Increased Rubisco Content in Transgenic Rice Transformed with the ‘Sense’ rbcS Gene

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Rice (Oryza sativa L.) plants with substantially increased Rubisco content were obtained by Agrobacterium-mediated transformation with the rice rbcS sense gene under the control of the rice rbcS promoter. The primary transformants were screened for the ratio of Rubisco to leaf-N content, and the transformants with >120% wild-type levels of Rubisco were selected. In the progeny of the selected lines of the transformants, the mRNA levels of one member of the rbcS gene family were increased from 3.9- to 6.2-fold, whereas those of other members of the rbcS gene family were unchanged. The total levels of rbcS mRNA were increased from 2.1- to 2.8-fold. The levels of rbcL mRNA were increased from 1.2- to 1.9-fold. Rubisco protein content was significantly increased by 30% on a leaf area basis. The ratio of Rubisco-N to leaf-N was also increased by 10–20%, irrespective of N treatment. The specific activity of Rubisco of Rubisco-N to leaf-N was also increased by 10–20%, significantly increased by 30% on a leaf area basis. The ratio increased from 1.2- to 1.9-fold. Rubisco protein content was rbcL from 2.1- to 2.8-fold. The levels of rbcS mRNA were increased from 3.9- to 6.2-fold, whereas those of other members of the rbcS gene family were unchanged. The total levels of rbcS mRNA were increased from 2.1- to 2.8-fold. The levels of rbcL mRNA were increased from 1.2- to 1.9-fold. Rubisco protein content was significantly increased by 30% on a leaf area basis. The ratio of Rubisco-N to leaf-N was also increased by 10–20%, irrespective of N treatment. The specific activity of Rubisco per unit of enzyme protein was not different. However, light-saturated photosynthesis was not enhanced even when the rate was measured at low [CO2] where Rubisco becomes limiting for photosynthesis. Some lines showed lower photosynthesis at high [CO2] (>60 Pa). We conclude that introduction of additional sense rbcS leads to overexpression of rbcS and that this overexpression slightly up-regulates the gene expression of rbcL at the transcript level and enhances the amount of Rubisco holoenzyme. However, overproduction of Rubisco protein does not improve photosynthesis.

Keywords: Nitrogen allocation — Overproduction — Photosynthesis — rbcS — Rice — Rubisco.

Abbreviations: CaMV, cauliflower mosaic virus; DTT, dithiothreitol; PPFD, photosynthetic photon flux density; RT–PCR, reverse transcription–PCR; TCA, trichloroacetic acid; UTR, untranslated region.

Introduction

Rubisco is the key enzyme responsible for photosynthesis and the most abundant leaf protein. Rubisco catalyzes two competing reactions, CO2 fixation in photosynthesis and the production of 2-phosphoglycolate in the photorespiratory pathway, and is a rate-limiting factor for both photosynthesis and photorespiration under conditions of saturating light and current atmospheric CO2 and O2 levels (Evans 1986, Makino et al. 1988). In addition, Rubisco accounts for 15–30% of total leaf-N content in C3 species (Makino et al. 1988, Evans 1989). Therefore, Rubisco plays a central role in both photosynthesis and N economy of the plant.

In higher plants, Rubisco is composed of eight small subunits, coded for by a nuclear multigene family (rbcS) (for a review, see Dean et al. 1989), and eight large subunits, coded for by a single gene (rbcL) in the chloroplast genome. Genetic modification of Rubisco content in higher plants has been established by transformation with a construct of an antisense rbcS. The first successful attempt to decrease Rubisco content by introduction of an antisense rbcS gene was done on tobacco (Rodermel et al. 1988), and the same approach has subsequently been used for tobacco (Hudson et al. 1992), a C4 plant Flaveria bidentis (Furbank et al. 1996), and rice (Makino et al. 1997b). Transgenic tobacco with antisense rbcS had lower levels of rbcS mRNA, normal levels of rbcL mRNA and coordinately decreased amounts of the large and small subunit proteins (Rodermel et al. 1988). In this transgenic tobacco, the amount of the large subunit was primarily adjusted to that of the small subunit at the level of rbcL mRNA translation initiation (Rodermel et al. 1996). Similar characteristics were found for the transgenic rice with antisense rbcS (Ishizuka et al. 2004).

On the other hand, little attention has been paid to whether transformation with a construct containing an rbcS gene in the sense orientation leads to overproduction of Rubisco. This question is interesting from the viewpoint of cross-talk between a nucleus and chloroplasts because it is not known whether the amount of a chloroplast protein encoded by both nuclear and chloroplast genes can be increased only by genetic manipulation of the nuclear gene. There are few data on transgenic plants transformed with a construct of an rbcS gene in the sense orientation. Valjakka et al. (2000) transformed silver birch (Betula pendula) with a

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sense *rbcS* gene under the control of a cauliflower mosaic virus (CaMV) 35S promoter. An increase in the level of neither *rbcS* mRNA nor Rubisco protein, however, was found (Sillanpää et al. 2005). Mitchell et al. (2004) transformed wheat (*Triticum aestivum*) with *rbcS* in sense and antisense orientations driven by the maize ubiquitin promoter, but did not find any overexpression of Rubisco. In the case with wheat investigated by Mitchell et al. (2004), introduction of the sense *rbcS* gene often resulted in decreased Rubisco content by co-suppression.

In the present study, we attempted to overproduce Rubisco by overexpression of *rbcS* in rice. Since the endogenous promoter of the rice *rbcS* gene (Kyozuka et al. 1993) may be suitable for such a purpose because of its strength and spatiotemporal expression pattern, this promoter was fused with the cDNA of the rice *rbcS* gene (Matsuoka et al. 1988). Rice plants were then transformed by mediation of *Agrobacterium tumefaciens* (Hiei et al. 1994). As a result, we successfully obtained several transgenic lines with overproduction of Rubisco.

The transformants were further selected at the T₁ progeny stage and their physiological properties were investigated in selected T₂ progeny. The selected transgenic lines were grown at different levels of N, and N partitioning into Rubisco and other N fractions was examined. The mRNA levels of each member of the *rbcS* gene family and *rbcL* were also determined. In addition, the rate of gas exchange was measured to examine the effects of overproduction of Rubisco on photosynthesis.

### Results

According to the database of full-length cDNA clones of rice (http://cdna01.dna.affrc.go.jp/cDNA) (Kikuchi et al. 2003), there are eight *rbcS* cDNA clones which derived from five independent *rbcS* genes, namely, *OsRBCS1*, *OsRBCS2*, *OsRBCS3*, *OsRBCS4* and *OsRBCS5* (Table 1). Whereas *OsRBCS1* is located on chromosome 2, the others are on chromosome 12. Except for *OsRBCS1*, homology among these *rbcS* genes is high. For example, similarities among the sequences of the full-length cDNAs, the coding regions of the cDNAs and the deduced amino acids derived from *OsRBCS2*, *OsRBCS3*, *OsRBCS4* and *OsRBCS5* are 69.9–76.6, 86.7–91.5 and 93.7–97.1%, respectively. Surprisingly, when the sequences of the amino acids, except for that of the transit peptide, were examined, the deduced sequences from these four genes were 100% identical. On the other hand, similarity between *OsRBCS1* and the other *rbcS* genes is not so high (53.1–57.4% in the sequence of full-length cDNA; 58.7–61.6% in the sequence of the coding region of the cDNA; 55.3% in the sequence of the deduced amino acids; and 55.4% in the sequence of the deduced amino acids except the transit peptide). The nucleotide sequences of the cDNA (Matsuoka et al. 1988) and the promoter (Kyozuka et al. 1993) used for the transformation were found to be identical to those of *OsRBCS2* (data not shown).

Thus, we transformed the *OsRBCS2* cDNA in sense and antisense orientation under the control of the *OsRBCS2* promoter by mediation of *Agrobacterium*. Forty-six lines of the sense *rbcS* transformant and 16 lines of the antisense *rbcS* transformant were obtained. Since the absolute amount of Rubisco content strongly depends on leaf-N content and leaf age, we screened for the Rubisco content to leaf-N ratio vs. leaf-N content in the uppermost, fully expanded leaves. The Rubisco content was determined spectrophotometrically after formamide extraction of Coomassie brilliant blue R-250-stained bands corresponding to the large and

### Table 1

The *rbcS* multigene family in rice data-mined from the full-length cDNA database of *Oryza sativa* L. cv. Nipponbare (Kikuchi et al. 2003)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus of transcription unit (TU ID)</th>
<th>Accession No. of cDNA</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cDNA (bp)</td>
<td>Amino acid</td>
</tr>
<tr>
<td><em>OsRBCS1</em></td>
<td>2,874,948–2,876,252 bp, chromosome 2 (R02-Cv3DPA-028008F)</td>
<td>AK059909</td>
<td>999</td>
</tr>
<tr>
<td><em>OsRBCS2</em></td>
<td>10,079,315–10,080,562 bp, chromosome 12 (R12-Cv3DPA-100007R)</td>
<td>AK061611</td>
<td>830</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AK119219</td>
<td>828</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AK121444</td>
<td>984</td>
</tr>
<tr>
<td><em>OsRBCS3</em></td>
<td>11,261,252–11,262,442 bp, chromosome 12 (R12-Cv3DPA-112004F)</td>
<td>AK068555</td>
<td>899</td>
</tr>
<tr>
<td><em>OsRBCS4</em></td>
<td>11,318,956–11,321,195 bp, chromosome 12 (R12-Cv3DPA-113005F)</td>
<td>AK068266</td>
<td>1999</td>
</tr>
<tr>
<td><em>OsRBCS5</em></td>
<td>11,275,929–11,277,138 bp, chromosome 12 (R12-Cv3DPA-112005F)</td>
<td>AK070257</td>
<td>845</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AK099574</td>
<td>947</td>
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</table>
small subunits of Rubisco separated by SDS–PAGE (Makino et al. 1985). For the sense \( rbcS \) transformants, a large variation in the Rubisco content to leaf-N ratio was found, but in a few transformants the Rubisco to leaf-N ratio was enhanced by about 20% compared with the wild-type levels (Fig. 1). On the other hand, most of the antisense transformants had a lower Rubisco content to leaf-N ratio than the wild-type plants.

We selected three transformants with 120% wild-type Rubisco (Sr-8), 130% wild-type Rubisco (Sr-26) and 120% wild-type Rubisco (Sr-35), respectively (indicated by the arrows in Fig. 1), and the \( T_1 \) seeds were obtained. The occurrence of the transgene in three lines (one copy for Sr-8 and two copies for Sr-26 and Sr-35) was confirmed by genomic DNA gel blot analysis (data not shown). The Rubisco to leaf-N content ratio was examined in the \( T_1 \) progeny (Fig. 2). Some lines of the \( T_1 \) progeny of Sr-26 and Sr-35 had more than 50% greater Rubisco content than the wild-type plants. Since both primary transformants had two copies of the transgene, the segregation of the \( T_1 \) population was complicated. Rubisco content was appropriately reduced in a few lines, which is probably caused by so-called ‘co-suppression’ or ‘homology-dependent gene silencing’. From these populations, we next selected three lines: one had 180% wild-type Rubisco (Sr-26-8), the second had 140% wild-type Rubisco (Sr-26-13) and the third had 155% wild-type Rubisco (Sr-35-4). All these lines were fully fertile and were allowed to self-fertilize. Their \( T_2 \) progeny were used for the following experiments.

The wild-type plants and selected \( T_2 \) transgenic lines were grown under different levels of N. Rubisco contents per unit of leaf area were significantly greater in the transgenic lines than in the wild-type plants in all N treatments except Sr-26-8 grown at 0.5 mM N (Fig. 3). When the Rubisco content was expressed as the ratio to leaf-N content, it was also found to be stably increased by 10–20% except in the case of Sr-35-4 grown at 2 mM N (Fig. 4A).

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**Fig. 1** Ratio of Rubisco-N to total leaf-N vs. total leaf N content in the uppermost, fully expanded leaves of the \( T_0 \) progeny of rice transformed with \( rbcS \) cDNA in the sense (open squares) or antisense (open triangles) orientation and wild-type (filled circles) rice plants. The arrows indicate the selected primary transformants with overproduction of Rubisco.

**Fig. 2** Relative ratio of Rubisco-N to total leaf-N in the uppermost, fully expanded leaves of the \( T_1 \) progeny of the selected primary transformants with sense \( rbcS \) cDNA. Data obtained with the wild-type rice are presented as means ± SD (\( n = 4 \)), and the mean is defined as 100% (WT). The arrows indicate the transgenic lines selected for the \( T_2 \) lines with overproduction of Rubisco.

**Fig. 3** Rubisco content per unit of leaf area in the uppermost, fully expanded leaves of the \( T_2 \) transgenic lines and the wild-type rice plants grown hydroponically with different N concentrations of 0.5, 2 and 8 mM. Data are presented as means ± SD (\( n = 4 \)). Statistical analysis was carried out among the plants grown at the same N concentration by ANOVA followed by Tukey’s test. Columns with the same letter were not significantly different (\( P \leq 0.05 \)).
Thus, although the Rubisco content decreased in these T2 progeny, compared with the levels of their T1 parents (see, Fig. 2), the expression of the sense rbcS transgene remained stable and no co-suppression occurred. On the other hand, the ratios of other soluble proteins-N and insoluble proteins-N tended to decrease in the transgenic lines (Fig. 4B, C). These results suggest the possibility that the increase in Rubisco content is accompanied by a decrease in other proteins including insoluble fractions in the transgenic lines. Our previous studies with rbcS antisense rice indicated that non-specific allocation of N from decreased Rubisco to other photosynthetic components occurs in antisense plants (Makino et al. 1997b). For these senserbcS transgenic lines, non-specific allocation of N to Rubisco may have occurred from other photosynthetic components. The trichloroacetic acid (TCA)-soluble N to leaf-N ratio did not differ between the wild-type plants and the transgenic lines (Fig. 4D).

Total RNA was extracted according to the method of Suzuki et al. (2004), and then the mRNA levels of each member of the rbcS gene family and those of rbcL were determined by semi-quantitative reverse transcription–PCR (RT–PCR). For these analyses, leaves which had emerged from the sheaths by about 50% were used because the levels of these mRNAs reached the maximum before full expansion (see Suzuki et al. 2001b). Since the respective members of the rbcS gene family have different nucleotide sequences in the 3′-untranslated regions (UTRs), such regions without similarity among the genes were amplified by RT–PCR. For the estimation of the mRNA levels of the total rbcS gene family, a partial sequence of the coding region with high similarity among the genes was amplified.

There was no difference in the total RNA content on a fresh weight basis between the wild-type plants and the transgenic lines (Fig. 5A). The level of a partial fragment derived from rRNA was also determined as an internal standard for mRNA analysis. The ratio of rRNA to total RNA levels was not different irrespective of genotype and N treatment (Fig. 5B). This indicates the validity of the RT–PCR method used here. The mRNA levels of the total rbcS gene family were increased from 2.1- to 2.8-fold in the transgenic lines (Fig. 6A). For each member of the rbcS gene family, however, the mRNA levels of OsRBCS2 were increased from 3.9- to 6.2-fold in the transgenic lines, but those of other members (OsRBCS3, OsRBCS4 and OsRBCS5) were unchanged (Fig. 6B–E). The mRNA levels of OsRBCS1 were only slightly detectable irrespective of genotype and N treatment (data not shown). These results indicate that the increase in the mRNA levels of total rbcS was caused only by the increase in the mRNA levels of OsRBCS2 which had been used as the transgene. The mRNA levels of rbcL were also increased in the transgenic lines, but the incremental ratio (1.2- to 1.9-fold) was smaller than that of rbcS. Although a few transgenic lines showed no statistical increase in the mRNA levels of rbcL, the ratio of the mRNA levels of rbcL to OsRBCS5 was statistically increased in all transgenic lines (Fig. 6G). Similarly, when the ratios of rbcL to OsRBCS3 or to OsRBCS4 were calculated, a statistically significant increase was also found for all lines (data not shown). These results indicate that the increase in the mRNA levels of rbcS led to an increase in the RNA levels of rbcL.

Relationships between the mRNA levels of total rbcS and rbcL and Rubisco contents were analyzed (Fig. 7). Both parameters are expressed on a different basis (leaf area for Rubisco protein levels and fresh weight for mRNA levels).
was examined at an irradiance of 1,200 mol m\(^{-2}\) s\(^{-1}\). The overproduced enzyme is catalytically active at the same temperature of 25 °C and an external CO\(_2\) partial pressure of 37 Pa (\(pC_a = 37\) Pa) between the wild-type plants and the transgenic lines (Fig. 8A). Similarly, the rates of CO\(_2\) assimilation at an intercellular CO\(_2\) partial pressure of 20 Pa (\(pC_i = 20\) Pa) did not differ between the genotypes (Fig. 8B). Some of the transgenic lines showed lower rates of CO\(_2\) assimilation at above 60 Pa CO\(_2\) of \(pC_i\) (Sr-26-8 at 0.5 and 2 mM N, Sr-35-4 at 8 mM N) (Fig. 8C). Fig. 8D shows typical examples of the rate of CO\(_2\) assimilation as a function of \(pC_i\) (A:C\(_i\) curve) in the plants grown with 2 mM N. The initial slope of the \(A:C_i\) curve was not different between the wild-type plants and the transgenic lines. According to the C3 photosynthetic model of von Caemmerer and Farquhar (1981) and Sharkey (1985), the photosynthetic rate at low CO\(_2\) partial pressures is limited by Rubisco capacity, whereas the rate at high CO\(_2\) is limited by electron transport capacity or by the capacity of starch and sucrose synthesis to regenerate Pi for photophosphorylation. Therefore, our results suggest that overproduction of Rubisco protein does not improve photosynthesis even at low [CO\(_2\)] where Rubisco becomes limiting for photosynthesis. The relationship between the CO\(_2\) assimilation rates at \(pC_i = 20\) Pa and the Rubisco contents was next analyzed (Fig. 8E). The measured rates of CO\(_2\) assimilation were lower for the same Rubisco content in the transgenic lines as in the wild-type plants. When the rate predicted from Rubisco activity at the stromal CO\(_2\) partial pressure obtained at \(pC_i = 20\) Pa was calculated from the Rubisco content and its kinetic constants on the assumption that all genotypes have the same CO\(_2\) transfer conductance between the intercellular air spaces and the carboxylation sites for the same rate of CO\(_2\) assimilation, the measured rate of CO\(_2\) assimilation was very close to the rate predicted from Rubisco activity in the wild-type plants (92.1 ± 7.7%). However, the measured rates of CO\(_2\) assimilation in Sr-26-8, Sr-26-13 and Sr-35-4 were 70.3 ± 9.4, 62.6 ± 10.1 and 72.5 ± 12.9% of the predicted rate, respectively.

**Discussion**

In the present study, the introduction of sense cDNA of OsRBCS2 to a nucleus led to a 2.1- to 2.9-fold increase in the mRNA level of total "rbcS" and resulted in a 1.3-fold increase in Rubisco content (Figs. 3, 4, 6). This small increase in Rubisco content was associated with a 1.2- to 1.9-fold increase in the mRNA levels of "rbcL" in chloroplasts (Fig. 6). Thus, the amount of Rubisco holoenzyme was enhanced only by transformation with an "rbcS" in the sense orientation. This means that a chloroplast protein encoded by both nuclear and chloroplast genes can be overproduced.

![Fig. 5](https://academic.oup.com/pcp/article-abstract/48/4/626/2756917/fig5)

The amount of total RNA (A) and the relative level of rRNA amplified by semi-quantitative RT-PCR (B) in the leaves expanding from the sheaths by about 50% in the selected T2 transgenic lines and the wild-type rice plants grown hydroponically with different N concentrations of 0.5, 2 and 8 mM. Data are presented as means ± SD (n = 4). Statistical analysis was carried out among the plants grown at the same N concentration by ANOVA followed by Tukey’s test. Columns with the same letter were not significantly different (\(P \leq 0.05\)).
Overproduction of Rubisco by sense rbcS in rice by transformation with a nuclear-encoded gene alone. However, it is poorly understood how overexpression of rbcS led to an increase in Rubisco protein content. Since the increase in the mRNA level of rbcS did not quantitatively lead to an increase in Rubisco content (Figs. 3, 5, 6), this suggests that the synthesis of the large subunit strongly limits the extent of the overproduction of Rubisco in the transgenic lines. Actually, a positive correlation was found between the levels of rbcL mRNA and Rubisco protein (Fig. 7). Thus, it is possible that the extent of the overproduction of Rubisco protein was limited by a slight up-regulation of rbcL mRNA. However, this up-regulation at the transcriptional level did not fully cooperate with the overexpression of rbcS. Of course, the possibility that the synthesis of the large subunit is also up-regulated at the translational level cannot be ruled out. In the antisense rbcS tobacco and the antisense rbcS rice, whereas lower levels of rbcS mRNA did not affect the levels of rbcL mRNA, the amounts of the large and small subunits were coordinately decreased (Rodermel et al. 1988, Ishizuka et al. 2004). In the case of transgenic tobacco, the synthesis of the large subunit was down-regulated at the level of

Fig. 6 The relative mRNA levels of each member of the rbcS gene family and rbcL in the leaves expanding from the sheaths by about 50% in the selected T₂ transgenic lines and the wild-type rice plants grown hydroponically with different N concentrations of 0.5, 2 and 8 mM. (A) Total rbcS, (B) OsRBCS2, (C) OsRBCS3, (D) OsRBCS4, (E) OsRBCS5, (F) rbcL, (G) the ratio of rbcL to OsRBCS5. The mRNA level of each gene was expressed on a leaf fresh weight basis, and the level in the wild-type plants at 2 mM N was defined as 1.0. Data are presented as means ± SD (n = 4). Statistical analysis was carried out among the plants grown at the same N concentration by ANOVA followed by Tukey’s test. Columns with the same letter were not significantly different (P ≤ 0.05).

Fig. 7 Relationships between the mRNA levels of total rbcS (A) and rbcL (B) in the leaves expanding from the sheaths by about 50%, and Rubisco content in the uppermost, fully expanded leaves of the selected T₂ transgenic lines and the wild-type rice plants. Open circles, wild-type; filled squares, Sr-26–8; filled triangles, Sr-26–13; filled inverted triangles, Sr-35–4. Data are taken from Figs. 3 and 6. For total rbcS mRNA, \( y = 1.58x + 0.48, r^2 = 0.93 \) (wild-type); \( y = 0.61x + 1.23, r^2 = 0.72 \) (transgenic lines). For rbcL mRNA, \( y = 1.06x + 1.13, r^2 = 0.69 \) (all genotypes).
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The translational initiation of rbcL mRNA by a decreased amount of the small subunit in chloroplasts (Rodermel et al. 1996). The results of Rodermel et al. indicated that the small subunit, either directly or indirectly, specifically affects the recruitment of ribosomes to rbcL mRNA. The factors that regulate translation initiation in chloroplasts are still unknown, but several nuclear-coded factors that bind to the 5′-UTR of some chloroplast mRNAs have been identified (for a review, see Rodermel 1999). In addition, a similar phenomenon has been observed in the synthesis of the cytochrome b6/f complex in Chlamydomonas (Choquet and Wollman 2002). Translation of the cytochrome f subunit was strongly attenuated when other subunits of the b6/f complex were...
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reduced as a result of a direct or indirect interaction between the 5'-UTR of the cytochrome \textit{f} mRNA and the C-terminal domain of the unassembled cytochrome \textit{f} subunit. If post-transcriptional regulation of \textit{rbcL} is controlled by the amount of the small subunit, factors that arrest translation initiation might be released due to increasing levels of the small subunit in the sense \textit{rbcS} transgenic lines. Thus, our present knowledge is insufficient to understand the integrated mechanism of Rubisco biosynthesis by nuclear–chloroplast interactions, but our results show that the introduction of sense \textit{rbcS} results in a significant increase in Rubisco content with overexpression of \textit{rbcS} and a small increase in the level of \textit{rbcL} mRNA.

Rubisco content also increased with increasing N supply (Fig. 3). When N is supplied, the amount of Rubisco protein increases relative to that of other photosynthetic components in many C3 species including rice (Evans and Terashima 1988, Makino et al. 1992, Nakano et al. 1997). This increase in Rubisco with the supply of N is associated with the increases in levels of \textit{rbcL} mRNA as well as \textit{rbcS} mRNA to a similar extent (Imai et al. 2005; Fig. 6F, G). This means that an increase in Rubisco resulting from the supply of N is caused not by a mediation of enhancement in gene expression of \textit{rbcS} but by direct increases in expression of both genes. In addition, Rubisco content in a leaf is affected by environmental factors during plant growth. However, correlation between Rubisco and leaf-N contents is always independent of such factors during plant growth. However, correlation between Rubisco and leaf-N contents is always independent of such environmental conditions, including light (Makino et al. 1997a, Miyake et al. 2005), temperature (Makino et al. 1994b) and CO\textsubscript{2} concentration (Nakano et al. 1997, Theobald et al. 1998). In addition, Rubisco content can be quantitatively accounted for by the amounts of N influx into the leaf during leaf development (Imai et al. 2005). These facts show that the amount of Rubisco synthesized is strictly controlled by expression of both \textit{rbcL} and \textit{rbcS} associated with N influx into a leaf. This is largely different from our artificial findings on the sense \textit{rbcS} transgenic rice. Thus, although our results suggest that overexpression of \textit{rbcS} directly or indirectly up-regulates the gene expression of \textit{rbcL} at a transcript level, it alone does not sufficiently function as a source of signals to regulate the expression of \textit{rbcL}.

In higher plants, the small subunits of Rubisco are coded in the form of a multigene family consisting of between two and 20 members, depending on the species (Rodermel 1999). Dean et al. (1989) described that the expression of the \textit{rbcS} genes varies depending on different organs and tissues and different developmental stages, and that it is controlled by environmental factors. In addition, Dedonder et al. (1993) reported that the expression of the \textit{rbcS} genes in \textit{Arabidopsis} is differently regulated by light. Yoon et al. (2001) found that the relative expression of each \textit{rbcS} gene differs between \textit{Arabidopsis} plants grown at different temperatures. These results suggest the possibility that an adaptation of Rubisco to environmental changes may occur through the specific expression of the \textit{rbcS} gene family. Recently, Yamori et al. (2006) have observed a difference in the in vitro kinetic properties of Rubisco purified from spinach grown at different temperatures. However, it is largely unknown how each individual gene contributes to the overall expression of the gene family.

Rice has five members of the \textit{rbcS} gene family (Table 1). Although the expression of \textit{OsRBCS1} was only slightly detectable, the other four genes (\textit{OsRBCS2}-\textit{OsRBCS5}) were constitutively expressed (Fig. 6). In the sense \textit{rbcS} transgenic lines, although the mRNA levels of \textit{OsRBCS2} were drastically increased, those of the other members were unchanged. This means that when one member of the gene family is overexpressed, the expression of other genes is not affected at the transcript level. However, with increasing N supply, the expression of each gene was enhanced to the same relative degree (Fig. 6). These results suggest that the expression of each member of the \textit{rbcS} gene family is not independently regulated. In addition, since the amino acid sequences, except for that of the transit peptide, deduced from these four \textit{rbcS} genes are 100% identical in rice at least, it is impossible for adaptation of Rubisco to environmental changes to occur through the specific expression of the \textit{rbcS} gene family.

Some attempts to introduce the \textit{rbcS} gene in the sense orientation have shown no increase in Rubisco content. Valjakka et al. (2000) and Sillanpää et al. (2005) introduced a sense \textit{rbcS} fused with the CaMV 35S promoter into silver birch. Mitchell et al. (2004) also transformed wheat with a sense \textit{rbcS} under the control of the maize ubiquitin promoter. Getzoff et al. (1998) produced transgenic \textit{Arabidopsis} with a construct of the sense \textit{rbcS} from pea under the control of the CaMV 35S promoter. In all the above studies, no increases in Rubisco content were observed. In silver birch, the levels of \textit{rbcS} mRNA did not increase either. In \textit{Arabidopsis}, although the pea \textit{rbcS} was expressed and assembled with the endogenous large subunit, the preliminary experiments showed that the Rubisco content was not increased. The reason for no increase in Rubisco protein in these plants is not known, but it is possible that the promoter used did not work efficiently. Mitchell et al. (2004) described that the strength of the maize ubiquitin promoter was not sufficient in wheat mesophyll tissues. Although the use of the CaMV 35S promoter has been successful in the antisense \textit{rbcS} tobacco (Rodermel et al. 1988, Hudson et al. 1992), its characteristics are constitutive and not always efficient in green tissues in rice (Tada et al. 1991). In addition, the synthesis of Rubisco is controlled by strictly spatiotemporal-specific gene expression of \textit{rbcS} and...
rub$L during leaf expansion (Suzuki et al. 2001b, Imai et al. 2005). Thus, the rice rbc$S promoter used for rice might have been effective for the expression of the rbc$S transgene.

Overproduction of Rubisco in the transgenic lines did not lead to an improvement of photosynthesis even under the conditions where Rubisco becomes limiting for photosynthesis (Fig. 8). The measured rate of CO$_2$ assimilation was lower than the rate predicted from the amount and kinetic constants of Rubisco in the transgenic lines, whereas those were close to each other in the wild-type plants. Furthermore, in spite of an increase in Rubisco content in the transgenic lines, there was no difference in the initial slope of the A:C$_1$ curve between the wild-type plants and the transgenic lines. Since the in vitro specific activity of Rubisco was not different (Table 2), these results indicate that the in vivo Rubisco activity in the transgenic lines was down-regulated even under the Rubisco-limited conditions. Actually, the activation state of Rubisco in the Sr-28-6 line was lower than that in the wild-type plants. Such a partial deactivation may be one of the reasons for no enhancement of photosynthesis in the transgenic line, but the factors that regulate the activation state of Rubisco are complicated. For example, it is possible that changes in N partitioning among photosynthetic components with a specific increase in Rubisco content gave rise to an imbalance between Rubisco and other components which limit photosynthesis. In addition, Rubisco activation is affected by changes in the stromal redox state and in the proton gradient through thylakoid membranes mediated by an alternative electron transport in the transgenic lines (see Makino et al. 2002). Further studies are necessary to clarify the mechanism of down-regulation of Rubisco activity in the rice rbc$S rice.

Genetic modification of crop Rubisco to enhance CO$_2$ fixation efficiency is an important strategy for improvement of photosynthesis (Parry et al. 2003). Our previous rice transformation with antisense rbc$S showed an increased photosynthetic rate only at elevated [CO$_2$] conditions for a given leaf-N content (Makino et al. 1992), overproduction of Rubisco in rice may have led to lower N investment for other components limiting photosynthesis. Actually, the insoluble-N content was smaller in the transgenic lines than in the wild-type plants (Fig. 4), and some transgenic lines showed lower rates of CO$_2$ assimilation at above 60 Pa CO$_2$ where Rubisco does not limit photosynthesis (Fig. 8). Therefore, to improve photosynthesis in rice, another strategy such as an enhancement of the catalytic performance of Rubisco may be necessary. Previous comparative studies of Rubisco activity among higher plants revealed that the specific activity of Rubisco in rice is significantly lower than that in other plants (Makino et al. 1985, Sage 2002). Thus, the lower specific activity of Rubisco in rice would make it possible to improve photosynthesis and N-use efficiency by introducing a more efficient Rubisco into rice.

**Materials and Methods**

**Vector construction of the sense or antisense rbc$S cDNA for Agrobacterium-mediated transformation**

The respective plasmids containing the sense rbc$S cDNA (pBIRS) and antisense rbc$S cDNA (pBIARS) were constructed with the rice rbc$S promoter and the rice rbc$S cDNA using the Ti plasmid pB101Hm into which a CaMV35S/hygromycin phosphotransferase gene (hpt) cassette (Clontech, Palo Alto, CA, USA) had been introduced by Kojima et al. (2000). A 3.6 kb fragment of the rice rbc$S promoter (Kyozuka et al. 1993) and the rice rbc$S cDNA (Matsuoka et al. 1988) was excised at the HindIII and SacI sites from the plasmid pRAS (Makino et al. 1997b), and the rbs$S cDNA fragment was inserted in the sense or antisense orientation, with respect to the rbc$S promoter, into the HindIII and SacI sites between the neomycin phosphotransferase gene (neo$II) and hpt in the pB101Hm from which the uid$A gene (encoding GUS) had been removed by Yamaya et al. (2002). After confirmation of the nucleotide sequence and orientation of the promoter and the cDNA, the plasmids were introduced into *A. tumefaciens* strain EHA101 as described in Kojima et al. (2000).

**Plant transformation and regeneration**

*Agrobacterium tumefaciens*-mediated transformation of rice (Hiei et al. 1994) was carried out as described by Kojima et al. (2000). Transformation with only pB101Hm was also adopted as a control. Rice (*Oryza sativa* L. cv Notohikari) was infected by co-cultivation with calli, derived from scutella, and *A. tumefaciens* EHA101 which had been transformed with pBIRS and pBIARS. The transformed cells were selected on R2-medium containing 9.5 μg hygromycin B, 0.0025% (w/v) carbenicillin and 9 μM 2,4-D. After 4-5 weeks, surviving callus clusters were regenerated. The plantlets obtained were grown at a photosynthetic photon flux density (PPFD) of 100 μmol quanta m$^{-2}$s$^{-1}$ and 26°C for 2-4 weeks, and then transferred to a 1.01 plastic pot containing N content and, consequently, N may have been optimally distributed between Rubisco and components limiting CO$_2$-saturated photosynthesis. However, the present rice with the sense rbc$S did not show an increase in the photosynthetic rate even at low [CO$_2$] conditions. Since rice originally has a greater Rubisco content than other plants (Makino et al. 1992), overproduction of Rubisco in rice may have led to lower N investment for other components limiting photosynthesis. Actually, the insoluble-N content was smaller in the transgenic lines than in the wild-type plants (Fig. 4), and some transgenic lines showed lower rates of CO$_2$ assimilation at above 60 Pa CO$_2$ where Rubisco does not limit photosynthesis (Fig. 8).

**Table 2** The specific activity of carboxylase per unit of Rubisco protein (mol CO$_2$ mol$^{-1}$ Rubisco s$^{-1}$) at 25°C in the leaves of the selected T$_2$ transgenic lines and the wild-type rice plants

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Sr-26-8</th>
<th>Sr-26-13</th>
<th>Sr-35-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity$^b$</td>
<td>10.9 ± 0.3</td>
<td>10.6 ± 0.6</td>
<td>11.5 ± 0.4</td>
<td>11.0 ± 0.9</td>
</tr>
</tbody>
</table>

$^a$The molecular weight of Rubisco is assumed to be 525,000 Da.
$^b$Data are presented as means ± SD (n = 3). The activity is not significantly different between the wild-type and transgenic plants by ANOVA followed by Tukey’s test (P ≤ 0.05).
nutrient solution in an isolated greenhouse under natural sunlight conditions. The plants were grown hydroponically (Makino et al. 1988) until maturity, and allowed to self-fertilize, then the T₁ seeds were collected.

Culture of the sense rbcS transformants

After selection of the transformants with substantially increased Rubisco content among the T₁ progeny, the T₂ progeny were used for experiments. Non-transformed rice (cv Notohikari) was also used as a control, and all plants were grown hydroponically in an isolated greenhouse. The greenhouse was maintained with a 14 h photoperiod (5:00–19:00) under natural sunlight conditions supplemented with six 400 W metal halide lamps, day/night temperature of 25/20°C and 60% relative humidity. Seeds were soaked in tap water, and then seedlings were grown on a net floating on tap water for 17 d. Four seedlings each were transplanted to 3.51 plastic pots containing nutrient solution as previously described by Makino et al. (1988). From 4 weeks after transplanting, plants were supplied with three N concentrations (mM): 0.5 (0.25 mM NH₄NO₃), 2 (1 mM NH₄NO₃) and 8 (2.0 mM NH₄NO₃ plus 4.0 mM NaNO₃). These solutions were renewed once a week and continuously aerated. The measurements of gas exchange and the determination of Rubisco protein and leaf-N were conducted on the uppermost, fully expanded leaves 2 weeks later. For determination of mRNAs of rbcS and rbcL, leaves which had emerged from their sheaths by at least 4 d after the renewal of the culture solution because N supply temporally affects the levels of rbcS and rbcL mRNAs (Imai et al. 2005). All collected leaves were weighed and frozen in liquid N₂ immediately, and then stored at –80°C until analysis.

Determination of Rubisco protein and leaf-N

Frozen leaves were homogenized in a chilled mortar with a pestle in 50 mM Na-phosphate (pH 7.5) containing 2 mM iodoacetic acid, 0.8% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol (Makino et al. 1988). Total leaf-N was determined from part of the homogenate before centrifugation. Soluble N and insoluble N were determined from the supernatant of the homogenate before centrifugation. Soluble N and insoluble N was calculated by subtracting soluble N from total leaf N. Insoluble N was calculated by subtracting soluble N from the supernatant of the homogenate centrifuged at 15,000 g for 5 min at 4°C. The remaining homogenate was used for the determination of Rubisco content. The Rubisco content was determined spectrophotometrically by formamide extraction of the Coomassie brilliant blue R-250-stained bands corresponding to the large and small subunits of Rubisco separated by SDS-PAGE using calibration curves made with Rubisco purified from rice leaves (Makino et al. 1985).

Extraction of total RNA and semi-quantitative RT–PCR analysis of the mRNAs of rbcS and rbcL

Total RNA was extracted according to the method of Suzuki et al. (2004) with slight modifications. A sample leaf was homogenized in a chilled mortar with a pestle and acid-washed quartz sand in the presence of liquid N₂. The resulting powder was suspended in the extraction buffer [100 mM Tris–HCl, 10 mM EDTA, 200 mM NaCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) lithium dodecylsulfate, pH 9.3] and weighed immediately. The suspension was further homogenized to destroy the leaf debris completely and treated sequentially with a mixture of chloroform and isoamylalcohol, and the phenol mixture. RNA in the supernatant was precipitated according to the method of Chomczynski and Mackey (1995), washed with 75% (v/v) ethanol and dissolved in a small amount of RNase-free water. The amount of extracted RNA was comparable with that extracted by the method of Suzuki et al. (2004), which can be used for determination of total RNA on a tissue weight basis. The extracted RNA was treated with DNase I (DNA-free, Ambion, Austin, TX, USA) and then reverse-transcribed (ExScript RT reagent kit, TAKARA, Ohtsu, Japan) with random hexamers according to the manufacturer’s instructions. An aliquot of the synthesized first-strand cDNA derived from 9.6 ng of total RNA was used for PCR amplification (total volume of 6 µl with Taq polymerase (PrimeSTAR, TAKARA) according to the manufacturer’s instruction. The oligonucleotide primer pairs, the number of PCR cycles and size of the PCR products were as follows: 5'-CAACTAAGCCGTACATCGT-3' and 5'-CCTCACCCAAAACATATACTG-3', 16 cycles, 208 bp for OsRBCS5; 5'-CAATGCGCTTGCTCTTAAC-3' and 5'-GGCGACGAAATTATCACAGATATG-3', 18 cycles, 168 bp for OsRBCS5; 5'-TACACACTATCGCTTATGCCT-3' and 5'-TGTGGACGATATGATG-3', 21 cycles, 121 bp for OsRBCS5. OsRBCS1, 15 cycles, 155 bp for OsRBCS5; 5'-TAAAGCGAGATGCACTACCTGTC-3', 21 cycles, 121 bp for OsRBCS5; 5'-CTCTGGCTAGCCT-3' and 5'-GGCGTAAAAGACCTTGTC-3', 21 cycles, 228 bp for rbcL. For an internal standard, an amplified DNA fragment with the Universal 18S standard (Ambion) was used. The ratio of the primers and the competitor was 4:6 and the number of PCR cycles was 12. It was confirmed by preliminary experiments that the numbers of PCR cycles were within the linear range of the amplifications. The amplified fragments were separated by electrophoresis on a 3.0% (w/v) agarose gel and the gel was stained with SYBR green-I (TAKARA, Ohtsu, Japan). The florescent intensity of the DNA fragment was measured with FLA-2000 (Fuji Film, Tokyo, Japan).

Gas exchange measurements

Gas exchange was determined with an open gas exchange system previously detailed by Makino et al. (1988). Measurements were made at a leaf temperature of 25°C, a PPFD of 1,700 μmol quanta m⁻² s⁻¹ and a leaf-to-air vapor pressure difference of 1.0–1.2 kPa. The measurement was initiated at pcH₂O = 37 Pa to obtain the steady state of the gas exchange rate, and the CO₂ pressure was lowered to obtain the rates at pC₀₂ = 20 Pa then raised to above 80 Pa. Gas exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981).

Assay of Rubisco activity

Rubisco activity was measured spectrophotometrically by coupling 3-phosphoglyceric acid formation with NADH oxidation according to Nishino et al. (2000). The green leaf was homogenized at 0–4°C in 50 mM HEPES-NaOH (pH 8.0) containing 20 mM MgCl₂ and 5 mM dithiothreitol (DTT). After centrifugation at 4°C for 10 s, a portion of the supernatant was injected in a reaction mixture containing 100 mM Bicine-NaOH
Overproduction of Rubisco by sense rbcS in rice

(pH 8.0) containing 15 mM MgCl₂, 5 mM DTT, 5 mM ATP, 5 mM phosphocreatine, 0.2 mM NADH, 10 mM NaHCO₃, 0.6 mM ribulose 1,5-bisphosphate, 20 mM glyceraldehyde-3-phosphate dehydrogenase, 10 U ml⁻¹ 3-phosphoglyceric acid kinase and 1 U ml⁻¹ creatine phosphokinase. The activity was measured from the supernatant after incubation with 20 mM NaHCO₃ and 20 mM MgCl₂. Rubisco content in the supernatant was determined spectrophotometrically by formamide extraction of Coomassie brilliant blue R on SDS–PAGE, as described above. Samples used for the Rubisco activation assay were collected from a leaf equilibrated at steady-state conditions in the gas exchange chamber. After gas exchange had reached the steady-state rate, the leaf in the chamber was taken and immediately frozen in liquid N₂. The activation state of Rubisco was determined according to Sage et al. (1993).

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References


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