Mechanism of High Photosynthetic Capacity in BC$_2$F$_4$ Lines Derived from a Cross between *Oryza sativa* and Wild Relatives *O. rufipogon*

Chisato Masumoto$^1$, Takashige Ishii$^2$, Tomoko Hatanaka$^2$ and Naotsugu Uchida$^2$

($^1$Graduate School of Science and Technology, Kobe University, 1-1, Rokkodai-cho, Nada-Ku, Kobe 657-8501, Japan; $^2$Faculty of Agriculture, Kobe University, 1-1, Rokkodai-cho, Nada-Ku, Kobe 657-8501, Japan)

Abstract: We found that several BC$_2$F$_4$ lines had high leaf photosynthetic rates under light-saturated and ambient CO$_2$ conditions. These lines are progenies of BC$_2$F$_1$ plants with high photosynthetic capacities which were generated by backcrossing between *Oryza rufipogon* (W630) and *O. sativa* cv. Nipponbare, as a recurrent parent. Some photosynthetic characteristics of the BC$_2$F$_4$ lines were investigated to identify the factors increasing photosynthetic rates. Photosynthetic rates of these lines under light-saturated conditions at 50 to 700 ppm CO$_2$ concentrations were higher than those in Nipponbare. The estimated-maximum photosynthetic rates under light-saturated and CO$_2$-saturated conditions in BC$_2$F$_4$ lines were also higher than that in Nipponbare. The photosynthetic rate under light-saturated and ambient CO$_2$ conditions was positively correlated with the carboxylation efficiency as an indicator of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity in vivo rather than stomatal conductance. Initial and total Rubisco activities in vitro tended to be higher in the BC$_2$F$_4$ lines than in Nipponbare. The content of active Rubisco calculated from the activation state of Rubisco was also higher in the BC$_2$F$_4$ lines than in Nipponbare. These results suggest that high photosynthetic capacities of BC$_2$F$_1$ plants can be maintained high in their progenies and high photosynthetic rates under light-saturated and ambient CO$_2$ conditions in the BC$_2$F$_4$ lines are achieved mainly by the high activity of Rubisco due to the high active Rubisco content.

Key words: Gas exchange rate, *Oryza rufipogon*, Photosynthesis, Rice, Rubisco, Rubisco activase, Sucrose synthesis.

Previously, we examined the photosynthetic capacity as O$_2$ evolution rate in BC$_2$F$_1$ populations generated by backcrossing between *Oryza rufipogon* (W630) and *O. sativa* cv. Nipponbare as a recurrent parent (Masumoto et al., 2004). We found that some BC$_2$F$_1$ plants had a higher photosynthetic capacity than Nipponbare, in spite of the low photosynthetic capacity of *O. rufipogon*. We then detected quantitative trait loci (QTL) responsible for the increase in photosynthetic capacity in *O. rufipogon* (Ishii et al., 2003). However, we could not identify the factors limiting photosynthetic capacity because of the wide genetic variations among BC$_2$F$_1$ populations.

The photosynthetic model shows that factors limiting photosynthetic rate vary with the intercellular CO$_2$ concentration (von Caemmerer and Farquhar, 1981; Sharkey, 1985). Under light-saturated and low-CO$_2$ conditions, the photosynthetic rate is mainly limited by the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C$_3$ plant (von Caemmerer and Farquhar, 1981). In rice leaves, the kinetics and the content of Rubisco can explain photosynthetic rate under ambient CO$_2$ (Makino et al., 1985). This Rubisco activity is controlled by Rubisco activase existing in the chloroplastic stroma (Portis, 1995). Fukayama et al. (1998) reported that the changes in Rubisco activity due to leaf aging in rice were influenced by Rubisco activase content. Photosynthetic rates at ambient CO$_2$ or CO$_2$-saturated concentrations are limited by capacities of RuBP regeneration. This RuBP regeneration capacity is affected by activities of electron transport and Pi regeneration (Sharkey, 1985; Stitt, 1986). In rice leaves, sucrose synthesis associated with Pi regeneration affects photosynthetic rate at a high CO$_2$ partial pressure under sufficient N supply conditions (Makino et al., 1994).

In this study, we investigated the photosynthetic characteristics of the BC$_2$F$_4$ lines with high photosynthetic rates to identify the factors increasing photosynthetic capacity. Gas exchange rate, the contents of Rubisco and Rubisco activase, and the activities of Rubisco and two enzymes of sucrose synthesis were examined.

Materials and Methods

1. Plant culture

We produced backcross populations (BC$_2$F$_1$) by crossing *O. rufipogon* (W630) from Myanmar with *O. sativa* cv. Nipponbare as a recurrent parent. The
BC$_2$F$_1$ plants that had higher O$_2$ evolution rates under saturated-light and 5% CO$_2$ conditions than Nipponbare were selected. Then 4 lines of their self-pollinated progenies, BC$_2$F$_4$ (M9-5-(1), M9-5-(2), P4-6-(1) and P4-6-(2)) were used in this study.

The seeds were sterilized with 1% sodium hypochlorite solution for 30 minutes and then soaked in tap water at 30 º C for 2 days. After germination, seedlings were planted in nursery boxes. When plants were at the 4-5 leaf stage, each seedling was transplanted to a 3 L pot. Each pot contained paddy soil and 1 g each of N, P and K. At about the 12.3-leaf stage, the 11th leaf blade of the main stem was used for the measurement of photosynthetic rate.

2. Photosynthetic rate measurement

Photosynthetic rate was determined as CO$_2$ gas exchange rate using an open gas analysis system. About 10 cm$^2$ of the center of 11th leaf blade attached to the plant was inserted into an acryl chamber and its net CO$_2$ uptake and transpirational H$_2$O loss were measured using an infrared gas analyzer (LI-7000, Li-Cor Inc, Lincoln, NE, USA). Light intensity was fixed at 1800 µmol quanta m$^{-2}$ s$^{-1}$, and leaf temperature was maintained at about 28 º C. First, photosynthetic rate at 350 ppm CO$_2$ (A$_{350}$) was measured in ten plants of each line. We then selected four plants from each line having a higher A$_{350}$ than Nipponbare and measured CO$_2$ gas exchange rates at 50, 100, 180, 220, 330, 350, 450 and 700 ppm CO$_2$. After the photosynthetic measurement, the leaf was exposed to the conditions for measurement of A$_{350}$ again. When the stable A$_{350}$ was attained, the leaf was rapidly frozen in liquid N$_2$ and stored at –80º C before enzyme assay. Intercellular CO$_2$ concentration (C$_i$) was calculated using the equation of von Caemmerer and Farquhar (1981). The carboxylation efficiency (CE) was calculated by fitting a linear regression to the initial slope of the A-C$_i$ response curve up to 200 ppm C$_i$. The maximum photosynthetic rate under light-saturated and CO$_2$-saturated (A$_{max}$) was estimated by the method of least squares with the Taylor expansion according to Uchida et al. (1988) adapted from Sakoda and Hiromi (1976). Eight values of C$_i$ and photosynthetic rate were used for this estimation of A$_{max}$.

3. Determination of Rubisco and Rubisco activase contents

Frozen leaf pieces were homogenized in 50 mM Na-phosphate buffer (pH 7.5) containing 5 mM DTT, 0.1 mM EDTA and 12.5% (v/v) glycerol with a small amount of PVPP and acid-washed quartz sand. The homogenate was centrifuged at 50,000 × g for 10 min at 4 º C. The supernatant was used for the determination of Rubisco and Rubisco activase contents. Rubisco and Rubisco activase contents were measured by enzyme-linked immunosorbent assay (ELISA) using rabbit polyclonal antibodies against rice Rubisco and Rubisco activase (Masumoto et al., 2004).

4. Activity of Rubisco

The activity of Rubisco was measured spectrophotometrically according to Du et al. (1996). Frozen leaf pieces were homogenized in a chilled mortar with insoluble PVPP, acid-washed quartz sand and extraction buffer containing 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 10 mM MgCl$_2$, 15 mM 2-mercaptoethanol, 0.4 mM ATP, 0.2% (w/v) BSA,
0.1% triton X-100 and 12.5% (w/v) glycerol within 30 s. The homogenate was centrifuged for 1 min at 14,000 g. The supernatant was used immediately for the determination of initial activity. The initial activity of Rubisco was assayed at 25º C for 1 min in 50 mM Hepes (pH 8.0) containing 20 mM MgCl₂, 10 mM NaHCO₃, 2.5 mM ATP, 10 mM KCl, 5 mM DTT, 5 mM phosphocreatine, 0.2 mM NADH, 0.6 mM RuBP, 6 U mL⁻¹ phosphoglycerate kinase, 6 U mL⁻¹ glyceraldehyde-3-phosphate dehydrogenase and 20 U mL⁻¹ phosphocreatine kinase. The activity of Rubisco was calculated from the rate of decrease in absorbance at 340 nm due to NADH oxidation. To measure the total activity, we transferred an aliquot of supernatant to a test tube, and made the Mg²⁺ and NaHCO₃ concentrations 20 mM and 10 mM, respectively. Rubisco was activated on ice for 10 minutes and determined as above.

5. Activities of cytosolic-1,6-bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS)

Frozen leaf pieces were homogenized in a chilled mortar with 50 mM Mops-KOH buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM EDTA, 2.5 mM DTT, 0.5% (w/v) BSA and 0.1% (v/v) Triton X-100. After centrifugation at 12,000 × g for 10 min, the supernatant was immediately passed through a Sephadex G-25 column previously equilibrated with the homogenation buffer without EDTA and Triton X-100. The eluate was used for enzyme assay. The cFBPase activity was assayed spectrophotometrically at 25°C by the method of Stitt et al. (1982). The activity of SPS under substrate-saturated conditions was assayed at 25°C by the method of Huber et al. (1989). The substrate-saturated conditions were 10 mM fructose-6-phosphate, 40 mM glucose-6-phosphate and 10 mM uridine diphosphate glucose. SPS activity was calculated from sucrose content determined by the anthrone method (Scott and Melvin, 1953).

### Results

Fig. 1 shows response curves of photosynthetic rate against external CO₂ (A-Ca) and intercellular CO₂ concentrations (A-Ci) under light-saturated conditions. The photosynthetic rates at 50 to 700 ppm external CO₂ and 45 to 600 ppm intercellular CO₂ concentrations in BC₂F₄ lines were higher than those in Nipponbare (Fig. 1). Apparent CO₂ compensation points in the BC₂F₄ lines were about 40 ppm similar to that in Nipponbare (Fig. 1A). Differences in photosynthetic rate between BC₂F₄ lines and Nipponbare tended to increase as CO₂ concentrations increased.

| Table 1. Comparison between Nipponbare and BC₂F₄ lines in the photosynthetic rate under light-saturated and 350 ppm CO₂ condition (Aₚ₅₀), the maximum photosynthetic rate (Aₘₐₓ) and the ratio of Aₚ₅₀ to Aₘₐₓ. |
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| n | Aₚ₅₀ (µmol CO₂ m⁻² s⁻¹) | Aₘₐₓ (µmol CO₂ m⁻² s⁻¹) | Aₚ₅₀/Aₘₐₓ (%) |
| Nipponbare | 3 | 25.1±0.4 | 51.6±3.5 | 48.7 |
| M9-5-(1) | 4 | 29.1±0.4** | 69.5±4.7** | 42.0 |
| M9-5-(2) | 4 | 30.0±1.5** | 68.1±4.8** | 44.2 |
| P4-6-(1) | 4 | 29.1±1.4** | 63.1±3.9* | 46.2 |
| P4-6-(2) | 4 | 28.4±0.9** | 63.2±8.1* | 45.5 |

Mean±SD.

* Aₘₐₓ was estimated by the method of least squares with the Taylor expansion.

** significant difference compared with Nipponbare value at 5% and 1% levels, respectively.

| Table 2. Comparison in carboxylation efficiency (CE) and stomatal conductance between Nipponbare and BC₂F₄ lines. |
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| n | CE (mol m⁻² s⁻¹) | Stomatal conductance (mol H₂O m⁻² s⁻¹) |
| Nipponbare | 3 | 0.124±0.004 | 0.48±0.06 |
| M9-5-(1) | 4 | 0.149±0.005** | 0.47±0.05 |
| M9-5-(2) | 4 | 0.151±0.007** | 0.59±0.08 |
| P4-6-(1) | 4 | 0.150±0.011** | 0.48±0.04 |
| P4-6-(2) | 4 | 0.150±0.010** | 0.47±0.06 |

Mean±SD.

** significant difference compared with Nipponbare value at 1% level.

0.1% triton X-100 and 12.5% (w/v) glycerol within 30 s. The homogenate was centrifuged for 1 min at 14,000×g. The supernatant was used immediately for the determination of initial activity. The initial activity of Rubisco was assayed at 25°C for 1 min in 50 mM Hepes (pH 8.0) containing 20 mM MgCl₂, 10 mM NaHCO₃, 2.5 mM ATP, 10 mM KCl, 5 mM DTT, 5 mM phosphocreatine, 0.2 mM NADH, 0.6 mM RuBP, 6 U mL⁻¹ phosphoglycerate kinase, 6 U mL⁻¹ glyceraldehyde-3-phosphate dehydrogenase and 20 U mL⁻¹ phosphocreatine kinase. The activity of Rubisco was calculated from the rate of decrease in absorbance at 340 nm due to NADH oxidation. To measure the total activity, we transferred an aliquot of supernatant to a test tube, and made the Mg²⁺ and NaHCO₃ concentrations 20 mM and 10 mM, respectively. Rubisco was activated on ice for 10 minutes and determined as above.

5. Activities of cytosolic-1,6-bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS)

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were increased.

Table 1 shows photosynthetic rates at 350 ppm CO$_2$ (A$_{350}$) and maximum photosynthetic rates (A$_{max}$) estimated by the method of least squares with the Taylor expansion. The A$_{350}$ was 13-20% higher in the BC$_2$F$_4$ lines than in Nipponbare (P<0.01). The estimated A$_{max}$ was higher in the BC$_2$F$_4$ lines than in Nipponbare, 32-35% higher in M9-5 lines (P<0.01) and 22% higher in P4-6 lines (P<0.05). The ratios of A$_{350}$ to A$_{max}$ (A$_{350}$/A$_{max}$) in BC$_2$F$_4$ lines were 42-46%, which were slightly lower than in Nipponbare.

The photosynthetic rate under light-saturated and ambient CO$_2$ conditions is determined by the activity of Rubisco and stomatal conductance. Table 2 shows the carboxylation efficiency (CE) as an indicator of Rubisco activity in vivo and the stomatal conductance. CE was calculated by fitting a linear regression to the initial slope of A-C$_i$ response curve up to 200 ppm C$_i$ (Fig. 1C). Formulas of these regression lines in Nipponbare, M9-5-(1), M9-5-(2), P4-6-(1) and P4-6-(2) lines were Y=0.124X−4.94, Y=0.149X−5.97, Y=0.151X−6.04, Y=0.150X−5.75 and Y=0.150X−6.22, respectively. The CE was significantly higher in the BC$_2$F$_4$ lines than in Nipponbare (Table 2, P<0.01). The stomatal conductance was 22% higher in M9-5-(2) than in Nipponbare. The stomatal conductance in the other BC$_2$F$_4$ lines was similar to that in Nipponbare. The correlation of A$_{350}$ and CE and stomatal conductance were examined. A$_{350}$ was positively correlated CE rather than stomatal conductance (Fig. 2), and the A$_{350}$ was higher in the BC$_2$F$_4$ lines than in Nipponbare at the same stomatal conductance (Fig. 2B).

We then measured contents of Rubisco and Rubisco activase and activity of Rubisco. There were some differences in these characteristics between BC$_2$F$_4$ lines and Nipponbare or among BC$_2$F$_4$ lines. The Rubisco contents in the BC$_2$F$_4$ lines were higher than in Nipponbare, about 20% and 10% increases in M9-5 and P4-6 lines, respectively (Fig. 3). The Rubisco activase content in M9-5-(2) line was 16% increased compared with that in Nipponbare (Fig. 3). The Rubisco activase contents of the other BC$_2$F$_4$ lines were about the same as that in Nipponbare. The initial activities of Rubisco in M9-5 and P4-6 lines were about 20% and 30% higher than that in Nipponbare (Fig. 4A). The total activities of Rubisco in BC$_2$F$_4$ lines except P4-6-(2) line were about 25% higher than that in Nipponbare (Fig. 4A). The activation state of Rubisco in Nipponbare, M9-5-(1) lines, M9-5-(2), P4-6-(1) and P4-6-(2) was 77%, 74%, 72%, 85% and 90%, respectively (Fig. 4A). The specific total activity of Rubisco was slightly higher in M9-5-(1) and P4-6-(1) than that in Nipponbare (Fig. 4B). The content of active Rubisco under light-saturated and 350 ppm CO$_2$
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The photosynthetic rate under light-saturated and CO₂-saturated conditions is limited by abilities of electron transport and Pi regeneration to regenerate RuBP (Sharkey, 1985; Stitt, 1986). Under sufficient nutrient conditions, the photosynthetic rates in rice leaves at a high CO₂ concentration are affected mainly by regeneration of Pi (Makino et al., 1994). Synthesis of sucrose is involved in the regeneration of Pi. We measured activities of cFBPase and SPS, key enzymes in sucrose synthesis (Fig. 5). The cFBPase activity was 10-17% higher in BC₂F₄ lines than in Nipponbare. On the other hand, the SPS activity was 6-10% lower in BC₂F₄ lines than in Nipponbare. The cFBPase activity tended to be high in M9-5-(1) and P4-6-(2) which had a low SPS activity.

Discussion

In this study, we examined the photosynthetic characteristics of BC₂F₄ lines, which had higher CO₂ gas exchange rates under light-saturated and ambient air conditions than their parental cultivar, Nipponbare, to investigate the factors increasing photosynthetic capacity. At 50 to 700 ppm of external CO₂ concentrations, the photosynthetic rates were higher in the BC₂F₄ lines than in Nipponbare (Fig. 1A). The photosynthetic rates at 350 ppm CO₂ (A₃₅₀) and estimated maximum photosynthetic rates (Aₘₐₓ) were significantly higher in the BC₂F₄ lines than in Nipponbare (Table 1). We estimated Aₘₐₓ by the method of least squares with the Taylor expansion, and the value of Aₘₐₓ was comparable to O₂ evolution rate obtained in our previous study (Masumoto et al., 2004). The ratio of A₃₅₀ to Aₘₐₓ tended to be lower in the BC₂F₄ lines than in Nipponbare because the difference between these lines and Nipponbare in Aₘₐₓ was larger than that in A₃₅₀. These ratios were similar to the ratio of O₂ evolution rate to CO₂ gas exchange rate in rice leaves (Xu et al., 1997). These results showed that the photosynthetic rates in the BC₂F₄ lines were high at not only ambient CO₂ concentration but also at 50 to 700 ppm CO₂ concentrations compared with those in Nipponbare. A high maximum photosynthetic capacity in BC₂F₄ plants might be maintained high in the BC₂F₄ lines.

The photosynthetic rate in the C₄ plant is affected by the photosynthetic activity in mesophyll cells, the open degree of stoma and the number of stomata. Regarding photosynthetic activity in mesophyll cells, the photosynthetic model shows that factors limiting the photosynthetic rate vary with the intercellular CO₂ concentration (von Caemmerer and Farquhar, 1981; Sharkey, 1985). Under low-C₄ and light-saturated conditions, the photosynthetic rate is largely
Rubisco activity. In this study, we used CE as an indicator of the activity of Rubisco in vivo. The CEs in the BC2F4 lines were significantly higher than that in Nipponbare (Table 2). The stomatal conductance in the BC2F4 lines except M9-5-(2) lines was similar to that in Nipponbare (Table 2). A positive correlation was found between CE and CO2 assimilation rate under light-saturated and ambient air conditions in the genus Oryza including various wild rice species (Yeo et al., 1994). In addition, the correlation coefficient between stomatal conductance and CO2 assimilation rate was high at the ripening stage in rice cultivars bred in Japan (Kuroda and Kumura, 1990). In this study, A50 was closely correlated with CE rather than stomatal conductance in Nipponbare and BC2F4 plants (Fig. 2), and A50 in the BC2F4 plants were higher than that in Nipponbare at the same stomatal conductance (Fig. 2B). These results indicated that the photosynthetic rates in the BC2F4 lines at light-saturated and ambient air conditions were affected mainly by the activity of Rubisco in vivo.

Next, we examined the content and activity of Rubisco. The content of Rubisco is also important for photosynthesis throughout the life span of rice leaf (Makino et al., 1983). The Rubisco contents in the BC2F4 lines were increased. We reported that the content of Rubisco were also increased in the BC2F1 population (Masumoto et al., 2004). These results showed that the increase in Rubisco content in the BC2F1 population was maintained in their progenies. The initial and total activities of Rubisco in the BC2F1 lines were not largely different from that in Nipponbare (Fig. 4A). Specific total activities of Rubisco in the BC2F4 lines were higher in the BC2F4 lines than in Nipponbare (Fig. 4B). The specific activity in O. rufipogon has been reported to be about the same as that in O. sativa (Makino et al., 1987). There was a difference in activation state of Rubisco between Nipponbare and BC2F4 lines (Fig. 4A). However, these activation states cannot be simply compared because of the difference in Rubisco content between Nipponbare and the BC2F4 lines (Fig. 3). The Rubisco contents in the BC2F4 lines would be surplus for the CO2 exchange rate under light-saturated and ambient CO2 conditions because O2 evolution rates under high CO2 conditions in rice leaves tend to be saturated at Rubisco content of around 3 g m−2 (Fukayama et al., 1996). So we calculated the content of active Rubisco from the activation state of Rubisco. The content of active Rubisco was higher in BC2F4 lines than in Nipponbare (Fig. 4C). These results suggest that high photosynthetic rates in the BC2F4 lines under light-saturated and ambient CO2 conditions are affected mainly by active Rubisco content.

Rubisco activase plays an essential role in regulating Rubisco activity. It promotes the activation of Rubisco by releasing binding inhibitors from holoenzymes or the active form of Rubisco (Wang and Portis, 1992; Robinson and Portis, 1988). Moreover, the turnover rate of the active site of Rubisco is maintained high by Rubisco activase (He et al., 1997). The content of Rubisco activase in rice leaves affects Rubisco activity under ambient CO2 and CO2-saturated conditions (Fukayama et al., 1998). The content of Rubisco activase was higher in M9-5-(1) line than in Nipponbare, but those in the other BC2F4 lines were about the same as that in Nipponbare (Fig. 3). This result suggested that the increase in active Rubisco content in M9-5-(1) line is caused by the increase in Rubisco activase content. There was a possibility that the Rubisco-activating activity of Rubisco activase might be higher in the other BC2F4 lines than in Nipponbare. The activity of Rubisco activase has been measured using the purified enzyme in many researches. However, there are few reports describing this activity in leaf extracts (Lan et al., 1992), and the differences in the activity of Rubisco activase among various plant species or interspecies have not been investigated yet. We need to measure the activity of Rubisco activase in rice leaf extracts and examine this activity in the BC2F4 lines.

Makino et al. (1994) reported that sucrose synthesis involving Pi regeneration affects the photosynthetic rate at high CO2 partial pressures in rice leaves with a sufficient N supply. We measured the activities of cFBPase and SPS, the key enzymes of sucrose synthesis (Fig. 5). In the BC2F4 lines, the cFBPase activity was higher but the SPS activity was lower than in Nipponbare. This tendency was also observed in BC2F1 lines (Masumoto et al., 2003). The cFBPase had a more significant effect on photosynthetic rate under CO2-saturated conditions than SPS using transgenic Arabidopsis with reduced contents of cFBPase or SPS (Strand et al., 2000). It cannot be concluded that the high cFBPase activity in the BC2F4 lines is responsible for the high photosynthetic rates under high or CO2-saturated concentrations in this study. The capacity of sucrose synthesis in BC2F4 lines may not be different from that in Nipponbare because they have higher cFBPase activity, but low SPS activity. We need to investigate this capacity by not only measuring the activities of enzymes but also measuring the amounts of metabolic intermediates.

In this study, we used BC2F4 lines with high photosynthetic rates under light-saturated and ambient air conditions. We found that the photosynthetic rates under light-saturated at 50 to 700 ppm CO2 concentrations in the BC2F4 lines were also higher than those in the parental cultivar, Nipponbare, and the high maximum photosynthetic capacity could be maintained in the BC2F4 lines. The high photosynthetic rates under light-saturated and ambient air conditions in the BC2F4 lines were achieved mainly by the high Rubisco activity due to the high active Rubisco content.
We are now carrying out a similar experiment using near-isogenic lines in which QTLs responsible for the O$_2$ evolution rate are substituted with those of O. rufipogon on a Nipponbare genetic background.

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* In Japanese.
** In Japanese. The title is translated by the present authors.
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