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Research article

The potential role of brassinosteroids (BRs) in alleviating antimony (Sb) stress in *Arabidopsis thaliana*

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ABSTRACT

Brassinosteroids (BRs) play a crucial role in improving plant resistance to various environmental stresses. In this study, we aimed to explore the potential role of BRs in protecting plants from antimony (Sb) toxicity. In the *in vitro* agar-plate culture experiments, the level changes of BR in wide-type plants and BR biosynthesis mutant *dwrf4-1* significantly affected the corresponding response of Arabidopsis to Sb stress. Increasing the BR content significantly enhanced Sb-induced root growth inhibition and lowering the BR level appeared to reduce the plant sensitivity to Sb stress. Foliar application of eBL, however, significantly decreased the Sb accumulation and peroxidation of membrane lipids, increased the contents of chlorophyll and proline, and further boosted and strengthened the antioxidant enzymes activities. These experiments demonstrated that BRs played an important role in regulating heavy metal stress responses in plants and exogenous foliar spray of eBL was an important method for alleviating toxicity of Sb.

1. Introduction

Heavy metal pollution has been accelerated along with the rapid development of modern industry. Because metal ions are non-biodegradable with long biological half-lives, soil heavy metal pollution has become one of serious environmental problems around world (Ali et al., 2013; Mahar et al., 2016; Rajewska et al., 2016). Antimony (Sb), a trace element with similar characteristics to arsenic (As), is toxic to both plants and humans (Feng et al., 2013; Li et al., 2018). Excess intake Sb can cause many diseases in humans, including respiratory disease and cardiovascular disease and even cancers (Filella et al., 2009). United States Environmental Protection Agency and the European Union therefore have listed Sb and its compounds as the primary pollutants since 1979 (Filella et al., 2002). During the last decades, the release of Sb into the soils, however, has become increasingly serious with the increase in mining, smelting and the extensive usage of Sb products (Okkenhaug et al., 2011; Li et al., 2017). Thus, how to rapid and effective remediate the emergent Sb pollution in soils has become one of the hot spots of research in recent years.

Phytoremediation has been considered to be an alternative promising technique to traditional physical-chemistry remediation methods, such as soil washing, physical separation, stabilization/solidification and thermal processes (Ali et al., 2013; Liu et al., 2018). Although this technology is an economic, effective and environment-friendly remediation strategy, it is only sustainable for low or moderate contaminated sites (Mahar et al., 2016; Sarwar et al., 2017). High levels of heavy metals in soils can significantly inhibit plant growth and development, reduce the photosynthetic rate, trigger the burst of reactive oxygen species (ROS) and cause oxidative damage (Feng et al., 2009, 2013; Filella et al., 2009; Pan et al., 2010; Chai et al., 2016). Therefore, reducing the toxic stresses of heavy metals on plants has been considered as a promising approach to introduce phytoremediation technique more applicable and efficient.

Brassinosteroids (BRs), one group of plant steroidal hormones, are well known for their critical roles in regulating plant physiological processes, such as seed germination, leaf epinasty, xylem differentiation and photomorphogenesis (Clouse and Sasse, 1998). Recently, emerging evidence also supports that BRs could improve the performance of plants under a broad range of environmental stresses like low or high temperature, drought, high salt, pathogen attack and heavy metal stress by increasing biomass and photosynthesis, strengthening antioxidant and detoxification potential as well as stimulating the expression of

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related genes (Bajguz and Hayat, 2009; Rajewska et al., 2016; Shahzad et al., 2018). Additionally, exogenous application of bioactive BRs (24-epibrassinolide, eBL and 28-homobrassinolide, HBL) has been observed to lighten the toxic impacts of heavy metals on plants (Ali et al., 2008b; Sharma et al., 2011; Soares et al., 2016; Zhou et al., 2018). BRs therefore have been considered to be a sort of potential candidates for "Assisted phytoremediation" in stressful conditions (Barbafieri and Tassi, 2011).

In view of the phytotoxicity of Sb and the protective role of BRs under metal stress, the present experiments were designed to explore the potential role of BRs in protecting plants from Sb toxicity. *Arabidopsis thaliana* was used as a model plant system in this study. Both *in vitro* agar-plate culture and hydroponic culture systems were performed. Exogenous application of 24-epibrassinolide (eBL, a bioactive BR) and brassinazole (Brz, a specific BR biosynthetic inhibitor) was used to modify the BR levels in wide-type Arabidopsis plants. Meanwhile, the interactive effects of eBL/Brz and Sb on root growth and Sb uptake, lipid peroxidation, accumulation of H_2O_2, photosynthetic machinery as well as antioxidant systems in Arabidopsis seedlings were investigated using a combination of morphological, biochemical and physiological approaches. We expected to provide more information regarding the contribution of BR-mediated mechanisms in improving heavy metal stress tolerance in plants and the feasibility of strengthen phytoremediation with exogenous application of BRs.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Wild-type (WT) Arabidopsis Col-0 and BR biosynthesis mutant *dwf4-1* were used in this study, which were obtained from Sunghwa Choe at Seoul National University, Seoul, South Korea (Choe et al., 1998). For *in vitro* agar-plate experiments, the seeds of Col-0 and *dwf4-1* were surfaced-sterilized before being evenly sprinkled on 1/2 MS medium containing 0.5% sucrose and 0.8% plant agar. After 3 days at 4 °C for stratification, the plates were vertically placed in a growth chamber at 22 (± 1) °C and a 16 h light/8 h dark cycle with a light intensity of 10000 lux (approximately 150 $\mu mol\ m^{-2}\ s^{-1}$) at 70% relative humidity. After germination, the seedlings of *dwf4-1* (2 days) were transferred to Sb ($C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$) containing plates (0–50 μM). Meanwhile, the seedlings of Col-0 were transferred to Sb containing plates (0–50 μM) with and without addition of 24-epibrassinolide (eBL, Sigma) or brassinazole (Brz, a specific BR biosynthesis inhibitor, TCI). After 6 days of vertical growth, the root lengths of seedlings were measured. 10^{-3} M stock solutions of eBL and Brz were prepared by dissolving them in dimethyl sulphoxide (DMSO) and further serial concentrations of eBL (0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM) and Brz (0.1 μM, 1 μM) were diluted by double distilled water (DDW). Each plate was photographed and the ImageJ software was employed to measure and analyze the root length of Arabidopsis seedlings (http://rsb.info.nih.gov/ij/).

For hydroponic experiments, Eppendorf tubes filled with 1/2 MS medium and 0.8% agar were prepared as holders for seed germination (Tocquin et al., 2003). Two days after seed germination, the tube bottoms were cut off to enable root to access the 1/2 Hoagland nutrient solution. After further 2 weeks hydroponic growth, seedlings with uniform size were selected and divided into four different groups: two groups were sprayed with deionized water and the other two groups were sprayed with 0.01 μM eBL and 1 μM Brz (an approximate volume of 5 mL), respectively. All seedlings were treated again with the respective solution after two days. Then, seedlings continued to grow for one week with 50 μM of Sb or without Sb in hydroponics solution and were described as: (1) control plants, (2) Sb-treated plants, (3) eBL + Sb-treated plants and (4) Brz + Sb-treated plants. The concentrations of Sb, eBL and Brz were selected on the basis of their effects on the Arabidopsis growth described above. Environmental conditions in the growth chamber for hydroponic experiments were the same as above. Finally, the treated seedlings were collected for the following analysis.

2.2. Measurement of Sb content

To explore the effects of BRs on Sb uptake in Arabidopsis, the aboveground parts of seedlings were harvested and dried in an oven at 80 °C for about 24 h. The dried samples (0.5 g) were then ground to a fine powder and digested with a mixture of H_2SO_4:HNO_3:$HClO_4$ (1:5:1). The total of Sb concentration in obtained digests was analyzed with an atomic absorption spectrophotometer (AA-6300; Shimadzu Co. Kyoto, Japan). The accuracy of the elemental analysis was assessed using standard reference material (GBW-07603) from the Center for Standard Reference of China.

2.3. Analysis of lipid peroxidation

To determine the degree of lipid peroxidation in Arabidopsis leaves, the malondialdehyde (MDA) content was measured by 2-thiobarbituric acid (TBA) reaction. Firstly, leaf sample (1 g) was homogenized with 5 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged for 10 min at 3000 × *g*. 1 mL supernatant was then mixed with 4 mL of 20% TCA containing 0.65% (w/v) TBA. The mixtures were heated at 95 °C for 25 min and immediately cooled on ice bath. After centrifugation, the absorbance of the supernatant was recorded at 440, 532, and 600 nm, respectively.

2.4. Determination of H_2O_2 content

The H_2O_2 content was determined according to Patterson et al. (1984). Briefly, 0.3 g leaf sample was homogenized in 3 mL cold acetone and centrifuged at 3000 × *g* for 20 min at 4 °C. Then, 1 mL supernatant was mixed with 0.1 mL 20% $TiCl_4$ and 0.2 mL NH_4OH. The mixture was centrifuged at 3000 × *g* for 10 min and the precipitate was dissolved with 3 mL of 1 M H_2SO_4. The absorption was measure at 410 nm.

2.5. Chlorophyll estimation

Chlorophyll content was measured following the method of Zlobin et al. (2015). Leaf sample (1 g) was homogenized with 5 mL 80% acetone at 4 °C. The homogenate was then filtered with Whatman filter paper and the absorbance of filtered solution was measured at 663 and 645 nm. The contents of Chl a, Chl b and total Chl were calculated as described by Arnon (1949).

2.6. Determination of proline content

The acid-ninhydrin method was used to estimate the proline content in Arabidopsis leaves. Leaf sample (0.5 g) was firstly homogenized with 5 mL 3% sulfosalicylic acid. After centrifugation (3000 × *g*, 8 min), 2 mL supernatant was added to equal volumes of glacial acetic acid and acid ninhydrin. The mixture was put at 100 °C for 60 min, and the slurry was cooled on ice bath. 4 mL of toluene blue was used to extract proline from samples before measuring the absorbance at 520 nm.

2.7. Antioxidant enzyme activity assay

About 0.3 g leaf tissue was homogenized with 3 mL ice-cold phosphate buffer (50 mM, pH 7.8) containing 0.2 M EDTA and 2% polyvinylpyrrolidone (PVP). The homogeneous mixture was centrifuged at 5000 × *g* for 10 min at 4 °C and the supernatant was used for the superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity measurements. The antioxidant enzymes activities were measured as

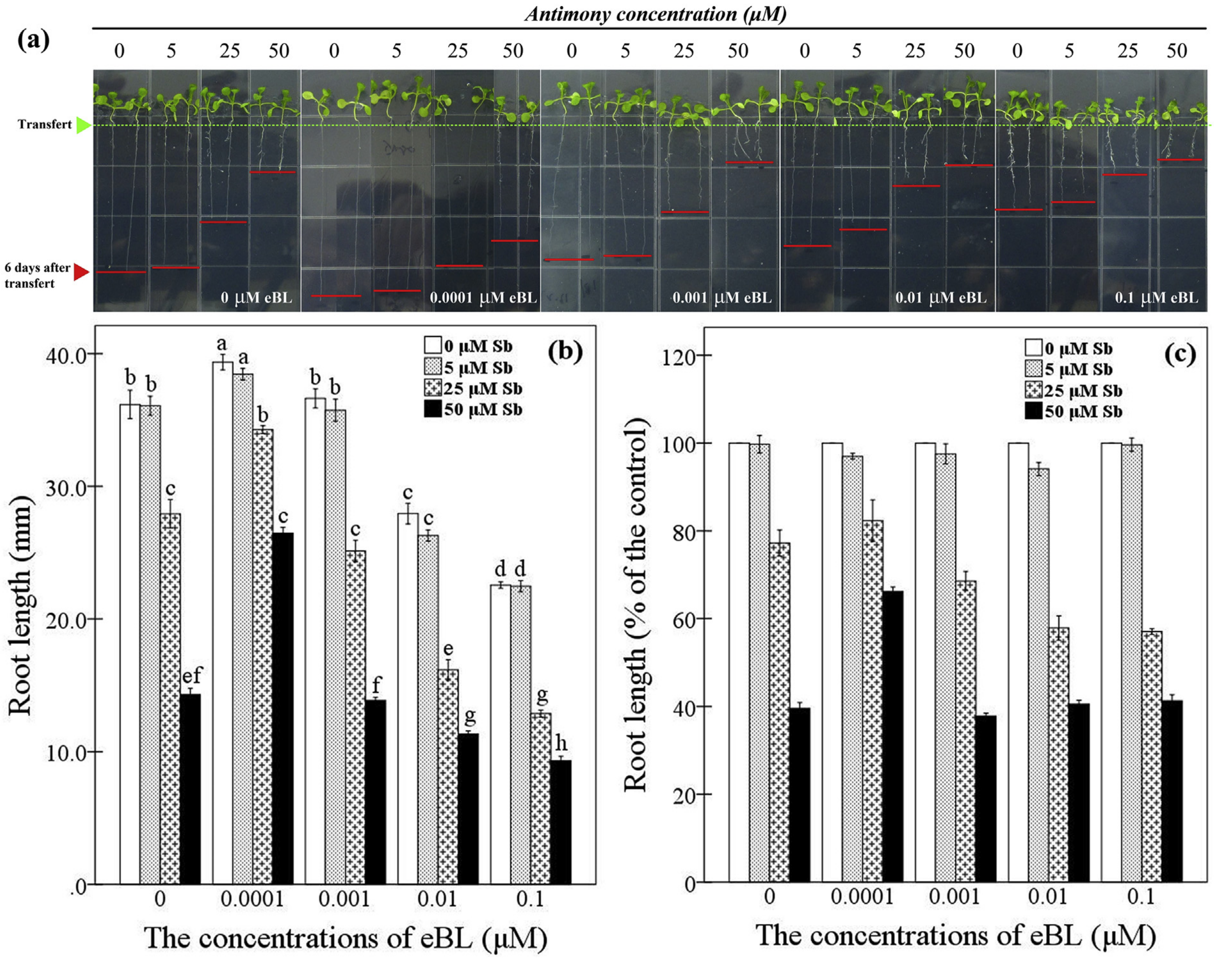

Fig. 1. Effects of exogenous 24-epibrassinolide (eBL) treatments on Arabidopsis seedlings response to Sb stress.

previously described by Feng et al. (2012).

2.8. Statistical analysis

All data were represented as mean ± standard deviation (SD) of at least three independent replicates. Statistical analysis was performed by one-way ANOVA in SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was defined as significant.

3. Results

3.1. The impact of modifying BR levels on root growth of Arabidopsis under Sb stress

Sb exposure caused noticeable toxicity in Arabidopsis seedlings. As shown in Fig. 1a and b, the root growth of Arabidopsis seedlings was significantly inhibited with increasing Sb concentrations in the growth media. Compared to the control, the root growth reduced about 23% and 60% at 25 μM and 50 μM Sb, respectively (Fig. 1c). Low levels of exogenous BRs stimulated the root growth of Arabidopsis seedlings. According to the results, application of eBL alone at 0.0001 μM significantly enhanced the root elongation of Arabidopsis plants compared with the control (Fig. 1b). However, BRs acted inhibitory if a threshold level was exceeded. The root length decreased by 23% and 37%, respectively, after exposure to 0.01 μM and 0.1 μM eBL (Fig. 1a and b). When both eBL and Sb were added to the growth media simultaneously, the toxic effect of Sb was considerably enhanced with the increasing of eBL concentrations, resulting in more drastically reduction of total root length (Fig. 1). When exposed to 25 μM Sb, the root inhibition increased from 18% for the plant treated with 0.0001 μM eBL to 42% in the presence of 0.01 μM eBL (Fig. 1c), indicating that increasing the BR level in Arabidopsis seedlings enhanced the plant sensitivity to the Sb.

When submitted to Brz alone, a progressive decrease in the root length of Arabidopsis seedlings was also observed in our experiment (Fig. 2a and b). However, application of Brz at 1 μM significantly reduced the inhibitory effect on root growth in Arabidopsis plants at all Sb concentrations (5, 25 and 50 μM) (Fig. 2c). Only 26% inhibition by 50 μM (60% inhibition in the control plants) was recorded. Furthermore, there were no significantly differences in the average root length between the control and Brz-treated plants at 5 μM and 25 μM Sb, indicating that lowering the BR level reduced the plant sensitivity to Sb toxicity.

In addition, the same kinds of experiments were also performed with *dwf4-1*, which is a BR biosynthesis mutant due to T-DNA insertion in *DWARF4* (Choe et al., 1998). The root length of *dwf4-1* was about 26% reduced compared with the WT plants in our growth condition (Fig. 3a and b). In the presence of Sb, there was only about 18.7% inhibition by 25 μM Sb versus 23% in the WT plants (Fig. 3c). When exposed to 50 μM Sb, the growth inhibition was still reduced in *dwf4-1* and it only reached 34% versus 60% in the WT plants. Importantly, the absolute root length of *dwf4-1* at 50 μM Sb was even significantly longer ($p < 0.05$) than that of the WT plants (Fig. 3a and b), indicating that reducing the BR level substantially enhanced the tolerance of Arabidopsis to high concentrations of Sb.

3.2. Effects of eBL and Brz treatments on Sb accumulation in Arabidopsis

As shown in Fig. 4, there was no Sb ion in the control plants, and the

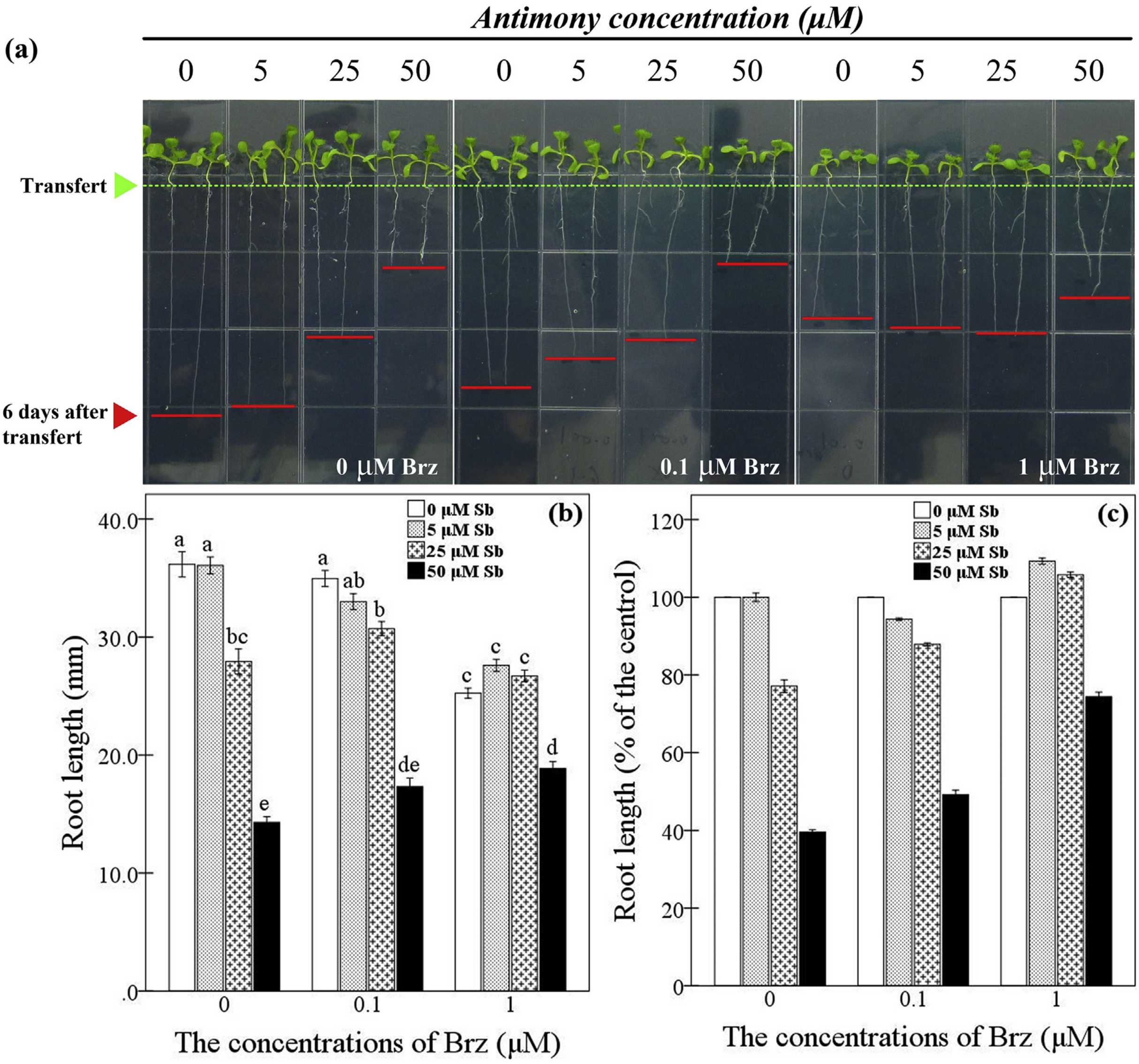

Fig. 2. Effects of exogenous brassinazole (Brz) treatments on Arabidopsis seedlings response to Sb stress.

content of Sb in Arabidopsis seedlings significantly increased after exposure to Sb (Fig. 4). After 7 days exposure to 50 μM Sb alone, the average Sb concentration in the aboveground parts of seedlings was 3.75 ± 0.05 mg/g. However, the accumulation of Sb metal in the plants was significantly blocked after foliar application of eBL. The average Sb content in eBL (0.01 μM)-treated plants decreased to around 3.30 ± 0.11 mg/g (Fig. 3). On the contrary, the Sb uptake was significantly increased after the Brz treatment. When the concentration of Brz was fixed at 1 μM, the average Sb content in Arabidopsis seedlings reached 4.12 ± 0.18 mg/g (Fig. 4).

3.3. Effects of eBL and Brz treatments on MDA and H_2O_2 contents in Arabidopsis leaves

As shown in Fig. 5a, there were no significant differences in MDA content between the control seedlings and seedlings treated with 50 μM Sb. Exogenous eBL supplementation, however, significantly reduced the level of MDA in Sb-treated plants (Fig. 5a). A decline of 15% in MDA content (51.79 ± 1.03 nmol/g) was observed in samples treated with combination of 50 μM Sb and 0.01 μM eBL as compared to samples growing in 50 μM Sb alone (60.7 ± 0.71 nmol/g). By contrast, the accumulation of MDA contents was significantly increased in the leaves of Arabidopsis when treated with Brz (Fig. 5a). The value of MDA content was elevated about 24% in samples with the combined treatment of 50 μM Sb and 1 μM Brz as compared to those growing in Sb alone. The H_2O_2 content in leaves increased after exposure to Sb in comparison to the control, although it is not significant at 50 μM Sb (Fig. 5b). However, foliar application of eBL induced a significant enhance in H_2O_2 content in leaves of Arabidopsis under Sb stress. Compared with seedlings treated by Sb alone, the value of H_2O_2 content was increased 27% after combination application of Sb (50 μM) and eBL (0.01 μM) (Fig. 5b).

3.4. Effects of eBL and Brz treatments on chlorophyll contents

As shown in Fig. 6, Sb treatment (50 μM) led to a marked decline in the contents of Chl a and total Chl in Arabidopsis leaves, which were reduced 15% and 11%, respectively in comparison with those values of the control seedlings. However, exogenous eBL application strongly mitigated this adverse effect in Sb-treated plants. Compared to those treated with Sb alone, the combined treatment of 0.01 μM eBL and 50 μM Sb significantly improved the total chlorophyll content. On the contrary, a decrease in the total chlorophyll content was observed for seedlings treated with 1 μM Brz and 50 μM Sb as compared to those

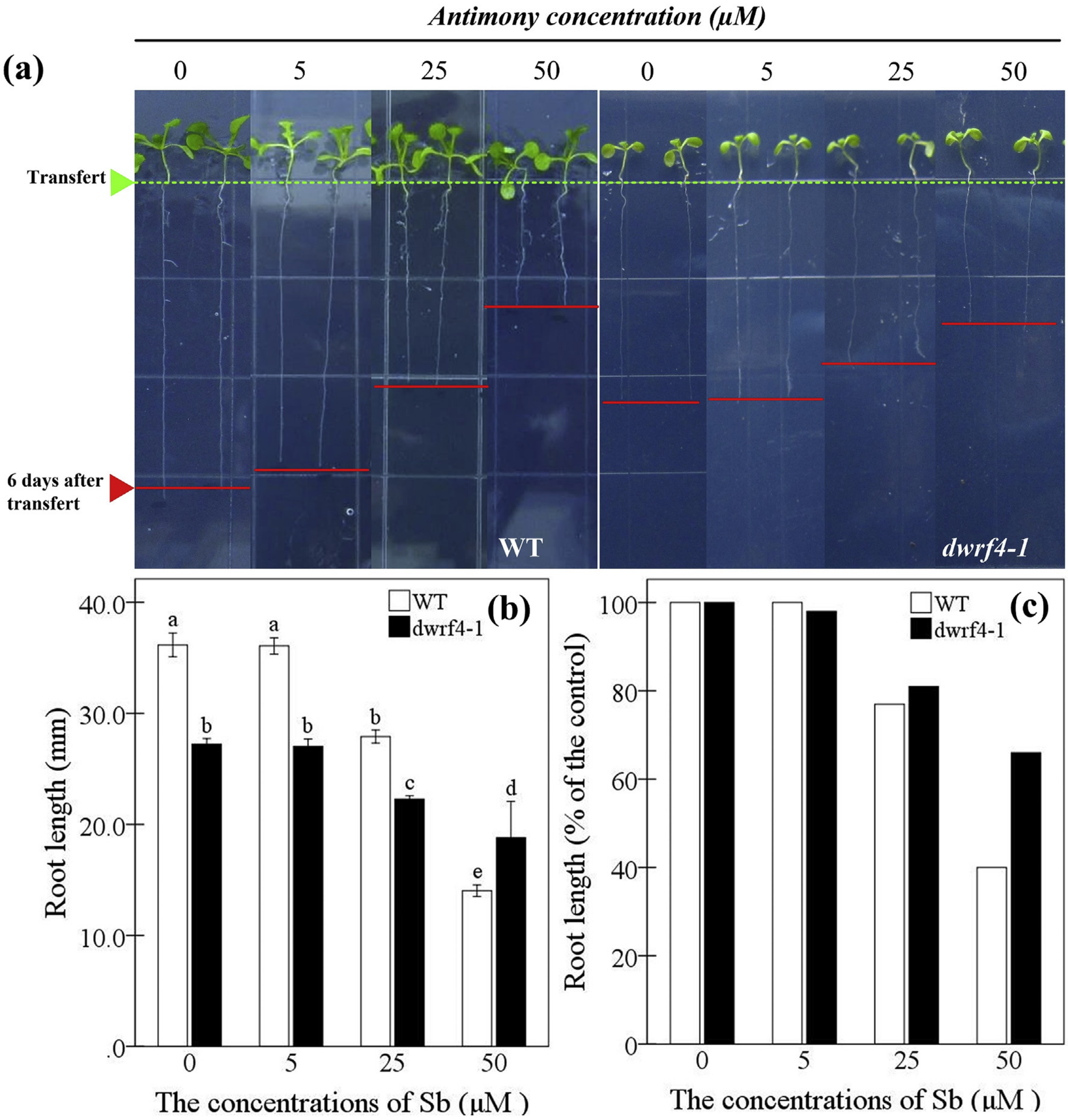

Fig. 3. The response of BR biosynthesis mutant *dwrf4-1* to Sb stress.

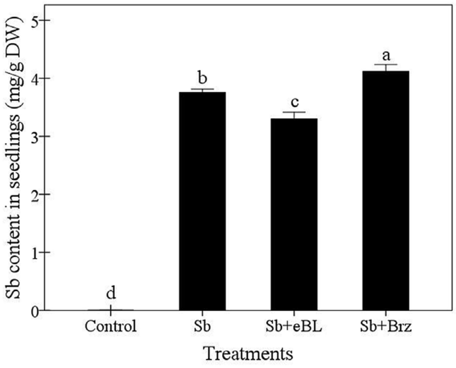

Fig. 4. Effects of exogenous eBL and Brz treatments on Sb accumulation in Arabidopsis seedlings.

treated with Sb alone, despite no statistically significant differences were detected.

3.5. Effects of eBL and Brz treatments on antioxidant enzyme activity and proline content in Arabidopsis leaves

As shown in Fig. 7, the activities of antioxidant enzymes and proline content in Arabidopsis leaves altered under Sb stress. Compared to those of the control, the activities of SOD and POD and proline content increased when the seedlings were treated with Sb (50 μM) (Fig. 7a, b and d). However, a significant decrease rather than increase of CAT activity was observed at 50 μM Sb (Fig. 7c). Exogenous eBL application (0.01 μM) triggered a significant enhancement in the enzymes activities and proline content compared to seedlings treated with Sb alone, which were increased by 62% (SOD), 46% (POD), 59% (CAT) and 22% (proline), respectively (Fig. 6). In contrast, exogenous foliar spray of Brz (1 μM) significantly decreased the activities of antioxidant enzymes and the content of proline by 40% (SOD), 53% (POD), 42% (CAT) and 43% (proline) (Fig. 7).

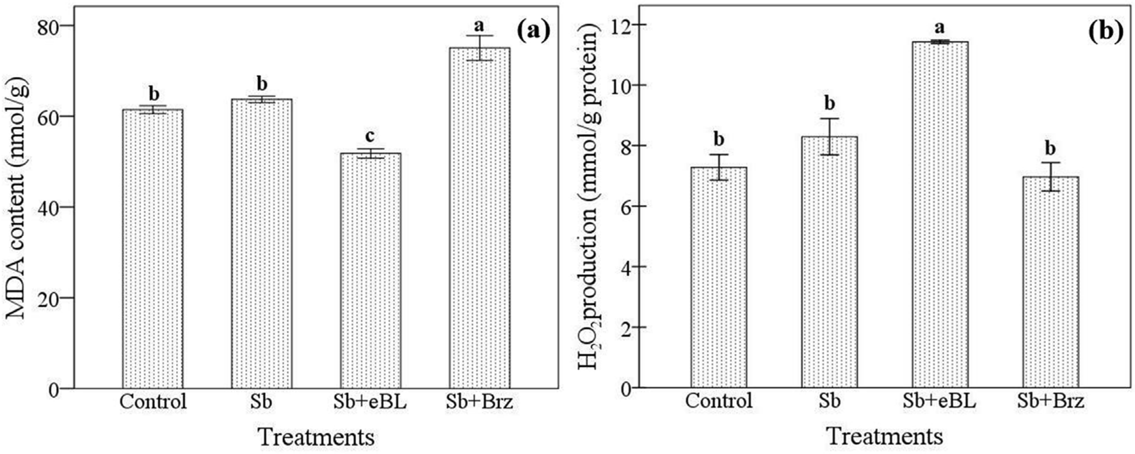

Fig. 5. Effects of exogenous eBL and Brz treatments on MDA and H_2O_2 contents in Arabidopsis leaves under Sb stress.

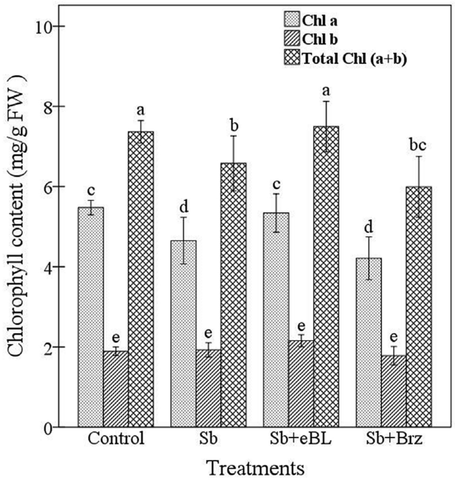

Fig. 6. Effects of exogenous eBL and Brz treatments on Chlorophyll contents in Arabidopsis leaves under Sb stress.

4. Discussion

The purpose of this study was to investigate the potential role of BRs in enhancing plant resistance to Sb stress. In the present study, Arabidopsis seedlings showed a considerable reduction in root growth and chlorophyll content as well as an enhancement in MDA and H_2O_2 contents after exposure to a high concentration of Sb, revealing the phytotoxic effect of Sb. In the *in vitro* culture experiments, the level changes of BRs in WT plants and BR biosynthesis mutant significantly affected the corresponding response of Arabidopsis to Sb stress. Increasing the level of BR in Arabidopsis seedlings significantly enhanced the inhibition effect of Sb on root growth. On the contrary, decreasing the BR level appeared to reduce the plant sensitivity to Sb stress and correspondingly lowered the Sb-induced root growth inhibition. In hydroponic culture experiments, foliar application of eBL, however, significantly decreased the total content of Sb in plants and the peroxidation of membrane lipids, increased the contents of chlorophyll and proline, and further boosted and strengthened the antioxidant defense systems. These experiments demonstrated that BRs played an important role in regulating heavy metal stress responses in plants and exogenous foliar spray of eBL was an important method for alleviating toxicity of Sb in plants (Shahzad et al., 2018; Zhou et al., 2018).

In contaminated soils, roots are the first part of plants to come in contact with heavy metals, and the inhibition of root growth therefore is the most prominent response observed in case of heavy metal stress (Malamy, 2005; Villiers et al., 2012). Although Sb is a non-essential element for plants, it still can be taken up by plants, causing significant root growth inhibition and biomass reduction (Pan et al., 2010; Feng et al., 2013). For example, the root growth of paddy rice was retarded under Sb stress (1–1000 μg/mL) and the length of root decreased maximum up to 92% compared with the control (He and Yang, 1999). Similarly, the total root length of maize (*Zea mays*) was only 13% of that of the control after 500 mg kg $^{-1}$ Sb treatment (Pan et al., 2010). In agreement with previous studies, the root length of Arabidopsis seedlings was significantly decreased with increased Sb stress (Fig. 1). The root length decreased to 60% (14.31 ± 1.53 mm) at the highest concentration of Sb (50 μM Sb) when compared to the control seedlings (36.17 ± 3.39 mm). This inhibitory effect on root growth might be due to certain physiological disruptions, e.g., inhibition of cell elongation and division, interferes with water relations and membrane permeability and causes nutrient deficiencies, such as N, P, Fe, P, K, Ca and Mg (Yusuf et al., 2012).

BRs, an emerging group of plant steroidal hormones, mediate many important physiological processes in plants (Clouse and Sasse, 1998). Although BRs play an important role in overall plant root development (Planas-Riverola et al., 2019), two opposite patterns about the role of BRs in the regulation of root growth have been observed during the past decade. On the one hand, mutants lacking BR compounds or BR receptors exhibited short roots, indicating that BR signaling was required for the normal root growth (González-Garcíaetal et al., 2011; Chaiwanon and Wang, 2015). On the other hand, short roots were also observed in plants treated with high concentrations of BRs (Müssig et al., 2003; González-Garcíaetal et al., 2011). For example, inhibitory effects of eBL were observed in *Lycopersicon esculentum*, *Brassica napus* and *Oryza sativa* seedlings (Özdemir et al., 2004). Meanwhile, a significant stimulation on root growth was also observed in *Zea mays* at the concentrations of 0.1–10 μM eBL (Romani et al., 1983). In Arabidopsis wild-type plants, low concentrations (0.05–0.1 nM) eBL promoted root elongation and growth inhibition occurred at higher BR levels (≥ 1 nM) (Müssig et al., 2003). Root growth stimulation at a low concentration of brassinolide (BL) (0.1 nM) also reported by Kim et al. (2007), whereas root inhibition increased as the concentration of BL increased. In accordance with previous studies, our data showed that eBL at a low concentration (0.0001 μM) significantly promoted the root elongation of Arabidopsis plants (Fig. 1). The root growth, however, was 23% and 37% reduced in the presence of 0.01 μM and 0.1 μM eBL, respectively ($p < 0.05$) (Fig. 1). Similarly, lowering the root elongation also observed in the BR biosynthesis mutant *dwf4-1* and Brz treated seedlings (Figs. 2 and 3). These results confirmed that BR homeostasis was vital for the normal development and growth of higher plants. Exogenously

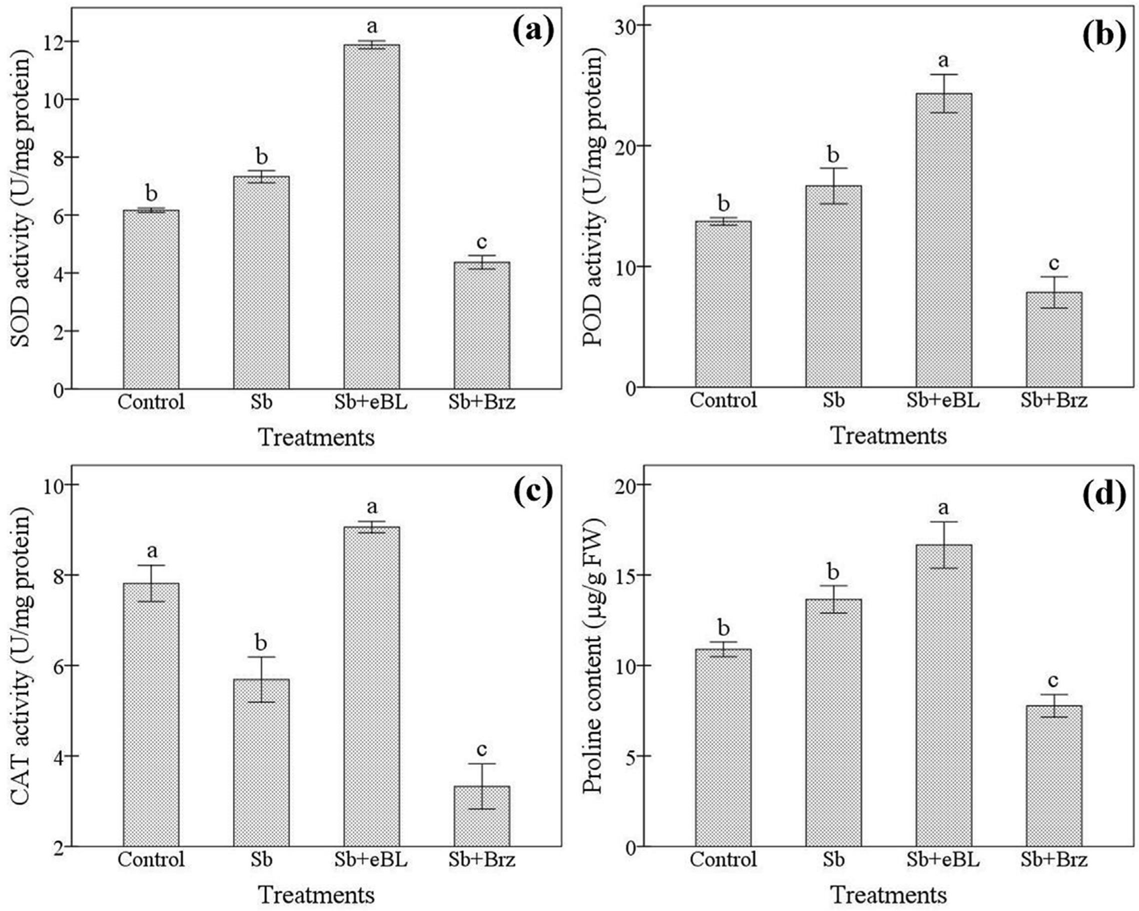

Fig. 7. Effects of exogenous eBL and Brz treatments on antioxidant enzyme activity and proline content in Arabidopsis leaves under Sb stress.

phytohormones override endogenous signaling networks, and an impaired balance of pathways may be detrimental (Müssig et al., 2003; Tanaka et al., 2005; Villiers et al., 2012).

In addition to their pivotal function in plant development and growth, BRs also can protect plants against heavy metal stress (Bajguz and Hayat, 2009; Rajewska et al., 2016). The root growth of *Vigna radiate* was severely inhibited under Al stress, and the root growth of the stressed seedlings significantly improved after the foliar spray of eBL (Ali et al., 2008a). In other studies, pre-soaking seeds with eBL or spraying with eBL could promote the root length of *Brassica juncea* in Pb, Cu, Zn, Mn, Co and Ni -treated seedlings (Ali et al., 2008b; Fariduddin et al., 2015; Kohli et al., 2018). Moreover, it was also reported that exogenous eBL application could effectively alleviate the metal stress in *Oryza sativa* and *Vitis vinifera* (Sharma et al., 2016; Zhou et al., 2018). In the present study, opposite results were observed in the *in vitro* agar-plate culture systems, although Sb toxicity was alleviated after exogenous application of eBL at a low concentration (0.0001 μM) (Fig. 1). The root growth inhibition induced by Sb in Arabidopsis enhanced with the increase of the BR level in Arabidopsis seedlings (Fig. 1). Meanwhile, reducing the BR level in *dwrf4-1* mutant and WT plants appeared to decrease the negative inhibition effects (Figs. 2 and 3).

The main reasons for this discrepancy in our study probably were: (1) the BR activity is dose dependent. The effects of BRs on root growth are strongly dependent on the concentration of BRs used. The beneficial effects of exogenous application of BRs may differ with differing concentrations of BRs and inhibition of root growth occurs if exceed a certain level (Müssig et al., 2003; González-Garcíaetal et al., 2011). This is similar to that for the effects of traditional plant growth regulators on plant growth and development, which exhibit a dose/response curve that is often bell shaped (Ashraf et al., 2010), indicating that they are potentially harmful at higher concentrations (Planas-Riverola et al., 2019). (2) The differences in the testing time. In our study, the root lengths were measured at seedling stage, and in other previous studies, older plants were used. In terms of eliciting the response, BRs are time specific and developing roots or primordia are more responsive to BRs than established or mature root tissues (Jones-Held et al., 1996). Moreover, recent modes proposed that the spatial distribution of the BR receptors determined the opposing impact of BRs on root growth (Fridman et al., 2014; Vragovic et al., 2015); (3) the differences in the experimental process. In our *in vitro* culture experiments, eBL and Sb were provided at the same time, whereas the plant seeds were firstly pre-soaked with eBL or sprayed with eBL and then provided the toxic metal in other studies. It is important to note that the effectiveness of BR application may also vary depending not only on plant species and the concentrations applied, but also the application model (Ashraf et al., 2010). In our experiments, addition of 0.01 μM eBL in the root growing media induced inhibited growth in hydroponically grown Arabidopsis seedlings. However, exogenous foliar application of 0.01 μM eBL did not significantly affected the plant biomass of Arabidopsis seedlings (Fig. S1); (4) In agreement with Villiers's hypothesis, there might be an interaction between Sb and BR signaling *in vivo*, which can adjust the sensitivity of plant to Sb stress (Villiers et al., 2012). In Arabidopsis, there might be a transcriptional Sb-BR cross-talk, and Sb-exposure-mediated activation in BRs signaling pathway could influence the plant responses to Sb through up-regulation and down-regulation of many Sb-stress-responsive genes (Villiers et al., 2012).

Besides the inhibited effect on the root growth, heavy metals can cause the formation of reactive oxygen species (ROS) in many plants, such as, hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical (•OH). Excessive ROS induced by metal stress damages biological active molecules, disrupts membrane through lipid peroxidation and ultimately results in cell death (Rajewska et al., 2016; Shahzad et al., 2018). MDA, the main cytotoxic product of membrane lipid peroxidation is a noted index to reflect the degree of stress. To remove the overproduced ROS, plants initiate their intrinsic enzymatic defense systems like SOD, CAT and POD as well as non-enzymatic defense mechanisms such as free amino acids especially proline to neutralize the enhanced ROS. Among these antioxidant enzymes, SOD is firstly enzyme responding for converting $O_2^{\cdot -}$ to H_2O_2, subsequently CAT and POD are engaged in catalyzing hydrolysis of H_2O_2 into H_2O and O_2 (Feng et al., 2009; Chai et al., 2016). Proline is a vital osmoprotectant and its accumulation under environmental stresses also plays a crucial role in the repair process of membrane, in scavenging of ROS and in metabolic signals (Szabados and Savoure, 2010).

Therefore, it was expected that the exposure to Sb could increase the level of MDA, H_2O_2 as well as proline and promote the activities of antioxidant enzymes in Arabidopsis. The enhanced leaf MDA content after Sb exposure has been observed in paddy rice (Feng et al., 2011), fern plants (Feng et al., 2009), *Miscanthus sinensis* (Xue et al., 2015) and *Boehmeria nivea* (Chai et al., 2016). In our hydroponic culture experiments, the MDA and H_2O_2 levels in Arabidopsis leaves were increased under Sb stress and meanwhile, the activities of antioxidant enzymes (SOD and POD) and the proline content were increased, indicating that Sb stress triggered the antioxidant response in Arabidopsis (Figs. 5 and 7). Exogenous foliar spray of eBL significantly alleviated Sb-induced oxidative damage, as evidenced by the decreased MDA level and the increased chlorophyll content in the leaves (Figs. 5 and 6). A significant increase of SOD, POD and CAT activities and considerable proline accumulation were also observed after eBL treatment, indicating that eBL could enhance the stress tolerance of Arabidopsis to Sb (Fig. 7). In addition, Brz-treated seedlings showed an opposite change trend (Fig. 7). Our results were similar to those of earlier studies, which reported that eBL-treated plants were more tolerant to heavy metals than untreated plants (Sharma and Bhardwaj, 2007; Rajewska et al., 2016). The mechanisms involved for enhancing tolerance against heavy metals by eBL might be either due to up-regulation in the expression levels of various antioxidant defense genes (Sharma et al., 2016) or due to the their function in blocking heavy metal uptake in plants (Bajguz, 2000; Sharma and Bhardwaj, 2007). Compared with Sb-stressed plants, a significant decrease of Sb content was observed in eBL-treated seedlings, which is consistent with the results as our expected (Fig. 4).

Interestingly, a significantly increased rather than decreased H_2O_2 content was found in eBL treated seedlings (Fig. 5). Depending on the degree of metal stress, dual roles of ROS has been reported in plants, including inducing lipid peroxidation at high ROS concentration or acting as signals for the activation of the stress response and defense pathways at low ROS concentration (Torres and Dangl, 2005). In plants, BRs-induced H_2O_2 accumulation has been considered as a central signaling molecule to response to various environmental stresses, which might mediate the transcriptional induction of defense or antioxidant genes (Nakagami et al., 2005; Xia et al., 2009).

5. Conclusion

The phytotoxic damage of Sb is manifested through reduced root length and chlorophyll content as well as the increased MDA and H_2O_2 contents in Arabidopsis. Depending on the concentration used and differences in the experimental procedures, the possible role of eBL in protecting plants from Sb toxicity is quite different. When eBL and Sb are provided at the same time, artificial alteration of the BRs levels in plants leads to a modulation of the plant sensitivity to Sb. Exogenous application of eBL may be beneficial at low concentrations and harmful if exceed a certain level. Meanwhile, exogenous foliar spray of eBL can significantly increase the tolerance of plants to Sb stress through improving the functions of enzymatic and non-enzymatic antioxidants. The present results open to new insights into plant resistance against heavy metals and may provide a theoretical basis for the exogenous application of BRs to counteract heavy metal stress in plants.

Conflicts of interest

No potential conflict of interest was reported by the authors.

Author contributions

Jiaokun Li designed the research and proposed the research proceeding. Chenchen Wu, Fang Li and Hui Xu performed the experiments. Weimin Zeng, Runlan Yu, Xueling Wu, Li Shen and Yuandong Liu analyzed the data. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.011.

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