

Enhancement of stress tolerance in cucumber seedlings by proanthocyanidins

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Abstract

Proanthocyanidins (PAs) are the main products of the flavonoid biosynthetic pathway in many plants. However, their biological function during environmental stresses in plants is rarely reported. In the present study, the effects of pretreatment with PAs on the response of cucumber (*Cucumis sativus* L.) seedlings to high irradiance (HI), polyethylene glycol (PEG), and cold stress were investigated. The PAs pretreatment alleviated stress-induced oxidative damage in plant cells and increased the activity of alternative oxidase (AOX) and content of abscisic acid (ABA). Furthermore, PAs-pretreated seedlings suffered less damage by the stress conditions, maintained higher content of chlorophyll *a+b* and AOX proteins in comparison with the control. Therefore, our findings suggest that PAs might contribute to plant tolerance to environmental stresses.

Additional key words: abscisic acid, alternative oxidase, chlorophyll, cold, *Cucumis sativus*, high irradiance, PEG.

Introduction

Environmental stresses cause significant loss of plant productivity (Gilbert 2012). Thus, improving the tolerance of crops against environmental stresses is critically important for agricultural and economic performance (Varshney *et al.* 2012).

Proanthocyanidins (PAs) are the end products of the flavonoid biosynthetic pathway in many plants. Over-accumulation of antioxidant flavonoids enhances tolerance to drought and oxidative stress in *Arabidopsis* (Nakabayashi *et al.* 2014). It has been reported that flavonoid accumulation also enhances plant tolerance against UV-B irradiation (Stracke *et al.* 2010, Kusano *et al.* 2011). Further, there is strong evidence that

anthocyanin accumulation in leaves protects against photoinhibitory damage caused by high irradiance (Gould *et al.* 2010, Zhang *et al.* 2010, Page *et al.* 2012). Therefore, it can be questioned whether exogenous PAs could effectively improve plant tolerance to environmental stresses.

Under normal conditions, reactive oxygen species (ROS) are kept at low levels but this balance is disrupted when the plant faces to biotic or abiotic stresses (Mittler 2002). To cope with the ROS excess, plants have developed a complex of ROS-removing mechanisms. Antioxidative enzymes, *e.g.*, superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase

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Abbreviations: ABA - abscisic acid; AOX - alternative oxidase; APX - ascorbate peroxidase; CAT - catalase; DAB - 3,3-diaminobenzidine; EL - electrolyte leakage; ETR - electron transport rate; F_v/F_m - variable to maximum chlorophyll fluorescence ratio; MDA - malondialdehyde; NBT - nitroblue tetrazolium; NPQ - non-photochemical quenching; PAs - proanthocyanidins; PEG - polyethylene glycol; POD - peroxidase; PS II - photosystem II; ROS - reactive oxygen species; RWC - relative water content; SHAM - salicylhydroxamic acid; SOD - superoxide dismutase; Valt - cyanide-resistant respiration rate; Vres - residual respiration rate; Vt - total respiration rate.

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(CAT) play a major role in keeping superoxide radicals and hydrogen peroxide at an appropriate level. Moreover, it has been reported that overexpression of genes encoding antioxidative enzymes or other antioxidants enhances stress tolerance remarkably (Lee *et al.* 2010, Xi *et al.* 2010). Furthermore, it has been reported that flavonoids, as ROS scavengers *in vitro*, also enhance drought tolerance *in vivo* (Nakabayashi *et al.* 2014). Thus, we questioned whether exogenous PAs could protect plants from stresses and the protection can be associated with regulation of antioxidant enzyme activities.

Abscisic acid (ABA) improves drought tolerance through the activation of ROS-scavenging enzymes (Lu

et al. 2009). Moreover, it has been reported that ABA maintains low ROS content through the activation of the alternative oxidase (AOX) pathway (Bahin *et al.* 2011). Furthermore, ABA signalling pathway and PAs are important for the response to oxidative stress during seed germination (Jia *et al.* 2012). Therefore, it can be questioned whether there is a crosstalk between PAs and ABA in promoting stress tolerance in plants.

The aim of the present study was to investigate whether exogenous PAs could effectively enhance cucumber seedlings tolerance to environmental stresses. Furthermore, the possible relationships between AOX, ABA, and ROS in alleviating stress-induced oxidative damage were investigated.

Materials and methods

The seeds of cucumber (*Cucumis sativus* L. cv. Jinrui No. 100) were obtained from the Sichuan Academy of Agricultural Science, Chengdu, China. Cucumber seedlings were grown until three-leaf stage in a growth chamber with day/night temperatures of 25/20 °C, a 16-h photoperiod, a photosynthetic photon flux density (PPFD) of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a relative humidity of 70 %. PAs were obtained from *Kikkoman Corporation* (Noda, Japan) and 0.025, 0.05, 0.1, or 0.2 % (m/v) solutions were prepared in water containing 0.02 % (v/v) *Tween* 20. Distilled water containing 0.02 % (v/v) *Tween* 20 was used as a control treatment. Seedlings were pretreated by foliar spraying with PAs and 24 h later, parts of seedlings were exposed to high irradiance (a fiber optic white light source of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Galvez-Valdivieso *et al.* 2009), PEG (18 %, m/v, PEG 6000), and cold stress (at 4 °C in a controlled growth chamber with a relative humidity of 70 %) for 3 d. The third leaf of cucumber seedlings was used for the following experiments.

To inhibit the activity of the AOX pathway, seedlings were sprayed with 1 mM salicylhydroxamic acid (SHAM), as this concentration is sufficiently low to avoid the possible side effects (Moller *et al.* 1988). For SHAM+PAs treatment, seedlings were first pretreated with 1 mM SHAM for 24 h and then exposed to environmental stresses as described earlier for another 24 h after being sprayed with 0.1 % PAs.

Respiratory oxygen consumption was measured using Clark-type electrodes (*Hansatech*, King's Lynn, UK) as described previously by Lei *et al.* (2010). Leaves (50 mg of fresh mass) were cut into small pieces, then pretreated with 10 cm^3 of deionized water for 15 min in order to eliminate wound-induced respiration. Measurements were done at 25 °C in a final volume of 1.5 cm^3 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 (0.5 mM *n*-propylgallate, nPG) were used. The

total respiration rate (V_t) was defined as O₂ uptake rate by cucumber leaves without any inhibitor. Then, 1 mM KCN was added to obtain the O₂ uptake rate defined as V_0 . The residual respiration (V_{res}) was estimated by measuring the rate of oxygen uptake in the presence of both 1 mM KCN and 0.5 mM nPG. The capacity of the alternative pathway (V_{alt}) was calculated by the formula: $V_{\text{alt}} = V_0 - V_{\text{res}}$. The V_{res} in experiment was always low, and negligible relative to other respiration rates. Therefore, V_{res} is not shown.

ABA was measured by a HPLC combined with enzyme-linked immuno-sorbent assay (ELISA) technique. Fresh leaves (50 mg) were ground using liquid nitrogen and extracted with 10 cm^3 of extraction buffer (80 %, v/v, aqueous methanol, and 40 mg dm^{-3} butylhydroxytoluene) at 4 °C in the dark for 24 h. At the beginning of the extraction, ³H-ABA was added to the extracts to measure the yield of the ABA purification (Julliard *et al.* 1994).

Total RNA was extracted from leaves according to Zhang *et al.* (2010). All gene expressions were measured by RT-qPCR. β -actin was used as internal control. The RT-qPCR primers are listed in Table 1 Suppl. The mRNA data were expressed as percentage of the corresponding β -actin transcription (Xu *et al.* 2012).

Total proteins were extracted in an ice bath with extraction buffer containing 50 mM Tris-HCl, pH 6.8, 5 % (v/v) mercapto-ethanol, 10 % (v/v) glycerol, 4 % (m/v) SDS, and 4 M urea. For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, 50 μg of protein from each sample was used. The proteins separated by SDS-PAGE were transferred to polyvinylidifluoride (PVDF) membranes, and then antisera to AOX (provided by Prof. Harvey Millar) were applied. The intensity of the signals of Western blot was analysed densitometrically by a thin-layer scanner (Xu *et al.* 2012).

The H₂O₂ content of leaves was measured as described by Velikova *et al.* (2000). Fresh leaves (200 mg) were homogenized in ice bath with 2 cm^3 of 0.1 %

(m/v) TCA. The homogenate was centrifuged at 12 000 g for 20 min. Then, 0.5 cm³ of supernatant was added to 0.5 cm³ of 10 mM potassium phosphate buffer (pH 7.0) and 1 cm³ of 1 M KI. The absorbance of supernatant was recorded at 390 nm. The content of H₂O₂ was calculated according to the standard curve.

Electrolyte leakage (EL) was measured as described Wang *et al.* (2011). Fresh leaves (0.2 g) were cut into pieces and placed in 10 cm³ of deionized water at room temperature. The conductivity (C₁) was determined after 1 h and then the samples were boiled at 100 °C for 15 min to achieve 100 % EL (C₂). The results were calculated according to the formula: EL = (C₁/C₂) × 100.

Lipid peroxidation was estimated by measuring the malondialdehyde (MDA) content as described by Cao *et al.* (2009). Fresh leaves (0.2 g) were homogenized with 5 % (m/v) trichloroacetic acid (TCA) on ice and centrifuged at 3 000 g and 4 °C for 10 min. The supernatants (1.5 cm³) were added to 1.5 cm³ of 0.67 % (m/v) thiobarbituric and incubated at 100 °C for 15 min. The cooled mixture was centrifuged at 4 000 g for 10 min. The absorbances of supernatants were recorded at 450, 532, and 600 nm.

Relative water content (RWC) is defined by the equation: RWC [%] = (FM - DM) / (WSM - DM) × 100, where, FM is fresh mass, WSM is mass of water saturated leaf, and DM is dry mass (Du *et al.* 2011).

A crude extract was prepared as described by Wang *et al.* (2011). Leaves (0.2 g) were ground with 2 cm³ of ice-cold 25 mM Hepes buffer (pH 7.8) containing 0.2 mM EDTA, 2 % (m/v) polyvinylpyrrolidone (PVP) and 2 mM ascorbate. The homogenates were centrifuged at 12 000 g and 4 °C for 20 min. The supernatants were used for the determination of enzymatic activities. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of nitro blue tetrazolium (NBT) chloride induced photochemical reduction (Stewart and Bewley 1980). One unit of SOD activity was defined as the amount of enzyme that produced a 50 % inhibition of

NBT reduction under the assay conditions. CAT and APX activities were measured as decreases in absorbances A₂₄₀ and A₂₉₀, respectively (Dong *et al.* 2014). Peroxidase (POD) activity was assayed as described Cao *et al.* (2009). One unit of POD activity was defined as the amount of the enzyme that caused an absorbance change of 0.01 unit per minute.

Leaf chlorophyll was extracted with 80 % (v/v) acetone from the fresh leaves and measured as described by Du *et al.* (2011). Chlorophyll fluorescence was measured at room temperature (25 °C) with an imaging pulse amplitude modulated fluorometer (IMAG-MINI; Heinz Walz, Effeltrich, Germany). Plants were dark-acclimated for 30 min. Minimal fluorescence (F₀) was determined during the weak irradiance, and maximal fluorescence (F_m) was measured after 0.8 s pulse of about 4 000 μmol m⁻² s⁻¹. F_v/F_m was calculated as F_m - F₀/F_m. An actinic radiation was then applied to obtain steady-state fluorescence yield (F_s), after which a second saturation pulse was applied for 0.7 s to obtain light-acclimated maximum fluorescence (F_{m'}). Non-photochemical quenching (NPQ) was calculated as (F_m/F_{m'}) - 1, respectively. Relative electron transport rate (ETR) was calculated as (1 - F_s/F_{m'}) × PAR, where PAR was the photosynthetically active radiations (Genty *et al.* 1989).

Superoxide and H₂O₂ were visually detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining, respectively, as described Lei *et al.* (2010). Cucumber leaves were vacuum infiltrated with NBT (0.5 mg cm⁻³) solutions for 2 h or DAB (2 mg cm⁻³) solutions for 8 h. Then, leaves were decolorized in boiling ethanol (95 %, v/v) for 20 min.

Experimental data were statistically analysed by analysis of variance (ANOVA) using SPSS v. 14.0 software (SPSS Inc., Chicago, IL, USA). Means were separated by least significant difference (LSD) tests at *P* ≤ 0.05.

Results

In order to investigate the effects of PAs pretreatment on plant responses to environmental stresses, cucumber seedlings were exposed to high irradiance (1000 μmol m⁻² s⁻¹), osmotic stress (18 %, m/v, PEG 6000), and cold stress (4 °C) for 3 d after being sprayed with different concentrations of PAs for 24 h. The results showed that cucumber seedlings pretreated with 0.1 % PAs showed an enhanced resistance to all three types of stress. More serious symptoms including curling, wilting, and yellowing of leaves or cotyledons were observed in the control seedlings after 3-d stress (Fig. 1A). Consistent with the observed symptoms in cucumber seedlings, the PAs-pretreated seedlings suffered less damage, accompanied with increased RWC and decreased EL and

MDA content when compared with the control seedlings under the same stress conditions (Fig. 1D,E,G).

Pretreatment with 0.1 % PAs for 24 h significantly enhanced the total respiration and alternative respiration rates compared with the control, but they decreased at 0.2 % PAs (Fig. 1 Suppl.). Significant increase in ABA content was observed at the 0.05 and 0.1 % PAs-pretreated seedlings as compared to the control (Fig. 1 Suppl.). Furthermore, pretreatment with 0.1 % PAs markedly decreased H₂O₂ content as compared to the control seedlings (Fig. 1 Suppl., Fig. 1B,F). In contrast, pretreatment with 0.2 % PAs did not alleviate stress-induced injury, and even more serious damage compared with the control seedlings was observed (Fig. 2 Suppl.).

Therefore, the concentration of 0.1 % PAs was used in the following study.

Antioxidant enzyme activities in the PAs-pretreated seedlings increased more sharply compared with the

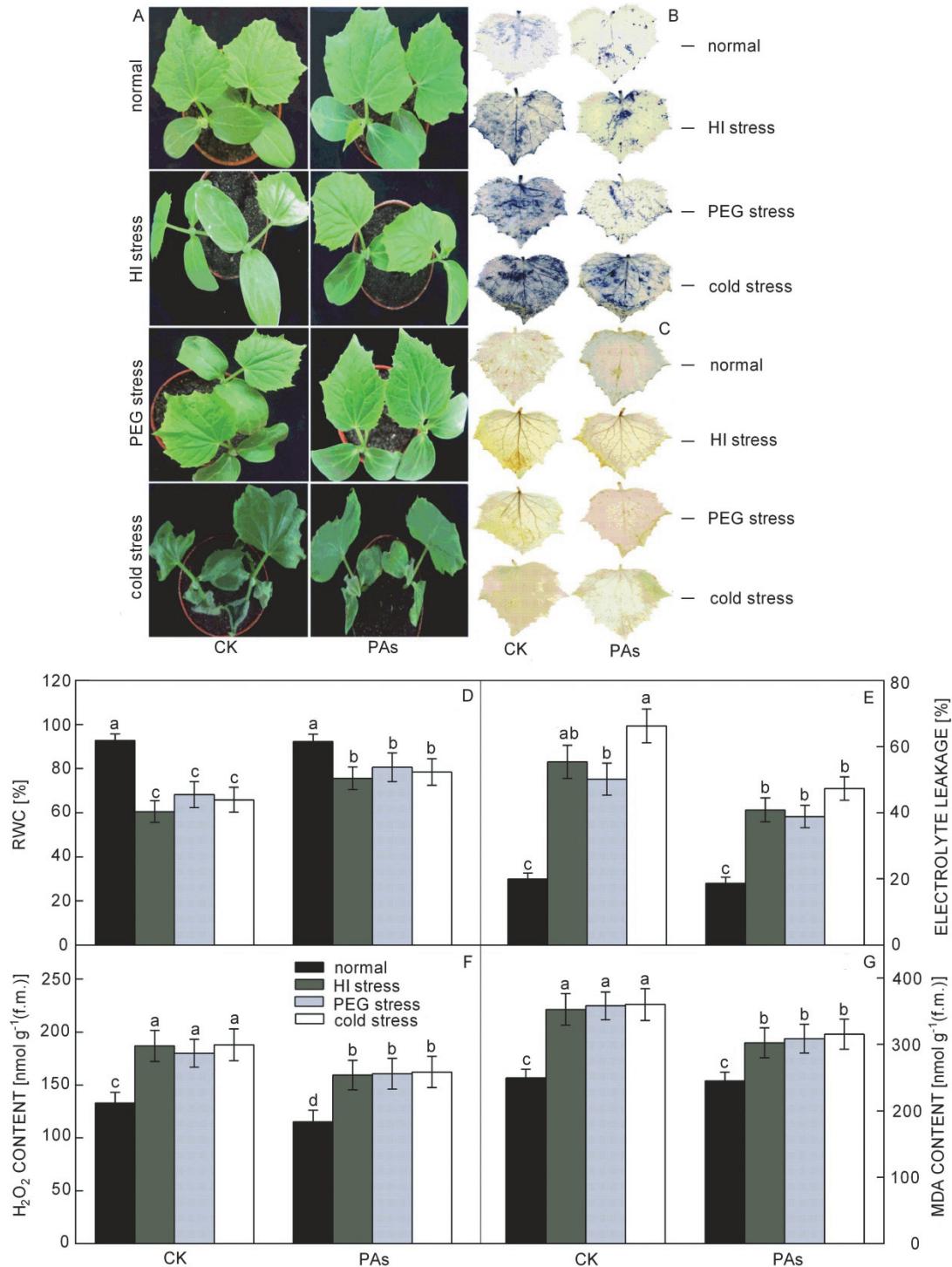


Fig. 1. A - Representative phenotypes of cucumber seedlings after 3 d of high irradiance ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$), PEG (18 %) and cold stress (4 °C) with or without 0.1 % PAs pretreatment. B - Superoxide was detected by 0.5 mg cm^{-3} nitroblue tetrazolium (NBT) staining. C - H₂O₂ was detected by 2 mg cm^{-3} 3, 3-diaminobenzidine (DAB) staining. Effects of 0.1 % PAs pretreatment on relative water content (RWC) (D), electrolyte leakage (EL) (E), H₂O₂ content (F) and malondialdehyde (MDA) content (G) in the cucumber seedlings under stress conditions for 3 d. Means \pm SDs of three biological replicates. Significant differences ($P \leq 0.05$) are denoted by different lowercase letters.

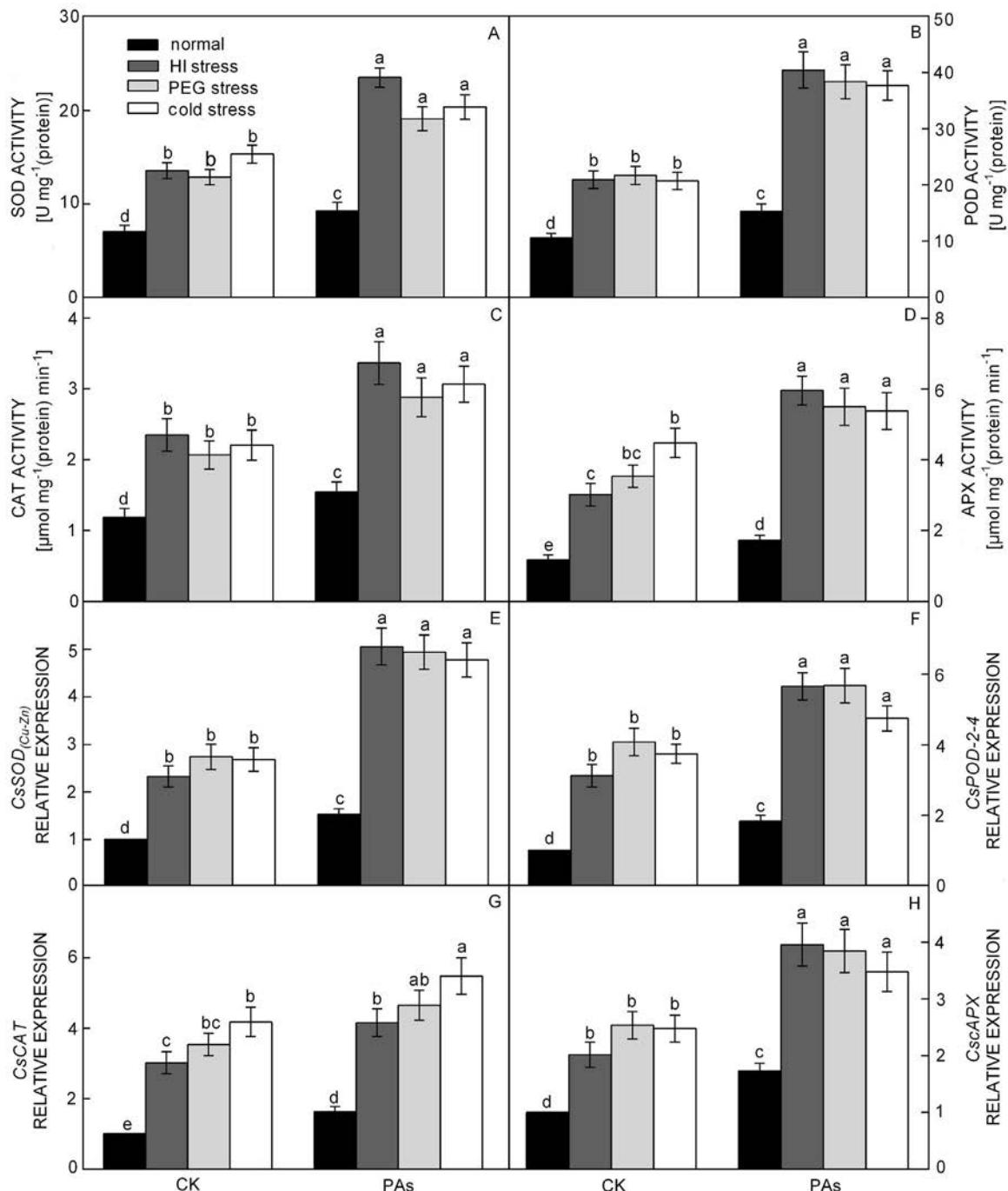


Fig. 2. Changes in the activities of superoxide dismutase (SOD) (A), peroxidase (POD) (B), catalase (CAT) (C), and ascorbate peroxidase (APX) (D) in cucumber seedlings under stress conditions for 3 d with or without 0.1 % PAs pretreatment. Transcription of *CsSOD_(Cu-Zn)* (E), *CsPOD-2-4* (F), *CsCAT* (G), and *CscAPX* (H) genes were determined by RT-qPCR. Means \pm SDs of three biological replicates. Significant differences ($P \leq 0.05$) are denoted by different lowercase letters (a, b, c, d, e) above the bars.

control seedlings after 3 d of stresses (Fig. 2). Moreover, transcripts of *CsSOD_(Cu-Zn)*, *CsPOD-2-4*, *CsCAT*, and *CscAPX* genes in the PAs-pretreated seedlings showed a more dramatic increase in comparison to the control seedlings after 3 d of stresses.

The total chlorophyll content remained higher in the PAs-pretreated seedlings than in the control seedlings

under all three types of stress (Fig. 3A). The function of photosystem II (PS II) was also investigated under stress conditions. The results showed that the photochemical efficiency of the PS II (F_v/F_m) and electron transport rate (ETR) were significantly higher in the PAs-pretreated seedlings than in the control seedlings under stress conditions (Fig. 3B,C). In contrast, the non-photo-

chemical quenching (NPQ) was significantly lower in the PAs-pretreated seedlings compared with the control seedlings (Fig. 3D).

The total respiration and cyanide (CN)-resistant respiration were both higher in the PAs-pretreated seedlings than in the control seedlings after all three types of stress for 3 d (Fig. 4A-D). Consistent with the determined CN-resistant respiration rate, the transcription and protein content of AOX participating enzymes were obviously higher in the PAs-pretreated seedlings, as

compared with the control (Fig. 4E,F). Furthermore, increase in ABA content was also induced by stress treatments with or without PAs pretreatment (Fig. 5D). However, it should be noted that ABA production and transcription of *CsABI1*, *CsABI2* and *CsABI3* were much higher in the PAs-pretreated seedlings than in the control seedlings after 3 d of stresses (Fig. 5).

The AOX inhibitor SHAM (1 mM) significantly affected the plant resistance to environmental stresses. Seedlings pretreated with 1 mM SHAM displayed more

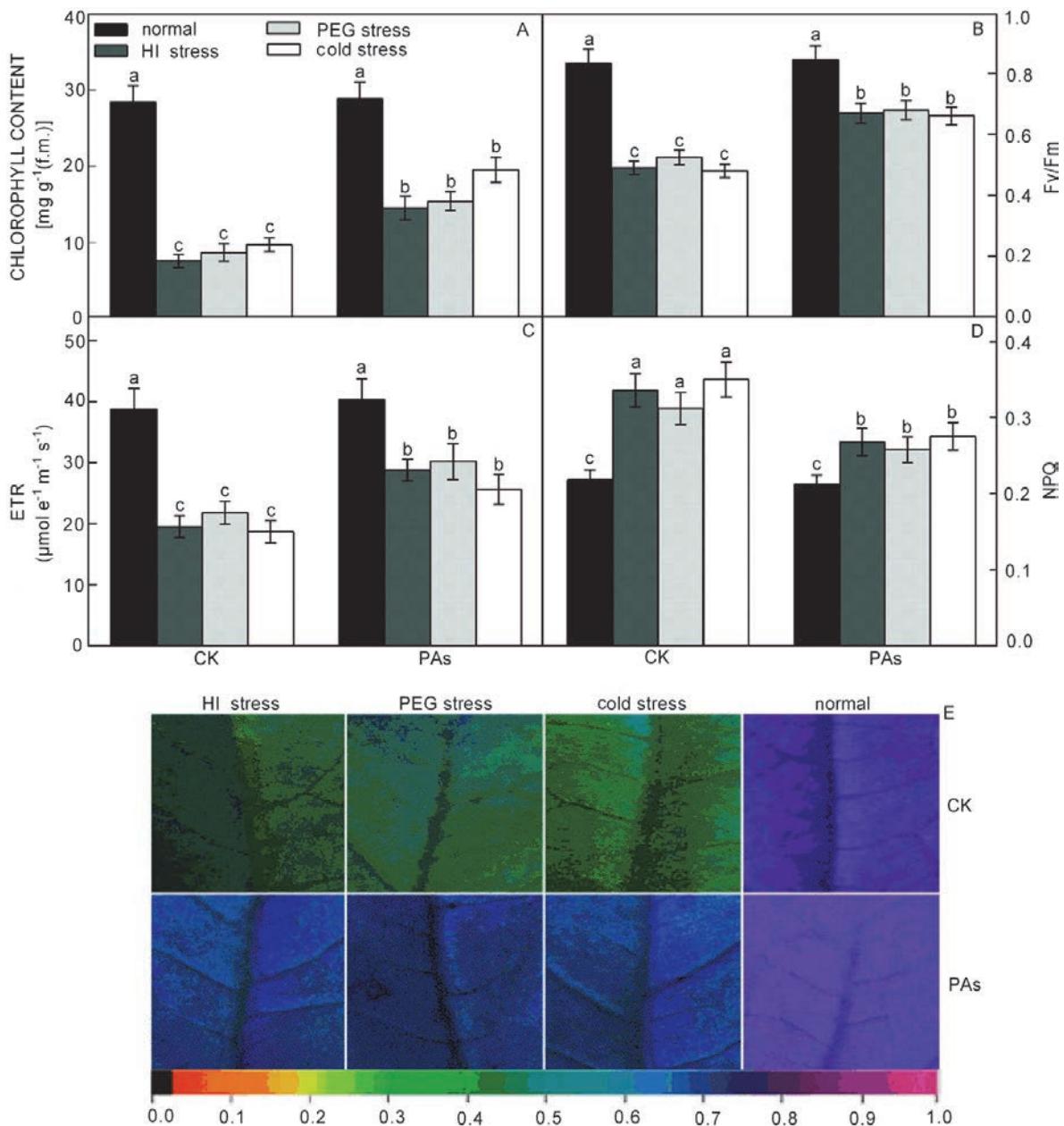


Fig. 3. Changes in total chlorophyll content (A), F_v/F_m (B), electron transport rate (ETR) (C), and non-photochemical quenching (NPQ) (D) of the cucumber leaves under stress conditions for 3 d with or without 0.1 % PAs pretreatment. Images of F_v/F_m (E) of the cucumber leaves under stress conditions for 3 d with or without 0.1 % PAs pretreatment. The false colour code depicted at the bottom of the image ranged from 0 (black) to 1.0 (purple). Means \pm SDs of three biological replicates. Significant differences ($P \leq 0.05$) are denoted by different lowercase letters.

severe damage after 3 d of HI, PEG, and cold stresses than the control. Application of 0.1 % PAs enhanced the 1 mM SHAM-pretreated seedlings stress tolerance (Fig. 3 Suppl.). Consistent with the H_2O_2 content, EL, and MDA content were lower in the control seedlings compared to

the 1 mM SHAM-pretreated seedlings, although all of them increased under stress conditions (Fig. 4 Suppl.). Meanwhile, the mRNA levels of AOX showed obviously higher in the PAs+SHAM-pretreated seedlings compared to the SHAM-pretreated seedlings (Fig. 3 Suppl.).

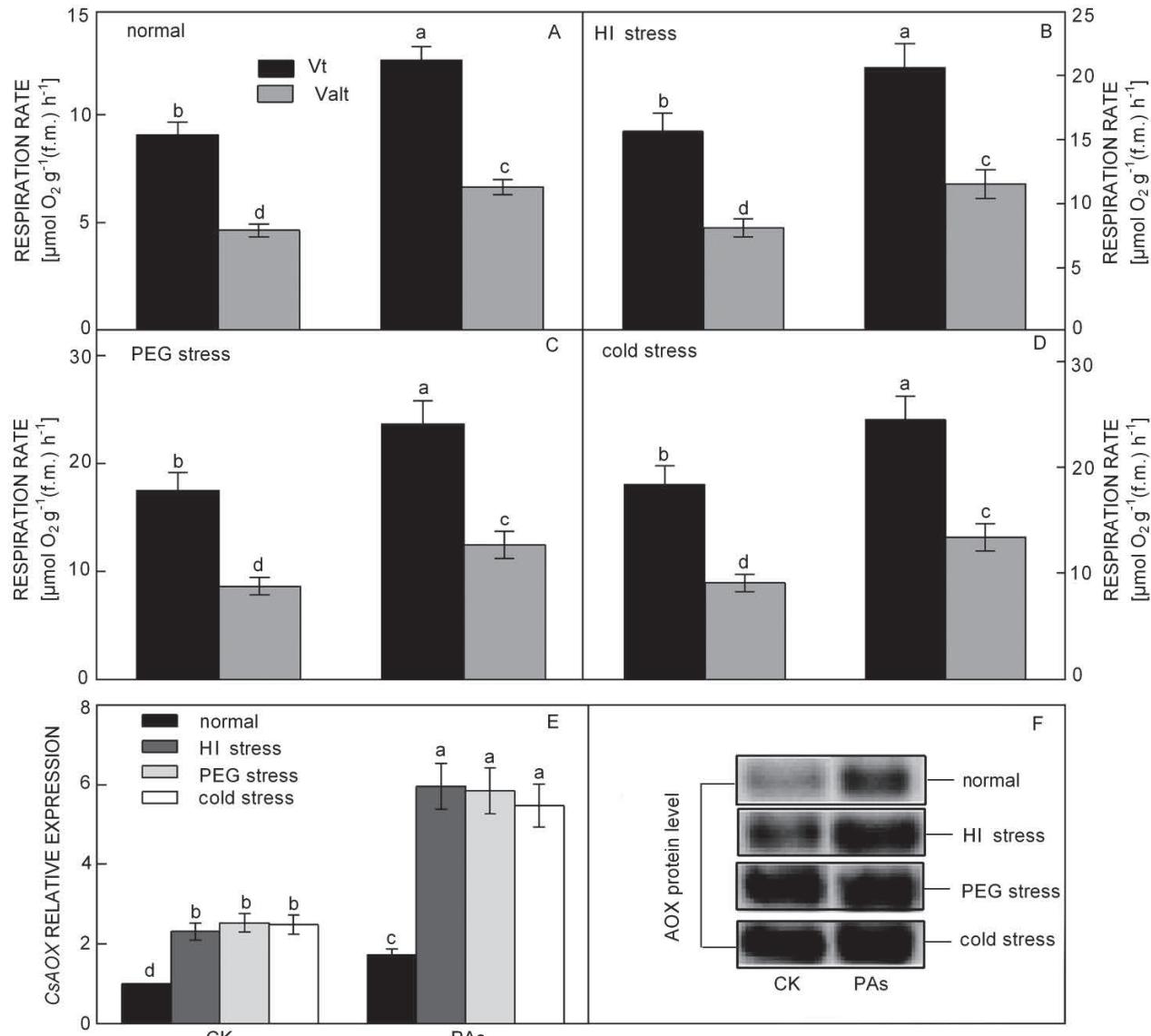


Fig. 4. Changes in respiration rate (A-D), transcripts of *CsAOX* (E) and AOX protein content (F) in cucumber seedlings under stress conditions for 3 d with or without 0.1 % PAs pretreatment. Total respiration (Vt) and cyanide-resistant respiration (Valt) were measured using Hansatech Clark-type electrodes. Transcription of *CsAOX* was determined by RT-qPCR. For Western blot analysis, proteins (50 μg) were separated by SDS-PAGE and then blotted with the AOA. Means \pm SDs of three biological replicates. Significant differences ($P \leq 0.05$) are denoted by different lowercase letters. HI - high irradiance, CK - control.

Discussion

PAs are present in leaves, seeds, barks, and fruits of many plants, where they provide protection against predation and pathogen attack (Dixon *et al.* 2005). Regulation of seed germination by PAs is mediated through ABA

signalling pathway (Jia *et al.* 2012). In this study, we found that 0.1 % PAs pretreatment enhanced cucumber seedlings tolerance to environmental stresses. However, the inhibited stress tolerance was observed with higher

PAs concentration pretreatment (Fig. 2 Suppl.). Hence, PAs might play dual role in response to stress. As mentioned in previous study, ABA is involved in the enhancement of plant resistance to stress conditions (Lu *et al.* 2009). We hypothesize that PAs act as a signalling molecules involved in the induction of ABA under stress conditions. The results showed higher ABA content in the PAs-pretreated seedlings than in the control seedlings after 3 d of stresses (Fig. 5D).

In this study, we found that cucumber seedling pretreated with 0.1 % PAs showed induced stress tolerance (Fig. 1). However, 0.1 % PAs pretreatment increased the activity of AOX (Fig. 1 Suppl.) and the

ABA content (Fig. 5). Therefore, it seemed that PAs pretreatment might directly affect AOX activity and ABA content, but not through the ROS. Meanwhile, environmental stress-induced ROS production (Fig. 1) might contribute to *CsAOX* expression. Furthermore, the transcription of *CsAOX*, *CsABI1*, *CsABI2*, and *CsABI3* were strongly up-regulated with PAs pretreatment, which were much higher than in the control after stresses (Fig. 4E, Fig. 5A-C). These findings are consistent with the previous studies that the enhanced AOX and ABA contribute to plant stress tolerance (Larkindale and Knight 2002, Van Aken *et al.* 2009).

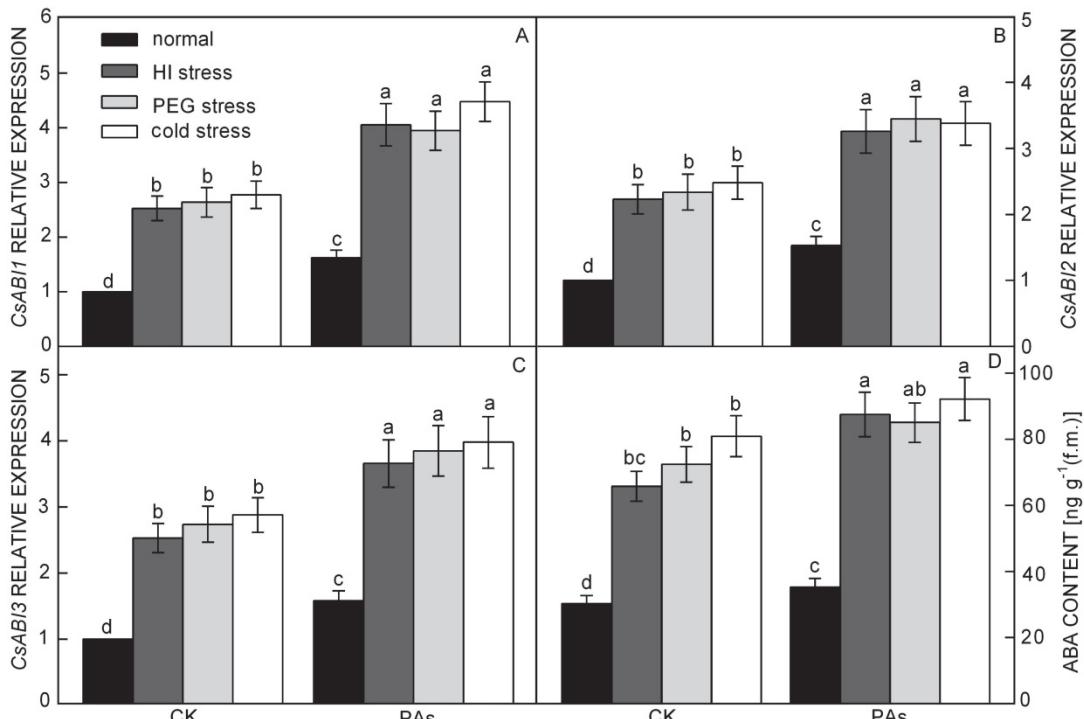


Fig. 5. Changes in transcription of *CsABI1*, *CsABI2*, or *CsABI3* determined by RT-qPCR (A-C) and ABA content (D) in cucumber seedlings under stress conditions for 3 d with or without 0.1 % PAs pretreatment. Means \pm SDs of three biological replicates. Significant differences ($P \leq 0.05$) are denoted by different lowercase letters.

It has been reported that the inhibition of AOX stimulates ROS production in plant mitochondria (Popov *et al.* 1997, Moller 2001), whereas overexpression of AOX results in lower ROS accumulation (Maxwell *et al.* 1999). Our work showed that the inhibition of AOX activity by SHAM caused a sharp increase in ROS production which led to more severe oxidative damage of plants in response to environmental stresses (Figs. 3 and 4 Suppl.). Therefore, it is reasonable to assume that the up-regulation of CN-resistant respiration by PAs pretreatment contributes to the lower ROS accumulation under stress conditions.

The antioxidant defence machinery protects plants against oxidative stress (Foyer and Noctor 2009, Moller and Sweetlove 2010) and the ROS-scavenging systems

might be activated in the PAs-pretreated seedlings. In the present study, we found that PAs pretreatment significantly enhanced the activities of antioxidant enzymes under environmental stresses (Fig. 2A-D). Moreover, the transcriptions of *CsSOD*_(Cu-Zn), *CsPOD*2-4, *CsCAT*, and *CsCAPX* were significantly up-regulated in the PAs-pretreated seedlings compared with the control seedlings (Fig. 2E-H). Therefore, it seems that the increase in antioxidant enzyme activities is one of the reasons for the lower ROS accumulation in the PAs-pretreated seedlings. We propose that the elevated ABA content in the PAs-pretreated seedlings (Fig. 5) might be involved in promoting the activities of antioxidant enzymes under environmental stresses.

It has been reported that up-regulation of the

mitochondrial AOX enhances photosynthetic electron transport under drought, whereas plants lacking AOX display lower photochemical efficiency and dissipate more excitation energy by NPQ during combined high irradiance and drought stress (Yoshida *et al.* 2006, Giraud *et al.* 2008). In our study, the total chlorophyll content, non-photochemical quenching (NPQ) and F_v/F_m in the PAs-pretreated seedlings were less affected by the stress conditions than in the control seedlings (Fig. 3A,B,D). Therefore, the data presented here suggest that the enhanced AOX by PAs pretreatment contributed to protection of chloroplasts under stress conditions.

In conclusion, suitable concentration of PAs enhanced

cucumber seedlings tolerance to environmental stresses. The results of our study indicate that both AOX and ABA were induced by PAs pretreatment. Then, the enhanced AOX contributed to the ROS avoidance and the protection of photosystems. Moreover, the increased ABA content was involved in the further induction of AOX and antioxidant enzymes activities. Therefore, our findings uncover a novel role of PAs in plant protection against environmental stresses and clarify the relationships between AOX, ROS, and ABA during stress conditions, although the detailed metabolic relations need further investigation.

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