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SHORT REPORT

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Rubisco small subunits of C_4 plants, Napier grass and guinea grass confer C_4-like catalytic properties on Rubisco in rice

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ABSTRACT

Overexpression of Rubisco small subunit (RbcS) of C_4 plant, sorghum (*sorghum bicolor*) was shown to enhance the catalytic turnover rate (k_{cat}) of Rubisco in rice (*Oryza sativa*). In this study, the effects of other Rubisco small subunits of C_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_{cat} and K_m for CO_2 (K_c) of Rubisco were increased in all transgenic lines. Interestingly, the k_{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_4 RbcS are effective approaches to alter the catalytic properties of Rubisco in rice.

Abbreviation: GgRbcS: guinea grass RbcS; K_c: K_m for CO2; k_{cat}: catalytic turnover rate; NpRbcS: Napier grass RbcS; RbcL: Rubisco large subunit; RbcS: Rubisco small subunit; $S_{c/o}$: CO_2/O_2 specificity.

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CO_2 fixation; C_4 plant; guinea grass; Napier grass; rice; Rubisco

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_2 fixation of photosynthesis, which considered to be one of the major bottleneck of plant productivity (Parry et al., 2013). The photosynthetic rate at present atmospheric CO_2 level is largely limited by Rubisco because of its low catalytic turnover rate (k_{cat}) and competing oxygenase reaction, which initiates energy wasteful photorespiration in C_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_3 plant. Unfortunately, the k_{cat} of Rubisco in C_3 plants is substantially lower than that in C_4 plants (Ishikawa, Hatanaka, Misoo, & Fukayama, 2009; Sage, 2002). Thus, C_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_3 plants shows higher affinity for CO_2 (i.e. lower K_m for CO_2, K_c) to reduce oxygenation reaction (Sage, 2002). The enhancement of k_{cat} usually decreases the affinity for CO_2, leading to increase in photorespiration. However, the photorespiration will be reduced under elevated CO_2 condition. Hence, the enhancement of k_{cat} as observed in C_4-type Rubisco can give a benefit for the photosynthesis and plant productivity of C_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_4 RbcS markedly enhanced the k_{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_{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_{cat} Rubisco (Sage, 2002). However, RbcS from a cold resistant C_3 plant, timothy (*Phleum pretense*) could not enhance the k_{cat} of Rubisco in rice (Fukayama et al., 2015). These results suggest that RbcS from high k_{cat} Rubisco does not always enhance the k_{cat} of low k_{cat} Rubisco.

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Supplementary data can be accessed here.

Although information of RbcS are still limited, we presumed that C_4 plants are likely to contain effective RbcS for the enhancement of k_{cat} of C_3 Rubisco. Among Rubisco in C_4 plants, Rubisco in Napier grass (*Pennisetum purpureum*) and guinea grass (*Megathyrsus maximus*) showed markedly higher k_{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 C_4–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 the full length mRNA sequence of RbcS

Total RNA was isolated from leaf blade of Napier grass and guinea grass using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). The first strand cDNA was synthesized from the total RNA using PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Otsu, Japan). RT-PCR was performed using primers designed at conserved region of *RbcS*. The PCR product was cloned into the pGEM-T Eazy vector (Promega, Madison, WI), and their nucleotide sequences were determined. Based on these nucleotide sequences, 5′RACE and 3′RACE were performed using a SMART RACE cDNA amplification kit (BD Bioscience, San Jose, CA) as described previously (Fukayama et al., 2015). Based on the sequences of RACE products, the coding sequences of Napier grass *RbcS* (*NgRbcS*) and guinea grass *RbcS* (*GgRbcS*) were amplified by RT-PCR. Primers used for RT-PCR, 5′RACE and 3′RACE were listed in Table S1.

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.

Determination of kinetic properties of Rubisco

Leaves were homogenized in extraction buffer containing 100 mM Bicine-NaOH, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM NaH_2PO_4, 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 $MgCl_2$ and 10 mM $NaHCO_3$ on ice for 15–20 min. Rubisco activity was determined at 28°C using [^{14}C] $NaHCO_3$ (specific activity, 37 MBq mmol^{-1}) by assaying the incorporation of ^{14}C into acid-stable products, as described previously (Ishikawa et al., 2009).

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

The k_{cat} of Rubisco (mol mol^{-1} s^{-1}) was calculated as ratio of *in vitro* maximum Rubisco activity to Rubisco catalytic sites. For determination of K_c, Rubisco activities were measured at six different $NaH^{14}CO_3$ concentrations (0.5–15 mM) as mentioned above. The K_c was calculated from the Hanes-Woolf plot.

To determine Rubisco CO_2/O_2 specificity ($S_{c/o}$), the CO_2 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 O_2 partial pressures ranging from two to 20%. The Sc/o was estimated by the slope of the regression lines of the dependence of CO_2 compensation point on the O_2 concentration (Laisk & Loreto, 1996).

Results

In this study, we studied the effects of RbcS from two C_4 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

set designated at a conserved region of *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 RbcS (NgRbcS) and guinea grass RbcS (GgRbcS) showing highest frequency in the RT-PCR products were determined by 5′RACE and 3′RACE. The phylogenetic tree analysis of deduced amino acid sequence showed that NpRbcS and GgRbcS classified into different clade from sorghum RbcS in C_4 plants (Figure 1(a)). Deduced amino acid sequences of NgRbcS and GgRbcS share 74.4 and 76.7% identity with rice *RbcS*, respectively. These identities were slightly higher than sorghum *RbcS* which shares 73.4% identity with rice *RbcS* (Ishikawa et al., 2011). Amino acid sequence of βA-βB loop has been suggested to be an important determinant of Rubisco kinetic properties (Spreitzer, 2003). Comparing the amino acid sequences of βA-βB loop, NgRbcS and GgRbcS contain two and three different amino acids with rice RbcS, and two and one different amino acids with sorghum 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 NpRbcS and GgRbcS were highly expressed in leaf blade (Figure 2), suggesting that these RbcSs are an usual photosynthetic RbcS and not an unusual non-photosynthetic RbcS reported in rice (Morita et al., 2014; Morita, Hatanaka, Misoo, & Fukayama, 2016).

NgRbcS and GgRbcS were overexpressed in rice. Transgenic plants showed the band of foreign C_4 RbcS as well as rice 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 NgRbcS and GgRbcS relative to total 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 RbcS or timothy RbcS (Fukayama et al., 2015; Ishikawa et al., 2011).

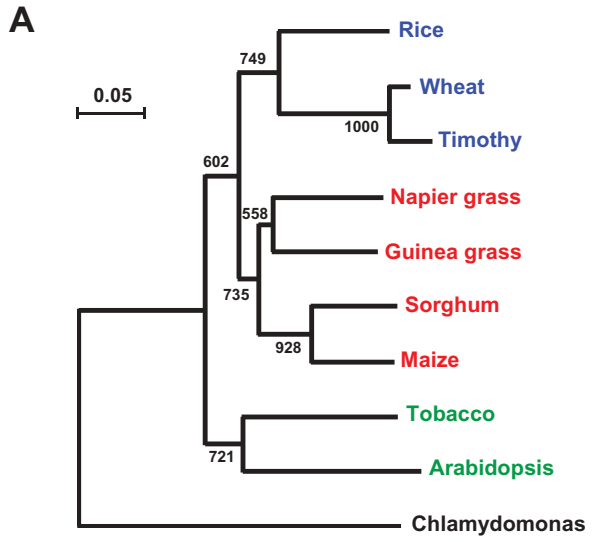

Figure 1. Comparison of the amino acid sequence of RbcS in plants. (a), The phylogenetic tree based on the amino acid sequence of RbcS. The phylogenetic tree was generated using the coding region of RbcS without chloroplast transit peptide by the N-J method. C_4 monocots, C_3 monocot, C_3 dicot are indicated 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 βA-βB loop of 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*, Cr206640.

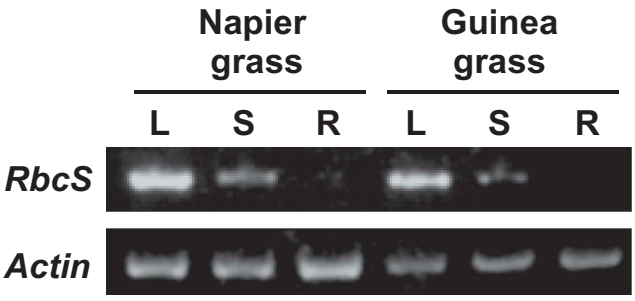

Figure 2. Expression analysis of RbcS by RT-PCR. The expressions of *NgRbcS* in Napier grass and GgRbcS in guinea grass were analyzed by RT-PCR. *Actin* was used as internal control. L, leaf blade; S, leaf sheath; R, root.

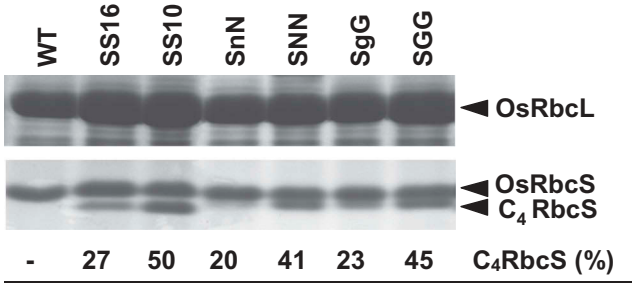

Figure 3. Expression analysis of RbcS by SDS-PAGE. Soluble proteins (10 μ) were separated by 15% SDS-PAGE and stained with Coomassie Blue. Fractions containing RbcL and RbcS are shown in upper and lower panels, respectively. Percent of introduced C_4RbcS expression is shown below the lower panel. WT, non-transgenic rice; SS16 and SS10, sorghum RbcS expression lines; SnN, heterozygous NgRbcS expression line; SNN, homozygous NgRbcS expression line; SgG, heterozygous GgRbcS expression line; SGG, homozygous GgRbcS expression line.

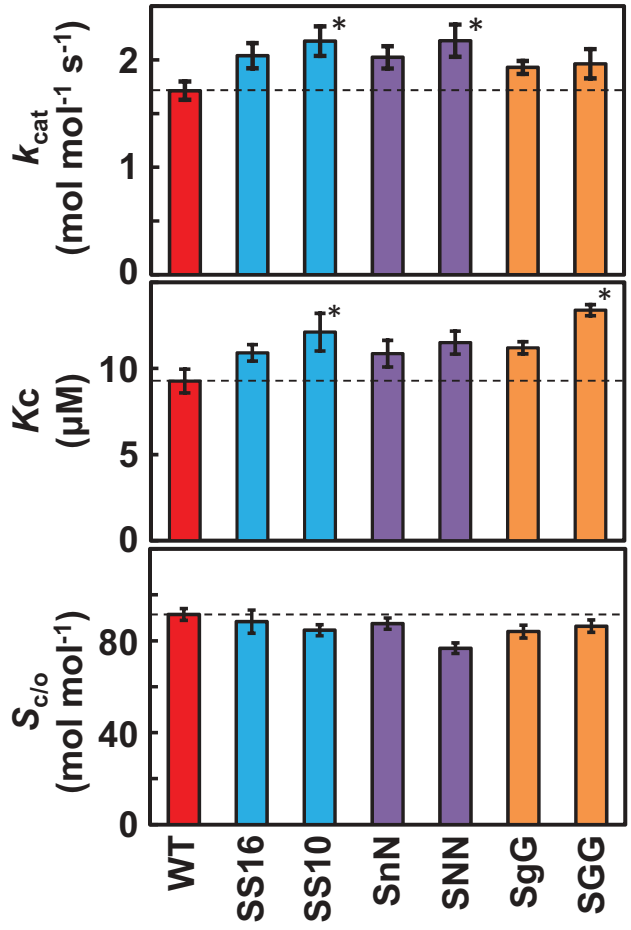

Figure 4. Kinetic properties of Rubisco. k_{cat} and K_c were determined by the measurement of Rubisco activity *in vitro*. $S_{c/o}$ was determined by the measurement of gas exchange rate. Values are the means ± SE of four to five biological replicates. *indicates the significant difference between WT and transgenic lines by student's t-test ($P < 0.05$). The plants are the same as in Figure 3.

The kinetic properties of Rubisco expressing NgRbcS and GgRbcS were analyzed using these transgenic lines (Figure 4). All transgenic plants showed higher k_{cat} and K_c of Rubisco than non-transgenic rice (WT). In particular, the k_{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_{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 $S_{c/o}$ 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 $S_{c/o}$.

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_{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_{cat} values of Rubisco in C_4 plants tested in this study were markedly higher than C_3 plants, whereas the k_{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_{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 C_4 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 C_4 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 C_4 RbcS.

In this study, we demonstrated that RbcS from C_4 plants containing high k_{cat} type-Rubisco would be effective to enhance the k_{cat} and carboxylation efficiency of rice Rubisco. Among C_4 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 C_4 RbcS. To make an accurate assessment of the effects of C_4 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 CO_2 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 C_4 RbcS are useful for the elucidation of the structure-function relationship of Rubisco and finally the improvement of photosynthetic capacity of C_3 plants in the future environment.

Disclosure statement

No potential conflict of interest was reported by the authors.

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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 Physiology and Plant Molecular 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 carbamylation. *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 CO_2 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 CO_2 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 CO_2-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 C_3 and C_4 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.