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# Exogenous salicylic acid alleviates the oxidative damage of *Arabidopsis thaliana* by enhancing antioxidant defense systems under high light

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## Abstract

To understand the protective roles of salicylic acid (SA) under high light, we investigated oxidative damage of *Arabidopsis thaliana* under high light in the presence or absence of SA. The results indicate that the high light led to an increase in the levels of proline, soluble sugars, reactive oxygen species, malondialdehyde, and electrolyte leakage, and a decrease in stomatal conductance (gs). Activities of six antioxidant enzymes increased significantly under the high light for 1 h. However, the high light for 3 h decreased the activities of peroxidase, superoxide dismutase, and catalase. In addition, we found that exogenous SA effectively improved antioxidant enzyme activities and significantly alleviated ROS accumulation and cell death in A. thaliana under the high light. Therefore, our results show that the high light caused a severe oxidative damage, and SA effectively alleviated the adverse effects of the high light on the plants by regulating the antioxidative defense system.

*Additional key words*: high light, salicylic acid, *Arabidopsis thaliana*, reactive oxygen species, antioxidant enzymes

## Introduction

Photosynthesis is a fundamental process for life on the earth and often limited by different environmental factors. In the past few decades, extreme weather conditions increased dramatically and significantly affected the growth of plants (Chen *et al.* 2017). Although light is the most elementary resource for photosynthesis in plants, low or high sunlight can limit photosynthetic performance. Many plants will be exposed to heat, dryness, and excessive irradiation under high light (Valladares and Niinemets 2008). A previous study indicated that high light limits plant productivity in both natural and agricultural systems (Verslues *et al.* 2006). A strong visible light usually causes retardation of plant growth, decrease in photosynthetic activity (Law and Crafts-Brandner 1999, Yamori *et al.* 2010), stomatal closure (Yamori *et al.* 2006a), rubisco deactivation (Yamori *et al.* 2006b), and decrease in yield (Vollenweider and Gunhardt-Goerg 2005, Wahid *et al.* 2007).

High light usually induces the generation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, which can cause damage to mitochondria and chloroplasts by disrupting cellular structure (Lu and Finkel 2008, Passos *et al.* 2010). Under high light, the main reason of free radical formation is due to limiting the ability for downregulating electron transport from photosystem (PS) II to PS I in thylakoid membranes of chloroplasts, and subsequently resulting in oxidative damage to photosynthetic structures including photosystems (Joliot and Johnson 2011, Zivcak

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*Abbreviations*: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; $c_i$ - intercellular $CO_2$ concentration; DAB - 3,3'-diaminobenzidine; DHA - dehydroascorbate; EL - electrolyte leakage; f.m. - fresh mass; GPX - glutathione peroxidase; GR - glutathione reductase; gs - stomatal conductance; GSH - reduced glutathione; GSSG - oxidized glutathione disulfide; HL - high light; MDA - malondialdehyde; NBT - nitroblue tetrazolium; POD - peroxidase; PS - photosystem; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase.
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*et al.* 2015). To scavenge the excessive ROS induced by high light, plants have evolved effective antioxidant systems consisting of non-enzymatic antioxidants (ascorbate and glutathione) and antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR) (Gill and Tuteja 2010). In addition, plants could also alleviate the oxidative damages by accumulating compatible solutes such as proline and soluble sugars (Ghoulam *et al.* 2002). A previous study reported that exogenously applied compounds could improve plant growth and increase the stress tolerance of plants by enhancing the activity of antioxidant enzymes (Gossett *et al.* 1994). Moreover, the tolerance to environmental stresses in plants is also associated with efficient regulation of photochemical conversion of light energy in chloroplasts by different photoprotective mechanisms (Kramer *et al.* 2004, Brestic *et al.* 2015). A previous report indicated that a key photoprotection process in PS II is non-photochemical quenching, by which excess light energy is harmlessly dissipated as heat (Demmig-Adams and Adams 2006).

Salicylic acid (SA) is an important phenolic compound and usually involved in plant defense response pathways under environmental stresses (Bartoli *et al.* 2013). Many studies indicated that SA could play an important role in pathogenesis-related gene expressions, systemic acquired resistance, and hypersensitive response (Ryals *et al.* 1996, Kunkel and Brooks 2002). In addition, SA is thought to be a growth regulator of plants under abiotic stresses such as ozone (Koch *et al.* 2000), heat (Clarke *et al.* 2004), drought (Nemeth *et al.* 2002), UV-B (Surplus *et al.* 1998), salinity (Borsani *et al.* 2001), and osmotic stress (Shim *et al.* 2003). Some studies also showed that SA is involved in the activation of stress-induced antioxidant system (Alam *et al.* 2014, Janda and Ruelland 2015). Previous studies have suggested that exogenous SA application could improve plant growth and yield (Hashmi *et al.* 2012), induce the activities of antioxidant enzymes (Ghasemzadeh and Jaafar 2013), and regulate some photosynthetic reactions (Arfan *et al.* 2007, Li *et al.* 2014). However, few studies have been performed to understand the regulatory roles of exogenous SA in alleviating the oxidative damage in *Arabidopsis thaliana* under high light.

In this work, the effects of exogenous SA on oxidative stress and antioxidant defense system in *A. thaliana* under high light were investigated by measuring the levels of ROS, callose accumulation, the activities of antioxidant enzymes, and the content of non-enzymatic antioxidants. Moreover, changes in the levels of compatible solutes and stomatal parameters under high light were also determined in *A. thaliana*. We demonstrated that SA may play an important regulatory role in the enhancement of resistance by regulating the antioxidant system in plants under environmental cues.

## Materials and methods

**Plants and stress treatments:** *Arabidopsis thaliana* wild type plants were grown in a growth chamber with an 8 h light/16 h dark cycle with a light intensity of 120 μmol $m^{-2}$ $s^{-1}$, and a temperature of 22 ± 1 °C. After four weeks, the plants were subjected to different treatments. Six treatments consisting of three different times (0, 1, and 3 h) under high light and two SA concentrations (0 and 0.3 mM) were conducted as follows: CK (distilled water); SA (distilled water with 0.3 mM SA); HL 1h (high light for 1 h); SA+HL 1h (high light for 1 h in the presence of 0.3 mM SA pretreatment); HL 3h (high light for 3 h); SA+HL 3h (high light for 3 h in the presence of 0.3 mM SA pretreatment). The SA was dissolved in ethanol with 0.1 % (v/v) Tween-20, and then double-distilled water was used to obtain 0.3 mM SA. The SA solution was sprayed on the *A. thaliana* leaves. After two days, high light (1000 μmol $m^{-2}$ $s^{-1}$) treatment was imposed on the *A. thaliana* plants. The light source was of white light and the irradiation was composed of the whole spectrum of wavelengths. During the HL treatment, the *A. thaliana* plants were placed in a control chamber with 22 °C and light passed through a 3 cm recirculation water system to avoid overheating.

**Measurements of proline and soluble sugars:** Proline content was measured according to a previous method (Bates *et al.* 1973) with minor modifications. Fresh leaf samples (0.5 g) from *A. thaliana* were homogenized with 5 $cm^3$ of 3 % (m/v) sulfosalicylic acid. The mixture was centrifuged, and the supernatant was removed. Then, 5 $cm^3$ of ninhydrin and 5 $cm^3$ of glacial acetic acid were added. The reaction mixture was put in boiling water for 1 h. Finally, the reaction was stopped by placing the mixture on ice. The absorbance of the mixture was obtained at 520 nm using a UV-visible spectrophotometer (*Hitachi-U2000*, Tokyo, Japan). Soluble sugars were extracted in boiling water, and their concentrations were determined according to the method of Farhad *et al.* (2011). Fresh leaves (0.5 g) from *A. thaliana* were homogenized with 5 $cm^3$ of 95 % (v/v) ethanol. Then, 3 $cm^3$ of anthrone (150 mg anthrone, 100 $cm^3$ of 72 % (m/v) sulphuric acid) was added to the 0.1 $cm^3$ of the mixture. The absorbance of the mixture was recorded at 625 nm using a UV-visible spectrophotometer.

**Measurement of ROS:** Hydrogen peroxide content was measured according to a modified method of Okuda *et al.* (1991). Leaves (0.5 g) were ground in ice bath with 5 $cm^3$ of 0.1 % (m/v) trichloroacetic acid. To a 0.5 $cm^3$ aliquot of the supernatant, 0.75 $cm^3$ of 1 M KI and 0.5 $cm^3$ of a 10 mM potassium phosphate buffer (pH 7.0) were added. The absorbance was recorded at 390 nm, and $H_2O_2$ content was expressed as nmol $g^{-1}$ (f.m.).

The production rate of superoxide anion radical ($O_2^{\cdot-}$) was determined according to a previous method (Elstner and Heupel 1976). Leaves (1 g) were homogenized in 3 $cm^3$ of a 65 mM potassium phosphate buffer with pH 7.8. After centrifugation (10 000 *g* at 4 °C for 15 min), the same potassium phosphate buffer (0.5 $cm^3$, pH 7.8) containing 10 mM hydroxylammonium chloride (0.1 $cm^3$) was added to the supernatant (0.5 $cm^3$). Then, 1 $cm^3$ of sulphanilic acid (58 mM) and 1 $cm^3$ of α-naphthyl amine (7 mM) were

added to the mixture. After incubation (25 °C for 20 min), an equal volume of chloroform was added to the mixture. The absorbance of the pink phase was recorded at 530 nm, and $O_2^{\cdot-}$ content was expressed as μmol $g^{-1}$ (f.m.)

**Measurements of lipid peroxidation and electrolyte leakage:** Lipid peroxidation in *A. thaliana* was assessed by measuring malondialdehyde (MDA) concentrations according to the method of Guidi *et al.* (2000). Fresh leaves (0.2 g) were grounded in 1.6 $cm^3$ 5 % (m/v) trichloroacetic acid, and then the homogenate was centrifuged at 12 000 *g* at 4 °C for 10 min. The supernatant (1.5 $cm^3$) was transferred into another centrifuge tube and 1.5 $cm^3$ of 0.67 % (m/v) thiobarbituric acid was supplemented. After centrifugation at 12 000 *g* for 10 min, the absorbances of the supernatant were obtained at 450, 532, and 600 nm. Then, MDA content was calculated by an equation: $MDA = [6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] \times (V / W)$, where A is absorbance, V is volume, and W is weight.

Electrolyte leakage (EL) of leaves was determined by a conductivity meter (*DDSJ-308A, Shanghai Precision Instruments Co., Ltd.*, China) according to a method of Chen *et al.* (2017). A conductivity measured after 15 min at 95 °C was considered as 100 %. Percent EL of the leaves was calculated by the initial conductivity / the absolute conductivity, multiplied by 100.

**Tissue staining:** Nitroblue tetrazolium (NBT) staining was used for histochemical localization of $O_2^{\cdot-}$ according to Yang *et al.* (2004) with some modifications. Leaves were vacuum infiltrated with a 6 mM NBT solution containing a 50 mM HEPES buffer (pH 7.5) for 1 min and then fixed with the NBT solution in the dark for 2 h. Then, the NBT staining solution was replaced by 95 % (v/v) ethanol. Chlorophyll was removed by boiling in 95 % (v/v) ethanol for 10 min. Finally, insoluble dark blue formazan compounds were formed by the $O_2^{\cdot-}$ accumulation.

Hydrogen peroxide ($H_2O_2$) was observed by using 3,3'-diaminobenzidine (DAB) staining. Leaves were vacuum infiltrated with a 5 mM DAB solution containing a 10 mM MES buffer (pH 3.8) for 5 min. Then, the leaves were immersed into the DAB solution in the dark for 8 h. The stained leaves were placed in a boiling solution (100 %, v/v, ethanol: 100 %, m/v, acetic acid: 100 %, v/v, glycerol; 3:1:1) for 15 min to remove chlorophyll. The DAB formed a deep brown polymerization product upon reaction with $H_2O_2$.

Trypan blue staining was carried out as described previously (Koch and Slusarenko 1990) with minor modifications. Leaves were cut and stained with a lactophenol-trypan blue solution (12.5 %, m/v, phenol; 12.5 %, v/v, glycerol; 12.5 %, m/v, lactic acid; 48 %, v/v, ethanol; and 0.025 %, m/v, trypan blue). The stained leaves were boiled in the lactophenol-trypan blue solution for 1 min and then decolorized in a chloral hydrate solution (75 g of chloral hydrate dissolved in 30 $cm^3$ of distilled water) for three days.

To observe the accumulation of callose, leaves were stained with aniline blue according to a method previously described (Lu *et al.* 2011). The leaves were fixed in 10 % (m/v) acetic acid in the dark overnight. Then, the samples were softened in 1 M NaOH overnight, washed several times with a 50 mM K-phosphate buffer (pH 7.5), and subsequently stained in 0.01 % (m/v) aniline blue for 15 min. Fluorescent images were obtained with a fluorescence microscope (*Bx53 System, Olympus Corporation*, Tokyo, Japan).

**Antioxidant systems:** For enzyme measurements, 0.5 g of fresh leaves was ground in a chilled extraction HEPES buffer (25 mM, pH 7.8) containing 0.2 mM $Na_2EDTA$, 2 mM ascorbate and 2 % (m/v) polyvinylpolypyrrolidone on ice. The homogenate was centrifuged at 12 000 *g* at 4 ºC for 20 min. Then, the supernatant was obtained for enzyme and non-enzyme antioxidant assays.

Peroxidase activity was assayed by detecting the increase of absorbance of guaiacol at 470 nm according to Kumar and Khan (1982). Superoxide dismutase activity was determined by its ability to inhibit the photochemical reduction of NBT according to Beauchamp and Fridovich (1971). Catalase activity was measured by following the consumption of $H_2O_2$ at 240 nm according to a method given by Chandlee and Scandalios (1984). Ascorbate peroxidase activity was estimated by monitoring ascorbate oxidation at 290 nm according to Nakano and Asada (1981). Glutathione peroxidase activity was determined by using consecutive glutathione reductase reaction according to Flohe and Gunzler (1984). Glutathione reductase activity was measured as a decrease in absorbance at 340 nm due to NADPH oxidation following Foyer and Halliwell (1976).

The ratio of reduced ascorbic acid/dehydroascorbate was analyzed as described by Kampfenkel *et al.* (1995). The ratio of reduced glutathione/oxidized glutathione was measured according to a method of Griffith (1980).

**Measurements of stomatal status:** Measurements of stomatal conductance ($g_s$) and intercellular $CO_2$ concentration ($c_i$) were done using a GSF-3000 photosynthesis system (*Heinz-Walz Instruments*, Effeltrich, Germany). The second leaves after high light and SA treatments were used for the determination with an *Arabidopsis* chamber 3010-A. The working conditions were set to a $CO_2$ concentration of 360 μmol $mol^{-1}$ and an irradiation of 1000 μmol $m^{-2}$ $s^{-1}$, a 70 % relative humidity at room temperature (Yamori *et al.* 2006a).

Leaves with the same position on four-week-old plants were used for stomatal aperture assay. After treatment with SA and high light in a growth chamber, leaf lower epidermis cells were stuck with adhesive plastic tapes and then tore off epidermal strips. The stomatal structure of the leaves was photographed with a fluorescence microscope (*Bx53 System, Olympus Corporation*, Tokyo, Japan) according to a method of Yu *et al.* (2016). All experiments were repeated at least four times.

**Statistical analysis:** A value of each treatment was from at least four independent replicates. Results are shown as means ± SDs (standard deviations). Statistical analysis was performed using the SPSS Statistics 19.0 software (IBM, Chicago, IL), and the comparison was done using the

Duncan multiplication range test. Different letters above the columns of figures indicate statistically significant differences among treatments at $P < 0.05$.

## Results

Proline and soluble sugar content: Osmolytes, such as proline and soluble sugars, usually accumulate and subsequently may help to reduce cellular injury from dehydration in plants in response to environmental stresses (Xin and Browse 1998). As shown in Fig. 1*A*,*B*, there were no obvious differences in the content of proline and soluble sugars between the SA-treated plants and the control. Under high light for 3 h, the content of proline and soluble sugars increased by 97.3 % and 45.8 %, respectively, compared with the control. Compared with the non-SA-treated plants under high light, SA treatment increased the content of proline and soluble sugars by 16.6 and 46.2 %, respectively, under high light for 3 h. In addition, we found that the two osmotic regulators of the SA-treated plants were generally equivalent to the control without high light (Fig. 1*A*,*B*).

Reactive oxygen species accumulation and lipid peroxidation: To investigate whether photodamage is accompanied with the production of ROS, we examined the levels of $O_2^{\cdot-}$ and $H_2O_2$ by NBT and DAB staining, respectively, under high light (Fig. 2). Staining shows that there were slight differences between the control and SA-treated plants without high light (Fig. 2). Under the high light in the absence of SA, *A. thaliana* leaves exhibited an intensive blue or yellow color compared with the control. However, the SA-treated plants were slightly stained compared with the non-SA-treated plants under the high light, especially for 3 h, suggesting a lower accumulation of ROS (Fig. 1 Suppl.).

To further confirm these results, we also quantified $O_2^{\cdot-}$ and $H_2O_2$ (Fig. 3*A*,*B*). The results are almost identical with those observed by staining with NBT and DAB. Compared with the control, $O_2^{\cdot-}$ production rate and $H_2O_2$ content increased by 27.3 %, and 51.8 %, respectively, under the high light for 3 h. However, SA treatment decreased content of $O_2^{\cdot-}$ and $H_2O_2$ by 18.2 % and 22.7 %, respectively, compared with the control. In addition, compared with the non-SA-treated plants, decreases in content of $O_2^{\cdot-}$ and $H_2O_2$ were 7.8 % and 24.2 %, respectively, in the SA-treated plants under the high light for 3 h.

Furthermore, we investigated oxidative damage by detecting the level of lipid peroxidation. Compared with the control, content of MDA and EL increased by 56.6 % and 37.6 %, respectively, under the high light for 3 h. The MDA and EL of the SA-treated plants decreased by approximately 11.5 % and 17.9 %, respectively, compared with the control. Nevertheless, compared with the non-SA-treated plants, SA treatment caused 23.6 % and 18.3 %, respectively, declines in MDA and EL under the high light for 3 h (Fig. 3*C*,*D*). These results demonstrate that SA might alleviate ROS accumulation and oxidative

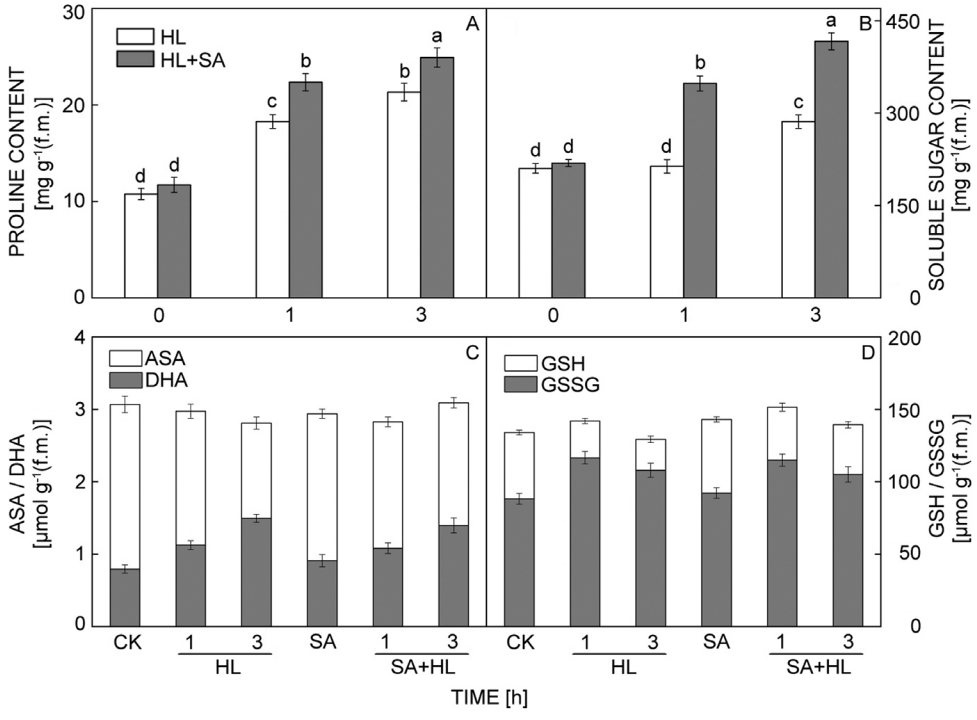


Fig.1. Effects of salicylic acid (SA) on proline (*A*), soluble sugar content (*B*), the ratios of ascorbic acid (AsA) to dehydroascorbate (DHA) (*C*), and reduced glutathione (GSH) to oxidaized glutathione disulfide (GSSG) (*D*) in *Arabidopsis thaliana* under high light. *Bars* represent standard deviations of four independent biological replicates. Different letters indicate significant differences ($P < 0.05$) according to the Duncan multiplication range test. CK - non-stressed plants; HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, and 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

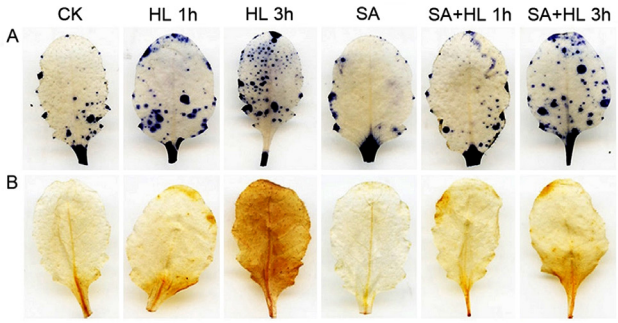


Fig. 2. Effects of salicylic acid (SA) on nitroblue tetrazolium (*A*) and 3,3'-diaminobenzidine staining (*B*) of *Arabidopsis thaliana* under high light. CK - non-stressed plants; HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, and 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

damage under the high light.

Cell death and callose accumulation: Excessive ROS can result in a progressive oxidative damage and ultimately cell death (Foyer and Shigeoka 2011). In the present experiment, tissue staining was used to investigate the degree of cell death under the high light in the presence and absence of SA. As expected, cell death and callose accumulation showed no significant differences between the control and the SA-treated leaves. However, the high light resulted in a severe cell death (trypan-blue staining) and obvious callose deposition (aniline-blue staining) in the absence of SA, especially under the high light for 3 h (Fig. 4). Compared with the HL-treated plants, the SA-pretreated plants showed a lower cell death and less callose deposition under the high light. These results indicate that SA pretreatment could effectively alleviate cell damage caused by the high light.

Activities of antioxidant system: Compared to the control, SA pretreatment did not result in significant changes in the activities of antioxidant enzymes and the content of non-enzymatic antioxidants under the control conditions. The activities of POD, SOD, and CAT increased apparently under the high light for 1 h compared with the control plants in the absence of SA (Fig. 5*A*-*C*). However, 3 h of the high light remarkably decreased the activities of these three antioxidant enzymes compared with the high light for 1 h. In addition, the high light led to an obvious increase in APX, GPX, and GR activities compared with the control plants, especially for 3 h (Fig. 5*D*-*F*). Furthermore, SA pretreatment effectively improved the activities of the six antioxidant enzymes under the high light (Fig. 5).

Additionally, the concentrations of DHA and GSSG significantly increased, whereas the content of AsA and GSH greatly decreased under the high light (Fig. 1*C*,*D*). When the high light was applied, the SA-treated plants showed significantly higher concentrations of AsA and GSH, and lower of DHA and GSSG compared with the non-SA-treated plants. Overall, these data suggest that SA might have important effects on the antioxidant defense system, which could be involved in alleviating high light-induced oxidative damage in *A. thaliana*.

Stomatal status: To determine whether SA has a protective role on stomata, stoma characteristics assays

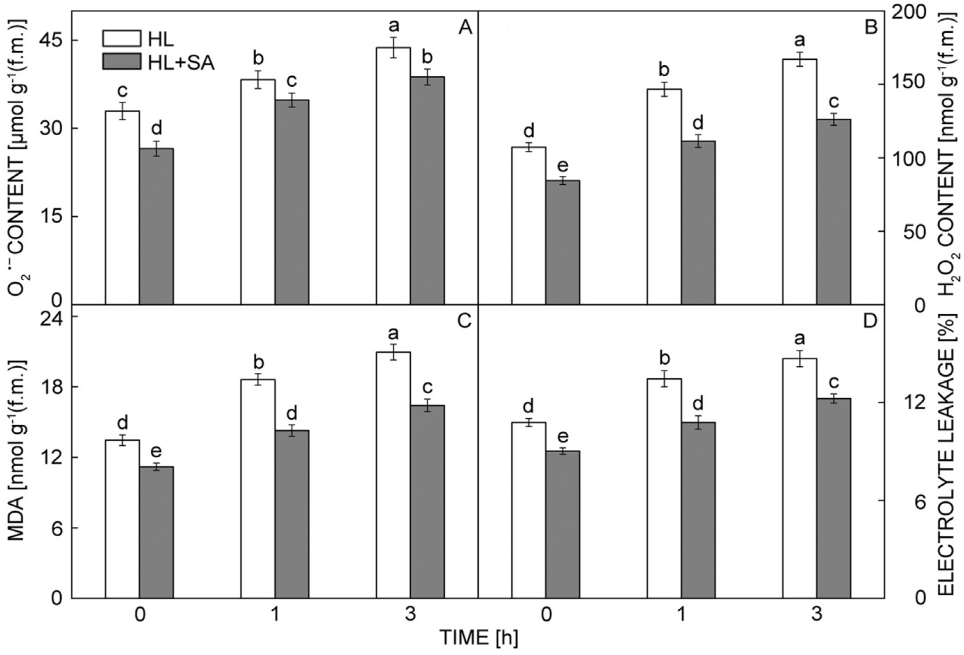


Fig. 3. Effects of salicylic acid (SA) on superoxide anion radicals ($O_2^{\cdot-}$) production rate (*A*), hydrogen peroxide ($H_2O_2$) content (*B*), malondialdehyde (MDA) content (*C*), and electrolyte leakage (*D*) in *Arabidopsis thaliana* under high light. Bars represent standard deviations of four independent biological replicates. Different letters indicate significant differences ($P < 0.05$) according to the Duncan multiplication range test. HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

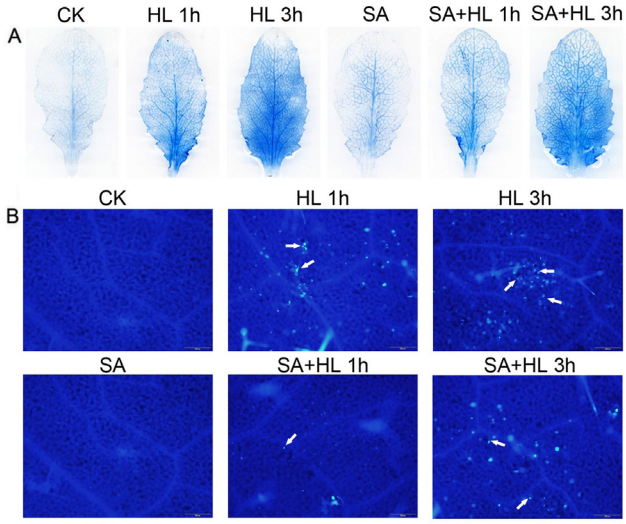


Fig. 4. Characterization of oxidative damage in *Arabidopsis thaliana* under high light in the presence or absence of salicylic acid (SA) pretreatment. The degree of cell death obtained from trypan blue staining (*A*). Callose accumulation obtained from aniline blue staining (*B*). The images are obtained with a fluorescence microscope. Bars = 100 µm. CK - non-stressed plants; HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

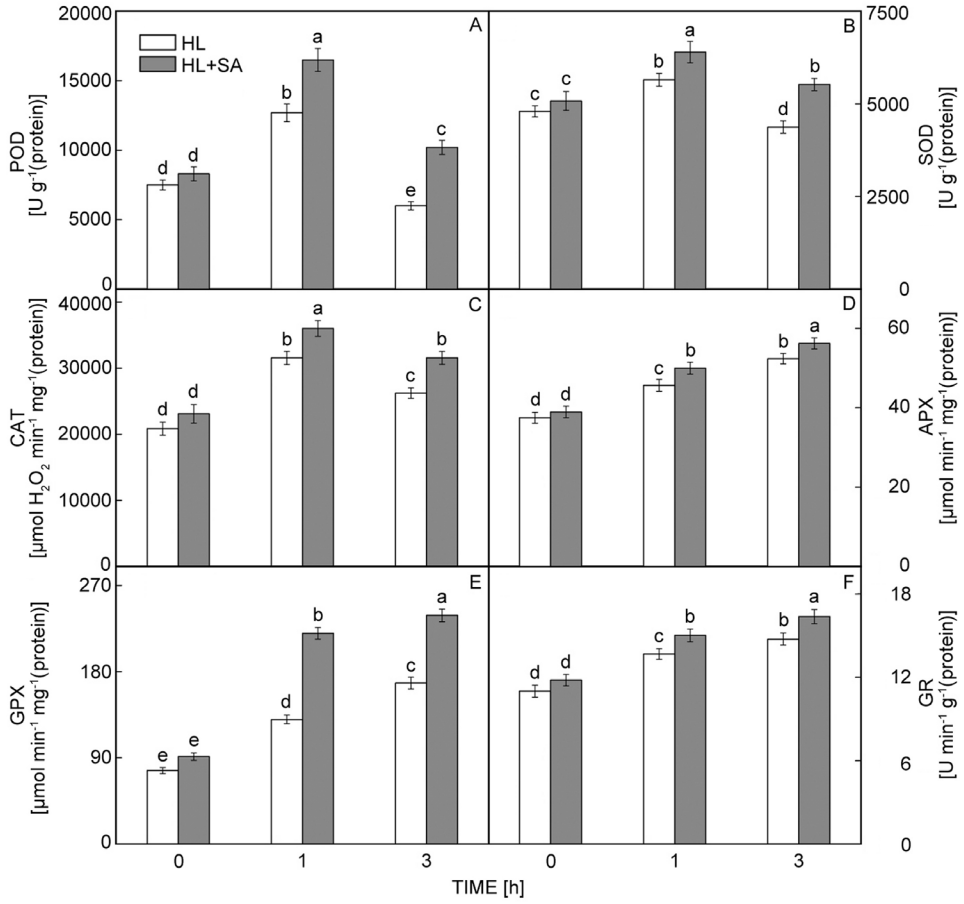


Fig. 5. Effects of salicylic acid (SA) on the specific activities of peroxidase (POD) (*A*), superoxide dismutase (SOD) (*B*), catalase (CAT) (*C*), ascorbate peroxidase (APX) (*D*), glutathione peroxidase (GPX) (*E*), and glutathione reductase (GR) (*F*) of *Arabidopsis thaliana* under high light. Bars represent standard deviations from four independent biological replicates. Different letters indicate significant differences ($P < 0.05$) according to the Duncan multiplication range test. HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

were performed in *A. thaliana* leaves under the high light in the presence and absence of SA. As shown in Fig. 6*A*, the high light resulted in a pronounced reduction in stomatal conductance with 46.0 % and 48.8 % under the high light for 1 and 3 h, respectively, compared with the control. Compared with the non-SA-treated plants under the high light, SA pretreatment increased stomatal conductance by approximately 55.6 % under the high light for 1 h. However, there was no significant difference in stomatal conductance and between the SA-treated plants and the control. Notably, intercellular $CO_2$ concentration under the high light in the presence and absence of SA was almost identical except for 3 h of the high light (Fig. 6*B*). Furthermore, the high light caused stomata closure. (Fig. 6*C*). Compared with the control, the stomata of *A. thaliana* leaves under the high light were mostly closed. However, SA pretreatment could effectively mitigate stomata closure and increase stomatal conductance. Therefore, these findings suggest that SA may play an important role in regulating stomatal aperture.

## Discussion

Changes in light intensity (*i.e.* random fluctuations in sunlight intensity) and duration (*i.e.* minutes within hours or days) can lead to stomata closure and severe cellular damage in many plants (Renata *et al.* 2017). However, SA may regulate many aspects of plant growth and development under abiotic stresses (Dempsey and Klessig 2017). Previous studies indicated that SA alleviates the adverse effects of salt stress on *Dianthus superbus* by activating photosynthesis and protecting morphological structures (Ma *et al.* 2017). Application of SA at moderate concentrations could increase tolerance to stress and PS II photochemistry and alleviate photooxidation of PS II (Chen *et al.* 2016; Cheng *et al.* 2016). In addition, a previous report also demonstrated that endogenous SA increases sharply during early stages of post-anthesis leaf senescence, and subsequently, photoprotection mechanisms including xanthophyll cycle-dependent excess energy dissipation was acted, thus avoiding the damage to PS II (Abreu and Munné-Bosch 2007). In the present study, the positive protective role of SA in the antioxidant defense system and oxidative damage in *A. thaliana* against high light was investigated.

Plants usually accumulate different types of solutes under environmental cues (Serraj and Sinclair 2002). These osmolytes may provide protective roles effectively by contributing to cellular osmotic adjustment, ROS detoxification and maintaining membrane integrity in plants exposed to environmental stresses (Bohnert and

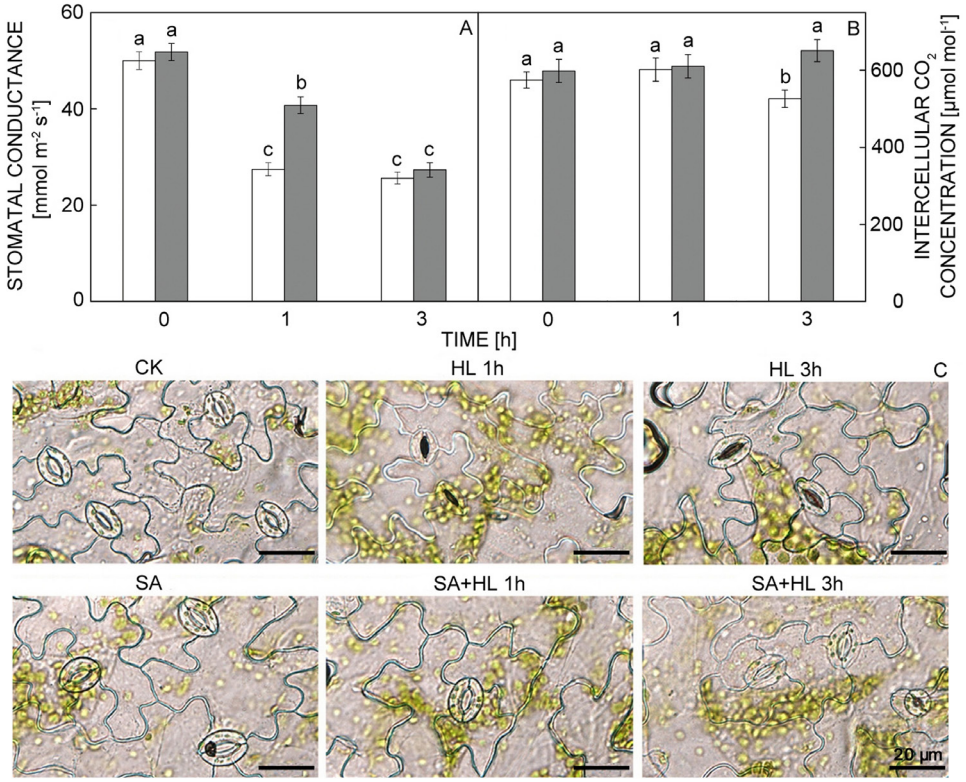


Fig. 6. Effects of salicylic acid (SA) on stomatal conductance (*A*), intercellular $CO_2$ concentration (*B*), and stomatal status (*C*) of *Arabidopsis thaliana* under high light. Stomatal conductance and intercellular $CO_2$ concentration were measured at an ambient $CO_2$ concentration of 360 µmol mol$^{-1}$ under a saturating light intensity of 1000 µmol (photons) m$^{-2}$ s$^{-1}$ at 25 °C. Bars represent standard deviations from four independent biological replicates. Different letters indicate significant differences ($P < 0.05$) according to the Duncan multiplication range test. Bars = 20 µm. CK - non-stressed plants; HL - high light; SA+HL - high light stress after SA pretreatment for 3 d; 0, 1, 3 h - high light stress for 0, 1, and 3 h in the presence or absence of SA pretreatment, respectively.

Jensen 1996, Ashraf and Foolad 2007). Proline and soluble sugars are the two most important organic solutes under stressful conditions (Pérez-Alfocea and Larher 1995). In this study, we found that the content of soluble sugars and proline increased significantly under the high light suggesting that the plants suffered the severe damage from the high light (Marcińska *et al.* 2012). Previous studies have shown that SA can alleviate a damage to plants by inducing the accumulation of osmotic regulators. This result was further demonstrated in our experiment.

In higher plants, ROS are usually generated as byproducts of various cellular activities, such as respiration and photosynthesis, especially under different environmental stresses (Apel and Hirt 2004). However, a low concentration of SA may alleviate ROS generation induced by abiotic stresses (Chen *et al.* 2016). Previous works have indicated that high light remarkably induces the accumulation of ROS in plants (Golan *et al.* 2010, Heyneke *et al.* 2013, Chen *et al.* 2017). These findings were further confirmed by our data suggesting that the high light could trigger excessive accumulation of ROS. A previous study reported that SA could protect cells and sub-cellular systems from ROS cytotoxic effects under salt stress (Ma *et al.* 2017). Consistently, our results indicate that the application of exogenous SA alleviated ROS accumulation under the high light. Under environmental stresses, MDA and EL are considered as important indicators in reflecting the level of lipid peroxidation (Shen *et al.* 2014) and permeability of membrane (Zhang *et al.* 2014). In our study, the high light significantly enhanced the content of MDA and EL indicating a high level of oxidative damage occurred in the membranes of plants. However, SA application could lower the amounts of MDA and EL under the high light, which was in accordance with the decrease in ROS accumulation. These findings suggest that SA might alleviate oxidative damage of high light to cell membranes.

Excessive accumulation of ROS may result in an irreversible oxidative damage to cellular components and eventually to cell death (Apel and Hirt 2004). Our previous work indicate that high light results in a remarkable cell death and callose deposition in *koLHCB6 Arabidopsis* (Chen *et al.* 2018). Consistent with the results of ROS production, our present study shows that the *A. thaliana* plants accumulated obvious cell death and callose depositions under the high light (Fig. 4). However, SA pretreatment effectively alleviated the degree of cell death because SA is a crucial determinant of cell death regulation (Vogelmann *et al.* 2012). Callose is deposited in the wall surrounding the plasmodesmata during plant development (Levy *et al.* 2007), and its accumulation is a typical spontaneous defense response (Wei *et al.* 2010). Our results obtained from aniline blue staining indicate that the high light led to the large deposition of callose, and the application of SA reduced callose deposition (Fig. 4*B*).

Plants have an effective antioxidant defense system mainly containing antioxidant enzymes and non-enzymatic antioxidants to alleviate oxidative damage caused by excessive ROS accumulation (Bowler and Montagu 1992). In the present study, all antioxidant enzyme activities markedly increased under the short-term high light (Fig. 5) indicating that plants can reduce oxidative damage by acting the antioxidant system rapidly under a short-term stress. It is probable that antioxidant enzymes are the main target of defense in plant cells (Renata *et al.* 2017). Our previous study indicated that the activities of POD, SOD, and CAT markedly decrease under a long-term heat and a high light co-stress in wheat seedlings (Chen *et al.* 2017). Our data obtained on *A. thaliana* plants exposed to the high light further demonstrate this result. Furthermore, higher enzyme activities of APX, GPX, and GR under the long-term high light (3 h) might be because these enzymes are the key antioxidant enzymes of $H_2O_2$ scavenging and are also involved in the ascorbate-glutathione cycle (Foyer *et al.* 1994, Foyer and Noctor 2009). Some previous studies have demonstrated that SA could increase activities of antioxidant enzymes to decrease ROS generation and lipid peroxidation under environmental stresses to plants (Idrees *et al.* 2011, Saruhan *et al.* 2012, Li *et al.* 2014). Consistent with these reports, our results clearly show that SA application may improve antioxidant enzyme activities effectively and subsequently lower the accumulation of ROS under high light. In addition, the ascorbate-glutathione cycle is regarded as an important part of the antioxidant defense system in plant response to oxidative stress (Zhang *et al.* 2015). In the present experiment, we found that the high light resulted in the decrease in the content of AsA and GSH, and the increase in the content of DHA and GSSG. A previous study showed that SA increases the content of AsA and GSH in salt-stressed wheat seedlings, which is associated with SA-induced salt tolerance (Wei 2013). Agnihotri *et al.* (2018) also indicated that SA boosts the ascorbate-glutathione cycle and thus helps to alleviate oxidative damage and damage to DNA under lead stress. Consistently, our results show that the SA-treated plants accumulated high concentrations of AsA and GSH, and low concentrations of DHA and GSSG under the high light suggesting that SA could improve the tolerance to high light by regulating the ascorbate-glutathione cycle. Therefore, the positive effects of SA on the ROS scavenging system could increase the resistance of plants under environmental stresses.

In order to adapt plants to the different environmental conditions, the status and the structure of stomata usually change (Casson and Hetherington 2010). Previous studies indicated that one of the important responses of plants to drought, salt stress, and high light are closed stomata, which limits gas exchange and thus decreases the ratio of $CO_2$ to $O_2$ (Bhargava and Sawant 2013, Rainer and Sergey 2018). Consistently, our results show that the *A. thaliana* plants decreased stomatal conductance and intercellular $CO_2$ concentration, and the stomata were mostly closed under the high light (Fig. 6). Ma *et al.* (2017) reported that SA could increase stomatal conductance and intercellular $CO_2$ concentration under salt stress. Our results obtained from the high light further demonstrate this finding. In addition, a previous study showed that SA accumulation plays an important role in stomatal closure and drought tolerance in *A. thaliana* (Miura *et al.* 2013). Ma *et al.* (2017) also found that SA could improve photosynthesis

and stoma of *Dianthus superbus* effectively under salt stress. In the present study, we found that SA application inhibits stomatal closure under the high light. Based on the above results, we speculate that SA probably plays a positive role in the stomatal morphology and structure to improve photosynthesis and the resistance to environmental stresses.

## Conclusions

The present study shows that the high light triggered an excessive accumulation of ROS and cell death, and subsequently resulted in a severe oxidative damage to *A. thaliana* plants. However, a low concentration of SA could alleviate accumulation of ROS, cell death, closed status of stomata, and callose deposition effectively by keeping high activities of antioxidant enzymes and high content of antioxidants under high light. Therefore, our results demonstrate that SA is a potential signaling molecule and can alleviate oxidative damage effectively by activating the antioxidant defense system under environmental stresses. However, in view of different results obtained from the existing studies on the role of SA in different plants exposed to environmental stresses, it needs to be further investigated whether SA application is an effective method in improving crop resistance to environmental stresses in the field.

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