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Brassinosteroid-induced CO_2 assimilation is associated with increased stability of redox-sensitive photosynthetic enzymes in the chloroplasts in cucumber plants

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ABSTRACT

Brassinosteroids (BRs) play important roles in plant growth, development, photosynthesis and stress tolerance; however, the mechanism underlying BR-enhanced photosynthesis is currently unclear. Here, we provide evidence that an increase in the BR level increased the quantum yield of PSII, activities of Rubisco activase (RCA) and fructose-1,6-bisphosphatase (FBPase), and CO_2 assimilation. BRs upregulated the transcript levels of genes and activity of enzymes involved in the ascorbate–glutathione cycle in the chloroplasts, leading to an increased ratio of reduced (GSH) to oxidized (GSSG) glutathione in the chloroplasts. An increased GSH/GSSG ratio protected RCA from proteolytic digestion and increased the stability of redox-sensitive enzymes in the chloroplasts. These results strongly suggest that BRs are capable of regulating the glutathione redox state in the chloroplasts through the activation of the ascorbate–glutathione cycle. The resulting increase in the chloroplast thiol reduction state promotes CO_2 assimilation, at least in part, by enhancing the stability and activity of redox-sensitive photosynthetic enzymes through post-translational modifications.

1. Introduction

Brassinosteroids (BRs) are a group of plant steroidal hormones that regulate a variety of plant growth and developmental processes [1]. A deficiency in BR biosynthesis or signaling results in extreme dwarf phenotypes, whereas the overexpression of the rate-limiting BR biosynthetic genes or the exogenous application of BRs increases both the yield and quality of crop plants [2,3]. Several groups, including ours, have recently reported that BRs enhance photosynthesis, which could provide an important mechanism for the observed beneficial effects of BRs on crop yields [3,4]. However, the mechanism by which BRs enhance photosynthesis is currently unclear.

A BR-induced increased CO_2 assimilation was associated with an increased content and activity of Rubisco activase (RCA) under the optimum growth conditions [5]. A number of enzymes, such as Rubisco, RCA, fructose-1,6-bisphosphatase (FBPase), and sedoheptulose-1,7-bisphosphatase (SBPase), involved in photosynthesis are subjected to redox regulation through the cleavage or formation of intramolecular disulfide bonds [6,7]. BRs trigger a rapid H_2O_2 accumulation at the apoplast and induce a nitric oxide (NO)-dependent increase in the activity of antioxidant enzymes in the leaf tissues of cucumber plants [8,9]. With regard to BR-induced photosynthesis, however, it is unclear whether BRs can exert their influence on the ascorbate–glutathione cycle, an important player in regulating the redox homeostasis of the chloroplasts. If this is the case, it should be resolved whether the BR-altered chloroplast redox homeostasis can lead to changes in the activity and stability of redox-sensitive photosynthetic enzymes involved in CO_2 assimilation. Here, we report that a BR-induced increase in the activity of the ascorbate–glutathione cycle was associated with an elevated glutathione redox state in the chloroplasts, contributing to the stability of redox-sensitive enzymes, such as RCA and FBPase, and, consequently, increased CO_2 assimilation.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; BRs, brassinosteroids; Brz, brassinazole; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EBR, 24-epibrassinolide; FBPase, fructose-1,6-bisphosphatase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDAR, monodehydroascorbate reductase; Φ_{PSII}, quantum efficiency of photosystem II; RCA, Rubisco activase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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2. Materials and methods

2.1. Plant materials and treatments

Cucumber (*Cucumis sativus* L. cv. Jinchun No. 3) plants were grown in growth chambers at a temperature of 25/17 °C (day/night), photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1}, and photoperiod of 12 h. Plants at the four-leaf stage were sprayed with water, 0.1 μM 24-epibrassinolide (EBR, Sigma Co., USA), 4 μM brassinazole (Brz, an inhibitor of BR biosynthesis) or 4 μM Brz plus 0.1 μM EBR. At one day after the treatments, CO_2 assimilation was determined in the 3rd leaf from the bottom. Leaf samples were harvested, frozen immediately in liquid nitrogen and stored at −80 °C before the biochemical and gene expression analyses.

2.2. Gas exchange and chlorophyll fluorescence measurements

The leaf gas exchange measurements were coupled with measurement of chlorophyll fluorescence using an open gas exchange system with an integrated fluorescence chamber head (LI-6400; LI-COR, Inc., NE, USA). Unless otherwise stated, gas exchange and chlorophyll fluorescence parameters were measured under the growth conditions at 25 °C, 80% relative humidity, and 1–1.3 kPa leaf-to-air vapor pressure deficit. The CO_2 assimilation versus intercellular CO_2 concentration (A/Ci) curves were measured at a PPFD of 600 μmol m^{-2} s^{-1}, whereas the CO_2 assimilation versus incident PPFD (A/PPFD) analyses were conducted at an ambient CO_2 concentration of 400 μmol mol^{-1}. The quantum efficiency of photosystem II (Φ_{PSII}) was measured simultaneously and calculated as described by Genty et al. [10].

2.3. Chloroplast isolation and assay for enzymatic and non-enzymatic antioxidants in the ascorbate–glutathione cycle

The chloroplasts were isolated from cucumber leaves using discontinuous Percoll density gradient methods, as previously described by Gruissem et al. [11]. The intactness of the purified chloroplasts was validated by measuring the O_2 production after the addition of potassium hexacyanoferrate [12]; the purified chloroplasts had intactness rates between 90.8% and 96.1%. The ascorbate peroxidase (APX, EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) activities were measured by the decrease in the absorbance at 290 nm and increase in the absorbance at 265 nm, according to Nakano and Asada [13]. The monodehydroascorbate reductase (MDAR, EC 1.6.5.4) activity was measured using 1 U of ascorbate oxidase, and the oxidation rate of NADH was followed at 340 nm [14]. The glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Foyer and Halliwell [15], as based on the rate of decrease in the absorbance of NADPH at 340 nm. The extraction and assay of reduced (AsA) and oxidized (DHA) ascorbate and reduced (GSH) and oxidized (GSSG) glutathione were performed as described by Rao and Ormrod [16].

2.4. Assay of FBPase and RCA activities

The fructose-1, 6-bisphosphatase (FBPase) activity was determined by monitoring the increase in A_{340} using an extinction coefficient of 6.2 mM^{-1} cm^{-1} [17]. The RCA activity was determined using a Rubisco Activase Assay Kit (Genmed Scientifics Inc., USA). To study the effects of the glutathione redox homeostasis on the RCA activity, RCA was purified from cucumber leaves at an approximate concentration of 0.2 mg mL^{-1}. This enzyme was incubated in an activation buffer with a mixture of GSH and GSSG at GSH/GSSG ratios of 10, 20, 30, 40, 50 and 100 for 2 h at 30 °C under an N_2 atmosphere in a vacuum oven [18]; incubation without the purified RCA served as the negative control.

2.5. RCA proteolytic assay

For the RCA proteolytic assay, purified RCA at 0.2 mg mL^{-1} in activation buffer was incubated with GSH, GSSG or the GSH + GSSG mixture as described above. Excess GSSG or GSH was eliminated by desalting using a Sephadex G-25 column equilibrated with the activation buffer. The proteolytic assays were conducted immediately. For the subtilisin and proteinase K digestion, 80 μL samples of RCA solution were mixed with 20 μL of the protease (2.5 μg mL^{-1}) in activation buffer [10 mM BTP, 10 mM DTT, and 0.2 mM ATP (pH 7.0)]. The mixtures were incubated in a water bath at 30 °C for different times, and the reaction was then stopped by the addition of 10 μL of 22 mM phenylmethylsulfonyl fluoride (PMSF) in 2-propanol and transferred to ice for 10 min. For SDS-PAGE, 55 μL of 3× SDS loading buffer [0.188 M Tris–HCl, 0.6 M 2-mercaptoethanol, 6% (w/v) SDS, 30% (v/v) glycerol, and 0.075% (w/v) bromophenol blue, pH 6.8] was added, and the mixture was boiled for 5 min. For native PAGE, 55 μL of 3× nondenaturing loading buffer [0.2 M Tris–HCl, 0.6 M 2-mercaptoethanol, 25% (v/v) glycerol, and 0.075% (w/v) bromophenol blue, pH 8.0] was mixed with the samples without boiling [18,19].

2.6. Total RNA extraction and gene expression analysis

The total RNA was extracted using Trizol according to the supplier's instructions. Any residual DNA was removed using a purification column. The total RNA (1 μg) was reverse-transcribed using 0.5 mg oligo (dT) 12–18 (Invitrogen, Carlsbad, CA, USA) and 200 units of Superscript II (Invitrogen) following the manufacturer's instructions. The gene-specific primers were designed based on the EST sequences for the corresponding genes in the chloroplast: *APX* – forward, 5′-ATGGGAAAGTGCTACCCTGTT-3′, and reverse, 5′-ACAATGTCCTGGTCCGAAAG-3′; *MDAR* – forward, 5′-CTCC TTATGAGCGTCCAG-3′, and reverse, 5′-GTGAAGCCTACAGCGACT-3′; *DHAR* – forward, 5′-GTGGCTCTTGGACACTTCAA-3′, and reverse, 5′-ATTCTGGAACGACTCTCGCT-3′; *GR* – forward, 5′-CTAAGCGTGTT GTGGTGCTT-3′, and reverse, 5′-ACTTTGGCACCCATACCATT-3′; and *actin* – forward, 5′-AAAGATGACGCAGATAAT-3′, and reverse, 5′-GAGAGATG GCTGGAATAG-3′. The quantitative real-time PCR was performed using the iCycler iQTM real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and the SYBR Green PCR Master Mix (Takara, Japan). The relative quantification of the mRNA levels is based on the method of Livak and Schmittgen [20].

3. Results and discussion

3.1. BR levels are related to CO_2 assimilation and PSII electron transport

To examine how BRs regulate the photosynthetic capacity, we sought to alter the cellular BR levels by the application of exogenous BR (EBR) and the BRs biosynthesis inhibitor (Brz) and to determine their effects on CO_2 assimilation and the quantum yield of PSII (Φ_{PSII}). The CO_2 assimilation rate and Φ_{PSII} were significantly decreased by Brz but increased by EBR, and the decrease of the CO_2 assimilation rate and Φ_{PSII} by Brz was restored by the subsequent application of EBR at all of the CO_2 concentrations and PPFDs applied (Fig. 1). The increase in the quantum yield of PSII electron transport in the EBR-treated leaves reflects the increased demand in the Calvin cycle for ATP and NADPH [4]. As the exogenous application of BRs can improve CO_2 assimilation under normal growth conditions, it appears that the endogenous BR levels are not

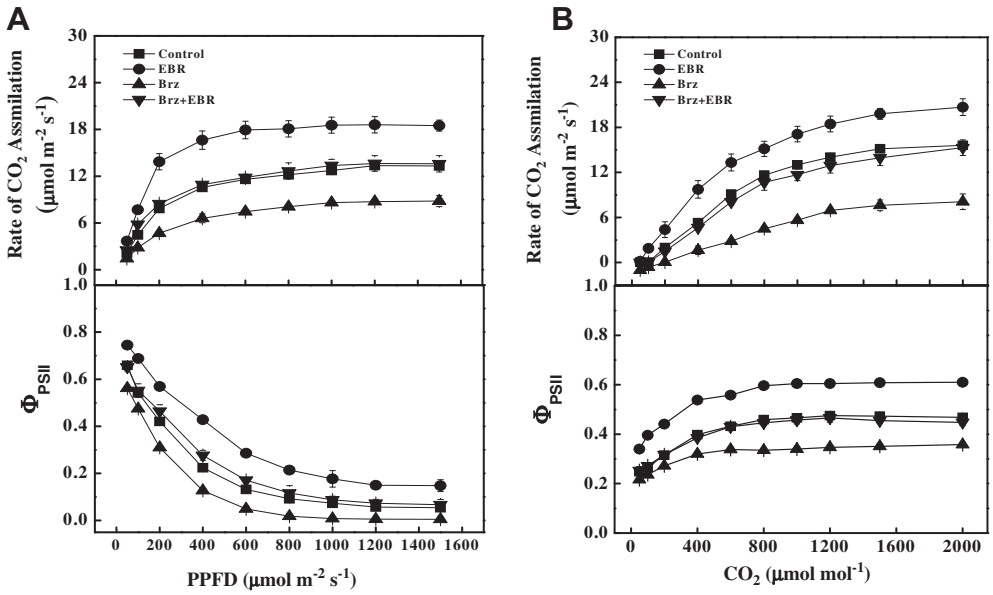

Fig. 1. Changes in the irradiance and intercellular CO_2 concentration response curves of the net CO_2 assimilation rate (A) and quantum efficiency of photosystem II (Φ_{PSII}), as influenced by the BR level in cucumber plants. The gas exchange and chlorophyll fluorescence parameters were measured under the growth conditions of 25 °C, 80% relative humidity, and 1–1.3 kPa leaf-to-air vapor pressure deficit. For the CO_2 assimilation versus intercellular CO_2 concentration (A/Ci) curve and the CO_2 assimilation versus incident PPFD (A/PPFD) analysis, a PPFD and CO_2 were applied at 600 μmol m^{-2} s^{-1} and 400 μmol mol^{-1}, respectively. The data are the means of four biological replicates (±SD).

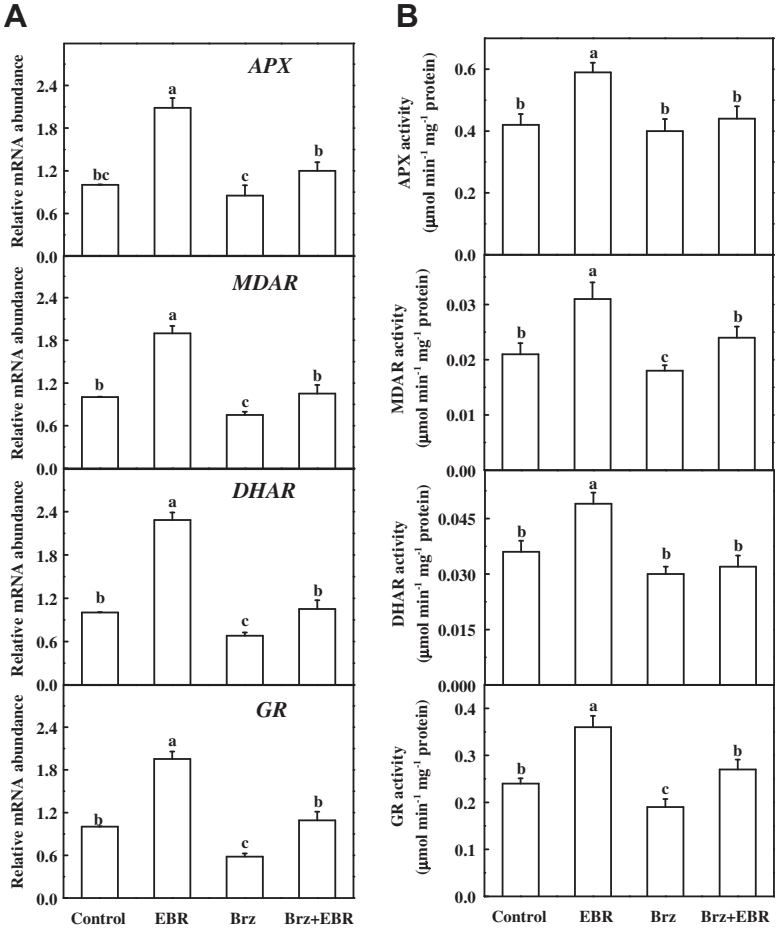

Fig. 2. Transcripts of *APX*, *MDAR*, *DHAR* and *GR* (A) and activity of APX, MDAR, DHAR and GR (B) in the chloroplasts isolated from cucumber leaves, as influenced by EBR and Brz treatment. Leaf samples were collected at 24 h after the EBR or Brz treatment. Data are the means of four biological replicates (±SD). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Tukey's test.

sufficient for optimum photosynthesis; therefore, the manipulation of the BR levels or signaling through biotechnological approaches may provide a feasible means to improve crop yields [3].

3.2. BR levels are related to the activity of the chloroplastic ascorbate–glutathione cycle and glutathione redox homeostasis

Previously, we found that BRs induced H_2O_2 accumulation in the apoplast [8]. To analyze further the BR-induced changes in the chloroplastic ascorbate–glutathione cycle and glutathione redox homeostasis, we examined the changes in the transcript levels and activity of enzymes involved in the ascorbate–glutathione cycle in the chloroplasts (Fig. 2A). The transcript levels of the *APX*, *MDAR*, *DHAR* and *GR* genes were all elevated in the EBR-treated plants but were reduced in the Brz-treated plants when compared to the control plants. Similar to the gene expression, the activities of APX, MDAR, DHAR and GR in the chloroplasts isolated from the EBR- and Brz-treated leaves were increased by 36–50% and decreased by 5–21%, respectively (Fig. 2B). Again, the Brz-induced decreases in the activities of these enzymes were recovered by the subsequent application of EBR. Furthermore, the EBR treatment resulted in a 30.1% increase in GSH, whereas the treatment with Brz resulted in a 23.0% decrease in GSH in the chloroplasts (Fig. 3A). In contrast, the EBR treatment decreased the GSSG content, whereas the Brz treatment increased the GSSG content significantly. Accordingly, the GSH/GSSG ratio in the chloroplasts was increased from 27.0 to 45.7 after the application of EBR but decreased to 11.8 after Brz application. Decreased GSH/GSSG ratio after the Brz treatment could also be restored by EBR. All of these results indicate that the redox status of glutathione is very sensitive to changes in GSSG. The BR-induced upregulation of GR is critical for maintaining the appropriate redox status of glutathione (Fig. 2B), as the reduced form GSH is continuously oxidized by the reactive oxygen species (ROS) produced from chloroplastic electron transport, even in the absence of cellular stress. By strengthening the AsA-GSH cycle, BRs are proposed to induce a reducing environment in the chloroplasts by increasing the GSH/GSSG ratio. These BR-induced redox changes are similar to those observed in salicylic acid (SA)-treated plants in which the inactivation of catalase and peroxidase by SA results in a concomitant increase in GR activity, along with increases in the GSH/GSSG ratio and CO_2 assimilation [21,22]. Furthermore, our results also suggested that the BR-induced changes in the redox state were not limited to the tissue level but also occurred in cellular compartments, such as the chloroplasts.

3.3. Effects of BR levels on the activity and stability of RCA and FBPase

Previously, we reported that increased BR levels induced an increase in the activation state of chloroplast-localized Rubisco and the content of RCA [4,5]. In the present study, we analyzed the changes in the activities of RCA and FBPase in plants with different BR levels (Fig. 3B). EBR application increased the activity of RCA, initial and total FBPase by 24.1%, 58.6% and 59.0%, respectively, whereas Brz decreased the activity of RCA and initial and total FBPase by 44.8%, 53.8% and 54.0%, respectively. Similarly, the Brz-induced decrease in the enzyme activities could be restored by subsequent application of EBR. Thus, a BR-induced change in the chloroplast glutathione redox homeostasis was closely associated with the activity of RCA and FBPase.

To determine whether the changes in the CO_2 assimilation induced by the increased BR levels are associated with the stability of the redox-sensitive enzymes as a result of reducing the chloroplast glutathione redox state, we performed *in vitro* assays to examine the changes in the stability and activity of the RCA large isomer (45 kDa) after the incubation with a defined concentration of GSH + GSSG and varying GSH/GSSG ratios. When the RCA large isomer (45 kDa) was incubated in a solution containing only GSH, RCA remained intact after 10 min and was degraded by proteinase K after 20 min of incubation (Fig. 4A). Conversely, when the enzyme was incubated in a solution containing only GSSG, RCA remained intact during the first 5 min but was degraded after 10 min of incubation (Fig. 4B). In addition, RCA was completely digested into smaller fragments by proteinase K at low GSH/GSSG ratios (1–20) after 8 min of incubation (Fig. 4C). However, when the ratio of GSH/GSSG was increased to 30–100, RCA remained largely intact during the same period of incubation with the proteinase. Furthermore, when RCA was incubated with a GSH and GSSG mixture, the activity declined with decreases in the GSH/GSSG ratios (Fig. 4D). These results suggest that reducing the glutathione redox state in the chloroplasts by elevating BR levels could lead to increased stability and activity of RCA, which would contribute to increased CO_2 assimilation. These results suggest a role of glutathione redox homeostasis in the regulation of redox-sensitive enzymes, probably at the post-translational level. It is highly likely that BRs influence many physiological processes by inducing a reducing environment in various cellular compartments, including the chloroplasts. Because the cellular redox states are involved in the regulation of defense against biotic and abiotic stress [23], it is also possible that the BR-induced changes in the redox

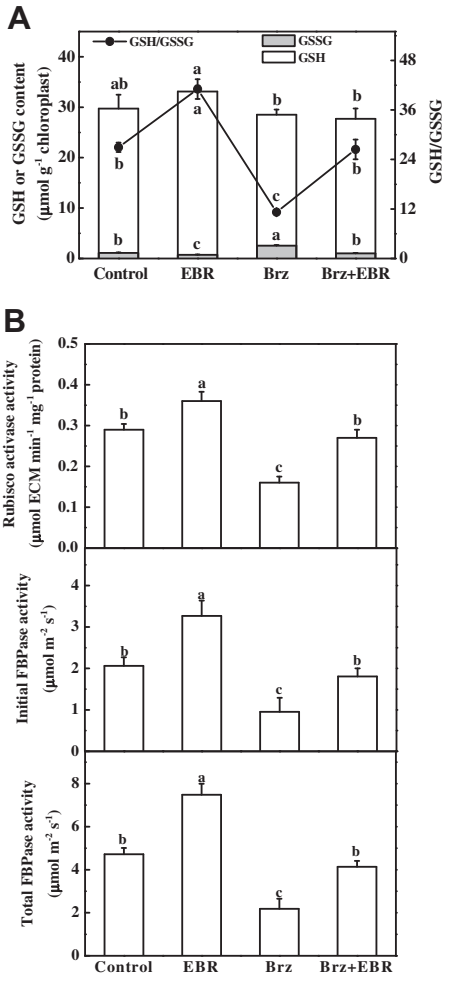

Fig. 3. Contents of GSH and GSSG and glutathione redox status in the chloroplasts (A) and activity of RCA and FBPase (B), as influenced by foliar EBR and Brz treatment. Leaf samples were collected at 24 h after the EBR or Brz treatment. Data are means of four biological replicates (±SD). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Tukey's test.

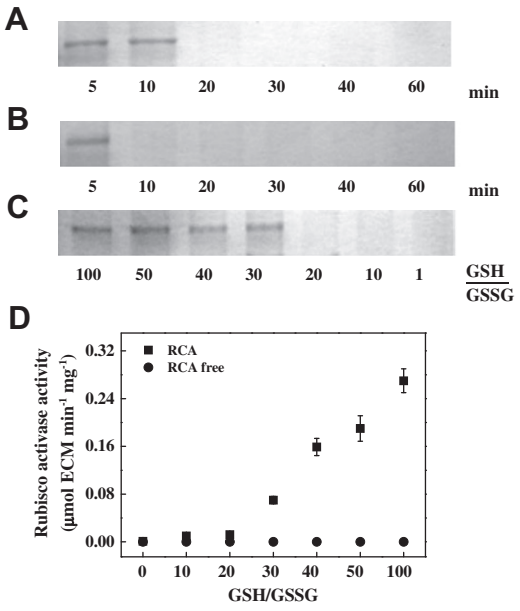

Fig. 4. *In vitro* assays of the RCA stability and activity, as influenced by the GSH/GSSG ratio in the incubation buffer. (A) RCA degradation in a buffer with GSH; (B) RCA degradation in a buffer with GSSG; (C) RCA degradation in a buffer with varying ratios of GSH to GSSG. 'Time' indicates the reaction duration for the proteolysis of RCA. In A–C, the purified RCA in activation buffer was incubated with GSH or GSSG alone or the different GSH/GSSG ratios indicated. Proteinase K was added to the activation buffer for the proteolytic assay. The products of RCA proteolysis were detected by SDS–PAGE after the reaction was stopped by the addition of a proteinase inhibitor (PMSF). (D) *In vitro* RCA activity, as influenced by the changes of the GSH/GSSG ratio in the assay solution. The reactions of the indicated GSH/GSSG ratios without purified RCA served as the negative controls.

homeostasis are important for both BR-induced photosynthesis and BR-induced disease resistance and stress tolerance [24,25]. If this is indeed the case, further studies to dissect the mechanisms by which BRs alter H_2O_2 and glutathione redox homeostasis will be of great interest in our understanding of the molecular basis for the wide range of biological activities of this important plant hormone.

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