The mesophyll anatomy enhancing CO₂ diffusion is a key trait for improving rice photosynthesis

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Abstract

Increases in rates of individual leaf photosynthesis (Pᵣ) are critical for future increases in yields of rice plants. Although many efforts have been made to improve rice Pᵣ with transgenic technology, the desired increases in Pᵣ have not yet been achieved. Two rice lines with extremely high values of Pᵣ were identified among the backcrossed inbred lines derived from the indica variety Takanari, one of the most productive varieties in Japan, and the elite japonica variety Koshihikari (Koshihikari/Takanari//Takanari). The Pᵣ values of the two lines at an ambient CO₂ concentration of 370 μmol mol⁻¹ as well as at a saturating concentration of CO₂ were 20–50% higher than those of the parental varieties. Compared with Takanari, these lines had neither a higher content nor a higher activity of ribulose 1,5-bisphosphate carboxylase/oxygenase when the leaf nitrogen contents were similar, but they did have high mesophyll conductance with respect to CO₂ flux due to their higher density and more highly developed lobes of mesophyll cells. These lines also had higher electron transport rates. The plant growth rates of these lines were higher than that of Takanari. The findings show that it is possible to increase Pᵣ significantly, both at the current atmospheric concentration of CO₂ and at the increased concentration of CO₂ expected in the future, using appropriate combinations of genetic resources that are available at present.

Key words: Leaf nitrogen content, mesophyll cell anatomy, mesophyll conductance, photosynthesis, Rubisco, stomatal conductance.

Introduction

In order to meet the demand for rice (Oryza sativa L.) of the rapidly growing Asian population, it is necessary to increase the grain yield per unit land area. Rice yields have more than doubled over the half century that has passed since modern semi-dwarf varieties with high harvest indices were released in the mid-1960s (Khush, 1999). However, as harvest indices are close to the theoretical maximum, increases in total biomass production are, now, the key to increases in rice yield (Mann, 1999). Massive increases in biomass production as a result of improvements in canopy architecture are unlikely because canopy architecture is now also close to optimal (Horton, 2000). Thus, improvements in rates of photosynthesis of individual leaves within the canopy have become the focus of current efforts to increase production of rice grains (Horton, 2000; Hubbart et al., 2007).

Many efforts to improve the rate of individual leaf photosynthesis (Pᵣ) in C₃ plants including rice have been made in previous studies with transgenic technology (von Caemmerer and Evans, 2010). Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the primary CO₂ fixation enzyme, and
the amount and kinetic properties of Rubisco strongly affect $P_n$. In addition, Rubisco of C_3 plants has a low catalytic turnover rate and competing oxygenase reaction, which initiates wasteful photorespiration (Whitney et al., 2011a). Transgenic rice that overexpresses the small subunit of Rubisco has been developed to increase the Rubisco content (Suzuki et al., 2007, 2009). In order to improve the catalytic turnover rate of Rubisco, transplastomic tobacco plants expressing Rubisco large subunits from Flaveria genus (Whitney et al., 2011b) and transgenic rice expressing the Rubisco small subunit from sorghum (Sorghum bicolor Moench) have been developed (Ishikawa et al., 2011). C_4 plants such as maize (Zea mays L.) and sorghum show high photosynthetic capacity due to their CO_2-concentrating mechanism, known as C_4 photosynthesis. Taniguchi et al. (2008) developed rice that expresses maize and sorghum genes for enzymes involved in C_4-type photosynthesis. However, these trials have failed to increase $P_n$ in rice because of some other factors that offset these enhanced effects. These findings might imply that another strategy is necessary to increase the rates of rice leaf photosynthesis.

It is widely acknowledged that $P_n$ is closely related to leaf nitrogen content (LNC) in rice (Evans, 1989; Makino et al., 1992). However, varietal differences in $P_n$ even under the same LNC were reported due to the difference in stomatal conductance ($g_s$; Hirasawa et al., 2010). The varietal difference in $g_s$ has not yet been fully considered in genetically improving rice $P_n$. Mesophyll conductance ($g_m$), with respect to the diffusion of CO_2 from the substomatal airspace to the chloroplast, might also be important for improving rice $P_n$ (Makino, 2011). The difference in $g_m$ can be correlated with anatomical characteristics and aquaporin activity (Hanba et al., 2004; Evans et al., 2009; Terashima et al., 2011). The surface area of chloroplasts exposed to intercellular airspace ($S_{in}$) and the cell wall thickness are the most important anatomical factors controlling $g_m$ (Tosens et al., 2012a, b; Peguero-Pina et al., 2012). Because most of the periphery of mesophyll cells is covered by chloroplast, $S_{in}$ in rice might be controlled by the surface area of mesophyll cells exposed to intercellular airspace ($S_{in}$; Sage and Sage, 2009). However, the differences in $g_m$ and parameters related to $g_m$ among rice varieties and lines have not been investigated yet.

There are varietal differences in $P_n$ of young expanded rice leaves, when measured under light-saturating and unstressed conditions, and values range from ~20 μmol m$^{-2}$ s$^{-1}$ to 30 μmol m$^{-2}$ s$^{-1}$ at an ambient CO_2 concentration of 370–400 μmol mol$^{-1}$ (Kanemura et al., 2007; Hirasawa et al., 2010; Jahn et al., 2011). A high-yielding indica variety, Takanari, has the highest recorded rate of leaf photosynthesis, namely ~30–33 μmol m$^{-2}$ s$^{-1}$ (Hirasawa et al., 2010). In contrast, Koshihikari, the most popular variety in Japan, has a relatively low $P_n$ of 25–28 μmol m$^{-2}$ s$^{-1}$. Among backcrossed inbred lines derived from a cross between Takanari and Koshihikari (Koshihikari/Takanari/Takanari), two rice lines were identified (BTK-a and BTK-b) with 20–50% higher values of $P_n$ than the parental lines. It is reported that the higher $g_m$ by altered anatomy of mesophyll contributed to the higher $P_n$ in the lines and the existing germplasm has the potential to increase rice $P_n$ to rates resembling those in maize leaves.

**Materials and methods**

### Plant materials

Rice plants, namely Koshihikari, Takanari, and BTK-a and BTK-b (BC_2F_1 and BC_2F_2 plants from Koshihikari/Takanari), were grown in the paddy field of the University Farm (35°40’N, 139°28’E). Seedlings at the fourth-leaf stage were transplanted to the paddy field in alluvial soil (clay loam) at a rate of 22.2 hills m$^{-2}$, with one plant per hill. As a basal dressing, manure was applied at a rate of ~15 t ha$^{-1}$ and chemical fertilizer was applied at a rate of 30, 60, and 60 kg ha$^{-1}$ for N, P_2O_5, and K_2O, respectively. One-third of the total nitrogen was applied as nitrogen sulphate; one-third as LP-50 elution-controlled urea (Chisso Asahi Fertilizer); and one-third as LPS-100 elution-controlled urea (Chisso Asahi Fertilizer). No top-dressing was applied.

For pot cultivation, BTK-a and BTK-b (BC_2F_1), as well as Takanari and Koshihikari, were grown in 12 litre pots filled with a mixture of paddy soil and Kanto diluvial soil (1:1, v/v) at a density of three hills per pot, with three plants per hill, outdoors. Basal fertilizer was applied at a rate of 0.5, 1.0, and 1.0 g per pot for N, P_2O_5, and K_2O, respectively, and additional fertilizer was applied at a rate of 0.3 g and 0.7 g of nitrogen per pot at the tillering stage and at the booting stage when the flag leaves had fully expanded, respectively. Nitrogen was also applied at rates of 0.3, 1.5, and 2.5 g to Takanari to change LNC at the booting stage. Maize plants (cv. Yumechikara) were also grown in 12 litre pots with fertilizer at a rate of 0.5, 1.0, and 1.0 g of N, P_2O_5, and K_2O, respectively, as a basal dressing.

### Gas exchange and chlorophyll fluorescence

Values of $P_n$ and $g_s$ were measured with a portable gas-exchange system (LI-6400; LI-COR) and an LED irradiation source (LI-6400-02R; LI-COR) at the full heading stage. The ambient concentration of CO_2 in the leaf chamber of LI-6400 was kept at 370 μmol mol$^{-1}$, the photosynthetically active radiation (PPFD) was 2000 μmol m$^{-2}$ s$^{-1}$, and the leaf-to-air vapour pressure difference was 1.3–1.6 kPa. The leaf temperature and air temperature were kept at 30 ºC for pot-grown plants and for field-grown plants, respectively, during measurements. $P_n$ was also measured at different values of intercellular concentration of CO_2 (C_i) by changing the ambient CO_2 concentration.

The $P_c$ is limited either by ribulose-1,5-bisphosphate (RuBP) carboxylation or by RuBP regeneration in response to CO_2 concentration according to the C_4 photosynthesis model (Farquhar et al., 1980). When $P_n$ is limited by the RuBP carboxylation rate, the rate of photosynthesis ($P_r$) is expressed as a function of the CO_2 concentration at the chloroplast stroma ($C_c$): 

$$ P_r=[V_{c_{\text{max}}} (C_c-G^*)] /[C_c+K_c(1+O/K_C)]-R_d $$

(1)

where $V_{c_{\text{max}}}$ is the maximum velocity of RuBP carboxylation, $K_c$ and $K_o$ are the Michaelis–Menten constants of Rubisco for CO_2 and O_2, respectively, $O$ is the O_2 concentration, and $G^*$ is the CO_2 compensation point in the absence of day respiration ($R_d$). When $P_r$ is limited by the RuBP regeneration rate, the rate of photosynthesis ($P_r$) is expressed as:

$$ P_r=[J_{\text{max}} (C_c-G^*)]/(4C_c+8G^*)-R_d $$

(2)

where $J_{\text{max}}$ (μmol m$^{-2}$ s$^{-1}$) is the maximum rate of electron transport. Values of the Rubisco kinetic constant and $G^*$ and their temperature responses were obtained from the C_4-based in vivo values of Bernacchi et al. (2002). The values of $K_c$, $K_o$, and $G^*$ at 30 ºC were 467.9 μmol mol$^{-1}$, 194.1 mmol mol$^{-1}$, and 44.0 μmol mol$^{-1}$, respectively. $R_d$ was estimated from the $P_n$–$C_c$ curve at $G^*$. The non-linear curve fitting as described by Sharkey et al. (2007) was used for estimating $C_c$, $V_{c_{\text{max}}}$ and $J_{\text{max}}$ and the model curves were expressed on a C_i basis in Fig. 2.
In addition, leaf gas exchange was determined at the same time as measurements of chlorophyll fluorescence using an LI-6400 system with an integrated fluorescence chamber head (LI6400-40). The conditions in the leaf chamber were those mentioned above, and steady-state fluorescence ($F_i$) and maximum fluorescence during a light-saturating pulse of ~8000 μmol m$^{-2}$ s$^{-1}$ ($F_{m}'$) were measured. The electron transport rate (ETR) was calculated as follows:

$$\text{ETR} = (F_{m}' - F_i) / F_{m}' \times PPFD \times \text{g_leaf} \times \beta$$  

Leaf absorbance ($g_{abs}$) and distribution of electrons between photosynthetic system I (PSI) and PSII ($\beta$) were assumed to be 0.85 and 0.5, respectively (Li et al., 2009). $g_m$ was calculated as follows (Harley et al., 1992):

$$g_m = P_d / (C_i - \Gamma \times \text{ETR} + 8(P_+ + R_0))/([\text{ETR} - 4(P_+ + R_0)])$$  

Here, $\Gamma$ and $R_0$ were obtained using CO$_2$ response curves with three different irradiances (Brooks and Farquhar, 1985) with the following equiation (von Caemmerer et al., 1994):

$$\Gamma = p* + R_0 / g_m$$  

where $p*$ is the intercellular CO$_2$ concentration at the intersection point. There was no significant difference in either $\Gamma$ or $R_0$ among Takanari, BTK-a, and BTK-b (Supplementary Table S1 available at JXB online), suggesting that these values were not involved in the difference in $g_m$. Estimations of $g_m$ were done at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$. Although it is known that $g_m$ varied in relation to the CO$_2$ concentration (Flexas et al., 2007), $g_m$ in Takanari did not change at $C_i < 500$ μmol mol$^{-1}$ (Supplementary Fig. S1). Then, the value of $g_m$ measured at an ambient CO$_2$ concentration was also used for estimating the $P_n$ versus $C_i$ initial slope at low CO$_2$ concentrations. The $C_i$ was calculated using the equation:

$$C_i = C_i - p_d / g_m$$  

Quantitation of Rubisco and nitrogen

Leaves were collected after completion of measurement of $P_n$, and they were stored at −80 °C prior to analysis. The area and fresh weight of each leaf were determined and each leaf was separated into two equal parts for separate quantification of Rubisco and nitrogen. The halves of leaves were homogenized separately using a mortar and pestle in a solution that contained 50 mM TRIS-HCl (pH 7.5), 1 μmM EDTA, 10 mM MgCl$_2$, 0.2% (v/v) Triton X-100, 10 mM 2-mercaptoethanol, and 5% (w/w) insoluble polyvinylpyrrolidone (Polyvlar VT, Wako). Each homogenate was centrifuged at 10 000 g for 10 min at 4 °C. The supernatant was used for quantitation of Rubisco by the single radial immunodiffusion method (Sugiyama and Hirayama, 1983) with rabbit polyclonal antibodies raised against purified Rubisco from rice. Nitrogen was quantified with a carbon and nitrogen analyser (MT700 Mark II; Yanako).

**Determination of Rubisco activity**

Leaves were collected after equilibration under steady-state conditions at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$. After the rate of gas exchange had stabilized, the leaf was removed from the chamber and immediately frozen in liquid nitrogen. Each leaf was powdered in liquid nitrogen and stored at −80 °C until analysis. Aliquots of ~20 mg fresh weight were weighed out at −180 °C and were extracted by vigorous shaking with 1 ml of extraction buffer, at an initial ~50-fold (w/v) dilution. The extraction buffer contained 20% (v/v) glycerol, 0.25% (w/v) bovine serum albumin, 1% (v/v) Triton X-100, 50 mM HEPES/KOH (pH 7.5), 10 μmM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin, and 0.5 mM diithiothreitol. Enzymatic activities were determined with a robotized platform (Multi Works 507; MS Technos) (Sulpice et al., 2007). The activity of Rubisco was expressed as the amount of the product 3-phosphoglycerate per unit leaf area per min. The values obtained with this method seemed to show the difference in the Rubisco carboxylation rate since the apparent O$_2$ inhibition was similar between Takanari and the two BTK lines (Supplementary Fig. S2 at JXB online). There was a close relationship between the $P_n$ and Rubisco initial activity in Takanari when the level of nitrogen application for Takanari was different (Supplementary Fig. S3).

**Analysis of leaf anatomy**

For light microscopy, leaves were fixed in formalin-acetic-alcohol and dehydrated through an ethanol series. They were then embedded in GMA-Quetol-523 resin (Quetol 523M; Nissin EM). Transverse and longitudinal sections of 5 μm thickness were cut on a sliding microtome (TU-213; Yamato Kohki) and stained with 1% toluidine blue. For electron microscopy, leaf samples of 1 × 2 mm were fixed in Karnovsky’s fixative (mixture of 4% paraformaldehyde and 5% glutaraldehyde in 50 mM phosphate buffer, pH 7.2) and post-fixed in 2% osmium tetroxide in the same buffer. Samples were dehydrated in a series of graded acetone and propylene oxide, and embedded in Spurr’s resin. Ultrathin 85 nm thick sections were cut with a diamond knife and placed on a 200 mesh copper grid. The grids were stained with 2% uranyl acetate for 20 min followed by lead citrate for 3 min. The sections were examined on a Hitachi H7500 transmission electron microscope at 100 kV, and photographed with a CCD camera (Advanced Microscopy Technique, USA) connected to the electron microscope.

**Anatomical characteristics**

were determined with computer-associated image analysis software (WinRoof Version 6; Mitami). Using transverse sections with the light microscope, the number of mesophyll cells between two small vascular bundles (CN, number μm$^{-1}$) was counted. The cell number/leaf area was calculated as a product of CN and the average number of cells per unit length obtained from longitudinal sections. The cross-sectional area ($A_{cell}$, μm$^2$) and the cell wall length ($L_{cell}$, μm) of a mesophyll cell were measured for >30 cells between the vascular bundles. Using the transverse sections prepared for electron microscopy, the length of the mesophyll cell wall exposed to the intercellular airspace ($L_{mes}$, μm), the total length of the mesophyll cell wall ($L_{mes}'$, μm), the length of chloroplasts exposed to the intercellular airspace ($L_{e}$, μm), and the fraction of intercellular airspace (%) were measured. The surface area of mesophyll cells exposed to the intercellular airspace ($S_{mes}$, m$^2$ m$^{-2}$) was calculated as:

$$S_{mes} = L_{cell} \times CN \times L_{mes} \times L_{mes}' \times F$$  

where $F$ is the curvature correlation factor (Thain, 1983). The $F$ value of 1.55 was used following Scaraf et al.,(2011) considering rice mesophyll cells as being prolate spheroids. The surface area of chloroplasts exposed to the intercellular airspace ($S_e$, m$^2$ m$^{-2}$) was calculated as:

$$S_e = L_{cell} \times L_{mes} \times S_{mes}$$  

**Plant growth analysis**

Plants grown in pots were separated into leaves and other parts. Leaf area was measured with an area meter (AAM-9; Hayashi Denko) immediately after separation of leaves from plants. Each group of plant parts was dried in a ventilated oven at 80 °C for at least 3 d. Plant growth rate, mean leaf area, and net assimilation rate were calculated as described elsewhere (Beadle, 1993).
Results

Photosynthetic rate of the BTK lines

The $P_n$ values of flag leaves were compared among BC$_1$F$_6$ and BC$_1$F$_7$ progeny lines derived from a cross between Takanari and Koshihikari at the full heading stage in the paddy field, and two lines, BTK-a and BTK-b, were identified with 20–25% higher $P_n$ than that of Takanari at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ (Fig. 1A, B). The value of the two lines was 40–50% higher than the $P_n$ values of Koshihikari. The values in the range from 37 μmol m$^{-2}$ s$^{-1}$ to 40 μmol m$^{-2}$ s$^{-1}$ are similar to that of maize that was grown in the field as well as those of some previous reports (Leakey et al., 2006; Markelz et al., 2011). To the authors’ knowledge, such a high value has not been reported in studies of varietal differences among $P_n$ values of rice leaves.

Causes for the high $P_n$ in BTK lines

The causes of the high $P_n$ of BTK-a and BTK-b, which were grown in pots, of BC$_1$F$_6$ progeny lines were investigated. At a CO$_2$ concentration of 370 μmol mol$^{-1}$, average rates of leaf photosynthesis were 38.8 μmol m$^{-2}$ s$^{-1}$ and 37.6 μmol m$^{-2}$ s$^{-1}$ for BTK-a and BTK-b, respectively (Fig. 1C), whereas the average rates for Takanari and Koshihikari were 30.8 μmol m$^{-2}$ s$^{-1}$ and 24.4 μmol m$^{-2}$ s$^{-1}$, respectively. When the response of $P_n$ to changes in $C_i$ was examined, it was found that the $P_n$ in the BTK lines was higher than that in Takanari at all $C_i$ levels, while $P_n$ in Koshihikari was consistently low compared with that in Takanari. When the CO$_2$ response curves were fitted according to the model of Sharkey et al. (2007), it was found that the $P_n$ in all genotypes was limited by the rate of RuBP carboxylation at low $C_i$, including the ambient CO$_2$ concentration and at $C_i > 350$ μmol mol$^{-1}$, respectively (Fig. 2). Then two different phases of $P_n$ were distinguished, i.e. phase I and phase II that were limited by the RuBP carboxylation rate and the RuBP regeneration rate, respectively. The reason for the high $P_n$ in the BTK lines was analysed by comparing it with that in Takanari at each phase.

To identify the limiting factor in phase I, values of $P_n$ and parameters associated with $P_n$ were compared at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ (Fig. 3). In order to change LNC, Takanari plants were top-dressed with nitrogen at different rates at the booting stage when the flag leaves had fully expanded. LNCS were higher in BTK-a and BTK-b than in Takanari, even at the same rate of nitrogen application (Fig. 3A). The increase in LNC might contribute somewhat to the higher $P_n$ in these lines than in Takanari even at the same LNC. Non-stomatal factors might be responsible for the higher $P_n$ values of BTK-a and BTK-b at the same LNC since there was no differences in $g_m$ between Takanari and the BTK lines (Fig. 3B). The initial slope of the $P_n$ versus $C_i$ curve, which is determined by the activity of Rubisco and $g_m$, was ~20% higher in the BTK lines than in Takanari at the same LNC (Fig. 3C). There was no difference in the relationship of Rubisco content versus LNC between Takanari and the two BTK lines (Fig. 3D). However, there were no differences in total activity and activation state of Rubisco per unit weight of protein among BTK-a, BTK-b, and Takanari, even though the $P_n$ values of the BTK lines were so much higher than that of Takanari at the same level of Rubisco (Fig. 4). These results suggest that the difference in $g_m$ is responsible for the difference in the carboxylation rate between Takanari and the BTK lines. In fact, the estimated value of $g_m$ was substantially higher in BTK-a and BTK-b than in Takanari, when plants with a similar LNC were compared (Fig. 5A). As the LNC was lower by ~30% and, therefore, the $g_m$ was significantly lower in Koshihikari than in Takanari, the characteristics of Koshihikari could
not be compared with those of other rice (data not shown). Furthermore, there was no difference in in vivo Rubisco activity among Takanari and the two BTK lines with similar LNC as the initial slopes of $P_n$ versus the concentration of CO$_2$ at the chloroplast ($C_c$) showed no significant difference among these rice lines (Fig. 5B).

**Fig. 2.** Relationships between the rate of photosynthesis ($P_n$) and the intercellular CO$_2$ concentration ($C_i$) from Fig. 1C with model curves according to Sharkey et al., (2007). The solid and dotted line indicate $P_n$ limited by RuBP carboxylation ($P_c$) and $P_n$ limited by RuBP regeneration ($P_r$), respectively. $P_n$ at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ is shown as a solid symbol.

**Fig. 3.** Relationships between leaf nitrogen content (LNC) and $P_n$ at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ (A), $g_s$ at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ (B), the initial slope of curves of $P_n$ versus $C_i$ (C), and Rubisco content (D) in Takanari (circles), BTK-a (diamonds), and BTK-b (triangles) of flag leaves. In order to change the levels of leaf nitrogen, nitrogen was applied at four different rates to Takanari at the booting stage when the flag leaves had fully expanded. Black symbols in Takanari indicate that the rate of nitrogen application was the same as in the two BTK lines. The initial slopes were estimated as the slopes of the linear regression between $C_i$ and $P_n$ from several points measured at a $C_i <$160 μmol mol$^{-1}$. Error bars indicate the SD for $n$=3–5.
To determine the causes of the higher $g_m$ in these lines, the anatomy of the mesophyll of these lines was analysed by comparing it with that of Takanari as well as of Koshihikari (Table 1). There was no difference in thickness of the mesophyll cell wall and fraction of the intercellular airspace among Takanari and BTK lines. The value of $S_c$ was much larger in BTK-a and BTK-b than in Takanari and Koshihikari. It was found that there was a close relationship between $S_c$ and $g_m$ (Supplementary Fig. S4 at JXB online). There was no significant difference in the proportion of chloroplast covering the mesophyll cell surface ($S_c/S_{mes}$) among Takanari and BTK lines (Table 1; Supplementary Fig. S5), indicating that the value of $S_c$ was controlled by the value of $S_{mes}$ in these plants. The number of mesophyll cells per leaf area was considerably larger in the BTK lines than in Takanari and Koshihikari, which was the result of the smaller size of mesophyll cells in BTK lines similar to that of Koshihikari and the greater thickness of mesophyll layer similar to that of Takanari. Mesophyll cells of BTK-a and BTK-b as well as Koshihikari had much more highly developed lobes than those of Takanari (Fig. 6). This caused the values of $L_{cell}$ of the two BTK lines to be similar to that of Takanari, despite the fact that the size of BTK mesophyll cells represented by $A_{cell}$ was smaller than that of Takanari mesophyll cells. The more highly developed lobes in BTK-a and BTK-b account for ~50% of the increases in $L_{cell}/A_{cell}$ over that of Takanari, while the smaller cell size accounts for the other half.

In phase II, where the $C_i$ was high, it is known that the rate of regeneration of RuBP is limited by the ETR in the chloroplast (Farquhar et al., 1980). The ETRs were far higher in BTK-a and BTK-b than in Takanari, irrespective of the LNC (Fig. 7).

**Contribution of elevated $P_n$ to plant growth**

Not only the $P_n$ of the flag leaf, but also $P_n$ values of the first and second leaves below the flag leaf were substantially higher in BTK-a and BTK-b than in Takanari (Fig. 8A). The
Table 1. Anatomical characteristics of leaf mesophyll cells.

<table>
<thead>
<tr>
<th></th>
<th>Takanari</th>
<th>BTK-a</th>
<th>BTK-b</th>
<th>Koshihikari</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophyll cell wall thickness (nm)</td>
<td>156 ± 2 a</td>
<td>155 ± 9 a</td>
<td>155 ± 6 a</td>
<td>157 ± 5 a</td>
</tr>
<tr>
<td>Intercellular airspace (% section)</td>
<td>22.6 ± 4.3 a</td>
<td>20.4 ± 2.5 a</td>
<td>22.0 ± 2.9 a</td>
<td>20.0 ± 4.9 a</td>
</tr>
<tr>
<td>S_c (m^2 m^-2)</td>
<td>19.0 ± 1.7 b</td>
<td>28.4 ± 1.6 a</td>
<td>27.6 ± 1.2 a</td>
<td>20.9 ± 1.5 b</td>
</tr>
<tr>
<td>S_{max} (m^2 m^-2)</td>
<td>21.2 ± 1.9 b</td>
<td>30.2 ± 1.7 a</td>
<td>29.4 ± 1.3 a</td>
<td>23.2 ± 1.6 b</td>
</tr>
<tr>
<td>S_c/S_{max}</td>
<td>0.89 ± 0.03 a</td>
<td>0.94 ± 0.06 a</td>
<td>0.94 ± 0.02 a</td>
<td>0.90 ± 0.06 a</td>
</tr>
<tr>
<td>Cell number/leaf area (×10^3 mm^-2)</td>
<td>36.7 ± 6.5 b</td>
<td>54.7 ± 4.3 a</td>
<td>56.1 ± 4.4 a</td>
<td>43.0 ± 2.9 b</td>
</tr>
<tr>
<td>Mesophyll layer thickness (μm)</td>
<td>109 ± 12 a</td>
<td>111 ± 12 a</td>
<td>123 ± 14 a</td>
<td>91 ± 5 b</td>
</tr>
<tr>
<td>A_{cell} (μm^2)</td>
<td>247 ± 26 a</td>
<td>202 ± 17 b</td>
<td>206 ± 24 b</td>
<td>184 ± 12 b</td>
</tr>
<tr>
<td>L_{cell} (μm)</td>
<td>78 ± 8 a</td>
<td>88 ± 4 a</td>
<td>82 ± 8 a</td>
<td>76 ± 4 a</td>
</tr>
<tr>
<td>L_{cell}/A_{cell} (μm μm^-2)</td>
<td>0.32 ± 0.02 c</td>
<td>0.43 ± 0.02 a</td>
<td>0.40 ± 0.01 b</td>
<td>0.41 ± 0.01 ab</td>
</tr>
</tbody>
</table>

S_c, surface area of chloroplasts exposed to the intercellular airspace; S_{max}, surface area of mesophyll cells exposed to the intercellular airspace; A_{cell}, cross-sectional area of each mesophyll cell; L_{cell}, cell wall length of each mesophyll cell. Values are means ±SD for n=4–7. Values followed by the same letters indicate no significant difference among rice lines at P < 0.05 (Tukey’s test).

net assimilation rates of the two BTK lines were higher than that of Takanari after tillering through ripening under conditions of minimum mutual shading, when pots were arranged at a distance from one another (Fig. 8C). Leaf area of these lines changed similarly to that of Takanari, although it was smaller in BTK-b at full heading (Fig. 8B, C). As a result, BTK-a and BTK-b showed a higher plant growth rate after tillering through ripening (Fig. 8C). The total dry weight in BTK-b at the final harvest was 23% higher than that in Takanari, while the short vegetative period of BTK-a resulted in BTK-b at the final harvest was 23% higher than that in Takanari and Koshihikari. However, the genetic variation of P_n among species was recognized in earlier studies (Warren, 2008; Niinemets et al., 2009; Barbour et al., 2010). Recently, Seafaro et al. (2011) reported that higher g_m in O. sativa than in Oryza meridionalis and Oryza australiensis is responsible for its higher P_n. No such difference in Rubisco activity was larger than that of Takanari and Koshihikari, and the factors which contributed to the high P_n were examined in this study (Fig. 4).

P_n is strongly influenced by the diffusion of CO2 from the atmosphere to the chloroplast in leaves under the atmospheric concentration of CO2. Varietal differences in stomatal conductance, which regulates the supply of CO2 from the atmosphere to the interior of the leaf, have often been observed even at a relatively small vapour pressure deficit (Ohsumi et al., 2007; Hirasawa et al., 2010). Stomatal conductance of indica varieties was larger than that of japonica varieties (Maruyama and Tajima, 1990). Recently, g_m, as well as g_s, has been recognized as an important modulator of P_n, especially in C3 plants (Warren, 2008; Makino, 2011; Terashima et al., 2011). Wide variations in g_m among species were recognized in earlier studies (Warren, 2008; Niinemets et al., 2009; Barbour et al., 2010). Recently, Seafaro et al. (2011) reported that higher g_m in O. sativa than in Oryza meridionalis and Oryza australiensis is responsible for its higher P_n. However, the genetic variation of g_m among O. sativa has not been examined.

P_n is higher in Takanari than in Koshihikari and many other japonica varieties. Recently, it has been proved that the higher P_n of Takanari can be attributed to the higher LNC and g_m, which are due to its elevated capacity for nitrogen accumulation in plants and the large hydraulic conductance of the root, respectively, compared with those of Koshihikari (Taylaran et al., 2011). BTK-a and BTK-b had high values of LNC and g_m, both of which were somewhat higher than in Takanari at the same rate of nitrogen application (Fig. 3). The two BTK lines showed significantly high P_n compared with Takanari for the same LNC (Fig. 3C). However, BTK-a and BTK-b showed neither a higher Rubisco content nor a higher Rubisco activity per unit weight of protein than Takanari (Fig. 4). Taking these findings together, it can be concluded that the larger g_m is a major cause of the higher P_n of BTK-a and BTK-b at an ambient CO2 concentration of 370 μmol mol^-1, as compared with

Discussion

In this study, two rice lines with extremely high values of P_n were identified among the backcrossed inbred lines derived from Takanari and Koshihikari, and the factors which contributed to the high P_n were analysed. According to the model of Farquhar et al. (1980), the rate of photosynthesis in C3 plants is limited by either the capacity of RuBP carboxylation (phase I) or the capacity of RuBP regeneration (phase II) depending on the CO2 concentration. First, the focus of this discussion is on the reason for the high rate of photosynthesis in the BTK lines in phase I under a low CO2 concentration including the current atmospheric CO2 concentration.

Many modulators of P_n are affected by LNC. It is well known that the Rubisco content of each leaf is closely correlated with the LNC (Makino et al., 1984) and there are significant varietal differences in LNC even at the same rate of nitrogen application (Kanemura et al., 2007; Hirasawa et al., 2010). This might be one reason why the causes of the varietal differences in P_n of rice are not fully understood, although they have been widely recognized.

There are some differences in terms of the Michaelis–Menten constant and the maximum carboxylation rate of Rubisco among Oryza species, but the variations among varieties are considered to be small (Makino et al., 1987). No differences were found in total activities and activation state of Rubisco per unit weight of protein among the rice lines examined in this study (Fig. 4).

P_n is strongly influenced by the diffusion of CO2 from the atmosphere to the chloroplast in leaves under the atmospheric concentration of CO2. Varietal differences in stomatal conductance, which regulates the supply of CO2 from the atmosphere to the interior of the leaf, have often been observed even at a relatively small vapour pressure deficit (Ohsumi et al., 2007; Hirasawa et al., 2010). Stomatal conductance of indica varieties was larger than that of japonica varieties (Maruyama and Tajima, 1990). Recently, g_m, as well as g_s, has been recognized as an important modulator of P_n, especially in C3 plants (Warren, 2008; Makino, 2011; Terashima et al., 2011). Wide variations in g_m among species were recognized in earlier studies (Warren, 2008; Niinemets et al., 2009; Barbour et al., 2010). Recently, Seafaro et al. (2011) reported that higher g_m in O. sativa than in Oryza meridionalis and Oryza australiensis is responsible for its higher P_n. However, the genetic variation of g_m among O. sativa has not been examined.
Takanari. C₄ plants have a unique system for concentrating CO₂ to very high levels in bundle sheath cells. In contrast, the value of $P_n$ of BTK-a and BTK-b was increased to the level of that of C₄ maize by increasing the diffusion of CO₂ from the intercellular air spaces to chloroplasts as well as the diffusion of CO₂ from the atmosphere to the intercellular spaces through stomata.

The larger $g_m$ of BTK-a and BTK-b can be attributed to the higher $S_t$ than that of Takanari (Table 1; Supplementary Fig. S4 at JXB online). The higher $S_t$ was caused by the higher $S_{mes}$, which is attributable to the higher density and more highly developed lobes of mesophyll cells in BTK-a and BTK-b (Table 1, Fig. 6). Although it is also reported that the wall thickness of mesophyll cells and the fraction of intercellular airspaces are related to the $g_m$ (Seafaro et al., 2011; Peguero-Pina et al., 2012), there is no difference in these two parameters among rice lines in this study (Table 1). It is known that rice leaves have mesophyll cells with well-developed lobes, which increase the ratio of the surface area to volume of mesophyll cells and, therefore, might increase $g_m$ (Chonan, 1967; Nobel, 1999; Sage and Sage, 2009). Since lobes of mesophyll cells are better developed in Koshihikari than in Takanari (Fig. 6), BTK-a and BTK-b might have inherited this trait from Koshihikari. Furthermore, the traits of the thicker layer of mesophyll and of the smaller size of mesophyll cells in the BTK lines might have been inherited from Takanari and Koshihikari, respectively (Table 1). Thus, the high $P_n$ values of BTK-a and BTK-b might be attributable to the cumulative effects of the distinguishing characteristics associated with photosynthesis in each of the two parental varieties. These suggest that it is possible to increase the $P_n$ of indica varieties, which show high $P_n$ compared with most japonica varieties, to the same degree as maize if the traits of smaller size and developed lobes of mesophyll cells are introduced into the indica varieties. Likewise, it might be possible to enhance the $P_n$ of japonica varieties to the level of that of maize if these japonica varieties have hydraulic conductance, nitrogen accumulation, and leaf thickness as high as the indica variety used in this study. The $S_t$ was much larger in BTK lines than in Takanari, while the Rubisco content of BTK lines was similar to that of Takanari (Table 1, Fig. 3D). There is a possibility that the size of chloroplasts may decrease in BTK lines (Li et al., 2009), but there was no clear evidence in the electron micrographs (Supplementary Fig. S5). This possibility should be analysed in future research.

With the focus on $P_n$ in phase II, possibilities were found that the BTK lines will display high $P_n$ beyond that of maize due to their high ETR under conditions of elevated atmospheric CO₂ concentration which are anticipated in the near future (Fig. 1C). The ETR in the BTK lines was even higher than that in Takanari for a given LNC (Fig. 7). From the
findings of previous studies (Price et al., 1995, 1998; Ruuska et al., 2000; Yamori et al., 2011), the levels of ATP synthase and/or the cytochrome b$_6$f complexes on the thylakoid membrane may be high in BTK lines irrespective of the LNC, and increases in them might be associated with the increase in the mesophyll cell density. The reason for the high ETR in the BTK lines should be uncovered in future research. Furthermore, the actual value of $P_n$ above a $C_i$ of 800 μmol mol$^{-1}$ was lower than the model curve that shows the rate of photosynthesis limited by the RuBP regeneration rate in all genotypes (Fig. 2). This indicates that the $P_n$ might have been limited by the utilization of triose phosphate under such a high CO$_2$ concentration (Sharkey, 1985). The difference in the rate of triose phosphate utilization among genotypes is to be investigated in future research.

This study showed that the $P_n$ of rice could be dramatically increased if a number of traits associating with $P_n$ originating from both Takanari and Koshihikari were combined. Due to recent advances in genomic technologies, whole-genome marker-assisted selection is becoming available (Fukuoka et al., 2010). Work is now being carried out to identify the quantitative trait loci (QTLs) for the anatomy of mesophyll cells of japonica varieties and QTLs for the hydraulic conductance, nitrogen accumulation, and leaf thickness of indica varieties. If the loci can be detected and the detailed functions of these genes clarified, it should be possible, in combination with conventional crossing, to increase the rates of rice leaf photosynthesis still further both under the current atmospheric CO$_2$ concentration and under the elevated CO$_2$ concentration in the future, even before introduction of photosynthesis-related genes from other plant species is exploited for enhancement of $P_n$.

**Supplementary data**

Supplementary data are available at JXB online.

*Figure S1.* Mesophyll conductance ($g_m$) relative to intercellular CO$_2$ concentration ($C_i$) in Takanari.

*Figure S2.* Apparent O$_2$ inhibition relative to leaf nitrogen content (LNC) of flag leaves.

*Figure S3.* Relationship between photosynthetic rate at an ambient CO$_2$ concentration of 370 μmol mol$^{-1}$ ($P_n$) and Rubisco initial activity of flag leaves.

*Figure S4.* Relationship between chloroplast surface area exposed to intercellular air space ($S_i$) and mesophyll conductance ($g_m$).
Figure S5. Transmission electron micrographs of transverse leaf sections of Takanari, Koshihikari, BTK-a, and BTK-b.

Table S1. The day respiration rate ($R_d$) and the CO$_2$ compensation point in the absence of $R_d$ (Γ*) of flag leaves of Takanari, BTK-a, and BTK-b.

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