Interactive effects of CO₂ enrichment and brassinosteroid on CO₂ assimilation and photosynthetic electron transport in *Cucumis sativus*

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**Abstract**

CO₂ enrichment and brassinosteroids (BR) both have positive impacts on photosynthesis and plant growth. To examine the interactive effect of CO₂ enrichment and BR on photosynthesis and plant growth, CO₂ assimilation, chlorophyll fluorescence quenching, carbohydrate metabolism, photosynthetic gene transcript and enzyme activity were analyzed in leaves of young plants of cucumber (*Cucumis sativus L.*) in response to a doubling of growing CO₂ level, foliar BR application alone or in combination. Both CO₂ elevation and application of BR increased shoot biomass, leaf area, CO₂ assimilation, total soluble sugar and starch contents, transcript for photosynthetic gene and activity for enzymes involved in Benson–Calvin cycle but a combination of the two treatments resulted in a more significant effect. Although an elevation of CO₂ level had little effects on quantum efficiency of PSII (Φ_{PSII}), it significantly increased the electron flux for photosynthetic carbon reduction [F_{RC} (PC)] but decreased electron flux for photorespiratory carbon oxidation [F_{CO} (PC)]. In contrast, BR treatment increased Φ_{PSII} and this increase in Φ_{PSII} was associated with increased F_{RC} (PC) and F_{CO} (PC). Furthermore, a combined treatment of CO₂ elevation and BR resulted in an additive effect on PSII electron flux. However, alternative electron flux was almost unaltered after CO₂ enrichment and BR treatment. Thus, short term CO₂ elevation did not induce a down-regulation of photosynthesis and there was an additive effect between BR and CO₂ on the enhancement of CO₂ assimilation in leaves of young cucumber plants.

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1. Introduction

The concentrations of atmospheric CO₂ are predicted to double the current levels of 380 µmol mol⁻¹ by the end of this century (Watson et al., 1990). If other growth conditions are optimal, the photosynthetic rate of C₃ plants is limited by the supply of CO₂ (Kimball, 1983; Kimball et al., 2002; Long et al., 2004). Accordingly, crop CO₂ enrichment has been a powerful practice in greenhouse industry for improving produce quality and increasing crop yield. Total dry matter production increases in many species of plants are correlated with increased CO₂ levels and are most pronounced during the early stages of vegetative growth and development (Chu et al., 1992). However, research effort has not kept pace with commercial development. Whilst a great deal of data is available on photosynthesis covering many varieties of plants, the role played by CO₂ is perhaps the least understood.

Elevated CO₂ levels may increase plant productivity due to enhanced CO₂ fixation, suppressed photorespiration and/or suppressed dark respiration (Bunce, 1990; Drake et al., 1997; Amthor, 2001). In plants with a C₃ photosynthetic pathway, the enzyme Rubisco catalyses the initial carboxylation and oxygenation reactions of ribulose-1,5-bisphosphate (RuBP) (Bowes, 1993). Rubisco is not CO₂-saturated at current atmospheric CO₂ levels in C₃ plants, and an increase in atmospheric CO₂ concentration will decrease photorespiration and increase photosynthesis since the balance of carboxylation and oxygenation depends on the CO₂ and O₂ ratio at the Rubisco site (Bowes, 1993; Drake et al., 1997). However, prolonged exposure to elevated atmospheric CO₂ will lead to a decreased photosynthetic capacity in many plant species (Sage et al., 1989). This acclimation response to elevated CO₂ is often accompanied by an increase in soluble carbohydrate pools and a decrease in Rubisco protein content, activity, and activation state (Bowes, 1993; Drake et al., 1997; Pfannschmidt, 2003). However, there are significant differences in the response of mature leaves and developing leaves to CO₂ elevation (Pearson and Brooks, 1995).

**Abbreviations:** BR, brassinosteroids; Fₑ/Fₘ, efficiency of excitation capture by open PSII center; Jₑ, alternative electron flux; Jₑ(PCO), electron flux for the photorespiratory carbon oxidation; Jₑ(PCR), electron flux for the photosynthetic carbon reduction; Jₑ(PC), total electron flux in PSII; qₑ, net CO₂ assimilation rate; PPFD, photosynthetic photon flux density; Φₑ(PC), quantum efficiency of PSII; qₑ, photochemical quenching coefficient; RuBP, ribulose-1,5-bisphosphate.

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Several studies showed that the mature leaves were more sensitive to the photosynthetic acclimation induced by elevated CO2 (Gesch et al., 1998; Sakai et al., 2006). To avoid photosynthetic acclimation and reduced growth vigour at the late growth stage, farmer prefer to introduce CO2 enrichment at the early growth stage instead of the whole growth stage in the commercial production. Until now, experiments on climate change under controlled environmental conditions have focused primarily on temperate plant species with a prolonged growth period (Sage et al., 1989; Gesch et al., 1998; Vu et al., 1999, 2001). Furthermore, there are close interactions between elevated CO2 and other environmental factors such as temperature, nutrients, water availability and ozone in the atmosphere (Prasad et al., 2003; Mateos-Naranjo et al., 2010). In addition, CO2 elevation had a significant effect on the levels of indole-3-acetic acid (IAA), gibberellins (GAs), cytokinins (CKs) and abscisic acid (ABA), and ethylene in the leaves or in the xylem sap (Yong et al., 2000; Li et al., 2002; Seneweera et al., 2003; Teng et al., 2006; Wang et al., 2009; Tao et al., 2010). However, a few studies to date have been conducted to examine the interactive effects of CO2 elevation and exogenous plant growth substances on the physiology of greenhouse crops such as tomatoes and cucumber although CO2 enrichment is widely used in their production.

Brassinosteroids (BRs) are a family of over 40 naturally occurring plant steroid hormones that are ubiquitously distributed in the plant kingdom (Clouse and Sasse, 1998; Bishop and Koncz, 2002; Krishna, 2003; Montoya et al., 2005). BRs play prominent roles in various physiological processes including the induction of a broad spectrum of cellular responses, such as stem elongation, pollen tube growth, xylem differentiation, leaf epinasty, root inhibition, induction of ethylene biosynthesis, proton pump activation, regulation of gene expression and photosynthesis, and adaptive responses to environmental stress (Clouse and Sasse, 1998; Dhaubhadel et al., 1999; Khripach et al., 2000; Krishna, 2003; Yu et al., 2004). As potent plant growth regulators, BRs are now widely used to enhance plant growth and yield of important agricultural crops (Khripach et al., 2000; Divi and Krishna, 2009). We have previously shown that application of exogenous BR increases photosynthetic CO2 assimilation in cucumber plants, which may provide an important mechanism for increased growth and yield in BR-treated plants (Yu et al., 2004). Recently, we found that BR increased whilst brassinazole (Brz), a specific inhibitor of BR biosynthesis, decreased the maximum Rubisco carboxylation rates ($V_{c_{max}}$), total and, to a greater extent, initial Rubisco activity and the Rubisco activation state (Xia et al., 2009a). Furthermore, BR upregulated whilst Brz downregulated the expressions of rbcl, rbcS and other Calvin–Benson cycle genes. In this regard, there is an intriguing possibility that BR and CO2 enrichment could have additive effects on photosynthesis by increasing RuBP carboxylation capacity by Rubisco and depressing photorespiration.

Cucumber is widely cultivated in greenhouse in the world. Due to its effectiveness in improving growth vigor and productivity, CO2 enrichment has been widely adopted in its commercial production around the world especially at the early plant growth stage. In the following study, we grew cucumber plants under two atmospheric CO2 levels with or without BR application to test the hypothesis that BR and CO2 enrichment could have additive effects on the increase in biomass accumulation and CO2 assimilation. Furthermore, carbohydrate accumulation, Calvin–Benson cycle gene transcripts and enzyme activity were analyzed to determine whether elevated CO2-induced photosynthetic acclimation occurs in young developing leaves and if so whether BR could alleviate elevated CO2-induced photosynthetic acclimation by modifying photosynthetic gene transcript and enzyme activity.

### 2. Materials and methods

#### 2.1. Plant materials and treatments

Seeds of cucumber (Cucumis sativus L. cv. Jinchun No. 3) were sown directly in a growth medium containing a mixture of peat, vermiculite and perlite (6:3:1, v:v:v) in plastic trays. Average day/night temperatures were 26/17°C in a greenhouse with natural sunlight as light source. When the first true leaf was fully expanded, seedlings were transplanted into plastic pots (15 cm diameter and 15 cm deep, one seedling per pot) and watered daily with half-strength Enshi nutrient solution (Yu and Matsui, 1997). After 3 weeks after sowing, the seedlings at the 3-leaf stage were set into Conviron E15 (Conviron, Manitoba, Canada) controlled environment growth chambers and allowed to acclimate for 2 days under 12-h photoperiod (8 am–8 pm), temperature of 25/18°C (day/night), photosynthetic photon flux density (PPFD) of 400 μmol m⁻² s⁻¹ above canopy and CO2 of 380 μmol mol⁻¹. Then, the seedlings were set into 4 growth chambers with four treatments: control (ambient CO2 of 380 μmol mol⁻¹); BR (ambient CO2 of 380 μmol mol⁻¹ + 0.1 μM 24-epibrassinolide); elevated CO2 (CO2 of 760 μmol mol⁻¹); and elevated CO2 + BR (CO2 of 760 μmol mol⁻¹ + 0.1 μM BR). The aqueous BR solution was made with a very low level of ethanol (0.01, v/v) and sprayed onto the leaves at day 1 and day 5. After 7 days, plants were harvested for biomass and leaf area analysis according to Xia et al. (2009a). Meanwhile, a subset of samples were taken, frozen immediately in liquid nitrogen and stored at −80°C before biochemical and molecular analysis. Each treatment had 8 plants with four replicates.

#### 2.2. Leaf gas exchange and chlorophyll fluorescence analysis

Leaf gas exchange measurements were coupled with measurements of chlorophyll fluorescence using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (LI-6400-40 leaf chamber fluorometer; LI-COR, Inc.) on the 4th leaves. For all cases unless otherwise stated, gas exchange and chlorophyll fluorescence parameters were measured under the growth conditions at 25°C, 80% relative humidity, 1–1.3 kPa leaf-to-air vapor pressure deficit, 380 or 760 μmol mol⁻¹ CO2 and 400 or 800 μmol m⁻² s⁻¹ incident PPFD, respectively. 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). Quantum efficiency of PSII (ΦPSII), efficiency of excitation capture by open PSII center (Fm'/Fm), and photochemical quenching coefficient (qP) were calculated as $(F_m' - F_o')/(F_m' - F_o) / (F_m' - F_o)$ and $(F_m' - F_o')/(F_m' - F_o)$, respectively (Genty et al., 1989; van Kooten and Snel, 1990).

#### 2.3. Estimation of the rate of alternative electron flow

The rate of electron transport through PSII ($J_e$[PSII]) was measured as described by Harley et al. (1992). The rate of oxygenation by Rubisco ($V_o$) was estimated following von Caemmerer and Farquhar (1981) and the rate of carboxylation by Rubisco ($V_c$) was estimated as described by Miyake and Yokota (2000). Under atmospheric conditions, the electron fluxes in the two cycles can be expressed as $J_e$(PCR) = $4 \times V_o$ and $J_e$(PCO) = $4 \times V_o$, respectively (Kratz and Edwards, 1992). An alternative flux, $J_a$, caused by electrons that are not used by the PCR and/or PCO cycles in the total electron flux driven by PSII, can be estimated from $J_e$(PSII) – $J_e$(PCR + PCO) (Miyake and Yokota, 2000; Zhou et al., 2004).
2.4. Measurement of total chlorophyll, soluble protein, and carbohydrates

Total chlorophyll content was determined by the method of Arnon (1949). Total soluble protein content was measured using Bradford reagent (Bradford, 1976). Freeze-dried samples were used for the determination of carbohydrate content. Sucrose, starch, and hexasaccharides were determined using a modified phenol-sulphuric acid method (Buyssse and Merckx, 1993). Soluble sugars were extracted from 200 mg of dried material with 50 mL of 80% ethanol (v/v), 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 (v/v) to hydrolyze starch. The supernatant was analyzed for starch content.

2.5. RNA extraction and real time RT-PCR for 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 cDNA template for real time RT-PCR was synthesized using a RevertAid™ first strand cDNA Synthesis Kit (Fermentas) from 2 µg total RNA purified using RNeasy Mini Kit (Qiagen). On the basis of EST sequences, the following gene-specific primers were designed and used for amplification: rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), 5′-ACCGATGGCTTACAGCTT-3′ and 5′-ATTCGAAATCTCCAGACG-3′; rbcS (ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit), 5′-ATGGTTCCCTCGGTTGA-3′ and 5′-CCTGGAATGCTGGTCG-3′; RCA (ribulose-1,5-bisphosphate carboxylase/oxygenase activase), 5′-GCTGACACACCAACCA-3′ and 5′-CATCCGACCTACGAG-3′; SBPase (sedoheptulose-1,7-bisphosphatase), 5′-CAGGTTATATCAA-TGTAG-3′ and 5′-GGACGTAGGAGGAAAGC-3′; PRK (ribulose-5-phosphate kinase), 5′-ACAGGTTGTAATGATGC-3′ and 5′-TTGTTTGGATGAAGTTGCTG-3′; TPI (triose-3-phosphate isomerase), 5′-ACTCTCCTGCTTGCTGC-3′ and 5′-AATTTGATGGTTGCTAAC-3′; PGK (glycerate-3-phosphate kinase), 5′-GAAGAAAACCTTGAACCAACC-3′ and 5′-AGAGAATAACGAAACCGA-3′; FBPase (fructose-1,6-bisphosphatase), 5′-GGGAGAACCCAGAAAAA-3′ and 5′-GGCTTTAGATGCCAAGA-3′; actin, 5′-TGAGCTCTGGTGATGTCTTA-3′ and 5′-CAATGGGAGATGCCTGAAAA-3′.

Real time RT-PCR was performed with an iCycler iQ Multi-color Real-time PCR Detection System (Bio-Rad, Hercules, CA). Each reaction (20 µL) consisted of 1 µL of diluted cDNA, 10 µL SYBR Green Supermix, and 0.1 µmol of forward and reverse primers. PCR cycling conditions were as follows: 95°C for 3 min and 40 cycles of 95°C for 10 s, 54°C for 45 s. Fluorescence data were collected during the 54°C step. Cucumber actin gene was used as an internal control. Relative gene expression was calculated as described by Livak and Schmittgen (2001).

2.6. Rubisco activase, FBPase, PKG and PRK activity determination

The frozen sample was homogenized using a chilled pestle and mortar with cooled extraction medium containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM MgCl2, 12.5% (v/v) glycerol, 10% PVP, and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 x g for 15 min at 4°C. Rubisco activase activity was determined by using a Rubisco Activase Assay Kit (Genmed Sciences Inc., USA) according to the manufacturer’s instruction. FBPase activity was determined by measuring the increase in A430g using an extinction coefficient of 6.2 mM−1 cm−1 (Scheibe et al., 1986). Initial activity was assayed immediately after homogenization. The assay mixture consisted of 0.1 M Hepes–NaOH (pH 8.0), 0.5 mM Na2EDTA, 10 mM MgCl2, 0.3 mM NADP+, 0.6 mM Fructose-1,6-bisP, 0.6 U Glc-6-P dehydrogenase from baker’s yeast (Sigma, USA), 1.2 U Glc-P-isomerase from baker’s yeast (Sigma, USA), and 100 µL of enzyme extract in a final volume of 1 mL. The reaction was initiated by the addition of enzyme extract. The activity of PKG was determined according to Hatch and Kagawa (1973). The reaction mixture consisted of 100 mM Hopes-KOH (pH 7.8), 10 mM MgCl2, 1 mM NaF, 1 mM KH2PO4, 4 mM phosphoglyceric acid, and 4 unit per mL triosephosphate isomerase, and 4 units per mL glyceraldehyde-3-phosphate dehydrogenase. Reactions were initiated by the addition of enzyme extract. 2 mM ATP and 0.1 mM NADH. For the assay of PRK, aliquots of extract were diluted and assayed by coupling the formation of ADP to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase (Kagawa, 1982).

2.7. Statistical analysis

The experimental design was a completely randomized block design with four blocks. The measurements were replicated four times and randomly arranged in each block. Analysis of variance was carried out according to the general linear model procedure of statistical analysis system (SAS). Differences between treatment means were separated by the Tukey’s test at P < 0.05 levels.

3. Results

3.1. Effects of CO2 enrichment and BR on plant growth

An elevation in the atmospheric CO2 level from 380 to 760 µmol mol−1 resulted in a 20.5% and 16.0% increase in leaf area and shoot biomass accumulation, respectively (Fig. 1). Similarly, plants received a foliar application of BR at 0.1 µM exhibited 22.6% and 20.6% increase in leaf area and shoot biomass accumulation, respectively, relative to that of control. Significantly, a combined treatment of CO2 enrichment and BR application further improved the plant growth, resulting in 49.0% and 40.2% increase in leaf area and shoot biomass, relative to that of the control, respectively. CO2 enrichment and BR treatment alone or in combination, however, had no significant effects on root biomass, whereas CO2 enrichment and BR treatment together led to a slight increase in root biomass accumulation.

3.2. Effects of CO2 enrichment and BR on total chlorophyll, protein and carbohydrate

We subsequently determined the effects of CO2 enrichment and BR application on contents of chlorophyll, total protein, and carbohydrates. Leaf total chlorophyll, soluble protein and carbohydrate contents were measured at 10 d after the BR and CO2 enrichment treatments. As shown in Table 1, total chlorophyll content was not significantly influenced by BR treatment but increased by CO2 enrichment. In contrast, soluble protein content was not altered by CO2 concentration but significantly increased by BR treatment. The soluble protein content for the plants under elevated CO2 concentration with BR treatment were 21.9% higher than that of control, respectively. Total soluble sugar content and starch contents were all significantly increased by CO2 enrichment and BR application. A combination of BR and CO2 enrichment resulted in a greater increase in the total soluble sugar content and starch content which increased by 77.0% and 91.4%, respectively, relative to the control. However, sucrose and hexose did not differ by the BR and CO2 enrichment treatments.
Table 1
Effects of CO2 enrichment and BR application on total chlorophyll, soluble protein and carbohydrate contents in cucumber leaves. All the measurements were made on leaves at 7 d after respective 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chlorophyll content (µg cm⁻²)</th>
<th>Soluble protein content (g m⁻²)</th>
<th>Total soluble sugar content (mg g⁻¹ DW)</th>
<th>Sucrose content (mg g⁻¹ DW)</th>
<th>Hexose content (mg g⁻¹ DW)</th>
<th>Starch content (mg g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amb CO2</td>
<td>26.38 ± 3.64b</td>
<td>6.85 ± 0.71b</td>
<td>27.32 ± 3.22c</td>
<td>12.74 ± 1.62ab</td>
<td>17.60 ± 2.03ab</td>
<td>25.90 ± 1.11d</td>
</tr>
<tr>
<td>Elev CO2</td>
<td>36.21 ± 1.66a</td>
<td>7.20 ± 0.51b</td>
<td>36.26 ± 2.13b</td>
<td>10.29 ± 0.24b</td>
<td>15.48 ± 1.67b</td>
<td>33.56 ± 2.43c</td>
</tr>
<tr>
<td>Amb CO2 + BR</td>
<td>27.62 ± 2.66b</td>
<td>8.11 ± 0.20a</td>
<td>40.92 ± 4.57b</td>
<td>13.53 ± 0.97a</td>
<td>20.33 ± 2.34a</td>
<td>38.30 ± 0.91b</td>
</tr>
<tr>
<td>Elev CO2 + BR</td>
<td>39.24 ± 2.68a</td>
<td>8.35 ± 0.36a</td>
<td>48.36 ± 1.99a</td>
<td>11.08 ± 1.25b</td>
<td>18.18 ± 0.75a</td>
<td>49.57 ± 2.05a</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of CO2 enrichment and BR application on biomass and leaf area in cucumber plants. All the measurements were made on leaves at 7 d after respective 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.

3.3. Effects of CO2 enrichment and BR on CO2 assimilation and chlorophyll fluorescence quenching

We subsequently investigated the effects of CO2 enrichment and BR on gas exchange and chlorophyll fluorescence quenching. Leaf gas exchange parameters were determined using plants under the growth conditions at 5 d after the CO2 enrichment and BR treatment. As shown in Fig. 2, a doubling of atmospheric CO2 level resulted in 44.1% increase in CO2 assimilation rate (∆F0). Similarly, BR treatment also significantly increased CO2 assimilation under ambient atmospheric CO2 conditions and the increase was close to that by CO2 enrichment. Interestingly, plants with BR application under CO2 enriched conditions showed highest CO2 assimilation rate, which was increased by 77.2% relative to the control. Furthermore, CO2 enrichment decreased stomatal conductance (gₛ) whilst an opposed effect was observed in BR treatment. BR treatment had little effect on intercellular CO2 concentrations (Ci) under ambient and CO2 enrichment condition.

Chlorophyll fluorescence quenching parameters were also measured under the growth conditions. CO2 elevation had little effects on quantum efficiency of PSII (ΦPSII), photochemical quenching coefficient (qP) and efficiency of excitation capture by open PSII center (Fv/Fm) whilst BR treatment significantly increased ΦPSII and qP but had little effects on Fv/Fm (Fig. 2). ΦPSII and qp for the BR treatment under elevated CO2 condition were slightly higher than those of BR treatment alone but the differences for Fv/Fm were not significant. Apparently, the increase in ΦPSII induced by BR was mostly attributed to that in qP, but not to Fv/Fm.

We further examined the effects of CO2 enrichment and BR treatment on electron flux in PSII. The total electron flux in PSII \( J_0(PSII) \) was divided into electron flux for photosynthetic carbon reduction \( J_e(PCR) \), electron flux for photosynthetic electron oxidation \( J_e(PCO) \), and alternative electron flux \( J_e \). CO2 enrichment had no significant effects on \( J_e(PSII) \) but \( J_e(PCR) \) and \( J_e(PCO) \) were significantly increased by 25.6% and decreased by 48.9%, respectively (Table 2). In comparison, BR significantly increased \( J_e(PSII) \) and this increase was attributed to the increase both in \( J_e(PCR) \) and \( J_e(PCO) \). Plants exposed to elevated CO2 with BR treatment exhibited highest \( J_e(PSII) \) and \( J_e(PCR) \), and \( J_e(PCO) \) was higher than the plants with CO2 enrichment alone. Meanwhile, CO2 enrichment and BR treatment did not significantly influence \( J_e \) (Table 2).

3.4. Effects of CO2 enrichment and BR on transcripts of photosynthetic genes

To further examine how CO2 enrichment and BR regulate photosynthesis, we analyzed transcript levels of eight Calvin–Benson cycle genes. These tested photosynthetic genes included those encoding ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL), ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS), triose-3-phosphate isomerase (TPI), glyceral-3-phosphate kinase (PGK), fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SPBase) and ribulose-5-phosphate kinase (PRK) in leaves after CO2 enrichment and BR application alone or in combination. As shown in Fig. 3, transcript levels for all the tested genes were up-regulated by both CO2 enrichment and BR application. Among them, the increase in transcript level for \( RCA, rbcL, rbcS, SBPase \) and \( PGK \) were more significantly induced by BR than by CO2 enrichment whilst those for \( TPI \) and \( PRK \) in CO2-enriched plants were comparable to those in BR-treated plants. Significantly, a combination of BR and CO2 enrichment treatment resulted in a more significant increase in the transcript level for all the tested genes, indicating that there

\[ \text{Equation} \]
Table 2
Effects of CO₂ enrichment and BR application on total electron flux in PSII (Jₑ[PSII]), the electron flux for photosynthetic carbon reduction (Jₑ[PCR]), electron flux for photorespiratory carbon oxidation (Jₑ[PCO]) and alternative electron flux (Jₑₐ) in cucumber leaves at 5 d after respective 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Jₑ[PSII] (µmol e⁻¹ m⁻² s⁻¹)</th>
<th>Jₑ[PCR] (µmol e⁻¹ m⁻² s⁻¹)</th>
<th>Jₑ[PCO] (µmol e⁻¹ m⁻² s⁻¹)</th>
<th>Jₑₐ (µmol e⁻¹ m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amb CO₂</td>
<td>94.79 ± 0.96c</td>
<td>68.14 ± 1.56c</td>
<td>18.53 ± 0.84b</td>
<td>8.12 ± 1.68a</td>
</tr>
<tr>
<td>Elev CO₂</td>
<td>100.84 ± 6.89bc</td>
<td>85.61 ± 6.61b</td>
<td>9.46 ± 0.65d</td>
<td>5.77 ± 5.48a</td>
</tr>
<tr>
<td>Amb CO₂ + BR</td>
<td>110.91 ± 3.87b</td>
<td>85.32 ± 4.07b</td>
<td>22.88 ± 1.96a</td>
<td>2.71 ± 2.44a</td>
</tr>
<tr>
<td>Elev CO₂ + BR</td>
<td>124.71 ± 7.79a</td>
<td>102.40 ± 6.85a</td>
<td>11.44 ± 0.74c</td>
<td>10.87 ± 10.70a</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of CO₂ enrichment and BR application on CO₂ assimilation rate (Pₐ), stomatal conductance (Gₛ), intercellular CO₂ concentration (Cᵢ), the quantum efficiency of PSII (Φₑ[PSII]), photochemical quenching coefficient (qₑ) and the efficiency of excitation capture by open PSII centers (Fₚ/Φₑ[PSII]) in cucumber leaves. All measurements were made under the growing conditions on the 4th leaves at 4 d after CO₂ enrichment and BR 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.

was an additive effect at the transcriptional level between BR and CO₂ concentration.

3.5. Effects of CO₂ enrichment and BR on activity of enzymes involved in the Benson–Calvin cycle

To further examine how CO₂ enrichment and BR enhance CO₂ assimilation at the protein level, we analyzed the activities of RCA, FBPase, PGK and PRK after CO₂ enrichment or BR treatment for 4 days. As observed in the changes in CO₂ assimilation and the gene transcript level, both CO₂ enrichment and BR treatment increased the activity for RCA, FBPase, PGK and PRK (Fig. 4). Again, a combination of CO₂ enrichment and BR treatment resulted in a more significant increase in the activity of these enzymes relative to BR or CO₂ enrichment alone. The activity of RCA, FBPase, PGK and PRK for BR + CO₂ enrichment treatment were 84.7%, 59.4%, 89.2% and 83.8% higher than those of the control, respectively.

4. Discussion

Our study showed that both CO₂ concentration and BR had positive effects on plant growth and leaf photosynthesis but the mechanisms are different. CO₂ elevation and BR application alone both increased biomass accumulation, CO₂ assimilation rate,
Fig. 3. Effects of CO₂ enrichment and BR application on Benson–Calvin cycle genes expression in cucumber leaves. All the measurements were made on leaves at 24 h after the 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.
transcript levels and activity for enzymes involved in Benson–Calvin cycle but the effects were more significant in combination. CO2 elevation increased photosynthetic carbon reduction by depressing photosynthetic carbon oxygenation whilst BR increased both photosynthetic carbon reduction and photosynthetic carbon oxygenation followed by increased PSII photochemical activity. To our knowledge, this is the first report of an additive effect between BR and CO2 on photosynthesis.

It was hypothesized that there may be an additive effect of CO2 enrichment and BR on plant growth and CO2 assimilation. Generally, our results support this hypothesis. Elevated CO2 and BR alone all significantly increased biomass accumulation and CO2 assimilation but a combination of CO2 enrichment and BR treatment resulted in a greater increase in CO2 assimilation and biomass accumulation in this study. There are many reports about the positive effects of elevated CO2 on plant growth and CO2 assimilation (Drake et al., 1997; Amthor, 2001). In peanut, a doubling of ambient [CO2] enhances leaf photosynthesis by 27% and seed yield by 30% (Prasad et al., 2003). However, both down-regulation and up-regulation of photosynthesis were observed in different plant species under elevated CO2 condition. Many plant species when grown for long periods in elevated CO2 exhibited decreased photosynthetic capacity, an acclimation response to elevated CO2 (Drake et al., 1997; Amthor, 2001). In cucumber, the increase in photosynthesis together with an increase in growth rate has been reported (Klaring et al., 2007). In this study, increases both in biomass accumulation and photosynthetic rate were observed under elevated CO2 condition. This finding was largely in agreement with these prior results.

An increase in BR level or the enhance of its signal component has been shown to increase photosynthesis in cucumber, tomato and rice (Yu et al., 2004; Wu et al., 2008; Xia et al., 2009a). In agreement with our early studies, BR significantly increased plant biomass accumulation and CO2 assimilation (Figs. 1 and 2). It is interesting to note that BR increased the biomass accumulation and CO2 assimilation to a degree similar to that by CO2 elevation. These results not only supported the important role of CO2 enrichment and BR in the regulation of photosynthesis, but also indicated the feasibility for the enhancement of plant growth and CO2 assimilation by their combined treatment since BR application is a convenient practice. The alleviative effects of CO2 enrichment and BR on other abiotic stress-induced decrease in photosynthesis have been frequently reported, to our knowledge, it is the first study reporting the additive effects of CO2 and BR on photosynthesis and plant growth under optimal growth conditions.

The positive effects of CO2 enrichment on CO2 assimilation are largely attributed to the inhibited oxygenation reaction of RuBP by Rubisco (Bowes, 1993; Drake et al., 1997). In supporting this, results from chlorophyll fluorescence analysis showed that $\Phi_{\text{PSII}}$ was not significantly influenced by CO2 elevation. A further measurement of electron allocation in PSII revealed that an elevation of CO2 from 380 to 760 µmol mol$^{-1}$ significantly increased $J_e$(PCR) but decreased $J_e$(PCO), but had no significant effects on $J_a$. Depression of photosynthesis by CO2 elevation has been well reported (Bowes, 1993; Drake et al., 1997). There are also evidences that CO2 elevation decreases nitrate reduction and oxidative stress in leaves which are driven by different alternative electron flux pathways (Zhou et al., 2004; Vurro et al., 2009; Bloom et al., 2010). However, we observed only a slight but not significant decrease in $J_a$ in plants raised at elevated CO2 conditions. Taken together, the increase in CO2 assimilation rate for the CO2 enrichment was attributable to the increased carboxylation at the expense of depressed
oxygenation by Rubisco (photospiration). In contrast, BR treatment resulted in a significant increase in ΦPSII and this increase was accompanied with a increase both in jps(Pet) and jps(PCO), and little changes in ja, suggesting that BR increased CO2 assimilation rate by increasing the activity of both carboxylation and oxygenation by Rubisco, leading to an increased demand for ATP and NADPH followed by an increased ΦPSII. Accordingly, BR increased CO2 assimilation by a path different to that for CO2 enrichment.

The molecular processes of elevated CO2 driven photosynthetic gene expression in plants are not well understood. In contrast with the mature leaves, we did not observe a down-regulation of both photosynthetic gene transcripts and enzyme activity under elevated CO2 condition in young plants. Carbohydrate source–sink balance under growth at elevated CO2 is believed to play a major role in the regulation of photosynthesis through feedback inhibition (Arp, 1991; Stitt, 1991). It has been repeatedly reported that long-term exposure to elevated CO2 resulted an accumulation of carbohydrates together with reduced transcript level of photosynthetic genes or activity of the related enzymes and this process is also associated with accelerated leaf senescence (Ludewig and Sonnewald, 2000). It is worth to note that the occurrence of down-regulation of photosynthetic gene transcripts and enzyme activity at elevated CO2 condition was largely dependent on plant and leaf developmental stage. In sorghum, the positive response of CO2 assimilation to elevated CO2 was greater in young leaves than in old leaves and elevated CO2 enhanced Rubisco activity in young leaves (Prasad et al., 2003). CO2 elevation led to an increase in photosynthesis and up-regulation of a series of photosynthesis-related genes and enzymes in sugarcane and populus (Vu et al., 2001, 2006; Taylor et al., 2005; De Souza et al., 2008). Fukayama et al. (2009) have reported down-regulation of genes associated with CO2 assimilation and up-regulation of genes encoding Rubisco activase, RuBP regeneration and starch synthesis in the leaves of rice grown under elevated CO2. Little decrease or even slight increase was observed in rbcS transcript level in the expanding leaves when the plants were switched from ambient CO2 condition to elevated CO2 condition (Gesch et al., 1998). Although both CO2 enrichment and BR induced an accumulation in carbohydrates, this did not lead to depression in CO2 assimilation, gene transcript and enzyme activity in young leaves of cucumber. Plants at an early developmental stage are expected to have reduced potential for acclimation to CO2 and loss of Rubisco activity or content due to higher sink capacity (Melkonian et al., 2005).

We have previously shown that enhancement of photosynthetic capacity by BR is associated with increased transcript levels for genes involved in Benson–Calvin cycle and increase in the Rubisco activation state (Xia et al., 2009a). Consistent with our earlier studies, we found significant increases in the transcript levels for the eight genes and in the activity for the four tested enzymes in the Benson–Calvin cycle. The mechanism by which BR improve photosynthesis is not well known. Recently, we found that BR could trigger a transient generation of reactive oxygen species (ROS) which may increase plant tolerance and CO2 assimilation by modifying redox signal system (Xia et al., 2009b, unpublished data). In agreement with the changes in CO2 assimilation, gene transcription and enzyme activity showed an additive response to CO2 enrichment and BR. Recently, there are accumulating reports about the alleviative effects of CO2 and BR alone on plant tolerance to stresses such as salt, low temperature, and ozone (Krishna, 2003; Ogweno et al., 2008; Xia et al., 2009b; Mateos-Naranjo et al., 2010). Since BR and CO2 enrichment in combination showed more positive effects on cucumber plants, it will be interesting to study these interactive effects of CO2 enrichment and BR under different stress conditions.

BR has been considered as one of the most potential plant hormones in regulating plant productivity (Divi and Krishna, 2009); it is, however, unlikely that BR operate alone in regulating plant productivity. Plant growth is known to be regulated by a set of plant hormones which is central to the integration of diverse environmental cues with plant’s genetic program. There are evidences that CO2 elevation increases the level of IAA, GAs, CTK and ethylene in plants, the relation between CO2 level and BR biosynthesis, however, is unknown. Plant hormones interact synergetically and/or antagonistically in many physiological processes such as hypocotyl elongation, seed germination and fruit development by affecting synthesis, transport or response such as the gene expression and the type of interaction often depends on the tissue, developmental stage and environmental conditions (Chen et al., 2004; Hardtke, 2007; Artseca and Artseca, 2008; Santner and Estelle, 2009; Zhang et al., 2009). It is, therefore, interesting to study the effects of CO2 elevation on BR biosynthesis and the interaction between BR and other hormones in terms of gene expression, enzyme activation and modification involved in photosynthesis under control and elevated CO2 conditions by using mutants for the signaling pathways of BRs and other hormones.

5. Conclusions

Our data demonstrated that young cucumber plant did not experience photosynthesis acclimation under CO2 enrichment condition. Both CO2 elevation and BR had positive effects on CO2 assimilation but they increased CO2 assimilation by different mechanisms. Furthermore, CO2 and BR had additive effects on plant growth, CO2 assimilation, photosynthetic gene transcripts and enzyme activity. Since young plants were used in the present study, it remains to be studied that whether BR could relieve the down-regulation of photosynthetic capacity induced by a long-term CO2 enrichment in mature leaves.

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