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Activation state of Rubisco decreases with the nitrogen accumulation during the reproductive stage in soybean [*Glycine max* (L.) Merr.]

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Abstract

The CO_2 assimilation rate, N content, Rubisco activation state, and protein distribution to Rubisco and Rubisco activase in the leaf were analyzed for four soybean genotypes at two growth stages under field conditions. The CO_2 assimilation rate was largely constant except for one genotype during the reproductive stage. Rubisco activation state significantly decreased with the increase of N content during this period in three of four genotypes. When standardized by total soluble protein amount, Rubisco activase amount was constant during this period in three genotypes. The results in the present study showed the deactivation of Rubisco with the N accumulation, which can result in the constant rate of CO_2 assimilation during the reproductive stage of soybean. The deactivation of Rubisco was attributed to an unknown mechanism other than to the deficiency of Rubisco activase.

Additional key words: leaf; nitrogen content; photosynthesis; ribulose-1,5-bisphosphate carboxylase/oxygenase; Rubisco activation state; soybean.

Introduction

Soybean is one of the most important sources of oil and protein production in the world (USDA 2012). An improvement in soybean production is needed to meet the increasing food demand, owing to the rapid growth of human population. It was reported that leaf photosynthetic capacity had the potential to improve the biomass productivity in crop plants (Long *et al*. 2006). However, promising targets for the enhancement of leaf photosynthetic capacity in soybean are still unclear.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the enzyme that catalyzes the initial step in photosynthetic carbon assimilation and photorespiratory pathways in the Calvin cycle (Woodrow and Berry 1988). Rubisco has been the primary target to improve leaf photosynthesis in crop plants, since it can limit leaf photosynthesis at the existing atmospheric CO_2 concentration (Farquhar *et al*. 1980). The limitation of photosynthesis by Rubisco results from the low catalytic turnover rate (k_{cat}) and competing oxygenation reaction of Rubisco in C_3 plants. To compensate for the disadvantages, large amount of Rubisco is accumulated in the leaves of C_3 plants (Evans 1989). It accounts for approximately 15–30% of total N to Rubisco in the leaf of some plant species (Makino *et al*. 1992).

Soybean is known to accumulate large amount of N in the leaf at the beginning of the seed-filling stage during the growth period (Osaki *et al*. 1988). In the previous study, we reported that leaf N and Rubisco content increased drastically, while the light-saturated CO_2 assimilation rate was relatively constant throughout the reproductive stage in soybean (Sakoda *et al*. 2016). Other studies showed the curvilinear relationship between the light-saturated CO_2 assimilation rate and leaf N content in some C_3 plants (Evans 1983, Sinclair and Horie 1989). This curvilinear relationship can be explained by the deactivation of Rubisco with the increase in the leaf N content as reported in apple (*Malus domestica* Borkh) and wheat (*Triticum aestivum* L.) (Mächler *et al*. 1988, Cheng and Fuchigami 2000). These results suggest that the activation state of Rubisco may decrease with the increase in leaf N content in soybean. However, it is not clear if the activation state of Rubisco is affected by the changes in leaf N content across different genotypes and growth stages in soybean.

Rubisco activase mediates the ATP-dependent removal of various inhibitory sugar phosphates from the Rubisco active site and regulates the activation of Rubisco (Portis

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Abbreviations: BSA – bovine serum albumin; C_i – intercellular CO_2 concentration; DAP – days after planting; DTT – dithiothreitol; g_s – stomatal conductance; k_{cat} – catalytic turnover rate; P_N – CO_2 assimilation rate; RbcL – large subunit of Rubisco; RcaI – large isoform of Rubisco activase; RcaII – small isoform of Rubisco activase; RuBP – ribulose-1,5-bisphosphate; R1 – beginning of flowering; R5 – beginning of seed filling.

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1992). The transgenic tobacco with decreased Rubisco activase content showed a substantial decrease in the activation state of Rubisco, demonstrating that Rubisco activase can be an essential factor regulating the activation state of Rubisco in plants (Jiang *et al.* 1994).

In the present study, we conducted a field experiment with four soybean genotypes that showed varied leaf photosynthetic capacity, N, and Rubisco content throughout the reproductive stage, as described by Sakoda *et al.* (2016). The gas-exchange parameters and leaf N content were measured at two growth stages in the reproductive stage. Simultaneously, the activation state of Rubisco and the distribution ratio of soluble protein to Rubisco and Rubisco activase in the leaf were investigated. The objective of the present study was to clarify the physiological basis for the inconsistency between the changes in leaf photosynthetic capacity and N content in soybean.

Materials and methods

Materials and cultivation of plants: Four soybean genotypes, the US commercial cultivar UA4805, the Japanese commercial cultivar Tachinagaha, PI 594409 A, and PI 603911 C, were cultivated at the Experimental Field of the Graduate School of Agriculture, Kyoto University, Kyoto, Japan (Lat. 35°2′N, Long. 135°47′E, and 65 m altitude; fulvic endoaquepts soil type). The sowing date was 4 July, 2016. The distance between rows and plants was 0.7 and 0.15 m, respectively. The fertilizers for N, P_2O_5, and K_2O were applied at 3, 10, and 10 g m^{-2}, respectively, before sowing. One experimental plot was established for each genotype, and each plot was composed of 24 plants in two rows. The days to the beginning of flowering (R1) and seed filling (R5) were recorded in all the genotypes.

Gas-exchange measurements were conducted using a portable gas-exchange system *LI-6400* (*LI-COR*, Lincoln, NE, USA). The CO_2 assimilation rate (P_N), the stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured in the field with a CO_2 concentration of 400 ppm, a PPFD of 1,500 μmol m^{-2} s^{-1}, and an air temperature of 33°C at the uppermost fully expanded leaf on 36 and 59 d after planting (DAP) (n = 4–6). The gas-exchange measurements were conducted from 09:00 to 13:00 h on the sunny day. The apparent mesophyll activity was calculated as the ratio of P_N to C_i.

Rubisco activity and activation state: To determine the k_{cat} of Rubisco in soybean, a leaf tissue (2.26 cm^2) was sampled from the uppermost fully expanded leaf of UA 4805 from 09:00 to 13:00 h on 36 DAP and frozen in liquid nitrogen. The leaf tissue was homogenized in 1 mL of extraction buffer [4 mM amino-*n*-caproic acid, 20 mM ascorbic acid, 0.8 mM benzamidine, 100 mL Bicine-NaOH, 5 mM dithiothreitol (DTT), 1 mM EDTA, 5 mM $MgCl_2$, and 2 mM NaH_2PO_4 at pH 8.0] with 0.4% (w/v) bovine serum albumin (BSA), 1% (w/v) polyvinylpolypyrrolidone, and quartz sand using a tilled pestle and mortar. The homogenate was centrifuged at 12,000 rpm for 2 min at 4°C. The supernatant was used for the determination of Rubisco activity and catalytic site. The extracted Rubisco was fully activated with 15 mM $MgCl_2$ and 10 mM $NaHCO_3$ on ice for 20 min. Rubisco total activity was measured at 30°C using [^{14}C] $NaHCO_3$ by assaying the incorporation of [^{14}C] in acid-stable products with some modifications, as described by Ishikawa *et al.* (2009). In the present study, 1 M HCl was added to the assay solution to stop the reaction. The Rubisco catalytic site was estimated by measuring the stoichiometric binding of [^{14}C] carboxyarabinitol-1,5-bisphosphate as described by Ishikawa *et al.* (2009). The k_{cat} of Rubisco was calculated based on Rubisco total activity and catalytic site.

The kinetic properties of Rubisco are known to be largely determined by Rubisco large subunit (RbcL) (Anderson and Backlund 2008). In the present study, genomic DNA was extracted from the leaf of four soybean genotypes using *DNA Prep Kit* (for plants) (*Cica Geneus*). Three partial fragments of *RbcL* were amplified by PCR using three pairs of primers;
RbcL1-F GTCGAGTAGACCTTGTTGTTTCG,
RbcL1-R AGCTCTACCATAATTCTTAGCGG,
RbcL2-F TGCGTGCTCTACGTCTGGAG,
RbcL2-R GTGAACATGATCTCCACCAG,
RbcL3-F ACTAGCTTGGCTCATTATTGCC,
RbcL3-R CCTTTTAGGAAAAGATTGGGCCG.

PCR products were purified by *FastGene Gel/PCR Extraction Kit* (*NIPPON Genetics Co, Ltd*). The base sequences of three fragments were determined to assemble the full-length sequence of *RbcL*. There was no difference in the full-length sequence of *RbcL* between four soybean genotypes as shown in Fig. 1S (*supplement*). In addition, Makino *et al.* (1987) showed the quite small variation of the k_{cat} of Rubisco among 25 varieties of rice. These facts support the consideration that the k_{cat} of Rubisco is similar in four soybean genotypes used in this study. We measured the k_{cat} of Rubisco in UA 4805 and assumed that it would be constant among soybean genotypes.

To measure Rubisco initial activity, the leaf tissue (2.26 cm^2) was sampled from the uppermost fully expanded leaf in all the genotypes after the measurements of P_N at 36 and 59 DAP and immediately frozen in liquid nitrogen. The leaf tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar. The leaf powder was suspended in 125 μl of extraction buffer [50 mM Bicine-NaOH, 20 mM ascorbic acid, 5 mM DTT, and 0.1 mM EDTA at pH 8.0] and subsequently centrifuged for a few seconds. The supernatant was used for the determination of Rubisco activity and catalytic site. The Rubisco total activity was calculated using the k_{cat} of Rubisco for UA 4805 and the catalytic site concentrations in each genotype. The activation state of Rubisco was calculated as the percentage of Rubisco initial activity to the total activity (n = 4–5).

Leaf nitrogen and proteins: For the quantification of leaf N content, the uppermost fully expanded leaf was sampled at 36 and 59 DAP in all the genotypes. The leaf area was measured with *LI-3100 area meter* (*LI-COR*, Lincoln, NE, USA). The leaves were dried at 70°C for 72 h, weighed, and ground to a coarse powder. The leaf N content was measured by Kjeldahl digestion followed by an indophenol assay (Vickery *et al*. 1946).

The supernatant obtained from the Rubisco initial activity analysis was used for the quantification of total soluble protein and Rubisco activase. In addition, the total soluble protein was extracted from the leaf of a rice cultivar, Nipponbare, as described by Ishikawa *et al*. (2011). The concentration of the total soluble protein was determined in soybean and rice using the Bradford assay with BSA as the standard (Bradford 1976). SDS-PAGE was conducted with 6.4 μg of total soluble protein with soybeans genotypes described by Ishikawa *et al*. (2011). Total soluble proteins (3.2 μg) extracted from rice was also applied as the standard to quantify Rubisco activase content of soybean genotypes using SDS-PAGE. The gel was subjected to immunoblotting with the antibody against Rubisco activase of rice. The immunoreactive band was detected using alkaline phosphatase, as described by Fukayama *et al*. (2006) (Fig. 2S, *supplement*). The relative amount of Rubisco activase was calculated in soybeans by comparing the detected bands corresponding to the large (RcaI) and small (RcaII) isoform of Rubisco activase of each soybean genotype with those of rice (n = 4–5). The images were analyzed using the gel analysis tool of *Image J* software (Schneider *et al*. 2012).

Statistical analysis: The analysis of variance (*ANOVA*) was applied to evaluate the difference of each parameter between 36 and 59 DAP in each genotype. In the present study, the statistical analysis was conducted using *R* (*R Development Core Team* 2010).

Results

Leaf photosynthetic capacity: UA 4805, Tachinagaha, PI 594409 A, and PI 603911 C reached R1 at 41, 35, 41, and 37 DAP; and R5 at 56, 48, 56, and 50 DAP, respectively (Table 1S, *supplement*).

The CO_2 assimilation rate (P_N) varied from 22.0 to 31.5 μmol(CO_2) m^{-2} s^{-1} at 36 DAP, and from 27.8 to 32.9 μmol(CO_2) m^{-2} s^{-1} at 59 DAP among four genotypes (Fig. 1*A*). The apparent mesophyll activity (P_N/C_i) varied from 0.079 to 0.111 μmol(CO_2) m^{-2} s^{-1} ppm^{-1} at 36 DAP, and from 0.094 to 0.117 μmol(CO_2) m^{-2} s^{-1} $μmol^{-1}$ mol at 59 DAP (Fig. 1*B*). P_N and P_N/C_i were mainly constant from 36 to 59 DAP in UA 4805, PI 594409 A, and PI 603911 C, whereas these parameters significantly increased in Tachinagaha. The g_s of Tachinagaha approximately doubled during this period, which mainly explained the increase of P_N in this genotype (Fig. 3S).

Leaf N content and the activation state of Rubisco: Leaf N content ranged from 1.55 to 1.79 g m^{-2} at 36 DAP, and from 2.14 to 2.39 g m^{-2} at 59 DAP among four genotypes (Fig. 2*A*). Leaf N content significantly increased from 36 to 59 DAP in all the genotypes. The catalytic turnover rate (k_{cat}) of Rubisco was 2.62 mol mol^{-1} s^{-1} in UA 4805 at 36 DAP. In the present study, this value of k_{cat} was used for the calculation of the activation state of Rubisco in each genotype. The activation state of Rubisco varied from 62.9–82.9% at 36 DAP, and from 55.3–60.0% at 59 DAP (Fig. 2*B*). The activation state of Rubisco significantly decreased from 36 to 59 DAP in UA 4805, Tachinagaha, and PI 603911 C.

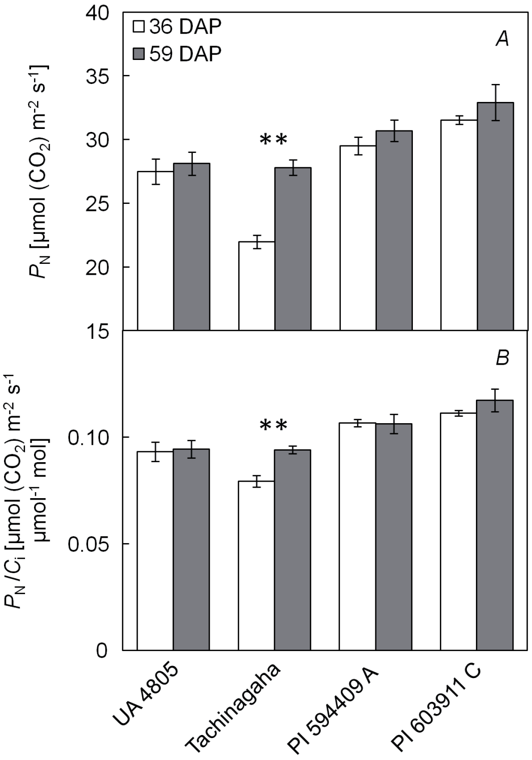

Fig. 1. Changes in CO_2 assimilation rate and apparent mesophyll activity of four soybean genotypes. The CO_2 assimilation rate (*A*; P_N) and apparent mesophyll activity (*B*; P_N/C_i) of the uppermost fully expanded leaf in UA 4805, Tachinagaha, PI 594409 A, and PI 603911 C on 36 (white bar) and 59 (gray bar) days after planting (DAP) were measured at a CO_2 concentration of 400 ppm, a PPFD of 1500 μmol m^{-2} s^{-1}, and an air temperature of 33°C. The *vertical bars* indicate the standard error (n = 4–6). ** – significant difference of each parameter between 36 and 59 DAP at $p<0.01$.

Protein distribution to Rubisco and Rubisco activase: The ratio of Rubisco to total soluble protein ranged from 0.47–0.57 g g^{-1} at 36 DAP, and was the similar at 59 DAP in four soybean genotypes (Fig. 3*A*). The ratio of Rubisco to total soluble protein significantly decreased from 36 to 59 DAP in UA 4805, PI 594409 A, and PI 603911 C, whereas it was largely constant in Tachinagaha. When standardized by the total soluble protein amount, the relative amount of Rubisco activase ranged from 0.90–1.09 at 36 DAP, and from 0.49–0.95 at 59 DAP (Fig. 3*B*). The Rubisco activase amount was largely constant from 36 to 59 DAP in UA 4805, PI 594409 A, and PI 603911 C, whereas it significantly decreased in Tachinagaha.

Discussion

In the present study, the P_N and the apparent mesophyll activity (P_N/C_i) were mostly constant from 36 to 59 DAP in UA 4805, PI 594409 A, and PI 603911 C, whereas leaf N content significantly increased in all the genotypes (Figs. 1, 2*A*). The activation state of Rubisco significantly decreased during this period in UA 4805, Tachinagaha, and PI 603911 C (Fig. 2*B*). These results showed that the activation state of Rubisco can decrease with the increase in the leaf N content, which can result in the constant rate of CO_2 assimilation during the reproductive stage of soybean. A negative correlation between the activation state of Rubisco and leaf N content was observed in wheat and apple with various N concentrations (Mächler *et al.* 1988, Chen and Fuchigami 2000). The present study shows that the deactivation of Rubisco with the N accumulation can be observed even across different growth stages in soybean with the same N concentration (Fig. 2).

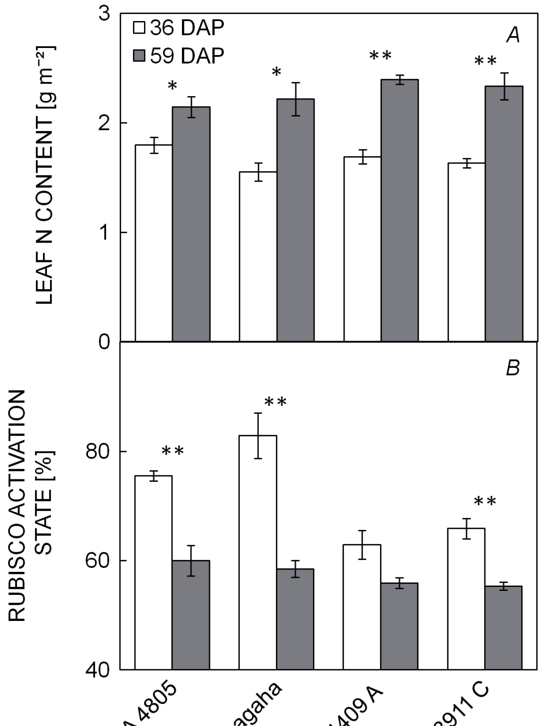

Fig. 2. Changes in the leaf N content and the activation state of Rubisco of four soybean genotypes. *A* – the leaf N content of the uppermost fully expanded leaf was determined in UA 4805, Tachinagaha, PI 594409 A, and PI 603911 C on 36 (white bar) and 59 (gray bar) days after planting (DAP). *B* – the activation state of Rubisco was estimated by measuring the Rubisco activity on 36 and 59 DAP in four soybean genotypes. The *vertical bars* indicate the standard error (n = 4–6). $^{*,\ **}$ – significant difference of each parameter between 36 and 59 DAP at $p<0.05$ and 0.01, respectively.

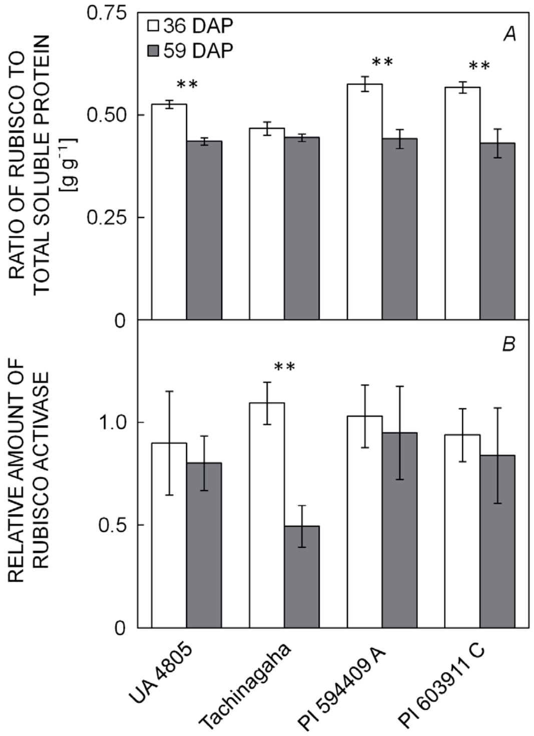

Fig. 3. Changes in the ratio of Rubisco to total soluble protein and the relative amount of Rubisco activase of four soybean genotypes. *A* – the ratio of Rubisco to total soluble protein was measured in the uppermost fully expanded leaf in UA 4805, Tachinagaha, PI 594409 A, and PI 603911 C at 36 (white bar) and 59 (gray bar) days after planting (DAP). *B* – when standardized by the total soluble protein amount, the Rubisco activase amount was measured in four soybean genotypes, as compared to that of rice as the standard at 36 and 59 DAP. The vertical bars indicate the standard error (n = 4–5). ** – significant difference of each parameter between 36 and 59 DAP at $p<0.01$.

In the transgenic tobacco, the decrease in Rubisco activase content resulted in the deactivation of Rubisco (Mate *et al.* 1993, Jiang *et al.* 1994). In the present study, the ratio of Rubisco to total soluble protein significantly decreased from 36 to 59 DAP, whereas the relative amount of Rubisco activase was largely constant in UA 4805, PI 594409 A, and PI 603911 C (Fig. 3). This suggests that the decrease in the activation state of Rubisco was attributed to an unknown mechanism other than to the deficiency of Rubisco activase per Rubisco amount in these three genotypes. Perdomo *et al.* (2017) suggests that the decrease in the activation state of Rubisco can be related not to Rubisco activase amount but to its activity under high temperature and water deficit conditions in crop plants. In the present study, the deactivation of Rubisco might be associated with the decrease in the activity of Rubisco activase. In Tachinagaha, on the other hand, the ratio of Rubisco content with the increase in leaf N content throughout the reproductive stage of the four soybean genotypes. Based on the observation of leaf N content, the increase in Rubisco content was expected among four genotypes, although Rubisco content was not shown in the present study. It was suggested that the change in Rubisco content affected the wide range of metabolites in the Calvin cycle and the energy status in chloroplasts

(Suzuki *et al*. 2012). The carbamylation state of Rubisco is affected by the activity of Rubisco activase. The activity of Rubisco activase is found to be sensitive to ATP/ADP ratio, since Rubisco activase requires ATP for its function and is inhibited by ADP (Portis *et al*. 1992). The activation state of Rubisco increased with the increase in ATP/ADP ratio in transgenic tobacco with decreased Rubisco content (Quick *et al*. 1991). The activity of Rubisco activase can be also affected by its redox change mediated by ferredoxin–thioredoxin, which can alter the sensitivity of Rubisco activase to the inhibition by ADP (Zhang *et al*. 2002). In addition, the activation state of Rubisco can be affected by stromal pH and metabolites such as ribulose-1,5-bisphosphate (RuBP) and phosphoglyceric acid (Servaites 1991, Portis *et al*. 2003). Overall, the deactivation of Rubisco observed in the present study might result from the above change in the Calvin cycle metabolites and energy status with increasing Rubisco content in soybean.

The activation state of Rubisco ranged 80–100% in other crop plants under high light and the ambient CO_2 condition, whereas it decreased to 50–60% during the reproductive stage with the increase in the leaf N content among soybean genotypes in the present study (Fig. 2*B*) (Carmo-Silva *et al*. 2012, Fukayama *et al*. 2012). It implies that the amount of Rubisco in soybean leaf can be excessive as compared to that of other components related to RuBP regeneration during this period.

In conclusion, we found a decrease in the activation state of Rubisco with the increase in leaf N content, which can result in the constant rate of CO_2 assimilation during the reproductive stage in soybean. The deactivation of Rubisco can be attributed to an unknown mechanism rather than to the deficiency of Rubisco activase per Rubisco amount in the leaf. Further investigation is needed to identify the mechanism to regulate the activation state of Rubisco in relation to the change in Rubisco content throughout the reproductive stage of soybean.

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