Translational downregulation of *RBCL* is operative in the coordinated expression of Rubisco genes in senescent leaves in rice

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Abstract

Rubisco gene expression was examined in detail in rice (*Oryza sativa* L.) leaves at different positions, i.e. expanding, mature, and senescent leaves. Rubisco small subunit (*RBCS*) synthesis and *RBCS* mRNA levels were maximal in expanding leaves and gradually became lower in mature and senescent leaves, with declines in those of the large subunit (*RBCL*) being relatively slower. The amount of synthesized *RBCL* per unit level of *RBCL* mRNA and polysome loading of *RBCL* mRNA declined in senescent leaves, whereas such phenomena were not observed for *RBCS*. These results suggested that gene expression of *RBCL* is downregulated at the level of its translation when a balance between *RBCL* and *RBCS* expression is disturbed by leaf senescence. It has been suggested that Rubisco protein is a positive regulator for *RBCL* mRNA level in expanding rice leaves, as judged from their stoichiometric relationship in *RBCS* transgenic rice plants. However, the ratio of the *RBCL* mRNA level to the amount of synthesized *RBCL* in senescent leaves was significantly higher than that in expanding leaves. Therefore, it is suggested that the decline in *RBCL* mRNA level in senescent leaves is not fully accounted for by that in the amount of synthesized *RBCS*. Effects of other factors such as the stability of *RBCL* mRNA may come into play.

Key words: gene expression, leaf senescence, rice, *RBCS*, *RBCL*, Rubisco.

Introduction

Rubisco (EC 4.1.1.39) is a key enzyme in photosynthesis and the most abundant leaf protein. It catalyses 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 at atmospheric levels of CO₂ and O₂ (Evans, 1986; Makino et al., 1988). Rubisco accounts for 15–30% of total leaf N content in C₃ species (Evans, 1989; Makino et al., 1992) and is important in both the C and N economy of the plant.

In higher plants, Rubisco is composed of eight small subunits, encoded by a nuclear multigene family (*RBCS*) (reviewed by Dean et al., 1989), and eight large subunits, encoded by a single gene (*RBCL*) in the chloroplast genome. It has been considered that expression of *RBCL* is modulated at the level of its translation for the coordinated expression between *RBCL* and *RBCS*. When the *RBCL* gene was suppressed by an antisense technique in tobacco (Rodermel et al., 1988; Hudson et al., 1992), a C₄ plant (*Flaveria bidentis*; Furbank et al., 1996), and rice (Makino et al., 1997), the amount of Rubisco holoenzyme declined. In *RBCS*-antisense tobacco, the *RBCL* mRNA level was unaffected, despite a substantial decline in *RBCS* mRNA level (Rodermel et al., 1988). Gene expression of *RBCL* was then downregulated primarily...
at the level of its translation initiation (Rodermel et al., 1996; Rodermel, 1999). Furthermore, it has been suggested that translation of RBCL is repressed by a repressor motif in unassembled RBCL protein that is otherwise not accessible (Wostrikoff and Stern, 2007). This autorregulation of Rubisco synthesis is similar to that first described for the cytochrome b6/f complex in Chlamydomonas, namely, control by epistasy of synthesis (CES; Kuras and Wollman, 1994; Choquet et al., 1998; Choquet and Vallon, 2000; Boulois et al., 2011).

In addition to tobacco, the translational modulation of RBCL is probably operative in Arabidopsis, as a decline in RBCL mRNA level has been found to be smaller than those in total RBCS mRNA level and the amount of Rubisco protein in rbcs mutants (Izumi et al., 2012).

In contrast, a different type of mechanism for the coordinated Rubisco gene expression has been found recently in rice. When the mRNA levels and protein synthesis of Rubisco subunits in young, expanding leaves were compared among RBCL-sense, RBCS-antisense, and wild-type plants, the RBCL mRNA level was observed to be tightly correlated not with RBCS mRNA levels but with the amount of synthesized RBCS protein (Suzuki and Makino, 2012). In contrast to tobacco, polysome loading of RBCL mRNA was relatively unaffected, even in RBCS-antisense plants. The RBCL mRNA level was then tightly correlated with the amount of synthesized RBCL protein, which was almost identical to that of RBCS protein synthesized. These results indicate that gene expression of RBCL is regulated at its transcript level in response to the availability of RBCS protein in young rice leaves.

It has been reported repeatedly that Rubisco gene expression changes in a coordinated manner during leaf development. In young leaves and/or developing leaf tissues, it has been shown that Rubisco is actively synthesized due to the accumulation of the mRNAs of Rubisco genes (wheat, Dean and Leech, 1982; barley, Nivison and Stocking, 1983; pea, Sasaki et al., 1987). During the life span of a leaf, the mRNA levels of both RBCS and RBCL have been found to decline with progress of leaf age in a number of plant species (amaranth, Nikolau and Klessig, 1987; maize, Loza-Tavera et al., 1990; bean, Bate et al., 1991; rice, Suzuki et al., 2001, 2009; eucalypt, Suzuki et al., 2010). Changes in Rubisco synthesis correspond approximately to those in the mRNA levels of Rubisco genes (Nikolau and Klessig, 1987; Bate et al., 1991; Suzuki et al., 2001, 2010). The balance between RBCS and RBCL expression may change during leaf development. For example, declines in RBCL mRNA level were slightly slower than those of RBCS (Nikolau and Klessig, 1987; Loza-Tavera et al., 1990; Bate et al., 1991; Suzuki et al., 2009, 2010). A similar trend was found when RBCL synthesis and RBCS synthesis were analysed separately (Nikolau and Klessig, 1987; Bate et al., 1991; Suzuki et al., 2010). However, how expression of Rubisco genes is coordinated is still unknown in relation to the differences in leaf age.

To examine this point, Rubisco gene expression was studied in detail in leaves at different positions, i.e. expanding, mature, and senescent leaves in rice. The amounts of synthesized RBCS and RBCL their corresponding mRNA levels were determined in these leaves and their relationships were analysed quantitatively. Polysome loading of the Rubisco genes was also analysed as an index for their translational status.

### Materials and methods

All experimental procedures have been described by Suzuki and Makino (2012). The followings are brief explanations.

#### Plant culture and 15N labelling

Rice (Oryza sativa L. cv Notohikari) plants were grown hydroponically in an isolated and temperature-controlled greenhouse. One plant was grown in a 1.1 litre plastic pot and the distance between the pots was about 20cm, which did not lead to heavy mutual shading. Plants were labelled with 15N for measurements of RBCS and RBCL synthesis for 2 d when the 11th leaves became one-third of their final length. The 11th, 10th, and 9th leaves were then collected, weighed, immediately frozen in liquid N2, and stored at –80ºC until analysis. All samples were collected between 11:00 and 13:00 h.

#### Rubisco determination

Rubisco content was determined by SDS-PAGE of leaf homogenate followed by formamide extraction of Coomassie Brilliant Blue R-250-stained bands corresponding to RBCS and RBCL using calibration curves prepared with purified rice Rubisco (Makino et al., 1985).

#### 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. The 15N abundances of these proteins were measured by emission spectrography (Yoneyama et al., 1975) using a 15N-analyser (N-151; JASCO, Tokyo, Japan) and the amounts of synthesized RBCS and RBCL were calculated as described by Mae et al. (1983).

#### RNA analysis

RNA preparation and polysome separation was carried out based on the methods of Suzuki et al. (2004) and Sugimoto et al. (2004), respectively, with slight modifications (Suzuki and Makino, 2012). The mRNA levels of Rubisco genes were determined by real-time quantitative PCR after reverse transcription (Ogawa et al., 2012).

#### Statistical analysis

Three independent plants were analysed per data plot and are shown as means ± standard error (SE) (n=3 biological replications). Dunnett’s test was performed with JMP (SAS Institute Inc., Cary, NC, USA).

### Results and discussion

#### Gene expression of RBCL becomes relatively stronger than that of RBCS in senescent rice leaves

Rubisco contents and the amounts of its subunits synthesized were determined in rice leaves at different positions. The 11th, 10th, and 9th leaves were young expanding leaves, the uppermost fully expanded mature leaves, and senescent leaves,
respectively. Rubisco content was lowest in the 11th leaves, highest in the 10th leaves, and became slightly lower in the 9th leaves (Fig. 1A). The amounts of synthesized RBCS and RBCL were highest in the 11th leaves and gradually decreased in the 10th and 9th leaves (Fig. 1B, C). The decline in RBCL synthesis was relatively slower than that of RBCS synthesis.

Although the molar ratio of RBCL synthesis to RBCS synthesis was almost identical in the 11th leaves, it became 1.4- and 2.2-fold higher in the 10th and the 9th leaves, respectively (Fig. 1D). This indicated that RBCL synthesis was excessive in comparison with RBCS synthesis in mature and senescent leaves. In spite of the changes in Rubisco synthesis, the relative band intensities between the two subunits on SDS-polyacrylamide gels did not differ, irrespective of leaf position (data not shown). This can be explained by the fact that the amount of excessive RBCL synthesized was much smaller than that of Rubisco holoenzyme (Fig. 1A–C).

Differences in the mRNA levels of total RBCS and RBCL among these leaf positions showed a trend similar to those in synthesis of the corresponding subunits (Fig. 2A, B).
decline in \( \text{RBCL} \) mRNA level with progress of leaf age was slower than that in the \( \text{RBCS} \) mRNA level. In the 11th leaves, the molar ratio of \( \text{RBCL} \) mRNA to total \( \text{RBCS} \) mRNA was 26 mol mol\(^{-1} \) and became 2.0- and 3.6-fold greater in the 10th and 9th leaves, respectively (Fig. 2C). These results indicated that gene expression of \( \text{RBCL} \) was relatively stronger than that of \( \text{RBCS} \) in senescent rice leaves, although the expression of these genes became inactive in contrast with those in young expanding leaves. A similar trend in protein synthesis and/or mRNA level of Rubisco subunits has been reported previously in a number of plant species (Nikolau and Klessig, 1987; Loza-Tavera et al., 1990; Bate et al., 1991; Suzuki et al., 2009, 2010). The amounts of total RNA and the levels of 18S rRNA per unit amount of total RNA, which is an internal standard for RT-PCR analysis, did not largely differ among leaves at different positions (data not shown).

**Translational downregulation of \( \text{RBCL} \) is operative in senescent leaves**

In order to examine relationships between the synthesis of Rubisco subunits and the corresponding mRNA levels, the ratios of protein synthesis to mRNA levels were calculated (Fig. 3). Data obtained from the young, expanding 11th leaves of \( \text{RBCS} \)-transgenic rice plants (Suzuki and Makino, 2012) were also analysed. RBCS synthesis per unit level of total \( \text{RBCS} \) mRNA in the 10th and 9th leaves tended to be slightly higher than that in the 11th leaves (Fig. 3A). A similar trend was observed in the 11th leaves of \( \text{RBCS} \)-antisense plants, whereas the RBCS synthesis/total \( \text{RBCS} \) mRNA ratios were clearly lower in \( \text{RBCS} \)-sense plants (see Suzuki and Makino, 2012). In contrast, RBCL synthesis per unit level of \( \text{RBCL} \) mRNA in the 9th leaves was significantly lower than that in the 11th leaves (Fig. 3B), whereas there was no statistically significant difference among other samples. These results suggested that gene expression of \( \text{RBCL} \) is downregulated post-transcriptionally in senescent rice leaves, whereas such regulation is unlikely to occur in the case of \( \text{RBCS} \).

In order to examine whether expression of Rubisco genes is modulated translationally, polysome loading of \( \text{RBCS} \) and \( \text{RBCL} \) mRNAs was analysed further after fractionation on a sucrose gradient as an index for translation initiation (Fig. 4). In the 10th and 9th leaves, profiles of polysome loading of \( \text{RBCL} \) genes were almost the same as in the 11th leaves (Fig. 4A–D). This was in accordance with the fact that the RBSC synthesis/total \( \text{RBCS} \) mRNA level ratio did not decline with progress in leaf age (Fig. 3A). However, in the case of \( \text{RBCL} \), distribution of its mRNA to the 9th fraction declined in the 10th leaves (Fig. 4E). A substantial shift to lighter fractions was also observed in the 9th leaves, showing the lesser polysome-bound status of \( \text{RBCL} \) mRNA. These results indicated that expression of \( \text{RBCL} \) is downregulated at the level of its translation initiation in senescent rice leaves. The distribution of total RNA was similar to that observed by Sugimoto et al. (2004) and was relatively unaffected by leaf position (Fig. 4F).

In \( \text{RBCS} \)-suppressed tobacco, gene expression of \( \text{RBCL} \) has been thought to be translationally downregulated in the CES manner. For instance, the \( \text{RBCL} \) mRNA level was relatively stable despite a drastic decline in \( \text{RBCS} \) mRNA level (Rodermel et al., 1988, 1996; Wostrikoff and Stern, 2007). Excessive, unassembled RBCL probably interacts with \( \text{RBCL} \) mRNA (Wostrikoff and Stern, 2007), leading to a decline in its polysome loading and inhibition of its translation initiation (Rodermel et al., 1996; Wostrikoff and Stern, 2007). Senescent rice leaves also had high levels of \( \text{RBCL} \) mRNA relative to \( \text{RBCS} \) mRNA (Fig. 2C), excessive synthesized \( \text{RBCL} \) (Fig. 1D), and reduced polysome loading of \( \text{RBCL} \) mRNA (Fig. 4E). Therefore, it is considered in rice that the CES regulation of \( \text{RBCL} \) is operative to prevent the imbalance between the expression of \( \text{RBCL} \) and \( \text{RBCS} \) caused by leaf senescence, whereas the expression of these genes is adjusted primarily at the level of \( \text{RBCL} \) mRNA when Rubisco is actively synthesized in young expanding leaves (Suzuki and Makino, 2012). These results showed that the transcriptional and translational regulations of \( \text{RBCL} \) are important for different reasons in the coordinated Rubisco gene expression during leaf development in rice. On the other hand, translational modulation plays a key role in tobacco (Rodermel et al., 1988, 1996; Wostrikoff and Stern, 2007) and probably also in *Arabidopsis* (Izumi et al., 2012). In addition, quantitative analysis has shown that the decline in the synthesis of Rubisco subunits was slightly slower than the amounts of synthesized...
Rubisco subunits per unit level of the corresponding mRNA, which became slightly lower in senescent leaves of eucalyptus (Suzuki et al., 2010). This suggests that both RBCS and RBCL expression is regulated post-transcriptionally. These observations imply an interspecific difference in the coordinated expression of Rubisco genes although the molecular basis for the difference is yet to be studied.

It is possible that the translational regulation of RBCL is operative to a greater extent when Rubisco synthesis becomes inactive earlier because of accelerated leaf senescence. For example, senescence of leaves at lower positions in a canopy of herbaceous plant species is enhanced to retranslocate the N compounds derived from protein degradation to leaves at the top. The resulting non-uniform N distribution within a canopy leads to increase in N-use efficiency of canopy photosynthesis, since high leaf-N content is not reflected in photosynthetic rate under low irradiance at lower positions in a canopy (reviewed by Hikosaka, 2005; Hirose, 2005; Terashima et al., 2005). Elevated atmospheric CO2 conditions induce a reduction in Rubisco protein and mRNAs (Nie et al., 1995; Miller et al., 1997; Gesch et al., 1998; Theobald et al., 1998; Onoda et al., 2005; Seneweera et al., 2011; Zhu et al., 2011). Possibly, these environmental conditions induce the translational regulation of RBCL. Moore et al. (1999) reported that polysome loading of RBCL mRNA in mature leaves of tobacco was reduced under elevated CO2 conditions, whereas that of RBCS mRNA was unaffected. The decline in the mRNA level of RBCL was greater than that of RBCS. These observations may be related to accelerated leaf senescence.

In mature leaves, the symptoms of translational modulation of RBCL were not clear (Fig. 4E), while RBCL synthesis was relatively excessive compared with RBCS synthesis (Fig. 1D). The reason for this phenomenon is still unclear. It is possible that there is a threshold in the ratio of the amount of excessive RBCL protein to RBCL mRNA level that can effectively elicit the CES regulation.

The decline in RBCL mRNA levels in senescent leaves is not fully accounted for by that in the availability of RBCS protein.

It has been indicated previously that gene expression of RBCL is adjusted primarily at the transcript level in response to the availability of RBCS protein in young, expanding rice leaves, as RBCL mRNA level and RBCS synthesis were tightly correlated with each other among RBCS-transgenic and wild-type rice plants, their ratios being almost constant (Suzuki and Makino, 2012). Here, the ratios of the RBCL mRNA levels to RBCS synthesis were calculated among rice leaves at different positions (Fig. 5). Data obtained previously from RBCS-transgenic rice plants (Suzuki and Makino, 2012) are also presented again. The ratios in leaves at the lower positions became gradually higher than that in the 11th leaves, leading to more RBCL mRNA per RBCS synthesis. On the other hand, the ratios in young expanding leaves of wild-type and the RBCS-transgenic plants were similar to each other. Therefore, the decline in RBCL mRNA levels with progress of leaf age was not fully accounted for by that in the availability of RBCS protein. It is possible that other factors such...
as the stability of $RBCL$ mRNA may have come into play. Klaff & Gruissem (1991) treated spinach leaves with orga-
nelle-specific translation inhibitors that forced mRNAs into a polysome-bound state or depleted mRNAs of ribosomes and found that $RBCL$ and $PSBA$ mRNAs were less stable when bound to polysomes relative to the polysome-depleted mRNAs. This agrees with our finding that $RBCL$ mRNA was less polysome-bound in the 10th and 9th leaves (Fig. 4E), sug-
ggesting that an increase in the stability of $RBCL$ mRNA led to a slow decline in its level.

In summary, it is suggested that gene expression of $RBCL$ is downregulated at the level of translation in the CES man-
ner to match that of $RBCS$ in senescent rice leaves. Although the regulation of $RBCL$ at the transcript level is operative
primarily in young expanding leaves, it may not be predominant in senescent leaves. It is of interest and should be studied how the transcriptional and translational regulations operate in the coordinated Rubisco gene expression in other plant species. For this purpose, it will be necessary to determine syn-
thesis and mRNA levels of Rubisco subunits and polysome loading of these mRNAs in leaves of different ages in a wide
range of plant species.

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RBCS multigene family, function to yield sufficient Rubisco content

Fig. 5. Relative ratios of $RBCL$ mRNA levels to the amount of synthesized RBCS. Data are taken from Figs 1 and 2. The value in the 11th leaves was defined as 1. Data obtained from young expanding 11th leaves in RBCS-sense (lines 26-8 and 35-4) and RBCS-antisense (line AS-71) rice plants (Suzuki and Makino, 2012) are also presented. Data are presented as means ±SE ($n=3$). An asterisk indicates a statistically significant difference compared with the value of wild-type plants by Dunnet’s test ($P <0.05$).
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