Availability of Rubisco Small Subunit Up-Regulates the Transcript Levels of Large Subunit for Stoichiometric Assembly of Its Holoenzyme in Rice[1]

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Rubisco is composed of eight small subunits coded for by the nuclear *RBCS* multigene family and eight large subunits coded for by the *rbcL* gene in the plastome. For synthesis of the Rubisco holoenzyme, both genes need to be expressed coordinately. To investigate this molecular mechanism, the protein synthesis of two subunits of Rubisco was characterized in transgenic rice (*Oryza sativa*) plants with overexpression or antisense suppression of the *RBCS* gene. Total *RBCS* and *rbcL* messenger RNA (mRNA) levels and RBCS and RbcL synthesis simultaneously increased in *RBCS*-sense plants, although the increase in total *RBCS* mRNA level was greater. In *RBCS*-antisense plants, the levels of these mRNAs and the synthesis of the corresponding proteins declined to a similar extent. The amount of RBCS synthesized was tightly correlated with *rbcL* mRNA level among genotypes but not associated with changes in mRNA levels of other major chloroplast-encoded photosynthetic genes. The level of *rbcL* mRNA, in turn, was tightly correlated with the amount of RbcL synthesized, the molar ratio of RBCS synthesis to RbcL synthesis being identical irrespective of genotype. Polysome loading of *rbcL* mRNA was not changed. These results demonstrate that the availability of RBCS protein up-regulates the gene expression of *rbcL* primarily at the transcript level in a quantitative manner for stoichiometric assembly of Rubisco holoenzyme.

A number of key photosynthetic components in chloroplasts are multisubunit protein complexes composed of both nucleus- and chloroplast-encoded subunits (Malkin and Niyogi, 2000). For their proper assembly and the formation of photosynthetic machinery, gene expression between these cellular compartments must be well coordinated, and photosynthetic organisms have signaling systems between the nucleus and chloroplasts for this purpose (Pesaresi et al., 2007; Woodson and Chory, 2008; Waters and Langdale, 2009; Stern et al., 2010). It is also understood that the availability of nucleus-encoded subunits modulates the gene expression of chloroplast-encoded subunits at translational levels.

The photosynthetic carbon-fixing enzyme Rubisco has been extensively used for the model system, as Rubisco is composed of only two kinds of subunits, a nuclear multigene family-encoded small subunit (RBCS) and a chloroplast-encoded large subunit (RbcL; Dean et al., 1989; Spreitzer, 2003). In higher plants and green algae (*Chlamydomonas reinhardtii*), Rubisco holoenzyme is a hexadecamer composed of eight RBCS and eight RbcL subunits. Rubisco is the most abundant leaf protein in C3 plants and catalyzes the first steps in photosynthesis and photorespiration (Ellis, 1979; Lorimer, 1981; Evans, 1989; Makino et al., 1992), these rates being determined by Rubisco activity under conditions of saturating light and current atmospheric CO_2 and O_2 levels (Evans, 1986; Makino et al., 1988).

When the gene expression of *RBCS* was suppressed in higher plants such as tobacco (*Nicotiana tabacum*; Rodermel et al., 1988; Hudson et al., 1992), the C4 plant *Flaveria bidentis* (Furbank et al., 1996), and rice (*Oryza sativa*; Makino et al., 1997), the amounts of RBCS and RbcL proteins declined in a coordinated manner. In *RBCS*-suppressed tobacco, polysome loading of *rbcL* mRNA was reduced without a change in *rbcL* mRNA level (Rodermel et al., 1996; Wostrikoff and Stern, 2007), suggesting repression of the translation of *rbcL* mRNA. It has been suggested that a repressor motif in unassembled RbcL protein, otherwise not accessible, interacts with *rbcL* mRNA for the repression of its translation (Wostrikoff and Stern, 2007). Translational suppression of *rbcL* has also been observed in *Chlamydomonas* spp. with suppression of *RBCS* gene by its deletion (Khrebtukova and Spreitzer, 1996). These results demonstrate that the gene expression of *rbcL* undergoes negative-feedback regulation at translational

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levels in response to the availability of RBCS protein. This mechanism is similar to that first described for the hierarchical synthesis of the cytochrome b_6f complex within a chloroplast in *Chlamydomonas* spp., namely, the control by epistasy of synthesis (Kuras and Wollman, 1994; Choquet et al., 1998; Choquet and Vallon, 2000; Boulouis et al., 2011).

A similar mechanism is operative for other photosynthetic components in *Chlamydomonas* spp. For example, in the synthesis of the F1 subunit of chloroplast ATP synthase, oligomers of chloroplast-encoded subunits α and β repressed the translation of subunit β when these subunits were not assembled with a nucleus-encoded subunit γ (Drapier et al., 2007). Synthesis of the chloroplast-encoded cytochrome *f* was repressed when a nucleus-encoded subunit Rieske iron-sulfer protein was deleted or its assembly with cytochrome *f* was disrupted by a point mutation (de Vitry et al., 2004). These studies imply that translational modulation of chloroplast-encoded genes plays a key role in the stoichiometric assembly of various chloroplast multimetric protein complexes.

On the other hand, when endogenous *RBCS* was overexpressed in rice, the *rbcL* mRNA level also increased concomitant with a drastic increase in the total *RBCS* mRNA level (Suzuki et al., 2007, 2009a, 2009b). Rubisco content increased to an extent similar to that of the *rbcL* mRNA level. In addition, the *rbcL* mRNA level declined to a level similar to that of total *RBCS* mRNA in *RBCS*-suppressed rice plants with the antisense technique or the RNA interference technique for individual *RBCS* genes (Suzuki et al., 2009a, 2009b; Ogawa et al., 2012). These results imply that the coordinated expression of two genes is not fully explained by the translational modulation of the *rbcL* gene.

To explore the molecular mechanism of Rubisco synthesis in detail, the coordinated gene expression of *RBCS* and *rbcL* was characterized in transgenic rice plants with an overexpression or suppression of *RBCS*. Young, expanding leaves were used as samples, because Rubisco synthesis is the most active and Rubisco degradation is inactive during the development of rice leaves (Mae et al., 1983; Makino et al., 1984; Suzuki et al., 2001). Syntheses of RBCS and RbcL proteins were separately determined, and their relationships with the mRNA levels of *RBCS* and *rbcL* were quantitatively analyzed. Polysome loading was also examined as an index for the translational status of these genes. We obtained evidence for the positive regulation of transcript levels of *rbcL* by the availability of RBCS protein in the synthesis of the Rubisco holoenzyme.

RESULTS AND DISCUSSION

The Synthesis of Rubisco Subunits Is Coordinated in a Quantitative Manner in *RBCS*-Transgenic Rice Plants

Rubisco activities, Rubisco and total nitrogen (N) contents, and Rubisco N/total N ratios were determined in *RBCS*-sense (lines 26-8 and 35-4; Suzuki et al., 2007), *RBCS*-antisense (line AS-71; Makino et al., 2000), and wild-type rice plants (Table I). Rubisco activities were 1.5- to 1.7-fold higher in *RBCS*-sense plants and 76% in *RBCS*-antisense plants in comparison with wild-type plants. Similar result was obtained for Rubisco contents. In order to examine whether Rubisco contents were specifically changed in the transgenic plants, total N contents and Rubisco N/total N ratios were measured. Total N contents were used as an index for organic N contents. It is shown that most of the organic N is used as protein N in leaf tissue (Peoples and Dalling, 1988). Total N did not greatly differ among the genotypes, although the values in transgenic plants were slightly higher. Rubisco N/total N ratios were 1.3- to 1.4-fold higher in *RBCS*-sense plants and declined to 65% in *RBCS*-antisense plants. These results indicated that the amount of Rubisco was specifically increased in *RBCS*-sense plants but declined in *RBCS*-antisense plants. Since expanding leaves were used as samples, the Rubisco N/total N ratios were different from our previous results obtained from the uppermost, fully expanded leaves of rice (Makino et al., 2000; Suzuki et al., 2007, 2009a). The Rubisco N/total N ratios in the uppermost, fully expanded leaves of the same plants were 25.9% ± 0.4%, 31.5% ± 0.6%, 30.8% ± 0.6%, and 11.0% ± 0.8% for wild-type plants, *RBCS*-sense plants (lines 26-8 and 35-4), and *RBCS*-antisense plants (AS-71), respectively. These values were equivalent to those in our previous studies.

The amounts of RBCS and RbcL synthesized per unit of leaf fresh weight were determined by the incorporation of ^{15}N label by these subunits. RBCS synthesis was 1.6- to 2.1-fold higher in *RBCS*-sense plants and about 55% in *RBCS*-antisense plants (Fig. 1A). The amounts of RbcL synthesis were almost identical to those of RBCS synthesis irrespective of genotype, being

Table I. *Rubisco activities, Rubisco protein and total N contents, and Rubisco N/total N ratios*

These values were determined in young, expanding leaves of wild-type plants and *RBCS*-sense (26-8 and 35-4) and *RBCS*-antisense (AS-71) plants. Asterisks indicate statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$). Data are presented as means ± SE ($n = 3$). FW, Fresh weight.

Line	Rubisco Activity	Rubisco Protein Content	Total N Content	Rubisco N/Total N
	mmol CO_2 kg^{-1} FW s^{-1}	*mmol active site kg^{-1} FW*	*mol kg^{-1} FW*	*%*
Wild type	0.126 ± 0.008	0.092 ± 0.002	0.423 ± 0.003	16.8 ± 0.2
26-8	0.184 ± 0.006*	0.127 ± 0.000*	0.446 ± 0.004	22.1 ± 0.2*
35-4	0.220 ± 0.024*	0.152 ± 0.016*	0.501 ± 0.017*	23.5 ± 1.8*
AS-71	0.096 ± 0.002	0.065 ± 0.001	0.471 ± 0.016	10.8 ± 0.5*

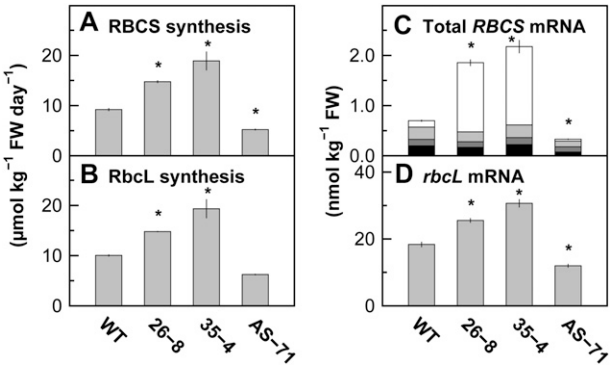

Figure 1. Protein synthesis and the mRNA levels of Rubisco. Protein synthesis of RBCS (A) and RbcL (B) and transcript levels of *RBCS* (C) and *rbcL* (D) were determined in expanding young leaves of wild-type (WT), *RBCS*-sense (26-8 and 35-4), and *RBCS*-antisense (AS-71) plants. Values are expressed per unit of leaf fresh weight (FW). Data are presented as means ± SE ($n = 3$). Asterisks indicate statistically significant differences from the values of wild-type plants by Dunnett's test ($P < 0.05$). In C, white, light gray, dark gray, and black columns represent transcript levels of *RBCS2*, *-3*, *-4*, and *-5*, respectively.

1.5- to 1.9-fold higher in *RBCS*-sense plants and about 60% in *RBCS*-antisense plants (Fig. 1B). These results indicate that the synthesis of Rubisco subunits is coordinated in a quantitative manner in young, expanding leaves of *RBCS*-sense and antisense rice plants to maintain stoichiometry between the subunits. It has been suggested in *Chlamydomonas* spp. that degradation of RBCS plays a role in the maintenance of stoichiometry between Rubisco subunits when the level of RbcL was decreased by blocking chloroplast protein synthesis (Schmidt and Mishkind, 1983). However, it was unknown how the synthesis and/or degradation of RbcL and RBCS are coordinated when RBCS is overproduced either in *Chlamydomonas* spp. or in higher plants.

The Transcript Level of *rbcL* Is Modulated to Synthesize RbcL Equivalent to RBCS

Four out of five members of the *RBCS* multigene family that are mainly expressed in leaf blades (Suzuki et al., 2007, 2009a, 2009b) were determined on a leaf fresh weight basis. In *RBCS*-sense plants, total *RBCS* mRNA levels were 2.7- to 3.1-fold higher than in wild-type plants, because of drastic increases in the mRNA level of the transgene *RBCS2* without changes in the levels of other members (Fig. 1C). *rbcL* mRNA levels also increased 1.5- to 1.9-fold of the wild-type level, but the difference was smaller than that in total *RBCS* mRNA levels (Fig. 1D). The total *RBCS* mRNA level in *RBCS*-antisense plants was about 50% of the wild-type level, because the mRNA level of each member of *RBCS* declined (Fig. 1C). The *rbcL* mRNA level also declined to about 65% of the wild-type level (Fig. 1D). The 18S ribosomal RNA per total RNA was determined as an internal standard, and no statistically significant difference was found among the genotypes (data not shown).

To examine the relationships between the synthesis of Rubisco subunits and the corresponding mRNA levels, the relationships between the amounts of RBCS and RbcL syntheses and their corresponding mRNA levels were analyzed (Fig. 2). In each graph, a dashed line passing through the origin and the wild-type data are drawn to quantitatively predict the amounts of protein synthesis as a function of the mRNA levels on the assumption that this relationship remains constant in the wild-type plants. When the relationships between Rubisco contents and the mRNA levels of total *RBCS* and *rbcL* at their maxima were analyzed in leaves of rice plants grown under different N nutrition levels, data approximately fell on the line passing through the origin and the control N data (Suzuki et al., 2007). Although RBCS synthesis was positively correlated with the total *RBCS* mRNA levels irrespective of genotype, RBCS synthesis measured in *RBCS*-sense plants was 60% to 64% of that predicted from the wild-type data, whereas RBCS synthesis measured in *RBCS*-antisense plants was almost identical to the predicted value (Fig. 2A). RbcL synthesis and the *rbcL* mRNA level were also positively correlated with each other irrespective of genotype (Fig. 2B). Measured RbcL synthesis was very close to that predicted from the wild-type data (95%–115%).

In addition, polysome loading of Rubisco transcripts was examined after fractionation with the Suc gradient as an index for their translational status. In wild-type plants, the pattern of mRNA distribution showed two peaks in *RBCS2*, *-3*, and *-5* and a single peak at lighter fractions in *RBCS4* (Fig. 3, A–D). In *rbcL*, a single peak with a small shoulder at lighter fractions was observed in the middle of the fractions (Fig. 3E). In *RBCS*-sense plants, substantial changes in the distribution pattern were not observed even in the transgene *RBCS2*. In *RBCS*-antisense plants, drastic changes were also not observed, although a slight shift to lighter fractions was observed in *RBCS2* (Fig. 3A), which was used for antisense suppression. Although a similar tendency

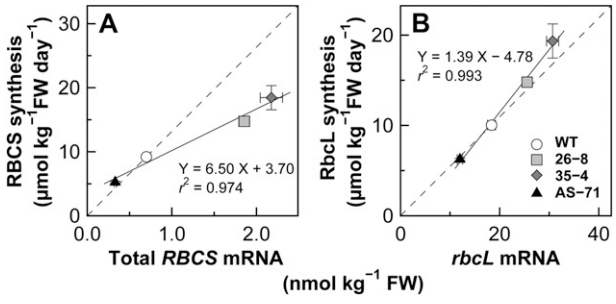

Figure 2. Relationships between protein synthesis and transcript levels of Rubisco. A, RBCS synthesis versus total *RBCS* mRNA level. B, RbcL synthesis versus *rbcL* mRNA level. Circles, squares, diamonds, and triangles represent the wild-type plant (WT), *RBCS*-sense line 26-8, *RBCS*-sense line 35-4, and *RBCS*-antisense line AS-71, respectively. Data are taken from Figure 1. A dotted line is drawn through the origin and data from wild-type plants. FW, Fresh weight.

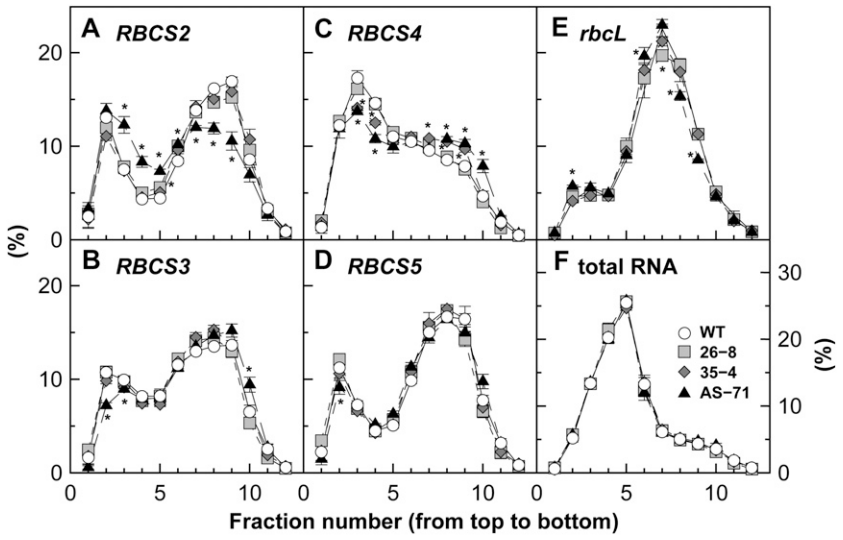

Figure 3. Polysome loading of Rubisco mRNAs. Transcript levels of each fraction were expressed as percentages of the sum of all fractions. A to E show the data of *RBCS2*, *-3*, *-4*, and *-5* and *rbcL*, respectively. F shows the distribution of total RNA among the fractions. Symbols are the same as in Figure 2. Asterisks indicate statistically significant differences from the values of wild-type plants (WT) by Dunnett's test ($P < 0.05$).

was found for *rbcL* (Fig. 3E), the extent was not as great as that observed by Rodermel et al. (1996) with *RBCS*-antisense tobacco, where the distribution of *rbcL* mRNA drastically shifted to lighter fractions. The amounts of total RNA were highest in fraction 5 irrespective of genotype (Fig. 3F). The distribution pattern was similar irrespective of genotype and was independent of the pattern of mRNA distribution.

Since mRNA levels and protein synthesis were tightly correlated to each other without a change in polysome loading in *rbcL*, it is indicated that RbcL synthesis is primarily determined by *rbcL* mRNA level in *RBCS*-transgenic rice plants. Since the amounts of RBCS and RbcL synthesized were almost identical irrespective of genotype (Fig. 1, C and D), it is also indicated that *rbcL* mRNA level is adjusted to synthesize RbcL equivalent to RBCS. It is understood that translational modulation of chloroplast-encoded subunits plays a key role in the assembly of chloroplast multimetric proteins (Rodermel et al., 1996; de Vitry et al., 2004; Drapier et al., 2007; Wostrikoff and Stern, 2007). In the case of Rubisco, it has been suggested that gene expression of *rbcL* undergoes negative-feedback regulation by excessive RbcL protein in tobacco (Rodermel et al., 1996; Wostrikoff and Stern, 2007). The reason for the discrepancy between these and our studies is not clear. It may be that transcriptional and translational regulations differ in importance in terms of Rubisco synthesis in different plant species. It has previously been suggested that these regulations are not mutually exclusive (Rodermel, 1999). For example, we have found in Arabidopsis (*Arabidopsis thaliana*) plants that the suppression of one or two major *RBCS* genes led to decreases in *rbcL* and total *RBCS* mRNA levels, although the decline in *rbcL* mRNA level was relatively smaller (Izumi et al., 2012). This finding suggests that modulation of *rbcL* mRNA level is also operative in Arabidopsis but that the extent is weaker than in rice and the translational regulation contributes to a greater extent. In addition, it has been suggested that nuclear factors are involved in the assembly of a specific chloroplast multisubunit protein complex at a posttranslational level. For example, maize (*Zea mays*) transposon mutants lacked the cytochrome b_6/f complex, although each subunit was normally synthesized (Voelker and Barkan, 1995). It is still unknown whether such a mechanism is operative for the assembly of Rubisco holoenzyme.

On the other hand, increases in the amounts of RBCS synthesized were much lower than those of the total *RBCS* mRNA levels in *RBCS*-sense plants (Fig. 2A), suggesting posttranscriptional down-regulation of RBCS synthesis. However, since polysome loading of *RBCS* mRNAs did not greatly differ among the genotypes (Fig. 3, A–D), it is unlikely, at least, that gene expression of *RBCS* is primarily modulated at translation initiation. Although the mechanism for the down-regulation of Rubisco synthesis in *RBCS*-sense plants is still unclear, it has been reported that the rate of translation was independent of polysome loading of the mRNA in some cases (Fütterer and Hohn, 1996). For example, protein synthesis of Rubisco rapidly declined when illuminated amaranth (*Amaranthus hypochondriacus*) seedlings were transferred to dark conditions (Berry et al., 1988). Light intensity did not affect polysome loading of the leaf catalase mRNA in rye (*Secale cereale*; Schmidt et al., 2002), although its protein turnover rate increased in a dose-dependent manner along with light intensity (Hertwig et al., 1992). In these cases, it has been suggested that translational initiation and elongation are modulated in a coordinated manner.

The Availability of RBCS Up-Regulates Transcript Levels of *rbcL*

In order to examine the interaction of gene expression of *RBCS* with that of *rbcL*, relationships between *rbcL* mRNA levels and the amounts of RBCS synthesized and *RBCS* mRNA level were analyzed. A dashed line passing thorough the origin and the wild-type

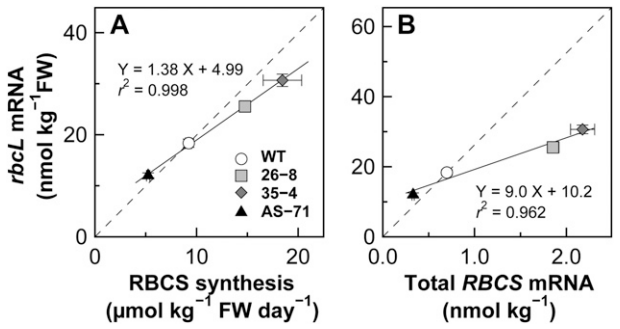

Figure 4. Relationships between *RBCS* and *rbcL*. A, *rbcL* mRNA level versus RBCS synthesis. B, *rbcL* mRNA level versus total *RBCS* mRNA level. Data are taken from Figure 1. Symbols and lines are the same as in Figure 2. FW, Fresh weight, WT, wild type.

data are also drawn in each graph. The *rbcL* mRNA level and RBCS synthesis were positively correlated with each other irrespective of genotype (Fig. 4A). Measured *rbcL* mRNA levels were close to those predicted from the wild-type data (83%–115%). On the other hand, although *rbcL* and total *RBCS* mRNA levels were also positively correlated with each other, *rbcL* mRNA levels measured in *RBCS*-sense plants were about 50% of the predicted values (Fig. 4B). These results show that *rbcL* mRNA level is tightly coupled not with total *RBCS* mRNA level but with the amounts of RBCS synthesized. *petA* (cytochrome *f* apoprotein), *atpB* (β-subunit of chloroplast ATP synthase), *psbA* (reaction center protein of PSII), and *psaA* (reaction center protein of PSI) were selected as reference genes of chloroplast-encoded major photosynthetic components. The expression levels of these genes were not affected by changes in the amounts of RBCS synthesized (Fig. 5). Therefore, it is indicated that the availability of RBCS protein primarily up-regulates *rbcL* mRNA level in a specific, quantitative manner for stoichiometric assembly of Rubisco holoenzyme in rice. This means that a nucleus-encoded subunit can play a role as a positive regulator for gene expression of its chloroplast-encoded assembly partner in the synthesis of chloroplast multimetric protein complexes, as predicted previously by Ellis (1977).

It is still an open question how the *rbcL* mRNA level is positively regulated by the RBCS protein. One possible explanation is enhancement of the transcription of *rbcL*. Chloroplast photosynthetic genes are transcribed by plastid-encoded RNA polymerase (PEP). PEP presumably requires nucleus-encoded σ factors, which determine the promoter specificity of the major PEP (Lysenko, 2007; Lerbs-Mache, 2011). Thus, the σ factors regulate the first step of chloroplast gene expression. However, the function of σ factors is likely to be redundant, and a specific one(s) for *rbcL* has not been reported. Protein factors such as CSP41 (Bollenbach et al., 2009) and NARA5 (Ogawa et al., 2009) have been reported to be involved in the transcription of *rbcL* via PEP in Arabidopsis, but they also affect the mRNA levels of genes for other chloroplast photosynthetic components. Thus, a pathway of specific transcriptional activation of *rbcL* has not yet been clarified. The stability of an mRNA also affects its steady-state level. In *Chlamydomonas* spp., nucleus-encoded factors that stabilize a specific chloroplast mRNA by preventing 5′-to-3′ exonucleolytic degradation has been found for genes encoding light-harvesting and electron transport components in the thylakoid membrane such as *petA* (Loiselay et al., 2008; Boulouis et al., 2011), *psbD* (Kuchka et al., 1989; Nickelsen et al., 1999), and *psbB* (Monod et al., 1992; Vaistij et al., 2000a, 2000b). An mRNA-stabilizing factor has also been found for *rbcL*, but its role in higher plants may be marginal because a defect in its ortholog in Arabidopsis only slightly affected the amount of Rubisco (Johnson et al., 2010). Thus, major factors that stabilize *rbcL* mRNA have not yet been identified in higher plants. Unknown factors may be involved in the process of the coordinated gene expression between *RBCS* and *rbcL*.

CONCLUSION

In summary, we demonstrated in young, expanding leaves of rice that the availability of RBCS protein

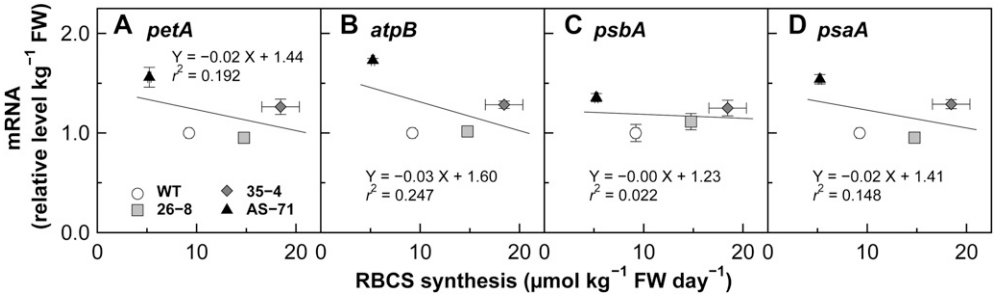

Figure 5. Relationship between mRNA levels of other chloroplast photosynthetic components and RBCS synthesis. A to D show the results of *petA*, *atpB*, *psbA*, and *psaA*, respectively. These transcript levels are expressed as relative values where the data from the wild-type plants (WT) are defined as 1. Symbols are the same as in Figure 2. Data are presented as means ± SE ($n = 3$). FW, Fresh weight.

primarily up-regulates *rbcL* mRNA level, which determines the amount of RbcL synthesized, and that, consequently, the stoichiometry between RBCS and RbcL is maintained. Which factor(s) mediates between RBCS protein and *rbcL* mRNA level and to what extent transcriptional and posttranscriptional processes contribute to adjustments of the expression of Rubisco genes are questions of interest to be studied. In addition, since the amount of Rubisco synthesized drastically changes during leaf development (Mae et al., 1983; Makino et al., 1984; Nikolau and Klessig, 1987; Bate et al., 1991; Suzuki et al., 2001, 2010; Imai et al., 2008), it is also of interest whether the *rbcL* mRNA level is regulated by RBCS protein in the leaves at different developmental stages.

MATERIALS AND METHODS

Plant Culture and ^{15}N Labeling

Rice (*Oryza sativa* 'Notohikari') was transformed with *RBCS2* complementary DNA in the sense orientation under the control of its own promoter (Suzuki et al., 2007). From these varieties, T3 progeny of lines Sr-26-8 and Sr-35-4 were used here. Rice transformed with *RBCS2* complementary DNA in the antisense orientation (line AS-71 with about 40% of wild-type Rubisco in mature leaves; Makino et al., 2000) and nontransformed rice 'Notohikari' were also used. Plants were grown hydroponically in an isolated and temperature-controlled greenhouse (Suzuki et al., 2009a) with slight modification. The greenhouse was maintained with a 14-h photoperiod (5 AM to 7 PM) under natural sunlight conditions supplemented with six 400-W metal halide lamps, day/night temperatures of 25°C/20°C, and 60% relative humidity. The final concentration of N in nutrient solution (Makino et al., 1988) was increased to 3 mM (1.5 mM NH_4NO_3). Plants were labeled with ^{15}N for the measurements of RBCS and RbcL synthesis. When 11th leaves became one-third of their final length, the plants were fed with nutrient solution without N. A solution of $(^{15}NH_4)_2SO_4$ (30.5 atom % excess) was then added to the nutrient solution to feed 0.94 mmol of N per plant. The next day, the pH of the nutrient solution was adjusted to 5.5 to 6.0 and the plants were fed with the same amount of $(^{15}NH_4)_2SO_4$ again. The following day, the 11th leaves were collected, weighed, immediately frozen in liquid N_2, and stored at −80°C until analysis. All samples were collected between 11 AM and 1 PM.

Determination of Total N and Rubisco Protein and Activity

Frozen leaves were homogenized in sodium phosphate buffer, and their N contents were determined with Nessler's reagent after Kjeldahl digestion as described by Suzuki et al. (2007). Rubisco content was determined by formamide extraction of 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 purified rice Rubisco (Makino et al., 1985) or by image analysis using Multi Gauge version 3.1 (Fuji Film). Rubisco activity was measured spectrophotometrically by coupling 3-phosphoglyceric acid formation with NADH oxidation at 25°C as described by Nakano et al. (2000) with slight modifications. Prior to the assay, sample homogenate was treated with Na_2SO_4 at 4°C for 30 min and then desalted (Parry et al., 1997). A final concentration of 250 mM Na_2SO_4 was found to be best for the removal of sugar-phosphate inhibitors with respect to Rubisco activity (data not shown). For desalting, a Zeba Spin Desalting Column (Thermo Scientific) was used.

Measurement of Rubisco Synthesis

RBCS and RbcL were purified by preparative SDS-PAGE (Suzuki et al., 2010). The amounts of RBCS and RbcL were calculated from the amounts of Rubisco holoenzyme and the ratio of molecular mass between RBCS and RbcL, since these subunits in unassembled form did not accumulate highly, even when gene expression of the other was suppressed (Rodermel, 1999). ^{15}N abundances of these proteins were measured by emission spectography (Yoneyama et al., 1975) using a ^{15}N analyzer (N-151; JASCO), and the amounts of RBCS and RbcL synthesized were calculated as described by Mae et al. (1983).

RNA Analysis

Sample leaves were homogenized with a small amount of acid-washed quartz sand in the presence of liquid N_2 using a mortar and pestle. An aliquot was used for the extraction of total RNA according to Suzuki et al. (2004) with slight modification (Suzuki et al., 2009a). Another aliquot was used for the analysis of polysome loading. Samples were prepared according to Sugimoto et al. (2004) with slight modifications. Heparin was removed from all the solutions used. A sample (0.8 mL) was layered onto 8 mL of Suc gradient (15%–55%) and centrifuged at 32,000*g* for 140 min at 4°C using an ultracentrifuge model 55P-72S equipped with a RPS40T rotor (Hitachi Koki). Twelve 0.69-mL fractions were collected by gentle pipetting from the top of the gradient and transferred into new tubes. After the addition of 69 μL of a solution of 5% (w/v) SDS, 0.2 M EDTA (pH 8), and 2 μL of nucleic acid carrier (Ethachinmate; Nippon Gene), RNA was extracted from each fraction from the step of chloroform-isoamyl alcohol extraction, all the steps being carried out at room temperature. The obtained RNA pellet was dissolved in nuclease-free water, isopropanol precipitated, washed with 75% (v/v) ethanol, and dissolved in 100 μL of nuclease-free water. RNA concentration was determined by A_{260}. The mRNA levels of *RBCS* multigene family and *rbcL* were determined by real-time quantitative PCR after reverse transcription (Ogawa et al., 2012). In the case of polysome analysis, equal volumes of RNA samples (less than 300 ng of RNA) were reverse transcribed with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). The *RBCS2* mRNA levels in *RBCS*-antisense plants were determined by amplification of the region including the open reading frame followed by agarose gel electrophoresis and SYBR Green detection (Suzuki et al., 2007). The primer pairs used for other than Rubisco genes were as follows: 5′-TGAATGTGGGTGCTGTTCTTATTT-3′ and 5′-TCGGGCGGCGCTAAT-3′ for *petA*; 5′-GGGAGCTGGAGTAGGTAAAACAGTA-3′ and 5′-CCCCCGTGAGCTTTAGCAA-3′ for *atpB*; 5′-GGCATACCATCAGAGAAACTTCCT-3′ and 5′-GTTGCAGCTGCTACTGCTGTTTT-3′ for *psbA*; and 5′-GAGGCTCATAAAGGCCCATTT-3′ and 5′-GAGCATGCCATGACGTTGTT-3′ for *psaA*.

Sequence data from this article can be found in GenBank/EMBL databases under the following accession numbers: *RBCS2*, Os12g0274700; *RBCS3*, Os12g0291100; *RBCS4*, Os12g0292400; *RBCS5*, Os12g0291400; *rbcL*, OrsajCp033; *petA*, OrsajCp041; *atpB*, OrsajCp032; *psbA*, OrsajCp002; and *psaA*, OrsajCp025.

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