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Full Length Article

Regulatory Mechanism of Photosynthesis that Depends on the Activation State of Rubisco under Sink Limitation

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ABSTRACT

Knowledge of regulatory mechanism of plant photosynthesis under sink-limitation occurring under various environmental conditions is still insufficient. In the present study, two different degrees of sink-limitation were imposed on soybean plants by removing either half or all developing pods and after that, photosynthetic rate and various other characteristics were investigated in fully expanded source leaves of the depodded and control plants. It was found that a larger degree of pod removal (sink-limitation) resulted in lower leaf photosynthetic rate, stomatal conductance and activation ratio (a percentage of initial activity to total activity) of Rubisco and higher leaf sucrose and starch contents without decreasing leaf intercellular CO_2 concentration and affecting leaf water, chlorophyll, total protein and Rubisco protein contents. In addition, there were agreements between activation ratios of Rubisco and photosynthetic rates of the depodded plants relative to controls. These results showed a regulatory mechanism (a down regulation mechanism) of leaf photosynthetic rate in soybean, which depends on the activation state (a decrease in activation ratio) of Rubisco rather than leaf Rubisco content or leaf intercellular CO_2 concentration under sink-limitation.

Key Words: Activation ratio; Photosynthetic rate; Pod removal; Rubisco; Sink-limitation; Soybean

INTRODUCTION

In plants, changes in the photosynthetic source-sink balance are an important factor that can regulate photosynthesis (Paul & Foyer, 2001). Elucidating the regulatory mechanism of photosynthesis under changes in the photosynthetic source-sink balance is important essentially to understand plant fundamental physiology. In agricultural aspect, understanding of the mechanism may be useful for example, in improving crop productivity. Soybean plants have often been used in studies conducting pod removal to understand the regulatory mechanism of photosynthesis under sink-limitation, which can occur under various environmental conditions (Paul & Foyer, 2001). The studies have implicated that sink-limitation imposed by pod removal can reduce leaf photosynthetic rate by decreasing leaf stomatal conductance or leaf Rubisco content (Setter & Brun, 1980; Wittenbach, 1982, 83; Xu *et al.* 1994). It is possible that a decrease in leaf stomatal conductance decreases leaf intercellular CO_2 concentration then reduces CO_2 supply for photosynthesis and decreases leaf photosynthetic rate, while a decrease in leaf Rubisco content can directly reduce photosynthetic CO_2 fixation. To understand the regulatory mechanism of photosynthesis under sink-limitation, it seems essential to analyze leaf intercellular CO_2 concentration, leaf Rubisco content, and activation ratio of Rubisco in leaf in experiment (s), since the concentration, content and ratio play essential roles in photosynthesis. However, in the studies conducting pod removal, either one or two, or all of the leaf CO_2 concentration, Rubisco content and activation ratio of Rubisco have not been investigated.

Until recently, manipulations other than pod removal have also been conducted with soybean to understand the regulatory mechanism of photosynthesis under changes in the photosynthetic source/sink balance. Deflowering was suggested to decrease leaf photosynthetic rate and leaf Rubisco content (Crafts-Brandner & Egli, 1987). In contrast, a study showed that deflowering decreased leaf photosynthetic rate and activation ratio of Rubisco in leaf (Sawada *et al.*, 1995). Petiole girdling, a manipulation giving an indirect sink limitation, was shown to decrease leaf photosynthetic rate and stomatal conductance (Setter & Brun, 1980). Exposure to continuous light, which is a manipulation giving a surplus source rather than a direct sink-limitation, was shown to decrease leaf photosynthetic rate and activation ratio of Rubisco in leaf (Sawada *et al.*, 1990). In contrast, high CO_2 treatment (70 Pa CO_2), a manipulation giving a surplus source, was shown to decrease leaf photosynthetic rate without decreasing activation ratio of Rubisco (Sawada *et al.*, 2001). However, there is a pioneer study showing both decreases in leaf photosynthetic rate and activation ratio of Rubisco in leaf following high CO_2 treatment (Sage *et al.*, 1989). I emphasize that except the pioneer study, studies conducting the above-mentioned manipulations also have not analyzed

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either two or all of leaf intercellular CO_2 concentration, Rubisco content and activation ratio of Rubisco.

To understand the regulatory mechanism of photosynthesis under sink-limitation, it may be a wise to obtain more information from the same crop plant, soybean by conducting the same manipulation experiment (s) for imposing sink-limitation. In the experiment(s), analyzing leaf intercellular CO_2 concentration, leaf Rubisco content and activation ratio of Rubisco in leaf is very important.

In the present study, soybean plants were used and two different degrees of sink-limitation were imposed on soybean plants by removing either half or all developing pods. After a period following the manipulations, photosynthetic rate and various other characteristics including leaf intercellular CO_2 concentration, Rubisco content and activation ratio of Rubisco were investigated in fully expanded source leaves of the depodded and control plants to analyze the regulatory mechanism of photosynthesis under sink-limitation.

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 growth chambers under a regime of 10 h light (24°C) and 14 h darkness (17°C). Light was supplied by incandescent lamps and the intensity was 400 μmol m^{-2} s^{-1} (400-700 nm) at the middle height of developed plants when they were depodded. Nutrients were supplied once a week with a 1,000-fold diluted solution of Hyponex (6-10-5 Type, Hyponex Co., OH, U.S.A.) and tap water was supplied in sufficient amounts. On day 55 after sowing, plants were divided into three groups (four plants each) and half and all developing pods were removed from the two groups of plants, respectively. Remaining one group was used as control plants without removing pods. On day 4 after pod removal, leaf photosynthetic rate, stomatal conductance and intercellular CO_2 concentration were measured in fully expanded fourth trifoliate leaf, which showed the highest photosynthetic rate among developed trifoliate leaves, at a light intensity of 1,000 μmol m^{-2} s^{-1}, air flow rate of 200 mL min^{-1}, air temperature of 24°C, relative humidity of 60% and CO_2 concentration of 350 μL L^{-1} using a portable photosynthetic analyzer (Cylus-1, Koito Industries, Ltd., Tokyo, Japan). Prior to measurements, plants were transferred to a different growth chamber whose light intensity and temperature were 600 μmol m^{-2} s^{-1} and 24°C, respectively and were placed under these conditions for one hour. After measuring photosynthetic rate, stomatal conductance and intercellular CO_2 concentration, leaf discs (a leaf disc, 1.79 cm^2) were cut off from the fourth trifoliate leaves. Leaf water content was analyzed by measuring the fresh weight of a part of leaf discs and the dry weight of discs were dried for 2 days at 75°C. The other analyses were performed with leaf discs that had been frozen immediately in liquid N after cutting and stored at -80°C (see below).

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). Rubisco was quantified as described by Makino *et al.* (1986). 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 in buffer (100 mmol L^{-1} HEPES-KOH, pH 7.8, 2 cm^3) was added to a cuvette containing 1.98 cm^3 of assay medium (100 mmol L^{-1} Bicine-KOH (pH 8.2), 20 mmol L^{-1} $MgCl_2$, 20 mmol L^{-1} $NaHCO_3$, 5 mmol L^{-1} creatin phosphate, 1 mmol L^{-1} ATP, 0.2 mmol L^{-1} NADH, 20 units creatin kinase, 20 units 3-phosphoglycerate kinase and 20 units glyceraldehyde-3-phosphate dehydrogenase), immediately followed by adding RuBP (final conc. 0.6 mm), then mixing well. For total activity, RuBP was added 5 min later (which gave the highest total activity), after 20 μL of the leaf disc extract was immediately combined with the assay medium.

Four plant samples were used for the analyses of photosynthetic rate, stomatal conductance and intercellular CO_2 concentration in the respective control and depodded plants. For each of the other analyses (except for the analysis of fresh weight & dry weight of leaf), four leaf disc samples, which were selected at random from frozen leaf discs, were used.

RESULTS AND DISCUSSION

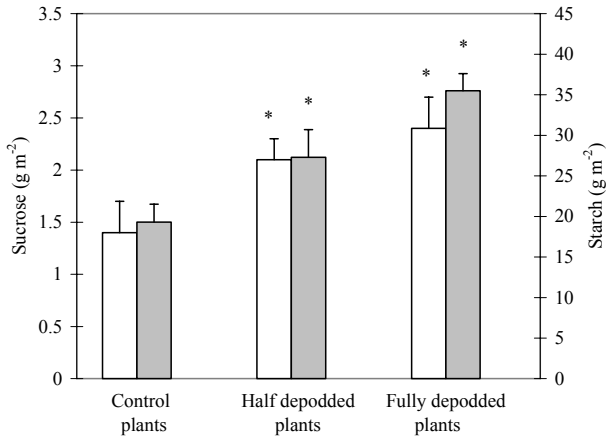
Table I shows the photosynthetic rate and various other characteristics in fully expanded fourth trifoliate leaves of control, half depodded and fully depodded soybean plants. The photosynthetic rate was lowest in fully depodded plants and then half depodded and control plants in that order. The stomatal conductance was also lowest in fully depodded plants followed by half depodded and control plants. The photosynthetic rates in fully depodded and half depodded plants relative to control plants were 31 and 70%, respectively and the stomatal conductance in fully depodded and half depodded plants relative to control plants were 43% and 73%, respectively. Therefore, it was considered that the sink-limitation imposed by pod removal induced the decrease in stomatal conductance, which then caused the decrease in photosynthetic rate by lowering the leaf intercellular CO_2 concentration. However, analyzed intercellular CO_2 concentrations in fourth trifoliate leaves of fully depodded and half depodded plants were not significantly lower than that in control plants (Table I). In contrast, the CO_2 concentration in fully depodded plants was significantly higher than that in control plants (Table I). These results suggest that CO_2 use in photosynthetic cells was more reduced rather than CO_2 supply through stomata by pod removal.

Table I. Leaf various characteristics in control, half and fully depodded soybean plants

	Control plants	Half depodded plants	Fully depodded plants
Photosynthetic rate[(a)]	10.2 ± 1.9	7.1 ± 0.1**	3.2 ± 0.5*
Stomatal conductance[(b)]	90.3 ± 4.1	65.6 ± 2.5*	38.7 ± 5.7*
Intercellular CO_2 concentration[(c)]	134.0 ± 20.7	147.3 ± 14.6***	194.9 ± 16.4*
Water[(d)]	155.4 ± 12.3	153.8 ± 7.7***	149.3 ± 10.6***
Chlorophyll[(d)]	0.51 ± 0.03	0.46 ± 0.06***	0.47 ± 0.07***
Total protein[(d)]	7.8 ± 0.6	7.9 ± 0.8***	8.0 ± 0.8***
Rubisco protein[(d)]	2.5 ± 0.3	2.6 ± 0.3***	2.4 ± 0.4***
Rubisco activity			
Initial[(e)]	37.6 ± 3.5	28.8 ± 6.1**	13.7 ± 4.4*
Total[(e)]	49.8 ± 7.2	45.7 ± 4.0***	43.7 ± 2.2***
Activation ratio (initial/total)[(f)]	76	63	31

Half or all developing pods were removed from two groups of growing soybean plants, which were selected at random. Pods of control plants were not removed. On day 4 after pod removal, the following characteristics were analyzed in fully expanded fourth trifoliate leaves. Values are means ± SD (n = 4). (a) μmol CO_2 m^{-2} s^{-1}. (b) mmol m^{-2} s^{-1}. (c) μL L^{-1}. (d) g m^{-2}. (e) μmol CO_2 m^{-2} s^{-1}. (f) %. *P < 0.01/**P < 0.05/***P > 0.05 (t-test) when compared with control plants.

Fig. 1. Leaf sucrose and starch contents of control, half and fully depodded soybean plants. Half or all developing pods were removed from two groups of growing soybean plants, which were selected at random. Pods of control plants were not removed. On day 4 after pod removal, sucrose and starch contents were analyzed in fully expanded fourth trifoliate leaves. Open and closed bars indicate sucrose and starch contents, respectively. Values are means ± SD (n = 4). *P < 0.01 (t-test) when compared with control plants



In fully depodded and half depodded plants, visible symptoms such as leaf bleaching or wilting were not observed. In fact, the contents of water, chlorophyll, total protein and Rubisco protein in fourth trifoliate leaves did not differ significantly between fully depodded, half depodded and control plants (Table I).

The initial activity of Rubisco in fourth trifoliate leaves was lowest in fully depodded and then half depodded and control plants in that order (Table I). In contrast, the total activity of Rubisco did not differ significantly between fully depodded, half depodded and control plants (Table I). As a result, the activation ratio (a percentage of initial activity to total activity) of Rubisco calculated from the initial and total activities of Rubisco was found to be lower in fully depodded and half depodded, compared to control plants (Table I). The activation ratios of Rubisco in fully depodded and half depodded plants relative to control plants were 41% and 83%, respectively and these values were roughly consistent with the photosynthetic rates in fully depodded (31%) and half depodded plants (70%) relative to control plants. When the relationship between activation ratio of Rubisco and photosynthetic rate was investigated using data from control, half depodded and fully depodded plants, a positive linear relationship was found to exist between the ratio and the rate [y (photosynthetic rate) = 0.149 x (activation ratio of Rubisco) – 1.621, $r^2 = 0.970$].

There is well known hypothesis of end product inhibition of photosynthesis, although the precise mechanism is still not clear (Paul & Pellny, 2003). 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 (Paul & Foyer, 2001). In a study using single-rooted soybean leaves, in which the effect of exposure to continuous light on photosynthetic rate and carbohydrate content was investigated, significant negative correlations were found to exist between leaf sucrose content and photosynthetic rate and leaf starch content and photosynthetic rate (Sawada *et al.*, 1986). Fig. 1 shows the contents of sucrose and starch in fourth trifoliate leaves of control, half depodded and fully depodded soybean plants. The contents of sucrose and starch were highest in fully depodded and then half depodded plants compared to control plants. Since the photosynthetic rate in fourth trifoliate leaves was lowest in the order of fully depodded, half depodded and then control plants, these results support end product inhibition of photosynthesis. When the mutual relationships between sucrose content and photosynthetic rate and starch content and photosynthetic rate were investigated using data from control, half depodded and fully depodded plants, negative linear relationships were shown to exist between the respective carbohydrate contents and photosynthetic rate [y (photosynthetic rate) = -6.544 x (sucrose) + 19.704, $r^2 = 0.917$; y (photosynthetic rate) = -0.432 x (starch) + 18.663, $r^2 = 0.996$].

In order to understand the regulatory mechanism of photosynthesis under sink-limitation, the present study explored two different degrees of sink-limitation by removing either half or all developing pods in soybean, and investigated photosynthetic rate and various other characteristics, including intercellular CO_2 concentration, Rubisco content and activation ratio of Rubisco, in fully expanded fourth trifoliate leaves of the half depodded, fully depodded and control plants. To my knowledge, there has been no study investigating the effect of different degrees of pod removal on leaf photosynthetic rate and various other

characteristics. In addition, in any manipulation experiment imposing sink-limitation other than high CO_2 treatment experiment, either one or two, or all of leaf intercellular CO_2 concentration, leaf Rubisco content and activation ratio of Rubisco in leaf, which must play essential roles in photosynthesis, have not been investigated. Results obtained from the present study show that the larger degree of pod removal (sink-limitation) resulted in lower leaf photosynthetic rate, stomatal conductance and activity and activation ratio of Rubisco and higher leaf sucrose and starch contents without decreasing leaf intercellular CO_2 concentration and affecting leaf water, chlorophyll, total protein and Rubisco protein contents. Results also showed that the activation ratios of Rubisco in fully depodded and half depodded plants relative to control plants were roughly consistent with the photosynthetic rates in those plants relative to control plants. In addition, it was observed that two different degrees of pod removal (sink-limitation) did not bring about visible damages such as leaf bleaching or wilting. Studies concerning pod removal in soybean plants to date have implicated that the sink-limitation imposed by pod removal can reduce leaf photosynthetic rate by decreasing leaf stomatal conductance or leaf Rubisco content (Setter & Brun, 1980; Wittenbach, 1982, 83; Xu *et al.,* 1994). A decrease in leaf stomatal conductance may reduce leaf photosynthetic rate by decreasing leaf intercellular CO_2 level. However, data in the present study show that in soybean, there is also a regulatory mechanism (a down regulation mechanism) of leaf photosynthetic rate that depends on the activation state (a decrease in activation ratio) of Rubisco rather than the leaf Rubisco content or leaf intercellular CO_2 concentration under the sink-limitation.

Manipulations giving surplus source rather than sink-limitation, exposure to continuous light and high CO_2 treatment were shown to decrease leaf photosynthetic rate and activation ratio of Rubisco in leaf (Sage *et al.*, 1989; Sawada *et al.*, 1990), similar to pod removal. Deflowering, a manipulation giving a direct sink-limitation, like pod removal, was also shown to decrease leaf photosynthetic rate and activation ratio of Rubisco in leaf (Sawada *et al.*, 1995). These manipulations and pod removal give rise to accumulation of carbohydrate (e.g., sucrose, starch) in leaf (Sawada *et al.*, 1986; Sage *et al.*, 1989; Xu *et al.*, 1994 & the present study). Existing evidence suggests inhibition of photosynthesis by accumulation of carbohydrate in leaf (see Paul & Pellney, 2003). Therefore, it is likely that leaf photosynthetic rate is regulated under both surplus source and sink-limitation through changes in the activation state of Rubisco by the same regulatory mechanism of photosynthesis that involves the leaf carbohydrate status, although the precise mechanism is still un-known.

With respect to the decline in activation ratio of Rubisco occurring under sink-limitation, inorganic phosphate may be important. It has been found using purified spinach Rubisco that *in vitro*, inorganic phosphate enhances the affinity of Rubisco for activator CO_2 (McCurry *et al.*, 1981; Anwaruzzaman, 1995; Marcus & Gurevitz, 2000). It has also been suggested that accumulated carbohydrate in leaf may reduce inorganic phosphate content within leaf chloroplasts by increasing various photosynthetic phosphorylated intermediates (Sawada *et al.*, 1989; Paul & Pellny, 2003). Rubisco exists within leaf chloroplasts. Therefore, it may be that under sink-limitation, accumulated carbohydrate in leaf gives rise to a reduction of inorganic phosphate content within leaf chloroplasts and as a result, activation ratio of Rubisco is reduced. Also, there may be a scenario that the reduction of inorganic phosphate content within leaf chloroplasts decreases photophosphorylation-mediated production of ATP on thylakoid membranes, then reduces the activity of Rubisco activase within the chloroplasts (Portis, 1992) and as a result, activation ratio of Rubisco is reduced.

Whether accumulated carbohydrate in leaf actually reduces inorganic phosphate content within leaf chloroplasts has not been verified probably because of technical difficulties in determining the phosphate content. A successful determination of the phosphate content is likely to bring about significant and essential information not only to solve the relationship between inorganic phosphate content within leaf chloroplasts and Rubisco activation state under sink-limitation but also to solve the regulatory mechanism of leaf photosynthetic rate that involves the leaf carbohydrate status under a variety of changes in the photosynthetic source/sink balance in future studies. Nevertheless, data in the present study demonstrate that there is a regulatory mechanism (a down regulation mechanism) of leaf photosynthetic rate that depends on the activation state (a decrease in activation ratio) of Rubisco rather than the leaf Rubisco content or leaf intercellular CO_2 concentration under a direct sink-limitation.

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