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The enhancement of salt stress tolerance by salicylic acid pretreatment in *Arabidopsis thaliana*

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Abstract

Salicylic acid (SA) is an important plant hormone involved in the activation of defense responses against environmental stresses. However, there are still large of unsolved mysteries about how SA pretreatment affects the establishment of plant stress tolerance. In this study, application of SA at different concentrations and different times were conducted to investigate their effects on the response of *Arabidopsis* seedlings to salt stress. The pretreatment with 10 or 20 μ M SA for more than 6 h promoted *Arabidopsis* seedlings resistance to salt stress. On the other hand, pretreatment with 200 μ M SA reduced *Arabidopsis* resistance to salt stress and aggravated oxidative damage to the seedlings. At all concentrations used, SA pretreatment inhibited the total respiration and promoted reactive oxygen species (ROS) generation. However, the ROS content in 10 or 20 μ M SA pretreated seedlings decreased to the basal level within 6 h and high activities of antioxidant enzymes and alternative oxidase were maintained. Notably, the SA-enhanced salt stress resistance was significantly impaired by blocking alternative oxidase (AOX) pathway. Our findings indicate that SA-mediated salt stress response is in a dose- and time-dependent manner and that the effects were related to the induction of AOX capacity and antioxidant system.

Additional key words: alternative oxidase, antioxidant enzymes, reactive oxygen species, resistance index.

Introduction

Environmental stresses are often limiting for plant productivity (Kumar *et al.* 2015). In order to cope with such adverse situations, plants have evolved well-developed mechanisms that help to perceive the stress signal and enable optimal growth response (Verma *et al.* 2016). Phytohormones, such as salicylic acid (SA), have been shown to play critical roles in helping the plants to adapt to adverse environmental conditions (Miura and Tada 2014, Sharma 2014). The SA is one of a diverse group of phenolic compounds, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, is synthesized by plants and is induced after infection by many pathogens or abiotic stress (Fragniere *et al.* 2011, Pál *et al.* 2013). It has been reported that endogenous SA content increased by water deficit, low temperature, salt

salinity, *etc.* (Scott *et al.* 2004, Sawada *et al.* 2006, Okuma *et al.* 2014). However, it is likely that the over-accumulation of endogenous SA triggers the overproduction of reactive oxygen generation (ROS), which results in stress sensitivity (Scott *et al.* 2004, Miura *et al.* 2013). Therefore, SA has been proposed to have a dual role in the regulation of plants response to stress conditions: lower SA accumulation plays a protective role in stress-induced oxidative damage, while higher amounts of SA have an adverse effects (Jayakannan *et al.* 2015). For instance, it was noted that SA-deficient *NahG* transgenic *Arabidopsis* plants displayed a higher adaptation to moderate salt stress, whereas the high-level SA *Arabidopsis* ecotype Cvi-0 showed a worse adaptation (Borsani *et al.* 2001, Cao *et al.* 2009).

Since SA is known to affect various physiological and biochemical activities of plants and has great potential to improve the stress tolerance of agriculturally important

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Submitted 6 March 2019, *last revision* 21 October 2019, *accepted* 2 December 2019.

Abbreviations: AOX - alternative oxidase; APX - ascorbate peroxidase; CAT - catalase; DI - damage index; GPX - guaiacol peroxidase; MDA - malondialdehyde; MOE - molecular operating environment; Qi - quinone reduction; ROS - reactive oxygen species; RI - resistance index; SA - salicylic acid; SHAM - salicylhydroxamic acid; SOD - superoxide dismutase.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (31400242, 31900242, 31500209, 31271686) and the Outstanding Youth Science and Technology Innovation Team of Hubei Province, China (T201732).

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crops, large number of studies were carried out to elucidate the effect of exogenous SA on growth, physiology, and stress response of plants grown under adverse conditions. Notably, it has been proposed that the utility of SA rely on the concentration of the applied SA and the developmental stage of the plants (Miura and Tada 2014). In many previous studies, SA was applied at micromolar concentrations (Norman *et al.* 2004, Ashraf *et al.* 2010) rather than at millimolar concentrations (Maxwell *et al.* 2002, Gilliland *et al.* 2003), showing to be beneficial for plant growth and plant tolerance to environmental stresses. For example, when the applied SA concentration is less than 10 μM , it could significantly alleviate the decline in photosynthesis caused by salt stress in many plants (Stevens *et al.* 2006, Szepesi *et al.* 2009, Wasti *et al.* 2012), but in the case of 1 - 5 mM SA application, the opposite effects occurred (Nazar *et al.* 2011). The research of Lee *et al.* (2010) showed that the application of more than 100 μM SA aggravated the inhibition of seed germination of *Arabidopsis* at high salinity, while less than 50 μM SA mitigated this inhibition.

In addition to the difference in exogenous SA concentration, SA pretreatment time was also different in previous studies. Generally, plants were pretreated with SA for 24 h followed by a variety of stress conditions (Khan *et al.* 2012, Palma *et al.* 2013, Singh *et al.* 2015). In addition, the application of SA in plants for 2 d (Palma *et al.* 2013), 7 d (Singh *et al.* 2015), 14 d (Bai *et al.* 2015), 21 d (Horváth *et al.* 2015) or more have also been implemented in the past. However, it is important to note that previous studies focused mainly on the effects of SA pretreatment on plant resistance after a certain concentration or a certain time. Less attention has been paid on the effects of different processing times of SA at a certain concentration on plants stress acclimation.

The aim of this study, therefore, is to further investigate the effects of pretreatment with different concentrations of SA on plant response to salt stress, and in particular to reveal the time effects of SA pretreatment on plant stress resistance. The changes in respiration rate, ROS production, and antioxidants enzyme activities were analyzed during SA pretreatment lasting 0 - 24 h. In addition, the resistance index (RI) was calculated to evaluate the differences in the ability of plants stress acclimation. To investigate the inhibitory effect of SA on cellular respiration, the possible docking sites of SA in cytochrome *bc1* were analyzed.

Materials and methods

Plant growth and chemical treatments: Seeds of *Arabidopsis thaliana* L. cv. Columbia (Col; wild-type) were surface-sterilized in 20 % (v/v) commercial bleach for 20 min, followed by six washes with sterile distilled water. The seeds were sown onto 1/2 MS agar plates for germination. The plates with the seeds were placed at 4 °C in the dark for 48 h to improve germination uniformity before transfer to growth chambers with 16-h photoperiod, an irradiance of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperatures 22/18 °C, and a 70 % relative humidity for 7 d. Then, the seedlings were transferred from MS agar plates to pots

containing *Vermiculite*, *Perlite*, and *Soilrite* mix (1:1:1 by volume). Pots were placed on the same condition in growth chamber.

For SA pretreatment, the four-week-old seedlings (rosette growth is almost complete) were sprayed with different concentrations of SA (*Sigma*, St. Louis, MO, USA) and the control seedlings were sprayed with deionized water. To study the affection of AOX inhibition on SA-mediated plant response to stress, 1 mM salicylhydroxamic acid (SHAM, *Sigma*), which is an inhibitor of alternative oxidase, was used in this experiment. For each treatment or independent experiment, at least 5 seedlings were used for experimental testing and analysis.

Salt stress treatment: To investigate the effects of SA pretreatment at different concentrations on plant response to salt stress, 200 mM NaCl was used in this study. Every two hours (from 0 to 24 h) after pretreatment with different concentrations of SA, at least five seedlings from each treatment were transferred to salt stress. The desired salinity was maintained by irrigating the soil with 200 mM NaCl and the trays were also filled with the same concentration of saline solution. The saline solution in the tray was replaced twice every day in order to ensure the consistency of NaCl concentration. All analyses performed after 5 d of salt stress treatment.

Leaf respiration measurements: Respiratory oxygen consumption was measured using Clark-type electrodes (*Hansatech*, King's Lynn, UK) based on the methods of Xu *et al.* (2012). The leaves (0.05 g) were cut into small pieces, then pretreated with 5 cm³ of deionized water for 15 min in order to eliminate wound-induced respiration. Measurements were done at 25 °C in a final volume of 2 cm³ of phosphate buffer (pH 6.8), and the cuvette was tightly closed to prevent diffusion of oxygen from the air. Inhibitors of the cytochrome pathway (1 mM KCN) and the alternative pathway (1 mM SHAM) were used. The AOX pathway respiration is defined as O₂ uptake rate in the presence of KCN that was sensitive to SHAM. Total respiration is defined as O₂ uptake rate by samples without any inhibitors.

Ion leakage was measured according to the method of Xu *et al.* (2012). After measuring the conductivity, the samples were boiled for 15 min to achieve 100 % ion leakage.

Malondialdehyde (MDA) content was measured according to the method of Sun *et al.* (2006). Approx. 0.2 g of fresh leaves were cut into small pieces and homogenized by the addition of 5 cm³ of 5 % (m/v) trichloroacetic acid (TCA) in an ice bath. The homogenate was transferred into a tube and centrifuged at 1 000 g and 4 °C for 10 min. Aliquots of supernatant and 0.5 % (m/v) thiobarbituric acid (TBA) in 20 % TCA solution were added into a new tube. This mixture was incubated at 98 °C for 40 min, 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 coefficient

of absorbance of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Hydrogen peroxide content of leaves was measured as described by Velikova *et al.* (2000). Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $12\,000 \text{ g}$ and 4°C for 20 min and 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The absorbance of the supernatant was read at 390 nm.

Measurement of leaf chlorophyll content: Leaf chlorophyll was extracted with 80 % (v/v) acetone from the fresh leaves and measured according to Lichtenthaler and Wellburn (1983).

Evaluation of damage index and resistance index of stressed seedlings: The damage index was categorized according to the methodology as follows:

Damage index (DI) = $[R_{\text{IL}} + R_{\text{MDA}} + R_{\text{H}_2\text{O}_2} + R_{\text{CHL}}] / 4$; IL - ion leakage, CHL - chlorophyll.

R_x : Decreased ratio of stressed samples relative to normal growing samples.

For R_{IL} , R_{MDA} , and $R_{\text{H}_2\text{O}_2}$ calculation, $R_x = 1 - (\text{value of stressed samples} / \text{value of normal samples})$. For R_{CHL} calculation, $R_x = (\text{value of stressed samples} / \text{value of normal samples}) - 1$.

Resistance index (RI) = DI (SA-pretreated samples) - DI (control samples). The RI of control seedlings is marked as number 0 (RI = 0). RI > 0 means that stress tolerance is better than the control. In contrast, RI < 0 means that the stress tolerance is worse than the control.

Antioxidant enzymes assay: A crude enzyme was extracted by the method of Wang *et al.* (2011). Leaves (0.5 g) were ground with 3 cm^3 of ice-cold 25 mM Hepes buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2 % (m/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at $12\,000 \text{ g}$ and 4°C for 20 min and the resulting supernatants were used for the determination of enzyme activities at 25°C . Protein content was determined according to Bradford (1976) with bovine serum albumin as standard.

The superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium (Stewart and Bewley 1980). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, $75 \mu\text{M}$ nitroblue tetrazolium (NBT), $2 \mu\text{M}$ riboflavin and 0.02 cm^3 of enzyme. One SOD unit was defined as the amount of enzyme that led to 50 % inhibition of NBT reduction.

The catalase (CAT, E.C. 1.11.1.6) activity was measured by monitoring the decrease in absorbance of H_2O_2 at 240 nm (Beers and Sizer 1952). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 15 mM H_2O_2 , and 0.02 cm^3 of enzyme extract. One CAT unit was defined as the amount of enzyme necessary to decompose $1 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$

under certain conditions.

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by the decrease in the absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada 1981). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA- Na_2 , $0.12 \text{ mM H}_2\text{O}_2$ and 0.020 cm^3 of enzyme extract. One APX unit was defined as the amount of enzyme that oxidizes $1 \mu\text{mol ascorbate min}^{-1}$ under certain conditions.

The guaiacol peroxidase (GPX, EC 1.11.1.9) activity was measured as the increase in the absorbance at 470 nm due to guaiacol oxidation (Uarrotta *et al.* 2016). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 19 mM H_2O_2 , 9 mM guaiacol and 0.02 cm^3 of enzyme extract. One GPX unit was defined as the amount of enzyme that produce $1 \mu\text{mol oxidized guaiacol min}^{-1}$ under certain condition.

Molecular docking: To investigate the possible binding sites of SA and the cytochrome *bcl* complex, the homology modeling of *Arabidopsis* cytochrome *bcl* complex subunit III (Swiss-Prot: P42792.2) was carried out by Swiss-Mode (<https://swissmodel.expasy.org/>) based on the X-ray structure of bovine cytochrome *bcl* complex (PDB id: 1PPJ). The protein structures alignment was performed by PyMOL 2.3.1. The ligand SA was prepared and minimized with molecular operating environment (MOE 2014.0901, Chemical Computing Group). Optimization and minimization of 3-D structure of the receptor were conducted using MOE software with addition of hydrogen atoms. Protonation was employed with protonating 3D programs. Then the protein-ligand docking compute was carried out and analyzed.

Statistical analysis: Statistical analysis of the results from experiments with three or more mean values used a one-way analysis of variance (ANOVA) as dictated by the number of main effects. The difference was considered to be statistically significant when $P < 0.05$.

Results

To investigate the effects of SA pretreatment at different concentrations and different time points against salt stress, the four-week-old *Arabidopsis* seedlings were pretreated with SA and then treated with salt stress (200 mM NaCl) for 5 d. The electrolyte leakage, MDA and H_2O_2 content, total chlorophyll content, and resistance index were evaluated and compared among different samples (Table 1 Suppl., Fig. 1 Suppl.). Results showed that lower concentrations of SA (10 and $20 \mu\text{M}$) significantly promoted plant salt stress tolerance, whereas pretreatment with higher concentrations of SA (especially $200 \mu\text{M}$) impaired plant resistance to salt stress. However, it should be noted that the promotion effects of lower concentration of SA on plant stress acclimation were in a time-dependent manner. For example, there was no significant difference between $10 \mu\text{M}$ SA-pretreated seedlings and the control seedlings if they were subjected to the salt stress after 2 or

4 h pretreatment. The best promotion effects of 10 μ M SA pretreatment in plant resistance to salt stress occurred after 8 - 18 h. In comparison, the 20 μ M SA-pretreated seedlings displayed stronger resistance to salt stress than the control as well as the 10 μ M SA-pretreated seedlings when they were exposed to salt stress after 6 h. However, if the seedlings were pretreated with 20 μ M SA only for 2 or 4 h, they were more vulnerable to damage than the control. Similarly, seedlings pretreated with 50 μ M SA for 2 to 14 h showed serious damage when exposed to salt stress, but, after 22 h of pretreatment with 50 μ M SA, the seedlings showed a little higher stress tolerance than the control (Fig. 1). In addition, 200 μ M SA pretreatment reduced the plant resistance to salt stress in each time point (Fig. 1). These results indicated that lower concentrations of SA pretreatment were beneficial for the plant tolerance to salt stress, but the promotion effects were time-dependent.

Previous work demonstrated that SA application may inhibit the plant mitochondrial respiration directly or indirectly (Xie and Chen 1999, Norman *et al.* 2004). Therefore, respiratory rate assay after different concentrations of SA pretreatment was investigated. As shown in Fig. 2A, all concentrations of SA pretreatment repressed the total respiration rates (V_t), but at different levels. It should be noted that V_t was also inhibited by the pretreatment with 10 μ M SA and 20 μ M SA during the first 4 h. In comparison, the V_t inhibition observed in the seedlings pretreated by 20 μ M SA was more severe than that observed in the seedlings pretreated by 10 μ M SA during the first 4 h, although they showed similar trend in V_t . There was significant V_t inhibition occurred in 50 μ M SA-pretreated seedlings during the first 10 h, and then the V_t gradually recovered but remained lower than control in the following time.

As shown in Fig. 2B, SA pretreatments inhibited the

V_t while promoted the AOX pathway respiration (V_{alt}). In contrast, the seedlings pretreated with 200 μ M SA had the highest induction rate of V_{alt} during the first 12 h but the lowest V_{alt} during the whole experiment (Fig. 2C). For 50 μ M SA-pretreated seedlings, the V_{alt} was induced significantly but the V_t was lower than in the 10 or 20 μ M SA-pretreated seedlings and control during the first 20 h. Interestingly, it should be noted that the V_{alt} of 20 μ M SA-pretreated seedlings was much higher than that of 10 μ M SA-pretreated seedlings during the whole experiment (Fig. 2B), although they maintained the same V_t as control after 6 h of SA pretreatments (Fig. 2A).

To investigate the effects of AOX inhibition on the SA-mediated plant salt stress acclimation, SHAM (an inhibitor for AOX) was used in the following experiment (Fig. 3). All the SHAM-pretreated seedlings were more sensitive to salt stress than the control and SA-pretreated seedlings. Moreover, the resistance to salt stress induced by 10 or 20 μ M SA was impaired by 1 mM SHAM, and the RI was even lower than in the control (Fig. 3). It should be noted that the seedlings pretreated with 50 μ M SA plus 1 mM SHAM exhibited terrible oxidative damage under salt stress, and the RI was five-fold lower than in the 50 μ M SA-pretreated seedlings and even two-fold lower than in the 200 μ M SA-pretreated seedlings (Fig. 3). Thus, the most serious oxidative damage (lowest RI) was observed in the seedlings pretreated with 200 μ M SA plus 1 mM SHAM (Fig. 3). These results indicated the importance of AOX in plant stress acclimation as well as in SA-mediated plant stress adaptation.

However, it should be noted that the plant resistance was slightly higher when the SHAM-pretreated seedlings were subjected to salt stress after 12 h (Fig. 3). Typically, the RI of SA (10 or 20 μ M) and SHAM co-pretreated seedlings increased gradually if they were transferred

Fig. 1. Effects of salicylic acid (SA) pretreatment at different concentrations and times on the salt stress resistance of *Arabidopsis*. Four-week-old seedlings were sprayed with different concentrations of SA and the control with water, and then every 2 h a group of seedlings was transferred to 200 mM NaCl (salt stress). After 5 d, the plant oxidative damage was measured for evaluating the resistance index (RI). Different colors represent different RIs of seedlings, of which green represents a better RI and red represents a weaker RI.

to salt stress after 12 h. Further, SA + SHAM pretreated seedlings exhibited better adaptation to the salt stress than the SHAM-pretreated seedlings, although their RI values were still lower than that of the SA-pretreated seedlings and even the control (Fig. 3). Accordingly, there should be some other resistance factors besides AOX, which were directly or indirectly induced by the lower concentrations of SA.

Fig. 2. Changes in respiration rates after pretreatment with different concentrations of salicylic acid (SA). Every two hours, V_t (A) and V_{alt} (B) were detected using Clark-type electrodes. In addition, the average data of V_{alt} and V_t during the first 24 h after SA pretreatment were calculated and the V_{alt}/V_t ratio was compared between different samples (C). V_t - total respiration, V_{alt} - alternative oxidase pathway respiration. Means $\pm$ SDs of three independent experiments. Asterisks represent statistical differences relative to a control at the same time point (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

It was shown that inhibition of mitochondrial respiration causes increased production of ROS, which could be scavenged by enzymatic antioxidants to protect plants from oxidative damage (Choudhury *et al.* 2017). Since SA pretreatments were shown to inhibit the V_t , thus the ROS production and the activities of antioxidant enzymes including SOD, CAT, APX, and GPX were investigated then. The results showed that SA pretreatments induced the H_2O_2 generation in a dose-dependent manner (Fig. 4A). Lower concentrations of SA (10 or 20 μM) increased the H_2O_2 generation during the first 8 h, but then decreased to the same level as control. The pretreatment with 50 μM SA induced higher amount of H_2O_2 than 10 or 20 μM SA, while less 200 μM SA pretreatment. The amount of H_2O_2 in 200 μM SA-pretreated seedlings was about four times higher than that of control seedlings during the first 12 h (Fig. 4A).

There were significant increases in activities of SOD, CAT, APX, and GPX after pretreatment with 10 or 20 μM SA for 4 h compared to control (Fig. 4B-E). Among them, the activities of SOD, CAT, and APX maintained high also after 10 h pretreatment. The activities of antioxidant enzymes in 20 μM SA-pretreated seedlings were higher than those of the 10 μM SA-pretreated seedlings, especially for the SOD and APX. Pretreatment with 50 μM SA also promoted the activities of SOD, CAT, APX, and GPX after 2 h, and they were a little higher than those of 10 or 20 μM SA-pretreated seedlings. Moreover, significant increases in the activities of antioxidant enzymes were found in 200 μM SA-pretreated seedlings during the first 10 h, but they decreased sharply after that, especially for the CAT, APX, and GPX.

Considering our results and previous reports mentioned that SA could inhibit cytochrome pathway respiration, especially the state 3 respiration (Norman *et al.* 2004), but little information is available on how SA affects the electron transport *via* the cytochrome pathway. Therefore, we tried to analyze the docking sites of SA in *Arabidopsis* cytochrome *bc1* complex. As shown in Fig. 2 Suppl., the homology model of *Arabidopsis* cytochrome *bc1* complex subunit 3 was generated and the protein-ligand (SA) docking computation was carried out. Results showed that SA can bind to the cytochrome *b* heme center and the gate of quinone reduction (Qi) tunnel (Fig. 5A,B), which is similar to the binding site of antimycin A (a typical inhibitor of cytochrome *bc1*). In addition, the chemical structure alignment showed that antimycin A has a headgroup consisting of 3-formylamino salicylate, that is similar to the chemical structure of SA (Fig. 5C).

Discussion

The effect of exogenous SA on the stress tolerance of plants is not always obvious (Horváth *et al.* 2007). In this study, efforts were made to improve our understanding of the influences of the different pretreatment times with different concentrations of SA on the response of *Arabidopsis* seedlings to salt stress induced by 200 mM NaCl for 5 d. Interestingly, we found that SA has both promotive and

inhibitory effects on plant salt stress tolerance, depending not only on the concentration applied but also on the duration of pretreatment.

Our study indicates that the transient and small amounts of ROS induced by lower concentrations of SA (10 and 20 μM) plays a pivotal role in plant stress resistance, although some previous studies have shown that lower concentrations of SA (0.01 - 0.05 mM), do not effectively induce ROS accumulation (Kumar *et al.* 2015). To explain this, we speculate that the time to detect ROS content is critical to understanding the relationship between SA and ROS production. In this study, the data showed that the ROS content in 10 or 20 μM SA-pretreated seedlings decreased to the same level as in the control after 6 h of pretreatment. Additionally, according to the result of SA docking computation with MOE, we found that SA can bind to the Qi site of cytochrome *bcl* complex, which is similar to the binding site of antimycin A. It is known that antimycin A, one of the first known and most potent inhibitors of the mitochondrial respiratory chain, binds to the Qi site of the cytochrome *bcl* complex (Huang *et al.* 2005, Ransac and Mazat 2010). Notably, antimycin A has a headgroup consisting of 3-formylamino salicylate, which is similar to the chemical structure of SA. So it is possible that SA affects Q-cycle by preventing the return of the electron from haem bH to Qi, which directly or indirectly affects the ROS production. The study of De Souza *et al.* (2011) proposed that SA inhibits the electron transport chain by inactivation of the semiquinone radical in the Q cycle. However, more research is needed to reveal the mechanism of SA-mediated respiration inhibition in cytochrome *bcl* complex and the protein crystallization with SA should be able to achieve it.

Additionally, the evidence in this study indicates that

SA does not only play a pro-oxidant role, but it also has an antioxidant role in concert with antioxidant enzymes. Consistent with previous studies, Horváth *et al.* (2007) reported that a rapid transient increase in ROS is followed by enhanced antioxidative capacity, which protects the plant from the severe damage. Similarly, Hossain *et al.* (2015) stated that H_2O_2 priming induces mild oxidative stress by disruption of cellular ROS homeostasis, but induces the accumulation of latent defense proteins including ROS-scavenging enzymes, resulting in a primed state and enhanced subsequent stress responses. Conversely, the enhanced sensitivity of plants to environmental stress was generally associated with the reduced ROS-scavenging ability (Das and Roychoudhury 2014, Hossain *et al.* 2015).

Pretreatment with 50 μM SA also stimulated the activities of antioxidant enzymes, but the seedlings were less adaptable to salt stress than the control seedlings, especially when the pretreated seedlings were subjected to salt stress after 2 to 12 h. This case might be attributed to the stronger inhibition of respiration rate and a higher rate of ROS generation observed in 50 μM SA-pretreated seedlings, compared to 10 or 20 μM SA-pretreated seedlings and control seedlings, leading to a decrease in the capacity to scavenge ROS within a short time and more serious oxidative damage happened when they suffered from salt stress. Higher concentrations of ROS cause an oxidative stress that the plant is unable to overcome and which may result in the death of the plant (Overmyer *et al.* 2003, Horváth *et al.* 2007). Consequently, it is not difficult to explain why the 200 μM SA-pretreated seedlings were more sensitive to salt stress because more ROS were generated compared to the other pretreated seedlings. Moreover, in 200 μM SA-pretreated seedlings, the initial delayed induction of activities of antioxidant enzymes and

Fig. 3. Comparison of salt stress resistance with or without (SHAM) treatment. The 4-week-old seedlings were pretreated with different concentrations of SA (10, 20, 50, 200 μM) with or without additional of 1 mM salicylhydroxamic acid (SHAM). Then, every 2 h the pretreated seedlings were transferred to 200 mM NaCl. The resistance index was evaluated after 5 d of salt stress. Means of three independent experiments.

rapid decline after 10 h may be one of the main reasons for the higher ROS accumulation in these seedlings. Under stressful conditions, when the level of ROS rises above the threshold value, oxidative burst associated with lipid peroxidation (*i.e.* MDA) and ion leakage is harmful to the plant survival (Das and Roychoudhury 2014, Pospíšil and Yamamoto 2017).

In this study, AOX was certainly involved in SA-mediated plant growth and stress tolerance. AOX is found in all plants and is insensitive to inhibitors of the cytochrome oxidase pathway (Moore *et al.* 2002). The involvement of AOX in the response to salinity stress has been evident in early studies where an increase in the AOX capacity was demonstrated to be responsible for decreasing ROS production in plant cells (Matos *et al.* 2009). It appears that there should be a link between AOX

capacity and ROS formation in response to salt stress of SA-mediated plants (Fig. 3 Suppl.). Here we found that inhibition of AOX activity by SHAM compromised the lower concentrations of SA-mediated salt stress resistance, suggesting that AOX played an important role in stress acclimation and also in SA-mediated stress tolerance. Since SA is an inhibitor of mitochondrial electron transport and it caused the concentration-dependent induction of AOX capacity, it appears that AOX contributes to reduce ROS accumulation caused by SA pretreatment. AOX is thought to be induced by SA and assist in minimizing the production of ROS by the respiratory chain (Maxwell *et al.* 2002). Nevertheless, the relationship between SA and AOX is still unclear, especially whether AOX is directly or indirectly induced by SA remains to be confirmed by further research.

Fig. 4. Changes in H_2O_2 content (A) and antioxidant enzyme activities (B-E) after pretreatment with different concentrations of salicylic acid (SA). Every 2 h the pretreated seedlings were transferred to 200 mM NaCl for 5 d. In this experiment, H_2O_2 content and activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) were determined. Means $\pm$ SDs of three independent experiments. Asterisks represent significant differences relative to a control at the same time point ($P < 0.05$).

Fig. 5. The possible docking sites of salicylic acid (SA) in *Arabidopsis* cytochrome *bc1* complex subunit 3. A cartoon model (A) and a surface model (B) of the docking sites of SA in a receptor protein. The molecular docking analysis was performed with the molecular operating environment (MOE2014.0901) software. In the cartoon model, the yellow-colored ligand is SA and the purple-colored one is antimycin A. In the surface model, residues of receptor are colored according to the electrostatic potential. C - Comparison of the chemical structure between SA and antimycin A.

Conclusions

In short, it is evident that application of SA might be beneficial for plant resistance to environmental stress but the utility of SA depends on the plant species, developmental stage, and the SA concentrations (Wani *et al.* 2016). However, in this study, not only the SA concentration but also pretreatment duration is importance for alleviating salinity stress. For example, pretreatment with 10 or 20 μ M SA contributed to the plants salt stress acclimation only if the SA pretreatment was not less than 6 h and not more than 24 h. Thus, our findings provide some valuable insights into the correlation between the establishment of plant stress resistance and SA applied concentrations and times, which are not only beneficial for further understanding the role of SA in plant response to stress, but also for the possible application of SA in improving the stress tolerance of important crops.

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