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Screening of High $k_{\text{cat}}$ Rubisco among Poaceae for Improvement of Photosynthetic CO$_2$ Assimilation in Rice

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Abstract: The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a major limitation of photosynthetic CO$_2$ assimilation in C$_3$ plants. In order to find useful Rubisco for improvement of photosynthesis in rice under elevated CO$_2$, we analyzed the catalytic turnover rate ($k_{\text{cat}}$) of Rubisco in Poaceae including C$_3$ alpine plants, C$_4$ cold-resistant plants and C$_4$ plants. Rubisco in these plants showed 1.1- to 2.8-fold higher $k_{\text{cat}}$ than that in rice. However, the most of high $k_{\text{cat}}$ Rubisco also showed a higher km for CO$_2$ (K$_c$) than that of rice, indicating that increase in $k_{\text{cat}}$ led to decrease in the affinity for CO$_2$. Rubisco in Festuca ovina, Phleum pratense and Sorghum bicolor showed relatively high $k_{\text{cat}}$ to K$_c$. Although the $k_{\text{cat}}$ of Rubisco in F. ovina and P. pratense was not so high (1.5-1.6 fold relative to rice), the K$_c$ was comparable to that in rice and the amino acid sequence of RbcL shared higher identity to that in rice than that in S. bicolor. By contrast, Rubisco of S. bicolor showed considerably high $k_{\text{cat}}$ (2.5-fold relative to rice), which is considered to be the most important factor for improvement of photosynthesis. In our estimation, the expression of high $k_{\text{cat}}$ Rubisco of F. ovina and S. bicolor in rice could significantly enhance CO$_2$ assimilation at Ci of 50 Pa, the level assumed to be reached by the middle of this century.

Key words: $k_{\text{cat}}$, Km, Oryza sativa L., Photosynthesis, Rice, Rubisco.
determinant of \( k_{cat} \) of Rubisco. From these view points, it is considered that one of the best ways to improve the photosynthetic capacity of rice in the near future atmospheric conditions is to find high \( k_{cat} \) Rubisco with high amino acid sequence similarity to rice Rubisco and substitute rice Rubisco with its \( RbcL \) using genetic recombination technology.

In this study, we analyzed the kinetic properties of Rubisco among Poaceae including \( C_4 \) alpine plants, \( C_3 \) cold-resistant plants and \( C_4 \) plants. In addition, we determined the sequences of \( RbcL \) and made a comparison of the deduced amino acid sequences among higher plants. Finally, the estimation of \( CO_2 \) assimilation rate was carried out to elucidate whether high \( k_{cat} \) Rubisco found in this study could enhance the photosynthetic performance of rice under elevated \( CO_2 \).

**Materials and Methods**

1. **Plant materials**

Rice (\( Oryza sativa \) L. cv. Nipponbare), \( C_4 \) alpine plants, \( C_3 \) cold-resistant plants and \( C_4 \) plants of Poaceae were grown and sampled at optimum conditions to obtain well developed leaves. Rice was planted on a paddy soil and grown in the greenhouse. Upper most fully expanded leaves were sampled in the middle of July 2007. The mature leaves of \( C_4 \) alpine plants, \( Anthoxanthum japonicum \) Hack., \( Calamagrostis longiseta \) Hack., \( Festuca ovina \) L. and \( Phleum alpinum \) L. were sampled in early June 2007 at the Botanical Garden of the Hakusan Alpine Plants Research Group (Hakusan city, Japan). The \( C_3 \) cold-resistant plants, \( Dactylius glomerata \) L. cv. Aikappu (orchard grass), \( Poa arctica \) R. Br. ssp. \( lanata \), \( Phleum pratense \) L. cv. Natsuyutaka (timothy) and \( Secale cereale \) L. cv. King Lye (rye) were planted on a commercial culture soil in late September, because they cannot grow well during summer under natural conditions. The mature leaves of these plants were sampled in the middle of December 2007. The \( C_4 \) plants, \( Zea mays \) L. cv. Golden×Bantam (maize), \( Sorghum bicolor \) L. Moench. cv. Tentaka (sorghum), \( Panicum maximum \) Jacq. cv. Natsuyutaka (guinea grass) were planted on a commercial culture soil and grown under natural condition. The mature leaves were sampled in the middle of July 2007. \( Pennisetum purpureum \) Schumach cv. Mericlone (napier grass) was grown in the experimental field of Kobe University and the mature leaves were sampled in the middle of July 2007. The mature leaves of \( Eleusine indica \) (L.) Gaertn., \( Miscanthus sinensis \) Anderss. and \( Setaria viridis \) P. Beauv. were sampled in the campus of Kobe University in the middle of September 2007. All of the leaves were collected at around 1100 in sunny days and immediately frozen in liquid nitrogen. Samples were stored in a –80°C freezer until use. All cultivated plants were watered and fertilized as needed.

2. **Determination of Rubisco activity and catalytic site**

About 2 cm\(^2\) of sampled leaf tissues (in case of rice, 1 cm\(^2\) of leaf tissues were used) were rapidly homogenized in 1 mL of extraction buffer (100 mM Bicine-NaOH, 1 mM EDTA, 5 mM MgCl\(_2\), 2 mM NaH\(_2\)PO\(_4\), 0.4% (w/v) BSA, 5 mM DTT, 4 mM amiono-n-caproic acid, 0.8 mM benzamidine, pH 8.0) using a chilled motor and pestle with a small amount of quartz sand. The homogenate was then centrifuged at 15,000 g for 2 min at 4°C. The supernatant was used for the determination of Rubisco activity and Rubisco catalytic site.

Rubisco activity was determined at 28°C using \([^{14}C]\) NaHCO\(_3\) by assaying the incorporation of \( ^{14}C \) into acid-stable products, as described by Kubien et al. (2003) with some modifications. Rubisco in the extract was activated by pre-incubation with 15 mM MgCl\(_2\) and 10 mM NaHCO\(_3\) on ice for 15–20 min. The reaction was started by the addition of activated Rubisco to the reaction mixture containing 100 mM Bicine-NaOH, 20 mM MgCl\(_2\), 1 mM EDTA, 5 mM DTT, 15 mM NaH\(^14\)CO\(_3\) (specific activity, 3.7 MBq mmol\(^{-1}\)) and 0.4 mM RuBP, pH 8.2. After 1 min, 1/2 vol. of formic acid was added to the reaction solution to stop the reaction. The acidified reaction mixtures were dried and acid-stable \( ^{14}C \) was measured by liquid scintillation.

Rubisco catalytic site concentrations were determined by measuring the stoichiometric binding of \([^{14}C]\) carboxy-D-arabinitol-1,5-bisphosphate (CABP) to Rubisco, based on the method of Butz and Sharkey (1989) with some modifications. Rubisco in the extracts was incubated with the buffer containing 50 mM Bicine-NaOH, 1 mM EDTA, 20 mM MgCl\(_2\), 15 mM NaHCO\(_3\) and 0.02 mM \( ^{14}C\)-CABP (specific activity, 1.85 GBq mmol\(^{-1}\), pH 7.8 at room temperature for 45 min to bind \( ^{14}C\)-CABP to Rubisco catalytic sites. Rubisco was precipitated with casein (2.0 mg mL\(^{-1}\)) as carrier by adding PEG 4000 and MgCl\(_2\) to final concentration of 20% (w/v) and 25 mM, respectively. After 30 min, the solution was centrifuged at 15,000 g for 15 min. The pellet was washed three times with washing buffer containing 50 mM Bicine, 15 mM MgCl\(_2\), 1 mM EDTA and 20% PEG4000, pH 7.8. Only at the first washing step, the pellet was thoroughly resuspended using a sonicator. The \( ^{14}C \) retained within the pellet was measured by liquid scintillation. The \( k_{cat} \) of Rubisco (mol \( ^{14}C \)/s) was calculated as the ratio of \( in vitro \) Rubisco activity to Rubisco catalytic sites. For determination of \( Km \) for \( CO_2 \) of Rubisco (Kc), Rubisco activities were measured at six different NaH\(^14\)CO\(_3\) concentrations (0.5-15 mM) as mentioned above and Kc was calculated from the Hanes-Woolf plot ([S]/v~[S] plot).

3. **Analysis of partial RbcL sequence**

Genomic DNA was extracted from leaf blades and purified by the CTAB method (Murray and Thompson, 1980). Partial fragments of \( RbcL \) were
amplified by PCR using a pair of primers (RbcL-F1 GAATCTTCTACTGATCATGGA, RbcL-R1 TCCCTTCTAATCTACCTACTA) which were designed in the region of RbcL highly conserved among higher plants and sequenced.

### Results and Discussion

All plants analyzed in this study showed significantly higher \( k_{\text{cat}} \) of Rubisco than that in rice and the values ranged from 1.1-to 2.8-fold relative to rice (Table 1). The \( k_{\text{cat}} \) of Rubisco in *Calamagrostis longiseta*, *Festuca ovina*, *Phleum alpinum*, *Poa arctica* and *Phleum pratense* were relatively high among C₃ plants and showed more than 1.4-fold relative to rice. Most of C₄ plants showed higher \( k_{\text{cat}} \) than C₃ plants. Among C₄ plants, *Panicum maximum*, *Pennisetum purpureum* and *Sorghum bicolor* showed more than 2.5-fold higher \( k_{\text{cat}} \) than that in rice. These findings are consistent with previous reports that C₄ plants and cold-resistant C₃ plants possess high \( k_{\text{cat}} \) or Vmax Rubisco (Seemann et al., 1984; Sage, 2002) and it is likely to be applicable to Rubisco among Poaceae (Table 1).

From each functional group, two species which showed high \( k_{\text{cat}} \) were selected to analyze Kc of Rubisco, namely, *F. ovina* and *P. alpinum* from C₄ alpine plants, *P. pratense* and *P. arctica* from C₃ cold-resistant plants, and *P. purpureum* and *S. bicolor* from C₄ plants (Table 2). The C₄ plants analyzed showed similar or slightly higher Kc than rice, while Kc of C₃ plants were significantly high; *P. purpureum* and *S. bicolor* showed 3.8- and 2.6-fold, respectively, higher Kc than rice. Yeoh et al. (1980; 1981) proposed that C₄ plants have a higher Kc than C₃ plants. In addition, it was suggested that there could be trade-off between \( k_{\text{cat}} \) and affinity for CO₂ in kinetics of Rubisco (von Caemmerer and Quick, 2000; Sage, 2002). This relation was clearly demonstrated by the significant positive correlation between \( k_{\text{cat}} \) and Kc in C₃ and C₄ plants.

### Table 1. \( k_{\text{cat}} \) of Rubisco from a variety of C₃ and C₄ plants in Poaceae.

| Species               | \( k_{\text{cat}} \) (mol mol⁻¹ s⁻¹) | Fold (relative to rice) |
|-----------------------|--------------------------------------|-------------------------|
| *Oryza sativa*        | 1.59±0.01                            | –                       |
| C₃ alpine plants      |                                      |                         |
| *Anthoxanthum japonicum* | 1.58±0.05**                          | 1.1                     |
| *Calamagrostis longiseta* | 1.92±0.11**                          | 1.4                     |
| *Festuca ovina*       | 2.05±0.07**                          | 1.5                     |
| *Phleum alpinum*      | 2.22±0.11**                          | 1.6                     |
| C₃ cold-resistant plants |                                      |                         |
| *Bromus inermis*      | 1.73±0.08**                          | 1.2                     |
| *Dactylis glomerata*  | 1.59±0.04**                          | 1.1                     |
| *Phleum pratense*     | 2.18±0.13**                          | 1.6                     |
| *Poa arctica*         | 1.93±0.04**                          | 1.4                     |
| *Secale cereale*      | 1.65±0.09*                           | 1.2                     |
| C₄ plants             |                                      |                         |
| *Eleusine indica*     | 2.73±0.09**                          | 2.0                     |
| *Miscanthus sinensis* | 3.34±0.29**                          | 2.4                     |
| *Panicum maximum*     | 3.48±0.05**                          | 2.5                     |
| *Pennisetum purpureum*| 3.92±0.06**                          | 2.8                     |
| *Setaria viridis*     | 2.17±0.24**                          | 1.6                     |
| *Sorghum bicolor*     | 3.50±0.09**                          | 2.5                     |
| *Zea mays*            | 3.05±0.03**                          | 2.2                     |

For individual species, \( k_{\text{cat}} \) values are presented as means (±SE) of three independent measurements. Significant differences between rice (*Oryza sativa*) and other plants were detected by one-way ANOVA. * and ** denote significant difference at P < 0.05 and P < 0.01, respectively.

### Table 2. Kc of Rubisco from C₃ and C₄ plants, which showed higher \( k_{\text{cat}} \) of Rubisco.

| Species               | Kc (µM) | Fold (relative to rice) |
|-----------------------|---------|-------------------------|
| *Oryza sativa*        | 9.7±1.05 | –                       |
| C₃ alpine plants      |         |                         |
| *Festuca ovina*       | 14.1±1.63* | 1.5                     |
| *Phleum alpinum*      | 21.6±1.07** | 2.2                     |
| C₃ cold-resistant plants |      |                         |
| *Phleum pratense*     | 16.4±1.28** | 1.7                     |
| *Poa arctica*         | 14.6±0.39** | 1.5                     |
| C₄ plants             |         |                         |
| *Pennisetum purpureum*| 37.2±1.35** | 3.8                     |
| *Sorghum bicolor*     | 25.6±1.27** | 2.6                     |

For individual species, Kc values are presented as means (±SE) of three independent measurements. Significant differences between rice (*Oryza sativa*) and other plants were detected by one-way ANOVA. * and ** denote significant difference at P < 0.05 and P < 0.01, respectively.

![Graph showing the relationship between \( k_{\text{cat}} \) and Kc for Rubisco from various plants.](image)

Fig. 1. Relationship between \( k_{\text{cat}} \) and Kc of Rubisco from plants used for the measurement of Kc. The data were obtained from Table 1 and 2. The regression line is indicated. The kinetic parameter of Rubisco in *Griffithsia monilis* is shown for comparison (\( k_{\text{cat}} = 2.6 \text{ mol mol}^{-1} \text{s}^{-1} \), Kc=9.3 µM, Whitney et al. (2001)).

\[ y = 0.093x + 0.599 \]
\[ R^2 = 0.904 \]
Fig. 2. Alignment of deduced amino acid sequence of RbcL. The partial amino acid sequences of RbcL from Gly-82 to Pro-141 (numbering based on the RbcL of Spinacia oleracea) are shown. The sequences of RbcL in Festuca ovina, Phleum alpinum, Phleum pratense, Poa arctica, Pennisetum purpureum and Sorghum bicolor were determined in this study. Asterisks and dots below the sequences represent identities and similarities, respectively. The region of βC-βD loop where RbcL interacts with Rubisco activase is indicated.

plants in Poaceae (Fig. 1). These results suggest that the higher $k_{cat}$ seems inevitably lead to a decreased affinity for CO$_2$. However, Whitney et al. (2001) reported that Rubisco from the red alga Griffithsia monilis showed relatively high specific activity and significantly high affinity for CO$_2$, suggesting that the improvement of both kinetic parameters in higher plants Rubisco is theoretically possible. Among Rubisco analyzed $K_c$, F. ovina, P. pratense and S. bicolor showed relatively high $k_{cat}$ to $K_c$ (Fig. 1). Rubisco in these plants would be a good candidate and useful for the improvement of photosynthesis by genetic recombination in rice.

Kinetic properties of Rubisco are considered to be largely determined by RbcL (Andersson and Backlund, 2008). We made a comparison of the deduced amino acid sequences of RbcL among the species used in this study and representative C$_3$ and C$_4$ plants (Figs. 2, 3). Over all amino acid sequence of RbcL is well conserved among higher plants, whereas there are some natural mutations. Unfortunately, the amino acid residues that determine high $k_{cat}$ or specificity for CO$_2$ have not been identified yet. As an example, one of the most variable regions including the βC-βD loop where Rubisco activase implicated to interact with RbcL (Ott et al., 2000) is shown in Fig. 2. Around this region, the differences in amino acid sequence common to the functional groups were found, for example, Ala-91 (numbering based on the RbcL of Spinacia oleracea) was Val in rice but Pro in C$_4$ plants, and Tyr-97 was Trp in C$_3$ alpine plant and C$_3$ cold-resistant plants. Also, some variations in amino acid sequence common to the functional groups were observed in the other region from Gly-82 to Arg-319 sequenced in this study. The significance or function of some amino acid residues in RbcL were investigated by site-directed mutagenesis and X-ray structural analysis (Andersson, 2008; Andersson and Backlund, 2008). However, the exact meaning of difference in amino acid sequence observed here is largely unclear. It is likely that small differences in amino acid sequence of RbcL common to the functional groups found in this study could significantly affect kinetic property of Rubisco.

Using partial amino acid sequences of RbcL, phylogenetic tree analysis was carried out to compare the sequence similarity among species (Fig. 3). Monocotyledonous and dicotyledonous plants were clustered into different groups, and monocotyledonous plants were divided into two clusters, namely, C$_3$ and
C₄ plants. Although the differences are small, RbcL of rice shares higher sequence identity with C₃ plants, S. bicolor (96.6%) than that of C₄ plant, S. bicolor (95.4%), suggesting that RbcL of F. ovina and P. pratense would be better candidate genes to improve photosynthesis of rice. However, Rubisco of S. bicolor showed considerably high kₐ and this property is considered to be the most important factor for screening a better Rubisco for rice under elevated CO₂. Using the model of von Caemmerer and Farquhar (1981), the simulation of photosynthesis in rice expressing Rubisco of F. ovina or S. bicolor were demonstrated as a function of Ci (Fig. 4). In this simulation, we assumed that the values of kₐ and Kc are substantial determinants of photosynthetic rate. It is also apparent from the model that the effects of kₐ and Γ* on photosynthetic rate is reduced with increasing CO₂ partial pressure. These observations would support the significance of our simulation focused on the photosynthetic rate under elevated CO₂. In our estimation, the expression of F. ovina or S. bicolor Rubisco in rice could slightly enhance the photosynthetic rate at typical current CO₂ partial pressure around Ci of 25 Pa. Photosynthesis was more enhanced by the Rubisco at a higher Ci and the CO₂ assimilation rates at Ci of 50 Pa were increased 21 and 40% by Rubisco of F. ovina and S. bicolor, respectively, suggesting that these rice would have an advantage in the near future atmospheric condition over wild-type rice. Moreover, it is considered that Rubisco activity becomes excess under elevated CO₂. Makino et al. (1997) reported that the specific reduction of Rubisco content by antisense improved the nitrogen use efficiency and stimulated the photosynthesis at elevated CO₂ in rice. If rice acquired the high kₐ Rubisco, a lesser amount of Rubisco would be sufficient to support photosynthesis. By expressing a proper amount of high kₐ Rubisco in rice, it should be possible to reallocate a large amount of nitrogen to the other photosynthetic proteins and could significantly enhance photosynthesis, nitrogen use efficiency and finally, crop productivity in the future.

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