RESEARCH PAPER

Interaction of brassinosteroids and polyamines enhances copper stress tolerance in *Raphanus sativus*

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Abstract

Brassinosteroids (BRs) and polyamines (PAs) regulate various responses to abiotic stress, but their involvement in the regulation of copper (Cu) homeostasis in plants exposed to toxic levels of Cu is poorly understood. This study provides an analysis of the effects of exogenously applied BRs and PAs on radish (*Raphanus sativus*) plants exposed to toxic concentrations of Cu. The interaction of 24-epibrassinolide (EBR, an active BR) and spermidine (Spd, an active PA) on gene expression and the physiology of radish plants resulted in enhanced tolerance to Cu stress. Results indicated that the combined application of EBR and Spd modulated the expression of genes encoding PA enzymes and genes that impact the metabolism of indole-3-acetic acid (IAA) and abscisic acid (ABA) resulting in enhanced Cu stress tolerance. Altered expression of genes implicated in Cu homeostasis appeared to be the main effect of EBR and Spd leading to Cu stress alleviation in radish. Ion leakage, in vivo imaging of H₂O₂, comet assay, and improved tolerance of Cu-sensitive yeast strains provided further evidence for the ability of EBR and Spd to improve Cu tolerance significantly. The study indicates that co-application of EBR and Spd is an effective approach for Cu detoxification and the maintenance of Cu homeostasis in plants. Therefore, the use of these compounds in agricultural production systems should be explored.

Keywords: Abscisic acid, brassinosteroids, comet assay, copper transporters, Cu homeostasis, Cu-sensitive yeast, indole-3-acetic acid, oxidative stress, polyamines.

Introduction

Copper (Cu) is an essential micronutrient for most biological organisms. It is a cofactor for a large array of proteins involved in diverse physiological processes, such as photosynthesis, the electron transport chain, respiration, cell wall metabolism, and hormone signalling (Bhakuni *et al.*, 2009; Andre *et al.*, 2010). Although Cu is essential at low concentration, excess Cu is cytotoxic due to its role in the catalysis of reactions which generate reactive oxygen species (ROS), ultimately leading to increased oxidative stress in plants (Andre *et al.*, 2010). Factors regulating Cu homeostasis play pivotal roles in tightly regulating intracellular Cu levels to avoid toxicity. In general, Cu uptake into the cytosol of plant cells is governed by a family of plasma membrane transporters named COPTs. At least six types of transport proteins (AtCOPT1–AtCOPT6), belonging to the Cu transport (CTR) family, have been recently characterized in *Arabidopsis thaliana* and rice (*Oryza sativa*) (Yuan *et al.*, 2011). The

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intracellular distribution of cytosolic Cu involves the participation of Cu chaperones (CCHs) and various Cu-binding proteins which deliver Cu to specific sites (Beauclair et al., 2010). Heavy metal ATPases (HMAs), such as HMA5, represent another group of proteins which play a key role in transmembrane transport of Cu in Arabidopsis (Andre et al., 2010). In addition to the activity of transporter systems that regulate Cu uptake, the cytotoxicity of Cu is also reduced by cellular antioxidant systems, compatible solutes such as glycinebetaines (GBs), proline (PL), polyamines (PAs), and sugar alcohols (Andre et al., 2010). The roles of phytochelatins (PCs) and metallothionenins (MTs) in metal detoxification have also been well characterized (Diwan et al., 2010).

Cu has emerged as a major environmental pollutant in the past few decades because of its excessive use in manufacturing and agricultural industries (Bouazizi et al., 2011). Cu build-up in the food chain has resulted in reports of Cu toxicity in humans due to the consumption of Cu-ladened food commodities (Chary et al., 2008; Desai and Kaler, 2008; Bouazizi et al., 2011). Radish (Raphanus sativus L.) tubers, which represent a rich source of anti-diabetics, antioxidants, and multipotent chemopreventive factors, are particularly prone to the toxic effects of high levels of Cu, resulting in a reduction in biomass and a detrimental impact on its nutritional profile (Baek et al., 2008; Sfaxi-Bousbih et al., 2010; Sun et al., 2010). Collectively, evidence indicates the clear need to minimize the accumulation of Cu and other heavy metals in the biosphere. A major focus on the metal tolerance of agricultural crops has been seen in the last decade.

In addition to their role in growth and development, the impact of phytohormones on abiotic stress tolerance has been well established (Peleg and Blumwald, 2010; Umezawa et al., 2010; Hadiarto and Tran, 2011; Nishiyama et al., 2011). Among the phytohormones, brassinosteroids (BRs) form a group of steroidal lactones that regulate various developmental and physiological processes, including cell elongation, morphogenesis, tissue differentiation, and reproduction (Clouse, 2011; Gudesblat and Russinova, 2011). BRs also confer both abiotic and biotic stress tolerance in plants (Bajguz et al., 2011; Choudhary et al., 2012a, b; Clouse, 2011; Gudesblat and Russinova, 2011). Molecular and signal transduction studies have established that the interactions of BRs with auxins (indole-3-acetic acid; IAA), jasmonates, abscisic acid (ABA), and ethylene play an important role in stress management (Divi et al., 2010). Among BRs, 24-epibrassinolide (EBR, an active BR) has been extensively used to improve heavy metal stress tolerance in plants (Wen et al., 2010). PA biosynthesis in plants is regulated by arginine decarboxylases (ADCs), 5-adenosyl methionine decarboxylase (SAMDC), and Spd synthases (SPDs) encoded by ADC1, ADC2, SAMDC, SPDS1, and SPDS3, respectively, while PA catabolism is controlled by a set of polyamine oxidases (PAOs). PAOs are involved in the back-conversion of higher PAs such as Spd and Spm to pyrroline, 1,3-diaminopropane, H2O2, and aminopropylpyrroline (Fincato et al., 2011; Hussain et al., 2011).

Cu stress mitigation with a large variety of chemical moieties has been widely documented in plants (Bajguz, 2010). Little information is available, however, on the exact mechanism of BR- and/or PA-induced Cu stress alleviation and Cu homeostasis in plants. In this report insight is provided into how EBR and Spd (a potent PA) confer Cu stress tolerance in radish. It is hypothesized that BRs and PAs impart Cu stress tolerance and maintain Cu homeostasis via similar mechanisms that impact both gene expression and the physiology of radish. The efficacy of a co-application of EBR and Spd versus their individual use for Cu detoxification and maintenance of Cu homeostasis in radish was examined.

Materials and methods

Plant materials and growth conditions

Radish seeds used in this study were obtained from Punjab Agriculture University, Ludhiana, India. Approximately 25 seeds were sown in autoclaved Petri dishes lined with Whatman No. 1 filter paper and supplied with Murashige and Skoog (MS) media with or without hormone and Cu treatments and kept at 20–25 °C with a 16 h photoperiod under fluorescent white light (175 µmol m−2 s−1) in a growth chamber.

Treatments

Two-day-old radish seedlings were treated on every other day until the day of harvest with 4 ml of MS medium solution containing EBR and Spd alone or together combined with a Cu solution at a final concentration of 10−6 M EBR, 1 mM Spd, and 0.2 mM CuSO4·5H2O. The Cu content of the MS medium under non-stressed conditions was 25 µg ml−1. The concentration of 0.2 mM Cu was selected as a stress treatment based on the IC50 previously determined for Raphanus in germination and growth tests (Choudhary et al., 2010). On the seventh day, seedlings were harvested (3.5–4.0 cm long hypocotyls) and subjected to a set of morphological, molecular, physiological, phytohormonal, and microscopic observations.

Morphological parameters

The growth of 7-day-old radish seedlings was assessed by recording shoot length (SL), root length (RL), and fresh weight (FW).

Yeast Cu stress tolerance assay

A yeast Cu stress tolerance assay was performed as previously described (Mu et al., 2011) (Supplementary Methods and Supplementary Tables S1, S2 available at JXB online).

Determination of Cu content

The Cu content in seedling tissue (2 g dry weight; DW) was determined by atomic absorption spectrometry using a 10% (v/v) HNO3 acid digestion procedure as previously described in Chow et al. (1995) and expressed as µg Cu g−1 DW.
Estimation of endogenous PAs, IAA, and ABA
Approximately 2 g FW of seedlings were used for the analysis of IAA and ABA as described previously (Supplementary Methods at JXB online) (Choudhary et al., 2010). The endogenous PA content was estimated according to the method of Choudhary et al. (2011) (Supplementary Methods).

Evaluation of antioxidant system parameters
Antioxidant levels
Endogenous levels of glutathione (GSH), PL, ascorbic acid (ASA), and total phenol (TP) were determined according to Sedlak and Lindsay (1968), Bates et al. (1973), Cakmak and Marschner (1992), and Choudhary et al. (2010), respectively. The total antioxidant status was evaluated by performing the 1,1-diphenyl-2-picrylhydrazyl (DPPH), inhibition of deoxyribose degradation (deoxyribose), and ferric-reducing antioxidant power (FRAP) assays as previously described (Choudhary et al., 2011).

Activities of antioxidant enzymes
The activities of antioxidant enzymes were determined by standard methods reported in Aebi (1983) for catalase (CAT) (EC 1.11.1.6), Kono (1978) for superoxide dismutase (SOD) (EC 1.15.1.1), Foyer and Halliwell (1976) for glutathione reductase (GR) (EC 1.6.4.2), Putter (1974) for guaiacol peroxidase (GPOX) (EC 1.11.1.7), Hossain et al. (1984) for monodehydroascorbate reductase (MDHAR) (EC 1.1.5.4), and Dalton et al. (1986) for dehydroascorbate reductase (DHAR) (EC 1.8.5.1). Total protein content was determined by the method of Lowry et al. (1951).

Cu detoxification factors
PC content was determined according to Doring et al. (2000). Photosynthetic pigments were determined by the method of Lichtenthaler (1987).

Stress indices
GB, H2O2, and total soluble sugars (TSS) were estimated according to Grieve and Grattan (1983), Choudhary et al. (2010), and Miller (1959), respectively. The extent of membrane damage in response to Cu stress, as measured by lipid peroxidation, was estimated according to Heath and Packer (1968). The loss of membrane permeability under Cu stress was evaluated by the ion leakage assay (He et al., 2008).

Localization of H2O2 and O2− under Cu stress
Detection and localization of H2O2 production in radish root tips under Cu stress was performed using confocal microscopy (Xia et al., 2011). The presence of O2− and H2O2 generated under Cu stress was performed using nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine hydrochloride (DAB) staining methods as described in Xia et al. (2009).

Single cell gel electrophoresis assay (comet assay)
Comet assay of the radish seedling was performed using the previously described method (Sakamoto et al., 2011).

Quantitative real-time polymerase chain reaction (RT-qPCR) analysis
RT-qPCR was performed using an iTcycler iQ Real-Time PCR detection system (Bio-Rad) as described previously (Choudhary et al., 2012a). Expression of 20 genes encoding enzymes involved in the uptake, assimilation, detoxification, and homeostasis of Cu, as well as the metabolism of PA, ABA, and IAA in radish was analysed in response to the application of EBR and/or Spd (Supplementary Table S3 at JXB online). The identification of specific radish genes was based on using their Arabidopsis orthologues for homology search against the expressed sequence tag (EST) databank of R. sativus available at www.plantgdb.org as previously described (Choudhary et al., 2012a). Specific primer pairs for each gene were designed as described in Le et al. (2011). The 26S rRNA (AY366932.1) was used as a reference gene in the expression analysis. The specificity of all primer pairs was verified by gel electrophoresis and sequencing of the corresponding amplicons. All the experiments were repeated three times using cDNAs prepared from two samples of radish tissues.

Statistical analysis
All experiments were performed in triplicate. Data shown are means ±SE of three replicates for each experiment where each replicate consisted of 25 pooled seedlings. One-way analysis of variance (ANOVA) was carried out using P < 0.05 as a measure of significance. All statistical calculations were performed using Sigma Stat.

Results
Effects of EBR and/or Spd on growth of radish under Cu stress
A significant 2- and 3-fold reduction in RL and SL, respectively, was observed in Cu-stressed seedlings compared with the unstressed control (Table 1; Supplementary Fig. S1 at JXB online). Individual applications of either EBR or Spd improved RL by 1.6- and 1.4-fold and SL by 2.5- and 2-fold, respectively, in seedlings subjected to Cu stress compared with seedlings exposed to just the Cu stress. Co-application of EBR and Spd increased RL and SL of seedlings under Cu stress by 2- and 3.2-fold compared with seedlings subjected to Cu stress alone (Table 1; Supplementary Fig. S1). No significant effect on RL and SL was observed in seedlings treated with either EBR or Spd alone when no Cu stress was applied compared with the untreated control. Co-application of EBR and Spd in the absence of Cu stress, however, was able to increase RL and SL by 1.6- and 1.4-fold, respectively, compared with the untreated control seedlings.

An ~1.8-fold reduction in FW was observed in seedlings under Cu stress compared with the untreated control. No significant increases in FW were noted in Cu-stressed seedlings that

Table 1. Effect of EBR and/or Spd with or without Cu stress on growth parameters of 7-day-old radish seedlings
Data presented are the mean ±SE. Different letters (a, b, c, and d) within a column indicate a significant difference from each other in all combinations (Tukey’s test, P < 0.05). Symbols '*' and '§' indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress, respectively (Tukey’s test, P < 0.05).
had received application of either EBR or Spd alone compared with seedlings subjected to Cu stress alone (Table 1). Combined application of EBR and Spd was more effective than the use of either compound alone in increasing FW, as indicated by a 1.7-fold improvement over seedlings subjected to a Cu stress alone (Table 1). Seedlings that had been treated with EBR or Spd alone without a Cu stress had no significant increase in FW compared with the untreated control. Interestingly, combined application of EBR and Spd in the absence of Cu stress was found to increase FW by 1.4-fold over the untreated control (Table 1). These data demonstrate that the co-application of EBR and Spd can improve the growth of radish seedlings under non-stress conditions as well as when they are subjected to Cu stress more effectively than when the compounds are used individually.

**EBR and Spd reduce Cu uptake, assimilation, and distribution to overcome Cu cytotoxicity**

Expression analysis of **COPT** genes was conducted in radish seedlings treated with EBR and/or Spd with or without Cu stress. The major increase in Cu uptake (9.9-fold) in radish seedlings subjected to Cu stress was associated with the up-regulation of **RsCOPT1** (6.5-fold) and **RsCOPT2** (11.3-fold), both of which are involved in the uptake of Cu (Fig. 1A). EBR applied singly under Cu stress reduced Cu uptake by 2.2-fold, which may be linked to the down-regulation of **RsCOPT1** (28.9-fold) and **RsCOPT2** (8.3-fold) compared with expression levels in seedlings subjected to Cu stress alone. An ~1.4-fold decrease in Cu uptake in Cu-stressed seedlings treated with Spd alone was associated with the repression of **RsCOPT1** (24.4-fold) expression compared with expression in seedlings subjected to Cu treatment alone (Fig. 1A). Furthermore, co-application of EBR and Spd reduced Cu uptake in 7-day-old, Cu-stressed seedlings to a significantly greater extent (3.8-fold) than when either compound was used alone. The significant decrease in Cu uptake in response to the combined application of EBR and Spd was associated with the dramatic down-regulation of **RsCOPT1** (220-fold) and **RsCOPT2** (6.9-fold) compared with seedlings subjected to Cu stress alone (Fig. 1A). No significant increases in Cu content and in the expression of **RsCOPT1** and **RsCOPT2** were observed in seedlings treated with either EBR or Spd alone with or without Cu stress compared with the untreated control (Fig. 1A).

HMAs play a central role in Cu assimilation in plants. In order to determine whether or not EBR and/or Spd mitigate Cu stress in radish seedlings by reducing Cu assimilation, their effect, applied either alone or together, on the expression of **RsHMA5**, a gene involved in Cu assimilation through interacting with CCHs (Andres-Colas et al., 2006), was examined. A significant increase in **RsHMA5** gene expression (7.16-fold) in seedlings subjected to Cu stress was observed, supporting the premise that there is an increase in Cu assimilation in seedlings subjected to Cu stress (Fig. 1B). In contrast, a significant reduction in **RsHMA5** expression (14.2-fold) was observed in Cu-stressed seedlings that had been treated with EBR, while Spd-treated seedlings had a 2.7-fold decrease in **RsHMA5** expression. Simultaneous application of EBR and Spd, however, produced a 3.9-fold decline in **RsHMA5** expression in Cu-treated radish seedlings (Fig. 1B). Statistically insignificant changes in **RsHMA5** expression were observed in seedlings treated with either EBR or Spd alone without Cu stress compared with untreated controls. Co-application of EBR and Spd to non-Cu treated seedlings significantly elevated **RsHMA5**

![Figure 1](https://academic.oup.com/jxb/article-abstract/63/15/5659/659335)

**Fig. 1.** Effect of EBR and/or Spd with or without Cu stress on (A) Cu content and expression of Cu transporter genes (**RsCOPT1** and **RsCOPT2**) and (B) Cu assimilation (**RsHMA5**) and Cu detoxification (**RsMT1C** and **RsCCH1**) genes in 7-day-old radish seedlings. Data presented are the mean ±SE. Different superscript letters (a, b, and c) indicate a significant difference from each other in all combinations (Tukey's test, \(P < 0.05\)). Symbols '*' and '§' indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd, respectively, under Cu stress (Tukey's test, \(P < 0.05\)).
Cu stress mitigation with spermidine and epibrassinolide in radish

EBR and Spd co-application efficiently manages the PA pool under Cu stress

PA levels are intricately regulated by a feedback mechanism that creates a balance between biosynthesis and catabolism. The accumulation of PAs is considered a basic strategy for the protection and survival of plants under abiotic stresses (Hussain et al., 2011). A significant increase in Put content (2.4-fold) in Cu-stressed radish seedlings was observed compared with the untreated control. The increase in Put was associated with the up-regulation of \( \text{RsADC2} \) (2-fold) and \( \text{RsSAMDC} \) (3.3-fold) and a minor decline in \( \text{RsADC1} \) compared with the untreated control (Fig. 2). These genes encode \( \text{RsADC2}, \text{RsSAMDC}, \) and \( \text{RsADC1} \) enzymes, which catalyse the formation of Put from arginine in plants. An ~1.6-fold increase in the Put level was observed in seedlings treated with EBR alone and subjected to Cu stress. The increase in Put was attributed to increased (6.3-fold) expression of \( \text{RsADC1} \) compared with Cu treatment alone. No significant gain in Put was noted in Spd-treated seedlings subjected to Cu stress. High levels of Put content (2.3-fold) in seedlings where EBR and Spd were co-applied and subjected to Cu stress was attributed to elevated \( \text{RsADC1} \) (7.3-fold) expression compared with seedlings treated with Cu stress alone (Fig. 2). An approximate 2.84- and 3.59-fold increase in Put content was observed in seedlings treated with either EBR or Spd alone without Cu stress. The increase in Put was associated with 1.4- and 1.9-fold increases in \( \text{RsSAMDC} \) expression compared with the untreated control. Increase in Put content by 2.7-fold in seedlings where EBR and Spd were co-applied but not subjected to Cu stress was associated with a 1.4- and 1.5-fold increase in \( \text{RsADC2} \) and \( \text{RsSAMDC} \) gene expression, respectively, when compared with the untreated control (Fig. 2).

The formation of Spd from Put is regulated by the \( \text{SPDS1} \) enzyme encoded by the \( \text{SPDS1} \) gene, while \( \text{PAO2} \) encoded by \( \text{PAO2} \) catalyses the breakdown of Spd and Spm. \( \text{PAO4} \), encoded by the \( \text{PAO4} \) gene, specifically catalyses the conversion of Spm to lower molecular weight products, thereby maintaining PA homeostasis. An enhanced Spd level (2-fold) in seedlings under Cu stress was directly connected with elevated expression of \( \text{RsSPDS1} \) (1.7-fold) and reduced expression of \( \text{RsPAO2} \) (5.7-fold) compared with the untreated control. An ~1.6-fold reduction in Spd concentration in Cu-stressed seedlings treated with EBR was associated with the down-regulation of \( \text{RsSPDS1} \) (6.5-fold) and the up-regulation of \( \text{RsPAO2} \) (2.9-fold), which resulted in more rapid breakdown of Spd compared with Cu treatment alone (Fig. 2). No significant reduction in Spd level was noted in seedlings treated with Spd alone and subjected to Cu stress compared with Cu stress alone. Moreover, co-application of EBR and Spd with Cu stress reduced Spd content (1.5-fold) by lowering the expression of \( \text{RsSPDS1} \) (6-fold) and increasing the expression of \( \text{RsPAO2} \) (4.5-fold) compared with Cu stress alone (Fig. 2). No significant change in Spd content was noted when EBR and Spd were applied individually with or without Cu stress compared with the untreated control (Fig. 2).

The conversion of Spd to Spm is catalysed by \( \text{SPDS3} \), an enzyme encoded by the \( \text{SPDS3} \) gene. In the current study, the major expansion in the Spm pool (29-fold) in seedlings subjected to Cu stress was associated with a 2.5-fold increase in \( \text{RsSPDS3} \) expression along with a significant decrease in both \( \text{RsPAO2} \) (5.7-fold) and \( \text{RsPAO4} \) (5.7-fold) expression compared with the untreated control (Fig. 2). An ~2.2-fold reduction in Spm content in Cu-stressed seedlings treated with EBR was linked to back-conversion of Spm, as indicated by 2.9- and 3.2-fold increases in \( \text{RsPAO2} \) and \( \text{RsPAO4} \) expression, respectively. A 3.8-fold decrease in Spm in seedlings treated with Spd alone can be attributed to a 1.6-fold decrease in \( \text{RsSPDS3} \) expression and a small increase in \( \text{RsPAO4} \) expression compared with the Cu treatment alone. A 4.7-fold reduction in Spm in seedlings where EBR and Spd were co-applied and subjected to Cu stress was found to be the result of a 5.3-fold reduction in \( \text{RsSPDS3} \) expression and a significant increase in \( \text{RsPAO2} \) (4.5-fold) expression compared with the Cu stress alone (Fig. 2).

Additionally, an ~4.4- and 3.72-fold reduction in Spm was found in seedlings treated with either EBR or Spd alone, respectively, without being subjected to Cu stress. The reduced levels of Spm were associated with significant decreases in \( \text{RsSPDS3} \) by 2.7- and 2.1-fold, and increases in \( \text{RsPAO2} \) (2.6-fold) and
RsP4O4 (2.1-fold) expression in EBR and Spd individually treated seedlings, respectively, when compared with the untreated control. The significant reduction in Spm content (7.2-fold) when EBR and Spd were applied in the absence of Cu stress was linked to a 4.1-fold decrease in RsSPDS3 expression and enhancement in expression of RsP4O2 (1.9-fold) and RsP4O4 (2.6-fold) compared with the untreated control (Fig. 2).

The findings demonstrated that co-application of EBR and Spd could affect the endogenous PA pool in seedlings under Cu stress through the selective regulation of PA metabolic genes. Modulation of the endogenous PA profile by co-application of EBR and Spd could be one of the possible mechanisms involved in the alleviation of Cu stress in radish seedlings. EBR and Spd co-application was able to modulate expression of PAO genes more effectively under Cu stress than their individual use. Additionally, EBR and Spd regulation of PA metabolism was specifically induced in response to Cu stress as no significant alteration in PA metabolism except for Spm pools was recorded under control conditions.

EBR and Spd modulate IAA and ABA profiles under Cu stress

IAA is involved in many vital physiological processes. Two independent routes of IAA biosynthesis have been established (Mashiguchi et al., 2011). These routes are regulated by a cascade of genes, such as CYTOCHROME P450 79B3 (CYP79B3), YUCCA1 (YUC1), and YUC3, encoding enzymes responsible for the conversion of tryptophan to IAAox (indole-3-acetaldoxime) and subsequently to IAN (indole-3-acetonitrile), and then finally to IAA (Mashiguchi et al., 2011).

A 4.5- and 2.7-fold reduction in free and bound IAA content, respectively, observed in seedlings subjected to Cu stress was associated with reduced expression of RsCYP79B3 (2.5-fold) and RsYUC1 (5.6-fold) compared with the untreated control (Fig. 3A). Decreased IAA biosynthesis (free or bound) in seedlings under Cu stress might be associated, at least in part, with the higher expression of RsYUC3 (2.8-fold) compared with the untreated control. Application of EBR alone was able to improve free (3.7-fold) and bound levels of IAA (2.4-fold) in seedlings under Cu stress mainly by the up-regulation of RsCYP79B3 (3.4-fold) compared with Cu treatment only. No significant increase in free and bound IAA was observed when seedlings were treated with Spd alone and subjected to Cu stress compared with Cu stress alone (Fig. 3A). The most significant increases in free (3.7-fold) and bound levels of IAA (2.4-fold) in seedlings under Cu stress mainly by the up-regulation of RsCYP79B3 (3.4-fold) compared with Cu stress only. No significant increase in free and bound IAA was observed when seedlings were treated with Spd alone and subjected to Cu stress compared with Cu stress alone (Fig. 3A). The most significant increases in free (4.2-fold) and bound IAA (2.7-fold) were observed when EBR and Spd were co-applied with Cu stress. The elevated levels of IAA were associated with the up-regulation of RsCYP79B3 (10-fold) and RsYUC1 (16.3-fold), and reduced expression of RsYUC3 (2.8-fold) compared with Cu stress alone (Fig. 3A). Application of EBR and Spd without Cu stress had no significant impact on the levels of free and bound IAA compared with the untreated control. A 1.4- and 1.7-fold increase in free and bound IAA was observed in seedlings treated with a combined application of EBR and Spd without Cu stress was attributed to 2.1-, 2.3-, and 2.6-fold increases in RsCYP79B3, RsYUC1, and RsYUC3, respectively, compared with the untreated control (Fig. 3A). These data provide evidence that the co-activity of
EBR and Spd can impact Cu detoxification through the stimulation of IAA biosynthetic genes more effectively under Cu stress than their individual use. Additionally, EBR and Spd application alone or in combination without Cu stress also enhanced free and bound IAA pools and improved seedling growth compared with untreated controls.

ABA production is controlled by the action of a number of biosynthetic enzymes, such as those encoded by ABA DEFICIENT 3 (ABA3), 9-cis-epoxycarotenoid dioxygenase (NCED1), Arabidopsis aldehyde oxidase 3 (AOA3), and catalytic enzymes encoded by CYP707A3 genes. Environmental stimuli specifically induce the expression of these genes and therefore impact ABA metabolism. Enhanced levels of free (4.3-fold) and bound (4-fold) ABA were observed in Cu-stressed seedlings compared with the untreated control. The higher levels of ABA were coincident with the up-regulation of RsABA3 (11-fold) and elevated expression of RsCYP707A3 (5-fold) compared with the Cu treatment alone. About a 1.7-fold decrease in bound ABA and an insignificant decrease in free ABA were observed in seedlings treated only with Spd and subjected to Cu stress. These results were associated with a 2- and 2.6-fold decrease in RsABA3 and RsAAO3 expression, respectively, and a 6.6-fold enhancement in RsCYP707A3 expression compared with Cu stress alone. No significant reductions in free and bound ABA content were observed in seedlings treated with both EBR and Spd and subjected to Cu stress compared with Cu stress alone (Fig. 3B). Individual applications of EBR and Spd without Cu stress resulted in no significant increases in free and bound ABA contents or in the expression of ABA metabolic genes compared with the untreated control. However, significant increases in free (1.67-fold) and bound (2-fold) ABA occurred in seedlings when EBR and Spd were co-applied without Cu stress and were associated with enhanced expression of RsABA3 (1.9-fold), RsNCED (1.6-fold), and RsAAO3 (1.9-fold) compared with the untreated control (Fig. 3B). These data indicate that EBR and Spd may ameliorate Cu stress and help to maintain Cu homeostasis through the selective induction of ABA metabolism.

Improved antioxidant status by EBR and Spd efficiently counteracts Cu stress

Antioxidant compounds and enzymes play important roles in managing oxidative stress in plants. Significant enhancements in the levels of GSH (1.8-fold), ASA (1.9-fold), PL (1.6-fold), and GB (2.8-fold) were found in response to Cu stress, while a 2.3-fold reduction in TP content was detected under the same conditions. No significant increases in GSH and PL were observed in the response of seedlings to individual application of EBR or Spd and being subjected to Cu stress compared with Cu stress alone. A 2.1- and 2-fold increase in ASA, and a 3- and 3.5-fold increase in TP were observed in seedlings receiving either EBR or Spd alone and subjected to Cu stress compared with Cu stress alone. GB was enhanced by 1.8-fold in seedlings receiving an EBR application and subjected to Cu stress. Co-application of EBR and Spd to seedlings subjected to Cu stress was found to increase GSH (1.9-fold), ASA (3.2-fold), PL (1.6-fold), GB (2.4-fold), and TP (4.7-fold) considerably compared with Cu

![Fig. 3. Effect of EBR and/or Spd with or without Cu stress on (A) IAA (free and bound) content and the expression of genes involved in the metabolism of IAA (RsCYP79B3, RsYUC1, and RsYUC3) and (B) ABA (free and bound) content (µg g⁻¹ FW) and the expression of genes involved in metabolism of ABA (RsABA3, RsNCED, RsAAO3, and RsCYP707A3) in 7-day-old radish seedlings. Data presented are the mean ±SE. Different superscript letters (a, b, c, and d) indicate a significant difference from each other in all combinations (Tukey’s test, P < 0.05). Symbols ‘*’ and ‘§’ indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress (Tukey’s test, P < 0.05).](https://academic.oup.com/jxb/article-abstract/63/15/5659/659335)
stress alone (Table 2), EBR applied alone without Cu stress resulted in considerable increases in GSH (2.9-fold), ASA (3-fold), and PL (1.6-fold) levels compared with the untreated control. A 4.9-, 1.7-, and 2.1-fold increase in ASA, PL, and TP was observed in seedlings treated with Spd alone without Cu stress compared with the untreated control. The co-application of EBR and Spd without a Cu stress considerably increased GSH (2.3-fold), ASA (6.4-fold), PL (2.5-fold), GB (2.3-fold), and TP (3.8-fold) compared with the untreated control (Table 2). These data indicate that the combined action of EBR and Spd could mitigate Cu stress by enhancing the antioxidant system more significantly than their individual effects. Furthermore, EBR and Spd co-application could also improve overall seedling performance under normal growth conditions by enhancing the antioxidant system in radish.

Various assays performed to estimate the total antioxidant status of radish seedlings under Cu stress also yielded significant results. An ~3.8-, 3.5-, and 3.3-fold decrease in DPPH, deoxyribose, and FRAP were noted in response to Cu stress. EBR applied to seedlings subjected to Cu stress alone increased the levels of DPPH, deoxyribose, and FRAP by 2.3-, 2.6-, and 1.8-fold, respectively, compared with Cu stress alone (Table 2). Seedlings treated with Spd alone and subjected to Cu stress also showed enhanced levels of DPPH (2.3-fold), deoxyribose (1.8-fold), and FRAP (1.7-fold) compared with Cu stress alone. Significant increases in DPPH (2.9-fold), deoxyribose (3.3-fold), and FRAP (2.6-fold) were observed in seedlings receiving a co-application of EBR and Spd and subjected to Cu stress compared with Cu stress alone (Table 2). Individual applications of EBR and Spd without Cu stress showed significant increases in DPPH (3.1- and 4.2-fold), deoxyribose (2.2- and 2.6-fold), and FRAP (2.3- and 2.64-fold) compared with the untreated control. Moreover, co-application of EBR and Spd without Cu stress also enhanced the levels of DPPH (4.5-fold), deoxyribose (2.5-fold), and FRAP (2.7-fold) compared with the untreated control (Table 2). These results demonstrate that the total antioxidant status of radish seedlings was significantly enhanced after the co-application of EBR and Spd when subjected to Cu stress or under control conditions compared with the individual applications of these compounds.

Regarding the activity of antioxidant enzymes, Cu stress resulted in significant increases in the activity of CAT (3.2-fold),

Table 2. Effect of EBR and/or Spd with or without Cu stress on the endogenous level of antioxidants and total antioxidant potential in 7-day-old radish seedlings

Data presented are the mean ± SE. Different letters (a, b, c, and d) within a column indicate a significant difference from each other in all combinations (Tukey’s test, P < 0.05). Symbols '*' and '§' indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress, respectively (Tukey’s test, P < 0.05).

GSH, glutathione; ASA, ascorbic acid; PL, proline; GB, glycine betaine; TP, total phenol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; Deoxyribose, inhibitor of deoxyribose degradation; FRAP, ferric-reducing antioxidant power assay.

| Treatment          | Control | Cu+EBR | Cu+Spd | Cu+EBR+Spd | EBR | Spd | EBR+Spd |
|--------------------|---------|--------|--------|------------|-----|-----|--------|
| GSH (µmol g⁻¹ FW)  | 23.8±1.68 | 42.7±2.62 | 49.7±1.85 | 51.5±2.22 | 82.0±3.45 | 62.8±3.29 | 53.8±3.55 |
| ASA (µg g⁻¹ FW)    | 0.80±0.14 | 1.5±0.39 | 3.2±0.23 | 3.0±0.34 | 5.0±0.34 | 0.3±0.05 | 0.1±0.04 |
| PL (µmol g⁻¹ FW)   | 2.5±0.16 | 4.0±0.15 | 4.8±0.12 | 4.3±0.11 | 6.5±0.15 | 4.2±0.12 | 6.2±0.34 |
| GB (µmol g⁻¹ FW)   | 11.8±1.13 | 32.6±3.58 | 59.1±2.75 | 41.3±2.25 | 77.1±2.98 | 15.4±1.62 | 16.4±1.26 |
| TP (µg g⁻¹ FW)     | 22.8±3.15 | 9.8±1.73 | 29.0±2.78 | 34.0±3.05 | 46.2±3.23 | 36.6±3.76 | 28.3±3.56 |
| DPPH               | 48.3±3.1  | 12.8±2.5 | 30.1±5.1 | 29.6±5.3 | 36.6±3.76 | 41.6±3.76 | 24.8±3.76 |
| Deoxyribose        | 44.3±4.68 | 12.6±3.21 | 32.5±2.86 | 23.2±3.56 | 45.7±3.81 | 45.7±3.81 | 127.4±9.1 |
| FRAP               | 56.1±3.75 | 17.2±2.78 | 32.1±4.41 | 29.5±3.19 | 146.1±4.9 | 127.4±9.1 | 151.5±10.1 |

Table 3. Effect of EBR and/or Spd with or without Cu stress on the activities of antioxidant enzymes in 7-day-old radish seedlings

Data presented are the mean ± SE. Different letters (a, b, and c) within a column indicate a significant difference from each other in all combinations (Tukey’s test, P < 0.05). Symbols '*' and '§' indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress, respectively (Tukey’s test, P < 0.05).

CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GPOX, guiacol peroxidase; MDHAR, monodehydro ascorbate reductase; DHAR, dehydroascorbate reductase.

| Treatment          | Protein (mg g⁻¹ FW) | CAT (µmol min⁻¹ mg⁻¹ protein) | SOD (µmol U mg⁻¹ protein) | GR (µmol min⁻¹ mg⁻¹ protein) | GPOX (µmol min⁻¹ mg⁻¹ protein) | MDHAR (µmol min⁻¹ mg⁻¹ protein) | DHAR (µmol min⁻¹ mg⁻¹ protein) |
|--------------------|---------------------|-------------------------------|---------------------------|-----------------------------|-----------------------------|---------------------------------|-------------------------------|
| Control            | 61.5±4.54           | 0.3±0.05                      | 1.3±0.09                  | 1.2±0.12                    | 2.5±0.10                    | 0.1±0.05                        | 0.17±0.08                     |
| Cu                 | 31.8±3.03           | 0.9±0.05                      | 3.9±0.11                  | 5.4±0.19                    | 4.6±0.25                    | 0.19±0.02                       | 0.39±0.07                     |
| Cu+EBR            | 48.1±1.94           | 2.8±0.19                      | 6.0±0.21                  | 1.4±0.26                    | 1.6±0.19                    | 0.20±0.03                       | 0.23±0.02                     |
| Cu+Spd            | 42.1±1.62           | 0.5±0.14                      | 4.6±0.24                  | 2.5±0.20                    | 1.3±0.09                    | 0.14±0.02                       | 0.25±0.03                     |
| Cu+EBR+Spd        | 54.9±2.17           | 2.2±0.15                      | 7.0±0.12                  | 4.1±0.35                    | 1.1±0.22                    | 0.35±0.09                       | 0.60±0.03                     |
| EBR                | 85.0±5.14           | 0.4±0.05                      | 1.7±0.16                  | 1.9±0.12                    | 3.1±0.32                    | 0.13±0.07                       | 0.14±0.03                     |
| Spd                | 62.8±2.29           | 0.3±0.06                      | 1.9±0.10                  | 1.6±0.16                    | 3.1±0.17                    | 0.15±0.05                       | 0.21±0.05                     |
| EBR+Spd           | 69.8±1.47           | 0.7±0.20                      | 2.2±0.17                  | 1.5±0.17                    | 1.6±0.25                    | 0.18±0.04                       | 0.22±0.01                     |
SOD (3.1-fold), GR (4-fold), GPOX (1.8-fold), MDHAR (1.7-fold), and DHAR (2.2-fold) in radish seedlings compared with the untreated control (Table 3). Enhanced activities of CAT (3.1-fold) and SOD (1.5-fold) and significantly decreased activities of GR (3.7-fold) and GPOX (3.5-fold) were observed in seedlings treated with EBR and subjected to Cu stress compared with Cu stress alone. No significant changes in the activity of either CAT or GR were noted in seedlings treated with Spd alone and subjected to Cu stress compared with Cu stress alone. Simultaneous application of EBR and Spd to seedlings subjected to Cu stress significantly lowered GPOX activity (4.3-fold) and increased the activity of CAT (2.5-fold) compared with Cu stress alone (Table 3). No significant increases in SOD, GR, GPOX, MDHAR, and DHAR activities were found in seedlings treated with either EBR or Spd alone or in combination without Cu stress compared with the untreated control. However, co-application of EBR and Spd without Cu stress resulted in a 2.3-fold increase in CAT activity compared with the untreated control (Table 3). No significant increases in SOD, GR, GPOX, MDHAR, and DHAR activities were found in seedlings treated with either EBR or Spd alone or in combination without Cu stress compared with the untreated control. Collectively, these data indicate that EBR and Spd application alone or together increases the activity of the antioxidant system of radish, improving Cu stress tolerance and the overall antioxidant activity in seedlings under non-stress conditions as well.

Simultaneous action of EBR and Spd enhances PC biosynthesis to mitigate Cu stress

The synthesis of PC represents a major metal and metalloid detoxification mechanism in plants (Meyer et al., 2011). In plants, the biosynthesis of PC involves the action of a key enzyme, phytochelatin synthase (PCS), encoded by the PCS gene. Hence, the expression pattern of RsPCS could be considered as a good indicator for PC accumulation and increased capacity to detoxify Cu. The present data indicate that a significant increase in PC (2.6-fold) in response to Cu stress was associated with an increase in the expression of RsPCS by 8.4-fold compared with the untreated control (Fig. 4). No significant increase in PC content was observed when EBR or Spd was applied alone to radish seedlings subjected to Cu stress compared with Cu stress alone. The increase in the PC level (1.7-fold) of seedlings treated with both EBR and Spd together and subjected to Cu stress was associated with a 2.6-fold increase in RsPCS expression compared with Cu stress alone (Fig. 4). No significant increases in PC content and RsPCS expression were observed in seedlings treated with either EBR or Spd alone or in combination without Cu stress compared with the untreated control (Fig. 4).

EBR and Spd co-action alleviates stress indices under Cu stress

A significant increase (7.4-fold) in electrical conductivity (EC) in response to Cu stress was linked to an increase (2.9-fold) in malondialdehyde (MDA; a product of lipid peroxidation), indicating a higher degree of membrane damage compared with the untreated control. An ~3-fold reduction in EC in EBR-treated seedlings subjected to Cu stress was connected to a slight decrease in MDA content compared with Cu stress alone (Fig. 4). A 1.7-fold decrease in EC was observed in Spd-treated seedlings subjected to Cu stress and was linked to only a small reduction...
in MDA compared with Cu stress alone. A 5-fold decrease in EC was observed in seedlings receiving a co-application of EBR and Spd and subjected to Cu stress, and was associated with a 2.2-fold reduction in MDA content compared with Cu stress alone (Fig. 4). No significant changes in EC value or MDA were observed in seedlings treated with EBR or Spd alone or in combination without Cu stress compared with the untreated control (Fig. 4).

An elevated level of endogenous H$_2$O$_2$ is an indicator of the intensity of oxidative stress. Production of H$_2$O$_2$ at the membrane level upon exposure to Cu stress is coupled to the activity of the enzyme, NADPH oxidase. A significant increase (3.5-fold) in H$_2$O$_2$ was linked to a 2.9-fold increase in RsNADPH expression in response Cu stress compared with the untreated control (Fig. 4). Approximately 1.7- and 1.6-fold reductions in H$_2$O$_2$ were observed in seedlings treated with either EBR or Spd, respectively, and subjected to Cu stress, and were found to be associated with slight decreases in RsNADPH expression compared with the untreated control (Fig. 4).

A 3- and 2.4-fold reduction in chlorophyll (Chl) a and Chl b content, respectively, was observed in radish seedlings in response to Cu stress compared with the untreated control (Table 4). Seedlings treated with either EBR or Spd alone exhibited higher levels of Chl a (1.7-fold for both compounds) and Chl b (1.9- and 1.8-fold, respectively) in response to Cu stress. Seedlings treated with EBR and Spd together and subjected to Cu stress showed increased levels of Chl a (2.8-fold), Chl b (3.2-fold), and carotenoids (Cart, 2-fold) compared with Cu stress alone. Individual applications of EBR and Spd in the absence of Cu stress also enhanced levels of Chl a (1.5-fold), Chl b (2.1-fold), and Cart (1.73-fold) compared with the untreated control (Table 4). Another stress indicator, TSS, was decreased ~2.1-fold in response to Cu stress compared with the untreated control (Table 4). Individual application of either EBR or Spd did not significantly alter the TSS content in Cu-stressed seedlings compared with Cu stress alone. Seedlings treated with EBR and Spd and subjected to Cu stress exhibited an increase in TSS (1.9-fold) compared with Cu stress alone. Seedlings treated with either EBR or Spd alone without Cu stress showed no significant increase in TSS compared with the untreated control. Co-application of EBR and Spd in the absence of Cu stress increased the TSS content of seedlings by 1.6-fold compared with the untreated control (Table 4).

Combined action of EBR and Spd strongly inhibits localization of H$_2$O$_2$ and O$_2^\cdot$ ions and DNA damage induced by Cu stress

Confocal scanning microscopy was used to detect the generation of H$_2$O$_2$ in radish seedlings in response to Cu stress and to evaluate the ability of the co-application of EBR and Spd to inhibit H$_2$O$_2$ generation in Cu-stressed seedlings. A significant production of H$_2$O$_2$ was observed in the root tips of Cu-stressed seedlings as indicated by the strong green fluorescence (Fig. 5A) compared with the untreated control. Application of either EBR or Spd reduced H$_2$O$_2$ production in response to Cu stress as evidenced by the weaker green fluorescence. A more remarkable decline in H$_2$O$_2$ generation was noted in root tips of Cu-stressed seedlings when EBR and Spd were applied together compared with Cu stress alone (Fig. 5A). In addition, both DAB and NBT staining of the cotyledons of radish seedlings subjected to Cu stress revealed the inhibitory influence of EBR or Spd applied alone on the production of H$_2$O$_2$ and O$_2^\cdot$ ions compared with Cu stress alone (Fig. 5B, 5C). A stronger inhibition of H$_2$O$_2$ and O$_2^\cdot$ production in seedlings exposed to Cu stress was noted when EBR and Spd were applied together compared with Cu stress alone. Application of EBR alone in the absence of Cu stress was observed to enhance H$_2$O$_2$ (Fig. 5A, 5B) and O$_2^\cdot$ (Fig. 5C)

### Table 4. Effect of EBR and/or Spd with or without Cu stress on photosynthetic pigments and soluble sugars in 7-day-old radish seedlings

|          | Chl a (µg g$^{-1}$ FW) | Chl b (µg g$^{-1}$ FW) | Cart (µg g$^{-1}$ FW) | TSS (µg g$^{-1}$ FW) |
|----------|------------------------|------------------------|-----------------------|----------------------|
| Control  | 62.8 ± 3.82 a          | 35.5 ± 2.06 a          | 36.4 ± 2.35 a         | 75.6 ± 2.97 a        |
| Cu       | 20.9 ± 0.98 b          | 15.0 ± 1.75 b          | 28.1 ± 3.91 b         | 35.9 ± 2.53 b        |
| Cu+EBR   | 36.4 ± 2.32 c          | 29.0 ± 1.03 a          | 45.6 ± 1.34 b         | 54.7 ± 2.97 b        |
| Cu+Spd   | 35.1 ± 2.12 c          | 26.6 ± 1.92 a          | 37.6 ± 2.65 b         | 41.8 ± 2.64 b        |
| Cu+EBR+Spd | 57.8 ± 3.28 a$^a$   | 47.7 ± 3.23 c$^a$     | 59.9 ± 3.21 c$^a$     | 69.1 ± 1.62 a$^a$    |
| EBR      | 59.1 ± 1.14 a          | 54.9 ± 1.52 c          | 67.1 ± 1.26 c         | 96.0 ± 1.48 a        |
| Spd      | 68.4 ± 2.89 a          | 57.6 ± 4.35 c          | 54.1 ± 3.55 b         | 80.6 ± 2.81 a        |
| EBR+Spd  | 93.3 ± 5.13 d          | 75.1 ± 3.41 d$^a$     | 63.1 ± 3.21 b         | 118.7 ± 3.52 c$^a$   |

Data presented are the mean ± SE. Different letters (a, b, c, and d) within a column indicate a significant difference from each other in all combinations (Tukey’s test, P < 0.05). Symbols ‘$^a$’ and ‘$^c$’ indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress, respectively (Tukey’s test, P < 0.05).

Chl a, chlorophyll a; Chl b, chlorophyll b; Cart, carotenoids; TSS, total soluble sugars.
production slightly compared with the untreated control. Spd application alone or in combination with EBR in the absence of Cu stress did not increase the generation of H$_2$O$_2$ (Fig. 5A, 5B) and O$_2^-$ (Fig. 5C) compared with the untreated control.

A comet assay was employed to examine the ability of EBR and Spd, applied individually or together, to protect DNA from Cu stress-induced damage. Excessive production of ROS, resulting from Cu stress, results in the degradation of DNA in the head of the comet and thus a concomitant gain in the tail DNA, which ultimately increases the tail moment of the comet. In the present investigation, a 1.2-fold reduction in comet head DNA was coupled with a 4.4-fold increase in tail DNA and a 7.3-fold increase in the tail moment, indicating a high level of DNA damage in response to Cu stress compared with the untreated control. When either EBR or Spd was applied alone to seedlings subjected to Cu stress no visible restoration of the comet head DNA and no reduction in comet tail DNA or tail moments was observed compared with Cu stress alone (Fig. 6A, 6B). However, co-application of EBR and Spd to seedlings subjected to Cu stress revealed a 1.1-fold gain in comet head DNA and significant reductions in both the comet tail DNA (2.3-fold) and tail moment (2.6-fold) compared with Cu stress alone. These results indicate a 71% inhibition of DNA damage compared with Cu stress alone. No significant changes in comet head DNA, comet tail DNA, or tail moment were observed in seedlings treated with EBR or Spd singly or in combination in the absence of Cu stress compared with the untreated control (Fig. 6A, 6B). These results together indicate that the co-action of EBR and Spd together offers greater protection against Cu stress-induced ROS damage of DNA compared with individual action of either component alone.

Discussion

The system in plants responsible for maintaining Cu homeostasis ensures the proper delivery of Cu to essential Cu-containing proteins while avoiding cytotoxicity when Cu is present in excess. Previous studies have shown that COPTs, CCHs, HMAs, and antioxidants play central roles in acquisition, distribution, assimilation, and detoxification of Cu in plants (Andres-Colas et al., 2010; Beauclair et al., 2010; Yuan et al., 2011). Despite this fact, very little information is available about the specific effects of plant growth regulators, including BRs and PAs, on the Cu homeostasis system in plants. This study has provided insight into how EBR and Spd regulate Cu homeostasis in the presence of excess Cu. Various methods for detoxifying excess Cu that are induced by the application of EBR and Spd together or individually were also elucidated.

The inhibitory effects of Cu stress on seedling growth were primarily associated with reduced Chl biosynthesis and altered hormonal levels. Although individual applications of EBR and Spd were able to improve growth of seedlings subjected to Cu stress, co-application of these compounds was the most effective in restoring growth to nearly that of unstressed seedlings (Table 1; Supplementary Fig. S1 at JXB online). The growth-promoting effects of BRs on seedlings under Cu stress may be linked to the general ability of BRs to promote cell elongation and cell cycle progression (Guo et al., 2009; Zhang et al., 2009; Gonzalez-Garcia et al., 2011) as well as the stimulation of genes encoding xyloglucanses and expansins (Gudesblat and Russinova, 2011). The role of Spd in plant growth and development has been widely documented under both normal and stress conditions (Wu et al., 2010; Hussain et al., 2011). Additional improvement in seedling growth with the co-application of EBR and Spd in the absence of Cu stress compared with the untreated control further indicates the coordinated interaction between BRs and PAs in plant growth.

Yeast has been widely used to study metal stress tolerance due to the availability of various mutants (Mu et al., 2011). Assays were performed utilizing yeast mutants (Supplementary Results at JXB online) to examine further the effects of EBR and/or Spd on Cu tolerance at the molecular level. Results demonstrated...
that co-application of EBR and Spd could enhance Cu stress tolerance in wild-type, $\Delta cup1$, and $\Delta sod1$ (Cu-sensitive) strains of yeast more effectively than their individual application (Supplementary Fig. S2A, B). Improved Cu stress tolerance was the result of the significant influence of EBR and Spd on the expression pattern of genes implicated in Cu uptake ($ScCTR1$, $ScCTR3$, and $ScCUP2$), assimilation ($ScCCC2$ and $ScSOD1$), and detoxification ($ScBSD2$) in wild-type, $\Delta cup1$, and $\Delta sod1$ strains (Supplementary Fig. S2B). These results indicate that EBR and/or Spd use a comprehensive mechanism to achieve Cu homeostasis in the presence of toxic levels of Cu (Supplementary Results).

Results of the present study demonstrated that excessive uptake of Cu ions by radish seedlings may be responsible for their poor growth and altered metabolism. EBR and Spd applied alone or in combination were able to reduce Cu uptake significantly, thereby improving seedling growth (Fig. 1A). These findings are consistent with a previous report (Bajguz, 2010) which showed that application of exogenous BRs could enhance the antioxidant system and growth of Chlorella vulgaris through a reduction in the uptake of Cu, Pb, and Cd. The expression profiles of $RsCOPT1$, $RsCOPT2$, and $RsHMA5$ and other genes involved in Cu detoxification ($RsPCS$ and $RsMT1C$) and assimilation ($RsCCHI1$) were examined in order to explore the factors responsible for reducing Cu uptake in radish (Fig. 1A, 1B). The results indicated that Cu stress caused differential induction of $RsCOPT$ genes in radish seedlings. The expression of $RsCOPT1$ and $RsCOPT2$ was increased many fold in whole seedlings subjected to Cu stress (Fig. 1A). The enhanced expression of $RsCOPT1$ and $RsCOPT2$ resulted in increased Cu uptake and Cu transportation from the root tip to the whole seedling, inducing high levels of oxidative stress by the generation of large amounts of H$_2$O$_2$ and O$_2^–$ (Fig. 5A–C) which led to poor growth. The co-application of EBR and Spd was the most effective in suppressing the Cu stress-induced expression of $RsCOPT1$ and $RsCOPT2$ in radish seedlings. These results are supported by the evidence that down-regulation of $AtCOPT1$ and $AtCOPT3$ resulted in a reduction in Cu uptake. $AtCOPT1$ is specific for Cu uptake at the root tip and its down-regulation resulted in reduced Cu uptake in Arabidopsis, whereas reduced expression of $AtCOPT3$ negatively affected the assimilation and distribution of Cu (Andres-Colas et al., 2010). Additionally, insignificant changes in the expression of $RsCOPT1$ and $RsCOPT2$ in seedlings treated with EBR and Spd either alone or in combination in the absence of Cu stress compared with the untreated control indicated that the down-regulation of $COPT$ genes by EBR and/or Spd in seedlings subjected to Cu stress was associated with reduced Cu levels (Fig. 1A).

HMA5 is a critical component responsible for metal detoxification in plant cells (Andres-Colas et al., 2006). High expression

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Fig. 6. Effect of EBR and/or Spd with or without Cu stress on comet tail length (A) and (B) percentage comet head and comet tail DNA and tail moment of 7-day-old radish seedling cell DNA. Data are presented as the mean ±SE. Different superscript letters (a, b, and c) within a column indicate a significant difference from each other in all combinations (Tukey’s test, $P < 0.05$). Symbols ‘*’ and ‘§’ indicate a significant difference for EBR+Spd versus EBR and EBR+Spd versus Spd under Cu stress, respectively (Tukey’s test, $P < 0.05$).

Control (negative, –ve) without Cu stress was used to compare DNA damage under normal conditions in 7-day-old radish seedlings. Control (positive, +ve) with Cu stress alone was used to calculate DNA damage in 7-day-old radish seedlings under Cu stress compared with the untreated control. (This figure is available in colour at JXB online.)
levels of RsHMA5 have been associated with efficient metal conjugation and transmembrane transport of Cu in plants (Andres-Colas et al., 2006). Enhanced expression of RsHMA5 was observed in seedlings in response to Cu stress. The most significant repression of RsHMA5 occurred in Cu-stressed seedlings with the application of EBR. The co-application of EBR and Spd also increased RsHMA5 expression, although the repressing effect was more evident with EBR applied alone (Fig. 1B). These observations indicated that EBR applied alone reduced the expression of RsHMA5 most significantly, resulting in less intracellular transport of Cu and thereby improved Cu stress management. As for the expression of RsMT1C and RsCCH1, only the expression of RsCCH1 was found to be enhanced several fold in Cu-stressed seedlings treated with EBR and Spd compared with Cu stress alone (Fig. 1B). More succinctly, co-application of EBR and Spd achieved more effectively maintained Cu homeostasis through selective modulation of genes involved in Cu uptake (RsCOP71 and RsCOP72), distribution (RsHMA5), and assimilation (RsCCH1) than their individual use. Furthermore, elevated expression of RsCCH1, noted when EBR and Spd were applied either individually or together in the absence of Cu stress, suggests that induction of RsCCH1 expression is dependent not only on the presence of Cu excess, but also on the presence of EBR and/or Spd. Reduction of Cu uptake by EBR and Spd treatments, which is associated with the down-regulation of RsCOP71, RsCOP72, and RsHMA5 and the up-regulation of RsCCH1, could be one of the mechanisms contributing to improved Cu detoxification when cytotoxic levels of Cu are present.

Cu stress significantly increased PA content in radish seedlings (Fig. 2), which is in agreement with observations of Cu-stressed wheat leaves (Groppa et al., 2007). Increased PA content was also previously reported in Cu-stressed radish seedlings (Choudhary et al., 2012a). These data suggest that elevated PA levels may play a positive role in the protection of plants against heavy metals. The present study demonstrated the combined effect of EBR and Spd in maintaining the appropriate PA pool in radish seedlings under Cu stress. EBR and Spd co-application influenced the expression of genes involved in PA biosynthesis and catabolism. Among the three PAs examined, co-application of EBR and Spd increased the Put content while reducing the levels of Spd and Spm in radish seedlings under Cu stress. The data demonstrated that EBR and Spd co-application could modulate the expression of RsADC1, RsADC2, and RsSAMDC genes to enhance the Put pool in seedlings subjected to Cu stress. EBR and Spd co-application, however, had stronger repressing effects on RsSPDS3 than their individual applications. Therefore, the combined effect of EBR and Spd reduced Spm pools in radish seedlings subjected to Cu stress more significantly than their individual effect (Fig. 2). Interestingly, under these conditions, the endogenous PA pool was found to be tightly regulated by altered expression of RsPAO2 and RsPAO4 encoding PA catabolic enzymes (Fig. 2). These findings are supported by a previous report demonstrating that co-application of EBR and Spd could up- or down-regulate the expression of RsADC and RsSPDS, respectively, to maintain the PA pool under Cr stress in radish seedlings (Choudhary et al., 2012a). Consistent with the present results, exogenous Spd was shown to regulate PA metabolism in Alternanthera philoxeroides by inhibiting the activities of ADC, ornithine decarboxylase, and PAOs, resulting in a decreased level of Put and enhanced levels of Spd and Spm, thus improving Cu stress tolerance (Xu et al., 2011). The present data further indicate that the induction of PA metabolic genes in seedlings under Cu stress is very specific to the co-application of EBR and Spd and is not achieved by their individual application. Additionally, it was observed that EBR and Spd application, either alone or in combination, in the absence of Cu stress could modulate the PA profile by affecting the expression of PA metabolic genes. These results suggest that EBR- and/or Spd-induced changes in PA profiles were not entirely dependent on Cu stress.

The effect of IAA on sunflower growth and heavy metal uptake in combination with ethylene diamine disuccinic acid has been previously reported (Fassler et al., 2010). More recently, the ability of auxins to increase Cd stress tolerance in Arabidopsis has been documented (Elobeid and Polle, 2012). In the present investigation, application of EBR and/or Spd, alone or together, improved seedling growth of radish subjected to Cu stress which could be partially attributed to enhanced synthesis of IAA (Fig. 3A). Up-regulation of RsCYP79B3 and RsYUC1 by EBR and Spd also demonstrated the combined effects of these compounds in overcoming Cu-induced oxidative stress by effectively maintaining the endogenous auxin level (Fig. 3A). Additionally, the enhanced expression of IAA metabolic genes along with a significant increase in IAA content, when seedlings were treated with a co-application of EBR and Spd in the absence of Cu stress, may, at least in part, contribute to the improved radish seedling growth compared with the unstressed controls (Fig. 3A, Table 1). The increase in ABA in response to Cu stress was associated with the up-regulation of RsABA3, RsNCED, and RsAAO3 (Fig. 3B). This result is consistent with a previous report which showed that BR application enhanced tolerance to Cu, Pb, and Cd stress by increasing endogenous levels of ABA, IAA, and zeatin in Chlorella vulgaris (Bagjuz, 2010). Since excessive production of ABA may signal the initiation of programmed cell death in plants, maintenance of its endogenous pool is crucial for the normal functioning of several physiological processes, such as the opening and closing of stomata, seed germination, and embryogenesis (Kim et al., 2010). The current study shows that co-application of EBR and Spd can regulate ABA metabolism through selective modulation of RsABA3, RsNCED, and RsAAO3 gene expression, and significant up-regulation of RsCYP707A3 (Fig. 3B). The net outcome of this selective up- and down-regulation of ABA metabolic genes is the maintenance of an ABA pool that contributes to Cu stress tolerance without impacting growth. Recently, co-application of EBR and Spd has been shown to regulate ABA levels in radish seedlings under Cr stress (Choudhary et al., 2012a). EBR and Spd also induce MTs indirectly in plants by stimulating an increase in ABA which results in an increase in MTs (Usha et al., 2009; Xue-Xuan et al., 2010). Importantly, co-application of EBR and Spd in the absence of Cu stress also increased ABA by the selective up-regulation of several ABA biosynthetic genes. These data provide evidence that the ABA content may be altered not only by Cu stress but also by the co-application of EBR and Spd (Fig. 3B), a result that indicates the existence of cross-talk among ABA, BRs, and PAs.

Antioxidant enzymes and compounds are the backbone of the oxidative stress response in plants. Elevated levels of antioxidants
upon application of hormones result in better oxidative stress management in plants (Andre et al., 2010; Thao and Tran, 2011). Data from the current experiments on radish revealed significant increases in antioxidants (GSH, PL, ASA, and TP), and altered activities of antioxidant enzymes (SOD, CAT, GPOX, GR, and APOX) when seedlings were treated with EBR and/or Spd and subjected to Cu stress (Tables 2, 3). These results are in agreement with a previous study that showed enhanced levels of GSH, ASA, PL, and TP in radish seedlings treated with EBR and/or Spd and subjected to Cr stress (Choudhary et al., 2012a).

Additionally, the current study demonstrated that the improvement in the total antioxidant status of radish seedlings under Cu stress was optimum when EBR and Spd were co-applied rather than with their individual use (Table 2).

Among the mechanisms that have evolved for metal detoxification in plants, the key role of PC has been widely accepted (Vurro et al., 2011). PC detoxifies heavy metals through conjugation and complex formation (Vurro et al., 2011). No significant increases in PC content or the expression level of RsPCS were observed in untreated seedlings and/or seedlings treated with EBR and/or Spd in the absence of Cu stress. However, a significant increase in PC was observed in response to Cu stress and a slightly greater PC increase was observed when seedlings were treated with a co-application of EBR and Spd and subjected to Cu stress. These results together indicate that PC induction is mainly dependent on cytotoxic levels of Cu and that the enhanced PC level induced by the co-application of EBR and Spd to seedlings subjected to Cu stress may contribute, at least in part, to Cu detoxification (Fig. 4). Significant up-regulation of RsPCS in seedlings treated with a co-application of EBR and Spd was determined to be responsible for the increase in PC in response to Cu stress (Fig. 4). These findings agree with previous observations reported by Vurro et al. (2011), indicating that PC could govern Zn and Cu homeostasis and Cd detoxification in Daucus carota parasitized by Cuscuta campestris. The authors further suggested that enhanced expression of PCS in response to Cd stress was responsible for the elevated PC level (Vurro et al., 2011).

ROS generated in response to Cu stress damage cell membranes by lipid peroxidation (Choudhary et al., 2010). The severalfold increase in ion leakage in response to Cu stress observed in the current study was linked to a significant increase in MDA (Fig. 4). Whereas a small decrease in MDA was observed in Cu-stressed radish seedlings treated with EBR and/or Spd, a significant decrease in ion leakage was also observed compared with seedlings exposed to Cu stress alone. These data are in agreement with the results of a recent report which demonstrated that EBR and Spd co-application could lower MDA levels and ion leakage in Cr-stressed radish seedlings more effectively than their independent use (Choudhary et al., 2012a). H$_2$O$_2$ is a potent ROS, generated in response to abiotic stress, such as Cu excess. The reduced production of H$_2$O$_2$ observed in Cu-stressed seedlings treated with EBR and/or Spd was potentially attributed to the down-regulation of RsNADPH (Fig. 4). The inhibitory effects of an excess of Cu on the synthesis of Chl $a$, Chl $b$, and Cart content are widely known (Padua et al., 2010). A significant reduction in Chl $a$, Chl $b$, and Cart levels was observed in response to Cu stress (Table 4). However, EBR and Spd together were able to mitigate the reduction of Chl $a$, Chl $b$, and Cart levels in Cu-stressed radish seedlings to nearly that of untreated controls. Moreover, application of EBR and Spd, either alone or in combination, in the absence of Cu stress was also shown to alter the levels of Chl $a$, Chl $b$, Cart, and TSS compared with the untreated control. These data indicate that EBR and Spd exert an influence on the levels of both of these pigments, as well as TSS, of seedlings under non-stressed or Cu-stressed conditions (Table 4). Anuradha and Rao (2009) also reported that EBR application protects the photosynthetic pigments of radish plants subjected to Cd stress. Similarly, Spd application was also observed to improve photosynthesis in Salvia natans exposed to Cd stress (Xu et al., 2008).

Confocal analysis of radish root tips, as well DAB and NBT staining of radish cotyledons, showed that the combination of EBR and Spd could more effectively inhibit H$_2$O$_2$ and O$_2$ generation in response to Cu stress compared with their individual use and when compared with Cu stress alone (Fig. 5A–C). Applications of EBR and Spd either alone or together slightly increased the production of H$_2$O$_2$ and O$_2$ under non-stress conditions compared with the untreated control (Fig. 5A–C). These results are supported by a previous report indicating that EBR could reduce the production of H$_2$O$_2$ in Cucumis sativus subjected to paraquat (Xia et al., 2009). Moreover, expression analysis in the current study indicated that the decline in H$_2$O$_2$ production resulting from the co-application of EBR and Spd was associated with the significant repression of RsNADPH in seedlings under Cu stress (Fig. 4). Additionally, as determined by the comet assay, co-application of EBR and Spd was more effective than either compound alone at limiting DNA damage resulting from Cu stress (Fig. 6A, 6B). The ability of castasterone (a type of BR) isolated from leaves of Centella asiatica to protect DNA from H$_2$O$_2$ has been previously reported (Sondhi et al., 2010).

Based on the results obtained in the current study, it can be suggested that Cu homeostasis in radish is brought about by the combined activity of EBR and Spd. These compounds act together in an additive or synergistic manner to impact the various regulatory mechanisms that have evolved to deal with cytotoxic levels of heavy metals such as Cu.

Supplementary data
Supplementary data are available at JXB online

Supplementary methods
Yeast Cu stress tolerance assay.
Estimation of endogenous IAA and ABA contents.
Estimation of endogenous PA contents.

Supplementary results
EBR and Spd applications enhance Cu stress tolerance in Cu-sensitive yeast strains.

Figure S1. Effect of EBR and/or Spd with or without Cu stress on growth of radish seedlings.
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Figure S2. Effect of EBR and/or Spd with or without Cu stress on the (A) growth and (B) expression of genes associated with Cu uptake (ScCCTR3 and ScCTR1), Cu assimilation (ScSOD1), and Cu homeostasis (ScBSD2, ScCC2, and ScCUP2) in wild-type, Δcup1, and Δsod1 strains of Saccharomyces cerevisiae grown on agar plates supplemented with Cu solution. Data presented are the mean ±SE.

Table S1. List of yeast strains used to test the effect of EBR and Spd on the survival of yeast subjected to Cu stress.

Table S2. List of gene-specific primers designed to amplify genes associated with Cu homeostasis and Cu detoxification factors in yeast. DNA coding sequences (CDS) were obtained at www.yeastgenome.org.

Table S3. List of gene-specific primers designed to amplify radish genes associated with PA, IAA, and ABA metabolism, Cu homeostasis, and Cu detoxification. ESTs were obtained from a databank available at www.plantgdb.org.

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