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Does Decrease in Ribulose-1,5-Bisphosphate Carboxylase by Antisense *RbcS* Lead to a Higher N-Use Efficiency of Photosynthesis under Conditions of Saturating CO_2 and Light in Rice Plants?[1]

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Rice (*Oryza sativa* L.) plants with decreased ribulose-1,5-bisphosphate carboxylase (Rubisco) were obtained by transformation with the rice *rbcS* antisense gene under the control of the rice *rbcS* promoter. The primary transformants were screened for the Rubisco to leaf N ratio, and the transformant with 65% wild-type Rubisco was selected as a plant set with optimal Rubisco content at saturating CO_2 partial pressures for photosynthesis under conditions of high irradiance and 25°C. This optimal Rubisco content was estimated from the amounts and kinetic constants of Rubisco and the gas-exchange data. The R_1 selfed progeny of the selected transformant were grown hydroponically with different N concentrations. Rubisco content in the R_1 population was distributed into two groups: 56 plants had about 65% wild-type Rubisco, whereas 23 plants were very similar to the wild type. Although the plants with decreased Rubisco showed 20% lower rates of light-saturated photosynthesis in normal air (36 Pa CO_2), they had 5 to 15% higher rates of photosynthesis in elevated partial pressures of CO_2 (100–115 Pa CO_2) than the wild-type plants for a given leaf N content. We conclude that the rice plants with 65% wild-type Rubisco show a higher N-use efficiency of photosynthesis under conditions of saturating CO_2 and high irradiance.

Rubisco is both the key enzyme of photosynthesis and the most abundant leaf protein. This enzyme catalyzes two competing reactions: CO_2 fixation in photosynthesis and the production of 2-phosphoglycolate in the photorespiratory pathway. Rubisco has a low rate of catalysis and is a rate-limiting factor for the maximum rate of photosynthesis at present atmospheric CO_2 pressures (Makino et al., 1985; Evans, 1986). Therefore, a great deal of leaf N is invested in Rubisco protein, and its amount accounts for 15 to 35% of total leaf N in C_3 species (Evans, 1989; Makino et al., 1992).

However, in spite of these important functions of Rubisco in plants, it does not always limit photosynthesis. For example, at elevated CO_2 or moderately low temperature, photosynthesis is limited by either electron transport capacity (Berry and Björkman, 1980; von Caemmerer and Farquhar, 1981) or the availability of Pi in the chloroplast for ATP synthesis (Sharkey, 1985; Sharkey et al., 1986; Labate and Leegood, 1988), and Rubisco is substantially deactivated (Sage et al., 1988). Similarly, at low irradiance, light-harvesting and electron transport capacities limit photosynthesis, and Rubisco is also down-regulated (Mott et al., 1984; Terashima and Evans, 1988; Sage et al., 1990). These findings indicate that the amount of Rubisco is clearly excessive under such environmental conditions. In addition, this amount of Rubisco is not necessarily optimized, even if plants become adapted to environments such as those with elevated CO_2 (Makino, 1994; Sage et al., 1995; Medlyn, 1996), low temperature (Makino et al., 1994b), or low irradiance (Lauerer et al., 1993). Therefore, for plants to have a higher efficiency of N use under conditions of "excess" Rubisco, it seems necessary to reduce N investment in Rubisco.

Genetic engineering using antisense technology has provided model plants with decreased Rubisco protein. Rodermel et al. (1988) used tobacco (*Nicotiana tabacum*) plants and first demonstrated that the amount of Rubisco could be reduced by transformation with an antisense gene to *rbcS*. Subsequently, Hudson et al. (1992) produced tobacco plants with decreased Rubisco according to the procedure of Rodermel et al. (1988). In addition, this antisense technique has been applied to the C_4 plant *Flaveria bidentis* (Furbank et al., 1996). These transgenic plants with reduced

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Abbreviations: CF_1, coupling factor 1; Chl, chlorophyll; *pCa*, ambient CO_2 partial pressure; *pCc*, chloroplastic CO_2 partial pressure; *pCi*, intercellular CO_2 partial pressure.

Rubisco have provided a new experimental system to evaluate the contribution of Rubisco itself to the control of leaf photosynthesis and plant growth (for reviews, see Stitt and Schulze [1994]; Andrews et al., 1995; Furbank and Taylor, 1995), but seldom have they been used to investigate the N economy of photosynthesis (Lauerer et al., 1993). In addition, such antisense transformation has not been undertaken for a major crop.

The purpose of this study was to examine whether a decrease in Rubisco content by antisense *rbc*S in rice plants leads to a higher N-use efficiency of photosynthesis under conditions of CO_2 enrichment. The global, atmospheric CO_2 concentration is rapidly increasing and is expected to double in the next 50 to 100 years; present-day levels of Rubisco will be excessive for such future environments. Our final aim is to construct rice plants that will perform better with low-N investment in elevated CO_2 atmospheres of the next century. In this study we first selected a transformant with optimal levels of Rubisco for high-CO_2 environments and examined the photosynthetic characteristics of the R_1 progeny of the selected primary transformant. We then analyzed N-use efficiency of photosynthesis under conditions of elevated CO_2.

MATERIALS AND METHODS

Construction of the *rbc*S Antisense Gene

The plasmid containing the *rbc*S antisense gene (pRAS) was constructed with the rice *rbc*S promoter, the rice *rbc*S cDNA, and the *nos* gene tail. A 2.8-kb fragment of the rice *rbc*S promoter excised at the *Hin*dIII and *Sal*I sites from the plasmid pRGN73 (Kyozuka et al., 1993) was cloned into the *Hin*dIII and *Sal*I sites of pUC119, of which the *nos* gene tail was previously inserted into the *Sac*I and *Eco*RI sites. The cDNA fragment for the rice Rubisco small subunit of 819 bp with 53-bp 5′ and 237-bp 3′ leader sequences was amplified by PCR in the presence of appropriate primers and pOsSSU1139 plasmid as a template (Matsuoka et al., 1988), and the PCR product was cloned into the plasmid pBluescript T-vector (Ichihara and Kurosawa, 1993). After the confirmation of nucleotide sequence and orientation, the *rbc*S cDNA fragment was excised at the *Xba*I and *Sal*I sites and inserted in the antisense orientation, with respect to the rice *rbc*S promoter, to create the plasmid pRAS.

Plant Transformation and Regeneration

The plasmid pDM302, containing the bacterial phosphinothricin acetyltransferase structural gene (*bar*), was used as the selectable marker in rice transformation by conferring resistance to a phosphinothricin-based herbicide. The *bar* gene was controlled by the rice actin 1 gene promoter and followed by the *nos* gene terminator. The plasmids pRAS and pDM302 were simultaneously introduced into mature embryos from rice (*Oryza sativa* L. cv Notohikari) seeds by bombardment (Shimada et al., 1995) with a Biolistic PDS-1000/He (Bio-Rad). The transformant cells were initially selected on Linsmaier-Skoog medium containing 15 μM bialaphos (commercial phosphinothricin-based herbicide, Meiji Seika, Tokyo, Japan) and 9 μM 2,4-D. After 9 to 11 weeks, surviving cell clusters were regenerated. The plantlets obtained were additionally selected with Linsmaier-Skoog medium containing 15 μM bialaphos at a PPFD of 100 μmol quanta m^{-2} s^{-1} (16-h photoperiod) and 26°C for 3 to 5 weeks and then transferred to a 3.5-L pot containing paddy soil in a greenhouse under natural sunlight conditions. About 90 bialaphos-resistant plants were obtained from 810 embryos, which had been bombarded.

The presence of the *rbc*S antisense DNA sequence in the genome of the resistant plants was verified by PCR. Total DNA was prepared from the bialaphos-resistant plants with the commercial DNA extraction reagent Isoplant (Nippon Gene, Tokyo, Japan) and used for PCR as a template. The PCR experiments were carried out according to the method of Sambrook et al. (1989). The two synthetic oligonucleotides used as the primers for PCR were 5′-GCGGGAGCCTGTGTGCAGAG and 5′-TGCATTCGTCCGTATCATCG, corresponding to the rice *rbc*S promoter sequence and the *rbc*S antisense cDNA sequence, respectively. The resulting 414-bp DNA fragment was detected in the genome of the transformed plants, whereas it was not observed in the genome of the untransformed plants. The plants identified by the presence of the *rbc*S antisense gene were then screened by SDS-PAGE for determination of Rubisco protein content (Makino and Osmond, 1991). The primary transformants were allowed to self-fertilize, and the R_1 seeds were collected.

Culture of the R_1 Antisense Plants

The R_1 seeds of the primary transformant with 65% wild-type Rubisco were used for this study. About 150 R_1 seeds and 15 wild-type seeds were germinated, and their seedlings were grown for 20 d on a Saran net (Asahikasei, Tokyo, Japan) floating on tap water adjusted to pH 5.5 in an air-conditioned greenhouse with a day/night temperature regime of 25/20°C under natural sunlight conditions. Each seedling was then transplanted to a 1-L plastic pot containing a nutrient solution in the same greenhouse. The basal nutrient solution was as previously described by Makino et al. (1988). After 2 to 3 weeks, 80 R_1 plants were screened by SDS-PAGE for Rubisco protein content, and then the antisense plants with decreased Rubisco and null (pseudo-wild-type) plants were transplanted separately to 3.5-L plastic pots (six plants per pot) containing the same nutrient solution in an environmentally controlled growth chamber. The chamber was operated with a 14-h photoperiod, 25/20°C day/night temperature, 60% RH, and a PPFD of 1000 μmol quanta m^{-2} s^{-1} at plant level during the daytime. Irradiance was provided by a combination of metal halide lamps (Toshiba, Yoko DF, Tokyo, Japan) and high-output fluorescent lamps (National FPR96 EX-N/A, Tokyo, Japan). After 1 week, plants were supplied with three N concentrations (mM): 0.5 (0.25 mM NH_4NO_3), 2.0 (1.0 mM NH_4NO_3), and 8.0 (2.5 mM NH_4NO_3 plus 3.0 mM $NaNO_3$). These solutions were renewed once a week and continuously aerated. Gas-exchange and biochemical assays (described below) were carried out on young, fully expanded leaves 3 to 4 weeks later. The plants used for the

assays were 85 to 92 d old. Wild-type plants were also grown hydroponically in the same growth chamber under the same conditions. The measurements were done on young, fully expanded leaves of the 70- to 80-d-old plants.

Gas-Exchange Measurements

Gas exchange was determined with an open gas-exchange system using a temperature-controlled chamber equipped with two fans. The system was previously detailed by Makino et al. (1988). Differences in the partial pressures of CO_2 and H_2O entering and exiting the chamber were measured with an IR gas analyzer (ASSA-1110, Horiba, Kyoto, Japan) and a dew point hygrometer (model 911, EG&G, Natick, MA), respectively. Measurements were made at a leaf temperature of 25°C, a PPFD of 350 or 1800 μmol quanta m^{-2} s^{-1}, and a leaf-to-air vapor pressure difference of 1.0 to 1.2 kPa. The first measurement was made at a *pCa* of 36 Pa to obtain steady-state readings of the photosynthetic rate and stomatal conductance, and then *pCa* was varied. The CO_2-saturated rate of photosynthesis was measured at a *pCi* of 80 to 90 Pa (corresponding to a *pCa* of 100–115 Pa). Gas-exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981).

Biochemical Assays

The amounts of Chl, total leaf N, Rubisco, Cyt *f*, and CF_1 were determined according to the method of Makino et al. (1994a). These determinations were made on the same leaf that had been used for the gas-exchange measurements. The leaf blade was homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 120 mM 2-mercaptoethanol, 2 mM iodoacetic acid, and 5% (v/v) glycerol. The total Chl and leaf N contents were measured from part of this homogenate. To solubilize membrane-bound Rubisco, a Triton X-100 solution at a final concentration of 0.1% (v/v) was added to a portion of the leaf homogenate (Makino and Osmond, 1991). After the sample was centrifuged the supernatant was treated with a lithium dodecyl sulfate solution (1.0% [w/v], final concentration) at 100°C for 90 s. The amount of Rubisco in this preparation was determined spectrophotometrically after formamide extraction of Coomassie brilliant blue R-250-stained subunit bands separated by SDS-PAGE. A calibration curve was made with Rubisco purified from rice leaves.

The remaining leaf homogenate was used for the determination of Cyt *f* and CF_1. The crude extracts were passed through cheesecloth and centrifuged at 2500*g* for 3 min. The precipitate was suspended in 100 mM Tris-HCl buffer (pH 8.6) and treated with a lithium dodecyl sulfate solution (2.0% [w/v], final concentration) at 100°C for 90 s. After the sample was centrifuged, Triton X-100 was added to the supernatant fraction to 12.5% (v/v), followed by storage at −35°C. The amounts of Cyt *f* and CF_1 in this preparation were determined by rocket immunoelectrophoresis, according to the method of Plumley and Schmidt (1983) with slight modification (Makino et al., 1994a). Polyclonal-monospecific antibodies against Cyt *f* and the α- and β-subunits of CF_1 were used according to the method of Hidema et al. (1991).

The nitrate pool was determined on oven-dried ground material of a subsample of each genotype. Free nitrate was extracted with 80% (v/v) ethanol at 80°C for 10 min. After evaporation of the ethanol, the extract was distilled in the presence of Devarda's alloy by the microdiffusion method of Conway (1950). Nitrate content was estimated by subtracting ammonium content in the presence of Devarda's alloy from the content in the absence of Devarda's alloy. The ammonium contents were measured with Nessler's reagent.

RESULTS

Selection of a Transformant with Higher N-Use Efficiency of Photosynthesis at Elevated CO_2

Rubisco efficiency for CO_2-saturated photosynthesis (the ratio of CO_2-saturated photosynthesis to the maximal Rubisco activity at pCc = 70 Pa) in wild-type plants was first estimated from the amounts and kinetic constants of Rubisco and the gas-exchange data. The purpose of this analysis was to elucidate how much Rubisco protein should be reduced to optimally distribute N between Rubisco and components limiting CO_2-saturated photosynthesis and to select an ideal antisense plant set for this study. Since the catalytic turnover rate of Rubisco measured in vitro is often lower than that predicted from gas-exchange measurements (von Caemmerer and Evans, 1991; Makino et al., 1994a; von Caemmerer et al., 1994), the kinetic constants of Rubisco, which had been corrected by the gas-exchange data (fig. 5B in Makino et al., 1994a), were used here.

Rubisco efficiency for CO_2-saturated photosynthesis was estimated to be from 70% down to 45% with increasing leaf N content (Fig. 1). This change in Rubisco efficiency is caused by a change in the balance between Rubisco and processes limiting CO_2-saturated photosynthesis with leaf N content; a relative increase in Rubisco content to electron transport components or Suc synthesis enzymes occurs with increasing leaf N content (Makino et al., 1994a). If N content of rice leaves grown at normal N levels is assumed to be about 120 mmol m^{-2}, it is necessary to reduce Rubisco to levels of about 55% to achieve 100% Rubisco efficiency. However, since N allocation from reduced Rubisco into other components limiting photosynthesis must be taken into consideration, the target level of Rubisco must be a little greater than 55%. In this study, therefore, we decided to select a transformant with 60 to 70% wild-type Rubisco as an ideal antisense plant set.

Rice was transformed with the rice *rbc*S antisense gene under the control of the rice *rbc*S promoter by bombardment. Twenty-three primary transformants were screened for the Rubisco to leaf N ratio versus leaf N content in young, fully expanded leaves. Since the amount of Rubisco changes greatly with leaf age and leaf N content, we did not screen for the absolute amount of Rubisco. For several transformants, the Rubisco to leaf N ratio was reduced by up to 30%, compared with wild-type levels (Fig. 2). We selected a transformant with 65% wild-type Rubisco (indicated by the arrow in Fig. 2), and the R_1 seeds were

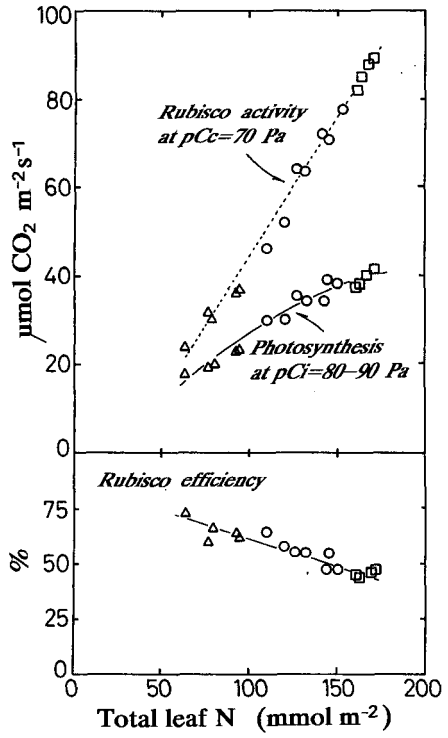

Figure 1. Rate of photosynthesis at pCi = 80 to 90 Pa, estimated Rubisco activity at pCc = 70 Pa, and Rubisco efficiency (the ratio of the photosynthetic rate at pCi = 80–90 Pa to the estimated Rubisco activity at pCc = 70 Pa), versus total N content in leaves of wild-type rice grown with 0.5 (△), 2.0 (○), and 8.0 (□) mM N concentrations. Photosynthesis was measured at a leaf temperature of 25°C, a PPFD of 1800 μmol quanta m^{-2} s^{-1}, a pCa of 100 to 115 Pa, and a leaf-to-air vapor difference of 1.0 to 1.2 kPa. Rubisco activity, v_c, was estimated from the Rubisco content and its kinetic constants at 25°C from rice (Makino et al., 1988) using the equation:

$$v_c = E\,V_c(pCc - 0.5\,V_o\,K_c/V_c\,K_o)/[pCc + K_c(1 + O/K_o)],$$

where E is the amount of Rubisco protein (μmol m^{-2}), V_c and V_o denote the maximum Rubisco activity of carboxylation and oxygenation, K_c and K_o are the Michaelis-Menten constants for CO_2 and O_2, pCc obtained at pCi = 80 to 90 Pa is assumed to be 70 Pa, and O is the partial pressure of O_2 in the chloroplast but assumed to be the same as in the atmosphere (21 kPa). The respective V_c and V_o values used here are 17.4 and 5.7 mol mol^{-1} Rubisco s^{-1}, which were multiplied by 1.12. The in vitro Rubisco activity needed to be 1.12-fold greater to account for the in vivo Rubisco-limited rate of photosynthesis (see fig. 5B in Makino et al., 1994a). K_c and K_o are 24 Pa and 28 kPa, respectively.

obtained. Table I shows the segregation of the Rubisco content of the R_1 population. Fifty-six plants had about 60% wild-type Rubisco and 23 plants were similar to the wild-type plants. Judging by an χ^2 test, this was not significantly different from a 3:1 ratio, corresponding to the expected Mendelian segregation. However, this segregation probably does not suggest that a single copy of the antisense gene is present in the selected primary transformant. Mendelian inheritance of transgenes in transformants produced by particle bombardment seems to be caused by a multicopy gene at a single locus (Register et al., 1994). Therefore, the population of the antisense plants with decreased Rubisco may not have segregated into two groups to the extent seen in tobacco plants with one copy of antisense *rbc*S (Quick et al., 1991; Masle et al., 1993). Rubisco content of the R_1 plants with decreased Rubisco was similar to that of the primary transformant. A slight difference between them may be caused by lower content of leaf N of the R_1 antisense plants, because this ratio also depends on leaf N content (Fig. 3). This R_1 population was used for this study.

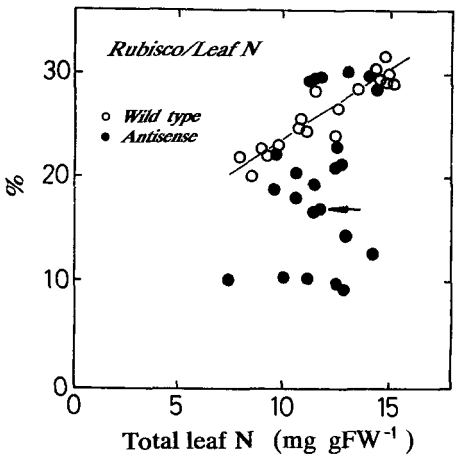

Figure 2. Ratio of Rubisco to total leaf N versus total N content in leaves from primary transformants (●) and wild-type rice (○). The arrow indicates the selected transformant. FW, Fresh weight.

Photosynthetic Characteristics at Normal CO_2 of the Selected Antisense Plants

Figure 3 shows the relationship between Rubisco content and leaf N content in leaves of three genotypes, i.e. wild-type, null (pseudo-wild-type), and antisense plants, grown with different N levels. A positive correlation between Rubisco and leaf N was also found for the antisense plants, and Rubisco content of the antisense plants was 65% of that in wild-type and null plants at the same leaf N content irrespective of N treatment. In addition, the increase in the ratio of Rubisco to leaf N with increasing leaf N was also observed in the antisense plants (Fig. 3, bottom). This response of Rubisco to leaf N content is commonly found in many C_3 species (Makino et al., 1992) and is considered to be required to maintain the co-limitation balance between Rubisco capacity and other processes limiting photosynthesis at normal atmospheric CO_2 pressures, because pCc is

Table I. *Segregation of the Rubisco to leaf N ratio of the R_1 progeny of the selected primary transformant*

Values are means ± SE.

Plant	No. of Plants	Rubisco/Leaf N		Leaf N	
		μmol mol⁻¹	*%*	*mmol g⁻¹ fresh wt*	*%*
Wild type	7	48 ± 2	100	1.02 ± 0.04	100
R_1 progeny	56	28 ± 4	58	0.93 ± 0.05	91
	23	47 ± 3	98	1.01 ± 0.09	99

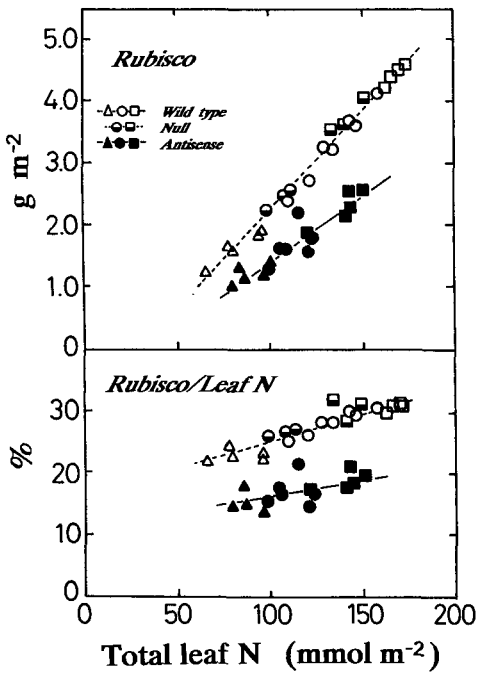

Figure 3. Rubisco content (top) and its ratio to total leaf N (bottom) versus total leaf N content. Wild-type plants are represented by open symbols, null plants (pseudo-wild-type) are represented by half-shaded symbols, and antisense plants are represented by closed symbols. The respective plants were grown hydroponically at a PPFD of 1000 μmol quanta m^{-2} s^{-1}, a day/night temperature of 25/20°C, and N concentrations of 0.5 (triangle), 2.0 (circle), and 8.0 (square) mM. $Y = 0.0334\ X - 1.07$, $r^2 = 0.99$ (wild-type and null plants); $Y = 0.0218\ X - 0.72$, $r^2 = 0.88$ (antisense plants)

reduced with increasing Rubisco by a CO_2-transfer resistance (for detailed discussion, see Evans and Terashima [1988]). Although this co-limitation balance was largely broken in the antisense plants, this response of Rubisco to leaf N was still conserved.

We next examined gas-exchange characteristics at normal CO_2 pressures in the antisense plants. The light-saturated rate of photosynthesis (at 1800 μmol quanta m^{-2} s^{-1}) was about 20% lower in the antisense plants than in the wild-type and null plants (Fig. 4). Although this decrease in photosynthesis was appreciably smaller than that predicted from the decrease (about 35%) in Rubisco content (Fig. 3), it was caused by higher stomatal conductance and *pCi* in the antisense plants. In fact, there was no difference in the relationship between light-saturated photosynthesis measured at $pCi = 20$ Pa and Rubisco content between the antisense and wild-type plants (Fig. 5). On the other hand, the light-limited rate of photosynthesis (at 350 μmol quanta m^{-2} s^{-1}) and its stomatal conductance and *pCi* did not differ at any leaf N content between the antisense and wild-type plants (Fig. 4).

Selected Antisense Plants Show a Higher N-Use Efficiency at Elevated CO_2

Figure 6 shows the relationships among Chl, Cyt *f*, and CF_1 contents versus leaf N content. There were apparently small increases in these components in the antisense plants compared with the wild type for the same leaf N content. Among them, the increase in Chl content was statistically significant ($P < 0.05$), and its incremental ratio exactly corresponded to the ratio of decreased Rubisco to total leaf N (7–12%, Fig. 3). This suggests that nonspecific allocation of N from decreased Rubisco (rather than a physiological response to compensate for less Rubisco) occurred, which resulted in the increase in other photosynthetic components for a given leaf N content. Furthermore, as expected from the increase in these components, the antisense plants showed higher rates of photosynthesis at elevated partial pressures of CO_2 (Fig. 7). These characteristics were especially evident in the high-N-grown antisense plants, but the low-N-grown plants showed only a slight difference. This may have been caused by a difference in Rubisco efficiency for CO_2-saturated photosynthesis among N treatments (Fig. 1). This Rubisco efficiency in the wild-type plants was greater in the low-N-grown plants than in the high-N-grown plants. This means that "excess" Rubisco at elevated CO_2 is smaller in the low-N-grown plants than in the high-N-grown plants. Therefore, a 35% reduction of Rubisco by antisense *rbc*S was a little too great for low-N-grown plants to optimize N allocation at saturating CO_2 partial pressures for photosynthesis. However, the results in Figure 7 clearly indicate that a 35% reduction of Rubisco leads to higher rates of CO_2-saturated photosynthesis, especially above 100 mmol N m^{-2} of leaf N content, and that the antisense plants selected here have a higher N-use efficiency of photosynthesis at saturating partial pressures of CO_2 and high irradiance.

DISCUSSION

Rubisco accounts for 15 to 35% of total leaf N in C_3 species and plays an important role in photosynthesis and the N economy of the plant. Therefore, it is not surprising that Rubisco has been taken as a specific target of antisense technology in several laboratories. The antisense plants obtained have been used to evaluate the interaction among Rubisco content, leaf photosynthesis, and plant growth (Stitt and Schulze, 1994; Andrews et al., 1995; Furbank and Taylor, 1995), but little attention has been paid to the antisense effect on the N economy in photosynthesis (Lauerer et al., 1993). In our antisense rice plants, N allocation from decreased Rubisco led to increases in the thylakoid components for a given leaf N content, and, consequently, N may have been optimally distributed between Rubisco and components limiting CO_2-saturated photosynthesis. Therefore, the antisense plants showed higher rates of CO_2-saturated photosynthesis (Fig. 7).

The allocation of N from Rubisco was clearer in the plants with the largest decrease in Rubisco. For example, the primary transformants with a 70% decrease in Rubisco showed approximately a 1.3-fold increase in Chl content, compared with the wild-type plants at the same leaf N content (data not shown). However, such transformants had lower rates of CO_2-saturated photosynthesis because N was not optimally distributed. On the other hand, in tobacco plants a decrease in Rubisco by antisense *rbc*S had

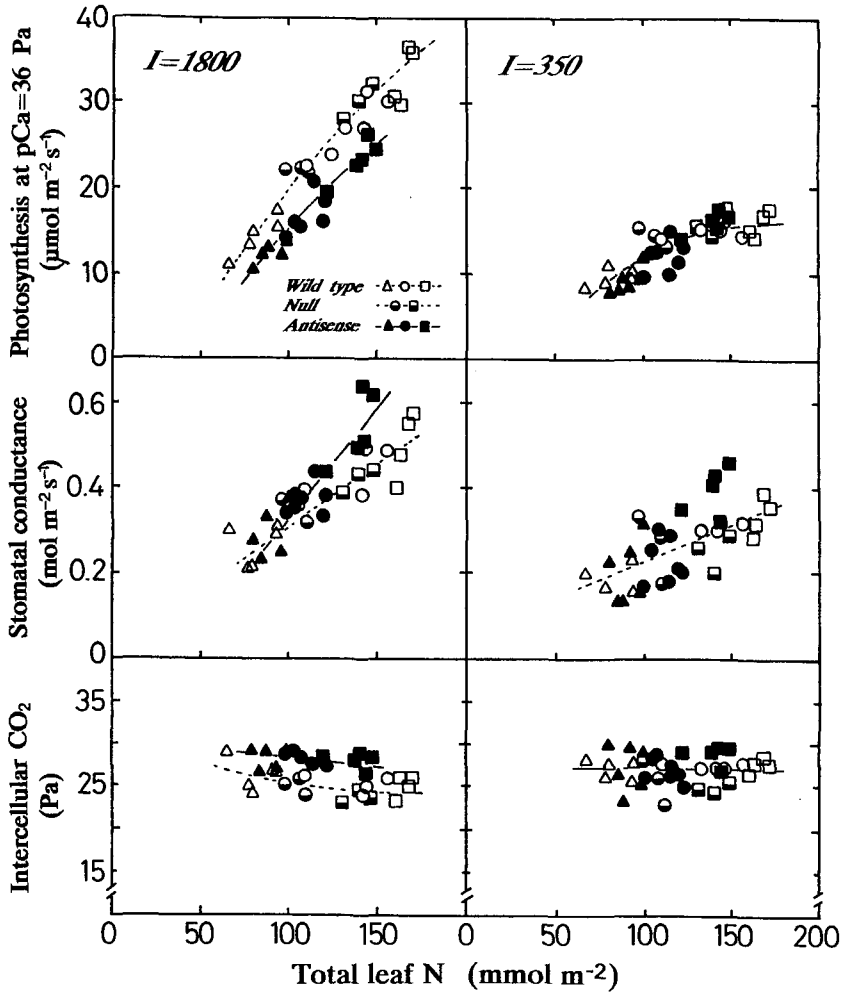

Figure 4. Rate of photosynthesis at *pCa* = 36 Pa, stomatal conductance, and *pCi* versus total leaf N content. Measurements were made at a PPFD of 1800 μmol quanta m^{-2} s^{-1} (left) or 350 μmol quanta m^{-2} s^{-1} (right), a leaf temperature of 25°C, and a leaf-to-air vapor pressure difference of 1.0 to 1.2 kPa. Symbols are the same as in Figure 3.

almost no effect on Chl content or other photosynthetic components (Quick et al., 1991; Hudson et al., 1992; Evans et al., 1994), and, therefore, it is not clear whether N allocation from Rubisco to other photosynthetic components occurred. In fact, the transgenic tobacco plants never had higher rates of photosynthesis than the wild-type tobacco plants, even if they were measured at high CO_2 partial pressures (Quick et al., 1991; Hudson et al., 1992; Masle et al., 1993; von Caemmerer et al., 1994). For the plants with severely decreased (70–80%) Rubisco, there were some indirect changes, such as decreased amounts of other components and enzymes of photosynthesis, and changed morphology and biomass allocation (Quick et al., 1991; Fichtner et al., 1993). These differences between rice and tobacco may be due to a species-dependent difference in N allocation in a leaf.

Two main differences between them have been observed. First, rice plants have a greater Rubisco content than tobacco plants. For example, in rice leaves, 22 to 33% of total N was present in Rubisco (Fig. 3; Makino et al., 1992, 1994a) compared with only 16 to 18% for tobacco leaves (Evans et al., 1994). Therefore, a decrease in Rubisco by the antisense gene in rice may have a greater effect on N partitioning into other components of photosynthesis. Second, rice plants do

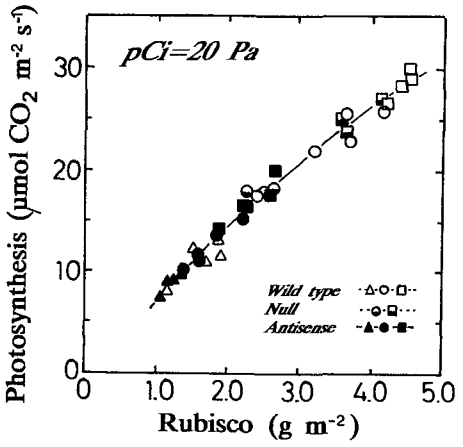

Figure 5. Relationship between rate of photosynthesis at *pCi* = 20 Pa and Rubisco content. Symbols are the same as in Figure 3. Measurements were made at a PPFD of 1800 μmol quanta m^{-2} s^{-1}, a leaf temperature of 25°C, a *pCa* of 26 to 32 Pa, and a leaf-to-air vapor pressure difference of 1.0 to 1.2 kPa.

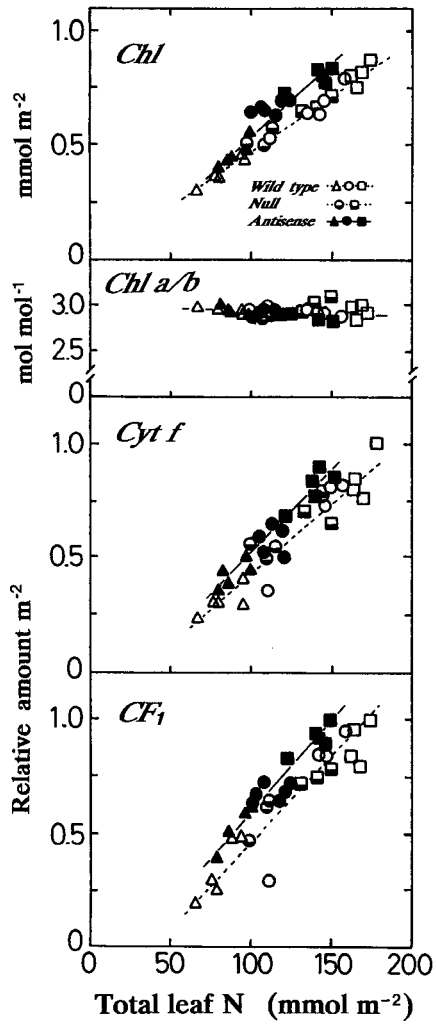

Figure 6. Chl content, Chl *a/b* ratio, Cyt *f*, and CF_1 (α- and β-subunits) contents versus total leaf N content. Symbols are the same as in Figure 3. For Chl, $Y = 0.00520X - 0.048$, $r^2 = 0.99$ (wild-type and null plants); $Y = 0.00639X - 0.078$, $r^2 = 0.90$ (antisense plants). For Cyt *f*, $Y = 0.00632X - 0.172$, $r^2 = 0.90$ (wild-type and null plants); $Y = 0.00644X - 0.145$, $r^2 = 0.88$ (antisense plants). For CF_1, $Y = 0.00733X - 0.263$, $r^2 = 90$ (wild-type and null plants); $Y = 0.00733X - 0.128$, $r^2 = 0.90$ (antisense plants).

not accumulate a nitrate pool in their leaves, even if they are grown with high nitrate. In rice leaves the ratio of nitrate to total N was only 0.3% (Table II), whereas it was 10 to 13% in tobacco leaves (Masle et al., 1993). Furthermore, the nitrate pool in the transgenic tobacco plants increased with decreasing Rubisco content, and its pool was much greater than the decrease in N invested in Rubisco protein (Fichtner et al., 1993; Masle et al., 1993; Stitt and Schulze, 1994). In contrast, there was no difference in the nitrate pool between the three genotypes of rice that we studied (Table II). These results suggest that genetic manipulation of Rubisco in rice directly affects N partitioning for other components of photosynthesis, whereas in tobacco it is almost completely compensated for by an increase in nitrate.

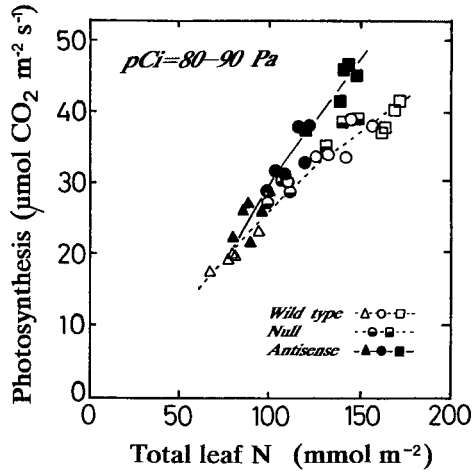

Figure 7. Rate of photosynthesis at *pCi* = 80 to 90 Pa versus total leaf N content. Symbols are the same as in Figure 3. Measurements were made at a PPFD of 1800 μmol quanta m^{-2} s^{-1}, a leaf temperature of 25°C, a *pCa* of 100 to 115 Pa, and a leaf-to-air vapor pressure difference of 1.0 to 1.2 kPa.

Lauerer et al. (1993) reported that transgenic tobacco plants with less Rubisco had a higher ratio of insoluble protein to total leaf protein or structural leaf dry weight and that some of them showed higher rates of photosynthesis in low irradiance. Since the plants do not necessarily optimize N allocation at low irradiance, they considered that the increased insoluble protein can lead to higher rates of light-limited photosynthesis. According to their data, the insoluble protein to total leaf protein ratio increased 1.5- to 2-fold in the plants with very low Rubisco content. This was interpreted as N allocation from decreased Rubisco by the antisense gene. However, if we consider the increase in the nitrate pool in tobacco transformants (see above), N allocation from Rubisco should have been much smaller. Masle et al. (1993) examined N distribution in transgenic tobacco plants and concluded that the N content of organic matter excluding Rubisco was not affected by the decrease in Rubisco. In fact, even if N allocation from Rubisco occurs, the amount of this N allocation is insufficient to account for their observed increases in insoluble protein. Our antisense rice plants did not show higher rates of photosynthesis when they were measured at low irradiance (Fig. 4), whereas they had greater content of thylakoid components, including Chl, for a given leaf N content. This may reflect the fact that light-limited photosynthesis is not necessarily limited by N. Indeed, a positive correlation between photosynthesis and N content is lost when irradi-

Table II. *Nitrate content and its ratio to total N content in leaves of three genotypes grown at 2 mM N (1 mM NH_4NO_3)*

Values are means ± SE (n = 4–5).

Plant	NO_3-N	Total Leaf N	NO_3-N/Leaf N
	mmol m^{-2}	*mmol m^{-2}*	%
Wild type	0.44 ± 0.02	126 ± 10	0.35
Null	0.46 ± 0.09	135 ± 12	0.34
Antisense	0.43 ± 0.07	122 ± 11	0.35

ance is limiting for photosynthesis (Hirose and Werger, 1987; Hikosaka et al., 1993).

Our results show that N investment in Rubisco can be optimized at elevated CO_2 by the antisense technology. However, it is unclear whether the wild-type plants are potentially able to acclimatize to elevated CO_2 and reduce Rubisco to the optimal levels when grown under conditions of CO_2 enrichment. Many studies of long-term acclimation to elevated CO_2 have shown a decrease in Rubisco (Sage et al., 1989; Rowland-Bamford et al., 1991; Jacob et al., 1995; Rogers et al., 1996). In addition, a decrease in transcript levels of *rbc*S, *rbc*L, and *rca* mRNA was found in the plants grown at elevated CO_2 partial pressures (Nie et al., 1995; van Oosten and Besford, 1996). However, it must be noted that total leaf N content is also reduced by CO_2 enrichment (Rowland-Bamford et al., 1991; Tissue et al., 1993; Rogers et al., 1996). Since photosynthesis is determined by absolute N content and N partitioning in a leaf, we need to evaluate changes in N allocation to Rubisco in relation to total leaf N content. Makino (1994) reported that growth CO_2 did not affect Rubisco content and electron transport activity for a given leaf N content. Similarly, Sage et al. (1995) found that the ratio of Rubisco content to electron transport activity was independent of growth CO_2. Rogers et al. (1996) observed that elevated CO_2 partial pressures reduced the absolute amount of soluble protein but did not affect the ratio of soluble protein to total leaf N content. In addition, Sage et al. (1989) reported that the deactivated Rubisco, immediately after exposure to high CO_2, does not recover during the subsequent prolonged exposure. If plants have an ideal acclimation, Rubisco activation should recover while the excess Rubisco protein decreases during long-term exposure to high CO_2.

On the other hand, there have been a few reports showing that Rubisco decreases relative to total leaf N during long-term exposure to high CO_2 when N is limiting for growth (Sage et al., 1989; Rowland-Bamford et al., 1991; Rogers et al., 1996). However, these findings remain uncertain because change in N content makes it difficult to evaluate change in N allocation to Rubisco and other components of photosynthesis. When N supply is low, N allocation to Rubisco is reduced relative to other components of photosynthesis (Evans and Terashima, 1988; Makino et al., 1992, 1994a), and this is independent of growth CO_2 (Makino, 1994). Thus, although long-term CO_2 enrichment leads to a decrease in Rubisco, the plants do not seem to optimize N allocation to Rubisco. We are therefore very interested in whether our antisense rice plants are more adaptive to high-CO_2 environments.

High yields of rice have strongly depended on large inputs of N fertilizer. Therefore, improvements in N-use efficiency are very important to maintain and increase the yield with reduced inputs of N fertilizer. Our final purpose is to construct rice plants that perform better with low-N input in future high-CO_2 environments. Our antisense plants remained stable through a generation. In addition, their size and heading time did not apparently differ from those of the wild-type plants when they were grown in present atmospheric CO_2 partial pressures. Further work aimed at examining growth performance in high-CO_2 environments is in progress.

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