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ORIGINAL PAPER

The alternative pathway in cucumber seedlings under low temperature stress was enhanced by salicylic acid

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Abstract The alternative pathway is a cyanide-resistant and non-phosphorylatory electron transport pathway in mitochondria of higher plants. Alternative oxidase (AOX) is the terminal oxidase of this pathway. Our present study investigated the effect of exogenous salicylic acid (SA) on alternative pathway in cucumber (*Cucumis sativus* L.) seedlings under low temperature stress. Results showed that during the process of low temperature stress, the alternative pathway capacity was enhanced as AOX expression increased in SA pretreated seedlings. Compared with seedlings without SA pretreatment, slower decrease of relative water content and lower levels of electrolyte leakage, H_2O_2 and malonyldialdehyde content were detected in SA pretreated seedlings. These results indicated that SA could alleviate the injury caused by low temperature on cucumber seedlings. Since the special protective functions of alternative pathway and AOX in plants, we suggested that the alternative pathway was related to SA-mediated plant resistance to environmental stresses such as low temperature.

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Keywords Alternative pathway · Alternative oxidase · Cucumber seedlings · Low temperature stress · Salicylic acid

Abbreviations

AOX	Alternative oxidase
DW	Dry weight
EL	Electrolyte leakage
MDA	Malonyldialdehyde
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid

Introduction

One difference between plant and animal mitochondria respiration is that there is a cyanide-resistant respiration and non-phosphorylatory electron transport pathway called the alternative pathway in plant mitochondria. It branches from the main respiratory electron transport chain after the site of ubiquinone pool, and bypasses the last few steps of the cytochrome respiratory pathway, with its terminal oxidase called alternative oxidase (AOX; Liang and Liang 1998). Researches on functions of the alternative pathway in plants have been continued for years. Early studies suggested that the alternative pathway consisted in thermogenic tissues of plant, produced heat, and then induced florescence (Vanlerberghe and McIntosh 1997). Moreover, functions of the alternative pathway in non-thermogenic tissues were also discovered. For example, maintaining stabilization of the respiratory electron chain and the tricarboxylic acid (TCA) cycle (Vanlerberghe and McIntosh 1994; Simons et al. 1999). Some studies also indicated that

AOX could scavenge reactive oxygen species (ROS) (Maxwell et al. 1999; Møller 2001), and played a role in the process of programmed cell death (PCD) in plants (Robson and Vanlerberghe 2002; Vanlerberghe et al. 2002). In addition, the alternative pathway was suggested to maintain the activation of NAD(P)H dehydrogenase and proton-pumping NADH dehydrogenase by keeping the flow of mitochondrial electrons. This process ensured sufficient ATP supply for rapid adaptation and the maintenance of plant growth rate homeostasis (Moore et al. 2002; Hansen et al. 2002; Fung et al. 2004). Our previous studies proved that the alternative pathway capacity and AOX expression in wheat could be influenced by water stress (He et al. 1999a, b). Furthermore, low temperature also enhanced the alternative pathway capacity and AOX expression in tobacco (Vanlerberghe and McIntosh 1992; Yan et al. 2004). Therefore, the alternative pathway and AOX were suggested to play a protective role against environmental stresses in plants (Zhao et al. 2007).

Salicylic acid (SA) is a phenolic compound known as a signal molecule in plant resistance to pathogens (Dempsey et al. 1999; Shah 2003; Beckers and Spoel 2006; Vasyukova and Ozeretskovskaya 2007). It takes part in the defense response to abiotic stresses such as drought, cold, and heat in plants (Senaratna et al. 2000; Yuan and Lin 2008). So, SA is a plant hormone plays important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses (Rajasekaran and Blake 1999; Singh and Usha 2003; Bari and Jones 2009; Okrent et al. 2009). In addition, SA was proved to be an inducer of the alternative pathway and AOX expression in plants (Rhoads and McIntosh 1992, 1993; Lei et al. 2008). Application of MeSA (methyl salicylate), the mobile form of SA (Park et al. 2007), also induced the expression of AOX gene (Fung et al. 2004, 2006). However, the relationship between the alternative pathway or AOX expression and SA is still unclear, especially under stress conditions.

In the present work, we investigated the effects of exogenous SA on alternative pathway respiration and low temperature tolerance in cucumber seedlings, with the purpose of studying the relationship between alternative pathway and SA-mediated plant tolerance. The expression and accumulation of AOX were also studied to understand how SA influences the alternative pathway.

Materials and methods

Plant material and treatments

Seeds of cucumber (*Cucumis sativus* L. cv. Chun Qiu Da Feng) were surface-sterilized with 1% NaClO for 10 min, and then germinated on water-moistened filter paper in dark for 24 h. The germinated seedlings were planted into sterilized sand and grown at 25°C under a 12-h photoperiod and photosynthetic photon flux density (PPFD) of 100 μM m^{-2} s^{-1} growth condition. Seedlings were cultivated with half-strength Hoagland's nutrient solution till the three-leaf stage (about 20 days). Then, half of them were pretreated with a single spray of SA (20 μM, pH 6.8) on leaves according to Shi et al. (2006). Other leaves sprayed with distilled water were used as control. After pretreated for 24 h, all cucumber seedlings were exposed to low temperature (10 ± 1°C) under same conditions of light and nutrient solution for 2, 4 and 6 days, respectively. The third leaf of cucumber seedlings were used for experiment.

Cucumber leaf respiration measurements

Respiration rate was measured according to Vanlerberghe et al. (2002). Leaves were cut into small pieces and were suspended in assay buffer (adjusted to about 2.5 mg dry weight ml^{-1}) in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 25°C. Inhibitors of the cytochrome pathway (1 mM KCN) and the alternative pathway (20 μM n-propyl gallate) were used. The alternative pathway capacity is defined as O_2 uptake rate in the presence of KCN that was sensitive to n-propyl gallate. Total respiration is defined as O_2 uptake rate by cucumber leaves without any inhibitor. Residual respiration (O_2 uptake by leaves in the presence of both KCN and n-propyl gallate) in our experiment was always low, and was negligible relative to other respirations. So the results were not shown.

Isolation and purification of cucumber leaf mitochondria

Mitochondria was isolated and purified according to Bartoli et al. (2006). Cucumber leaves were homogenized in 75 mM MOPS buffer (pH 7.5) containing 600 mM sucrose, 4 mM EDTA, 0.2% (w/v) polyvinyl-pyrrolidone (PVP)-40, 8 mM cysteine, and 0.2% (w/v) BSA. Homogenate was centrifuged at 3,000×*g* for 10 min, and then the supernatant was centrifuged at 16,000×*g* for 10 min. The resulting pellet was resuspended in 10 mM MOPS buffer (pH 7.2) containing 300 mM sucrose, and then layered onto Percoll gradients consisting of 20% Percoll (7.5 ml)/45% Percoll (2.5 ml), centrifuged at 26,000×*g* for 15 min. Mitochondria, recovered at the inferface between the 20 and 45% Percoll layers, was washed twice with MOPS buffer (pH 7.2). All steps were performed under 4°C. Protein concentration was determined by the Bradford

method using bovine serum albumin as a standard (Bradford 1976).

SDS–PAGE and western blot analysis

For SDS–PAGE and western blot analysis, 50 μg of protein from each sample was pretreated with sample buffer (10% [v/v] β-mercaptoethanol, 20% [v/v] glycerol, 4% [w/v] SDS, 0.005% [w/v] bromophenol blue and 50 mM Tris, pH 6.8) and boiled for 3 min, electrophoresed in 15% polyacrylamid and then transferred to a nitrocellulose membrane according to Yan et al. (2002). After transfer, the nitrocellulose membrane was immune blotted according to Sambrook et al. (1989). The primary antibody was AOA (dilution 1:100), the AOX monoclonal antibody (Elthon et al. 1989), provided by Dr. Jian-Ping Yu (Michigan State University). And the secondary antibody was horse anti-mouse IgG alkaline phosphatase conjugate (dilution 1:500). The digital data of band intensity was analyzed densitometrically by scanning the blots with a thin-layer scanner.

Preparation of total RNA and northern blot analysis

Total RNA was extracted from approximately 0.5 g of liquid nitrogen powdered leaf tissue by 500 μl of extraction buffer (20 mM Tris–HCl, pH 8.0, 1% [w/v] SDS, 200 mM NaC1, 5 mM EDTA) and phenol–chloroform (1:1, v/v) according to Xi et al. (2006). The resulting RNA was precipitated by 4 M LiCl at 4°C and dissolved in diethylpyrocarbonate-treated distilled water. RNA concentration was determined spectrophotometrically.

For northern blot analysis, equal amount of total RNA (30 μg) of each sample was separated on formaldehyde agarose gels, and then transferred to nylon membrane for subsequent probe hybridization according to Sambrook et al. (1989). DNA fragment of Arabidopsis *Aox1* gene was used as probe for RNA blot analysis, since there is high homology among *Aox1* genes across species (Clifton et al. 2006; Considine et al. 2002). The radioative labeling of the probe with [α-^{32}P]-dCTP was carried out using a Nick-Translation Labeling Kit (TaKaRa, Dalian, China) according to the manufacturer's instruction. The intensity of the signal was analyzed densitometrically by scanning the blots with a thin-layer scanner.

Relative water content measurements

The result was calculated according to Morgan's (1984) formula with some modified: (FW − DW)/(SW − DW) × 100%. Where FW means fresh weight, SW means saturated fresh weight measured after floating discs on distilled water in the dark overnight and DW means dry weight measured after oven drying at 80°C for 24 h.

Electrolyte leakage measurements

Electrolyte leakage (EL) was measured according to Liu et al. (2006): after measuring the electrical conductivity, the sample was boiled at 100°C for 15 min to achieve 100% EL. The relative conductivity of plasma membranes was calculated according to the ratio of electrical conductivity before and after boiling.

Malonyldialdehyde content measurements

The Malonyldialdehyde (MDA) content was measured by the method of Duan et al. (2006). Approximately 0.2 g of fresh leaves were cut into small pieces and homogenized by the addition of 5 ml of 5% trichloroacetic acid (TCA) in an ice bath. The homogenate was transferred into a tube and centrifuged at 1,000×g for 10 min at 4°C. Aliquots of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added into a new tube. The mixture was incubated in boiled water for 40 min, and then cooled to room temperature and centrifuged at 8,000×g for 5 min. The supernatant was subjected to analysis with the spectrophotometer. The MDA content was calculated from the subtracted absorbance (A_{535}–A_{600}) using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide content measurements

Hydrogen peroxide (H_2O_2) content was determined according to Sun et al. (2006). Approximately 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 ml of 0.1% (w/v) TCA. The homogenate was transferred into a tube and centrifuged at 12,000×g for 20 min at 4°C. 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbancy of supernatant was read at 390 nm. The content of H_2O_2 was determined by a standard curve.

Statistical analysis

The results were means of five independent measurements by separately grown and separately treated cucumber seedlings, and were statistically evaluated using the standard deviation and t-test methods. The difference was considered to be statistically significant when $P < 0.05$.

Results

The alternative pathway capacity

The alternative pathway capacity represents the maximal ability of plant to transport respiratory electron flow along

the alternative pathway when cytochrome pathway was inhibited. When plant grows in normal environmental condition, the alternative pathway respiration level is very low. However, many types of stresses could enhance the alternative pathway capacity. In the present study, we investigated response of the alternative pathway respiration in cucumber seedlings to SA pretreatment and low temperature stress (Fig. 1). Low temperature steadily enhanced the alternative pathway capacity (Fig. 1a) and its proportion in total respiration (Fig. 1b). But the alternative pathway capacity, as well as its proportion, increased significantly in SA pretreated seedlings compared to control during the process of low temperature stress (Fig. 1a, b). These results suggested that SA pretreatment enhanced the alternative pathway capacity in cucumber seedlings under low temperature stress.

AOX protein level

Alternative oxidase (AOX) in cucumber mitochondria is a homodimeric protein composed of two 36-kDa subunits (Juszczuk et al. 2007). We examined its level in order to determine the effects of SA and low temperature on AOX protein accumulation (Fig. 2). Equal amount of isolated mitochondria (50 μg) was used for protein analysis. Result of western blot showed that AOX protein level increased steadily during low temperature stress, and the level increased significantly in SA pretreated seedlings compared to control (Fig. 2a, b). These results suggested that AOX protein level was increased by SA pretreatment in cucumber seedlings under low temperature, and was consistent with the situation in alternative pathway capacity test.

AOX transcript level

Although there are different gene subfamilies of AOX in plants, most studies have focused on the relationship between *Aox1* gene expression and stress adaptation (Vanlerberghe and McIntosh 1997), since other subfamilies usually express constitutively or developmentally but have no response to stress (Considine et al. 2002). Thus, we examined *Aox1* mRNA to investigate the effects of SA and low temperature on AOX transcript level in cucumber seedlings (Fig. 3). Equal amount of total RNA (30 μg) was used for northern blot, and rRNA was used as loading control. It was shown that *Aox1* transcript level increased steadily during low temperature stress, and the level increased significantly in SA pretreated seedlings compared to control (Fig. 3a, b). These results were consistent

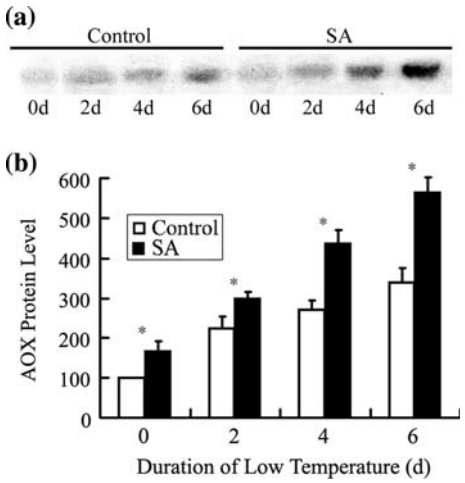

Fig. 2 Accumulation of AOX protein in response to SA in the third leaf of the cucumber seedlings under low temperature stress. Mitochondria were isolated from the two groups of cucumber leaves treated as described in Fig. 1. **a** Western blot analysis of mitochondrial protein separated by SDS–PAGE. Equal amounts (50 μg) of mitochondrial protein was loaded in each lane and blotted with the AOA. **b** Comparison of AOX protein level in SA pretreated seedlings with control. The results were expressed as percentages of control at 0 day. *Bars* represent the standard deviations of five independent measurements. Statistically significant different at $*P < 0.05$

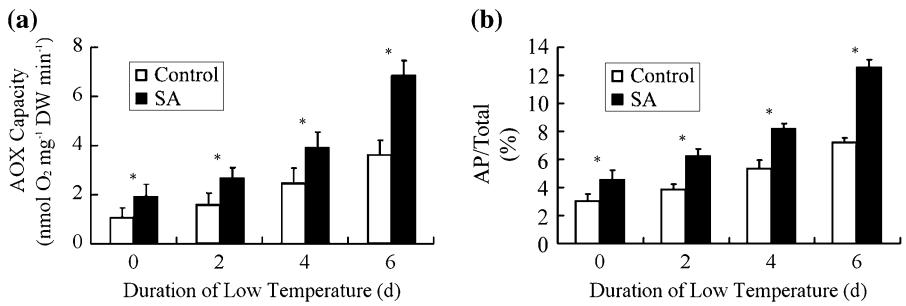

Fig. 1 Effects of SA on the alternative pathway capacity in the third leaf of cucumber seedlings under low temperature stress. Cucumber seedlings were divided into two s groups, SA: pretreated with a single spray of 20 μM SA for 24 h; Control: pretreated with a single spray of distilled water for 24 h. Both groups were then transferred to low temperature treatment (10 ± 1°C). O_2 uptake by the alternative pathway was measured at different duration of low temperature treatment. **a** AOX capacity; **b** Capacity proportion of the alternative pathway took up in total respiration. *AP* alternative pathway; *Total* total respiration. *Bars* represent standard deviations of five independent measurements. Statistically significant different at $*P < 0.05$

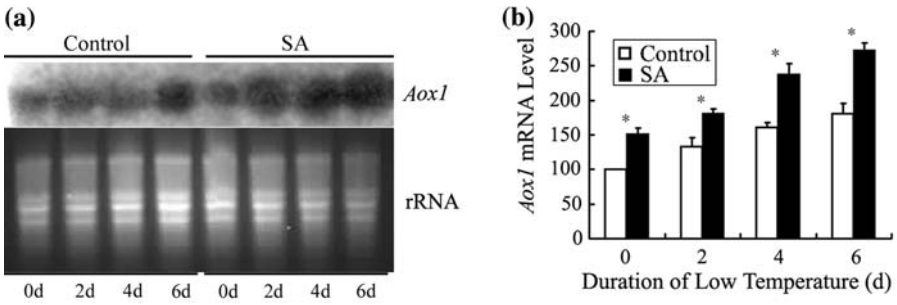

Fig. 3 Accumulation of AOX transcript in response to SA in the third leaf of cucumber seedlings under low temperature stress. Total RNA was isolated from cucumber leaves treated as described in Fig. 1. **a** Northern blot analysis in accumulation of AOX transcript. Equal amounts (30 μg) of total RNA was loaded in each lane and AOX transcript was detected by hybridization with specific DNA probe. rRNA was used as loading control. **b** Comparison of AOX transcript level in SA pretreated seedlings with control. The results were expressed as percentages of control at 0 day. *Bars* represent the standard deviations of five independent measurements. Statistically significant different at $*P < 0.05$

with the situation of AOX protein level, and the alternative pathway capacity as well.

H_2O_2 content, RWC, EL and MDA content

Hydrogen peroxide (H_2O_2) is an important type of ROS in plant cells, and index such as relative water content (RWC), EL and MDA content can imply the injury degree in plants caused by environmental stresses. In order to make sure whether SA play a role in reducing low temperature injury of cucumber seedlings, we compared the level of H_2O_2 content, RWC, EL and MDA content in both SA pretreated and untreated seedlings at different temporal points during the process of stress (Fig. 4).

Hydrogen peroxide (H_2O_2) content, EL and MDA content presented a similar change during the process of low temperature treatment. As shown in Fig. 4a, c, d, H_2O_2 content, EL and MDA content were all enhanced dramatically under low temperature stress. Furthermore, their levels increased significantly in control compared to SA pretreated seedlings, especially in the later stage of low temperature treatment. RWC of cucumber seedlings presented a continuous decrease during the stress process. And the data decreased significantly in control compared to SA pretreated seedlings, especially at 4 and 6 days (Fig. 4b). We also examined these parameters under normal temperature at 0, 2, 4, and 6 days, respectively. The results were almost unchanged, they were not shown.

To sum up, low temperature treatment induced a distinct injury in cucumber seedlings, but SA pretreatment alleviated the injury degree. All of these results indicated that SA played a role in the resistance of cucumber seedlings against low temperature stress.

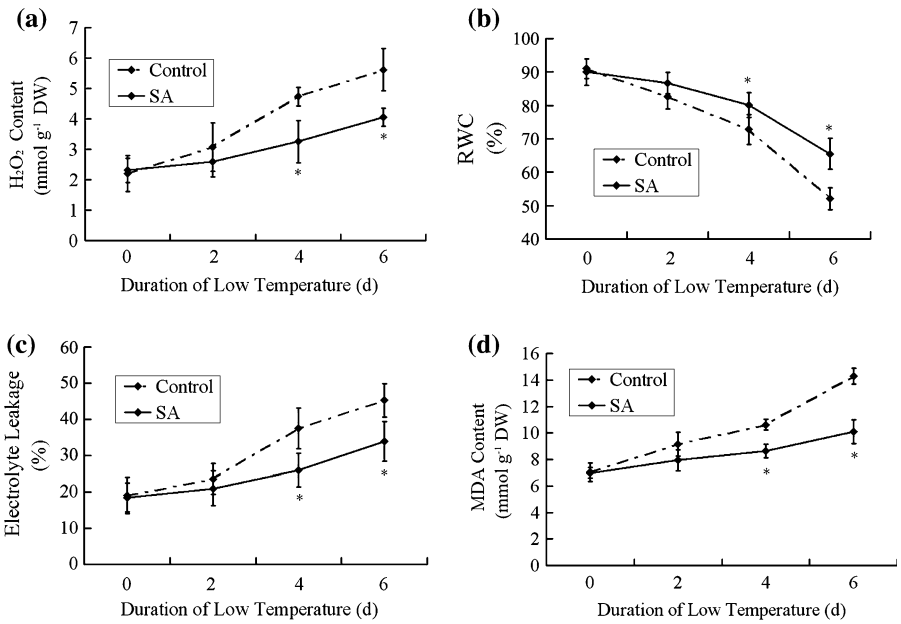

Fig. 4 Effects of SA on H_2O_2 content (**a**), RWC (**b**), EL (**c**) and MDA content (**d**) in the third leaf of cucumber seedlings under low temperature stress. Cucumber seedlings were treated as described in Fig. 1. *Bars* represent standard deviations of five independent measurements. Statistically significant different at $*P < 0.05$

Discussion

The growth rate of plant was dependent upon a constant supply of ATP. Under various environmental stresses, such as drought, low temperature and UV-induced oxidative stress, electron transport chain through the cytochrome pathway may be inhibited. This process could activate the alternative pathway in mitochondria (He et al. 1999a, b; Yan et al. 2004; Zhao et al. 2007). That means the relative activity of the cytochrome pathway and the alternative pathway plays an important role in maintaining plant growth rate homeostasis under environmental stresses by providing constant ATP (Hansen et al. 2002; Moore et al. 2002). Thus, the alternative pathway and AOX were suggested to play a protective role against environmental stresses (Zhao et al. 2007). In our present work, alternative pathway capacity and AOX expression in cucumber seedlings were also detected to be enhanced by low temperature stress.

Salicylic acid (SA) has been proved to induce AOX expression and the alternative pathway respiration in plant mitochondria (Rhoads and McIntosh 1992, 1993). It was also indicated that SA-induced resistance to virus could be mediated by a salicythydroxamic acid (SHAM)-sensitive signaling pathway, in which AOX was probably involved (Chivasa et al. 1997; Chivasa and Carr 1998). Furthermore, Gilliland et al. (2003) indicated that SA-induced resistance resulted from the activation of multiple antiviral mechanisms, a subset of which was influenced by AOX. Ordog et al. (2002) examined the effect of AOX expression on the SA-induced resistance in plants, and drew a conclusion that AOX was not a critical component of plant viral resistance but might play a role in the hypersensitive response. The inhibition of electron transfer and ATP synthesis in mitochondria also induced the transcription of AOX gene (Saisho et al. 2001). Thus, as an inhibitor of both ATP synthesis and O_2 uptake in plants by inhibiting complex I of mitochondrial electron transport chain and certain enzymes (e.g., aconitase) in the TCA cycle, SA was suggested to regulate AOX gene expression (Vanlerberghe and McIntosh 1994, 1996; Xie and Chen 1999). In addition, SA-induced inhibition of mitochondrial functions would cause the accumulation of ROS (Xie and Chen 1999; Norman et al. 2004). ROS, such as H_2O_2, could lead to an increased level of citrate that serves as a signal for enhancing AOX gene expression and the alternative pathway capacity (Vanlerberghe and McIntosh 1994, 1996). In our present work, the data of O_2 uptake rate demonstrated that the alternative pathway capacity in the SA pretreated seedlings was enhanced obviously compared to control. Our results also indicated that the level of the 36-kDa AOX protein and its transcripts in cucumber seedlings were increased by SA pretreatment. These results showed that SA pretreatment induced an enhancement of the expression and accumulation of AOX, consequently, the enhancement of alternative pathway capacity.

Former studies suggested that exogenous SA at high concentrations would be cytotoxic to plant cells (Allan and Fluhr 1997; Anderson et al. 1998; Kawano et al. 1998). However, SA at relatively low level which was not sufficient to cause cell death could play a role in plant defense response (Xie and Chen 1999). And the expanding work by Norman et al. (2004) showed that lower concentrations of SA could be accumulated to higher concentrations in cells and even low concentrations of SA did cause transient changes in mitochondria. SA would not increase H_2O_2 content because under the low SA level, the electron transport chain was uncoupled, not inhibited. According to our previous studies (Lei et al. 2008), 20 μM SA treatment enhanced the alternative pathway respiration and AOX expression in tobacco which grew in normal environmental temperature. In our present work, 20 μM SA also enhanced AOX expression and the alternative pathway capacity in cucumber seedlings before low temperature treatment (0 day in Figs. 1, 2, and 3). However, H_2O_2 content, RWC, EL and MDA content in cucumber seedlings were not influenced obviously by SA pretreatment until low temperature was applied (0 day in Fig. 4). These results suggested that 20 μM SA, which was not harmful to cucumber seedlings under normal temperature, had the ability to induce an increase of the alternative pathway capacity by enhancing expression and accumulation of AOX, without the increase of H_2O_2 or ROS level.

Exogenous SA was considered to play an important role in plant resistance to various environmental stresses such as chilling, heat and water stress (Senaratna et al. 2000). And EL, H_2O_2 and MDA content in cucumbers leaves were all enhanced by chilling stress according to Hu et al. (2006). During the whole process of low temperature stress in our present work, these indexes were also affected. But SA pretreated seedlings always presented slower decrease of RWC and lower level of EL, H_2O_2 and MDA content compared to control. These results indicated that application of 20 μM SA alleviated the injury caused by low temperature in cucumber seedlings. Since the alternative pathway and AOX were suggested to have protective functions against environmental stresses (He et al. 1999a, b; Yan et al. 2004; Zhao et al. 2007), we proposed that the alternative pathway may play a role in SA-mediated plant tolerance to low temperature stress.

It is interesting that a low level of exogenous SA can only alleviate low temperature stress, but not other abiotic stresses. Significantly drought, salt or metal stress tolerances usually need more SA, ranging from 0.1 to 0.5 mM (Yuan and Lin 2008). The possible thermogenic role of the alternative pathway may be accounted as the reason. Since

the alternative pathway is a non-phosphorylating respiration pathway, it wastes two of the three energy coupling sites which are part of the cytochrome pathway and the residual energy is released as heat (Vanlerberghe and McIntosh 1997). But in the high thermostatic capacity of the cold room we were not able to detect a temperature increase during the whole period of low temperature stress. In this regard, Calegario et al. (2003) suggested that although most plants, with the notable exception of thermogenic plant, clearly do not produce enough metabolic heat to raise the temperature of whole plant. However, respiratory heat may have a pronounced local effect at the subcellular level and a local increase in temperature around the individual mitochondrion may be of physiological importance (Calegario et al. 2003).

In conclusion, our results showed that SA pretreatment induced a significant increase in the alternative pathway capacity of cucumber seedlings under low temperature stress. It was indicated to be the result of SA-enhanced expression and accumulation of AOX. SA also alleviated the cold injury caused by low temperature, and increased capacity of the alternative pathway was suggested to be involved in this phenomenon.

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