Putrescine metabolism modulates the biphasic effects of brassinosteroids on canola and *Arabidopsis* salt tolerance

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Funding information

This research was funded by the National Natural Science Foundation of China (31702167 and 31672141), the China Postdoctoral Science Foundation (2018M641030), the Fundamental Research Funds for the Central Universities

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.13757

(2452018125), and the Natural Science Basic Research Plan in Shaanxi Province of China (2019JM-014).

Running Head

BR-Put crosstalk in regulating salt tolerance

ABSTRACT

Brassinosteroids (BRs) are known to improve salt tolerance of plants, but not in all situations. Here, we show that a certain concentration of 24-epibrassinolide (EBL), an active BR, can promote the tolerance of canola under high salt stress, but the same concentration is disadvantageous under low salt stress. We define this phenomenon as hormonal stress-level-dependent biphasic (SLDB) effects. The SLDB effects of EBL on salt tolerance in canola are closely related to H_2O_2 accumulation, which is regulated by polyamine metabolism, especially putrescine (Put) oxidation. The inhibition of EBL on canola under low salt stress can be ameliorated by repressing Put biosynthesis or diamine oxidase activity to reduce H_2O_2 production. Genetic and phenotypic results of *bri1-9*, *bak1*, *bes1-D*, and *bzr1-1D* mutants and over-expression lines of *BRI1* and *BAK1* in *Arabidopsis* indicate that a proper enhancement of BR signaling benefits plants in countering salt stress, whereas excessive enhancement is just as harmful as a

deficiency. These results highlight the involvement of crosstalk between BR signaling and Put metabolism in H_2O_2 accumulation, which underlies the dual role of BR in plant salt tolerance.

Key-words: brassinosteroids; polyamines; hydrogen peroxide; biphasic effect; salt stress

INTRODUCTION

Canola (*Brassica napus* L.) is an essential edible oil crop worldwide, and its production is severely influenced by multiple abiotic stresses, including salinity. Salt stress, one of the most significant abiotic stresses, seriously affects plant growth and development, which is a major limiting factor for crop yields worldwide. Soil salinity first causes osmotic stress, which inhibits plant growth of young leaves; then, ion toxicity occurs, thus accelerating leaf senescence (He et al., 2005; Ijaz et al., 2019; Liang, Ma, Wan, & Liu, 2018; Munns, James, & Lauchli, 2006; Munns & Tester, 2008). More than 800 million hectares of the world's arable lands are severely affected by salt (FAO, 2000), especially in arid and semi-arid areas (Ma et al., 2017; Munns & Tester, 2008). To survive in saline soils, plants have evolved a variety of morphological, physiological,

biochemical and molecular mechanisms to protect themselves from toxicity, for example, restoring ion balance in cells, synthesizing osmotic adjustment substances, activating antioxidant enzyme activity and regulating the expression of genes under salt stress (Isayenkov & Maathuis, 2019; Liang et al., 2018; Robles & Quesada, 2019). Previously, we found that 24-epibrassinolide (EBL), which is one of the most active brassinosteroids (BRs), can enhance salt tolerance of canola via regulation of ion homeostasis and osmotic adjustment (Liu et al., 2014), but the underlying mechanisms remain unclear.

BRs are a class of phytohormones that are ubiquitous in the plant kingdom and play essential roles in regulating plant growth and development in an array of physiological, developmental and cellular processes, including alleviating various abiotic stresses such as salt, drought, cold and heavy metal stresses (Jakubowska & Janicka, 2017; Jiang et al., 2013; T. W. Kim et al., 2009; Peres et al., 2019; Tanveer, Shahzad, Sharma, Biju, & Bhardwaj, 2018; Tanveer, Shahzad, Sharma, & Khan, 2019). Salt stress induced growth inhibition is associated with the enhanced generation of hydrogen peroxide (H_2O_2) as well as superoxide (O^{2-}) in the mitochondria, chloroplasts and peroxisomes of plant cells (Ijaz et al., 2019). Excessive reactive oxygen

species (ROS) production can lead to severe damage of proteins, lipids and nucleic acids. BR can significantly enhance antioxidant capacity by decreasing the generation of ROS, which cause lipid peroxidation, DNA damage and protein oxidation under saline conditions (Abd Allah, Alqarawi, Hashem, Wirth, & Egamberdieva, 2018; Kaur et al., 2016; Rady, 2011), but the underlying mechanisms are poorly understood.

Polyamines (PAs) are small aliphatic nitrogenous bases with two or more amino groups widely distributed in eukaryotic organisms. Triamine spermidine (Spd), tetraamine spermine (Spm), and their diamine obligate precursor putrescine (Put) are three major PAs in plants, each of which play essential roles in important physiological and developmental processes such as organogenesis, embryogenesis, cell division, floral development, leaf senescence and fruit maturation processes (Chen, Shao, Yin, Younis, & Zheng, 2018; Seifi & Shelp, 2019). In addition, PAs also have different functions in responses to various biotic and abiotic stresses, such as salt, cold, drought, heavy metal and high temperature stresses (Chen et al., 2018; López-Gómez et al., 2017; Soudek, Ursu, Petrova, & Vanek, 2016; Tanou et al., 2014; Wu, Fu, Sun, Xiao, & Liu, 2016; Zhuo, Liang, Zhao, Guo, & Lu, 2018). PAs exhibit essential roles in balance of cellular ROS levels (Gupta, Sengupta,

Chakraborty, & Gupta, 2016; Miller, Suzuki, Ciftci-Yilmaz, & Mittler, 2010). PAs have ROS scavenging capacity or can enhance antioxidant ability of plants under stresses (Ha et al., 1998). In contrast, the catabolic pathway of PA degradation can increase levels of H_2O_2 and other by-products such as NO, γ-aminobutyric acid (GABA) and acrolein. The enzymes involved in the catabolic pathway include PA oxidases (PAOs), diamine oxidases (DAOs), aminopropyl transferase and γ-aminobutyric acid transaminase (Gupta et al., 2016). Among them, PAO and DAO can oxidize Spd/Spm and Put, respectively, to induce H_2O_2 accumulation (Gemes et al., 2016; Saha et al., 2015). Previous studies by ourselves and others have found that the accumulation and balance of PAs respond positively to BR in enhancing salt tolerance of tomato and lettuce plants (Serna et al., 2015; Zheng et al., 2015). Accordingly, whether PA metabolism plays an important role in BR regulation of salt tolerance and whether it is related to the accumulation of H_2O_2 is worth exploring.

With above issues in mind, we identified the effects of EBL on salt-stressed canola during seed germination. Surprisingly, EBL has contrasting effects under low and high salt stress conditions. Owing to the dual role of PAs on cellular H_2O_2 accumulation, we hypothesize that PA metabolism may modulate the biphasic effects of EBL on canola salt tolerance.

Physiological analyses of accumulations of H_2O_2 and PAs and activities of PAO and DAO as well as external applications of a ROS scavenger, PA biosynthesis inhibitor and a diamine oxidase inhibitor were conducted to uncover the underlying processes. We thus demonstrate that the biphasic effects of EBL on canola salt tolerance are mainly related to H_2O_2 regulation by Put metabolism. Furthermore, genetic and phenotypic analyses of *Arabidopsis* mutants and overexpression lines for the genes *BRI1(BRASSINOSTEROID-INSENSITIVE 1), BAK1*(*BRI1-ASSOCIATED RECEPTOR KINASE 1*), *BES1* (*BRI1-EMS-SUPRESSOR 1*), and *BZR1* (*BRASSINAZOLE-RESISTANT 1*) were conducted to better understand the role of crosstalk of BR signalling and Put in regulating H_2O_2 accumulation of plants under salt stress.

METHODS

Plant materials and growth conditions

The canola N1 seeds (*Brassica napus* L. cv. 'Nanyanyou 1') used in this study were obtained from the College of Resources & Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu, China. After being surface sterilized with 5% NaClO for 10 min and washed five times with sterile ddH_2O, 50 healthy seeds were sown on Whatman No.1 filter paper in autoclaved Petri

dishes and supplied with deionised water with or without NaCl, 24-epibrassinoide (EBL), the ROS scavenger dimethylthiourea (DMTU), the polyamine biosynthesis inhibitor D-arginine (D-Arg) and the diamine oxidase inhibitor aminoguanidine (AG) and kept at 25°C in darkness. The germination rate and lengths of hypocotyls and radicals were determined. All *Arabidopsis thaliana* materials used in this study, including the BR-receptor mutant *bri1-9*, the co-receptor mutant *bak1*, the gain-of-function *bes1-D* and *bzr1-D* mutants (which both display constitutive BR responses), and the *BRI1* and *BAK1* overexpression lines, were in the Columbia-0 (Col-0) background. Seeds were surface sterilized with 75% ethanol and washed five times with sterile ddH_2O. For germination experiments, 40 healthy sterilized seeds were sown on 1/2 MS medium [1% (*w*/*v*) agar, 1% (*w*/*v*) sucrose] with or without NaCl. For seedling experiments, 3-day-old seedlings with uniform growth were transferred onto 1/2 MS agar plates with or without NaCl and Put for another 7–20 days of growth, or 2-week-old seedlings grown on 1/2 MS agar plates were transferred to 1/4 Hoagland solution with or without NaCl for another week of hydroponic culture. For H_2O_2 staining experiments, healthy 3-d-old seedlings were transferred to 1/2 MS agar plates with or without NaCl and Put for another week of treatment. Plants were grown at 22°C under long-day

conditions (16-h illumination of 150 μmol/m^2/s, and 8-h dark cycle).

H_2O_2 measurements

The concentration of H_2O_2 in seeds was determined using a minor modified eFOX method (Cheeseman, 2006; Ortiz-Espin et al., 2017). First, 100-mg samples of seeds collected at different times during germination were homogenized in liquid nitrogen, mixed with 1 mL of ice-cold acid acetone containing 0.13% H_2SO_4 and frozen again with liquid nitrogen. After defrosting, the mixture was centrifuged at 12000 × *g* for 10 min at 4°C. The supernatant was mixed (1:5, *v*/*v*) with the eFOX reagent [250 μM ferrous ammonium sulphate; 100 μM sorbitol; 100 μM xylenol orange; 1 %(*v*/*v*) ethanol and 25 mM H_2SO_4] and incubated for 45 min, and the H_2O_2 level was determined by measuring the difference in absorbance between 550 and 800 nm.

Histochemical H_2O_2 Staining

The histochemical staining of H_2O_2 was performed as the method described by Xia et al. (2009) with minor modifications. Plant samples were vacuum infiltrated with 1 mg mL^{-1} DAB (50 mM Tris-acetate, pH 3.8) solution containing 0.05% (*v*/*v*) Tween 20, incubated at 25°C for 24 h in darkness, rinsed in 80% (*v*/*v*) ethanol for 10 min at 70°C, mounted in lactic acid/phenol/water (1:1:1, *v*/*v*/*v*) and photographed.

Determination of polyamines

PAs were extracted and assayed using the method we described previously (Zheng et al., 2015). In brief, 1 g of fresh sample was homogenized with 6 mL of ice-cold 5% (*v*/*v*) perchloric acid in an ice bath, extracted at 4°C overnight and centrifuged at 27,000 × *g* for 20 min at 4°C. For the derivatization of PAs, 1 mL of extract was combined with 1 mL of 1% dansyl chloride and 1 mL of saturated Na_2CO_3. The derivatization was conducted at 60°C for 15 min, and stopped by adding 0.5 mL of 10% proline. After cooling to room temperature, the dansylated PAs were extracted by toluene sufficiently and dried by nitrogen gas blowing at 40°C. Then, the dansylated PAs were re-dissolved in 1 mL of acetonitrile and analysed by HPLC (Agilent Technologies 1200 Series, Santa Clara, CA, USA) on a reverse-phase C18 column (Agilent Zorbax SB-C18 column, 5 μm, 4.6 mm × 250 mm) using an acetonitrile to water gradient (60–70% acetonitrile for 5.5 min, 70–80% for 1.5 min, 80–100% for 2 min, 100% for 2 min, 100–70% for 2 min, and 70–60% for 2 min at a flow rate of 1.5 mL min^{-1}). The injected volume was 20 μL, and the column temperature was 30°C. Eluted peaks were detected using a spectrofluorometer (Agilent FLD Cell, 8 μL, Max press 2 MPa, excitation 365 nm, emission 510 nm), recorded and integrated by an attached computer using the Agilent 1200 Series HPLC

software. PA content was expressed in nmol g^{-1} FW.

Enzyme analysis

Activity levels of DAO (EC 1.4.3.6) and PAO (EC 1.5.3.11) were determined using the method described by Quinet et al. (2010) with minor modifications. First, 1-g samples of seeds at different points in germination were homogenized with 1 mL of ice-cold 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM dithiothreitol at 4°C. The homogenate was centrifuged at 16,000 × *g* for 20 min at 4°C, and the supernatant was transferred into an ice-cold tube. The residue was extracted twice for 10 min with 500 µL of 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM NaCl at 4°C. To determine the DAO activity, 100 µL of extract was combined with 50 µL of catalase (1000 U mL^{-1}, pH 7.5), 250 µL of 0.1% 2-aminobenzaldehyde (pH 7.5), 350 µL of 50 mM potassium phosphate buffer (pH 7.5), and 250 µL of 10 mM Put in 50 mM potassium phosphate buffer (pH 7.5). PAO activity was determined using a reaction that was similar to that used for DAO determination, except the pH was 6.0 and Spd was used instead of Put. The reaction was conducted at 30°C for 3 h, stopped with 10% (*v*/*v*) perchloric acid and centrifuged at 6500 × *g* for 15 min. The product of Δ-pyrroline was determined by absorbance at 430 nm. Control reactions were carried out with

inactive enzymes prepared by blotting the extract for 20 min. The enzyme activities were expressed as pmol Δ-pyrroline $min^{-1}g^{-1}$ FW using an extinction coefficient of 1.86×10^3 mol^{-1} cm^{-1}.

RT-qPCRs

Total RNA from plantlets was extracted using the Eastep® Super Total RNA Extraction Kit (Promega LS1040; Promega Corporation, Madison, WI, USA). First-strand cDNA was synthesized with HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, R323; Vazyme Biotech Co., Ltd., Nanjing, China), according to the manufacturer's instructions. RT-qPCR amplifications and measurements were performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711) on a LightCycler 480II (Roche, Basel, Switzerland). The sequences of the primers used for PCR amplifications were designed using Primer 5 software (Table S1).

RESULTS

EBL has contrasting effects on low- and high-salt-stressed canola closely related to H_2O_2 levels

In salt-soil agricultural production, whether crop seeds can be effectively germinated into seedlings that break through the soil is the most important prerequisite for crop productivity. We assessed the effects of EBL on the

germination of salt-stressed canola. The seed germination rate was significantly inhibited by increasing salt concentrations in canola (Fig. S1a). Interestingly, we discovered that the exogenous application of EBL can significantly increase the seed germination rate of canola seeds under high salt stress (≥200 mM), but this treatment decreases germination rates under low salt stress (≤150 mM) (Fig. 1), with the effect of treatment with 10^{-6} M EBL (EBL7) being most obvious (Figure S1b-f). In addition, hypocotyl enlargement was observed to accompany increased salt tolerance in canola (Fig. S2).

Further, we measured the H_2O_2 content of canola seedlings under high and low salt conditions. As germination days continued, the H_2O_2 content was progressively higher (Fig. S3). The non-salt stressed canola, which also had much better germination rates and growth statuses, accumulated much more H_2O_2 than salt-stressed canola (Fig. S3). Because the germination rate of seeds was so low, it was difficult to determine the H_2O_2 content of the germinated seedlings under high salt stress conditions directly. Accordingly, we determined the H_2O_2 contents of seeds at different times during germination, and the relative H_2O_2 content of the germinated seedlings were calculated by dividing the H_2O_2 contents of seeds by the germination rate (Fig. 2a). On the other hand, H_2O_2 histochemical staining was conducted to confirm

the relative H_2O_2 content results (Fig. 2b). Consistent with seed germination under high salt conditions, EBL7 treatment effectively reduced the accumulation of H_2O_2 in canola seedlings; in contrast, under low salt treatment, EBL7 treatment significantly increased H_2O_2 content (Fig. 2). To explore whether the dual effect of EBL on seed germination was related to H_2O_2 accumulation, we observed that the inhibition of EBL on the length of hypocotyls and radicles of canola seedlings could be reversed by treatment with a H_2O_2 scavenger (DMTU) under low salt conditions; however, under high salt conditions, DMTU further alleviated the toxic effect of salt stress on the lengths of hypocotyls and radicles of canola seedlings under EBL7 treatment (Fig. 3, S4).

Put oxidation determines the effects of EBL on H_2O_2 accumulation in salt-stressed canola

Owing to the dual role of PAs in maintaining cellular ROS homeostasis, we speculate that PAs may mediate the biphasic effects of EBL on H_2O_2 accumulation under salt stress. To investigate the effect of EBL on PA accumulation in salt-stressed canola during seed germination, we measured PA content under 0 mM and 250 mM NaCl treatment with or without EBL7. Put content was significantly decreased under 0 mM and 250 mM NaCl treatments

with EBL7 within 7 d, but Spd and Spm contents were increased under the same conditions, although there was an obvious decline on the second day under the 0 mM NaCl treatment (Fig. S5). However, this cannot explain why EBL has contrasting effects on H_2O_2 accumulation in low- and high-salt-stressed canola. Thus, we measured Put and Spd+Spm contents in different tissues including cotyledons, upper/lower-hypocotyls and radicles under 0, 100 and 200 mM NaCl conditions. Notably, the effect of EBL on Put accumulation exhibited contrasting effects under low and high salt conditions (Fig. 4a). In cotyledons, EBL decreased Put accumulation under low salt conditions (0 and 100 mM NaCl), but promoted Put accumulation remarkably under high salt conditions (200 mM NaCl); however, the opposite trend was observed in the hypocotyls and radicles. EBL mainly promoted the accumulation of Spd+Spm in different tissues under both low and high salt stress conditions, which was consistent with PA content in whole seedlings (Fig. 4b, S5), implying that the contrasting effects of EBL on salt-stressed canola during seed germination was more related to Put than Spd or Spm. As the H_2O_2 distribution was consistent with Put accumulation with or without EBL7 under low and high salt conditions (Fig. 2b, 4a), Put oxidation may

determine the regulation of H_2O_2 regulated by EBL in the salt-stressed canola during germination.

External application of D-Arg, an established Put biosynthesis inhibitor (Hao, Kitashiba, Honda, Nada, & Moriguchi, 2005), can effectively reduce the level of H_2O_2 under salt stress with EBL7 and even led to the disappearance of H_2O_2 under high concentrations of D-Arg (Fig. 5a), indicating that H_2O_2 was mainly produced by oxidation of PAs under salt stress. However, 10^{-4} M D-Arg did not obviously promote the elongation of hypocotyls and radicles, but did significantly decrease H_2O_2 levels, thus improving germination rates of canola seeds under 100 mM NaCl supplemented with EBL7 (Fig. 5b-d). AG, a specific inhibitor of DAO (Yang, Guo, & Gu, 2013), could effectively relieve the inhibition of EBL on seed germination and growth under low salt stress (100 mM NaCl), corresponding to the reduction of H_2O_2 in seedlings (Fig. 5b,c,e,f). These results suggest that the inhibition of EBL on low-salt stressed canola can be reversed by repressing Put biosynthesis or Put oxidation. To further confirm our hypothesis, DAO and PAO were analysed (Fig. 6, S6). DAO activity was upregulated remarkably under low salt stress (0 and 100 mM NaCl) with EBL7, but downregulated under high salt conditions (200 mM NaCl) with EBL7 (Fig. 6); however, PAO activity was upregulated in a manner that was not

salt concentration dependent (Fig. S6), which was consistent with the Put and Spd+Spm contents of hypocotyls and radicles (Fig. 4). These results indicate that the contrasting effects of EBL on the H_2O_2 accumulation in low- and high-salt-stressed canola are mainly determined by Put oxidation.

BR signalling affects salt tolerance of *Arabidopsis* coupled with H_2O_2 accumulation regulated by Put

To further confirm the functional characteristics of BR signalling in response to salt tolerance and its relationship with Put metabolism, we first conducted a series of phenotypic assessments by using multiple genetic materials with effects mediated by the BR signalling pathway. RT-qPCR analysis showed that transcription of the BR receptor gene *BRI1* and the BRI1 co-receptor gene *BAK1* were significantly induced by salt stress, indicating that the BR signalling pathway positively regulates salt stress (Fig. S7). During seed germination, we found that *bri1-9*, *bes1-D*, and *bzr1-1D* mutants exhibited a dual germination phenotype of low-salt tolerance (≤100 mM NaCl) and high-salt sensitivity (≥150 mM NaCl) compared with the wild-type Col-0 control (Fig. S8a-e). The *bak1* mutant also had higher germination rates than Col-0 under lower NaCl concentrations (<200 mM), but, similar to *bri1-9*, its growth status was no better than that of Col-0 (Fig. S8a,d). However, the BRI1 and BAK1

overexpression lines not only demonstrated higher germination rates, but also had a better growth status than Col-0 plants under salt stress (Fig. S8a,c,d). Interestingly, the salt sensitivity of some plants changed at the seedling stage. For example, the *bes1-D* mutant was seriously defective in root elongation even under normal conditions, but demonstrated better leaf growth under the 150 mM NaCl treatment (Fig. S8f); *bak1* seeds had a higher germination rate, but their seedlings were more sensitive than Col-0 plants to 150 mM NaCl or even lower salt conditions (Fig. S8f,h). The *bri1-9 and bzr1-1D* seedlings were still more sensitive, and the BRI1 and BAK1 overexpression lines also showed more tolerance than Col-0 to high salt stress conditions (Fig. S8f,g).

Further, H_2O_2 staining assays of the different genetic materials were performed under 0–150 mM NaCl conditions. Consistent with the phenotypic analysis, a stronger staining density of H_2O_2 was detected in root tips of *bri1-9*, *bak1*, *bes1-D* and *bzr1-1D* mutants, but lower H_2O_2 staining was observed in overexpression lines of BRI1 and BAK1 under salt stress (Fig. 7a, S10). Furthermore, we analysed the transcription levels of Put synthesis enzyme genes (*AtADC1* and *AtADC2*) and the Put oxidase enzyme gene (*AtAO1*) under 0–150 mM NaCl conditions (Fig. 7b-d), but H_2O_2 accumulation was poorly explained by these genes separately (Fig. 7a-d); however, the pattern

fits well with the value of *(AtADC1+AtADC2)*AtAO1* (Fig. 7a,e). This indicates that H_2O_2 accumulation is co-modulated by Put biosynthesis and oxidation. Then, we assessed the effects of Put on growth via the BR signalling pathway under salt stress, but no significant differences were observed (Fig. S9); however, H_2O_2 levels in roots were markedly disturbed (Fig. S10). H_2O_2 accumulation was significantly decreased in wild-type plants by exogenous Put application (Fig. S10a), but the H_2O_2 accumulation of *bri1-9* and *bak1* mutants was maintained at a high level, which was not affected by Put treatment (Fig. S10b,d). Put application decreased H_2O_2 accumulation in BRI1 and BAK1 overexpression lines at appropriate doses, but increased under high Put concentrations (Fig. S10c,e). The *bes1-D* mutants accumulated higher H_2O_2 levels under normal conditions, with lower levels under the 50 mM NaCl treatment, though they were also not as affected by Put as the *bri1-9* and *bak1* mutants (Fig. S10b,d,f). Higher H_2O_2 levels were observed in *bzr1-1D* mutants, which were slightly influenced by Put (which promoted and reduced H_2O_2 accumulation under low and high salt conditions, respectively) (Fig. S10f). In general, the above results confirmed the essential function of BR signal transduction in the regulation of salt resistance in plants, exhibiting a complex relationship with H_2O_2 regulated by Put.

DISCUSSION

In the process of regulating plant growth and development, phytohormones often have biphasic effects (Lin, Wang, Wang, Zaharia & Abrams, 2005; Teale, Paponov & Palme, 2006; Walton et al., 2012). It is reported that an appropriate concentration of EBL can induce stomatal opening, whereas high concentrations of EBR induce stomatal closure, and this is closely related to dynamic changes in H_2O_2 and the redox status of guard cells (Xia et al., 2014). In this study, EBL also showed a similar effect on seed germination under normal conditions (Fig. S1b). We found a more complex phenomenon: the same concentration of EBL (10^{-6} M) had opposite effects on seed germination and seedling establishment under low and high salt stress, i.e. low-salt inhibition and high-salt promotion (Fig. 1, S2). We define this phenomenon as hormonal stress-level-dependent biphasic (SLDB) effects.

Put metabolism modulates the SLDB effects of BRs on plant salt tolerance

The concentration of BR required for normal growth and development of plants is very low. Exogenous application of nM-scale concentrations of BR affects cell expansion, vascular differentiation and so on, but high concentrations of BR are sometimes detrimental to plants (Clouse, 1998; Mussig, Shin, &

Altmann, 2003; Wei & Li, 2016). *DWARF4* (*DWF4*) encodes a C-22 hydroxylase that is essential for BR biosynthesis, and its promoter serves as a focal regulating point in maintaining homeostasis of endogenous bioactive BR pools (H. B. Kim et al., 2006). Expression of *StDWF4* can be induced by salt stress and over-expression or RNAi of *StDWF4* can respectively increase or decrease salt stress tolerance in potato plants (Zhou et al., 2018). Transcriptomic analysis shows that several gene families related to BR metabolism, including *DWF4*, were significantly enriched in the comparison of salt tolerance between two poplar species (Zhang et al. 2019). *Brassica napus* plants that overexpressed the BR biosynthesis gene *DWF4* displayed significantly better tolerance to dehydration stress (Sahni et al., 2016). Therefore, the optimum concentration of BRs required by plants may gradually increase as stress intensifies to enhance their resistance to stress, but the optimum concentration may be excessive under another low-stress condition, which is detrimental to plants and thus leads to the emergence of SLDB effects (Fig. S11a).

Previous studies have shown that PAs interact with BR in enhancing plant stress tolerance (Choudhary, Oral, Bhardwaj, Yu, & Tran, 2012; Serna et al., 2015; Zheng et al., 2015). PAs are acknowledged regulators of plant stress

responses, and Spd and Spm are generally considered to play more important roles than Put (Liu, Wang, Wu, Gong, & Moriguchi, 2015). Whereas, Put has specific functions that differ from those of Spd and Spm in countering abiotic stresses, such as potassium deficiency (Cui, Pottosin, Lamade & Tcherkez, 2020). Overexpression of *ADC*, an arginine decarboxylase gene encoding a key enzyme involved in Put synthesis, can improve stress tolerance of plants, but it should be considered that toxic effects of Put over-production have also been observed, and the deleterious effects may be caused by enhanced DAO activity and excessive ROS production (Cui, Pottosin, Lamade & Tcherkez, 2020). Here, we found that exogenous EBL can promote the accumulation of Spd and Spm, but decrease the level of Put, and the transformation of Put to Spd and Spm was beneficial to plant salt tolerance; however, when plants did not require large amounts of BR under low salt stress, excessive application of EBL promoted Put oxidation and produced large amounts of H_2O_2, which harmed plants. Thus, the contrasting roles of Put in countering salt-stress and generating H_2O_2 likely mediate the SLDB effects of BR (Fig. S11a).

BR signal transduction regulates plant salt tolerance with SLDB effects

The regulatory roles of BR in plant salt tolerance have been assessed by exogenous hormone application (Serna et al., 2015), but evidence of its

genetic effects have been lacking so far. In BR signal transduction, BRI is the BR receptor and BAK1 acts as a coreceptor interacting with BRI, and BES1 and BZR1 are the key transcription factors for BR-regulated gene expression downstream of the BR signalling cascades (Kim & Wang, 2010). Cui et al.(2012) showed that *bri1-9* mutants are sensitive to salt stress under 125 mM or 150 mM NaCl treatment at germination, and the wild-type phenotype could be partially rescued by a mutation in UBC32, a stress-induced functional ubiquitin conjugase localized to the ER membrane. Our results also showed that the *bri1-9* mutant was sensitive to high salt stress (≥ 150 mM NaCl), but it had a higher germination rate than wild-type seeds under lower salt conditions (≤100 mM NaCl), similar to that of the *bak1* mutant (Fig. S8a,c,g). A similar dual phenotype was also observed among the *bes1-D* and *bzr1-1D* mutants (Fig. S8b,e), which is consistent with the SLDB effects of BR on canola.

BZR1 has dual roles in BR homeostasis and growth responses (He et al., 2005). A dominant *bzr1-D* mutation causes constitutive activation of many BR-dependent processes, and it recovers root growth earlier and has higher growth rates during the recovery phase than the wild-type under salt stress conditions (Geng et al., 2013). However, when grown in light, *bzr1-1D* mutants have reduced cell elongation and reduced levels of BR, perhaps as a

consequence of feedback regulation (He et al., 2005). Under long-day conditions, the *bzr1-1D* mutant also showed salt sensitivity under 150 mM NaCl treatment, where germination rate was lower and growth status was worse than that of the wild-type (Fig. S8e,f). In addition to BZR1, there may be other signalling pathways involved the salt tolerance regulation role of BR. Like *bzr1-1D*, *bes1-D* is a gain-of-function mutant, but the leaves of *bes1-D* seedlings were healthier than wild-type leaves under salt stress (Fig. S8f). Unlike the *bri1-9* and *bak1* mutants, the BRI1- and BAK1-overexpression lines were tolerant to high salt stress (Fig. S8). These findings indicate that BR signal transduction plays an important role in regulating plant salt tolerance (Fig. S11b), and there are SLDB effects observed at the germination stage.

BR signalling may reduce salt-induced H_2O_2 increases and have crosstalk with Put

ROS are often increased by salinity and play a dual role in plants through their function as signalling molecules at low levels and as toxic molecules at high levels (Miller et al., 2010). BR can regulate ROS homeostasis to control root growth or enhance plant stress tolerance (Lv et al., 2018; Xia et al., 2009). The *det2-9* mutant is defective in BR synthesis and displayed an overaccumulation of superoxide anions (O_2^-), and its increased O_2^- levels

inhibited root growth (Lv et al., 2018). Here, we also found the *bri1-9* and *bak1* mutants accumulated more H_2O_2 in their root tips than did wild-type plants, especially under salt stress conditions (Fig. 7a). In contrast with the mutants, the overexpression lines of BRI1 and BAK1 displayed lower H_2O_2 levels than wild-type plants (Fig. 7a). However, the *bes1-D* and *bzr1-1D* mutants that showed constitutive BR responses also accumulated dramatically high levels of H_2O_2, like *bri1-9* and *bak1* mutants, compared with the wild-type control, and *bes1-D* always displayed a brevis radix phenotype (Fig. 7a, S8f, S9, S10a,S10f). BRs play essential roles in root growth, but both an excess and a lack of BR compounds or BR signalling are disadvantageous to primary root growth and development (Planas-Riverola et al., 2019). These findings suggest that, rather than simply increasing BRs levels or enhancing BR signalling, the correct balance of BR levels for an appropriate enhancement of BR signalling appears to enhance the control of H_2O_2 burst and survival under salinity (Fig. S11c).

PA tissue concentrations and the expression of genes encoding PA enzymes can be modulated by BRs, which indicates the potential for crosstalk between BR signalling and PAs in regulating abiotic stress tolerance of plants (Choudhary, Oral, Bhardwaj, Yu, & Tran, 2012; Serna et al., 2015; Zheng et al.,

2015). Our results showed that BR signalling could modulate the expression of Put synthesis genes *AtADC1/2* and the Put oxidase enzyme gene *AtAO1*, which together determined the accumulation of H_2O_2 under salt stress (Fig. 7). Interestingly, overexpression of *AtADC2*, as well as mutation in *BUD2* and *ACL5* (two genes that encode enzymes necessary for PA biosynthesis), can cause dwarfism, which is also observed in many BR mutants (Alcázar, García-Martínez, Cuevas, Tiburcio, & Altabella, 2005; Ge et al., 2006; Imai et al., 2006; Noguchi et al. 1999). The ATHB8/ACL5–BUD2 transcription module not only regulates auxin-related genes but also the BR receptor gene *BRL2* (Baima et al., 2014; Ceserani, Trofka, Gandotra, & Nelson, 2009). These imply that Put or PAs in general may also modulate BR signalling but currently available information is rather limited. Here, we found that H_2O_2 accumulation in the genetic materials of BR signalling could be disturbed by external Put application (Fig. S10). Overall, the mutants that are insensitive to exogenous BR, including *bri1-9*, *bak1*, *bes1-D* and *bzr1-1D*, were also duller than the wild-type or the overexpression lines of BRI1 and BAK1. Although proper Put application could reduce H_2O_2 accumulation in roots under salt stress, Put was more likely to exceed the optimal concentration in the BRI1 and BAK1 overexpression lines than in wild-type plants, which leads to an increase in

H_2O_2 via Put catabolism. H_2O_2 may in turn cause oxidative stress but may also participate in BR signalling via BZR1 oxidation and thus favour BR-related stress tolerance (Tian et al., 2018; Xia et al., 2009). BR, PAs and H_2O_2 have specific signaling pathways in regulating plant stress tolerance, but little is known about their potential relationships (Gupta, Sengupta, Chakraborty, & Gupta, 2016; Planas-Riverola et al., 2019). Here, we show that salt stress triggers crosstalk between BR signalling and Put, where the dual role of Put in H_2O_2 accumulation are likely regulated by BR signalling (Fig. S11d).

SLDB effects occur in the BR regulation of plant salt tolerance, and this phenomenon is closely related to H_2O_2 regulation by Put metabolism. A proper increase in BR level or BR signalling enhances the ability of plants to counter salt stress, whereas excess levels are just as harmful as deficient levels.

ACKNOWLEDGMENTS

We are grateful to Prof. Cun Wang of the College of Life Sciences, Northwest A&F University for providing *Arabidopsis* mutants and overexpression lines. This work was supported by the National Natural Science Foundation of China (31702167 and 31672141), the China Postdoctoral Science Foundation Grant (2018M641030), the Fundamental Research Funds for the Central Universities (2452018125), and the Natural Science Basic Research Plan in Shaanxi

Province, China (2019JM-014). The authors declare no competing financial interests.

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FIGURE LEGENDS

FIGURE 1 The phytohormone 24-epibrassinolide (EBL) has contrasting effects on the germination of low- and high-salt-stressed canola. Seeds were germinated in the presence of NaCl (0, 100, 150, 200, 250 mM) and/or EBL7 (10^{-6} M EBL) treatment. Seed germination rates were recorded on the (a) 1st, (b) 3rd, and (c) 7th days of germination. Radicle emergence was used as the

morphological marker for germination. Asterisks denote statistically different values according to independent sample *t*-tests (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). Mean ± SD values ($n = 4$) are presented.

FIGURE 2 The phytohormone 24-epibrassinolide (EBL) has contrasting effects on H_2O_2 accumulation in low- and high-salt-stressed canola. (a) Relative H_2O_2 contents of seedlings germinated in the presence of NaCl (0, 100, 150, 200, 250 mM NaCl) and/or EBL7 (10^{-6} M). The H_2O_2 contents in seeds on the 7th day of germination were determined, and the relative H_2O_2 contents in the germinated seedlings were calculated by dividing the H_2O_2 contents in seeds by the germination rate. Asterisks denote significantly different values according to independent sample *t*-tests (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). Mean ± SD values ($n \geq 4$) are presented. (b) Histochemical staining of H_2O_2 in 7-day-old seedlings germinated in the presence of NaCl (0, 100, 200 mM) and/or the EBL7 (10^{-6} M EBL) treatment.

FIGURE 3 The inhibition of 24-epibrassinolide (EBL) on low-salt-stressed canola is reversed by reducing H_2O_2 levels. Dimethylthiourea (DMTU) is a H_2O_2 scavenger. Seeds were germinated in the presence of NaCl (0, 100, 150,

200, 250 mM), EBL7 (10^{-6} M EBL) and/or DMTU (0.5 M). Lengths of (a) hypocotyls and (b) radicles were measured on the 7th day of germination. Different letters above bars indicate significant differences between treatments ($P < 0.05$; Duncan's test). Mean ± SD values ($n = 10$) are presented.

FIGURE 4 The phytohormone 24-epibrassinolide (EBL) has contrasting effects on tissue concentrations of putrescine (Put) but not spermidine (Spd) or spermine (Spm) in low- and high-salt-stressed canola. (a) Tissue concentrations of Put under treatments of NaCl (0, 100, 200 mM) and/or EBL7 (10^{-6} M EBL). On the 7th day of germination under the indicated conditions, polyamines (PAs) in different parts of germinated seedlings, including the cotyledon, the upper half of the hypocotyl (U-hypocotyl), the lower half of the hypocotyl (U-hypocotyl), and the radicle, were extracted and determined. (b) Total contents of Spd and Spm in different tissues were calculated. Asterisks denote statistically different values according to independent sample *t*-tests (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). Mean ± SD values ($n = 3$) are presented.

FIGURE 5 The inhibition of 24-epibrassinolide (EBL) on low-salt stressed canola is ameliorated by repressing the biosynthesis or oxidation of putrescine

(Put). (a) Histochemical staining of H_2O_2 in the 7-day-old seedlings germinated in the presence of 200 mM NaCl, EBL7 (10^{-6} M EBL), and the Put synthesis inhibitor D-Arg (0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M). (b) Histochemical staining of H_2O_2 in 7-day-old seedlings germinated in the presence of 100 mM NaCl, EBL7 (10^{-6} M), D-Arg (10^{-4} M), and aminoguanidine (AG; 10^{-2}, 10^{-4}, 10^{-6} M). AG is a specific inhibitor of diamine oxidase (DAO). (c) Germination phenotype of canola in the presence of 100 mM NaCl, EBL7 (10^{-6} M EBL), D-Arg (10^{-4} M), and AG (10^{-2}, 10^{-4}, 10^{-6} M). Images were captured on the 3rd day of germination. (d, e) Germination rate of canola in (c) was recorded at the indicated times of germination. Radicle emergence was used as the morphological marker for germination. Mean ± SD values (n = 4) are presented. (f) Hypocotyl and radicle lengths of germinated seedlings in (c) were measured on the 7th day of germination. Different letters above bars indicate significant differences between treatments (P < 0.05; Duncan's test). Mean ± SD values (n = 20) are presented.

FIGURE 6 The phytohormone 24-epibrassinolide (EBL) has contrasting effects on the activities of diamine oxidase (DAO) in low- and high-salt-stressed canola. Seeds were germinated in the presence of NaCl (0, 100, or 200 mM)

and/or EBL7 (10^{-6} M EBL). DAO was extracted from seeds at indicated germination time points, and DAO activities were subsequently determined. Asterisks denote statistically different values according to independent sample *t*-tests (**P* < 0.05, ***P* < 0.005, ****P* < 0.001). Mean ± SD values (*n* ≥ 10) are presented.

FIGURE 7 Analysis of H_2O_2 histochemical staining and the expression of putrescine (Put) metabolic genes involved in brassinosteroid (BR) signaling in plants under salt stresses. (a) H_2O_2 histochemical staining of wild-type (Col-0), *bri1-9*, *bak1*, BRI1 overexpression (BRI1-OE), BAK1 overexpression (BAK1-OE), *bes1-D*, and *bzr1-1D* seedlings after treatment with NaCl (0, 50, 150 mM) for 7 days. H_2O_2 was mainly stained in roots, and images of root tips were taken with microscope. (b–d) Expression levels of Put metabolism genes, including (b) *AtADC1*, (c) *AtADC2*, and (d) *AtAO1*, which are involved in BR signaling, in plants after treatment with NaCl (0, 50, 150 mM) for 7 days. Expression values were normalized to *ACTIN2* expression levels. Different letters above bars indicate significant differences between treatments (*P* < 0.05; Duncan's test). Mean ± SD values (*n* = 3) are presented. (e) The value of (*AtADC1* + *AtADC2)* × *AtAO1*, which was calculated with the average means of the relative expression levels of *AtADC1*, *AtADC2*, and *AtAO1* in (b–d).