| Title | Rubisco small subunits of C4 plants, Napier grass and guinea grass confer C4-like catalytic properties on Rubisco in rice |
|-------|----------------------------------------------------------------------------------------------------------|
| Author(s) | Fukayama, Hiroshi / Kobara, Takashi / Shiomi, Keita / Morita, Ryutaro / Sasayama, Daisuke / Hatanaka, Tomoko / Azuma, Tetsushi |
| Citation | Plant Production Science, 22(2):296-300 |
| Issue date | 2019-04-03 |
| Resource Type | Journal Article / 学術雑誌論文 |
| Resource Version | publisher |
| Rights | © 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. |
| DOI | 10.1080/1343943X.2018.1540279 |
| JaLCDOI | |
| URL | http://www.lib.kobe-u.ac.jp/handle_kernel/90006001 |

PDF issue: 2020-05-06
Rubisco small subunits of C₄ plants, Napier grass and guinea grass confer C₄-like catalytic properties on Rubisco in rice

Hiroshi Fukayama, Takashi Kobara, Keita Shiomi, Ryutaro Morita, Daisuke Sasayama, Tomoko Hatanaka & Tetsushi Azuma

To cite this article: Hiroshi Fukayama, Takashi Kobara, Keita Shiomi, Ryutaro Morita, Daisuke Sasayama, Tomoko Hatanaka & Tetsushi Azuma (2019) Rubisco small subunits of C₄ plants, Napier grass and guinea grass confer C₄-like catalytic properties on Rubisco in rice, Plant Production Science, 22:2, 296-300, DOI: 10.1080/1343943X.2018.1540279

To link to this article: https://doi.org/10.1080/1343943X.2018.1540279

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

View supplementary material

Accepted author version posted online: 08 Nov 2018.
Published online: 03 Dec 2018.

Submit your article to this journal

Article views: 176
Rubisco small subunits of C\textsubscript{4} plants, Napier grass and guinea grass confer C\textsubscript{4}-like catalytic properties on Rubisco in rice

Hiroshi Fukayama\textsuperscript{a}, Takashi Kobara\textsuperscript{a}, Keita Shiomi\textsuperscript{a}, Ryutaro Morita\textsuperscript{b, \textdagger}; Daisuke Sasayama\textsuperscript{a}, Tomoko Hatanaka\textsuperscript{a} and Tetsushi Azuma\textsuperscript{a}

\textsuperscript{a}Graduate School of Agricultural Science, Laboratory of Tropical Plant Science, Kobe University, Kobe, Japan; \textsuperscript{b}Graduate School of Agricultural Science, Laboratory of Crop Science, Kobe University, Kobe, Japan

**ABSTRACT**

Overexpression of Rubisco small subunit (RbcS) of C\textsubscript{4} plant, sorghum (sorghum bicolor) was shown to enhance the catalytic turnover rate ($k_{\text{cat}}$) of Rubisco in rice (Oryza sativa). In this study, the effects of other Rubisco small subunits of C\textsubscript{4} plants, Napier grass (Pennisetum purpureum) and guinea grass (Megathyrsus maximus) on kinetic properties of Rubisco in rice were studied. The expression levels of Napier grass RbcS (NgRbcS) and guinea grass RbcS (GgRbcS) proteins accounted for 41\% and 45\% of total RbcS, respectively in homozygous overexpression lines. The $k_{\text{cat}}$ and $K_{m}$ for CO\textsubscript{2} ($K_{c}$) of Rubisco were increased in all transgenic lines. Interestingly, the $k_{\text{cat}}$ was markedly higher in NgRbcS homozygous line, whereas $K_{c}$ was notably higher in GgRbcS homozygous line. Although its effects depend on species, these results suggest that the introduction of C\textsubscript{4} RbcS are effective approaches to alter the catalytic properties of Rubisco in rice.

**Abbreviation:** GgRbcS: guinea grass RbcS; $K_{c}$: $K_{m}$ for CO\textsubscript{2}; $k_{\text{cat}}$: catalytic turnover rate; NpRbcS: Napier grass RbcS; RbcL: Rubisco large subunit; RbcS: Rubisco small subunit; $S_{\text{CO}_2}$: CO\textsubscript{2}/O\textsubscript{2} specificity.

**Introduction**

The crop productivity should be substantially improved to meet the demand for food in the near future. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the initial CO\textsubscript{2} fixation of photosynthesis, which considered to be one of the major bottlenecks of plant productivity (Parry et al., 2013). The photosynthetic rate at present atmospheric CO\textsubscript{2} level is largely limited by Rubisco because of its low catalytic turnover rate ($k_{\text{cat}}$) and competing oxygenase reaction, which initiates energy wasteful photorespiration in C\textsubscript{3} plants (von Caemmerer & Quick, 2000). Many important crops such as rice (Oryza sativa), potato (Solanum tuberosum) and soybean (Glycine max) are classified as C\textsubscript{3} plant. Unfortunately, the $k_{\text{cat}}$ of Rubisco in C\textsubscript{3} plants is substantially lower than that in C\textsubscript{4} plants (Ishikawa, Hatanaka, Misoo, & Fukayama, 2009; Sage, 2002). Thus, C\textsubscript{3} plants need to accumulate large amount of Rubisco in leaves, leading to low nitrogen use efficiency (Makino et al., 1992). In contrast, Rubisco from C\textsubscript{4} plants shows higher affinity for CO\textsubscript{2} (i.e. lower $K_{m}$ for CO\textsubscript{2}, $K_{c}$) to reduce oxygenation reaction (Sage, 2002). The enhancement of $k_{\text{cat}}$ usually decreases the affinity for CO\textsubscript{2}, leading to increase in photorespiration. However, the photorespiration will be reduced under elevated CO\textsubscript{2} condition. Hence, the enhancement of $k_{\text{cat}}$ as observed in C\textsubscript{4}-type Rubisco can give a benefit for the photosynthesis and plant productivity of C\textsubscript{3} plants in the future environment.

Rubisco is composed of the large subunit (RbcL) and small subunit (RbcS). RbcL is present in the chloroplast DNA, while RbcS is encoded by a multigene family in the nucleus (Dean, Pichersky, & Dunsmuir, 1989). RbcL contains most of important amino acids necessary for catalysis (Andersson & Backlund, 2008). In contrast, the function of RbcS is still obscure. However, we showed that the overexpression of *Sorghum bicolor* C\textsubscript{4} RbcS markedly enhanced the $k_{\text{cat}}$ of Rubisco in rice (Ishikawa, Hatanaka, Misoo, Miyake, & Fukayama, 2011). In addition, *OsRbcS1*, a member of rice RbcS gene family that is not expressed in photosynthetic cell also enhanced the $k_{\text{cat}}$ of Rubisco in rice (Morita, Hatanaka, Misoo, & Fukayama, 2014). These results clearly demonstrated that RbcS can be an important determinant of kinetic properties of Rubisco. Another functional group, cold-resistant plants also have acquired a high $k_{\text{cat}}$ Rubisco (Sage, 2002). However, RbcS from a cold resistant C\textsubscript{3} plant, timothy (*Phleum pretense*) could not enhance the $k_{\text{cat}}$ of Rubisco in rice (Fukayama et al., 2015). These results suggest that Rubisco from high $k_{\text{cat}}$ Rubisco does not always enhance the $k_{\text{cat}}$ of low $k_{\text{cat}}$ Rubisco.
Although information of RbcS are still limited, we presumed that C₄ plants are likely to contain effective RbcS for the enhancement of $k_{\text{cat}}$ of C₃ Rubisco. Among Rubisco in C₄ plants, Rubisco in Napier grass (Pennisetum purpureum) and guinea grass (Megathyrsus maximus) showed markedly higher $k_{\text{cat}}$ than that from rice (Ishikawa et al., 2009). In this study, we isolated and overexpressed RbcS from Napier grass and guinea grass in rice, and found that these C₄-type RbcS could be effective to improve the kinetic properties of Rubisco in rice.

**Materials and methods**

**Plant materials and growth conditions**

Napier grass (cv. Merkeron) and guinea grass (cv. Natsuyutaka) were grown in the experimental field of Kobe University. Rice (cv. Nipponbare) and transgenic rice lines were grown in soil under natural light conditions in a temperature-controlled greenhouse (30°C day/25°C night). Rice seedlings at the leaf stage of 4.5 were transplanted into 1 L pots filled with paddy soil containing a chemical fertilizer (N:P:K = 8:8:8) at 0.3 g N per pot. At 10.5 leaf stage, the 9th leaf blades were sampled at 10:30–11:30 on sunny days, immediately frozen in liquid nitrogen and stored at −80°C until required.

**Determination of kinetic properties of Rubisco**

Leaves were homogenized in extraction buffer containing 100 mM Bicine-NaOH, 1 mM EDTA, 5 mM MgCl₂, 2 mM NaH₂PO₄ 0.4% (w/v) BSA, 5 mM DTT, 4 mM amino-n-caproic acid and 0.8 mM benzamidine, pH 8.0, using a chilled mortar and pestle with a small amount of quartz sand. The homogenate was then centrifuged at 15,000 × g for 2 min at 4°C. Rubisco in the supernatant was activated by pre-incubation with 15 mM MgCl₂ and 10 mM NaHCO₃ on ice for 15–20 min. Rubisco activity was determined at 28°C using [¹⁴C] NaHCO₃ (specific activity, 37 MBq mmol⁻¹) by assaying the incorporation of ¹⁴C into acid-stable products, as described previously (Ishikawa et al., 2009).

Rubisco catalytic site concentration was determined by measuring the stoichiometric binding of [¹⁴C] carboxyarabinitol-1,5-bisphosphate (CABP; specific activity, 1.85 GBq mmol⁻¹) as described previously (Ishikawa et al., 2009).

The $k_{\text{cat}}$ of Rubisco (mol mol⁻¹ s⁻¹) was calculated as a ratio of in vitro maximum Rubisco activity to Rubisco catalytic site activities. For determination of $K_c$, Rubisco activities were measured at six different NaH¹⁴CO₃ concentrations (0.5–15 mM) as mentioned above. The $K_c$ was calculated from the Hanes-Woolf plot.

To determine Rubisco CO₂/O₂ specificity ($S_{c/o}$), the CO₂ compensation points were measured by the gas exchange rates using an open gas-exchange system (LI-6400, Li-Cor, Lincoln) under the condition of leaf temperature of 28°C, a photosynthetic photon flux density (PPFD) of 1,200 μmol quanta m⁻² s⁻¹, a leaf-to-air vapor pressure difference of 1.0–1.2 kPa and four different O₂ partial pressures ranging from two to 20%. The Sc/o was estimated by the slope of the regression lines of the dependence of CO₂ compensation point on the O₂ concentration (Laisk & Loreto, 1996).

**Results**

In this study, we studied the effects of RbcS from two C₄ plants, Napier grass and guinea grass on Rubisco kinetics in rice. Because mRNA sequences of RbcS from Napier grass and guinea grass were not found in published genome database, RT-PCR was carried out using primer

**Plasmid construction and transformation of rice**

The coding sequences with almost full length 3′UTR of NgRbcS and GgRbcS were cloned into the binary vector pIG121Hm containing the chlorophyll a/b-binding protein (Cab) promoter of rice. These constructs were introduced into rice (cv. Nipponbare) via Agrobacterium-mediated gene transfer. Hygromycin-resistant transgenic rice plants were regenerated and grown in soil. The level of transgene expression was screened by SDS-PAGE after staining with Coomassie blue as described previously (Fukayama et al., 2015). The transgenic lines showing higher expression levels were used for further analysis.
set designated at a conserved region of \( \text{RbcS} \) among Poaceae and obtained ten and seven different sequences from Napier grass and guinea grass, respectively. The full length mRNA sequences of Napier grass \( \text{RbcS} \) (Ng\( \text{RbcS} \)) and guinea grass \( \text{RbcS} \) (Gg\( \text{RbcS} \)) showing highest frequency in the RT-PCR products were determined by 5’T\( \text{RACE} \) and 3’T\( \text{RACE} \). The phylogenetic tree analysis of deduced amino acid sequence showed that Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) classified into different clade from sorghum \( \text{RbcS} \) in \( \text{C}_4 \) plants (Figure 1(a)). Deduced amino acid sequences of Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) share 74.4 and 76.7% identity with rice \( \text{RbcS} \), respectively. These identities were slightly higher than sorghum \( \text{RbcS} \) which shares 73.4% identity with rice \( \text{RbcS} \) (Ishikawa et al., 2011). Amino acid sequence of \( \beta \text{A-}\beta \text{B} \) loop has been suggested to be an important determinant of Rubisco kinetic properties (Spreitzer, 2003). Comparing the amino acid sequences of \( \beta \text{A-}\beta \text{B} \) loop, Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) contain two and three different amino acids with rice \( \text{RbcS} \), and two and one different amino acids with sorghum \( \text{RbcS} \), respectively (Figure 1(b)). These differences in amino acids can expect different effects on Rubisco kinetic properties. Semi quantitative RT-PCR analysis indicated that Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) were highly expressed in leaf blade (Figure 2), suggesting that these \( \text{RbcSs} \) are an usual photosynthetic \( \text{RbcSs} \) and not an unusual non-photosynthetic \( \text{RbcS} \) reported in rice (Morita et al., 2014; Morita, Hatanaka, Misoo, & Fukayama, 2016).

Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) were overexpressed in rice. Transgenic plants showed the band of foreign \( \text{C}_4 \) \( \text{RbcS} \) as well as rice \( \text{RbcS} \) in SDS-PAGE with Coomassie blue staining (Figure 3). Transgenic lines showing higher expression level were used for subsequent analyses. These transgenic lines exhibited a normal growth behavior and fertility. The expression levels of Ng\( \text{RbcS} \) and Gg\( \text{RbcS} \) relative to total \( \text{RbcS} \) were 41% and 45%, respectively in homozygous lines (Figure 3). The expression levels of transgene in these lines were similar level compared to those of previous reports overexpressing sorghum \( \text{RbcS} \) or timothy \( \text{RbcS} \) (Fukayama et al., 2015; Ishikawa et al., 2011).

![Figure 1](image-url)  
Figure 1. Comparison of the amino acid sequence of \( \text{RbcS} \) in plants. (a), The phylogenetic tree based on the amino acid sequence of \( \text{RbcS} \). The phylogenetic tree was generated using the coding region of \( \text{RbcS} \) without chloroplast transit peptide by the N-J method. \( \text{C}_4 \) monocots, \( \text{C}_3 \) monocot, \( \text{C}_4 \) dicot are indicated in red, blue and green. The bootstrap values calculated as per mil for 1,000 replications are shown at nodes. (b), Alignment of amino acid sequence of \( \beta \text{A-}\beta \text{B} \) loop of \( \text{RbcS} \). The amino acids different from rice are colored red. Accession numbers: rice, Os12g0291100; wheat, KT288199; timothy, AB976028; sorghum, AB564718; Napier grass, LC390054; guinea grass, LC390055; maize, NM_001111824; Arabidopsis thaliana, AF325004; tobacco, KM025335; Chlamydomonas reinhardtii, C206640.)
The kinetic properties of Rubisco expressing NgRbcS and GgRbcS were analyzed using these transgenic lines (Figure 4). All transgenic plants showed higher $k_{\text{cat}}$ and $K_c$ of Rubisco than non-transgenic rice (WT). In particular, the $k_{\text{cat}}$ was significantly increased in homozygous NpRbcS line (SNN) which was comparable to a high expression line of sorghum RbcS (SS10). In contrast, $K_c$ was greatly increased in homozygous GgRbcS line (SGG). These findings suggest that the effects of RbcS on the Rubisco kinetics would differ between NpRbcS and GgRbcS. Carboxylation efficiencies ($k_{\text{cat}}/K_c$) of WT, SS10, SNN, and SGG were 0.184, 0.180, 0.189, and 0.147, respectively. Because the expression level of foreign RbcS in SNN was lower than those in SS10 and SGG, these results imply that NpRbcS can be most effective to improve the kinetic properties of Rubisco for carboxylation.

The $S_{\text{c/o}}$ in transgenic lines overexpressing NgRbcS or GgRbcS were marginally lower than that in WT, whereas these differences were not statistically significant. These results suggest that the incorporation of NpRbcS and GgRbcS to Rice Rubisco would not cause large-scale effects on the $S_{\text{c/o}}$.

**Discussion**

In this study, we showed that C_4 RbcS from Napier grass and guinea grass as well as sorghum can confer C4-like high $k_{\text{cat}}$ type catalytic properties on Rubisco in rice. In contrast, the introduction of RbcS from pea (Pisum sativum L.) into Arabidopsis thaliana (Getzoff, Zhu, Bohnert, & Jensen, 1998) and timothy RbcS into rice (Fukayama et al., 2015) did not significantly affect on the kinetic properties of Rubisco. First of all, the $k_{\text{cat}}$ values of Rubisco in C_4 plants tested in this study were markedly higher than C_3 plants, whereas the $k_{\text{cat}}$ values of Rubisco in pea and timothy were not as high as C_4 plants. Considering these observations, the introduction of RbcS from plants showing markedly higher Rubisco $k_{\text{cat}}$ is considered to be effective to change the catalytic properties of Rubisco in C_3 plants.
Although the amino acid sequences of RbcSs from C₄ plants used in this study shared high identity among them, these showed different effects on the kinetics of Rubisco in rice (Figure 4). Spreitzer (2003) reported that βA-βB loop of RbcS can be an important region affecting the kinetics of Rubisco. All effective RbcSs to enhance the $k_{cat}$ of Rubisco in rice contain Ser55 which is substituted for His in rice (Figure 1). However, the amino acid sequences of this region are quite similar among these three C₄ plants. Although the effects of RbcS on Rubisco kinetics were different between Napier grass RbcS and guinea grass RbcS as shown in this study (Figure 4), these amino acid sequences in βA-βB loop differ only one amino acid, namely, Arg56 in Napier grass is substituted for Thr in guinea grass. There should be a lack of information to conclude that this marginal difference has different effects on Rubisco. It is also possible that the difference in amino acid sequence other than βA-βB loop may also affect the kinetics of hybrid Rubisco consisting of rice Rbcl and C₄ RbcS.

In this study, we demonstrated that RbcS from C₄ plants containing high $k_{cat}$ type-Rubisco would be effective to enhance the $k_{cat}$ and carboxylation efficiency of rice Rubisco. Among C₄ plant RbcSs, Napier grass RbcS can be most effective to improve these parameters for photosynthesis. However, we analyzed the kinetics of chimera Rubiscos consisting of rice RbcS and C₄ RbcS. To make an accurate assessment of the effects of C₄ RbcSs, rice RbcS should be knocked down or knocked out in our transgenic plants. At the same time, this way will lead to the reduction of Rubisco content. To improve the photosynthetic nitrogen efficiency and capacity under elevated CO₂ condition, we presume that the reduction of Rubisco content to an appropriate level as well as the increase in $k_{cat}$ will be necessary. We consider that our transgenic plants expressing C₄ RbcS are useful for the elucidation of the structure-function relationship of Rubisco and finally the improvement of photosynthetic capacity of C₃ plants in the future environment.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by JSPS KAKENHI under grant number [22114511] for H.F.

**ORCID**

Hiroshi Fukayama http://orcid.org/0000-0002-3466-8899

Ryutaro Morita http://orcid.org/0000-0003-3016-9504

**References**

Andersson, I., & Backlund, A. (2008). Structure and function of Rubisco. *Plant Physiology and Biochemistry*, 46, 275–291.

Dean, C., Pichersky, E., & Dunsmuir, P. (1989). Structure, evolution, and regulation of RbcS genes in higher plants. *Annual Review of Plant Biology*, 40, 415–439.

Fukayama, H., Koga, A., Hatanaka, T., & Misoo, S. (2015). Small subunit of a cold-resistant plant, timothy, does not significantly alter the catalytic properties of Rubisco in transgenic rice. *Photosynthesis Research*, 124, 57–65.

Getzoff, T. P., Zhu, G., Bohnert, H. J., & Jensen, R. G. (1998). Chimeric Arabidopsis thaliana ribulose-1,5-bisphosphate carboxylase/oxygenase containing a pea small subunit protein is compromised in carboxylation. *Plant Physiology*, 116, 695–702.

Ishikawa, C., Hatanaka, T., Misoo, S., & Fukayama, H. (2009). Screening of high $k_{cat}$ Rubisco among Poaceae for improvement of photosynthetic CO₂ assimilation in rice. *Plant Production Science*, 12, 345–350.

Ishikawa, C., Hatanaka, T., Misoo, S., Miyake, C., & Fukayama, H. (2011). Functional incorporation of sorghum small subunit increases the catalytic turnover rate of Rubisco in transgenic rice. *Plant Physiology*, 156, 1603–1611.

Laisk, A., & Loreto, F. (1996). Determining photosynthetic parameters from leaf CO₂ exchange and chlorophyll fluorescence: Ribulose-1,5-bisphosphate carboxylase/oxygenase specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate, and mesophyll diffusion resistance. *Plant Physiology*, 110, 903–912.

Makino, A., Sakashita, H., Hidema, J., Mae, T., Ojima, K., & Osmond, B. (1992). Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nutrition and their possible relationships to CO₂-transfer resistance. *Plant Physiology*, 100, 1737–1743.

Morita, K., Hatanaka, T., Misoo, S., & Fukayama, H. (2014). Unusual small subunit that is not expressed in photosynthetic cells alters the catalytic properties of Rubisco in rice. *Plant Physiology*, 164, 69–79.

Morita, K., Hatanaka, T., Misoo, S., & Fukayama, H. (2016). Identification and expression analysis of non-photosynthetic Rubisco small subunit, OsRbcS1-like genes in plants. *Plant Gene*, 8, 26–31.

Parry, M. A., Andralojc, P. J., Scales, J. C., Salvucci, M. E., Carmo-Silva, A. E., Alonso, H., & Whitney, S. M. (2013). Rubisco activity and regulation as targets for crop improvement. *Journal of Experimental Botany*, 64, 717–730.

Sage, R. F. (2002). Variation in the $k_{cat}$ of Rubisco in C₃ and C₄ plants and some implications for photosynthetic performance at high and low temperature. *Journal of Experimental Botany*, 53, 609–620.

Spreitzer, R. J. (2003). Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. *Archives of Biochemistry and Biophysics*, 414, 141–149.

von Caemmerer, S., & Quick, W. P. (2000). Rubisco: Physiology in vivo. In R. C. Leegood, T. D. Sharkey, & S. von Caemmerer (Eds.), *Photosynthesis: Physiology and metabolism* (pp. 85–113). Dordrecht: Kluwer Academic Publishers.