Effect of Sink-Limitation on Leaf Photosynthetic Rate and Related Characteristics in Soybean Plants

Minobu Kasai, Hideki Nakata, Hiroya Seino, Daisuke Kamata and Toshifumi Tsukiyama
(Department of Biofunctional Science, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori 036-8561, Japan)

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Soybean plants are often used in the experiments involving pod removal to understand how sink-limitation regulates photosynthesis. Elucidating the regulatory mechanism of photosynthesis in plants through changes in the source-sink balance is important to understand fundamental plant physiology and may also be useful in studies for creating crop plants with higher productivity. It has been reported in soybean that pod removal decreases the photosynthetic rate (Mondal et al., 1978; Wittenbach, 1982, 1983; Xu et al., 1994), transpiration (Wittenbach, 1982, 1983), stomatal conductance (Xu et al., 1994), and the activity and amount of ribulose-1,5-bisphosphate carboxylase (Rubisco) (Mondal et al., 1978; Wittenbach, 1982, 1983; Crafts-Brandner and Egli, 1987; Xu et al., 1994), and increases the content of soluble sugars (Mondal et al., 1978; Xu et al., 1994) and starch (Mondal et al., 1978; Wittenbach, 1982; Xu et al., 1994) in leaf. In these reports, the effect of pod removal was investigated more than one week after the removal. To fully understand how sink-limitation regulates photosynthesis, it is important to know the effect of pod removal within a short period(s) in the same crop plant, soybean. The effect of short-term sink-limitation on photosynthesis and its regulatory mechanism may be different from that of the long-term sink-limitation. There is a report showing that pod removal reduced the leaf photosynthetic rate and stomatal conductance of soybean plants at 48 h after the treatment (Setter and Brun, 1980). In that report, since pod removal only slightly affected photosynthetic rate of leaf slices, which had been prepared to eliminate the stomatal effect, it was concluded that pod removal reduced leaf photosynthetic rate through stomatal closure. We investigated in the present study the effect of pod removal on photosynthetic rate and various related characteristics in leaves of soybean plants mainly on day 3 after pod removal. We also investigated the effect of pod removal on the amount of ribulose-1,5-bisphosphate (RuBP) that was bound to protein in the leaf extract. The amount of RuBP bound to protein in leaf extract was previously found to correlate positively with the amount of uncarbamylated, inactive Rubisco (Brooks and Portis, 1988), which very tightly binds RuBP (Jordan and Chollet, 1983).

Materials and Methods

Soybean (Glycine max L. Merr. cv. Tsurunoko) seeds were sown in plastic pots (13.5 cm in height, 12.5 cm in diameter) that contained almost equal volumes of vermiculite and sand, and were grown in a growth chamber under a regime of 10 hr light (25°C) and 14 hr darkness (17°C). Light was supplied from incandescent lamps, and the intensity was 440 μmol photons m⁻² s⁻¹ (400−700 nm) at the mean middle height of the plants when they were depodded. Nutrients were supplied twice a week with a 1,000-fold diluted solution of Hyponex (6-10-5 Type (N :P :K =6 :10 :5), Hyponex Co., OH, U.S.A.), and tap water was supplied sufficiently. On day 45 after sowing, developed, actively growing pods of the plants, whose growth stage was between R5 and R6 (Egli and Crafts-Brandner, 1996), were all removed from half of the plants, and the depodded plants and the remaining plants with pods (control) were grown under an extended photoperiod (14 h), which might somewhat improve the source-to-sink balance, in the same growth chamber. On day 3 after pod removal, plants were transferred to another growth chamber in which the light intensity and temperature were 650 μmol photons m⁻² s⁻¹ (at the mean middle height of the plants) and 25°C, respectively. After one hour, photosynthetic rate, stomatal conductance, and intercellular CO₂ concentration were measured in fully expanded trifoliolate leaves, which were the fourth position from the lowest trifoliolate leaves in the plants, at a light intensity of 1,000 μmol photons m⁻² s⁻¹, air flow rate of 200 mL min⁻¹, air temperature of 25°C, relative humidity of 60% and CO₂ concentration of 350 μL L⁻¹ using a photosynthetic analyzer (Cylus-1,
Table 1. Leaf characteristics in control and depodded soybean plants on day 3 after pod removal.

|                          | Control plants | Depodded plants |
|--------------------------|----------------|-----------------|
| Photosynthetic rate**    | 31.8 ± 2.2     | 18.8 ± 2.7**    |
| Stomatal conductance**   | 696.0 ± 94.1   | 325.8 ± 51.4**  |
| Leaf intercellular CO₂ concentration** | 182.3 ± 29.2 | 190.5 ± 19.9NS |
| Leaf water**             | 145.9 ± 15.0   | 149.4 ± 24.3NS  |
| Leaf chlorophyll**       | 0.56 ± 0.04    | 0.58 ± 0.09NS   |
| Leaf protein**           | 9.8 ± 0.8      | 9.9 ± 1.2NS     |
| Leaf Rubisco**           | 3.1 ± 0.4      | 3.0 ± 0.7NS     |

Rubisco activity

|                          | Initial**      | Initial/Total (activation ratio)** |
|--------------------------|----------------|-----------------------------------|
|                          | 29.6 ± 4.5     | 44.0 ± 4.8                        |
|                          | 16.2 ± 2.6**   | 38.7 ± 2.9**                      |
|                          | 67             | 42                                |
| Leaf protein-bound RuBP**| 1.2 ± 0.1      | 2.5 ± 0.4**                       |
| Leaf sucrose**           | 1.5 ± 0.4      | 2.3 ± 0.2**                       |
| Leaf starch**            | 26.7 ± 4.4     | 17.1 ± 2.3**                      |

Half of the soybean plants grown in a growth chamber were depodded on day 45 after sowing, and on day 3 after depodding, the following characteristics were analyzed in fully expanded fourth trifoliate leaves. Data are means ± SD (n = 4). (a) μmol (CO₂) m⁻² s⁻¹. (b) mmol m⁻² s⁻¹. (c) μL L⁻¹. (d) g m⁻². (e) μmol (CO₂) m⁻² s⁻¹. (f) %. (g) nmol (RuBP) mg (leaf total protein)⁻¹. **, significantly different at P < 0.01 (t-test) as compared with control plants which were not depodded. NS, not significantly different at P > 0.05.

Koito Industries, Ltd., Tokyo, Japan). Investigations on day 2 after pod removal showed that the fourth trifoliate leaves had the highest photosynthetic rate in both control and depodded plants. After measuring photosynthetic rate, stomatal conductance, and intercellular CO₂ concentration, leaf discs (a leaf disc, 1.79 cm²) were cut off from the leaves for various analyses. Leaf water content was analyzed by measuring the fresh weight of leaf discs and dry weight of discs that had been dried for 2 days at 75°C. The other analyses, which are described below, were performed with leaf discs that had been frozen immediately after cutting in liquid nitrogen and stored at −80°C.

Chlorophyll was quantified by the method of Mackinney (1941). Total protein was extracted as described by Makino et al. (1986) and quantified by the method of Bradford (1976) using bovine serum albumin as the standard. Rubisco was quantified as described by Makino et al. (1986) using purified spinach Rubisco as the standard. Sucrose and starch were quantified as described by Sawada et al. (1999). The activity of Rubisco was determined enzymatically essentially as described by Cheng and Fuchigami (2000). For the initial activity, 20 μL of a leaf extract obtained by homogenizing a leaf disc with buffer (100 mM HEPES-KOH, pH 7.8, 2 mL) was added to a cuvette containing 1.98 mL of assay medium (100 mM Bicine-KOH, pH 8.2, 20 mM MgCl₂, 20 mM NaHCO₃, 5 mM creatin phosphate, 1 mM ATP, 0.2 mM NADH, 20 units of creatin kinase, 20 units of 3-phosphoglycerate kinase, and 20 units of glyceraldehyde-3-phosphate dehydrogenase), immediately followed by adding RuBP (final conc., 0.6 mM), then mixing well. For the total activity, RuBP was added 5 min later (which gave the highest total activity), after 20 μL of the leaf disc extract was combined with the assay medium.

The amount of RuBP bound to protein in the leaf extract was determined essentially as described by Brooks and Portis (1988). A leaf extract (800 μL) obtained by homogenizing a leaf disc with buffer (100 mM HEPES-KOH, pH 7.8, 1 mL) was centrifuged (100 g, 1 min) after loading onto a column containing Sephadex G-50 (bed volume before centrifugation, 4 mL) that had been equilibrated with the same buffer. The eluent from the column that lacked free RuBP (500 μL) was centrifuged (10,000 g, 10 min) after mixing with an acid solution (5.5 M HClO₄, 50 μL) to precipitate protein in the eluent. The resulting supernatant was centrifuged (10,000 g, 10 min) after neutralizing to pH 5.6 with K₂CO₃, and RuBP in the supernatant was determined in the assay medium used for the determination of activity of Rubisco using purified spinach Rubisco (0.5 unit) instead of leaf disc extract.

To examine whether pod removal could give the sink-limitation to soybean plants, we examined the growth of pods for three days after pod removal in a separate experiment. Pods were taken from the control plants with pods on day of pod removal and day 3 after pod removal, and the fresh weight and dry weight of these pods were measured. Dry weight of pods was measured after 2 days of drying at 75°C. Four plant samples were used for the analysis of photosynthetic rate, stomatal conductance,
intercellular CO$_2$ concentration and leaf water content and for obtaining frozen leaf disc samples in each of control and depodded plants, and for the measurement of fresh weight and dry weight of pods. For each of the other analyses, four leaf disc samples, which were selected at random from frozen discs, were used.

**Results and Discussion**

On day 3 after pod removal, photosynthetic rate in depodded soybean plants was significantly lower ($t$-test, $P<0.01$) than that in control plants (Table 1). Stomatal conductance in depodded plants was also significantly lower ($P<0.01$) than that in control plants (Table 1). The level of stomatal conductance in depodded plants relative to control plants was 47%, and it was almost consistent with the level of photosynthetic rate in depodded plants relative to control plants, which was 59%.

These results suggest that the lower photosynthetic rate in depodded plants could result from an increase in inactive Rubisco content, but not from a decrease in leaf intercellular CO$_2$ concentration due to a lower stomatal conductance or a decrease in leaf Rubisco content. Setter and Brun (1980) observed that pod removal reduced the leaf photosynthetic rate and stomatal conductance at 48 h after the treatment in soybean plants. They also observed that the leaf photosynthetic rate was only slightly affected by pod removal when measured with leaf slices in a solution containing sorbitol (0.25 M), Mes-KOH (20 mM, pH 6.7), KNO$_3$ (5 mM), Ca(NO$_3$)$_2$ (2 mM), MgCl$_2$ and

![Fig. 1. Growth of pods in control soybean plants during experimental period of pod removal. The pods of control soybean plants were taken on day 45 after sowing, which was the day of pod removal, and day 3 after the pod removal, and the fresh weight and dry weight of pods were determined. Open and closed bars indicate the fresh weight and dry weight of pods, respectively. Vertical bars denote SD (n = 4). **, significantly different at $P<0.01$ ($t$-test) when compared with pod weight on the day of pod removal.](image-url)
KHCO₃ (<10 mM). Although they did not investigate leaf intercellular CO₂ concentration, they concluded that pod removal reduced leaf photosynthetic rate through stomatal closure. We cannot receive simply their conclusion to the observed lower photosynthetic rate in depodded soybean plants in the present study, since, although stomatal conductance in depodded plants was lower than that in control plants, leaf intercellular CO₂ concentration in depodded plants did not differ from that in control plants (Table 1). We investigated photosynthetic rate, stomatal conductance and leaf intercellular CO₂ concentration on day 2 (approximately 48 h) after pod removal in a separate experiment. Photosynthetic rate and stomatal conductance in depodded plants were significantly lower ($P<0.01$) than those in control plants (29.0 ± 2.9 μmol (CO₂) m⁻² s⁻¹ and 660.6 ± 59.5 mmol m⁻² s⁻¹ (n = 4) in control plants, 22.5 ± 2.0 μmol (CO₂) m⁻² s⁻¹ and 412.7 ± 71.7 mmol m⁻² s⁻¹ (n = 4) in depodded plants), while leaf intercellular CO₂ concentration in depodded plants did not differ significantly ($P>0.05$) from that in control plants (185.0 ± 12.8 μL L⁻¹ (n = 4) in control plants, 180.0 ± 15.8 μL L⁻¹ (n = 4) in depodded plants). It is suggested that in depodded plants, a decrease in leaf intercellular CO₂ concentration did not result from lower stomatal conductance because of an increase in the inactivation of Rubisco that fixes CO₂.

There have been a number of data showing the negative correlation between photosynthetic rate and leaf sucrose or starch content, suggesting end product inhibition of photosynthesis, although the mechanism of inhibition is not yet clear. For example, data from studies using soybean, cotton, sunflower, or sorghum plants show that there is robust correlation between high content of leaf starch and low assimilation rate (see Paul and Foyer, 2001). Studies using single-rooted soybean leaves showed that continuous exposure of leaves of the plants to light or cooling the roots decreased the leaf photosynthetic rate and that there was significant negative correlation between leaf photosynthetic rate and the content of leaf sucrose (Sawada et al., 1986, 1987). In the present study, leaf sucrose content in depodded plants was higher than that in control plants. In contrast, leaf starch content in depodded plants was lower than that in control plants (Table 1). Since photosynthetic rate in depodded plants was lower than that in control plants, the results in the present study implicate the end product inhibition of photosynthesis via sucrose.

In the present study using soybean, in which the effect of pod removal was investigated mainly on day 3 after pod removal, pod removal could give the significant sink-limitation to the plants and resulted in the lower leaf photosynthetic rate, stomatal conductance, activity of Rubisco, and the higher content of leaf sucrose. These effects of pod removal corresponded to those reported by previous studies using soybean in which the effect of pod removal was investigated more than one week after depodding treatment (Mondal et al., 1978; Wittenbach, 1982, 1983; Crafts-Brandner and Egli, 1987; Xu et al., 1994). However, whereas in the previous studies, pod removal resulted in the decrease in leaf Rubisco content (Wittenbach, 1982, 1983; Crafts-Brandner and Egli, 1987), that in the present study did not affect significantly leaf Rubisco content. We observed in a separate experiment that leaf Rubisco content in depodded soybean plants was significantly lower ($P<0.05$) than that in control plants at one week after pod removal (2.8 ± 0.3 g m⁻² (n = 4) in control plants, 2.2 ± 0.4 g m⁻² (n = 4) in depodded plants). Therefore, it seems likely that leaf Rubisco content in soybean plants decreases under a longer term sink-limitation after pod removal. In the previous studies, the effect of pod removal on leaf intercellular CO₂ concentration and the amount of RuBP bound to protein in leaf extract was not investigated. In the present study, data from the investigations further showed that the lower photosynthetic rate under the sink-limitation imposed by pod removal could result from an increase in leaf inactive Rubisco content, but not from a lower leaf intercellular CO₂ concentration. We observed that depodded soybean plants had similar contents of leaf protein, chlorophyll and water, as compared to control plants. Therefore, data in the present study suggest that leaf photosynthetic rate of soybean plants can be regulated through the inactivation of Rubisco under a short term sink-limitation.

Studies using purified Rubisco have shown that inorganic phosphate enhances the affinity of uncarbamylated, inactive Rubisco for activator CO₂ (McCurry et al., 1981; Anwaruzzaman et al., 1995; Marcus and Gurevitz, 2000). Studies using single-rooted soybean leaves implicate that accumulation of sucrose in leaf may decrease the content of inorganic phosphate in leaf chloroplasts, in which Rubisco exists, through increases in the contents of photosynthetic phosphorylated intermediates that may occur through feed-back inhibition of sucrose phosphate synthase by sucrose (see Sawada et al., 1989, 1990). It is now well known that ATP is required for the catalytic action of Rubisco activase, which dissociates RuBP from RuBP-bound, uncarbamylated inactive Rubisco in leaf chloroplasts (see Crafts-Brandner and Salvucci, 2000). Recently, H₂O₂, a reactive oxygen species, has been suggested to be a factor causing the inactivation of Rubisco (Zhou et al., 2006). Therefore, it is of interest to elucidate whether inorganic phosphate, ATP, and/or H₂O₂ are involved in the inactivation of Rubisco under sink-limitation described above. It may be that sink-limitation of pod removal carried out for soybean plants in the present study, which resulted in a decreased leaf photosynthetic rate and increased leaf sucrose content, resulted in an inactivation of
Rubisco through change in the content(s) of inorganic phosphate, ATP, and/or H$_2$O$_2$ in leaf chloroplasts. Although the method(s) determining accurately the concentrations of inorganic phosphate, ATP, or H$_2$O$_2$ within the chloroplasts of intact plants under light conditions has not been established, the successful determination in the future, together with the analysis of the activity of Rubisco activase, may provide substantial information for solving the mechanism of the inactivation of Rubisco under sink-limitation.

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