Whole-Plant Growth and N Utilization in Transgenic Rice Plants with Increased or Decreased Rubisco Content under Different CO₂ Partial Pressures

Emi Sudo¹, Yuji Suzuki¹ and Amane Makino¹,²,*

¹Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai, 981-8555 Japan
²CREST, JST, Gobancho, Chiyoda-ku, Tokyo, 102-0076 Japan
*Corresponding author: E-mail, makino@biochem.tohoku.ac.jp; Tel, +81-22-272-8769; Fax, +81-22-272-8765.
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Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) strongly limits photosynthesis at lower CO₂ concentration [CO₂] whereas 10 Rubisco limitation is cancelled by elevated [CO₂]. Therefore, increase or reduction in Rubisco content by transformation with a sense or an antisense RBCS construct are expected to alter the biomass production under different CO₂ levels. RBCS-sense (125% Rubisco of wild-type) and -antisense (35% Rubisco of wild-type) rice (Oryza sativa L.) plants were grown for 63 days at three different CO₂ levels: low [CO₂] (28 Pa), normal [CO₂] (40 Pa) and elevated [CO₂] (120 Pa). The biomass of RBCS-sense plants was 32% and 15% greater at low [CO₂] and normal [CO₂] than that of the wild-type plants, respectively, but did not differ at elevated [CO₂]. Conversely, the biomass of RBCS-antisense plants was the smallest at low [CO₂]. Thus, overproduction of Rubisco was effective for biomass production at low [CO₂]. Greater biomass production at low [CO₂] in RBCS-sense plants was caused by an increase in the net assimilation rate, and associated with an increase in the amount of N uptake. Furthermore, Rubisco overproduction in RBCS-sense plants was also promoted at low [CO₂]. Although it seems that low [CO₂]-growth additionally stimulates the effect of Rubisco overexpression, such a phenomenon observed at low [CO₂] was mediated through an increase in total leaf N content. Thus, the dependence of the growth improvement in RBCS-sense rice on growth [CO₂] was closely related to the degree of Rubisco overproduction which was accompanied not only by leaf N content but also by whole plant N content.

**Keywords**: Biomass • Low and elevated [CO₂] • Nitrogen • Oryza sativa • RBCS • Rubisco overproduction.

**Abbreviations**: LAR, leaf area ratio; NAR, net assimilation rate; PPFD, photosynthetic photon flux density; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RGR, relative growth rate.

**Introduction**

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (EC 4.1.1.39) is both the key enzyme of photosynthesis and the most abundant leaf protein. This enzyme catalyzes two competing reactions, CO₂ fixation in photosynthesis and the oxygenation in the photorespiratory pathway, and is a rate-limiting factor for potential photosynthesis under the present atmospheric air conditions (Evans 1986, Makino et al. 1997a). In addition, it has been found that large amounts of N are invested in Rubisco protein, accounting for 15–30% of the total leaf N content in C₃ species (Evans 1989, Makino et al. 1992). Thus, Rubisco is closely related to potential photosynthesis as well as to the N economy in higher plants.

The impact of genetic modification of Rubisco content in a leaf on photosynthesis and biomass production has been examined. Rubisco is composed of eight small subunits, coded for by a nuclear multigene family (RBCS), and eight large subunits, coded for by a single gene (rbcL) in the chloroplast genome. Genetic modulation of Rubisco content was first established by transformation using an antisense RBCS construct in tobacco (Rodermel et al. 1988, Hudson et al. 1992), a C₄ plant Flaveria bidentis (Furbank et al. 1996) and rice (Makino et al. 1997a). In RBCS-antisense tobacco and rice, the photosynthetic rate at the present CO₂ levels declined in parallel with a decrease in Rubisco content (Hudson et al. 1992, Makino et al. 1997a) and the whole plant biomass was also decreased (Masle et al. 1993, Makino et al. 2000). On the other hand, little attention has been paid to whether transformation with a sense RBCS construct leads to overproduction of Rubisco. Meanwhile, although we successfully overproduced Rubisco by introducing sense RBCS with its own promoter (Suzuki et al. 2007), such an overproduction of Rubisco in rice did not quantitatively lead to photosynthesis improvement (Makino and Sage 2007). In addition, when the leaf had the same N content, there was no difference in the photosynthetic rate between the wild-type and RBCS-sense rice with increased Rubisco content (Suzuki et al. 2007 and 2009). Neither did the whole plant biomass increase significantly when the plants were grown under the present atmospheric air conditions (Suzuki et al. 2009). An overproduction of Rubisco reduced N allocation to other N components (Suzuki et al. 2007 and 2009) and caused a partial deactivation of Rubisco in vivo (Makino and Sage 2007, Suzuki et al. 2009).

Rubisco strongly limits photosynthesis at lower [CO₂] whereas Rubisco limitation is cancelled by elevated [CO₂] (von Caemmerer and Farquhar 1981). Consistently, in RBCS-antisense tobacco and rice, growth suppression was...
ameliorated when they were grown at elevated [CO₂] (Masle et al. 1993, Makino et al. 2000). Thus, increase and reduction in Rubisco content are expected to alter the dependence of biomass production on growth [CO₂] because Rubisco limitation in photosynthesis is strongly affected by [CO₂]. In addition, Rubisco content is also affected by growth [CO₂]. Many studies have shown that CO₂ enrichment reduces Rubisco content and that the decrease in Rubisco content by CO₂ enrichment is greater than that of other photosynthetic components (for a review, see Sage and Coleman 2001). Thus, it is possible that the RBCS-sense or -antisense effect is amplified or suppressed in RBCS-transgenic rice plants under the conditions of different CO₂ levels.

In the present study, we analyzed RBCS-sense and -antisense rice plants grown at three different CO₂ levels: low [CO₂] (28 Pa), normal [CO₂] (40 Pa) and elevated [CO₂] (120 Pa). Biomass, N content and biomass allocation were examined at 63 d after germination and the growth rate was analyzed. In addition, Rubisco and Chl contents were examined in the uppermost fully expanded leaves and their relationships to leaf N content were analyzed. Finally, we examined how overproduction and reduction of Rubisco affect the whole plant growth in different CO₂ environments.

Results

In this study, we used RBCS-sense rice with 125% wild-type Rubisco (line S-26-8, Suzuki et al. 2007), RBCS-antisense rice with 35% wild-type Rubisco (line AS-71, Makino et al. 2000) and wild-type (non-transgenic) rice. The whole plant biomass and N contents including roots were examined at the 63 d after germination (Fig. 1). Irrespective of genotype, total biomass increased with elevation of growth [CO₂]. The biomass of RBCS-sense plants was 32% and 15% greater at low [CO₂] and normal [CO₂] than that of wild-type plants, respectively, whereas no difference was observed between them at elevated [CO₂] (Fig. 1A). Thus, the low [CO₂] growth stimulated growth improvement in the RBCS-sense rice plants. On the other hand, the growth of RBCS-antisense plants was severely suppressed at low [CO₂] but the growth was ameliorated at elevated [CO₂] (Fig. 1A). Total plant N content was also greater in the RBCS-sense plants than in wild-type plants at low [CO₂] and there was no difference at elevated [CO₂] between them (Fig. 1B). Similarly, the smallest N content was observed for the RBCS-antisense rice plants. These alterations of plant N content were similar to those of biomass production. Actually, the difference in biomass production per N content was extremely small (Fig. 1C). Thus, greater biomass was always associated with greater N uptake.

Table 1 shows several growth parameters of three genotypes at 63 d after germination. Tiller number of RBCS-sense plants was the greatest irrespective of growth [CO₂] and the

![Fig. 1](https://academic.oup.com/pcp/article-abstract/55/11/1905/2755961/1)
slight increase in the NAR was shown at normal [CO₂] (Fig. 2A, B). The LAR of RBCS-antisense plants was higher at normal and elevated [CO₂] (Fig. 2C). Thus, these growth analyses suggested that an increase in the whole-plant biomass in RBCS-sense plants grown at low [CO₂] was mainly caused by an increase in net assimilation capacity. The increased LAR in RBCS-antisense plants may have been one of the compensation phenomena for suppressed photosynthesis due to the Rubisco-antisense effect (Fig. 2C).

The accumulation of sucrose and starch was examined in each plant organ of the three genotypes (Fig. 3). In RBCS-sense plants, carbohydrate concentration (sucrose + starch) was greater in leaf blades and sheaths at normal [CO₂] and in sheaths at elevated [CO₂] than in wild-type rice (Fig. 3A, B). On the other hand, the concentrations in each plant organ at low [CO₂] were similar to those in wild-type plants (Fig. 3A–C). At the whole plant level, carbohydrate contents of RBCS-sense plants was higher irrespective of growth [CO₂] (Fig. 3D). For RBCS-antisense plants, carbohydrate concentrations in each plant organ were dramatically declined at low and normal [CO₂] while such a decline in carbohydrate accumulation was somewhat ameliorated at elevated [CO₂] (Fig. 3A–C). At the whole plant level, carbohydrate content was higher in RBCS-sense plants and lower in RBCS-antisense plants irrespective of growth [CO₂] (Fig. 3D).

Rubisco, Chl and total leaf N contents were examined on the uppermost fully expanded leaves of 63- to 70-day-old plants (Fig. 4). Low [CO₂]-growth stimulated Rubisco overproduction. At low [CO₂], Rubisco content in RBCS-sense plants increased by 43%, compared with wild-type plants, while a 23% increase in Rubisco was observed in RBCS-sense plants at normal and elevated [CO₂] (Fig. 4A). On the other hand, low [CO₂] did not increase Rubisco content in RBCS-antisense plants but elevated [CO₂] enhanced Rubisco content. This is because N uptake in RBCS-antisense plants largely increased with elevating [CO₂] (Fig. 1B). Chl content in RBCS-sense plants did not differ at low [CO₂] although it decreased at normal and elevated [CO₂] (Fig. 4B). Total leaf N content was significantly increased in RBCS-sense plants grown only at low [CO₂] (Fig. 4C). To clarify
Whether such an increase in Rubisco content observed in RBCS-sense plants grown at low [CO2] is associated with increases in total leaf N content, Rubisco and total leaf N contents in RBCS-sense plants grown under low and elevated [CO2] conditions were compared with those in the three genotypes grown with different N levels under normal [CO2] conditions (Fig. 5). A regression line between Rubisco and total leaf N contents depended on each genotype, and the ratio of Rubisco to total leaf N increased with increasing leaf N content irrespective of genotype (Fig. 5A, B). In addition, the data obtained with RBCS-sense plants grown at low and elevated [CO2] fell on the same line with RBCS-sense plants grown with different N levels under conditions of normal [CO2] (Fig. 5A, B). These results clearly indicate that although it looks like that low [CO2]-growth stimulatesRubisco overproduction, such a phenomenon is mediated through an increase in total leaf N content in rice grown at low [CO2]. For RBCS-antisense plants, as described above, elevated [CO2] increased Rubisco content (Fig. 4A). The data obtained with RBCS-antisense plants at elevated [CO2] also fell on the same line with RBCS-antisense plants grown at normal [CO2] (Fig. 5A, B). Thus, Rubisco content in the RBCS-transgenic rice plants appears to be also determined by leaf N content as in wild-type rice plants.

**Discussion**

Rubisco more strongly limits photosynthesis at low [CO2] whereas such Rubisco limitation is cancelled at elevated [CO2] (von Caemmerer and Farquhar 1981). Our results consistently indicate that biomass production was greater in RBCS-sense rice plants grown at low [CO2] while the enhancement of biomass production was eliminated at elevated [CO2] (Fig. 1A). This was caused by an increase in NAR, leading to an increase in RGR (Fig. 2A, B). Thus, overproduction of Rubisco clearly has an advantage for growth at low [CO2]. As one of possible explanations for this, we consider that overproduction of Rubisco directly causes a greater biomass through small enhancement of potential photosynthesis. Actually, the photosynthetic rates at low and normal [CO2] were a little higher in RBCS-sense plants than in wild-type plants when the rates are expressed per unit of Chl or per unit of leaf area (Makino and Sage 2007), although the rate per leaf N content did not differ (Suzuki et al. 2007). On the other hand, the photosynthetic rate at elevated [CO2] did not differ or was lower in the RBCS-antisense rice plants (Makino and Sage 2007). This may be the reason for cancellation of enhancement of biomass at elevated [CO2]. Furthermore, greater biomass at low [CO2] was associated with higher leaf N content as well as a large increase in Rubisco content (Fig. 4A, C), which may be additional explanations for greater NAR. Such increases in Rubisco and leaf N contents were accompanied by an increase in whole plant N content (Fig. 4C, Fig. 1B). Thus, the biomass enhancement of RBCS-sense rice plants may have also been achieved via a large increase in Rubisco content, which can consequently result in an increase in leaf N content and an increase in whole plant N content.

However, it is not known how a large increase in Rubisco content is related to a promotion of plant N uptake in RBCS-sense plants grown at low [CO2]. Of course, greater biomass requires more N uptake. It is possible that overproduction of Rubisco leads to an enhancement of sink capacity for N uptake because Rubisco can be a large N pool in leaves. Similarly, in RBCS-antisense rice plants with decreased Rubisco content, total plant N content largely decreased. However, although
Rubisco content by elevated [CO2] led to greater changes in Rubisco contents in both RBCS-sense and antisense plants grown at elevated [CO2] (Figs. 4A, 5). Thus, changes in total leaf N content by [CO2] led to greater changes in Rubisco contents in both RBCS-sense and antisense plants. This means that Rubisco content in RBCS-transgenic rice plants is also determined by leaf N content as in wild-type rice plants.

We have previously suggested that N re-allocation from reduction of Rubisco content to RuBP regeneration related components is one of the strategies for an improvement of biomass at elevated CO2 levels (Makino et al. 2000). In the present study, it was demonstrated that overproduction of Rubisco was effective at lower CO2 levels. However, the overproduction of Rubisco led to a decline in N allocation to other N components (Suzuki et al. 2007, 2009). Leading to a partial deactivation of Rubisco in vivo (Makino and Sage 2007, Suzuki et al. 2009). The mechanism(s) of a partial deactivation of Rubisco is not known in RBCS-sense rice plants, but such deactivation of Rubisco frequently occurs to maintain the balance between the in vivo capacities of Rubisco and other processes limiting photosynthesis when the photosynthetic limitation shifts to other processes rather than Rubisco (Mott et al. 1984, Sage et al. 1988). Our metabolome analysis with RBCS-sense rice plants indicates that the levels of 3-phosphoglycerate, sedoheptulose 7-phosphate and some photorespiratory metabolites increased but that RuBP, ATP and ADP levels were not affected (Suzuki et al. 2012). From these results, it is considered that RuBP regeneration independent of ATP supply possibly limits photosynthesis. The candidates are some Calvin cycle and/or photorespiratory enzymes. Another possibility is that Rubisco activase limitsRubisco activation in RBCS-sense rice plants. Although several studies showed that Rubisco activase levels are in excess of that required for photosynthesis (Jiang et al. 1994, Mate et al. 1996, Yamori et al. 2012), Fukayama et al. (2012) recently observed a negative correlation between Rubisco and Rubisco activase contents in transgenic rice plants with increased or decreased Rubisco activase content. Their results suggest that Rubisco activase content decreases in RBCS-sense plants. Thus, it is possible that a limitation by Rubisco activase occurs in RBCS-sense plants.

**Figure 4** shows that low [CO2]-growth stimulated Rubisco overproduction. However, this response was attributed to responses related to an increase in total leaf N content in RBCS-sense plants grown at low [CO2] rather than direct responses to low [CO2]-growth, because the ratio of Rubisco to total leaf N in RBCS-sense plants increased with increasing leaf N content irrespective of growth [CO2] (Fig. 5). We previously found that when total leaf N content increases, Rubisco content always increases relative to other photosynthetic N components in many C3 species including rice (Makino et al. 1992 and 1994). On the other hand, elevated [CO2] growth frequently reduced Rubisco content, and this decrease in Rubisco content by elevated [CO2] was always greater than that of other photosynthetic N components (for a review, see Makino and Mae 1999). However, these phenomena were also simply explained by a decrease in total leaf N content by CO2 enrichment in rice (Nakano et al. 1997) and wheat (Theobald et al. 1998). For RBCS-antisense plants, elevated [CO2] increased Rubisco content. However, this was also explained by an increase in total leaf N content in RBCS-antisense plants grown at elevated [CO2] (Figs. 4A, 5). Thus, changes in total leaf N content by [CO2] led to greater changes in Rubisco contents in both RBCS-sense and antisense plants. This means that Rubisco content in RBCS-transgenic rice plants is also determined by leaf N content as in wild-type rice plants.

RBCS-sense plants grown at elevated [CO2] had still greater Rubisco content (Fig. 4A), total plant N content at elevated [CO2] did not differ between wild type and RBCS-sense rice plants (Fig. 1B). The reason for this discrepancy in N demand in RBCS-sense plants between low and elevated [CO2] is not known, but we previously found that CO2 enrichment frequently leads to a decline in N demand (Makino et al. 1997b). Meanwhile, RBCS-sense plants grown at normal [CO2] showed declined levels of many amino acids (Suzuki et al. 2012) and accumulation of carbohydrates in this study (Fig. 3A, B), which are typical phenomena for N deficiency. This change in C/N balance could possibly enhance N demand in RBCS-sense rice plants.

**Figure 5** Relationships between Rubisco content, the ratio of Rubisco N to total leaf N content and total leaf N content in the uppermost fully expanded leaves of RBCS-transgenic rice plants grown at 40 Pa CO2. (A) Rubisco content and (B) the ratio of Rubisco N to total leaf N content versus total leaf N content in leaves of wild-type (open symbols), RBCS-sense (blue symbols) and RBCS-antisense (red symbols) plants. Plants were grown at 40 Pa CO2 with three N concentrations of 0.5 (▲), 2.0 (●) and 8.0 (■) mM. For Rubisco content, Y = 0.0269X – 0.569; r2 = 0.95 (wild-type), Y = 0.0393X – 1.33; r2 = 0.99 (RBCS-sense), Y = 0.0170X – 0.776; r2 = 0.92 (RBCS-antisense). For Rubisco ratio to total leaf N content, Y = 0.0415X + 19.9; r2 = 0.50 (wild-type), Y = 0.0952X + 19.6; r2 = 0.93 (RBCS-sense), Y = 0.0762X + 2.69; r2 = 0.71 (RBCS-antisense). Orange symbols represent the data of three genotypes grown at different CO2 levels of 28 (▲), 40 (●) and 120 (■) Pa. These data are taken from Fig. 4A and 4C.
In conclusion, overproduction of Rubisco is effective for biomass production at low \([\text{CO}_2]\). Greater biomass in rice plants with overexpressed RBCS is always associated with an increase in the amount of N uptake. Since this increase in N uptake leads to a greater increase in Rubisco content as well as leaf N content, these factors have an additional advantage for growth at low \([\text{CO}_2]\). However, a partial deactivation of Rubisco occurs in rice plants with overproduced Rubisco (Makino and Sage 2007, Suzuki et al. 2009). This may be because overproduction of Rubisco disrupts the in vivo balance between RuBP carboxylation and RuBP regeneration or in vivo balance between Rubisco and Rubisco activase. If recovery from Rubisco deactivation in rice plants with overproduced Rubisco is successful, further photosynthetic improvement as well as biomass enhancement can be achieved by overproduction of Rubisco.

**Materials and Methods**

**Plant materials and growth conditions**

Rice (Oryza sativa L. cv. Notohikari) was transformed with OsRBCS2 cDNA in the sense orientation under the control of its own OsRBCS2 promoter, and transgenic lines with substantially increased Rubisco content were selected (Suzuki et al. 2007). From these varieties, T3 progenies of the line Sr-26-8 (with about 125% of wild-type Rubisco) were used here. Non-transformed rice (Oryza sativa L. cv. Notohikari) and transgenic rice transformed with OsRBCS2 cDNA in the antisense orientation (AS-S1 line with about 35% of wild-type Rubisco; Makino et al. 2000) were also used as controls.

Plants were grown hydroponically with continuous aeration in an environmentally controlled growth chamber (LPH-0.5P-SH; NK system, Osaka, Japan). The chamber was maintained with a 15 h photoperiod, 60% relative humidity and a photosynthetic photon flux density (PPFD) of 1000 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) at plant level during the daytime. Three \(\text{CO}_2\) treatments were imposed with strict \(\text{CO}_2\) regulation: low \([\text{CO}_2]\) (28 Pa), normal \([\text{CO}_2]\) (40 Pa) and elevated \([\text{CO}_2]\) (120 Pa). Seeds of three genotypes were soaked in tap water at 30 \(^\circ\)C for 2 d, and the seedlings were grown on a plastic net floating on tap water for 21 d. Four seedlings each were then transplanted to 12 3.5-L plastic pots containing nutrient solution (4 pots per genotype). The basal hydroponic solution was renewed once a 2 d, and the seedlings were grown on a plastic net floating on tap water for 21 d.

**Determinations of Rubisco, Chl and total leaf N contents**

Uppermost fully expanded leaves from 63- to 70-day-old plants were immediately homogenized with a pestle and in a chilled mortar in 50 mM Na-phosphate buffer (pH 7.0) containing 2 mM iodoacetic acid, 120 mM 2-mercaptoethanol and 5% (v/v) glycerol. Total leaf N was determined from the gel (Makino et al. 1994) using calibration curves made with Rubisco purified from rice leaves. The Chl content was determined by the method of Arnon (1949). The N content was determined with Nessler’s reagent after Kjeldahl digestion (Makino et al. 1994).

**Statistical analyses**

Data are presented as the mean ± S.E. Tukey-Kramer’s HSD test was performed with JMP (SAS Institute Inc., Cary, NC, USA).

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**References**


