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A Small Decrease in Rubisco Content by Individual Suppression of *RBCS* Genes Leads to Improvement of Photosynthesis and Greater Biomass Production in Rice Under Conditions of Elevated CO_2

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Rubisco limits photosynthesis at low CO_2 concentrations ($[CO_2]$), but does not limit it at elevated $[CO_2]$. This means that the amount of Rubisco is excessive for photosynthesis at elevated $[CO_2]$. Therefore, we examined whether a small decrease in Rubisco content by individual suppression of the *RBCS* multigene family leads to increases in photosynthesis and biomass production at elevated $[CO_2]$ in rice (*Oryza sativa* L.). Our previous studies indicated that the individual suppression of *RBCS* decreased Rubisco content in rice by 10–25%. Three lines of BC_2F_2 progeny were selected from transgenic plants with individual suppression of *OsRBCS2*, 3 and 5. Rubisco content in the selected lines was 71–90% that of wild-type plants. These three transgenic lines showed lower rates of CO_2 assimilation at low $[CO_2]$ (28 Pa) but higher rates of CO_2 assimilation at elevated $[CO_2]$ (120 Pa). Similarly, the biomass production and relative growth rate (RGR) of the two lines were also smaller at low $[CO_2]$ but greater than that of wild-type plants at elevated $[CO_2]$. This greater RGR was caused by the higher net assimilation rate (NAR). When the nitrogen use efficiency (NUE) for the NAR was estimated by dividing the NAR by whole-plant leaf N content, the NUE for NAR at elevated $[CO_2]$ was higher in these two lines. Thus, a small decrease in Rubisco content leads to improvements of photosynthesis and greater biomass production in rice under conditions of elevated CO_2.

Keywords: Biomass • Elevated $[CO_2]$ • *Oryza sativa* • Photosynthesis • *RBCS* • Rubisco.

Abbreviations: LAR, leaf area ratio; NAR, net assimilation rate; NUE, nitrogen use efficiency; PPFD, photosynthetic photon flux density; RGR, relative growth rate; RNAi, RNA interference; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the first steps of the Calvin–Benson cycle in photosynthesis and the C_2 oxidative photosynthetic cycle in photorespiration. These two reactions are often rate-limiting steps in light-saturated net photosynthesis. In addition, Rubisco accounts for 15–30% of total leaf nitrogen (N) content in C_3 species (Evans 1989, Makino et al. 1992, Evans and Poorter 2001). Thus, Rubisco plays a central role in photosynthesis as well as in the N economy of a plant. However, the activity and regulation of Rubisco are not always optimal for photosynthesis and biomass production in various environments (Parry et al. 2013, Carmo-Silva et al. 2015). For example, under elevated $[CO_2]$ conditions, the photosynthetic rate is limited by either electron transport capacity (Farquhar et al. 1980) or the regeneration of Pi during starch and sucrose synthesis (Sharkey 1985, Sage 1990), and Rubisco protein is an excessive N component for photosynthesis (Makino et al. 1997). Therefore, to improve nitrogen use efficiency (NUE) for CO_2-enriched photosynthesis, it is necessary to optimize Rubisco content at elevated $[CO_2]$.

We previously produced a series of transgenic rice with decreased Rubisco content by transformation with rice *RBCS* antisense cDNA and selected a plant with optimal Rubisco content for CO_2-saturated photosynthesis (Makino et al. 1997). Transgenic rice with 65% wild-type Rubisco content as an 'ideal' line at CO_2-saturated photosynthesis showed 20% lower rates of photosynthesis at 36 Pa CO_2, but 5–15% higher rates at 100 Pa CO_2. In this selected line, non-specific reallocation of N from decreased Rubisco to other components limiting photosynthesis occurred, and consequently N may have been optimally distributed between Rubisco and other components limiting photosynthesis. However, biomass production of the selected line was not greater than that of the wild-type lines, even under elevated $[CO_2]$ conditions (Makino et al. 2000). This was due to the relatively strong Rubisco antisense effect during the seedling stages. Although the biological reason is still not known, the antisense suppression of *RBCS* was not stable and gradually decreased with plant growth. Therefore, we introduced another gene suppression technique to control the amount of Rubisco during the life span of the plant. We obtained transgenic rice plants with decreased Rubisco by the RNA interference (RNAi) approach. Rice has five *RBCS* genes, and four out of these five genes (*OsRBCS2*–*OsRBCS5*)

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have been found to be actively expressed in leaf blades (Suzuki et al. 2009). We successfully suppressed individual *RBCS* genes by RNAi using the 3′-untranslated region as a trigger sequence because homology is low. Rubisco content in each *RBCS* suppression line decreased to 75–90% of the wild-type levels, irrespective of growth stages from the seedling stages to the reproductive stages (Ogawa et al. 2012). Although Rubisco content in these lines was slightly higher than the optimal content at elevated [CO_2], which we had estimated before (Makino et al. 1997), we expected that these transgenic lines might have higher NUE under the conditions of elevated [CO_2].

In the present study, we obtained BC_2F_2 progeny of three lines of *RBCS*-RNAi rice plants, and first examined photosynthesis at different [CO_2] in these transgenic lines by transformation with individual *RBCS*-RNAi grown at normal [CO_2] conditions. Secondly, we investigated the whole-plant growth of these progeny at different [CO_2] environments. Thirdly, photosynthetic characteristics were examined at the single-leaf level, and then biomass production and its NUE at the whole-plant level were analyzed under low and elevated [CO_2] conditions.

Results

In the present study, BC_2F_2 progeny of transgenic lines with individual suppression of *RBCS* by RNAi (*RBCS2*-RNAi, *RBC3*-RNAi and *RBCS5*-RNAi lines) were used. Since some growth suppression, which may have been caused by somaclonal variations during plant transformation, was observed for T_1 progeny, the selected homozygous T_2 line was backcrossed twice with wild-type lines and then the BC_2F_2 generation was obtained by self-fertilization of the BC_2 generation. During these processes, the BC_2 generation of the *RBCS4*-RNAi lines became sterile. The null lines were also obtained from the same BC_2F_2 generation

Rubisco content and its ratio to total leaf N content decreased to 70% of the wild-type level in the *RBCS2*-RNAi and *RBC3*-RNAi lines and to 90% of the wild-type level in the *RBCS5*-RNAi line (**Fig. 1A, B**), whereas total leaf N contents did not differ among all genotypes (**Fig. 1C**). These suppression levels were similar to those observed for T_2 progeny in our previous studies (Ogawa et al. 2012), and co-ordination between *RbcL* and *RBCS* expression was also observed in these individual *RBCS*-RNAi lines (data not shown). This means that RNAi suppression of individual *RBCS* remained stable in the BC_2F_2 generation. On the other hand, Chl content tended to increase slightly in the *RBCS*-RNAi lines (**Fig. 1D**). Similarly, insoluble N content and soluble protein other than Rubisco also tended to increase slightly in the *RBCS2*-RNAi and *RBCS3*-RNAi lines (**Supplementary Fig. S1**).

We measured the rates of CO_2 assimilation at three different CO_2 partial pressures of 28, 40 and 120 Pa in these individual *RBCS* suppression lines grown at 40 Pa CO_2 (**Fig. 2**). While the rates of CO_2 assimilation at 28 and 40 Pa in the individual *RBCS* suppression lines were lower than those of the wild-type and null lines, the rates at 120 Pa were significantly higher in the individual *RBCS* suppression lines. Typical examples of the response of CO_2 assimilation to intercellular CO_2 partial pressures (A–C_i curve) are shown in **Fig. 3**. Whereas the initial slope was higher in the wild-type and null lines, the rates at elevated [CO_2] were higher in the individual *RBCS* suppression lines. These results indicate that a small decrease in Rubisco content leads to an increase in photosynthesis at the single-leaf level at elevated [CO_2]. Similar results were also observed for *RBCS* antisense rice with 65% wild-type Rubisco in our previous study (Makino et al. 1997).

We next examined the whole-plant biomass including roots in these individual *RBCS* suppression lines grown at three different CO_2 partial pressures of 28, 40 and 120 Pa (**Fig. 4**). The biomass at 28 Pa CO_2 of the *RBCS3*-RNAi and *RBC5*-RNAi lines was clearly smaller than that of the wild-type and null lines (**Fig. 4A**), but was significantly greater at 120 Pa CO_2 (**Fig. 4C**). For the *RBCS2*-RNAi line, a similar trend was also found although the difference was not significant. For the *RBCS2*-RNAi and *RBCS3*-RNAi lines, the biomass was not different at 40 Pa CO_2.

Growth analysis was done between 21 and 84 d after germination (**Fig. 5**). Although the relative growth rate (RGR), which had been calculated as total dry weight increment per dry weight per day, did not differ among genotypes at 28 Pa CO_2 (**Fig. 5A**), the RGR was significantly higher at 120 Pa CO_2 in three *RBCS*-RNAi lines than in the wild-type and null lines (**Fig. 5D**). Among them, this higher RGR in the *RBCS3*-RNAi and *RBC5*-RNAi lines was caused by a higher net assimilation rate (NAR) (**Fig. 5E**), but not by the leaf area ratio (LAR) (**Fig. 5F**). A similar trend was also found for the *RBCS2*-RNAi line, although the difference was not significant. Thus, these results indicate that a small decrease in Rubisco content by suppression of individual *RBCS* is significantly effective for biomass improvement by enhanced NAR at elevated [CO_2].

Starch content was examined in leaf blades and sheaths in all genotypes at 84 d after germination (**Fig. 6**). For growth at 28 Pa CO_2, while a decrease in starch content was observed in the leaf sheath in the *RBCS3*-RNAi and *RBCS5*-RNAi lines (**Fig. 6B**), an increase in starch content was found in the leaf blades in the same RNAi lines at 120 Pa CO_2 (**Fig. 6C**). This increase in starch content may have been reflected by a significant enhancement of photosynthesis in the *RBCS3*-RNAi and *RBCS5*-RNAi lines grown at 120 Pa CO_2.

Whole-plant N content was slightly lower in the *RBCS3*-RNAi and *RBC5*-RNAi lines grown at 28 Pa CO_2 (**Fig. 7A**), but there was no difference at 120 Pa CO_2 among all genotypes (**Fig. 7C**). The ratio of N allocation into leaf blades to that into whole plants was higher in the 28 Pa CO_2 treatments than in the 12 Pa CO_2 treatments, but there were no differences among all genotypes when the plants were grown at the same CO_2 partial pressure (**Supplementary Table S1**). NUE was next analyzed for NAR. The NUE for NAR was estimated by dividing the NAR by the average leaf N content between 21 and 84 d (**Fig. 5**). Although NUE for NAR was lower at 28 Pa CO_2 in the *RBCS3*-RNAi and *RBCS5*-RNAi lines (**Fig. 7B**), it was significantly higher at 120 Pa CO_2 in the same RNAi lines than in the wild-type and null lines (**Fig. 7D**). Since the average leaf N content was not different among genotypes at 120 Pa CO_2 (**Fig. 7C**;

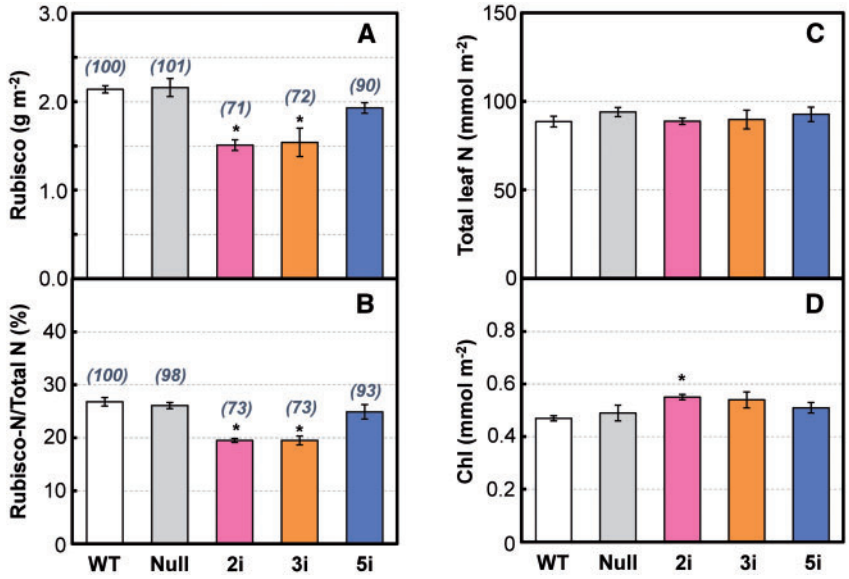

Fig. 1 Rubisco, total leaf N and Chl contents in leaves of the wild-type and transgenic rice plants grown at 40 Pa CO_2. Rubisco content (A), the ratio of Rubisco N to total leaf N content (B), total leaf N content (C) and Chl content (D) in uppermost, fully expanded leaf blades of the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) *and RBCS5* (5i), respectively. Values above each column are the ratio relative to the data obtained with the wild-type plants (A, B). The vertical bar on each column indicates the SE (n = 3–4). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

Supplementary Table S1), this increase in NUE was concluded to be mainly due to the enhancement of NAR (**Fig. 5E**). Since CO_2 assimilation rate per unit of N content at the level of a single leaf also tended to be higher at 120 Pa CO_2 (**Figs. 1C**, **2C**), higher NUE for biomass in the individual *RBCS*-RNAi lines may have been accompanied by greater NUE for CO_2 assimilation at elevated [CO_2].

Discussion

Our results clearly indicate that a 10–20% decrease in Rubisco content by individual suppression of the *RBCS* multigene family in rice leads to photosynthetic improvements and greater biomass production at elevated [CO_2]. In addition, when NUE for ther NAR at the whole-plant level was estimated at elevated [CO_2], this parameter was also higher in two lines of individual *RBCS*-RNAi lines. We have previously reported that *RBCS* antisense rice with 65% wild-type Rubisco content as an 'ideal' line at CO_2-saturated photosynthesis showed 5–15% higher rates of photosynthesis at elevated [CO_2] for the same N content (Makino et al. 1997). In this *RBCS* antisense line, non-specific reallocation of N from decreased Rubisco to other components limiting photosynthesis occurred at elevated [CO_2] and, consequently, N was optimally distributed between Rubisco and other components limiting photosynthesis. For the individual *RBCS*-RNAi lines, similar phenomena, in addition to Rubisco, may have tended to increase slightly (**Fig. 1D**; **Supplementary Fig. S1**). Therefore, whereas Rubisco content in the individual *RBCS*-RNAi lines was slightly higher than the optimal content which had been previously estimated at elevated [CO_2] (65% wild-type Rubisco level; Makino et al. 1997), the present lines could also have higher rates of photosynthesis at 120 Pa CO_2.

We did not measure CO_2 assimilation rate at each growth [CO_2] level from the plants grown at different [CO_2]. Although it is possible that plants easily acclimate to different growth CO_2 levels, our previous studies indicate that growth CO_2 levels do not affect N partitioning into key photosynthetic components including Rubisco in rice plants including *RBCS* transgenic rice (Nakano et al. 1997, Sudo et al. 2014). Furthermore, in the present study, since total leaf N content did not differ among genotypes grown at 120 Pa CO_2 (**Fig. 7C**; **Supplementary Table S1**), the photosynthetic characteristics in the plants grown at 40 Pa CO_2 should not have been different from those in the plants grown at 120 Pa CO_2.

For the individual *RBCS*-RNAi lines grown at 28 Pa CO_2, although whole-plant N content decreased (**Fig. 7A**), LAR was slightly increased (**Fig. 5C**). Such an increase in LAR was considered to be a compensation phenomenon associated with suppression of photosynthesis at low [CO_2]. A preferential N investment into leaf blades was found for all genotypes grown at 28 Pa CO_2 (**Supplementary Table S1**). However, such compensation effects were insufficient for whole-plant growth to recover fully (**Fig. 4A**). On the other hand, when these *RBCS*-RNAi lines were grown at elevated [CO_2], whole-plant N content increased to the same level as that of the wild-type plants (**Fig. 7C**), and the increase in LAR was not found (**Fig. 5F**). This means that a small decrease in Rubisco content by individual suppression of *RBCS* was adaptive for growth at elevated [CO_2]. Similar responses have also been observed between *RBCS* antisense and wild-type rice plants (Makino et al. 2000)

In our *RBCS* antisense rice, however, the final biomass did not exceed that of wild-type plants (Makino et al. 2000). The reason for this phenomenon was considered to be that the antisense effect was stronger than expected during the early stage of the plants, resulting in severe reduction of the initial

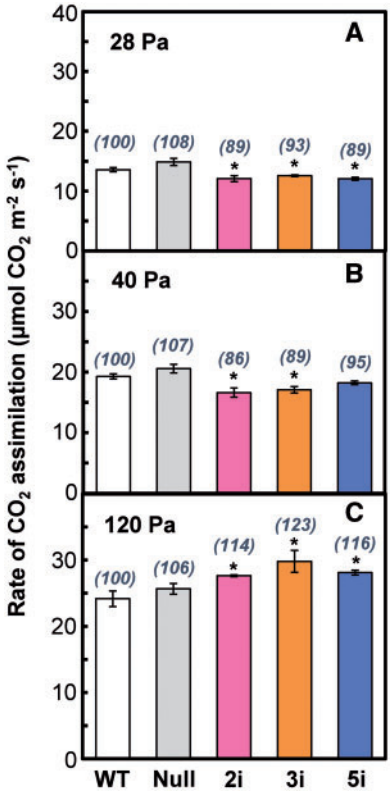

Fig. 2 Rate of CO_2 assimilation measured in leaves of the wild-type and transgenic rice plants grown at 40 Pa CO_2. Measurements were made at a leaf temperature of 25 °C, a PPFD of 1,500 μmol quanta m^{-2} s^{-1} and three different CO_2 partial pressures of 28 Pa (A), 40 Pa (B) and 120 Pa (C) in the uppermost, fully expanded leaf blades of the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) and *RBCS5* (5i), respectively. Values above each column are the ratio relative to the data obtained with the wild-type plants. The vertical bar on each column indicates the SE (n = 3–4). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

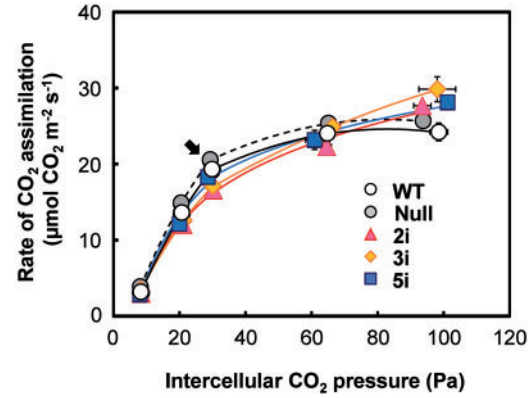

Fig. 3 Rate of CO_2 assimilation as a function of the intercellular CO_2 partial pressure in leaves of the wild-type and transgenic rice plants grown at 40 Pa CO_2. Measurements were made at a leaf temperature of 25 °C and a PPFD of 1,500 μmol quanta m^{-2} s^{-1} in the uppermost, fully expanded leaf blades of the wild-type (open circle), null (gray circle) and transgenic rice plants with individual suppression of *RBCS2* (triangle), *RBCS3* (diamond) and *RBCS5* (square), respectively. The arrow indicates the points obtained at an external [CO_2] of 40 Pa. Data are presented as means (n = 3–4).

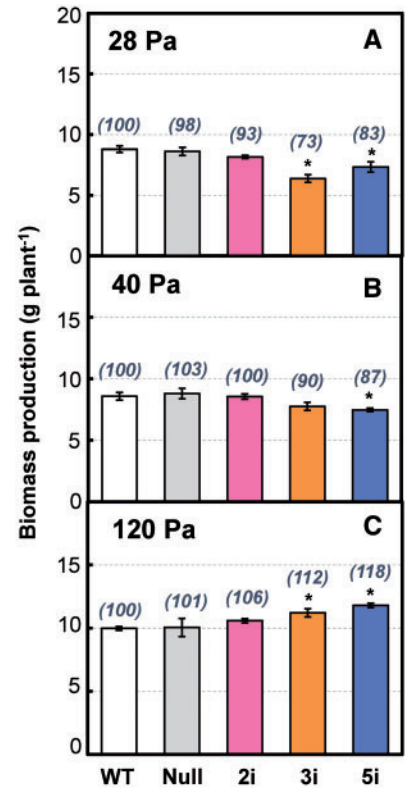

Fig. 4 Biomass production in the wild-type and transgenic rice plants at final harvest (84 d after germination). Total biomass production including roots in the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) and *RBCS5* (5i) grown at three different CO_2 partial pressures of 28 Pa (A), 40 Pa (B) and 120 Pa (C). Values above each column are the ratio relative to the data obtained with the wild-type plants. The vertical bar on each column indicates the SE (n = 3–4). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

biomass. On the other hand, such suppression effects depending on growth stages were not observed for the individual *RBCS*-RNAi lines (Ogawa et al. 2012). Rubisco content in each *RBCS*-RNAi line remained at 75–90% of the wild-type levels irrespective of growth stages from seedling to reproductive stages. Therefore, in the present study, a small decrease in Rubisco content by individual suppression of the *RBCS* multigene family successfully led to greater biomass production at elevated [CO_2].

We previously found that suppression of one *RBCS* gene by RNAi did not affect the transcript levels of other *RBCS* genes (Ogawa et al. 2012). Similarly, overexpression of one *RBCS* gene also did not affect the transcript levels of other *RBCS* genes (Suzuki et al. 2007). These results indicate that expression of each *RBCS* gene is independently controlled and does not affect the expression of other *RBCS* genes. In addition, four out of five *RBCS* genes are used for Rubisco accumulation in leaf blades, and these four *RBCS* genes encode all identical mature RBCS protein (Suzuki et al. 2009). Therefore, the individual suppression of *RBCS* in rice could be useful for photosynthetic

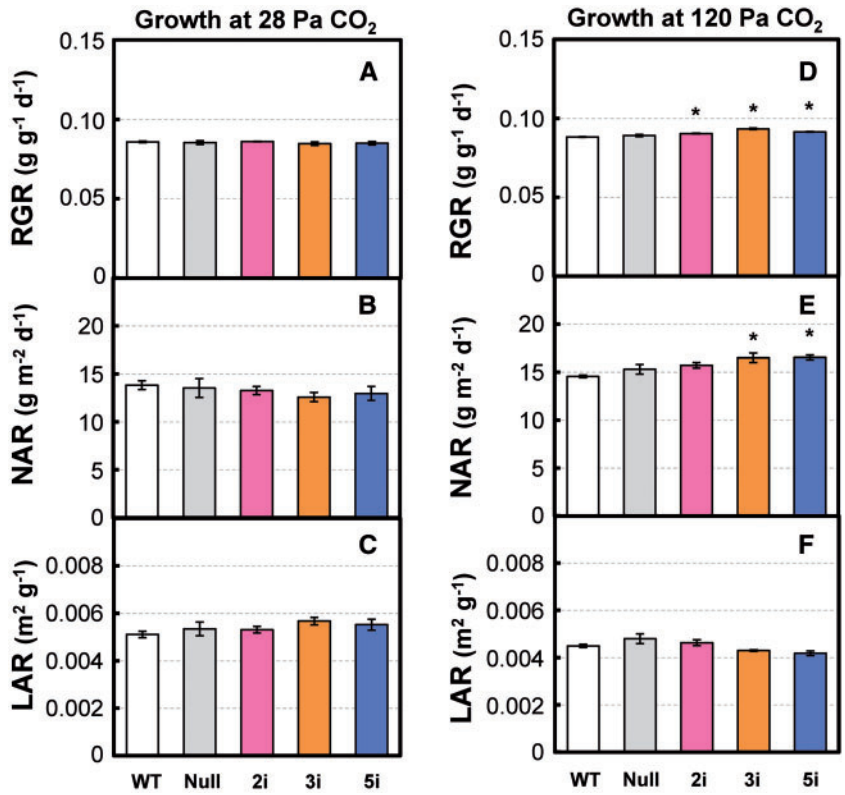

Fig. 5 Growth analysis between 21 and 84 d after germination in the wild-type and transgenic rice plants. RGR (A, D), NAR (B, E) and LAR (C, F) were estimated in the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) and *RBCS5* (5i) grown at 28 Pa CO_2 (left panel) and 120 Pa CO_2 (right panel). The vertical bar on each column indicates the SE (n = 4–6). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

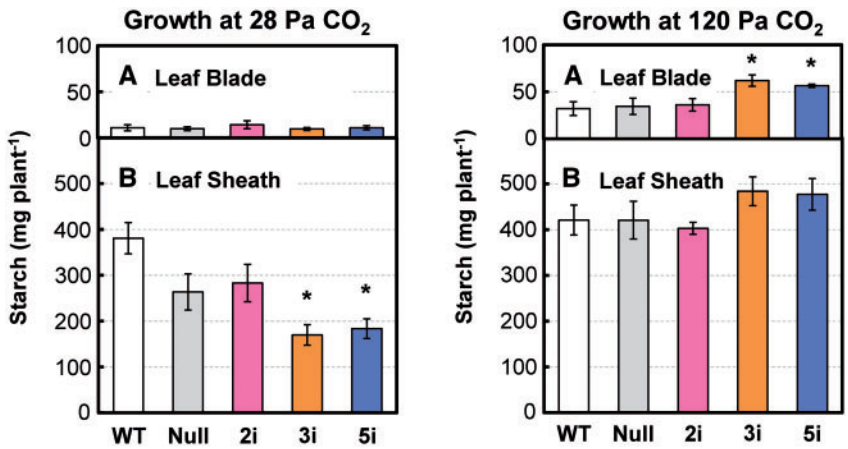

Fig. 6 Starch content in the leaf blades and sheaths of the wild-type and transgenic rice plants at final harvest (84 d after germination). Starch content was determined in the leaf blades (A, C) and leaf sheaths (B, D) of the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) and *RBCS5* (5i) at 28 Pa CO_2 (left panel) and 120 Pa CO_2 (right panel). Plants were harvested in the middle of the day (11:00 and 15:00 h). The vertical bar on each column indicates the SE (n = 4). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

engineering to address elevated [CO_2]. However, it is not known whether this RNAi suppression targeting to each *RBCS* gene can be generally applied to optimize Rubisco content at elevated [CO_2] in other crops and plants. There may be species-dependent differences in how members of the *RBCS* multigene family determine Rubisco accumulation. In iceplants (DeRochrer and Bohnert 1993), French beans (Sawabridge et al. 1996) and *Eucalyptus* plants (Suzuki et al. 2010), the transcript levels of one *RBCS* gene are predominant. Wheat has >22 *RBCS* genes, and one subfamily containing two major *RBCS* genes accounts for most of the total *RBCS* transcripts (Galili et al. 1998). These reports suggest that a limited number of

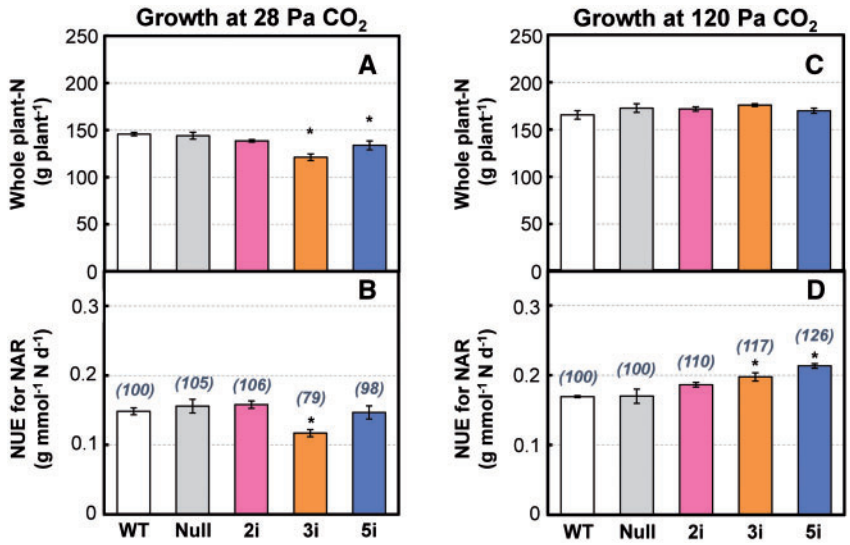

Fig. 7 Whole-plant N and N-use efficiency (NUE) for net assimilation rate at the whole-plant level (NAR) in the wild-type and transgenic rice plants. Whole-plant N including roots in the plants grown at 28 Pa CO_2 (A) and 120 Pa CO_2 (C). NUE for NAR (B, D) was estimated by dividing NAR (**Fig. 4**) by the average leaf N content in all leaf blades between 21 and 84 d in the wild-type (WT), null (Null) and transgenic rice plants with individual suppression of *RBCS2* (2i), *RBCS3* (3i) and *RBCS5* (5i) grown at 28 Pa CO_2 (B) and 120 Pa CO_2 (D). Values above each column are the ratio relative to the data obtained with the wild-type plants (B, D). The vertical bar on each column indicates the SE (n = 3–4). The asterisks show statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$).

RBCS genes determine the accumulation of Rubisco protein. Additionally, in Arabidopsis (Cheng et al. 1998, Yoon et al. 2001, Izumi et al. 2012) and coffee plants (Marraccini et al. 2011), expression of each member of the *RBCS* multigene family is differently regulated and varies depending on the growth conditions. Thus, the approach using RNAi suppression targeting to individual *RBCS* genes is possibly useful only for limited plant species to optimize Rubisco content for projected future environments.

Conclusion

In the present study, we demonstrated that a small decrease in Rubisco content is a useful strategy for photosynthesis improvements at elevated [CO_2] and leads to greater biomass production under conditions of elevated [CO_2]. We showed that the RNAi approach to individual members of the *RBCS* multigene family may also be available for a small decrease in Rubisco content in rice during the life span of the plant. Since large amounts of N are invested in Rubisco, a small decrease in N allocation to Rubisco could lead to higher NUE for photosynthesis and biomass production. Presently, high yields of rice strongly depend on N application. On the other hand, the application of high levels of N fertilizer has a negative environmental impact (Cassman et al. 1998, Cassman et al. 2003). Therefore, improvements in NUE are very important for reduction of the negative environmental impact of N application in agriculture. The results of our attempt indicate that our transgenic rice can be one of the model crops which perform better under low N input conditions in high CO_2 environments expected in the near future.

Recent targeted genome editing using artificial nucleases such as the clustered regularly interspersed short palindromic repeats (CRISPR)/Cas system is a powerful tool for developing valuable new traits in plants (Woo et al. 2015, Ma et al. 2016). These technologies could also be useful for individual knockout in the *RBCS* multigene family. Based on such technologies, further photosynthetic improvement and biomass enhancement by more strictly controlling Rubisco content in response to future environmental changes will hopefully be achieved.

Materials and Methods

Plant materials

BC_2F_2 progeny of transgenic plants with suppression of *OsRBCS2*, 3, 5 and the null lines from the same BC_2F_2 generation, and wild-type rice plants (*Oryza sativa* L. cv. Notohikari) were used in this study. T_1 progeny of transgenic plants with decreases in Rubisco contents (Ogawa et al. (2012) were allowed to self-fertilize. T_2 progeny were backcrossed twice with wild-type plants to obtain a BC_2 generation. The resulting BC_2F_2 generation and null lines were obtained by self-fertilization of the BC_2 generation.

Plant culture

All plants were grown hydroponically in an isolated and environmentally controlled growth chamber as described in Sudo et al. (2014) with slight modifications. The chamber was controlled with a 14 h photoperiod, a 27/22°C day/night temperature, 60% relative humidity and a photosynthetic photon flux density (PPFD) of 1,000 µmol quanta m^{-2} s^{-1} during the daytime. Samples grown under 40 Pa [CO_2] were used for the measurement of leaf photosynthesis. To analyze whole-plant growth and biomass production, plants were grown at 28 Pa [CO_2] and 120 Pa [CO_2], respectively. The seedlings were grown on a plastic net with tap water (pH 5.5) during the first 21 d and were then individually transplanted to 1.0 liter plastic pots containing a nutrient solution. Before transplantation, the introduction of an *RBCS*-RNAi construct was checked according to the method of Prior et al. (2006) as described in Ogawa et al. (2012).

The basal nutrient solution was as previously described by Makino et al. (1994). The solution was renewed once a week, and its concentration was increased depending on the plant growth as follows: one-third, between 21

and 35 d after germination; half, between 35 and 42 d after germination; two-thirds, between 42 and 49 d after germination; full, between 49 and 84 d after germination.

Measurement of photosynthesis

Rates of photosynthetic CO_2 assimilation were determined with a portable gas exchange system (Li-6400XT, Li-COR). Measurements were done on the 10th or 11th leaves of the main stems just after full expansion at a leaf temperature of 25°C, a PPFD of 1,500 µmol quanta m^{-2} s^{-1} and a leaf-to-air vapor pressure difference of 1.0–1.2 Pa. Measurement was first done at an ambient [CO_2] (pCa) of 40 Pa to reach the steady state of the CO_2 exchange rate. The CO_2 partial pressure was then lowered to obtain the rates at a pCa of 28 Pa. Finally, the CO_2 partial pressure was raised to a pCa of 120 Pa. After measurement of photosynthesis, fresh weight and leaf area were measured, and the leaves were immediately frozen in liquid nitrogen and stored at − 80°C until analysis.

Determination of Rubisco, Chl, total N and leaf N distribution

Frozen leaves were immediately homogenized with a pestle in a chilled mortar in 50 mM Na-phosphate buffer (pH 7.5). Total N, soluble protein N, insoluble N and trichloroacetic acid (TCA)-soluble N were determined with Nessler's reagent after Kjeldahl digestion according to Makino et al. (1994). The amount of Rubisco was determined spectrophotometrically by formamide extraction of the Coomassie Brilliant Blue R-250-stained subunit bands from the gel, using calibration curves made with Rubisco purified from leaf blades of rice. Chl content was assessed by the method of Arnon (1949).

Growth analysis and determination of N and starch

Four plants were sampled per each [CO_2] treatment between 11:00 and 15:00 h, at the 21st and 84th day after germination. After measurements of leaf blades, leaf sheaths and roots were oven-dried separately at 80°C for at least 1 week. Stems had not developed at day 84. RGR, LAR and NAR were calculated from total dry weight and leaf area, respectively (Tazoe et al. 2016).

Total N content of dried samples was determined as previously described by Makino et al. (1994). Starch content was measured as previously described by Nakano et al. (1997). Starch and soluble sugar fractions were separated by 80% ethanol at 80°C. Starch content in the ethanol-insoluble fraction was determined using an F-kit for starch (Wako). NUE for growth rate was estimated by dividing the NAR by total leaf N content per total leaf area of the whole plant, as described by Nagai and Makino (2009).

Statistical analysis

Data are presented as the mean ± SE. Dunnett's test was performed with JMP.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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