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Hydrogen peroxide functions as a secondary messenger for brassinosteroids-induced CO_2 assimilation and carbohydrate metabolism in *Cucumis sativus**

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Abstract: Brassinosteroids (BRs) are potent regulators of photosynthesis and crop yield in agricultural crops; however, the mechanism by which BRs increase photosynthesis is not fully understood. Here, we show that foliar application of 24-epibrassinolide (EBR) resulted in increases in CO_2 assimilation, hydrogen peroxide (H_2O_2) accumulation, and leaf area in cucumber. H_2O_2 treatment induced increases in CO_2 assimilation whilst inhibition of the H_2O_2 accumulation by its generation inhibitor or scavenger completely abolished EBR-induced CO_2 assimilation. Increases of light harvesting due to larger leaf areas in EBR- and H_2O_2-treated plants were accompanied by increases in the photochemical efficiency of photosystem II (Φ_{PSII}) and photochemical quenching coefficient (q_P). EBR and H_2O_2 both activated carboxylation efficiency of ribulose-1,5-bisphosphate oxygenase/carboxylase (Rubisco) from analysis of CO_2 response curve and in vitro measurement of Rubisco activities. Moreover, EBR and H_2O_2 increased contents of total soluble sugar, sucrose, hexose, and starch, followed by enhanced activities of sugar metabolism such as sucrose phosphate synthase, sucrose synthase, and invertase. Interestingly, expression of transcripts of enzymes involved in starch and sugar utilization were inhibited by EBR and H_2O_2. However, the effects of EBR on carbohydrate metabolisms were reversed by the H_2O_2 generation inhibitor diphenyleneodonium (DPI) or scavenger dimethylthiourea (DMTU) pretreatment. All of these results indicate that H_2O_2 functions as a secondary messenger for EBR-induced CO_2 assimilation and carbohydrate metabolism in cucumber plants. Our study confirms that H_2O_2 mediates the regulation of photosynthesis by BRs and suggests that EBR and H_2O_2 regulate Calvin cycle and sugar metabolism via redox signaling and thus increase the photosynthetic potential and yield of crops.

Key words: Metabolism, Photosynthesis, Reactive oxygen species, Rubisco, Sucrose
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1 Introduction

In this century, the world faces great challenges such as an exploding human population, global warming, environmental pollution, and shortages of fresh water and arable land. It is predicted by the US Population Division that the global population will increase to 9.1 billion by 2050 (Population Division

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of the Department of Economic and Social Affairs of the United Nations Secretariat, 2009). For the survival of this large population, it is a priority to increase global crop yields, which are mostly determined by the photosynthetic potential of the crops (Long *et al*., 2006). In C3 plants, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes primary carbon fixation, in which CO_2 is converted to 3-phosphoglycerate. Phosphoglycerate is then phosphorylated and reduced by adenosine triphosphate (ATP) and triphosphopyridine nucleotide (NADPH), which are generated in the thylakoid membrane at the expense of the proton motive force generated by photosynthetic electron transport. The product triose phosphate is exported from the chloroplast via the chloroplast envelope phosphate (Pi) transporter to the cytosol for sugar metabolism or is used for starch synthesis or recycling to ribulose-1,5-bisphosphate (RuBP) in chloroplast (Sonnewald *et al*., 1994; Furbank and Taylor, 1995). It is clear that there are many constraints limiting the increase of photosynthetic potential from the light reaction through the Calvin cycle and sugar metabolism. During the past thirty years, great efforts have been made to increase photosynthesis efficiency, with special emphasis on the modification of the biochemical characteristics of Rubisco, an enzyme with dual functions of RuBP carboxylation and oxygenation (Parry *et al*., 2003). Researchers have focused on engineering the key enzymes used in the Calvin cycle and sugar metabolism via plant biotechnology (Galtier *et al*., 1995; Miyagawa *et al*., 2001; Lefebvre *et al*., 2005). However, most of these studies failed to significantly increase photosynthetic rate and crop yield (Sinclair *et al*., 2004). It is likely that the regulation of photosynthesis is far more complex than is commonly understood.

The regulation of photosynthesis by phytohormones is an approach that figures prominently in research aimed at increasing crop yields. In recent years, brassinosteroids (BRs), a new type of phytohormones, have shown exciting promotion effects on crop yield in both field and greenhouse trials (Khripach *et al*., 2000). Over-expression of genes involved in BR biosynthesis and reduction of plant BR level in BR deficient mutants or by application of inhibitors of BR biosynthesis resulted in enhanced plant growth, reduced rate of photosynthetic CO_2 assimilation, and lower sink strength and carbohydrate metabolism, respectively (Choe *et al*., 2001; Schluter *et al*., 2002; Asami *et al*., 2003; Wu *et al*., 2008). Wu *et al*. (2008) generated a BR biosynthetic gene over-expressing rice lines using roots and shoots specific promoters and observed significant increases in biomass accumulation and seed yield. They attributed the larger size of seeds and higher yield per plant to the increased flow of photoassimilates from source leaves. We have previously shown that BR enhances photosynthesis by directly regulating the activation state of Rubisco (Yu *et al*., 2004). However, the mechanism by which BR regulates Rubisco and increases photosynthesis is still unclear.

To date, the major framework for BR signaling in plant growth and development has been established, which includes the receptor brassinosteroid-insensitive 1 (BRI1) and associated signaling cascade components BRI1-associated receptor kinase 1 (BAK1), BR-signaling kinase 1 (BSK1), BRI1 suppressor 1 (BSU1), and BZR1/2 (Clouse, 2011). A surprisingly wide range of genes involved in cellular processes, environmental responses, development and hormonal responses have been shown to be directly regulated by BZR1 (Sun *et al*., 2010). However, no clues have been provided as to the role they play in the regulation of photosynthetic genes. It is highly likely that enzymes of carbon metabolism are regulated posttranscriptionally by BRs, or there are missing links between BRs and expression of photosynthetic genes.

We have previously shown that BR induced a transient oxidative burst, with hydrogen peroxide (H_2O_2) accumulation in the apoplast of mesophyll cells of cucumber (Xia *et al*., 2009b). H_2O_2 has been implicated as a second messenger in several plant hormone responses (Laloi *et al*., 2004; Kwak *et al*., 2006). Abscisic acid-induced stomata closure was dependent on H_2O_2 production in the cell membrane and chloroplast (Zhang *et al*., 2001; Kwak *et al*., 2003) and H_2O_2 crosstalks with Ca^{2+}, nitric oxide, and cyclic guanosine monophosphate (cGMP) (Desikan *et al*., 2004). Interestingly, the kinase domain of BRI1 receptor contains a GC-domain, which potentially generates cGMP (Kwezi *et al*., 2007). This prompts us to consider the link between H_2O_2 and BR signaling. Recently, we have proposed evidence suggesting that H_2O_2 is required for BR-induced expressions of photosynthetic genes, which are involved in light harvesting, electron transport, and the Calvin cycle (Jiang *et al.*, 2012). It is extremely important to note that the activities of certain enzymes in CO_2 assimilation are regulated by the redox potential via the ferredoxin/thioredoxin system (Schüermann and Buchanan, 2008). Although reactive oxygen species (ROS) such as H_2O_2 could cause posttranslational modifications to proteins and inhibition of enzymes at a high level, evidence suggests that ROS are involved as signal transducing messengers in the regulation of enzyme activities by thioredoxins (Kamata and Hirata, 1999). An appropriate level of H_2O_2 accumulation during plant acclimation to cold, high light, and drought resulted in increased content and redox state of glutathione (Foyer *et al.*, 1997). Up-regulation of genes involved in cysteine and glutathione biosynthesis by H_2O_2 further confirm the role of ROS in modulation of cellular redox state (Queval *et al.*, 2009). Glutathione is potentially involved in regulating enzyme activity via electron input into the redox network or protein glutathionylation (Meyer and Hell, 2005; Rouhier *et al.*, 2008).

In this study, we compared the effects of 24-epebrassinolide (EBR) and H_2O_2 treatments on the growth and photosynthesis of cucumber seedlings. We found that EBR and H_2O_2 can both cause similar increases in the carboxylation capacity of Rubisco and potential maximum rate of CO_2 assimilation. In addition, accelerated carbohydrate metabolism was also involved in EBR- or H_2O_2-induced photosynthesis. Pretreatment with inhibitors of production or scavenger of ROS indicated that ROS act as mediators for the enhancement of photosynthesis.

2 Materials and methods

2.1 Plant materials and treatments

Cucumber (*Cucumis sativus* L. cv. Jinchun No. 3) seeds were germinated in a growth medium of peat, vermiculite, and perlite (6:3:1, v/v/v) in a glasshouse. Cucumber seedlings were transplanted into plastic pots (15 cm diameter and 15 cm deep) when the first true leaf was fully expanded. The seedlings were watered daily with half-strength Enshi nutrient solution (Yu and Matsui, 1997) and kept in growth chambers. The growth conditions were as follows: a 12-h photoperiod, a temperature of 25 °C:17 °C (day:night), and a light intensity of 600 μmol/(m^2·s).

Treatment with EBR (Sigma, Santa Clara, USA) at 0.1 μmol/L or H_2O_2 at 5 mmol/L was performed by foliar spraying of cucumber seedlings at the four-leaf stage using distilled water as a control. For analysis of the role of H_2O_2 in EBR-induced up-regulation of CO_2 assimilation, we pretreated cucumber seedlings with 5 mmol/L dimethylthiourea (DMTU; a H_2O_2 and OH· scavenger) (Fox, 1984) or 100 μmol/L diphenyleneodonium (DPI; an inhibitor of NADPH oxidases which produce H_2O_2 (Hancock and Jones, 1987) and could be up-regulated by EBR (Xia *et al.*, 2009b)), and then treated the plants with 0.1 μmol/L EBR 8 h later. For all the treatments, the chemicals were applied to all leaves. For all cases, the solutions were applied at the rate of 80 ml per m^2 leaf area.

2.2 Gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange measurements were coupled with measurements of chlorophyll fluorescence using an open gas exchange with an integrated fluorescence chamber head system (LI-6400, LI-COR, Inc., Lincoln, NE, USA) on the 3rd leaves. Light-saturated rate of CO_2 assimilation (A_{sat}) was measured at ambient CO_2 concentration of 360 μmol/mol and saturating photosynthetic photon flux density (1 000 μmol/(m^2·s)) with a leaf temperature of (25±1.5) °C and relative air humidity of 80%–90%. An assimilation versus intercellular CO_2 concentration (A/C_i) curve was measured according to von Caemmerer and Farquhar (1981). Assimilation was measured as described by Yu *et al.* (2004). The maximum Rubisco carboxylation rates ($V_{c,max}$) and maximum RuBP regeneration rates (J_{max}) were estimated from the A/C_i curves using the method of Ethier and Livingston (2004). Stomatal limitation (l), the proportion of photosynthesis that is limited by stomatal conductance, was calculated according to Farquhar and Sharkey (1982).

Chlorophyll fluorescence parameters were calculated on the basis of the light-adapted fluorescence measurements as described by Zhou *et al.* (2004) and Ogweno *et al.* (2008). Photochemical efficiency of photosystem II (Φ_{PSII}), efficiency of excitation capture by open PSII center (F_v'/F_m'), and photochemical quenching coefficient (q_P) were calculated as $(F_m'-F_s)/F_m'$, $(F_m'-F_o')/F_m'$, and $(F_m'-F_s)/(F_m'-F_o')$, respectively (Genty *et al*., 1989; van Kooten and Snel, 1990). F_o, F_m, F_v: minimal, maximal, and variable fluorescence yields; F_m', F_v', F_s, maximal, variable, and steady-state fluorescence yield in a light-adapted state.

2.3 Measurements of total chlorophyll, soluble proteins, and carbohydrate content

Total chlorophyll content was determined by the method of Arnon (1949). Total soluble protein content was measured using Bradford (1976) reagent. Freeze-dried samples were used for the determination of carbohydrate content. Sucrose, starch, and hexose contents were determined using a modified phenol-sulfuric acid method (Buysse and Merckx, 1993). Soluble sugars were extracted from 200 mg of dried material with 50 ml of 80% ethanol, using five extraction steps. The supernatant was analyzed for hexose, sucrose, and total soluble sugars. The residue was boiled for 3 h in 10 ml 2% HCl to hydrolyze starch. The supernatant was analyzed for soluble sugars released from starch by acid hydrolysis (Yu *et al*., 2004).

2.4 Measurement of H_2O_2

Leaf samples (0.5 g) were ground in liquid nitrogen and 2 ml of 0.2 mol/L $HClO_4$. After thawing, the mixture was transferred to a 10-ml plastic tube and another 2 ml of 0.2 mol/L $HClO_4$ was added. The homogenate was centrifuged at 2700×*g* for 30 min at 4 °C and the supernatant was collected, adjusted to pH 6.0 with 4 mol/L KOH, and then centrifuged at 110×*g* for 1 min at 4 °C. The supernatant was placed onto an AG1x8 prepacked column (Bio-Rad, Hercules, CA) and H_2O_2 was eluted with 4 ml double-distilled H_2O. Recovery efficiencies of H_2O_2 from different samples were determined by analyzing duplicate samples to which H_2O_2 was added during grinding at a final concentration of 50 μmol/L. The sample (800 μl) was mixed with 400 μl reaction buffer containing 4 mmol/L 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) and 100 mmol/L potassium acetate at pH 4.4, 400 μl deionized water, and 0.25 U of horseradish peroxidase (HRP). H_2O_2 content was measured at optical density at 412 nm (OD_{412}) (Willekens *et al*., 1997).

2.5 Measurement of enzyme activity of carbohydrate metabolism

Sucrose phosphate synthase (SPS) and sucrose synthase (SS) were extracted at 0–4 °C according to Lowell *et al*. (1989). SPS activity was assayed at 37 °C by the method of Hubbard *et al*. (1989) and assayed essentially as described previously (Yu *et al*., 2004). Colour development of the reaction solution was measured at 620 nm and the SPS activity was calculated. SS was assayed in both the synthetic (SS-s) and cleavage (SS-c) directions with the method of Lowell *et al*. (1989). The increase in absorbance at 520 nm was measured (Yu *et al*., 2004). Acid invertase (AI) was extracted as described by Miron and Schaffer (1991). The reaction mixture that consisted of 4% sucrose, 50 mmol/L sodium acetate buffer (pH 4.5), and an aliquot of enzyme solution in a total volume of 1 ml. Reducing groups formed in the reaction mixture were measured (Endo *et al*., 1990).

2.6 Measurement of Rubisco activity

For measurements of Rubisco activity, frozen leaf samples were ground to a fine powder in liquid nitrogen and then extracted in a solution containing 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L $MgCl_2$, 12.5% glycerin, 10% polyvinylpyrrolidone (PVP), and 10 mmol/L β-mercaptoethanol. After centrifugation at 15000×*g* for 15 min at 4 °C, the total Rubisco activity was assayed according to Lilley and Walker (1974) and Sharkey *et al*. (1991), and the analysis was performed essentially as described previously (Yu *et al*., 2004). The oxidation of NADH was followed by changes in absorbance at 340 nm for 90 s.

2.7 Total RNA extraction and gene expression analysis

Total RNA was isolated from cucumber leaves using TRIZOL reagent (Sangon, China) according to the instructions supplied by the manufacturer. After extraction, total RNA was dissolved in diethyl pyrocarbonate-treated water. The complementary DNA (cDNA) template for real time polymerase chain reaction (RT-PCR) was synthesized using a RevertAid™ first strand cDNA synthesis kit (Fermentas, Burlington, Canada) from 2 μg total RNA purified using RNeasy mini kit (Qiagen, Hilden, Germany).

For quantitative RT-PCR (qRT-PCR) analysis, PCR products were amplified in triplicate using iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA) in 25 μl qRT-PCR reactions, an iCycler iQ 96-well RT-PCR detection system (Bio-Rad), iCycler software to calculate threshold cycle values and cucumber actin as an internal control. On the basis of expressed sequence tag (EST) sequences, the following gene-specific primers were designed and used for amplification: *BAM* (β-amylolytic enzyme), 5′-ACCGAATATGGCGAATTCTT-3′ and 5′-TGCCAATGAATTCCTGCTAC-3′; *SUS* (sucrose synthase), 5′-AAATTGGCCATTCAGGTTGT-3′ and 5′-CTAGTGGTTGTGGCTGGAGA-3′; *Invertase*, 5′-CTGGAAAGAATATGGTGGCA-3′ and 5′-CCCAAGAGAAGGATCGAGAG-3′; *UGDH* (UDP-glucose 6-dehydrogenase), 5′-CCTACCCATCTTCAGCCAAT-3′ and 5′-TCCTTGTTGCTTCATACGC-3′; *actin*, 5′-TGGACTCTGGTGATGGTGTTA-3′ and 5′-CAATGAGGGATGGCTGGAAAA-3′. Relative gene-expression was calculated as described by Livak and Schmittgen (2001).

2.8 Statistical analysis

Tukey's test was used for testing the mean difference between treatments by using the SPSS statistical software package. A *P*-value <0.05 was considered statistically significant.

3 Results

3.1 Time course of CO_2 assimilation and H_2O_2 accumulation in leaves after EBR treatment

We first compared the time courses of the effects of EBR on A_{sat} and H_2O_2 accumulation (Fig. 1). A_{sat} was significantly increased 3 h after EBR treatment. A_{sat} reached the highest level 6 h after EBR treatment and then gradually declined. At 72 h after treatment, there was no significant difference of A_{sat} between control and EBR-treated leaves. Elevated H_2O_2 content was observed as early as 1 h after application of 0.1 μmol/L EBR. Thus, elevation of H_2O_2 preceded the increase in A_{sat} in EBR-treated plants. Induction of H_2O_2 by EBR was sustained until 72 h after EBR treatment.

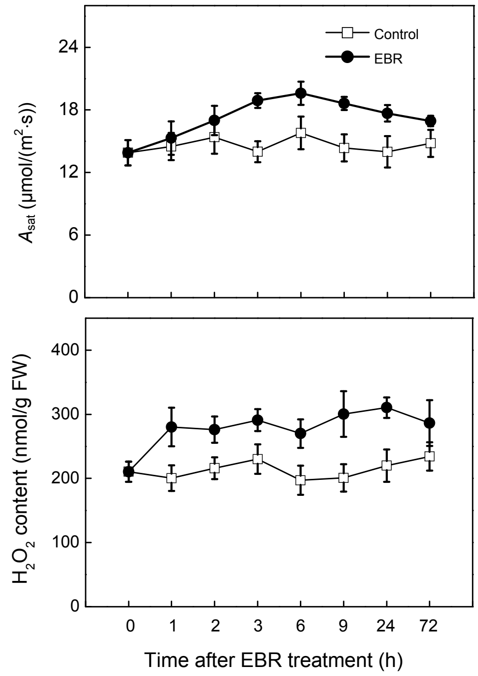

Fig. 1 Time course responses of A_{sat} and content of H_2O_2 to EBR
Four-week-old cucumber plants were treated with distilled water or EBR at 0.1 μmol/L at 9 am. CO_2 assimilation rate at saturated light (A_{sat}) was determined at 1 000 μmol/(m^2·s) light intensity and 25 °C. Measurements were taken at 1, 2, 3, 6, 9, 24, and 72 h after EBR treatment. Data are expressed as mean±standard deviation (SD) (*n*=4). FW: fresh weight

3.2 Effects of EBR and H_2O_2 on the growth of cucumber seedlings

To investigate whether EBR-induced H_2O_2 is involved in the enhanced plant growth, we first compared the effects of EBR and H_2O_2 on leaf mass, leaf area, total chlorophyll, and soluble protein contents. As expected, EBR significantly increased leaf area and leaf mass after 3 d of treatment (Table 1). Similarly, H_2O_2 slightly increased leaf area and mass after 3 d of treatment, although the difference was not statistically significant in some cases. Neither EBR nor H_2O_2 had significant effects on total chlorophyll content. Interestingly, EBR significantly increased the total soluble protein content after 1 d of treatment although there were some exceptions. In contrast, H_2O_2 had no effects on the total soluble protein content as compared to the control.

Table 1 Effects of EBR and H_2O_2 treatments on leaf mass per area, leaf area, total chlorophyll, and soluble protein contents in cucumber leaves

Time after treatment (h)	Treatment	Leaf area (cm^2/plant)	Leaf mass per area (g FW/m^2)	Total chlorophyll content (μg/cm^2)	Soluble protein content (g/m^2)
3	Control	314.3±15.6^a	191.8±10.2^a	33.1±1.2^a	7.39±0.48^a
	EBR	324.6±27.3^a	189.6±7.8^a	31.8±1.2^a	8.01±0.38^a
	H_2O_2	321.0±14.7^a	187.8±9.5^a	32.5±1.3^a	7.49±0.32^a
24	Control	417.6±24.7^a	193.6±11.1^a	30.9±2.1^a	7.41±0.40^b
	EBR	433.6±20.6^a	190.8±8.2^a	31.2±1.8^a	8.29±0.44^a
	H_2O_2	434.6±9.1^a	186.5±11.8^a	30.5±2.4^a	7.44±0.13^b
72	Control	472.0±15.5^b	189.6±13.5^a	32.5±0.9^a	7.58±0.39^a
	EBR	531.0±26.0^a	192.1±9.8^a	30.2±1.9^a	8.32±0.45^a
	H_2O_2	519.6±25.2^{ab}	193.2±9.6^a	31.6±3.2^a	7.56±0.30^a
120	Control	540.8±14.7^b	194.5±8.0^a	33.2±1.6^a	7.39±0.29^b
	EBR	603.4±11.5^a	188.6±12.2^a	32.8±2.0^a	8.31±0.54^a
	H_2O_2	588.6±43.9^{ab}	188.8±7.2^a	30.6±2.1^a	7.68±0.40^{ab}
168	Control	630.7±21.2^b	189.9±7.6^a	29.2±2.6^a	7.21±0.39^b
	EBR	696.6±23.0^a	190.1±6.5^a	27.6±2.5^a	8.18±0.40^a
	H_2O_2	660.8±43.2^{ab}	191.3±9.0^a	28.6±1.8^a	7.41±0.39^{ab}

EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L was used, respectively. Values are expressed as mean±SD (n=4). Significant differences (P<0.05) between treatments are indicated by different letters according to Tukey's test. FW: fresh weight

3.3 Effects of EBR and H_2O_2 on the CO_2 assimilation and chlorophyll fluorescence quenching

To examine whether enhanced plant growth by EBR and H_2O_2 treatment was associated with increased photosynthesis, we analyzed parameters of gas exchange and chlorophyll fluorescence after EBR and H_2O_2 treatments, respectively. Fig. 2a shows that the maximum carboxylation rate of Rubisco ($V_{c,max}$)increased rapidly at 3 h after treatment with either EBR or H_2O_2. $V_{c,max}$ values peaked at 24 h after treatment and then declined. Interestingly, the effects of H_2O_2 on $V_{c,max}$ declined more rapidly than those of EBR treatment. $V_{c,max}$ for the H_2O_2-treated plants had no significant difference with control from 3 d afterward, whereas the effects of EBR sustained until 5 d after treatment. Changes of maximum potential rate of electron transport (J_{max}) showed trends similar to those of $V_{c,max}$ except that the induction rate of J_{max} was a little lower than that of $V_{c,max}$ by EBR and H_2O_2 treatments (Fig. 2b). EBR and H_2O_2 treatment, however, had no significant effects on l (Fig. 2c).

Actual Φ_{PSII} for control leaves remained almost constant throughout the experiment (Fig. 2d). Compared with control leaves, EBR- or H_2O_2-treated leaves exhibited significantly higher Φ_{PSII} values 3 h after treatment. The value of EBR-treated plants reached maximum at 1 d after treatment. In contrast, the value for the H_2O_2-treated plants began to decline at 1 d. Φ_{PSII} of both EBR- and H_2O_2-treated plants declined rapidly after 1 d of treatment and showed no significant difference with control afterward. Changes of q_P almost paralleled that of Φ_{PSII} (Fig. 2e), whereas no changes in the efficiency of energy capture by open PSII reaction centers (F_v'/F_m') were observed (Fig. 2f). Thus, the increase in Φ_{PSII} was mainly due to a significant increase in q_P.

3.4 Effects of EBR and H_2O_2 on the activity of Rubisco

Changes of $V_{c,max}$ reflected the in vivo carboxylation states of Rubisco. To further investigate whether EBR and H_2O_2 increased photosynthesis by regulating activity of Rubisco, we determined total Rubisco activity and initial Rubisco activity. Table 2 shows that EBR and H_2O_2 had no significant effects on total Rubisco activity, whereas it significantly induced initial activity and activation state of Rubisco. In comparison, the effect of EBR on the initial activity and activation state of Rubisco was more pronounced than that of H_2O_2.

3.5 Role of H_2O_2 in the EBR-induced CO_2 assimilation

To determine whether EBR-induced H_2O_2 accumulation played a role in CO_2 assimilation, we

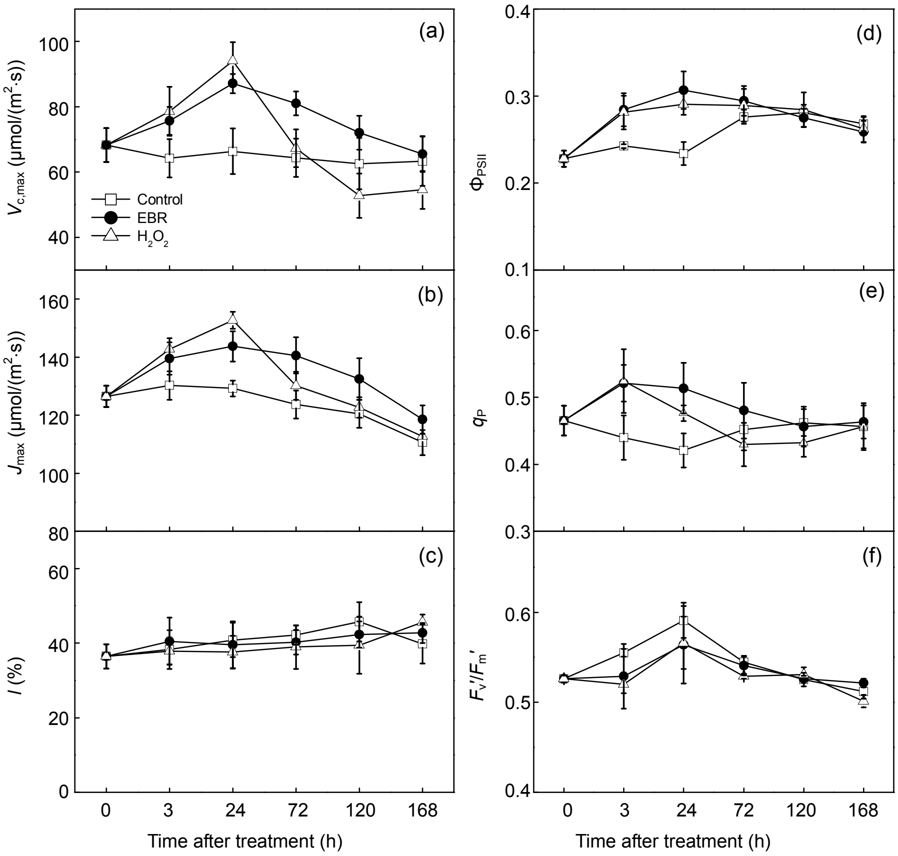

Fig. 2 Changes in maximum carboxylation rate of Rubisco ($V_{c,max}$) (a), maximum RuBP regeneration rates (J_{max}) (b), stomatal limitation (l) (c), photochemical efficiency of photosystem II (Φ_{PSII}) (d), photochemical quenching coefficient (q_P) (e), and the efficiency of excitation capture by open PSII centers (Fv'/Fm') (f) for control, EBR-treated, and H_2O_2-treated plants

Measurements were taken at 1, 2, 3, 6, 9, 24, and 72 h after treatment with 0.1 μmol/L EBR or 5 mmol/L H_2O_2, respectively. Data are expressed as mean±SD (n=4)

Table 2 Effects of EBR and H_2O_2 on total Rubisco activity, initial Rubisco activity, and Rubisco activation rate

Treatment	Total Rubisco activity (μmol/(m²·s))	Initial Rubico activity (μmol/(m²·s))	Rubisco activation rate (%)
Control	40.37±5.57[a]	13.90±1.10[c]	34.43[c]
EBR	47.75±6.37[a]	33.69±2.47[a]	71.12[a]
H_2O_2	43.74±6.97[a]	23.24±0.29[b]	53.13[b]

Samples were taken at 24 h after treatment with EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L, respectively. Values are expressed as mean±SD (n=4). Significant differences (P<0.05) between treatments are indicated by different letters according to Tukey's test

analyzed the changes of potential maximum rate of photosynthetic rate (A_{max}) at saturating photosynthetic photon flux density (PPFD) of 1 500 μmol/(m²·s) and CO_2 of 2 000 μmol/mol in plants pretreated with the DPI and DMTU which inhibits NADPH oxidase-dependent H_2O_2 production and accumulation, respectively, before EBR treatment. The high concentration of CO_2 used in this study excluded the limitation of carboxylation by Rubisco, and therefore, the induction of A_{max} was related to mechanisms other than regulation of activity of Rubisco. Fig. 3 shows that A_{max} was significantly increased as compared to control by EBR and H_2O_2 treatments. Importantly, inhibition of H_2O_2 production or scavenging of EBR-induced H_2O_2 by DPI and DMTU, respectively, completely abolished the effects of EBR on A_{max}.

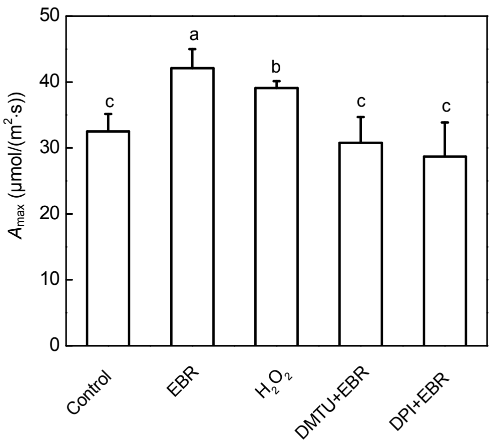

Fig. 3 Effects of EBR and H_2O_2 and pretreatments with DPI and DMTU on the A_{max} of CO_2

A_{max} was determined at saturating PPFD of 1 500 μmol/(m^2·s) and CO_2 of 2 000 μmol/mol. Measurements were taken at 24 h after treatment with EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L, respectively. DPI at 100 μmol/L and DMTU at 5 mmol/L were used, respectively. Values are expressed as mean±SD (n=4). Means denoted with different letters showed statistically significant difference (P<0.05) according to Tukey's test

3.6 Effects of EBR and H_2O_2 on carbohydrate metabolism

Photosynthesis is also limited by inorganic phosphate supply to chloroplasts, which is accompanied by triosephosphate transport and sucrose metabolism. Therefore, we determined the involvement of EBR and H_2O_2 on carbohydrate metabolism. Treatment of EBR significantly increased total soluble sugar, sucrose, hexose and starch contents (Table 3). The change of sucrose was most significant, with an increase of about 30%. Treatment with H_2O_2 had similar effects on contents of the above carbohydrates. Importantly, pretreatment, with DPI and DMTU completely inhibited the induction of carbohydrates by EBR. Increases in carbohydrate metabolite contents were accompanied by increases in acid invertase activity (AI) and activity of SS-s in EBR- or H_2O_2-treated leaves, which increased by 82% and 31%, or 116% and 23%, respectively (Table 4). However, EBR and H_2O_2 had no influence on the activities of SPS and SS-c direction. Again, pretreatments with DPI and DMTU completely inhibited the induction of activities of AI and SS-s by EBR.

Consistent with changes of AI, the transcription of invertase was induced significantly by H_2O_2 treatment but was only slightly increased by EBR treatment (Fig. 4). Meanwhile, EBR and H_2O_2 significantly inhibited the expressions of *BAM* and *UGDH*, encoding β-amylolytic enzyme and UDP-glucose 6-dehydrogenase, which are involved in starch and glucose metabolism, respectively. In contrast, EBR and H_2O_2 had no significant effects on transcript of *SUS*. Importantly, pretreatments of DPI and DMTU reversed the effects of EBR on the expressions of *BAM* and *UGDH*. These results suggest that sugar metabolism is one of the factors that drive the flux of CO_2 assimilation after EBR or H_2O_2 treatment, and H_2O_2 is involved in regulation of carbohydrate metabolism by EBR.

4 Discussion

In this study, we found that both EBR and H_2O_2 treatments induced an up-regulation of CO_2 assimilation and carbohydrate metabolism. Foliar application of EBR resulted in a H_2O_2 accumulation while

Table 3 Effects of EBR and H_2O_2 and pretreatments with DPI and DMTU on the contents of total soluble sugar, sucrose, hexose, and starch after 24 h treatments

Treatment	Content (mg/g DW)			
	Total soluble sugar	Sucrose	Hexose	Starch
Control	42.12±1.90^b	10.88±0.96^b	26.76±0.47^b	22.41±0.55^b
EBR	51.88±3.37^a	14.16±1.40^a	30.79±0.82^a	28.50±1.01^a
H_2O_2	52.26±1.90^a	13.44±0.34^a	31.19±0.62^a	29.32±0.48^a
DMTU+EBR	40.34±1.64^b	11.31±1.11^b	23.19±0.97^c	19.00±3.31^c
DPI+EBR	42.99±0.58^b	10.33±0.09^b	23.83±0.40^c	19.65±1.13^{bc}

DPI at 100 μmol/L and DMTU at 5 mmol/L were applied 8 h before EBR treatment, respectively. Samples were taken at 24 h after treatment with EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L, respectively. Values are expressed as mean±SD (n=4). Significant differences (P<0.05) between treatments are indicated by different letters according to Tukey's test. DW: dry weight

Table 4 Effects of EBR and H_2O_2 and pretreatments with DPI and DMTU on the activities of SPS, AI, SS-s and SS-c directions after 24 h treatments

Treatment	SPS activity (μmol/(h·g FW))	AI activity (μmol/(h·g FW))	SS-s activity (μmol/(h·g FW))	SS-c activity (μmol/(h·g FW))
Control	34.23±3.21^{a}	17.66±2.11^{c}	37.71±1.05^{b}	18.26±1.20^{a}
EBR	29.59±5.01^{a}	32.10±2.64^{a}	49.51±3.02^{a}	22.80±3.02^{a}
H_2O_2	38.23±4.55^{a}	38.06±5.10^{a}	46.20±4.27^{a}	24.78±5.23^{a}
DMTU+EBR	30.00±4.20^{a}	19.00±4.19^{bc}	29.55±2.19^{c}	20.58±3.41^{a}
DPI+EBR	37.60±3.90^{a}	23.44±3.25^{b}	33.41±5.22^{bc}	17.28±2.19^{a}

DPI at 100 μmol/L and DMTU at 5 mmol/L were applied 8 h before EBR treatment, respectively. Samples were taken at 24 h after treatment with EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L, respectively. Values are expressed as mean±SD (n=4). Significant differences (P<0.05) between treatments are indicated by different letters according to Tukey's test. FW: fresh weight

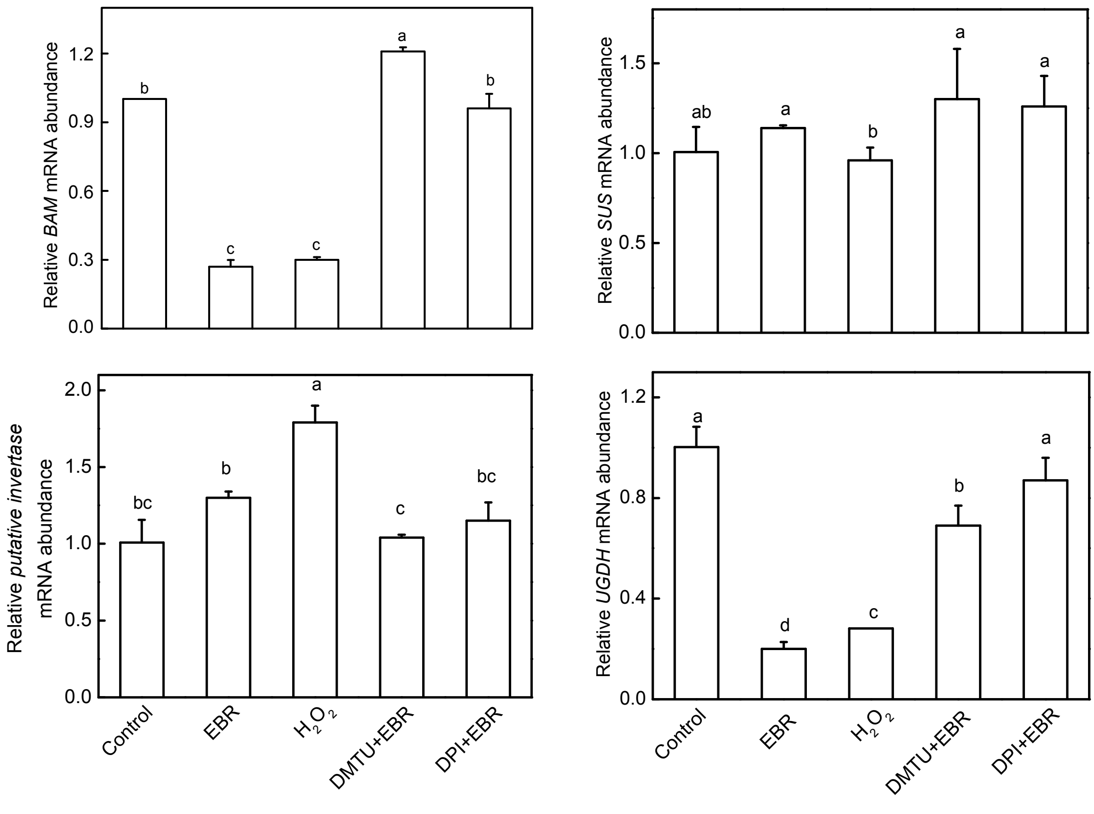

Fig. 4 Effects of EBR and H_2O_2 and pretreatments with DPI and DMTU on transcripts of *BAM* (β-amylolytic enzyme), *SUS* (sucrose synthase), *invertase*, and *UGDH* (UDP-glucose 6-dehydrogenase) in cucumber leaves

Leaf samples were taken at 24 h after treatment with EBR at 0.1 μmol/L or H_2O_2 at 5 mmol/L, respectively. DPI at 100 μmol/L and DMTU at 5 mmol/L were used respectively. Data are expressed as mean±SD (n=4). Means denoted with different letters showed statistically significant difference (P<0.05) according to Tukey's test

the inhibition of the H_2O_2 accumulation completely abolished EBR-induced CO_2 assimilation and carbohydrate metabolism. These results suggest that H_2O_2 functions as a secondary messenger for the EBR-induced changes in CO_2 assimilation and carbohydrate metabolism.

The effects of H_2O_2 and EBR on plant growth and photosynthesis were comparable, which led us to consider whether the effects of EBR were mediated by ROS. Previously, we have shown that EBR induces the transient induction of H_2O_2 accumulation in the apoplast by activating plasma membrane NADPH oxidase (Xia *et al*., 2009b). Inhibition of ROS production or scavenging of H_2O_2 significantly inhibited EBR-mediated stress tolerance, which suggests that ROS may act as a second messenger of BR signaling. Recently, we have shown that plant ROS content was highly correlated with endogenous BR content in cucumber and accumulation of ROS increased steadily with increasing concentration of exogenously applied EBR (Jiang *et al*., 2012). Interestingly, the enhancement of CO_2 assimilation after EBR treatment was abolished by pretreatment with DPI or DMTU whilst DPI or DMTU at the applied concentrations had no significant effects on CO_2 assimilation (Jiang *et al*., 2012). In this study, inhibition of H_2O_2 accumulation inhibited the effects of EBR on A_{max}, accumulation of carbohydrates, and induction of sugar metabolism enzymes. This further confirmed the role of ROS in EBR-mediated plant growth and photosynthesis.

Both EBR and H_2O_2 increased the $V_{c,max}$, Φ_{PSII} and potential maximum photosynthetic rate (Figs. 2 and 3). Photosynthetic CO_2 assimilation is limited by several physiological processes. Stomatal movement controls the entry of CO_2 into the cell, photosynthetic electron transport supplies reducing power of ATP and NADPH for CO_2 reduction, Rubisco is the rate limiting enzyme of the Calvin cycle and sugar metabolisms regulate the balance of triosephosphate transport and Pi translocation (Paul *et al*., 1992; Fryer *et al*., 1995; Allen and Ort, 2001). In this study, EBR and H_2O_2 had no influence on the l value, which excluded the involvement of stomatal limitation (Fig. 2). Our previous research indicates that EBR enhances photosynthetic CO_2 assimilation by regulating the expressions and activities of Rubisco and Rubisco activase (Xia *et al*., 2009a). Consistent with these results, EBR and H_2O_2 treatments both caused increased $V_{c,max}$ and initial Rubisco activity, but had no effects on total activity of Rubisco. This indicates that EBR and H_2O_2 mainly regulate the activation state of Rubisco, possibly through the action of Rubisco activase. EBR and H_2O_2 treatments also significantly increased the Φ_{PSII}. Φ_{PSII} is determined by q_P and F_v'/F_m'. From our analysis, the inductions of Φ_{PSII} by EBR and H_2O_2 were mainly attributed to q_P but not related to F_v'/F_m'. This is consistent with the fact that there were no differences in chlorophyll contents between control and EBR or H_2O_2-treated leaves (Table 1). q_P has been proposed to be controlled by the demand of ATP and NADPH of Calvin cycle (Nogues and Baker, 2000). The increased activity of the Calvin cycle induced by EBR and H_2O_2, therefore, contributed to the increased demand and the increased q_P.

It was interesting to note that A_{max} varied between control and EBR- or H_2O_2-treated leaves (Fig. 3). At saturating PPFD and CO_2 concentration, the capacity for sucrose synthesis ultimately limits the maximal rates of photosynthesis by restricting the rate at which inorganic phosphate can be recycled to support electron transport and carbon fixation in the chloroplast (Stitt, 1986). Increased A_{max} for the EBR- or H_2O_2-treated leaves suggests that triose-phosphate transport and Pi translocation were in a new balanced state in EBR or H_2O_2 treated leaves, which could probably be attributed to accelerated carbohydrate metabolisms. An increase in A_{max} was accompanied by increases in sucrose, soluble sugars, and starch contents, together with significant increases in acid invertase and sucrose synthase (Tables 3 and 4) and reductions in the expression of genes of carbohydrate utilization such as *BAM* and *UGDH* (Fig. 4). These results suggest that EBR and H_2O_2 increased carbohydrate metabolism and kept efficient supply of Pi to the chloroplast. Ozaki *et al*. (2009) determined the effects of periodic soil H_2O_2 drenching on carbohydrate metabolism in leaves and fruits of melon and observed that appropriate dose of H_2O_2 increased the contents of fructose, glucose, sucrose and starch with concomitant increases of sucrose phosphate synthase and invertase in leaves or fruits. The discrepancy in the effects on SPS between our findings and those of Ozaki *et al*. (2009) may be caused by differing application methods and concentration of H_2O_2 used, because the H_2O_2 signal could be attenuated rapidly in plant cells due to efficient antioxidant system (Neill *et al*., 2002). Single application of EBR and H_2O_2 in our experiment system triggered a transient stimulus of metabolism, whereas the periodic application to soil in their application system could ensure sustained translocation of H_2O_2 signal from roots. The increase of activity of invertase suggests for the enhanced sucrose synthesis and vice versa, as shown by increases in hexose in EBR- or H_2O_2-treated leaves. The increase of invertase was related to enhanced sink strength (Roitsch and Gonzalez, 2004). Ozaki *et al*. (2009) proposed that increases of sugars in melon fruits were related to enhanced carbon flux from source leaves. It is likely that sink strength was increased by EBR and H_2O_2 treatments, which increased the demand for photo assimilates and CO_2 assimilation.

Plants have evolved an efficient oxidation and reduction (redox) system to control their metabolic fluxes (Dietz, 2008). Ferredoxin/thioredoxin is well known to be involved in the regulation of Calvin cycle activity and sugar metabolism including fructose-1,6-bisphosphatase (FBPase), SPS, and SUS (Ruelland and Miginiac-Maslow, 1999; Buchanan *et al.*, 2002; Marino *et al.*, 2008). Although higher level of ROS could induce oxidative stress in the cells, ROS could be induced as second messengers during response to EBR at modest concentrations. BRs have been shown to activate the reduction of glutathione pool, resulting in reductive cellular redox state (Jiang *et al.*, 2012). Interestingly, EBR had little effects on Rubisco content and transcript levels of SUS and AI, but significantly increased activities of these enzymes, which suggests a role of posttranscriptional regulation. Several studies have shown that sugar metabolism enzymes are subject to direct thiol modification in plants (Balmer *et al.*, 2003; Hendriks *et al.*, 2003; Ito *et al.*, 2003; Lemaire *et al.*, 2004). The photosynthesis increase was strictly dependent on the concentration of EBR, which is mostly related to appropriate redox states. It is probable that the reducing redox state induced by EBR application favors the activation of sugar metabolism enzymes.

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