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 O₂ 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.

Keywords: Aging, Oryza sativa L., Photosynthesis, Rice, Rubisco, Rubisco activase, Senescence.

Photosynthesis in rice leaves are mainly restricted by Rubisco content because its activity is much lower than the other photosynthetic enzymes⁴⁰ and limitation caused by RuBPy regeneration does not occur throughout the life of the leaf.⁹. Enzymic properties of rice Rubisco are characterized by low maximum specific activity and high affinity for substrate CO₂ compared to those of other higher plants.¹² These kinetic parameters and amounts of Rubisco can explain CO₂ assimilation rates at various intercellular CO₂ concentrations under the saturating irradiance.⁶ On the other hand, Rubisco activation is regulated by light intensity¹¹, and its potential activation state varies depending on the leaf age.²⁵ Therefore, it is considered that Rubisco activation in a rice leaf is closely related to its
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) carbamilation of the amino group of a lysin residue near the Rubisco active site\(^{15}\) and (ii) dissociation of RuBP tightly bound to inactive Rubisco\(^{23}\). 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 CO\(_2\) assimilation suggested the presence of some activators\(^{22}\). 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 leaves\(^{40}\). 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 Mac *et al*\(^{40}\). 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 H\(_2\)SO\(_4\). 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 CO\(_2\) assimilation was determined as O\(_2\) evolution\(^{40}\) using a gasphase oxygen electrode (Hansatech; CB1D, LS2, LD-1). The portion about 5 cm\(^2\) 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 O\(_2\) 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% CO\(_2\), 20% O\(_2\) and 75% N\(_2\), saturated water vapor, and illuminance of 2,400 \(\mu\)mol photon m\(^{-2}\)s\(^{-1}\). 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 Arnon\(^{11}\). 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 Bradford\(^{29}\) 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*\(^{40}\). Soluble leaf
extracts were applied to agar gels (1.2%) containing 0.9% (w/v) NaCl, 0.1% (w/v) NaN₃ 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 μl/l H₂O₂, 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 O₂ evolution rate (OER) in the 10th leaf blade on the main stem of rice from its emergence through senescence. The OER increased slightly from 3 days after leaf emergence, and attained its maximum (34 μmol m⁻² 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 O₂ 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% CO₂, 20% O₂ and 75% N₂, saturated water vapor and illuminance of 2,400 μmol photon m⁻² 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
shows the relation between the \textit{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 \textit{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 leaf\cite{10}. 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 components. This may reflect that Rubisco is a protein little turned over, and that the degradation during leaf aging proceeds before chloroplast disintegration\cite{11}. 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 \textit{vice versa}\cite{12}.

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\cite{13,14,15} and saturated CO$_2$ level\cite{16}. 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 \textit{in vivo} Rubisco capacity\cite{17}. In a rice leaf, the \textit{in vitro} activity of completely activated Rubisco would be just sufficient to account for the \textit{in situ} photosynthetic rate at intercellular CO$_2$ concentration\cite{18}. If that is the case, as the CO$_2$ concentrations at carboxylation site was lowered by CO$_2$ transport resistance\cite{19}, 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 Rubisco\cite{20}. We found that the efficiency of carboxylation at low intercellular CO$_2$ concentration (i.e. amount of active Rubisco or \textit{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 activase\cite{21}. 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 intensity\cite{22}, and that control of photosynthesis is largely shared between Rubisco and Rubisco activase\cite{23}. 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 regeneration. As the activity of Rubisco activase and therefore Rubisco activation is dependent on the ATP production and electron transport, 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 activase. 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.

References
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* In Japanese with English abstract or summary.
** In Japanese.
*** In Japanese. The title is translated by the present authors.