici accumulated much less H_2O_2, compared to Beijing jietou, upon chilling stress, exogenous SA could eliminate the H_2O_2 production caused by chilling stress in both cultivars, suggesting that SA may play an important role in preventing cucumber seedlings from chilling injury caused by oxidative stress. To our knowledge, this study is the first to report that SA could impact the H_2O_2 production caused by chilling in cucumber as demonstrated by histochemical approaches. It has been well-known that chilling causes oxidative damage of plants due to increased ROS production (Prosad *et al.*, 1994; Zhou *et al.*, 2006; Xu *et al.*, 2008), which is considered primarily responsible for lipid peroxidation of cellular membranes caused by environmental stresses (Apel and Hirt, 2004). Together with previous reports, the present study strongly demonstrates that SA exerts its function by inducing the antioxidant machinery to prevent chilling injury through counteracting the oxidative stresses imposed on chilled seedlings of cucumber, including induction of antioxidant enzyme activity and elimination of ROS production.

Abreu M. E. and Munné-Bosch S. (2009), Salicylic acid deficiency in NahG transgenic lines and sid2 mutants increases seed yield in the annual plant *Arabidopsis thaliana*. J. Exp. Bot. **60**, 1261–1271.

Alonso A., Queiroz C. S., and Magalhães A. C. (1997), Chilling stress leads to increased cell membrane rigidity in roots of coffee (*Coffea arabica* L.) seedlings. Biochim. Biophys. Acta Biomemb. **1321**, 75–84.

Anderson M. D., Prasad T. K., Martin B. A., and Stewart C. R. (1994), Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance. Plant Physiol. **105**, 331–339.

Apel K. and Hirt H. (2004), Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55**, 373–399.

Bajji M., Kinet J. M., and Lutts S. (2002), The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul. **36**, 61–70.

Borsani O., Valpuesta V., and Botella M. A. (2001), Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. Plant Physiol. **126**, 1024–1030.

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

Cao Y., Zhang Z. W., Xue L. W., Du J. B., Shang J., Xu F., Yuan S., and Lin H. H. (2009), Lack of salicylic acid in *Arabidopsis* protects plants against moderate salt stress. Z. Naturforsch. **64c**, 231–238.

Clarke S. M., Mur L. A. J., Wood J. E., and Scott I. M. (2004), Salicylic acid dependent signaling promotes basal thermotolerance but is not essential for acquired thermotolerance in *Arabidopsis thaliana*. Plant J. **38**, 432–447.

Dat J. E., Lopze-Delgado H., Foye C. H., and Scott I. M. (1998), Parallel change in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol. **116**, 1351–1357.

Dhindsa R. S., Pulmb-Dhindsa P., and Thorpe T. A. (1981), Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. **32**, 93–101.

Fujita M., Fujita Y., Noutoshi Y., Takahashi F., Narusaka Y., Yamaguchi-Shinozaki K., and Shinozaki K. (2006), Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. **9**, 436–442.

Gong M., Li Y. J., and Chen S. Z. (1998), Abscisic acid-induced thermo-tolerance in maize seedlings is mediated by calcium and associated with antioxidant systems. J. Plant Physiol. **153**, 88–96.

Janda T., Szalai G., Tari I., and Paeli E. (1999), Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea may* L.) plants. Planta **208**, 175–180.

Janda T., Horváth E., Szalai G., and Páldi E. (2007), Role of salicylic acid in the induction of abiotic stress tolerance. In: Salicylic Acid – A Plant Hormone (Hayat S. and Ahmad A., eds.). Springer, Dordrecht, The Netherlands, pp. 91–150.

Kang H. M. and Saltveit M. E. (2002), Chilling tolerance of maize, cucumber and rice seedling leaves

and roots are differentially affected by salicylic acid. Physiol. Plant. **115**, 571–576.

Kang G. Z., Wang C. H., Sun G. C., and Wang Z. X. (2003a), Salicylic acid changes activities of H_2O_2-metabolizing enzymes and increases the chilling tolerance of banana seedlings. Environ. Exp. Bot. **50**, 9–15.

Kang G. Z., Wang Z. X., and Sun G. C. (2003b), Participation of H_2O_2 in enhancement of cold chilling by salicylic acid in banana seedlings. Acta Bot. Sin. **45**, 567–573.

Kochba J., Lavee S., and Spiegel-Roy P. (1977), Differences in peroxidase activity and isoenzymes in embryogenic and non-embryogenic 'Shamouti' orange ovular callus lines. Plant Cell Physiol. **18**, 463–467.

Kudoh H. and Sonoike K. (2002), Irreversible damage to photosystem I by chilling in the light: cause of the degradation of chlorophyll after returning to normal growth temperature. Planta **215**, 541–548.

Lee D. H. and Lee C. B. (2000), Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. **159**, 75–85.

Lu M. H., Li X. M., Chen J. F., Chen L. Z., and Qian C. T. (2005), Study on chilling tolerance of cucumber during germination and expression of lysine decarboxylase gene. Sci. Agric. Sin. **38**, 2492–2495.

Malamy J., Carr J. P., Klessig D. F., and Raskin I. (1990), Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science **250**, 1002–1004.

Metwally A., Finkemeier I., Georgi M., and Dietz K. J. (2003), Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiol. **132**, 272–281.

Moore K. and Roberts L. J. (1998), Measurement of lipid peroxidation. Free Radic. Res. **28**, 659–671.

Orozco-Cardenas M. and Ryan C. A. (1999), Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA **96**, 6553–6557.

Prosad T. K., Anderson M. D., Martin B. A., and Stewart C. R. (1994), Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6**, 65–74.

Rao M. V. and Davis R. D. (1999), Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. Plant J. **17**, 603–614.

Raskin I. (1992), Role of salicylic acid in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**, 439–463.

Rider J. E., Hacker A., Mackintosh C. A., Pegg A. E., Woster P. M., and Casero R. A. Jr. (2007), Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids **33**, 231–240.

Shah J. (2003), The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6**, 365–371.

Sharma Y. K., Leon J., Raskin I., and Davis K. R. (1996), Ozone induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense related transcripts and induced resistance. Proc. Natl. Acad. Sci. USA **93**, 5099–5104.

Shen W., Nada K., and Tachibana S. (2000), Involvement of polyamines in the chilling tolerance of cucumber cultivars. Plant Physiol. **124**, 431–439.

Xie Z., Zhang Z. L., Hanzlik S., Cook E., and Shen Q. X. J. (2007), Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic acid-inducible WRKY gene. Plant Mol. Biol. **64**, 293–303.

Xiong L., Schumaker K. S., and Zhu J. K. (2002), Cell signaling during cold, drought, and salt stress. Plant Cell **14**, S165–S183.

Xu P. L., Guo Y. K., Bai J. G., Shang L., and Wang X. J. (2008), Effects of long-term chilling on ultrastructure and antioxidant activity in leaves of two cucumber cultivars under low light. Physiol. Plant. **132**, 467–478.

Yang Y. N., Qi M., and Mei C. S. (2004), Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. Plant J. **40**, 909–919.

Zhang W., Jiang B., Li W., Song H., Yu Y., and Chen J. (2009), Polyamines enhance chilling tolerance of cucumber (*Cucumis sativus* L.) through modulating antioxidative system. Sci. Hort. **122**, 200–208.

Zhou Y. H., Yu J. Q., Mao W. H., Huang L. F., Song X. S., and Nogués S. (2006), Genotypic variation of *Rubisco* expression, photosynthetic electron flow and antioxidant metabolism in the chloroplasts of chill-exposed cucumber plants. Plant Cell Physiol. **47**, 192–199.