Relationships between Photosynthetic Activity and the Amounts of Rubisco Activase and Rubisco in Rice Leaves from Emergence through Senescence*

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Abstract : Photosynthetic activity determined as O_2 evolution (OER) and the amounts of Rubisco activase, Rubisco, total soluble protein and chlorophyll were investigated in the 10th leaf of rice, *Oryza sativa* L. cv. Nipponbare, in relation to its aging. The Rubisco activase content increased until the 17th day after leaf emergence, at which time it attained its maximum and accounted for 1.43% of total soluble protein ; thereafter, it decreased rapidly. This change was most remarkable compared to the other leaf constituents examined. The Rubisco content had already reached its maximum 3 days after leaf emergence and had begun to decrease earliest among the leaf constituents. The OER depended linearly on the leaf Rubisco content below the value of 3 g m^{-2} , but tended to saturate above this value. On the contrary, the amount of Rubisco activase and OER were linearly correlated during the life span of the leaf. The *in vivo* Rubisco activity, as the OER per unit Rubisco content, increased exponentially with the increased Rubisco activase/Rubisco ratio. These results show that the amount of Rubisco activase is closely related to the photosynthetic rates in rice leaf from its emergence through senescence, and suggest that *in vivo* Rubisco activity can be restricted by Rubisco activase content, particularly when the leaf is young and accumulates excess Rubisco.

Key words : Aging, *Oryza sativa* L., Photosynthesis, Rice, Rubisco, Rubisco activase, Senescence.

イネ葉の加齢に伴う光合成能力の変化と Rubisco activase および Rubisco 含量との関係: 深山 浩・内田直次**・東 哲司・安田武司** (神戸大学大学院自然科学研究科, **神戸大学農学部)

要 旨 : 日本型水稻の日本晴を供試し, 主稈第 10 葉の加齢に伴う最大光合成能力(酸素電極法)の変化と Rubisco activase, Rubisco, 全可溶性タンパクおよびクロフィルの各含量の変化を調べ, それらの間の関係について検討した. Rubisco activase 含量は最大値を示す 17 日目まで徐々に増加し, その後急速に減少した. その変化は葉内成分中最も大きかった. また, 全可溶性タンパクに占める割合は最大で 1.43%であった. Rubisco 含量は出葉後 3 日目で既に最大値であり, 低下しはじめる時期が葉内成分中最も早かった. 光合成速度は 3 g m^{-2} 以下の Rubisco 含量と比例関係にあるが, その値を越えると飽和する傾向にあった. 一方, Rubisco activase 含量との間には葉の一生を通して高い正の相関が認められた. そして, Rubisco に対する Rubisco activase の含量比が高まると, Rubisco の *in vivo* での比活性(単位 Rubisco 量当りの光合成速度)は高くなった. 以上の結果から, Rubisco activase 含量はイネ葉の加齢に伴う光合成能力の変化と密接に関わっており, 特に葉が若く Rubisco が過剰にある場合, *in vivo* の Rubisco 活性が Rubisco activase による量的な制限を受けているものと考えられる.

キーワード : イネ, 加齢, 光合成, Rubisco, Rubisco activase, 老化.

Photosynthesis in rice leaves are mainly restricted by Rubisco content because its activity is much lower than the other photosynthetic enzymes⁸⁾ and limitation caused by RuBP regeneration does not occur throughout the life of the leaf¹¹⁾. Enzymic properties of rice

Rubisco are characterized by low maximum specific activity and high affinity for substrate CO_2 compared to those of other higher plants¹²⁾. These kinetic parameters and amounts of Rubisco can explain CO_2 assimilation rates at various intercellular CO_2 concentrations under the saturating irradiance¹⁰⁾. On the other hand, Rubisco activation is regulated by light intensity¹¹⁾, and its potential activation state varies depending on the leaf age²⁵⁾. Therefore, it is considered that Rubisco activation in a rice leaf is closely related to its

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photosynthetic rate.

Early investigations on this topic showed that the lacking of Rubisco activation in a mutant of *Arabidopsis thaliana* was correlated with the absence of Rubisco activase composed of two chloroplast polypeptides^{17,18}. Now, it is known that Rubisco activase plays a central role in Rubisco activation. The most important roles of Rubisco activase are (i) carbamylation of the amino group of a lysin residue near the Rubisco active site¹⁵ and (ii) dissociation of RuBP tightly bound to inactive Rubisco²⁷. While much attention has focused on the biochemistry and molecular biology of Rubisco activase since its discovery, less attention has paid to understanding the control that Rubisco activase exerts on the photosynthetic process. So, it is important to investigate quantitative relationships between Rubisco activase and photosynthetic rate.

In a rice leaf, kinetic analysis of CO₂ assimilation suggested the presence of some activators²². Thus, as an approach to studying the activation mechanisms of photosynthesis, we had purified Rubisco activase from rice leaves, and showed the existence of this enzyme in rice leaves⁴. In this study, we examined the ontogenetic changes in photosynthetic activity and the amounts of Rubisco activase and other leaf constituents in a rice leaf, and discussed the possible roles of Rubisco activase in regulating the photosynthesis.

Materials and Methods

1. Plant culture and sampling

Japonica rice (*Oryza sativa* L. cv. Nipponbare) was used in this study. Plants were water cultured under natural conditions. Seeds were sterilized with 1% sodium hypochlorite solution for 30 minutes and then soaked in tap water at 25°C for 2 days. After germination, seedlings were placed on a plastic net with a styrofoam frame floating on tap water. When plants were at 4-5 leaf age, each seedling which was supported with a sponge at its base, was transferred to a 42-liter box at a density of 6×7 cm on nutrient solution according to Mae *et al.*⁶. The concentration of culture solution was progressively increased depending on plant growth. The solution was renewed every 3 days and its pH was adjusted to 5.0-5.5 by adding of 1N H₂SO₄. The 10th

leaf blades on the main stems were used as samples and were collected once a week from 9.5 leaf age until full senescence. All the results were expressed as the mean of five replicates.

2. Photosynthetic measurement

The maximum rate of CO₂ assimilation was determined as O₂ evolution²⁶ using a gas-phase oxygen electrode (Hansatech; CB1D, LS2, LD-1). The portion about 5 cm² between 13 cm and 18 cm from the tip of the 10th leaf blade was taken as a source of a leaf piece (two strips). The leaf piece was floated on deionized water overnight because the higher and stable O₂ evolution rates could be obtained by this treatment in a pre-experiment. Photosynthetic measurements were conducted under following conditions: air temperature of 25°C, gas mixture of 5% CO₂, 20% O₂ and 75% N₂, saturated water vapor, and illuminance of 2,400 μmol photon m⁻²s⁻¹. Measurement started after pre-illumination for 10 min in air stream of the same conditions described above. The measured leaf was frozen in liquid nitrogen and stored at -80°C until required.

3. Extraction and determination of chlorophyll and soluble protein

Frozen leaf pieces (about 50 mg) were homogenated in 4 ml extraction buffer (50 mM Na-phosphate, 5 mM DTT, 0.1 mM EDTA, 12.5% glycerol, pH7.5) in a chilled mortar and pestle containing a slight amount of PVPP and acid washed quartz sand. For chlorophyll determination, one ml of this homogenate containing cell debris was collected stirring with pestle, and then added 4 ml of 100% acetone. After incubation at 4°C for one hour (h) in darkness, this suspension was centrifuged at 5,000 × g for 10 min. The supernatant was collected and assayed according to the method of Arnon¹¹. The remaining homogenate in the mortar was centrifuged at 30,000 × g for 10 min at 4°C. The supernatant was used to determine the amounts of total soluble protein, Rubisco activase and Rubisco. Total soluble protein was determined by the method of Bradford²¹ using BSA as a standard.

4. Determination of Rubisco content

Rubisco content was determined by single radial immuno diffusion using rabbit polyclonal antibodies against rice Rubisco purified according to Makino *et al.*⁹. Soluble leaf

extracts were applied to agar gels (1.2%) containing 0.9% (w/v) NaCl, 0.1% (w/v) NaN_3 and 1.0% Rubisco antiserum. The gels were incubated at 25°C for 4 days and then washed with 0.9% (w/v) NaCl solution. Then, the gels were stained in a solution containing 0.25% (w/v) Coomassie Brilliant Blue R-250, and destained in a solution of 25% methanol, 5% ethanol and 7% acetic acid. The resulting diameters were measured and compared with standards from purified rice Rubisco (0.6-2.4 μg) that had been applied in duplicate on each gel.

5. Determination of Rubisco activase content

Rubisco activase content was determined by enzyme linked immunosorbent assay (ELISA) using rabbit polyclonal antibodies against rice Rubisco activase purified as reported previously⁴⁾. Leaf extracts were diluted to 1:50 with 10 mM Na-phosphate buffer, pH 7.2, and then applied to microtiter plate for ELISA (Corning; Cell Wells). After it was incubated at 4°C for one night, non-specific protein binding sites were blocked with T-PBS (20 mM Na-phosphate, 150 mM NaCl, 0.05% Tween 20) containing 5% BSA for 3h at 25°C. Afterwards, rabbit polyclonal antibodies against rice Rubisco activase diluted to 1:1000 with T-PBS was added to the plate and incubated at 25°C for 2h. Peroxidase conjugated rabbit IgG (H+L)-goat antibody (Wako Pure Chemical) was applied to each well and incubated at 25°C for 2h. After each process described above, the plate was washed with T-PBS completely. Substrate solution (0.1 M citrate, 0.2 M Na-phosphate, 1 mM ABTS, 150 $\mu\text{l/l}$ H_2O_2 , pH 5.0) was applied to the plate to start the reaction. After incubation at 25°C for about 30 min, the reaction was stopped with 0.25 M citric acid solution. The optical absorbance at 415 nm was measured by a micro plate reader (Tosoh; A4) and the resulting absorbance was compared with standards from purified rice Rubisco activase (0.05-0.40 ng) in duplicate.

Results

Figure 1 shows the change in photosynthetic O_2 evolution rate (OER) in the 10th leaf blade on the main stem of rice from leaf emergence through senescence. The OER increased slightly from 3 days after leaf emer-

gence, and attained its maximum (34 $\mu\text{mol m}^{-2}\text{s}^{-1}$) on the 17th day, and then decreased rapidly. Thirty-eight days after leaf emergence, the leaf blade almost lost its activity.

The amount of Rubisco activase in the leaf blade changed in a similar way as the OER (Fig. 2). Rubisco activase content attained its maximum 17th days after leaf emergence, and then declined rapidly to nearly zero 38 days after leaf emergence. The ratio of Rubisco activase as a percentage of total soluble protein was the highest 17 days after leaf emergence and was negligible 38 days after leaf emergence.

The patterns of changes in the contents at each sampling day as a percentage of the values 3 days after leaf emergence differed remarkably among the leaf constituents (Fig. 3). Rubisco content had already reached its maximum at the onset of the experiment, 3 days after leaf emergence, and then decreased steadily. Moreover, the start of significant decline in Rubisco was the fastest among all the constituents. The contents of total soluble protein and chlorophyll were almost constant from 3 to 17 days after leaf emergence and then decreased. The changes in the Rubisco activase content were the most remarkable

Fig. 1. Changes in the photosynthetic O_2 evolution rate in the 10th leaf blade on the main stem of rice from its emergence through senescence. Photosynthetic activity was measured by an oxygen electrode under the following conditions; air temperature of 25°C, gas mixture of 5% CO_2 , 20% O_2 and 75% N_2 , saturated water vapor and illuminance of 2,400 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$. Bars in the figure indicate standard errors of the mean ($n=5$).

Fig. 2. Changes in the Rubisco activase content in the 10th leaf blade on the main stem of rice from its emergence through senescence. Rubisco activase content was determined by ELISA using purified rice Rubisco activase as standard. The numbers in parentheses indicate the ratio of Rubisco activase to total soluble protein content. Bars in the figure indicate standard errors of the mean ($n=5$).

Fig. 3. Changes in the relative contents of leaf constituents in the 10th leaf blade on the main stem of rice from its emergence through senescence. The values at each sampling day were represented for percentage of the values 3 days after leaf emergence. The amounts of Rubisco activase (●), Rubisco (■), total soluble protein (▲) and chlorophyll (◆) were 72.5 mg m⁻², 3.92 g m⁻², 6.76 g m⁻² and 373 mg m⁻², respectively.

Fig. 4. Correlation between photosynthetic O₂ evolution rate and Rubisco content. **means significance at 1% level. Data were obtained from Figs. 1 and 3.

Fig. 5. Correlation between photosynthetic O₂ evolution rate and Rubisco activase content. ***means significance at 0.1% level. Data were obtained from Figs. 1 and 2.

among the leaf components; the value varied from 130% to 0.5% depending on the leaf age. At 38 days after leaf emergence, Rubisco activase almost disappeared, whereas about 20% of the initial values of the other leaf constituents remained.

Based on these results, we examined the relationships between the OER and the amounts of Rubisco and Rubisco activase

(Figs. 4 and 5). Although a linear correlation was observed between the OER and Rubisco at amounts less than 3 g m⁻², the OER tended to saturate against Rubisco at values higher than this. In contrast, the OER and the amount of Rubisco activase were linearly correlated during the life span of the leaf. Its correlation coefficient of linear regression was 0.995 (significant at $p=0.001$). Figure 6

Fig. 6. Correlation between Rubisco activity *in vivo* and the ratio of Rubisco activase to Rubisco content. The Rubisco activity was defined as the photosynthetic rate per unit Rubisco content. r^2 shows coefficients of determination. Data were obtained from Figs. 1, 2 and 3.

shows the relation between the *in vivo* Rubisco activity and Rubisco activase/Rubisco ratio. The former was calculated as the OER per unit Rubisco content, and the latter as percentages of Rubisco activase to Rubisco contents. The *in vivo* Rubisco activity increased exponentially with an increase of the ratio of Rubisco activase to Rubisco.

Discussion

Rubisco activase content varied with the age of a rice leaf, and the maximum content of Rubisco activase as a percentage of total soluble protein was 1.43% (Fig. 2). This value is comparable to about 2% of spinach leaf¹⁶⁾. The amount of Rubisco activase changed most remarkably among the leaf constituents examined in this experiment (Fig. 3), and the changing pattern was similar to that of the OER (Fig. 1). On the other hand, Rubisco content had already reached its maximum 3 days after leaf emergence and then began to decrease earlier than the other leaf components. This may reflect that Rubisco is a protein little turned over, and that the degradation during leaf aging proceeds before chloroplast disintegration⁷⁾. Therefore, our results may causally reflect that the synthesis or degradation processes are different between Rubisco and Rubisco activase, though they are both chloroplastic enzymes, and support

the recent findings that Rubisco expression was not strongly coupled with that of Rubisco activase, and *vice versa*⁵⁾.

We examined the relationships between the maximum rate of photosynthesis and the amounts of Rubisco and Rubisco activase. The photosynthetic rate at saturated CO_2 concentration and light intensity depended linearly on leaf Rubisco content below the value of 3 g m^{-2} , but tended to saturate above this value (Fig. 4). This curvilinear relationship was also observed at ambient CO_2 level^{10,13,25)} and saturated CO_2 level²¹⁾. In these studies, the amount of Rubisco departing from linearity with the rate of photosynthesis was variable and ranged from 1.5 to 4.0 g m^{-2} . Although this inconsistency may be mainly caused by different analytical methods and conditions, it possibly related that the activation states of Rubisco varied due to the leaf condition used as materials. At low CO_2 and light saturation, photosynthesis is largely dependent on *in vivo* Rubisco capacity²⁴⁾. In a rice leaf, the *in vitro* activity of completely activated Rubisco would be just sufficient to account for the *in situ* photosynthetic rate at intercellular CO_2 concentration¹⁰⁾. If that is the case, as the CO_2 concentrations at carboxylation site was lowered by CO_2 transport resistance²²⁾, contribution of some activators like Rubisco activase should be taken into consideration. In rice plants grown under natural conditions, Rubisco activation states varied with leaf age and were lower in a young leaf because of its excess amount of Rubisco²⁵⁾. We found that the efficiency of carboxylation at low intercellular CO_2 concentration (i.e. amount of active Rubisco or *in vivo* Rubisco activity) tended to be saturated against Rubisco at values higher than 3 g m^{-2} , whereas the efficiency was almost linearly correlated to the amount of Rubisco activase²³⁾. Moreover, several reports on transgenic tobacco plants with an antisense gene directed against the mRNA for Rubisco activase revealed that low Rubisco activation states caused a depression of photosynthesis at ambient CO_2 concentrations and saturated light intensity¹⁴⁾, and that control of photosynthesis is largely shared between Rubisco and Rubisco activase⁵⁾. These reports indicate that Rubisco is not always fully activated even in light saturation, and that Rubisco activase plays a essential role in

regulating the photosynthetic rate at low CO₂ concentrations.

In general, light and CO₂-saturated photosynthesis is limited by Pi regeneration capacity. If Pi level decline enough, photophosphorylation can become inhibited, reducing ATP synthesis and in turn RuBP regeneration¹⁸⁾. As the activity of Rubisco activase and therefore Rubisco activation is dependent on the ATP production and electron transport^{3,20)}, it is possible that the content of Rubisco activase closely correlates to photosynthetic activity. In this study at saturated CO₂ concentration and light intensity, the photosynthetic rate did not respond to the Rubisco content higher than 3 g m⁻² (Fig. 4) but to the amount of Rubisco activase (Fig. 5). This result suggests that Rubisco activase restricts *in vivo* Rubisco activity and the maximum photosynthetic rate, although the extent of Pi regeneration capacity is unknown so far. Thus, we examined the relationships between *in vivo* Rubisco activity and Rubisco activase/Rubisco ratio (Fig. 6). As a result, we found a relation that the *in vivo* Rubisco activity increased exponentially with the increased Rubisco activase/Rubisco ratio. The importance of this ratio was also recognized by the *in vitro* experiment, in which the final Rubisco specific activity achieved was dependent on the concentration of both Rubisco and Rubisco activase¹⁶⁾. Our ratios of Rubisco activase to Rubisco were within the range of it in this *in vitro* analysis.

Therefore, it is concluded that photosynthetic activity of naturally aging rice leaf changes along with *in vivo* Rubisco activity, which can be restricted by the amount of Rubisco activase because of excess amounts of Rubisco accumulated prior to Rubisco activase, especially in the developing stage.

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* In Japanese with English abstract or summary.

** In Japanese.

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