Effects of Salicylic Acid on Alternative Pathway Respiration and Alternative Oxidase Expression in Tobacco Calli

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Z. Naturforsch. **63 c**, 706–712 (2008); received March 3/April 15, 2008

The alternative pathway (AP) respiration of plants is a cyanide-resistant and non-phosphorylating electron transport pathway in mitochondria. Alternative oxidase (AOX) is the terminal oxidase of the AP and exists in plant mitochondria as two states: the reduced, non-covalently linked state or the oxidized, covalently cross-linked state. In the present study, the effects of 20 μM exogenous salicylic acid (SA) on both AP activity and AOX expression in mitochondria of tobacco (*Nicotiana rustica* L. cv. yellow flower) calli were investigated. The results showed that SA treatment enhanced the AP activity. During the process of SA treatment, the AP activity increased dramatically and achieved the peak value after 8 h of treatment. Then it declined until 16 h, and maintained a steady level between 16 and 24 h. Changes in both the total AOX protein level and the reduced state were in accordance with the AP activity, but the oxidized state changed differently. The *aox1* gene transcript level also showed a similar change as the AP activity and AOX protein level. The induction of AOX expression by low concentrations of SA was inferred through a reactive oxygen species (ROS)-independent pathway. These results indicate that the enhancement of AP activity in response to SA is correlated to the expression of AOX, and the reduced, non-covalently linked state of AOX plays an important role during this process.

Key words: Alternative Pathway (AP), Alternative Oxidase (AOX), Salicylic Acid (SA)

Introduction

A difference between plant and animal mitochondrial respiration is that there are two main electron transport pathways in plant mitochondria: one is the cytochrome pathway (CP) and the other the alternative pathway (AP) with its terminal oxidase called alternative oxidase (AOX) (Liang and Liang, 1998). The AP is a cyanide-resistant respiration and non-phosphorylating electron transport pathway. Branching from the cytochrome chain at the ubiquinol pool, it bypasses two of the three energy-conserving sites (complexes III and IV), and the potential energy of the system is lost as heat. So, the accumulation of AOX protein and enhancement of AP respiration were initially considered to cause the increase in the temperature of plant tissues (Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997). Afterwards, AP and AOX have many other functions, such as maintaining stabilization of the respiratory electron chain and the tricarboxylic acid (TCA) cycle (Vanlerberghe and McIntosh, 1994), scavenging reactive oxygen species (ROS) in plant cells (Maxwell *et al*., 1999; Møller, 2001), playing a role in the process of programmed cell death (PCD) (Vanlerberghe *et al*., 2002), and maintaining plant growth rate homeostasis (Hansen *et al*., 2002; Moore *et al*., 2002).

Salicylic acid (SA) is a phenolic compound known to be an important signal molecule in plant cells. It has been indicated to regulate many physiological processes in plants, for example, thermogenesis in which the AP is involved (Raskin *et al*., 1987; Liang and Liang, 1998). By previous studies, we found that the increase in the AP of potato tuber slices is associated with the increase in endogenous SA (Wen and Liang, 1994a), and exogenous SA treatment resulted in enhancement of the AP and increase in temperature (Wen and Liang, 1994b; Wen *et al*., 1995). Other studies

also proved that SA is an inducer of the AP and AOX expression in plants (Rhoads and McIntosh, 1992, 1993; Yuan and Lin, 2008). Furthermore, application of methyl salicylate (MeSA), the mobile form of SA (Park *et al*., 2007), also induced the expression of *aox* genes (Fung *et al*., 2004, 2006).

The AOX protein locates in the inner mitochondrial membrane as two states: the reduced, noncovalently linked state and the oxidized, covalently cross-linked state (Umbach and Siedow, 1993; Umbach *et al*., 1994; Vanlerberghe and McIntosh, 1997). The reduction of the intermolecular disulfide bonds at the conserved cysteine residues in the oxidized state resulted in a more active state of AOX leading to increased AP activity (Umbach and Siedow, 1993; Vanlerberghe *et al*., 1998). Therefore, we infer that changes in the relative content of these two AOX protein states may affect the activity of the AP. In addition, although there were numerous studies on effects of SA on AP respiration and AOX expression, researches on the relationship between the allosteric mechanism of AOX and AP respiration in response to SA treatment are rare. In the present work, we studied the effects of SA on AP activity. The expression of AOX, especially the accumulation of both states of the AOX protein, was also investigated to understand how SA affects AP respiration.

Materials and Methods

Plant material and SA treatment

Tobacco (*Nicotiana rustica* L. cv. yellow flower) calli were dedifferentiated from seedling leaves on a basal Murashige and Skoog medium supplemented with 2,4-D (2 mg/L), 6-BA (0.5 mg/L), 3% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8). The calli grew in a growth chamber at 25 °C and were subcultured once a month. When grown to the 15-day stage, calli were incubated with 2 mL sterile SA solution (20 μM) for 0, 4, 8, 12, 16, 20 and 24 h, respectively. Control samples were treated with sterile water parallel with SA-treated samples.

Tobacco calli respiration measurement

The respiration rate was measured according to Li *et al*. (2001) with some modifications. Tobacco calli were placed in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 25 °C. Inhibitors of the CP (1 mM KCN) and the AP [20 μM *n*-propyl gallate, according to Vanlerberghe *et al*. (2002)] were used. The O_2 uptake in the absence of any respiration inhibitors presented the total respiration, and that in the presence of both KCN and *n*-propyl gallate showed the residual respiration. The AP activity representing the extent of actual operation of AP respiration was obtained from total respiration minus O_2 uptake in the presence of *n*-propyl gallate. The CP activity was calculated from the O_2 uptake in the presence of *n*-propyl gallate minus residual respiration.

Isolation and purification of tobacco calli mitochondria

Mitochondria were isolated and purified from tobacco calli treated with SA for different times based on the procedure of Yan *et al*. (2002). Tobacco calli were homogenized in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 300 mM mannitol, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 0.1% (w/v) bovine serum albumin (BSA), and 0.5% (w/v) polyvinyl-polypyrrolidone (PVPP). The homogenate was centrifuged at 1,000 $\times$ *g* for 15 min, and the supernatant was then centrifuged at 10,000 $\times$ *g* for 15 min. The resulting pellet was resuspended in 10 mM Tris-HCl (pH 7.2), 300 mM mannitol, 1 M EDTA, and 1% (w/v) BSA. Samples were layered onto percoll gradients, then centrifuged at 40,000 $\times$ *g* for 30 min. Mitochondria, recovered in percoll, were washed with 10 mM Tris-HCl (pH 7.2), 300 mM mannitol, and 1 M EDTA. All steps were below 4 °C. Protein concentrations were determined by the Bradford method using BSA as a standard (Bradford, 1976).

SDS-PAGE and Western blot analysis

Mitochondrial proteins were separated by reducing and non-reducing SDS-PAGE according to Vanlerberghe *et al*. (1998) and Yan *et al*. (2002). For reducing SDS-PAGE, 50 μg of proteins from each sample were pretreated with an equal volume of sample buffer [50 mM Tris-HCl (pH 6.8), 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.005% (w/v) bromophenol blue]. Omitting the β-mercaptoethanol from the sample buffer performed non-reducing SDS-PAGE analysis. All of these samples were boiled for 3 min. SDS-PAGE analysis was performed and then proteins were transferred to a nitrocellulose membrane. After transfer, the blot was probed with a 1:100 dilution of a monoclonal antibody (AOA) raised against the *Sauromatum guttatum* AOX

protein (Elthon *et al.*, 1989) by the standard method of Sambrook *et al.* (1989). The secondary antibody was goat-anti-mouse IgG conjugated with alkaline phosphatase (diluted 1:500). The colour was developed by reaction with colour buffer supplemented with 5% (w/v) *ϱ*-nitro blue tetrazolium chloride and 5% (w/v) 5-bromo-4-chloro-indolyl phosphate. 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 tobacco calli by 500 μL of extraction buffer [20 mM Tris-HCl (pH 8.0), 1% (w/v) SDS, 200 mM NaCl, 5 mM EDTA and 1:1 (v/v) phenol/chloroform] 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 concentrations were determined spectrophotometrically.

For Northern blot analysis, equal amounts of total RNA (30 μg) were separated on formaldehyde agrose gels, and then transferred to a nylon membrane for subsequent probe hybridization. A PCR amplification product of tobacco (*Nicotiana rustica* L. cv. yellow flower) *aox1* cDNA fragment was used as a probe. The primers used to generate the *aox1* probe were: 5′-GGAGAAAGCGGTGGTGATGT-3′ and 5′-TAACCCATAGTGATTCGGTG-3′. The Northern blot hybridization was performed with a DIG-labeled probe, and positive signals were visualized following the instruction manual of DIG Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). The intensity of the signals was analyzed densitometrically by scanning the blots with a thin-layer scanner.

Statistical analyses

The results were means of five independent measurements 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

Effects of SA treatment on the AP activity

The AP activity represents the ability of the plant to transport a respiratory electron flow along AP respiration. In the present work, in order to avoid undulation of AP respiration and AOX expression during the photoperiod, tobacco calli for experiment were harvested simultaneously. We recorded the index of AP activity in control samples to confirm that AP respiration did not change with water treatment (Fig. 1, control). As shown in Fig. 1, SA enhanced the activity of the AP in tobacco calli. During the process of SA treatment, the AP activity increased dramatically and achieved the peak value after 8 h of treatment, and then declined gradually till 16 h. Between 16 and 24 h, the AP activity maintained a stable level, which was a little higher than the data at 0 h.

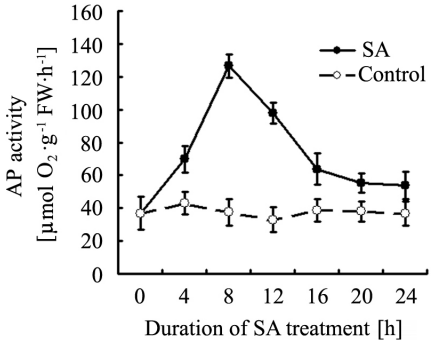

Fig. 1. Effects of SA on the AP activity. Tobacco calli were treated with 20 μM SA for different durations, and samples harvested at each point of duration were used. AP activity in control samples was studied to confirm that AP respiration did not change with water treatment. Bars represent the standard deviations of five independent replicates.

Effects of SA treatment on the allocation of respiration electron flux between the CP and AP

Since the AP is an electron transport chain which branches after the site of ubiquinone and shares the common ubiquinone pool with the CP, they may impact on each other. We compared the changes in contributions of the CP and AP to total respiration during SA treatment in order to observe the effects of SA on allocation of respiration electron flux between the CP and AP (Fig. 2). In the beginning of SA treatment, the proportion of electron flux distributed from the CP was much higher than that of the AP, suggesting that the CP was the main electron transport pathway in tobacco calli under normal growth conditions. However, during SA incubation, the proportion of the CP declined quickly and reached the rock bottom

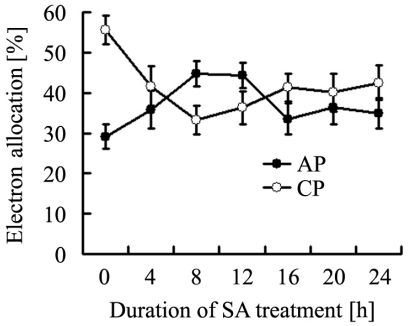

Fig. 2. Effects of SA on the allocation of respiration electron flux between the CP and AP. The allocation of respiration electron flux between CP and AP was recorded by the percentages of CP and AP take from total respiration. Bars represent the standard deviations of five independent replicates.

at 8 h. On the contrary, the proportion of the AP was obviously enhanced by SA and achieved the peak value after 8 h of treatment. But it declined with longer SA incubation and that of CP increased. Then, data of both pathways reached a close level at 16 h. Between 16 and 24 h, both proportions shared a similar and stable level, indicating that the CP and AP achieved homeostasis.

Effects of SA treatment on the AOX protein level and states

There are two states of AOX protein existing in tobacco mitochondria: the 35 kDa monomer (reduced state) and the 70 kDa disulfide linked dimer (oxidized state). The 35 kDa monomer is much more active than the 70 kDa dimer (Vanlerberghe *et al.*, 1995). We examined the AOX protein total level and the levels of these two states in tobacco calli in order to determine the effects of SA on AOX protein accumulation and allosteric mechanism. In tobacco mitochondria, β-mercaptoethanol was used to reduce the disulfide link, so the AOX protein existed as 35 kDa form and implied the total level. In samples without β-mercaptoethanol treatment, the AOX protein existed as both the 35 and 70 kDa forms, *i.e.* reduced state and oxidized state. As shown in Fig. 3a, SA enhanced the total level of the AOX protein. The total level increased dramatically and achieved the peak value at 8 h (about 2-fold of the beginning), then declined gradually and reached a stable level at 16 h. However, during the process of SA treatment, changes in the 35 and 70 kDa levels were not parallel. The 35 kDa protein level reached a peak at 8 h, but the level of the 70 kDa AOX protein represented a continue increase and achieved the peak value at 16 h. In addition, after 8 h, the 35 kDa AOX protein level declined gradually and reached a stable level at 16 h; the level of the 70 kDa AOX protein decreased after 16 h (Fig. 3b). These results suggest that effects of SA on the 35 kDa but not 70 kDa AOX protein level are consisted with the AP activity.

Effects of SA treatment on the aox1 transcript level

In the present study, we also studied the transcript level of *aox1*, the nuclear gene encoding

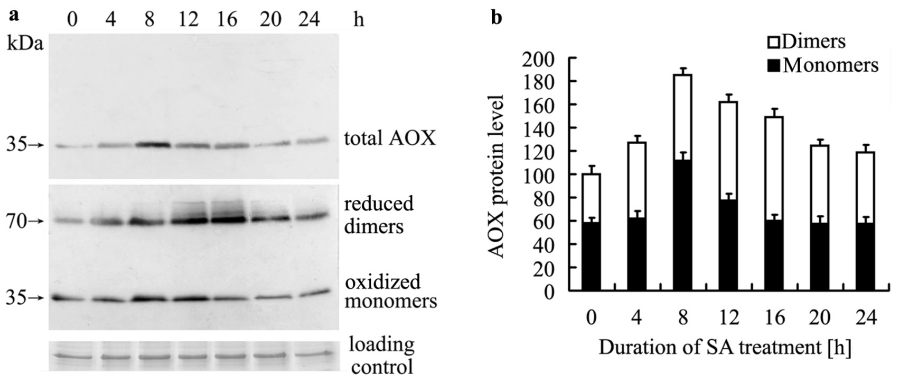

Fig. 3. Effects of SA on the AOX protein level. AOX protein was detected by Western blot. Equal amounts (50 μg) of mitochondrial protein were loaded in each lane and blotted with the AOA. An uncertain protein was used as loading control. (a) Levels of total AOX protein, reduced and oxidized AOX protein states. (b) Relative AOX protein level in SA-treated samples. The total protein level at 0 h of SA treatment was defined as 100%. Bars represent the standard deviations of five independent replicates.

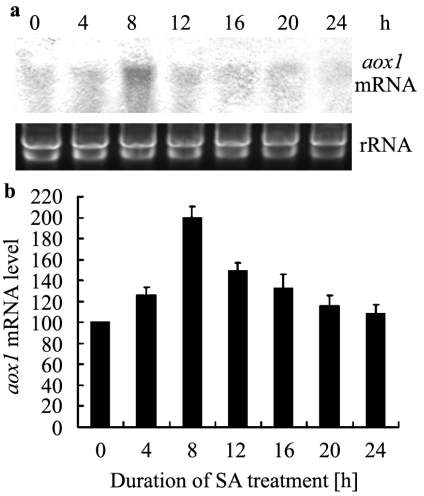

Fig. 4. Effects of SA on the *aox1* transcription level. (a) *aox1* mRNA was detected by Northern blot. Equal amounts (30 μg) of total RNA were loaded in each lane and blotted with the specific DNA probe. rRNA was used as loading control. (b) Relative *aox1* transcript level in SA-treated samples. The level at 0 h of SA treatment was defined as 100%. Bars represent the standard deviations of five independent replicates.

AOX. As shown in Fig. 4, SA treatment enhanced the level of *aox1* transcript dramatically. During SA incubation, the *aox1* transcript level increased quickly and achieved the peak value at 8 h, with a level about 2-fold the one of the beginning. But it decreased steadily and reached a stable level after 16 h of treatment. These results were similar to the changes in the AOX protein level. It was suggested that the enhancement of AOX protein accumulation was based on the increased transcription of *aox1* gene under SA treatment.

Discussion

The AP is a cyanide-resistant respiration and non-phosphorylatory electron transport pathway, and its activity in plant mitochondria is always consistent to the expression of terminal AOX. Previous studies showed that AOX expression could be induced by ROS formation, especially by H_2O_2 (Wagner, 1995; Vanlerberghe and McIntosh, 1996). Many studies demonstrated that SA is an inhibitor of H_2O_2-scavenging enzymes, such as catalase and ascorbate peroxidase (Durner and Klessig, 1995; Rao *et al.*, 1997). Therefore, SA-induced AOX expression was suggested to be associated with H_2O_2 accumulation in plant cells. However, a ROS-independent pathway, in which tricarboxylic acid (TCA) cycle intermediates were involved, was also proved to regulate AOX expression (Gray *et al.*, 2004). For example, exogenously supplied TCA cycle citrate, malate and 2-oxoglutarate caused rapid and dramatic increases in the steady-state level of *aox1* mRNA without significant increases in ROS levels (Gray *et al.*, 2004). In addition, the inhibition of electron transfer and ATP synthesis in mitochondria also induced the transcription of AOX (Saisho *et al.*, 2001; Norman *et al.*, 2004). As an inhibitor of both ATP synthesis and O_2 uptake in plant mitochondria by inhibiting complex I of the mitochondrial electron transport chain and certain enzymes in the TCA cycle (*e.g.* aconitase, whose inhibition would cause an increased citrate level), SA was suggested to regulate the AOX gene expression (Vanlerberghe and McIntosh, 1994; Norman *et al.*, 2004). Thus, two main pathways could be inferred in SA-induced AOX expression: one involving SA-caused ROS accumulation and the other being ROS-independent.

In the present study, 20 μM SA was enough to induce an increased AP activity (Fig. 1), but not to stimulate a visible accumulation of H_2O_2 (H_2O_2 content maintained stable during the whole process, data not shown.). We observed the effects of SA on *aox1* mRNA and AOX protein levels. Results showed that during the process of SA treatment, both mRNA and protein levels were increased with a peak value at 8 h (Figs. 3 and 4). These changes were in accordance with that of AP activity. Therefore, we considered that 20 μM SA in our work regulated the AOX expression mainly through the ROS-independent pathway, but not by accumulation of H_2O_2. Consequently, the activity of AP respiration and the allocation of respiration electron flux were influenced.

The AOX protein exists in the inner membrane of plant mitochondria as two states: a reduced, non-covalently linked form and an oxidized, covalently cross-linked form (Umbach and Siedow, 1993; Umbach *et al.*, 1994; Vanlerberghe and McIntosh 1997). The activity of the reduced form was proved to be 4- to 5-fold higher than that of the oxidized state (Moore *et al.*, 1995), and could be stimulated by certain salts of organic acids, such as pyruvate and citrate, which allosterically activate AOX (Liang and Liang, 1998; Gray *et al.*, 2004). In our present study, SA treatment enhanced the level of the total AOX protein. But the changes in two states of the AOX protein were

different (Fig. 3). Since SA was suggested to affect the TCA cycles and/or the mitochondrial electron transport, we considered that certain intermediate organic acids might cause the different changes in two states of the AOX protein. The present results showed that changes in the reduced state of the AOX coincided with the changes in the AP activity. However, changes in the oxidized state varied from those of the AP activity, and reached the peak value after a longer incubation period of SA (Fig. 3). Therefore, we suggested that it was the reduced state but not the oxidized state playing an important role in the response of the AP activity to SA.

In conclusion, the present results indicate that exogenous treatment with 20 μM SA could regulate the mitochondrial respiration in tobacco calli through a ROS-independent pathway of AOX expression. SA treatment enhanced the expression of the AOX both in mRNA and protein levels. The effects of SA on the AP activity were mainly based on the accumulation of the reduced state of the AOX protein.

Acknowledgements

The authors thank Dr. Thomas E. Elthon (University of Nebraska, USA) for his generous gift of AOA. This work was supported by grants from the National Natural Science Foundation of China (30670166), Key Project of Chinese Ministry of Education (108110), New Century Excellent Talents Program (NCET-05-0786), and Sichuan Science and Technology Foundation (04ZQ026-036, 2006J13-088).

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