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Amelioration of oxidative damage in *Solanum melongena* seedlings by 24-epibrassinolide during chilling stress and recovery

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Abstract

The effects of foliar application of 24-epibrassinolide (EBR) on chlorophyll content, oxidative damage, and antioxidant capacity in eggplant (*Solanum melongena* L.) seedlings during a short-term chilling stress and subsequent recovery were investigated. The eggplant seedlings pretreated with 0.1 μM EBR were exposed to 5 °C for 12 h in the dark and then recovered under a normal temperature for another 12 h. The results show that EBR alleviated a chilling-induced oxidative stress reflected by a decrease in malondialdehyde and H_2O_2 content, $O_2^{\bullet-}$ production rate, and an increase in chlorophyll content and activities of superoxide dismutase, guaicol peroxidase, and catalase. The EBR pretreatment also promoted activities of ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, as well as the accumulation of ascorbic acid (AsA) and glutathione (GSH) involved in the AsA-GSH cycle. Moreover, after the12 h recovery, EBR plus chilling treated leaves showed no significant changes in content of MDA and chlorophyll *a*, lower reactive oxygen species (ROS) content, and greater activities of AsA-GSH cycle enzymes than in seedlings only chilled. The results suggest that EBR alleviated an oxidative damage caused by the dark chilling stress and accelerated a recovery rate mainly through increasing the ROS scavenging system including the AsA-GSH cycle.

Additional key words: antioxidant system, AsA-GSH cycle, brassinosteroids, eggplant, short-term chilling.

Introduction

Chilling stress is one of the major limiting factors affecting plant growth and productivity (Chinnusamy *et al*. 2006, Caffagni *et al*. 2014). A common phenomenon of chilling stress is an overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$), resulting in oxidative stress in the plant (Mittler 2002). ROS are highly toxic and they are the major contributing factors to chilling-induced injury (Liu *et al*. 2009, Genisel *et al*. 2013). To balance ROS metabolism, plants have evolved a specific defence system including antioxidative enzymes like peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), and nonenzymatic antioxidants like ascorbate (AsA) and glutathione (GSH) (Mittler 2002, Ramakrishna and Rao 2013, Xi *et al*. 2013). APX, GR, MDHAR, DHAR, AsA, and GSH are important components of the ascorbate-glutathione (AsA-GSH) cycle which is a key element in the network of biochemical reactions for the efficient elimination of ROS (Foyer and Noctor 2005, Kang *et al*. 2013). Accumulating evidence indicate that the antioxidant defence system plays a general role in the tolerance capacity of plants against chilling stress (Foyer *et al*.

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Abbrevations: APX - ascorbate peroxidase, AsA - ascorbate, BRs - brassinosteroids, CAT - catalase, Chl - chlorophyll, DHAR - dehydroascorbate reductase, EBR - 24-epibrassinolide, GR - glutathione reductase, GSH - reduced glutathione, MDA - malondialdehyde, NBT - nitroblue tetrazolium chloride, $O_2^{\bullet-}$- superoxide anion radical, POD - peroxidase, ROS - reactive oxygen species, SOD - superoxide dismutase.
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2002, Liu *et al*. 2009, Fariduddin *et al*. 2011, Genisel *et al*. 2013, Xi *et al*. 2013, Wu *et al*. 2014).

Brassinosteroids (BRs) are a group of over 40 naturally occurring plant steroid hormones which play an essential role in plant growth and development as well as in responses to environmental factors including chilling stress (Kartal *et al*. 2009, Ding *et al*. 2012, Li *et al*. 2012, Ramakrishna and Rao 2012, Ahammed *et al*. 2013, Fariduddin *et al*. 2014, Zhang *et al*. 2014). Previous studies have shown that BRs-induced tolerance is associated with a protection of photosynthesis, activation of the antioxidant system and alteration in gene expression (Bajguz and Hayat 2009, Fariduddin *et al*. 2014). However, the mechanisms for the BRs-mediated stress relief are still poorly understood (Hu *et al*. 2010). Although some studies have reported that BRs can protect plants from chilling damage (Liu *et al*. 2009, Fariduddin *et al*. 2011, Genisel *et al*. 2013, Xi *et al*. 2013, Wu *et al*. 2014), there are few reports on the role of BRs in response to short-term dark chilling stress and recovery. BRs-induced tolerance to chilling stress does not only happen during the stress period, but also occurs during the subsequent recovery under optimum growth conditions.

Eggplant is an important agricultural plant that has high sensitivity to chilling. Nonetheless, information about chilling tolerance induced by EBR in eggplant is scanty. In our previous study, we found that 0.1 μM EBR is the optimum concentration to alleviate a cold-caused eggplant growth inhibition by regulating photosynthesis and the antioxidative systems (Wu *et al*. 2014). However, it is not sure whether the AsA-GSH cycle is involved in EBR-induced tolerance to cold stress. To further explore the potential mechanism of EBR in promoting cold tolerance, eggplants were exposed to a short-term dark chilling stress and recovery and the role of EBR in chilling resistance was investigated. Accordingly, the chlorophyll content, lipid peroxidation, ROS metabolism, and antioxidant capacity including AsA-GSH cycle were determined.

Materials and methods

Seeds of eggplant (*Solanum melongena* L. cv. Huqie 9) were rinsed thoroughly with distilled water and germinated on moist filter paper in an incubator at 28 °C. Uniformly germinated seeds were selected and sown in plastic pots filled with a 1:1 mixture of peat and *Vermiculite*, one plant per pot. Seedlings were grown in a greenhouse under a 12-h photoperiod, an irradiance of 600 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a relative humidity of 65 - 70 %. All plants were irrigated with a half-strength Hoagland nutrient solution every 2 d.

Treatments by 24-epibrassinolide (EBR, *Sigma*, St. Louis, USA) and chilling were conducted when the eggplant seedlings were at the fourth or fifth-true leaf stage. Healthy plants of a similar size were chosen and then sprayed with 0.1 μM EBR or distilled water as control. One day after spraying, the plants were transferred to a cold chamber (25/5 °C). The controls were grown at 25/20 °C. The chilling treatments lasted 12 h in the dark and then the plants were recovered at the beginning of the photoperiod (6:00 h) to the control growth chamber. The four treatments were designed as: *1*) control, 0 μM EBR and 25/20 °C, *2*) EBR, 0.1 μM EBR and 25/20 °C, *3*) CS, 0 μM EBR and 25/5 °C, and *4*) CS+EBR, 0.1 μM EBR and 25/5 °C. After 0, 6, 12 h, and 24 h, the third leaves were sampled and immediately frozen in liquid N_2, and then stored at -80 °C for further analyses.

For determination of chlorophyll (Chl) content, fresh leaves from each group (0.2 g) were homogenized in 95 % (v/v) ethanol and then the homogenate was filtered through filter paper. The absorbances of the resulting solution was read by a spectrophotometer *DU730* (*Beckman*, Coulter, USA) at 649 and 665 nm. The chlorophyll content was calculated according to Knudson *et al*. (1977).

$O_2^{\bullet-}$ production rate was measured as described by Zhao *et al*. (2008) by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$. A standard curve with NO_2^- was used to calculate the production rate of $O_2^{\bullet-}$ from the chemical reaction of $O_2^{\bullet-}$ and hydroxylamine. The content of H_2O_2 was determined by monitoring the absorbance (A_{410}) of a titanium peroxide complex according to the method described by Patterson *et al*. (1984).

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) production by the thiobarbituric acid reaction as described by Jiang and Zhang (2001).

For enzyme assays, frozen leaves (0.2 g) were ground with 3 cm^3 of an ice-cold 50 mM phosphate buffered saline (PBS) buffer (pH 7.8) containing 0.2 mM EDTA and 2 % (m/v) polyvinylpyrrolidone (PVP; analytical grade). The homogenates were centrifuged at 12 000 *g* and 4 °C for 20 min and the supernatants were used for the determination of enzymatic activities. SOD activity was assayed by the nitroblue tetrazolium (NBT) method of Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme required to cause a 50 % inhibition of the reduction of NBT as monitored at 560 nm. POD activity was measured according to Hammerschmidt *et al*. (1982) by monitoring the rate of guaiacol oxidation at 470 nm. CAT was assayed as described by Durner and Klessing (1996), and the activity was determined as a decrease in the absorbance at 240 nm for 1 min following the decomposition of H_2O_2. APX and DHAR activities were measured by a decrease in absorbance at 290 nm and an increase in absorbance at 265 nm, respectively (Nakano and Asada 1981). GR activity was measured according to the method of Foyer and Halliwell (1976) which depends on the rate of

decrease in the absorbance of NADPH at 340 nm. MDHAR activity was measured by using 1 U ascorbate oxidase and the oxidation rate of NADH was followed at 340 nm (Hossain *et al*. 1984).

For determination of antioxidants content, fresh leaves (0.2 g) were ground to a fine powder in liquid N_2 and then homogenized in 1 cm^3 of an ice-cold 0.5 mM Na-phosphate buffer (pH 7.8) and the equal volume of 10 % (m/v) trichloroacetic acid was added for the AsA assay, or the equal volume of 10 % (m/v) sulfosalicylic acid was added for the GSH assay. The crude extracts were centrifuged at 12 000 *g* and 4 °C for 20 min, and the supernatant was used for further determinations. AsA content was measured according to changes in absorbance at 412 nm after the addition of ascorbate oxidase according to the method of Arakawa *et al*. (1981). GSH content was assayed by following the change in absorbance at 412 nm after the addition of 5-dithio-bis-(-2-nitrobenzoic acid) according to the method of Griffiths (1980).

Proline content was measured according to the method of Bates *et al*. (1973) after the extraction of leaves (0.5 g) at room temperature with a 3 % (m/v) 5-sulfo-salicylic acid solution.

Each value in results is a mean ± standard deviation (SD) from a minimum of three replicates. Differences between treatments were considered statistically significant when $P < 0.05$ according to the Duncan's multiple range test using the *SPSS* package *v. 11* (*SPSS*, Chicago, IL, USA).

Results

The eggplant seedlings showed a rapid decrease in the content of Chl *a* and Chl *b* during chilling (Fig. 1). After the 12-h chilling stress, the content of Chl *a*, Chl *b*, and total Chl decreased by 11.1, 55.7, 21.6 %, respectively, compared to the control. However, after 12 h of recovery, Chl *a* recovered to the control level in the CS+EBR plants (Fig. 1*A*).

The chilling stress significantly increased the MDA

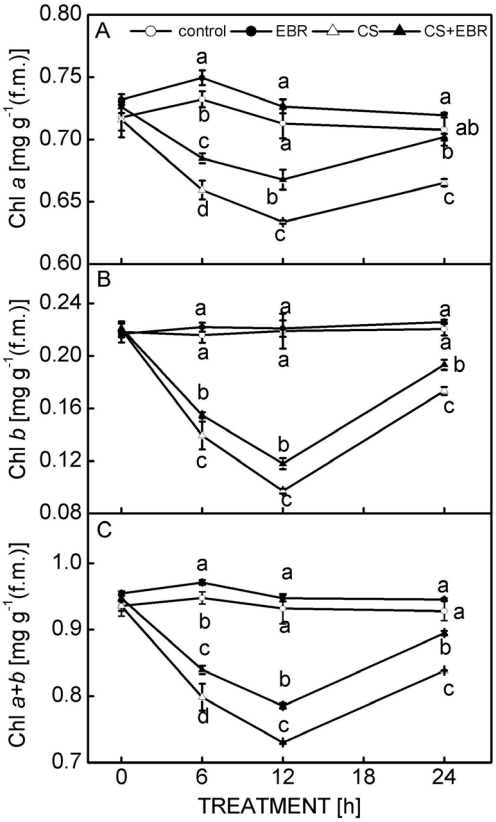

Fig. 1. Effects of EBR on Chl *a* (*A*), Chll *b* (*B*), and Chl a + *b* (*C*) content in eggplant seedlings subjected to dark chilling (5 °C) for 12 h and then recovery at 25 °C and an irradiance of 600 µmol m^{-2} s^{-1} for ddditional 12 h. Means ± SD, $n = 3$. Means marked with different letters at the same time indicate significant differences between treatments at $P < 0.05$ according to the Duncan's multiple range test.

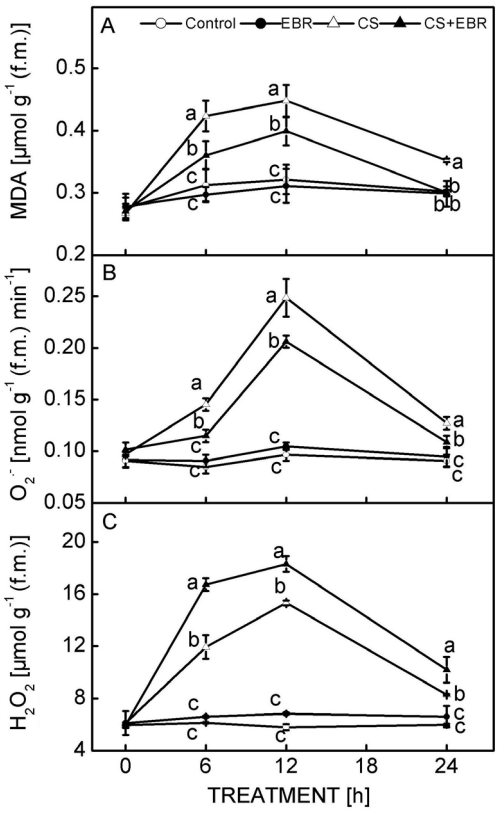

Fig. 2. Effects of EBR on MDA content (*A*), $O_2^{\bullet-}$ production rate (*B*), and H_2O_2 content (*C*) in eggplant seedlings subjected to dark chilling (5 °C) for 12 h and then recovery at 25 °C and irradiance of 600 µmol m^{-2} s^{-1} for additional 12 h. Means ± SD, $n = 3$. Means marked with different letters at the same time indicate significant differences between treatments at $P < 0.05$ according to the Duncan's multiple range test.

content, $O_2^{\bullet-}$ production rate, and H_2O_2 content reaching the highest values after the 12-h stress (Fig. 2). After 12 h of recovery, these parameters of the CS plants sharply decreased but not to the control values. Interestingly, the CS+EBR plants showed a lower content of MDA and H_2O_2, and a lower $O_2^{\bullet-}$ production rate under the chilling stress or recovery period than the CS plants. Importantly, the MDA content of the CS+EBR plants restored to the control values (Fig. 2*A*). Under the normal conditions, exogenous EBR showed no effect on these parameters (Fig. 2).

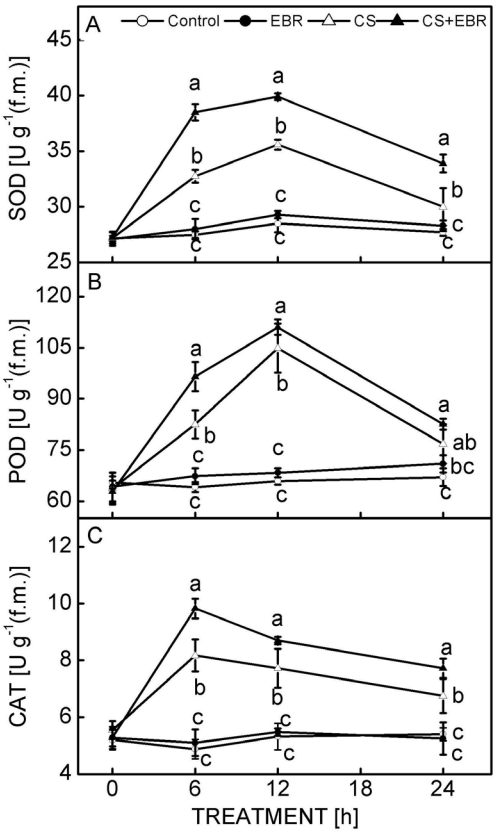

Fig. 3. Effects of EBR on the activities of SOD (*A*), POD (*B*), and CAT (*C*) in eggplant seedlings subjected to dark chilling (5 °C) for 12 h and then recovery at 25 °C and an irradiance of 600 μmol m^{-2} s^{-1} for additional 12 h. Means ± SD, $n = 3$. Means marked with different letters at the same time indicate significant differences between treatments at $P < 0.05$ according to the Duncan's multiple range test.

Under the chilling stress, the activities of SOD and POD increased significantly reaching the maximum values at 12 h (Fig. 3*A*,*B*), whereas the CAT activity increased up to the maximum value at 6 h (Fig. 3*C*). The activities of SOD, POD and CAT decreased gradually but did not reach the control values. The EBR treated plants maintained higher SOD, POD, and CAT activities during chilling and the subsequent recovery period. After the 12-h recovery, the SOD, POD and CAT activities of the CS+EBR plants were 13.0, 7.5, and 14.4 % higher than those of the CS plants. The activities of SOD, POD and CAT did not change upon the exposure to the EBR treatment alone (Fig. 3*A*,*B*,*C*).

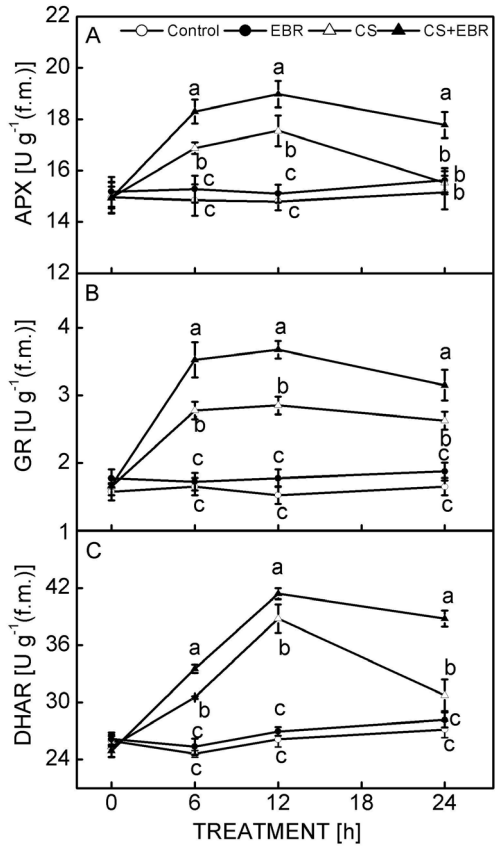

Fig. 4. Effects of EBR on the ctivities of APX (*A*), GR (*B*,) and MDHR (*C*) of eggplant seedlings subjected to dark chilling (5 °C) for 12 h and then recovery at 25 °C and an irradiance of 600 μmol m^{-2} s^{-1} for additional 12 h. Means ± SD, $n = 3$. Means marked with different letters at the same time indicate significant differences between treatments at $P < 0.05$ according to the Duncan's multiple range test.

The APX, GR, and DHAR activities significantly increased after the chilling stress reaching the maximum values at 12 h. The EBR pretreatment significantly increased the APX, GR, and DHAR activities during the chilling stress and subsequent recovery. After the 12-h recovery, the APX, GR, and DHAR activities were 14.5, 20.0, and 26.1 % higher at CS+EBR compared to the CS plants. The EBR pretreatment exerted a non-significant influence on the activity of these enzymes under the normal conditions (Fig. 4*A*,*B*,*C*).

Compared with the control, the content of AsA and GSH was significantly enhanced during the chilling stress and recovery and the EBR application further increased the AsA and GSH content. Under the normal growth conditions, EBR slightly increased the AsA and GSH content at 12 h (Fig. 5*A*,*B*).

The proline content increased sharply during the chilling stress and remained high during the subsequent recovery. After 6, 12, and 24 h of the treatment, the proline content was 2.0, 5.0, and 4.5-fold higher in the

CS than in control plants. EBR further increased the proline content by 16.2, 22.5, and 24.2 % at the 6-h and 12-h chilling stress and 12-h recovery, respectively. The EBR application showed no influence on the proline content under the normal conditions (Fig. 5*C*).

Discussion

Although many investigators have studied the role of BRs in plants under chilling stress, most employed plants were subjected to the full diurnal cycle of chilling stress (Liu

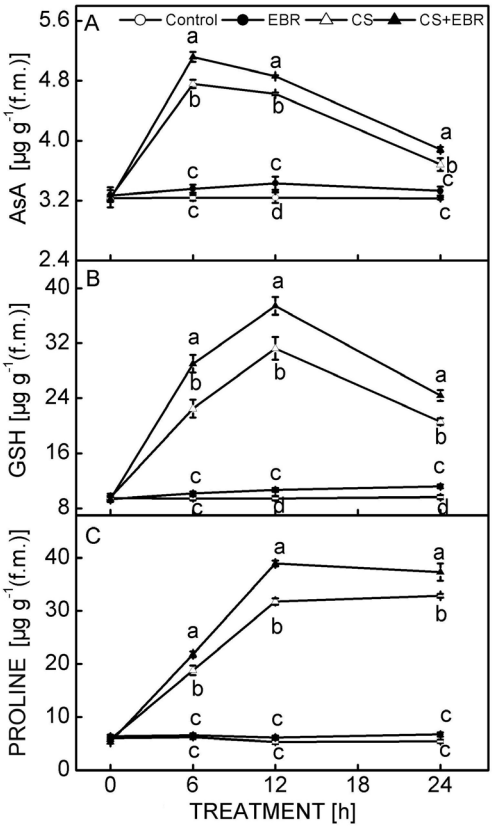

Fig. 5. Effects of EBR on the content of AsA (*A*), GSH (*B*), and proline (*C*) of eggplant seedlings subjected to dark chilling (5 °C) for 12 h and then recovery at 25 °C and an irradiance of 600 µmol m^{-2} s^{-1} for additional 12 h. Means ± SD, $n = 3$. Means marked with different letters at the same time indicate significant differences between treatments at $P < 0.05$ according to the Duncan's multiple range test.

et al. 2009, Hu *et al*. 2010, Fariduddin *et al*. 2011, Jiang *et al*. 2013, Xi *et al*. 2013, Wu *et al*. 2014). In comparison to recovery from chilling during daytime, the recovery from chilling in the dark proceeds faster (Jun *et al*. 2001). The photosynthetic capacity declines in chilling-stressed plants (Hu *et al*. 2010, Fariduddin *et al*. 2011, Jiang *et al*. 2013), and the content of Chl is a good criterion to estimate the effect of stress on the photosynthetic apparatus. In this study, it was observed that the content of Chl *a* and Chl *b* significantly decreased during 12 h of dark chilling (Fig. 1), which is in agreement with Ibrahim and Bafeel (2008) and Soares-Cordeiro *et al*. (2010). However, Garstka *et al*. (2007) showed that a total Chl content in dark-chilled and subsequently recovered leaves of pea and tomato are not altered significantly. The change of Chl content may depend upon species, its cold tolerance, and the experimental arrangement. The low Chl content in dark chilled eggplant leaves might result from destruction of chloroplast structure (Garstka *et al*. 2007), decreased Chl synthesis, or increased Chl degradation. Many reports as well as our previous study have shown that an EBR application ameliorates a cold-induced reduction in Chl content (Hu *et al*. 2010, Singh *et al*. 2012, Wu *et al*. 2014). In the present study, the EBR application mitigated a decrease in Chl caused by the short-term chilling stress (Fig. 1) indicating that EBR could protect chloroplasts and maintain a higher photosynthetic capacity during short-term dark chilling and subsequent recovering. An EBR application may directly or indirectly stimulate the Chl biosynthesis or reduce the chlorophyllase activity (Xia *et al*. 2009). In addition, an EBR-maintained high Chl content can be due to a lower ROS content as result of efficient scavenging ROS (Genisel *et al*. 2013).

It is well documented that chilling stress can induce an excessive production of ROS leading to oxidative damage to cell membranes (Genisel *et al*. 2013, Wu *et al*. 2014). The inactivation of photosynthesis by dark chilling is related to the damage of the membranes caused by ROS (Ibrahim and Bafeel 2008). The present study shows that dark chilling accelerated the accumulation of MDA (an indicator of lipid peroxidation) in parallel with a marked increase in $O_2^{\bullet-}$ production rate and H_2O_2 content, whereas the EBR treatment resulted in a significant decline in these parameters, especially MDA (recovered to the control level). A similar result was also found in the study of cucumber seedlings treated with EBR under a chilling stress (Hu *et al*. 2010). Our results suggest that EBR protected the plant from oxidative damage during the dark chilling stress, quickly repaired membrane lipid peroxidation during the recovery period, and thereby enhanced the plant chilling tolerance. In response to chilling-induced oxidative injury, plants have evolved a complex antioxidant defence system (Mittler 2002, Ramakrishna and Rao 2013, Xi *et al*. 2013). Recently, effects of BRs on the antioxidant system under various biotic and abiotic stresses have been reviewed (Bajguz and Hayat 2009, Fariduddin *et al*. 2014). In the present work, the activities of SOD, POD, and CAT significantly increased during the 12-h chilling stress, and the EBR application further increased these activities, which provided an additional power to balance ROS metabolism. The results suggest that EBR could play a

positive role in the protection against chilling-induced oxidative damage through increasing the activities of antioxidative enzymes like SOD, POD, and CAT. It was reported that a treatment with EBR or 28-homobrassinolide increases the activities of antioxidant enzymes under a chilling stress (Fariduddin *et al*. 2011, Xi *et al*. 2013). Xia *et al*. (2009) reported that BRs-induced stress tolerance is associated with an increased expression of genes encoding antioxidant enzymes, such as *SOD*, *CAT*, *POD*, *GR*, and *APX*, in leaves of cucumber.

In plant cells, the AsA-GSH cycle plays a major role in ROS metabolism. In this cycle, APX utilizes AsA to detoxify H_2O_2, and AsA is oxidized to monodehydroascorbate (MDHA) which can be converted to AsA by NADPH-dependent MDHAR. MDHA may also disproportionate non-enzymatically to AsA and dehydroascorbate (DHA). DHAR utilizes GSH as reductant to reduce DHA to AsA. Oxidized glutathione (GSSG) is reconverted to GSH by GR (Ramakrishna and Rao 2013). In this study, EBR alone slightly increased the content of AsA and GSH (Fig. 5*A*,*B*), which is supported by Jiang *et al*. (2013) and Ramakrishna and Rao (2013) who stated that EBR maintains an elevated redox state of AsA and GSH accompanied by increased activities of APX, MDHAR, DHAR, and GR in radish. During dark chilling and subsequent recovering, AsA and GSH were sharply promoted together with an increase in the activities of APX, DHAR, and GR (Fig 4*A*,*C*,*B*) which were further enhanced by the EBR application. It was also reported that an EBR treatment can increase an AsA and GSH content in plants under a chilling stress (Liu *et al*. 2009, Xi *et al*. 2013). All these results suggest that BRs can accelerate the AsA-GSH cycle under a low temperature, and a high content of AsA and GSH may alleviate oxidative damage caused by chilling stress.

Proline accumulation represents another adaptive mechanism of plants to unfavorable environments (Fariduddin *et al*. 2011). Accumulating proline has multiple protective functions, such as osmotic adjustment, protein and biomembrane stabilization, ROS-scavenging, redox buffering, and so on (Theocharis *et al*. 2012). In the present study, the content of proline in eggplant was further induced by the EBR application during the chilling stress and recovery. Similar results have been reported in cucumber (Fariduddin *et al*. 2011) and grapevine (Xi *et al*. 2013) under chilling conditions. However, effects of BRs on proline metabolism have been poorly addressed. An EBR-induced accumulation of proline under stress may be associated with a reduction in proline utilization due to a minimum protein formation, proline degradation, and enhancement in proline formation due to the stimulation of Δ^1-pyrroline-5-carboxylate synthetase (Zhang *et al*. 2014).

In conclusion, exogenous EBR could alleviate short-term dark chilling-induced oxidative damage and improve chilling tolerance of the eggplant through enhancing the antioxidant enzyme activities, especially the ASA-GSH cycle, to suppress the accumulation of ROS and subsequent membrane lipid peroxidation. Our future research will be focused on molecular mechanisms involved in plant responses to chilling stress after EBR treatment.

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