한국생물공학회지 제 11권 제 3호
Korean J. Biotechnol. Bioeng.
Vol. 11. No. 3. 295-302(1996)

Kidney Bean에서의 고 CO_2 농도에 의한 Rubisco의 Activation과 Carbamylation의 감소

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Decrease of the Activation and Carbamylation of Rubisco by High CO_2 in Kidney Bean

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ABSTRACT

The measurements of rubisco parameters are important in photosynthetic studies. In this experiment, we used photometric assay method to detect these major parameters, such as activity, carbamylation and amount of rubisco. The main advantages of this method are very simple and as sensitive as conventional methods which usually produce radioactive waste. In this study, with kidney bean (*Phaseolus vulgaris* L.) leaves grown at normal CO_2 (350 ppm) and high CO_2 (650 ppm), we investigated the effect of CO_2 concentration on activation and carbamylation of rubisco by measuring the rubisco activity, carbamylation rate and amount of rubisco using a dual beam (334 nm and 405 nm) spectrophotometer, and analyzed the polypeptide profiles of rubisco by SDS-PAGE. When CO_2 concentration was raised from 350 ppm to 650 ppm, all parameters of rubisco were decreased : 41.2 $\mu M/m^2/s$ and 52.2 $\mu M/m^2/s$ to 27.4 $\mu M/m^2/s$ and 46.1 $\mu M/m^2/s$ for initial and total rubisco activity, respectively; from 79% to 58.9 % for carbamylation rate; from 1.94 $\mu M/m^2$ to 1.58 $\mu M/m^2$ for amount of rubisco. These results suggest that the decrease in rubisco activity at high CO_2 was caused by carbamylation. The analysis of the preparation by SDS-PAGE showed two major polypeptides at 50 and 14.5 kD which were identified as the large and the small subunits of rubisco. There were no differences in the intensity compared high CO_2 to normal CO_2 in both 50 kD and 14.5 kD bands. We also found that these inhibitory effects of CO_2 were reversible. When high CO_2 was switched to normal CO_2, the parameters of rubisco changed were almost the same as normal rubisco parameters. These data provide an evidence that activity of rubisco was recovered by CO_2 concentration of 350 ppm.

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INTRODUCTION

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) comprises up to 50% and 70% of the soluble leaf protein in plants(1, 2) and of the total soluble protein in the stroma (3), respectively. The ribulose 1,5-bisphosphate (RuBP) as the substrate and 3-phosphoglycerate as the product of the carboxylase reaction of rubisco are the main sugar-phosphates in chloroplasts, which are important organic substances for development and growth of plant(4).

The bifunctional enzyme, rubisco catalyzes both the carboxylation of RuBP, which is the initial step in photosynthetic carbon reduction, and its oxygenation, which is the first reaction of the photorespiratory carbon oxidation cycle(5).

The enzyme rubisco is activated by one ion of Mg^{2+} and two molecules of activator CO_2(2, 6, 7) which is distinct from the substrate CO_2(8, 9). The activator CO_2 has been trapped on the large subunit of rubisco, and the metal ion has been bound directly to amino acids of the large subunit (7, 10, 11). A carbamate is formed by the reaction of the ε-amino group of Lys-201 within the active site on the large subunit with CO_2 in a slow and reversible fashion, followed by a rapid binding of Mg^{2+} to the carbamate in a second reversible reaction to form the active ternary complex(12). Although this process can occur spontaneously *in vitro*, rubisco activation *in vivo* is catalyzed by rubisco activase(13) in an ATP-dependent reaction(14).

Phosphate is also an essential factor for the activation and the carbamylation of rubisco by enhancing the binding of the activator CO_2 to the active sites both *in vivo* and *in vitro*(15-21).

The binding of inhibitors such as CA1P (carboxyarabinitol 1-bisphosphate) and CABP (carboxyarabinitol 1,5-bisphosphate) to catalytic sites has been shown to regulate the activity of rubisco by blocking catalysis(22, 23). CABP provides an excellent tool for determining the number of reaction sites on rubisco. It binds tightly and specifically to the active enzyme-CO_2-Mg^{2+} sites of rubisco. Although CABP does bind to both activated sites and inactive sites, the binding is not nearly as tight to inactive sites(24).

The measurements of rubisco parameters, that is, activity, carbamylation and amount of rubisco, are important to photosynthesis studies. These characteristics of rubisco are measured under several different environmental conditions which include changes in photon flux density (PFD), partial pressure of CO_2 and partial pressure of O_2. However, it is unclear whether the effects of environmental factors on the fluctuation of rubisco activity are mediated by carbamylation or amount of rubisco enzyme. To pursue the answer to this question, we studied the effect of CO_2 on the activity, carbamylation rate and amount of rubisco using a dual beam spectrophotometer, and analyzed the polypeptide profiles of rubisco by SDS-PAGE.

MATERIALS and METHODS

Plant culture

Kidney bean (*Phaseolus vulgaris* L.) plants were grown in Conviron E-15 growth chambers. Plants were illuminated by metal-halide plus incandescent lamps at a peak level of 800 (pot level) to 1,200 (top of mature plants) $\mu M/m^2/s$ PFD, 350 ppm (normal) and 650 ppm (high) CO_2 for the entire 24-hr period, and 26℃ for the 16-hr day and 18℃ at night with a relative humidity of 60%. Plants were watered manually with half-strength Hoagland's solution(25) everyday. Plants were grown in 6L pots using a soil-less potting mix (Metromix 360; W. R. Grace and Co., Cambridge, MA, U.S.A.).

Chemical syntheses

The RuBP used in this study was synthesized enzymatically from ribose 5-phosphate, phosphoribuloisomerase, ATP, and phosphoribulokinase as described by Horecker *et al.*(26). Briefly, 100 mM ATP was added and 1 unit/ml phosphoribuloisomerase and phosphoribulokinase, and 4 mM DTT (final concentration) were added to

5 ml of 100 mM ribose 5-phosphate and 100 mM $MgCl_2$ solution, pH 6.8. After the reaction was completed, the solution was acidified with 3.5% $HClO_4$, kept on ice for 5 min, and then centrifuged for 5 min to remove the enzymes. The pH was adjusted to 5 with 2 M KOH, 150 mM HEPES, and 10 mM KCl. After removal of potassium perchlorate by centrifugation, the synthesized RuBP was kept at −80℃.

As described by Collatz *et al.*(27), to make CABP, RuBP was reacted with 10% molar excess of NaCN at room temperature. After 16 hrs of reaction, 88% formic acid was added to stop the reaction, and the mixture was dried under vacuum in a rotary evaporator.

All chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S. A.).

Extraction of leaves for rubisco analysis

Leaf punches, around 1.5 cm^2 were frozen and held at −80℃ until analyzed. For analysis, the leaf piece was ground in a Ten-Broeck glass homogenizer with 1 M DTT, 0.1% Triton X-100 (v/v), 1.5% insoluble PVPP (w/v) and 2 ml extraction buffer. The extraction buffer was 100 mM Bicine pH 7.8, 20 mM $MgCl_2$, 4 mM amino *n*-caproic acid, 0.8 mM benzamidine, 1 mM EDTA, 0.02% BSA (w/v), 150 μM $NaHCO_3$. The resulting homogenized solution was transferred to a microcentrifuge tube and centrifuged for 10 sec at 8,000 g in a Beckman microfuge tube. Initial extracts were assayed to determine initial and total rubisco activity. Care was taken to keep all solutions as close to 0℃ as possible during the preparation of the samples for initial and total activity measurement.

Assay of rubisco activity

Following the procedure of Sharkey *et al.*(28), the activity was measured by coupling the activity of rubisco to NADH oxidation using phosphoglycerate (PGA) kinase and glyceraldehyde-3-phosphate (GAP) dehydrogenase. The oxidation of NADH was measured in a dual beam spectrophotometer (Sigma ZFP 22).

For the assay of initial rubisco activity, 5 μL of initial extract was added to a spectrophotometer cuvette containing 745 μL assay buffer at 25℃. The assay buffer was 50 mM Bicine pH 8.0, 15 mM $MgCl_2$, 1 mM EDTA, 2 M NaCl, 1 M $NaHCO_3$, 1 M DTT, 20 mM RuBP, 10 mM NADH, 0.5 M phosphocreatine, 1 unit/μL of phosphocreatine kinase, 0.5 M ATP, 1 unit/μL of PGA kinase and GAP dehydrogenase. The assay solution was allowed to stand. After mixing for 5 min, optical density was read at 334 nm and at 405 nm.

Extracts for the measurements of total activity and amount of rubisco were prepared by adding 10 μL of 1 M $NaHCO_3$ and 20 μL of 1 M $MgCl_2$ to 1 mL of initial extract mentioned above. For the assay of total rubisco activity, 5 μL of the extract plus 745 μL of assay buffer were mixed to read OD values. For the measurement of rubisco amount, different amount of 5 μM CABP was added to 100 μL of mixture of initial extracts for total rubisco activity assay before reading OD values.

To calculate the rubisco activity we used the following formula :

$$OD\ A_{334} - A_{405} \times \frac{mol}{extinction\ coefficient} \times \frac{assay\ vol}{sample\ vol} \times \frac{extraction\ vol}{area\ punch} \times \frac{1}{2} \times \frac{1}{60sec}$$

in which extinction coefficient is 6,190L/mol, assay volume is 750 μL, sample volume is 5 μL, extraction volume is 2 mL, and punch area is 1.5 cm^2.

Purification of rubisco

Rubisco was purified from kidney bean leaves as described by Wang *et al.*(29). Kidney bean leaves (100g) were deribbed, frozen in liquid nitrogen, and ground to a fine powder. The frozen leaf powder was slowly added to 150 mL of extraction buffer [20 mM BTP (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA, 0.4 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 0.01 mM

leupeptin] with continuous stirring after the ice was melted, and the solution could be filtered. Then the leaf slurry was filtered through two layers of cheesecloth and one layer of Miracloth and centrifuged for 15 min at 30,000g. $(NH_4)_2SO_4$ powder was added slowly into the supernatant fluid with continuous stirring to 35% saturation. After 30 min, the supernatant of centrifugation for 10 min at 8,000 g was brought to 55% saturation of $(NH_4)_2SO_4$ by addition of the salt powder. The precipitate was collected by centrifugation for 10 min at 10,000g. The pellet was dissolved in 10 mL of buffer A [40 mM Tris (pH 7.5), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, and 10 mM DTT], and PEG-10,000 was added to a final concentration of 17%. The resulting precipitate was collected by centrifugation for 10 min at 10,000g, resuspended in 10 mL of buffer A, and loaded onto a 100 mL Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. The column was eluted with 150 mL of the same buffer containing 0.1 M NaCl at a flow rate of 2 mL/min before continuing with 400 ml of a linear gradient from 0.1 to 0.5 M NaCl. Fractions of rubisco with more than 95% purity were pooled and used for SDS-PAGE.

All purification procedures were carried out at 4℃.

Electrophoresis

SDS-PAGE was performed with 13% gels using the method of Laemmli(30). Gels were stained with Coomassie blue R 250 to visualize the proteins.

RESULTS AND DISCUSSION

CO_2 serves as an environmental stimulus which regulates development in higher plant. The activity of rubisco *in vivo* is modulated to maintain the efficiency of CO_2 fixation under environmental conditions such as light(31), partial pressure of CO_2(32) and partial pressure of O_2(33). In this study, we have investigated the effect of high CO_2 concentration on rubisco activity by measuring several parameters including initial and total activity upon extraction, carbamylation rate, and the total amount of enzyme present in a leaf. Photometric assay method(28) was employed in measuring these parameters because this method is simple and does not use radioactive isotopes.

Table 1. Initial and total activities of rubisco determined on leaves of kidney bean in normal and high CO_2. The concentration of CO_2 for the normal and the high CO_2 treatment were 350 ppm and 650 ppm, respectively. Tissue was sampled at 50 days after seedling in the middle of the light period; 10 leaves were sampled per plant. *Values represent the mean ±SE.

Parameters	Normal CO_2	High CO_2
Initial activity ($\mu M/m^2/s$)	41.2±3.3	27.4±5.9*
Total activity ($\mu M/m^2/s$)	52.2±4.0	46.1±2.1

As shown in Table 1, total activity is higher than initial activity in both normal and high CO_2 concentration. The addition of CO_2 and Mg^{2+} results in activation of inactive rubisco by binding Mg^{2+} to rubisco-CO_2 complex.

When CO_2 concentration was raised from 350 ppm to 650 ppm, rubisco activity in high concentration of CO_2 was lower than that in normal concentration of CO_2. In normal CO_2 concentration, initial and total rubisco activity were 41.2 and 52.2 $\mu M/m^2/s$, respectively. In high CO_2 concentration, however, initial and total rubisco activity were decreased to 27.4 and 46.1 $\mu M/m^2/s$, respectively(Table 1). Decrease in rubisco activity at high CO_2 concentration was also observed in transgenic tomato by Micallef *et al.* (34): Sucrose phosphate synthase-transformed line (SSU-9) and kanamycin-resistant control line (Tcon-1). Vu *et al.*(35) reported that there were some differences between the control treatment (330 μL CO_2/L) and the highest CO_2 growth treatment (800 μL CO_2 / L) in both the

Table 2. Carbamylation rate and amount of rubisco in leaves of kidney bean grown at normal and high CO_2. Carbamylation rate was calculated using the following formula: initial activity/total activity × 100. *Values represent the mean ±SE.

Parameters	Normal CO_2	High CO_2
Carbamylation (%)	79.0±1.3	58.9±7.4*
Amount of rubisco ($\mu M/m^2$)	1.94±0.12	1.58±0.07

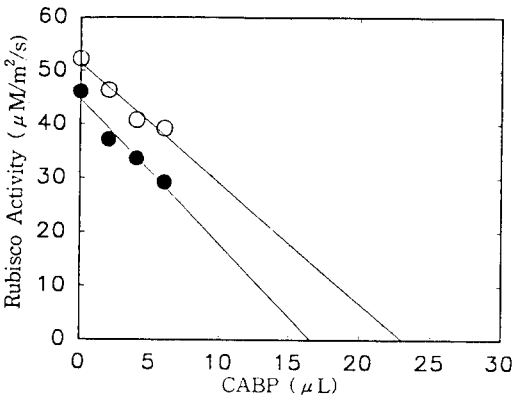

Fig. 1. A plot of rubisco activity versus CABP. The CABP is 2, 4, and 6 μL of 5 μM CABP added to 100 μL of mixture of initial extract, respectively. ○—○, normal CO_2; ●—●, high CO_2.

activity of HCO_3^-/Mg^{2+}-activated and nonactivated rubisco in soybean.

Because decreases in initial and total rubisco activity in high concentration of CO_2 were suspected due to decreased carbamylation rate, we calculated the carbamylation rate from the data of initial and total rubisco activity. As shown in Table 2, 79% of the carbamylation rate in normal CO_2 concentration decreased to 58.9% in high CO_2 concentration. Carbamylation rate was decreased according to increase of CO_2 concentration.

Rubisco activase promoted carbamylation of rubisco at physiological CO_2 concentrations in the presence of RuBP(36, 37) and prevented the deactivation of rubisco(13, 38, 39). In the absence of rubisco activase, only 20 to 40% of rubisco catalytic sites are carbamylated under physiological conditions, leading to a significant inhibition of photosynthesis(36, 37). However, full carbamylation of rubisco can occur if CO_2 levels are increased well above ambient in the absence of RuBP (above 100 μM)(12). In the presence of RuBP and the absence of rubisco activase, carbamylation of rubisco from C_3 plants was inhibited, even at high concentrations of CO_2(39, 40). For these reasons, we postulate that decrease in carbamylation rate in high CO_2 concentration is due to decrease in either activity itself or amount of rubisco activase.

In further studies, to answer this question, with rubisco activase purified from kidney bean grown both at 350 ppm and 650 ppm CO_2 concentration, we are going to measure not only the activity of rubisco activase itself, but also the amount of activase by employing both Western blot analysis and ELISA using the antibody.

Effect of CABP on rubisco activity was studied by incubation of rubisco extract with CO_2 and Mg^{2+}, followed by addition of different amounts of CABP (Fig. 1). The rationale of this experiment is follows: In the carboxylation reaction catalyzed by rubisco, CABP is capable of binding specifically to carbamylated and Mg^{2+}-complexed catalytic sites of the rubisco holoenzyme(41). EPR and NMR studies have shown that the carboxyl group at the C-2 position interacts with the Mg^{2+} ion bound at the catalytic site. This carboxylic group is thought to occupy the position of the substrate CO_2 in 3-keto-2-CABP(42). In this study, decrease in rubisco activity as increasing CABP concentration seems to be caused by inhibitory effect of c-2-carboxy chemicals interacting with the metal ions bound to catalytic site of the enzyme. CABP concentration at which rubisco activity was completely inhibited was extrapolated by plotting each CABP concentration and corresponding rubisco activity (Fig. 1). Compared to normal CO_2 concentration, less amount of CABP was required to block rubisco activity in high CO_2

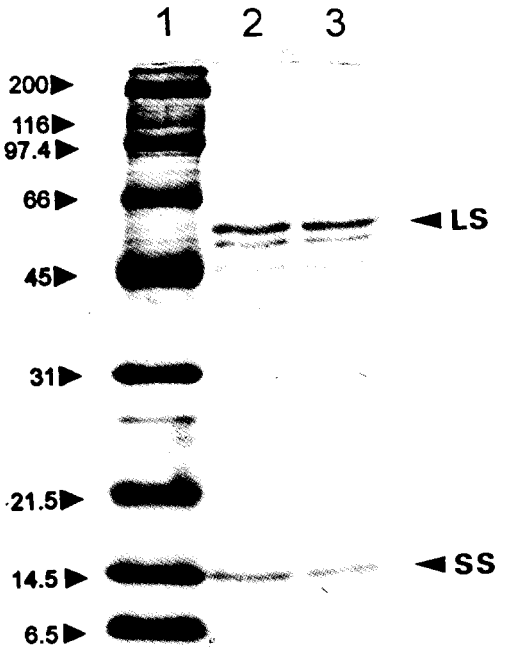

Fig. 2. SDS-PAGE analysis of purified rubisco from kidney bean. Lanes contained 4 μg protein. Lane 1, maker protein: 200, rabbit skeletal muscle myosin; 97.4, phosphorylase b; 66, bovine albumin; 45, egg albumin; 31, bovine carbonic anhydrase; 21.5, soybean trypsin inhibitor: 14.5, hen egg white lysozyme; 6.5, bovine pancrease aprotinin; lane 2, normal CO_2; lane 3, high CO_2. LS, large subunit; SS, small subunit.

concentration.

The measure of rubisco amount is based on the titration of rubisco activity by CABP concentrations(28). The rationale of this measurement are: First, CO_2 fixation was controlled by the amount of rubisco that is active(43). Second, the rubisco activity was associated with a amount of rubisco protein(44).

Under the assumption that decrease in rubisco activity is related with rubisco amount, we calculated rubisco amount from CABP concentration. 1.94 $\mu M/m^2$ of rubisco amount at 350 ppm CO_2 concentration decreased to 1.58 $\mu M/m^2$ at 650 ppm of CO_2 concentration (Table 2).

Table 3. Carbamylation and amount of rubisco in switch kidney bean. Rubisco parameters were measured after switch CO_2 concentration from 650 ppm to 350 ppm. Switch means that high CO_2 transfer to normal CO_2. Tissue was sampled at 10 days after transfer to normal CO_2 in the middle of light period; 10 leaves were sampled per plant. Rate of recovery was calculated a: switch value/normal CO_2 value × 100. *Values represent the mean ±SE.

Parameters	Switch	Rate of recovery
Carbamylation (%)	77.4±1.87*	98.0%
Amount of rubisco ($\mu M/m^2$)	1.9±0.26	97.9%

To verify that increased CO_2 concentration causes decrease in rubisco amount, we have preliminarily tested SDS-PAGE rubisco profile in kidney bean leaves grown at normal CO_2 and high CO_2. Two major polypeptides of 50 and 14.5kD detected by SDS-PAGE analysis were identified as the large and the small subunits of rubisco, respectively (Fig. 2). Two additional bands were present on the gel, which were presumably to be the rubisco activase. Unexpectedly, there were no differences in the density comparing high CO_2 to normal CO_2 in the both 50kD and 14.5kD bands. We assume that this preliminary experiment is not accurate enough to distinguish slight difference of rubisco amount. Therefore, we are going to perform ELISA using the antibodies for large and small subunit to clarify this ambiguity in the future experiment.

In the last experiment, we measured rubisco parameters after switch CO_2 concentration from 650 ppm to 350 ppm. As shown in Table 3, 77.4% of carbamylation rate after CO_2 switch corresponds to 98.0% of recovery rate because carbamylation rate of the plant grown in normal CO_2 from the beginning was 79% (Table 2). Likewise, 97.9% of the rubisco amount of the plant in

control was recovered in the CO_2-switched plant because the rubisco amount of control and CO_2-switched plant were 1.94 $\mu M/m^2$ and 1.9 $\mu M/m^2$, respectively. Although it is expected to maintain the inhibitory effect by 650 ppm CO_2 concentration, these values seem to be very close to the values obtained from the sample leaf grown at normal CO_2 concentration from the beginning. Therefore, inhibitory effects of high CO_2 concentration on the carbamylation rate and amount of rubisco were completely recovered at normal CO_2 concentration.

In conclusion, the decrease in rubisco activity at high CO_2 was caused by both carbamylation and amount of rubisco and these inhibition effect of CO_2 was reversible at normal CO_2 concentration.

요 약

Rubisco의 parameter들을 측정하는 것은 광합성 연구에 대단히 중요한데, 본 연구에서는 방사성동위원소를 사용하는 방법이 아닌 photometric assay 방법을 사용하여 parameter를 측정하였다. Rubisco의 activation과 carbamylation에 미치는 고 CO_2 농도의 효과를 연구하기 위하여, 정상 CO_2 농도(350 ppm)와 고 CO_2 농도(650 ppm)에서 기른 kidney bean (*Phaseolus vulgaris* L.) 잎을 각각 재료로 하여, dual beam(334 nm와 405 nm) spectrophotometer를 사용하여 rubisco의 initial activity와 total activity, carbamylation rate 및 量을 측정 비교하였으며, SDS-PAGE에 의해 rubisco의 전기영동 profile을 분석하였다.

정상 CO_2 농도에서의 initial activity와 total activity는 41.2 $\mu M/m^2/s$와 52.2 $\mu M/m^2/s$이며, 고 CO_2 농도에서는 27.4 $\mu M/m^2/s$와 46.1 $\mu M/m^2/s$로서, 350 ppm에서 650 ppm으로 CO_2 농도를 증가시키면 rubisco의 initial activity와 total activity가 감소되었다. 또한 carbamylation율도 정상 CO_2 농도에서는 79%이며, 고 CO_2 농도에서는 58.9%로서, CO_2 농도의 증가에 따라 감소되었다. Rubisco의 量은 정상 CO_2 농도에서는 1.94 $\mu M/m^2$임에 반해 고 CO_2 농도에서는 1.58 $\mu M/m^2$로서, CO_2 농도는 증가되었는데 그 量은 감소되었다. 이와 같이 고 CO_2 농도에서 rubisco의 activity가 감소되는 것은 rubisco의 carbamylation에 기인되는 것으로 생각된다.

SDS-PAGE 분석에서 50 kD 분자량의 large subunit와 14.5 kD의 분자량을 가지는 small subunit를 동정하였는데, 고 CO_2 농도와 정상 CO_2 농도의 50 kD와 14.5 kD band의 intensity를 비교하면 두 구 사이에 큰 차이가 발견되지 않았다.

고 CO_2 농도에서 정상 CO_2 농도로 switch한 rubisco의 parameter는 정상 CO_2 농도에서의 parameter와 거의 비슷한 값을 나타내었는데, 이는 350 ppm의 정상 CO_2 농도에 의해 rubisco의 activity가 회복되었음을 의미한다.

ACKNOWLEDGMENTS

We thank Dr. Thomas D. Sharkey (Dept. of Biology, University of Wisconsin-Madison) for generous supply of kidney bean seeds and for his advice on this research project. The present studied were a part of the research project "Environmental Adaptation and Variation of Higher Plants" supported by the Basic Science Research Institute Program, Ministry of Education in Korea, 1994, Project No. BSRI-94-4404.

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