Study on Brassinosteroid-Enhanced Sugar Accumulation in Cucumber Epicotyls

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Abstract: Exogenous epibrassinolide (EBR) treatment promoted elongation of cucumber epicotyls and at the same time, enhanced sugar accumulation in the epicotyls. This accumulation was concomitant with the elongation. Accordingly, it was suggested that the sugar accumulation is closely related to the promotion of elongation. Furthermore, the components of the soluble sugar were researched. By EBR treatment, the amount of glucose was remarkably increased, although sucrose was not affected. The amount and transport of 14C-assimilates in EBR-treated cucumber were analyzed. EBR treatment had no effect on the amount of 14CO2 assimilation but they promoted the transport of 14C-photosynthates to epicotyls. In addition, the amount of 14C-labeled glucose transported to epicotyl was remarkably increased. In conclusion, these changes enhanced by EBR seem to be a cause of glucose accumulation in cucumber epicotyls.

Key words: Brassinosteroid, Cucumber, Elongation, Epibrassinolide, Epicotyl, Sugar accumulation

Brassinosteroids (BRs) are a plant growth-regulating substance with steroidal lactone. It is known that BRs have various physiological activities and are widely distributed in plant kingdom. Today, BRs are recognized as a plant hormone.

BRs promote elongation of growing hypocotyls, epicotyls and petiole of various plants. In this study, it was found that epibrassinolide (EBR) of BRs, promoted elongation and sugar accumulation of cucumber epicotyls. The sugar accumulation occurred at the same time as the elongation. Accordingly, it was suggested that there is a close relation between them. It has already been reported that exogenous soluble sugars enhanced elongation of detached epicotyls of Pismum sativum grown in darkness. Soluble sugars seem to play an important role in promoting of elongation. This study was carried out to analyze the accumulation and transport of 14C-sugars in EBR-treated cucumber epicotyl.

Materials and Methods

1. Plant materials

Seeds of cucumber (Cucumis sativus L. cv. Tokiwajibai) were sown in vermiculite under natural light in a greenhouse which temperature was kept at 20-32°C. After 7 to 10 d, young seedlings were transferred to solution culture with nutrient solution of Otsuka No. 1 and No. 2 (Otsuka Kagaku Co., Ltd.) medium. After 3 to 5 d, seedlings of about 20 mm length epicotyl were selected for next experiments.

Epibrassinolide ((22R, 23R, 24R)-2α, 3α, 22, 23-tetrahydoroxo-B-homo-7-oxa-5α-ergostan-6-one) was donated from Nippon Kayaku Co., Ltd. EBR was stored in 99.5% ethanol and was diluted as required for individual experiments. Roots of 10 seedlings were immersed in either 200 ml of aqueous epibrassinolide solution (0.1 μg mL -1) or the same volume of distilled water. They were placed in...
a growth chamber of 14 h of light (light intensity at leaf surface, 33 W m⁻²) and 10 h of dark, at 25°C. After appropriate incubation, the length and fresh weight of the epicotyls were measured, and then the epicotyls were used for the following analyses.

2. Determination of the amount of soluble sugar

The epicotyls were homogenized in 0.1 M Tris-HCl buffer (pH 7.5) with a mortar and pestle on ice. The homogenate was centrifuged at 2000 g for 20 min at 4°C. The supernatant was used for determination of the amount of soluble sugar by the phenol-sulfuric acid methods.²⁹

3. Analysis of sugars by gas chromatography (GC)

The epicotyls were homogenized in 80% ethanol, extracted at 80°C for 30 min and then centrifuged at 2500 g for 10 min. After decanting the supernatant, two more extractions were made. Three supernatant fractions were combined and evaporated at 40°C under reduced pressure. According to the method of Sweeley et al.,²⁸ the sample was trimethylsilylated for identification of sugars by GC. The silylation agent was a mixture of pyridine, trimethylchlorosilane and hexamethyldisilazane (10 : 2 : 1, v/v/v). The column was a 2 m-long glass tube with inner diameter of 1.8 mm filled with 2% Silicone OV-17 on 80/100 Chromosorb WAW DMCS. The column temperature was increased from 140°C to 170°C with a temperature gradient of 2°C/min and from 170°C to 270°C with a temperature gradient of 4°C/min. Henicosane was used as internal standard for determination of the amounts of sucrose, glucose and fructose.

4. Determination of the amount of starch

Starch in the dried residue was hydrolyzed according to the method of Emerson and Koller³⁰ and its amount was determined by the method of Nelson-Somogyi.³¹

5. Assay of phosphorylase activity

The epicotyls were homogenized in 5 mM Tris-HCl buffer (pH 7.5) on ice, and the homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was used as crude enzyme. The assay was made according to the method of Kamogawa et al. with slight modification.

6. ¹⁴CO₂ assimilation

After 4 h of EBR treatment, two EBR-treated plants and two control plants were exposed to ¹⁴CO₂ in a acrylic exposure chamber. The effective volume of the chamber was 2,500 cm³. A schale containing 37 kBq of [¹⁴C] Na₂CO₃ was kept inside the chamber. The light intensity at leaf surface was 100 W m⁻² and the temperature was 25–30°C. The ¹⁴CO₂ was generated by injecting 20% acetate acid, and an electric fan was kept inside the chamber to mix the ¹⁴CO₂ in the chamber. An exposure was for 10 min. After the exposure, the plants were harvested soon or after 6 h or 20 h of chase period (Fig. 4). For the chase period, they were placed in the growth chamber described above. The harvested plants were separated into different tissues. After the fresh weight of them was measured, they were cut into small pieces and dried at 60°C overnight. Each of the pieces was added to 10 ml of scintillation fluid (Toluene-PPO) and the radioactivity was counted with a liquid scintillation counter (Beckman, LC 6000 SC).

7. Fractionation of ¹⁴C-photosynthates

The photosynthates were fractionated into different components by using ion-exchange chromatography according to Dickson² with some modifications. ¹⁴C-labelled plants were made using the same method described in the paragraph of ¹⁴CO₂ assimilation, but 74 kBq of [¹⁴C] Na₂CO₃ was used instead of 37 kBq of [¹⁴C] Na₂CO₃. An exposure was 20 min and the chase period was 20 h. Five-tenths g fresh weight of the epicotyls of the ¹⁴C-labelled plants was frozen in liquid N₂, and they were extracted in 6 mL methanol : chloroform : water (12 : 5 : 3, v/v/v). The contents were centrifuged and this extraction process was repeated twice. The pellet was dried at 60°C overnight and referred as the residue fraction. The combined supernatants were added chloroform and water. After centrifuged, two phases were formed. The bottom chloroform layer was referred as the fraction of pigments and lipids and the top methanol-water layer was further fractionated. A cation exchanger column (Dowex 50-X8) was arranged over an anion exchanger column (Dowex I-X8). With the columns, the methanol-water fraction was separated into the fractions of sugars, amino acids and organic acids. These fractions were evaporated to desired volume at 40°C under
Fig. 1. Effects of EBR treatment on elongation of cucumber epicotyls. Values are the means ± S.D. \( n = 6 \).

Fig. 2. Effects of EBR treatment on soluble sugar contents of cucumber epicotyls. Values are the means of 4 or more replications ± S.D.

Fig. 3. Effects of EBR treatment on glucose and sucrose contents of cucumber epicotyls.

reduced pressure. The radioactivity in each fraction with scintillation fluids (Toluene-PPO or Scintisol 500) was measured using the liquid scintillation counter described above.

8. Separation into sucrose, glucose and fructose

After the fraction of sugars has been concentrated by drying and redissolving in a little 50% ethanol, it was separated into sucrose, glucose, fructose and others by thin-layer chromatography (TLC). TLC was carried out on a glass plate coated with cellulose. The sample was developed with n-butanol: acetic acid : water \((12:3:5, v/v/v)\) for about 3h. Diphenylamine-aniline-phosphoric acid was used for detection. Each spot on the TLC plate was scraped and counted by the liquid scintillation counter with scintillation fluid (Toluene-PPO).

Results

EBR treatment stimulated elongation of cucumber epicotyls (Fig. 1). The stimulation of elongation began after 6 h of EBR treatment and the effect was biggest after 12–24 h of the treatment and became smaller after 48 h. On the other hand, EBR treatment enhanced soluble sugar accumulation in epicotyls (Fig. 2). The increase of the soluble sugar content by EBR treatment was biggest after 12–24 h of EBR treatment and was concomitant with the stimulation of elongation. GC analysis of the sugars in epicotyls revealed that sucrose, glucose, fructose and myo-inositol were the major sugars. EBR treatment remarkably increased the amount of glucose and also minor increase of fructose amount, but sucrose was not affected by EBR (Fig. 3).

The increase of glucose may be indebted to EBR-stimulated enzymolysis of starch. Therefore, the effects of EBR treatment on the amount of starch and phosphorylase activity were researched. EBR treatment had little effect on the amount of starch per epicotyl and the phosphorylase activity was not affected by EBR treatment (data not shown).

In order to know whether EBR treatment promoted the photosynthesize transpor to epicotyl, effects of EBR treatment on \(^{14}C\) distribution to the different plant tissues were researched. Fig. 4 shows the time course of this experiment. EBR treatment had little effect on the amounts of \(^{14}C\)-assimilates by photosyn-
Table 1. Effects of EBR treatment on $^{14}$C distribution in major biochemical fractions of cucumber epicotyls 20 h after $^{14}$CO$_2$ assimilation. Values are expressed as $10^3$ dpm/g fresh weight and percentage of total radioactivity in epicotyl.

<table>
<thead>
<tr>
<th>Chemical fraction</th>
<th>Radioactivity ($\times 10^3$ dpm/gFW)</th>
<th>Control (%)</th>
<th>EBR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars</td>
<td>25.6 (9.3)</td>
<td>46.7 (13.4)</td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td>11.3 (4.1)</td>
<td>22.3 (6.4)</td>
<td></td>
</tr>
<tr>
<td>Organic acids</td>
<td>46.5 (16.9)</td>
<td>57.9 (16.6)</td>
<td></td>
</tr>
<tr>
<td>Pigments and lipids</td>
<td>14.7 (5.3)</td>
<td>30.1 (8.6)</td>
<td></td>
</tr>
<tr>
<td>Residue</td>
<td>177.0 (64.3)</td>
<td>191.2 (54.9)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>275.1 (100)</td>
<td>348.1 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Effects of EBR treatment on $^{14}$C distribution in different plant tissues of cucumber 0 h (A), 6 h (B) and 20 h (C) after $^{14}$CO$_2$ assimilation. The radioactivity in each tissue is expressed as the percentage of total radioactivity recovered. Values are the means of 4 or more replications ± S.D.

Fig. 6. Effects of EBR treatment on $^{14}$C distribution in neutral sugars of cucumber epicotyls 20 h after $^{14}$CO$_2$ assimilation.

thesis. Soon after $^{14}$C assimilation, radioactivity was distributed only to the primary leaf and the cotyledon (Fig. 5-A). Six h after $^{14}$C uptake, radioactivity was distributed to all tissues of the plant, but EBR treatment had only a little effect on the distribution (Fig. 5-B). But 20 h after the assimilation, the percentage of radioactivity in the epicotyl was remarkably increased by EBR treatment (Fig. 5-C).

$^{14}$C-compounds transported to the epicotyl were analyzed, that is, effects of EBR treatment on $^{14}$C distribution in the five chemical fractions were researched. EBR treatment especially increased each radioactivity of sugars, amino acids and pigments and lipids (Table 1). While each radioactivity of organic
acids and residue was slightly increased.

Furthermore, the effects of EBR treatment on $^{14}C$ distribution in sucrose, glucose, fructose and others were researched in detail. EBR treatment remarkably increased the radioactivity of glucose and fructose in epicotyls, but had little effect on sucrose and others (Fig. 6).

**Discussion**

In this study, it became obvious that exogenous EBR treatment enhanced sugar accumulation in cucumber epicotyls during elongation. Besides, EBR-enhanced increase of soluble sugar was concomitant with promotion of the elongation. So it is presumed that the sugar accumulation is strongly connected to the elongation stimulated by EBR treatment. Glucose and fructose were increased by EBR, while sucrose was hardly affected. The big increase of glucose of soluble sugar was attributed to the sugar accumulation in epicotyls.

Gibberellin (GA) stimulated elongation of the subhook of *Pisum sativum* epicotyl and also enhanced sugar accumulation$^{12,13}$. GA enhanced sugar accumulation in internode of *Phaseolus vulgaris* grown in light during promoting of elongation$^{8,10}$; the concentration of hexose sugars as glucose and fructose was increased, but sucrose decreased. The possibility of rate-limiting in cell enlargement *in vivo* by the availability of hexose is supported by observations of Broughton and McComb$^{1}$ that glucose or glucose-1-phosphate can mimic the effects of GA on cell and internode growth in *Pisum sativum*. Sucrose was much less effective. These authors concluded that the principal effect of GA was to increase the provision of hexose substrates through its action on enzyme activity. In short, hexose increased with GA enhances elongation. Also in the process of EBR-enhanced sugar accumulation, increase of hexose sugars as glucose and fructose was observed, and it is possible to think that the increased hexose enhanced elongation just as GA.

Starch amount per epicotyl and activity of phosphorylase related with starch metabolism were not affected by EBR treatment. Therefore, EBR-enhanced soluble sugar accumulation is directly unrelated to enzymolysis of starch in epicotyls.

As mentioned above, sugar accumulation is an important factor of GA-enhanced elongation which was caused by promoting of sugar transport to growing tissues. In *Phaseolus vulgaris* grown in light, GA promoted transport of $^{14}C$-labelled sucrose from primary leaf to growing internode$^{10}$. In seedlings of *Pisum sativum* grown in darkness, sugar transport from cotyledon or other mature tissues to growing subhook of the epicotyl was promoted by GA$^{11}$. It was possible that the EBR-enhanced increase of soluble sugar in epicotyls during the promoting of elongation was also due to promoting of sugar transport to epicotyl, because the effects of EBR treatment in this study were similar to those of GA. The amount of $^{14}CO_2$ assimilates were hardly affected by EBR treatment. Twenty h after $^{14}CO_2$ assimilation, EBR treatment, however, increased the percentage of radioactivity in epicotyl. These results indicate that the transoort of $^{14}C$-assimilates to epicotyl was promoted by EBR treatment. Radioactivity of the sugar fraction was also increased by EBR treatment. It was mainly due to the increase of radioactivity in glucose.

Therefore, it is clear that the transport of sugar created newly by photosynthesis to epicotyl was promoted by EBR treatment, and also at the epicotyl, the amount of glucose in the sugar was increased by EBR treatment. In conclusion, these changes enhanced by EBR treatment seem to be a cause of glucose accumulation which plays an imortant role in promoting of elongation in cucumber epicotyls.

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