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Expression level of Rubisco activase negatively correlates with Rubisco content in transgenic rice

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Author Comments:	Dear Dr. Bricker, Thank you very much for your e-mail on March 27, 2018, concerning our article entitled “Expression level of Rubisco activase negatively correlates with Rubisco content in transgenic rice” submitted to Photosynthesis Research (PRES-D-18-00006). We are also thankful to you and the reviewers for valuable comments. As you and the reviewers suggested, we carried out immunoblot analysis to determine the protein levels of Calvin-cycle enzymes. These data are newly included in the revised manuscript. The reviewer 3 suggested that there is a critical problem in the Coomassie Blue method to determine Rca in this study. Therefore, these data (Supplemental Fig S3 in previous manuscript) and its description were omitted from the manuscript. I hope that all the reviewer’s criticisms are answered in the revised manuscript. I should be obligated if you would consider the revised manuscript acceptable for publication in Photosynthesis Research. Sincerely yours Hiroshi Fukayama

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Abstract:

The relationship between ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase (Rca) levels was studied using transgenic rice overexpressing maize Rca (OX-mRca) and knockdown transgenic rice expressing antisense Rca (KD-Rca). The ratio of Rubisco to total soluble protein was lower in OX-mRca, whereas it was higher in KD-Rca than in WT, indicating that Rca expression was negatively correlated with Rubisco content. The expressions of other Calvin-Benson-Bassham cycle enzymes such as sedoheptulose-1,7-bisphosphatase and phosphoribulokinase analyzed by immunoblotting did not show such a negative correlation with Rca, suggesting that the effect of Rca on protein expression may be specific for Rubisco. Although Rubisco content was decreased in OX-mRca, the transcript levels of the Rubisco large subunit (OsRbcL) and the Rubisco small subunit mostly increased in OX-mRca as well as in KD-Rca. Additionally, polysome loading of OsRbcL was slightly higher in OX-mRca than it was in WT, suggesting that the OsRbcL translation activity was likely stimulated by overexpression of Rca. 35S-methionine labeling experiments demonstrated that there was no significant difference in the stability of newly synthesized Rubisco among genotypes. However, 35S-methionine labeled Rubisco was marginally decreased in OX-mRca and increased in KD-Rca compared to the WT. These results suggest that Rca negatively affects the Rubisco content, possibly in the synthesis step.

Response to Reviewers:

Responses to reviewers

We appreciate helpful comments by the reviewers. In the light of these comments, we have revised the manuscript. Answers to the comments and changes in the manuscript are as follows.

Reviewer 1

We are thankful for your favorable comments.

Reviewer 2

Thank you for valuable comments on our manuscript.

1. We do not have the antibodies against PGA kinase and FBPase. Instead, we obtained the antibodies raised against another three Calvin-cycle enzymes, aldolase, SBPase and PRK from Agrisera. SBPase and PRK are considered to be Calvin-cycle specific enzymes and suitable for this experiment. We carried out immunoblot analysis and clearly showed that the expression of Rubisco activase did not affect on the protein levels of aldolase, SBPase and PRK. These results are shown in Fig. 2. Concomitantly, the data of PGA kinase and FBPase activities are moved to the supplemental Fig. 3S. The corresponding texts in Abstract and Results sections are revised.

2. As you pointed out, reference Jin et al. (2006) is corrected.

Reviewer 3

We appreciate helpful comments on our manuscript.

As you pointed out, we produced the maize Rca overexpression line and the barley Rca overexpression lines. Because the expression level of Rca in the maize Rca lines was higher than that in the barley Rca lines, the decrease in Rubisco content in the maize Rca line was more marked than that in the barley Rca lines. Thus, we decided to use the maize Rca line in this study. We describe this point in the results section of revised manuscript.

1. I understand that there is a critical problem in the Coomassie Blue method to

	determine Rca in our experiment. Therefore, these data (Supplemental Fig S3 in previous manuscript) and its description were omitted from the manuscript. The expression level of Rca in Supplemental Fig. S1 was determined by immunoblot analysis. 2. This point was also pointed out by the reviewer 2. We carried out immunoblot analysis of three Calvin-cycle enzymes as mentioned in the response to the reviewer 2.
Additional Information:	
Question	**Response**

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Expression level of Rubisco activase negatively correlates with Rubisco content in transgenic rice

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Abstract

The relationship between ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase (Rca) levels was studied using transgenic rice overexpressing maize Rca (OX-mRca) and knockdown transgenic rice expressing antisense *Rca* (KD-Rca). The ratio of Rubisco to total soluble protein was lower in OX-mRca, whereas it was higher in KD-Rca than in WT, indicating that Rca expression was negatively correlated with Rubisco content. The expressions of other Calvin-Benson-Bassham cycle enzymes such as sedoheptulose-1,7-bisphosphatase and phosphoribulokinase analyzed by immunoblotting did not show such a negative correlation with Rca, suggesting that the effect of Rca on protein expression may be specific for Rubisco. Although Rubisco content was decreased in OX-mRca, the transcript levels of the Rubisco large subunit (*OsRbcL*) and the Rubisco small subunit mostly increased in OX-mRca as well as in KD-Rca. Additionally, polysome loading of OsRbcL was slightly higher in OX-mRca than it was in WT, suggesting that the *OsRbcL* translation activity was likely stimulated by overexpression of Rca. ^{35}S-methionine labeling experiments demonstrated that there was no significant difference in the stability of newly synthesized Rubisco among genotypes. However, ^{35}S-methionine labeled Rubisco was marginally decreased in OX-mRca and increased in KD-Rca compared to the WT. These results suggest that Rca negatively affects the Rubisco content, possibly in the synthesis step.

Keywords: photosynthesis, polysome loading, rice, Rubisco, Rubisco activase, protein turnover.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the first step of photosynthetic CO_2 assimilation and photorespiration (Ogren and Bowes 1971). In C_3 plants, Rubisco capacity is a major limiting factor of photosynthesis due to its low catalytic turnover rate and competing oxygenation reaction (Farquhar et al. 1980). Consequently, C_3 plants invest large amounts of Rubisco, dedicating approximately 15-30% of total leaf nitrogen in this single enzyme (Evans 1989; Makino et al. 1992). These characteristics make Rubisco a potential target for genetic engineering to improve photosynthesis and nitrogen use efficiency in plants.

To become catalytically functional, Rubisco needs to be activated by the binding of CO_2 to the lysine residue near the active site (carbamylation), followed by the binding of Mg^{2+} (Lorimer 1981; Portis 2003). In plants, some sugar phosphates, such as the substrate ribulose-1,5-bisphosphate (RuBP), a misfire product xylulose-1,5-bisphosphate, and a nocturnal regulatory compound carboxyarabinitol 1-phosphate (CA1P), bind to Rubisco active sites and inactivate it. Rubisco activase (Rca), an AAA+ family protein (ATPases associated with various cellular activities), mediates the activation of Rubisco by facilitating the ATP-dependent removal of these inhibitory sugar phosphates from the Rubisco active site (Portis 2003). This action is necessary for spontaneous carbamylation of Rubisco active sites *in vivo* and thus essential for photosynthetic CO_2 assimilation (Portis 2003). Many plants including rice contain large (45-46 kDa) and small (40-42 kDa) isoforms of Rca (Portis 2003). Unlike the small isoform, the large isoform has a C-terminal extension with redox sensitive Cys residues (Zhang and Portis 1999). The large isoform of Rca is regulated by chloroplast redox state via thioredoxin-f in response to varying light intensity (Zhang and Portis 1999).

Recombinant DNA technology has been used to address the extent of the limitation of photosynthesis imposed by Rubisco and Rca. Reduction of Rubisco has been shown to have a significant level of control over photosynthetic rate (Hudson et al. 1992). In the case of Rca, severe reductions to below 20% of wild type were necessary to affect the steady-state photosynthetic rate (Eckardt et al. 1997; Jiang et al. 1994; Mate et al. 1996; Masumoto et al. 2012). Additionally, overexpression of Rca did not enhance the photosynthetic rate in rice (Fukayama et al. 2012). These observations imply that Rca levels are higher than is required for the steady-state photosynthesis. In contrast, Rca levels is a major limiting factor of non-steady state photosynthesis after step increase in light intensity (Yamori et al. 2012). Moreover, Rca levels may indirectly affect the photosynthetic rate. Interestingly, it has been reported that Rubisco content was significantly increased in leaves of antisense Rca transgenic tobacco and rice (Mate et al. 1993; He et al. 1997; Jin et al. 2006). In addition, we previously showed that overexpression of Rca from maize and barley led to significant decreases in Rubisco content in transgenic rice (Fukayama et al. 2012). These results imply that Rca levels can negatively affect Rubisco content. In transgenic rice overexpressing Rca, the transcripts of Rubisco large subunit (*RbcL*) and Rubisco small subunit (*RbcS*) did not decrease, even though Rubisco content was significantly decreased (Fukayama et al. 2012). Therefore, Rca might affect the post-transcriptional step of Rubisco biosynthesis or the stability of Rubisco. However, the detailed mechanism of this phenomenon remains to be elucidated.

In this study, the mechanism how Rca affects Rubisco content was further studied using transgenic rice overexpressing Rca genes from maize and Rca antisense transgenic rice. We showed that the synthesis of Rubisco possibly after translation was negatively correlated with Rca expression. The possible mechanism and physiological significance of this interaction between Rubisco and Rca is discussed.

Materials and Methods

Plant materials and growth conditions

Rice (*Oryza sativa* L. cv Nipponbare) plants, antisense Rca transgenic rice plants with reduced amounts of Rca (KD-Rca, Masumoto et al. 2012) and transgenic rice plants overexpressing maize Rca (OX-mRca, Fukayama et al. 2012) were used in this study. All transgenic plants were derived from Nipponbare and homozygous T3-T4 generations. Rice seeds were sterilized with 1% (w/v) sodium hypochlorite solution and sown in seedling trays filled with nursery soil. The rice seedlings at the 4.5 leaf stage were transplanted into 1-L pots supplemented with a chemical fertilizer (N:P:K = 8:8:8) at 0.3 g N per pot. Rice plants were grown under natural light conditions in a temperature-controlled greenhouse (28°C day/23°C night) or grown in a growth chamber on a 28°C day/23°C night, with a 14 h photoperiod under illumination at a photon flux density of 500 µmol m^{-2} s^{-1}.

SDS-PAGE, Blue Native-PAGE and immunoblotting

Leaf tissues were homogenized in extraction buffer (50 mM Hepes-KOH pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.01 mM leupeptin, 1 mM phenylmethylsulfonylfluoride, 10% (w/v) glycerol, and 5% (w/v) polyvinylpolypyrrolidone) using a chilled motor and pestle with a small amount of quartz sand. The homogenates were centrifuged at 15,000 × g for 5 min at 4℃. The supernatants were collected as crude soluble protein extracts. The proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 or subjected to immunoblotting using antibodies raised against rice Rca (Fukayama et al. 2012), fructose-1,6-bisphosphate aldolase (Agrisera), sedoheptulose-1,7-bisphosphatase (Agrisera), and phosphoribulokinase (Agrisera). Blue native-PAGE was carried out using a precast gradient gel (NativePAGE Novex 3-12% Bis-Tris Gel, Life Technologies) according to the manufacturer's instructions as described previously (Ishikawa et al. 2011). The gels were stained with Coomassie Brilliant Blue R-250 or subjected to immunoblotting using antiserum raised against rice Rubisco. Immunoreactive bands were visualized using alkaline phosphatase as described previously (Masumoto et al. 2010) or using peroxidase with the ECL Select Western Blotting Detection Kit (GE Healthcare).

Determination of Rubisco, Rca and total soluble protein

Leaf tissues were homogenized in extraction buffer (50 mM Hepes-KOH pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 4 mM amino-n-caproic acid, 0.8 mM benzamidine, 0.05% (v/v) Triton X-100, 5% (w/v) glycerol, and 0.1% (w/v) polyvinylpolypyrrolidone) using a chilled motor and pestle with a small amount of quartz sand. The homogenate was then centrifuged at 15,000 × g for 5 min at 4°C. The supernatant was used for determination of Rubisco and total soluble proteins. Rubisco content was determined using [^{14}C] 2-carboxyarabinitol 1,5-bisphosphate (CABP) as described previously and calculated by assuming a stoichiometry of 6.5 molecules of CABP bound per molecule of Rubisco (Ishikawa et al. 2011). Total soluble protein was determined by a dye-binding method (Bradford 1976) with bovine serum albumin as the standard.

RT-PCR analyses

Total RNA was isolated from leaf tissues using the RNeasy Plant Mini Kit (Qiagen). DNA in the total RNA solution was digested by deoxyribonuclease (Nippon Gene). The 1st strand cDNA was synthesized from total RNA with oligo $(dT)_{18}$ and random hexamers as primers using PrimeScript II 1st Strand cDNA Synthesis Kit (Takara). Quantitative RT-PCR was performed essentially as described by Suzuki et al. (2009) using the gene specific primers listed in Supplementary Table S1. PCR was carried out using SYBR Premix Ex Taq GC (Takara) and Thermal Cycler Dice TP800 (Takara) according to the manufacturer's instructions. Expression of the actin gene (*Actin*, AB047313) was examined as an internal control.

Polysome loading analysis

The analysis of polysome loading was carried out according to the methods of Suzuki and Makino (2012). Sample leaves (200 mg) were ground in liquid N_2 using a mortar and pestle. The resultant leaf powder was suspended in 1 mL of polysome extraction buffer (200 mM Tris-HCl pH 9.0, 200 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, 25 mM EGTA, 20 mM DTT, 0.1 mg ml^{-1} chloramphenicol, 0.025 mg ml^{-1} cycloheximide, 2% (v/v) polyethylene-10-tridecyl, 1% (v/v) Triton X-100 and 800 units mL^{-1} Ribonuclease Inhibitor (Takara)). After 10 min on ice, the suspension was centrifuged at 15,000 g for 5 min. The supernatant was collected and sodium deoxycholate was added to a final concentration of 0.5% (w/v). After 5 min on ice, the mixture was centrifuged at 15,000 g for 5 min. The supernatant (0.85 mL) was layered onto an 8-mL continuous sucrose gradient solution (15%–55%) and centrifuged at 32,000 g for 140 min at 4°C using an ultracentrifuge (OPTIMA L, Beckman) equipped with a SW40Ti rotor. Twelve 0.69-mL fractions were collected by gentle pipetting from the top of the gradient solution and added 69 μL of a solution containing 5% (w/v) SDS and 0.2 M EDTA (pH 8), and 2 μL of nucleic acid carrier (Ethachinmate, Nippon Gene). RNA was extracted by the chloroform-isoamyl alcohol extraction method as described Sugimoto et al (2004). The obtained RNA pellet was dissolved in nuclease-free water. mRNA levels of *OsRbcL*, *OsRbcS2* and *Actin* were determined by qRT-PCR as described above.

^{35}S Pulse–Chase Labeling

The fourth leaf blades of 4.5 leaf stage seedlings were soaked in ^{35}S-labeling solution (10 mM Tris-HCl pH 7.5, 216 μCi mL^{-1} ^{35}S-methionine (Muromachi Yakuhin)) for 1 h under illumination in the growth chamber. After ^{35}S-labeling, the leaf blades were rinsed with distilled water. Sections of leaf blades (4 cm) were sampled at 1, 3 and 7 days after ^{35}S-labeling, immediately frozen in liquid nitrogen and stored at -80˚C until use. Leaf soluble proteins were extracted as described in the determination of Rubisco content and separated by SDS-PAGE. The gels were stained with Coomassie blue. The RbcL bands were cut out and treated with 30% (w/v) hydrogen peroxide at 50˚C over night. The mixture was cooled at room temperature and added the same volume of 2 M HCl and 10 times volume of Hionic-Fluor scintillation cocktail (PerkinElmer). The radioactivily of ^{35}S in the RbcL band was determined by liquid scintillation counting. The radioactively of leaf soluble protein extract was also determined.

Results

Rca content is negatively correlated with Rubisco content

In this study, we used the transgenic rice plants overexpressing maize Rca (OX-mRca, Fukayama et al. 2012) and expressing antisense *Rca* (KD-Rca, Masumoto et al. 2012). We previously produced the barley Rca overexpression lines as well as the OX-mRca lines (Fukayama et al. 2012). Because the expression level of Rca in the OX-mRca lines was higher than that in the barley Rca overexpression lines, the decrease in Rubisco content in the maize Rca line was more marked than that in the barley Rca overexpression lines. Thus, we decided to use the maize Rca line in this study.

The expression of Rca in these transgenic lines was analyzed by immunoblotting (Fig. 1a). Two types of Rca, i.e., the large isoform (OsRcaI) and the small isoform (OsRcaII) were detected in non-transgenic rice (WT). In addition, an Rca band smaller than OsRcaII was also detected possibly because of limited N-terminal proteolysis *in vivo* (Vargas-Suárez et al. 2004). The molecular mass of maize Rca is similar to that of OsRcaII. Thus, the band intensity corresponding to OsRcaII was substantially increased in OX-mRca. In contrast, the band intensities of both large and small isoforms were markedly decreased in KD-Rca. The sum of Rca band intensities in leaf blades at different positions (4-7th leaf) was quantified by NIH-image software after immunoblotting (Fig. 1b). In this experiment, the 7th leaf was the youngest developing leaf and the 4th leaf was the oldest leaf. As expected, band intensities of Rca were higher in OX-mRca and lower in KD-Rca than those in WT for all leaves. Rca band intensities in KD-Rca decreased with leaf age and were lowest in the 4th leaf blade, whereas these values were relatively constant in WT. In contrast, Rca band intensities in OX-mRca tended to increase with leaf age and were highest in the 5th leaf blade. Therefore, the differences in expression levels of Rca among genotypes were greater in mature and older leaves than those in young developing leaves.

In our previous study, we showed a negative correlation between Rca content and Rubisco content (Fukayama et al. 2012). In this study, the ratio of Rubisco to total soluble protein (Rubisco/protein) was analyzed in the same leaf extract used for immunoblot analysis of Rca (Fig. 1c). For all leaf positions, the ratio of Rubisco/protein was significantly decreased in OX-mRca compared to that in WT. In contrast, the ratio of Rubisco/protein was higher in KD-Rca than that in WT, and these differences were statistically significant in older leaves. However, independent of leaf position, Rca expression was negatively correlated with

Rubisco/protein (Fig. S1). Because Rubisco content was significantly lower in OX-mRca compared to that in WT, the photosynthetic rate was decreased in OX-mRca (Fig. S2), as observed in a previous report (Fukayama et al. 2012).

Protein levels of other Calvin-Benson-Bassham cycle enzymes

To clarify whether changes in protein content were specific to Rubisco, the protein levels of three Calvin-Benson-Bassham cycle enzymes, fructose-1,6-bisphosphate aldolase, sedoheptulose-1,7-bisphosphatase and phosphoribulokinase were determined by immunoblotting (Fig. 2). There were no significant differences in the protein levels of all these enzymes among genotypes. In addition, the activities of other two Calvin-Benson-Bassham cycle enzymes, phosphoglycerate kinase and fructose-1,6-bisphosphate phosphatase were determined (Fig. S3). PGA kinase activities were not significantly different in transgenic lines compared to WT. There has been no evidence of activity regulation by covalent modification in PGA kinase. Thus, this result suggest that the protein level of PGK would be similar among genotypes. FBPase activity in KD-Rca was lower than that in WT. Because the photosynthetic rate of KD-Rca was substantially lower than that of WT and OX-mRca (Fig. S2), the down-regulation of FBPase activity may occur in KD-Rca as reported in antisense RbcS transgenic rice plants (Ishizuka et al. 2004). Overall, these results suggest that the protein levels of Calvin-Benson-Bassham cycle enzymes do not undergo similar changes as Rubisco, i.e. they negatively correlate with Rca level in transgenic lines.

Expression of RbcS and RbcL

Rice contains one *RbcL* gene (*OsRbcL*) in the plastome and five *RbcS* genes (*OsRbcS1*-*OsRbcS5*) in the nuclear genome. Because *OsRbcS1* was not expressed in photosynthetic organs (Morita et al. 2014), expression analysis of this gene was omitted in this study. Rather, the transcript levels of *OsRbcL* and *OsRbcS2*-*OsRbcS5* were analyzed by qRT-PCR (Fig. 3). Although Rubisco contents were decreased in the leaves of OX-mRca, the expression levels of *OsRbcS2*-*OsRbcS5* in OX-mRca were significantly higher than those in WT in all analyzed leaves. The expression of *OsRbcL* was slightly increased in OX-mRca compared to that in WT. These increases in gene expression were largely consistent with previous results (Fukayama et al. 2012). In addition to the overexpression line, the expression of these genes was also higher in KD-Rca than that in WT. These responses may indicate a compensation effect of decreased Rubisco activity that occurred due to reduced Rubisco content in OX-mRca and decreased Rubisco activation in KD-Rca. Considering these findings, the difference in Rubisco content in Rca transgenic plants did not result from the transcriptional step but from a post-transcriptional step in Rubisco biosynthesis or through its degradation.

Polysome loading of OsRbcL and OsRbcS2

Among the rice *RbcS* gene family, *OsRbcS2* is a major *RbcS* and mostly contributes to the Rubisco content (Ogawa et al. 2012). Thus, the post-transcriptional regulation of *OsRbcS2* and *OsRbcL* was analyzed by polysome loading (Fig. 4 and S4). The mRNA distribution of *OsRbcS2* and *Actin* fractionated by sucrose density gradient centrifugation was detected as a single peak around fraction 8-10 in all genotypes. Essentially, there were no differences in the polysome distribution of *OsRbcS2* and *Actin* among the genotypes. The mRNA distribution of *OsRbcL* showed two peaks around fraction 2-3 and fraction 7-9. The lighter polysome peaked at 2-3 was lower in Ox-mRca (Fig. 4), whereas this trend was not reproducible in another set of samples (Fig. S4). The heavier polysomes peaked at fraction 7-9 in OX-mRca was slightly higher than that in KD-Rca and WT. These findings suggest that the translational activity of *OsRbcL* was slightly enhanced in OX-mRca. Therefore, the negative correlation between Rubisco and Rca may not be explained by altered translation of *RbcL* or *RbcS*.

Turnover of Rubisco

Pulse-chase labelling with ^{35}S-methionine was used to study the turnover of RbcL expressed in leaves of transgenic rice. The expression levels of RbcL were largely constant during this experiment (Fig. 5a). The percent of labeled RbcL per labeled soluble protein in all genotypes was almost constant during the time course of this experiment (Fig. 5b). However, the percent of labeled RbcL in KD-Rca was always higher than that in WT. In contrast, the percent of labeled RbcL in OX-mRca was slightly lower than that in WT. Therefore, the levels of ^{35}S-methionine incorporation into RbcL were higher in KD-Rca and lower in OX-mRca compared to those in WT. Importantly, this trend was observed from the early period of the experiment, one day after ^{35}S-labeling. Thus, it is likely that the synthesis rate of RbcL was higher in KD-Rca and lower in OX-mRca than that in WT, and after synthesis, the stability of RbcL did not differ among genotypes.

RbcL-chaperonin complex and Rubisco biosynthesis-related genes

When Rubisco synthesis is suppressed by the knockout of Rubisco assembly chaperons, such as Rubisco accumulation factor 1 (RAF1), the RbcL-chaperonin complex can be detected by Native-PAGE above the Rubisco band (Feiz et al. 2012). To stabilize the RbcL-chaperonin complex, a crosslinking treatment was carried out using formaldehyde. Rubisco bands were decreased to a similar degree by the crosslinking treatment in all genotypes (Fig. 6). The putative RbcL-chaperonin complex was detected by Native-PAGE/immunoblot analysis using antibodies raised against rice Rubisco. Although Rubisco bands were decreased, the bands of the putative RbcL-chaperonin complex were marginally increased by crosslinking treatment, especially in KD-Rca. However, no substantial differences in the band intensities of the RbcL-chaperonin complex were observed among genotypes.

The expression of genes required for Rubisco biosynthesis, such as RAF1, Rubisco accumulation factor 2 (RAF2), bundle sheath defective 2 (BSD2), chaperonin 60α1 (Cpn60α1) and RbcX (Brutnell et al. 1999; Saschenbrecker et al. 2007; Feiz et al. 2012; Kim et al. 2013; Feiz et al. 2014) were analyzed by qRT-PCR. The expression levels of these genes were not significantly different between transgenic lines and WT (Fig. S5). These findings indicate that Rca does not affect the expression of Rubisco biosynthesis related genes.

Discussion

In this study, we showed a significant decrease in Rubisco content in the leaves of transgenic rice overexpressing Rca and a significant increase in Rubisco content in leaves of an Rca antisense line (Figs. 1, S1). This result is consistent with previous reports showing a reduction in Rubisco in an Rca-overexpressing transgenic rice (Fukayama et al. 2012) and an increase in Rubisco content in knockdown transgenic plants (Mate et al. 1993; He et al. 1997; Jin et al. 2006). Therefore, it is likely that Rca has a negative effect on Rubisco content. To explain this negative effect, we can propose two possible mechanisms, namely, Rca may accelerate the degradation of Rubisco or inhibit its synthesis.

Considering the degradation of Rubisco, the susceptibility of Rubisco to proteolysis can differ depending on the activation state. It has been reported that binding of sugar phosphate inhibitors such as CA1P and RuBP significantly protected Rubisco against proteolytic degradation (Khan et al. 1999). Indeed, the activation state of Rubisco is high in Rca overexpression lines and low in knockdown lines compared to that of WT (Fukayama et al. 2012; Yamori et al. 2012). Thereby, Rubisco in Rca overexpression lines showing a high activation state would be more prone to be attacked by proteases than in knockdown lines. Therefore, it is possible that the expression level of Rca influences the activation state and leads to changes in the stability of Rubisco, which may affect the content of Rubisco in transgenic rice. Nevertheless, ^{35}S-methionine-labeled RbcL showed a similar stability among genotypes (Fig. 5). In addition, the activation level of Rubisco is usually high in RbcS knockdown plants (Makino and Sage 2007). However, Rubisco deficiency substantially delayed the decrease in Rubisco content during senescence in Rubisco knockdown rice (Ishizuka et al. 2004). In short, higher activation state does not always led to accelerate the decrease in Rubisco. From these observation, Rubisco stability related to the activation state is not considered to be an essential point of action of Rca regarding Rubisco content.

Rubisco has been suggested to be more stable than Rca *in vivo* (Yamaoka et al. 2016). The *Rca* transcript is highly expressed in plants, and its expression is higher than *RbcS* in rice (Yamaoka et al. 2016). However, the protein level of Rca is substantially lower, and less than three percent as abundant as Rubisco in rice (Fukayama et al. 1996). During leaf senescence, the expression of *RbcS* transcript rapidly decreased, whereas a significant level of *Rca* transcript was maintained in senescent leaves (Yamaoka et al. 2016). These observations imply that Rca is a labile protein *in vivo* and must be highly and continuously expressed to compensate for its instability. In fact, Rca protein is more susceptible to proteolysis than Rubisco in leaf extracts (Fukayama et al. 2010). Therefore, it is likely that the accumulation of Rca may involve a significantly higher cost, than we expected due to its instability. Furthermore, Rca can hydrolyze ATP even in the absence of Rubisco (Portis 2003). Because Rca is present in a large excess over normal photosynthesis in the overexpression line, this might waste a large amount of ATP. The ATPase activity of Rca may further increases the energy cost to maintain a higher expression level of Rca protein in the overexpression line. This can negatively affect the content of Rubisco. However, if this is the case, photosynthetic proteins other than Rubisco should also be altered in Rca transgenic rice. In this study, the protein levels of Calvin-Benson-Bassham cycle enzymes such as fructose-1,6-bisphosphate aldolase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase did not show a negative correlation with the expression level of Rca (Fig. 2). The differences in Rubisco content also seems to be rather specific in antisense Rca transgenic tobacco (He et al. 1997). Therefore, the effects of Rca are not universal and may possibly be limited to Rubisco. This hypothesis cannot explain the limited effect to Rubisco observed in Rca transgenic plants. Thus, the degradation or stability of Rubisco is not presumed to be a major factor affecting Rubisco content in Rca transgenic lines.

With respect to synthesis, the transcripts of *RbcS* and *RbcL* were both up-regulated in OX-mRca and KD-Rca (Fig. 3). This seems to be a compensation response to decreased photosynthesis caused by reduced Rubisco content or activation. Accumulation of sugar is known to down-regulate photosynthetic genes, including Rubisco (Jang et al. 1997). Thus, inversely, the decreased photosynthetic rate in OX-mRca and KD-Rca led to a decrease in the sugar content in leaves, and may up-regulate *RbcS* and *RbcL*. Up-regulation of *RbcS* and *RbcL* is inconsistent with the decreased Rubisco content in OX-mRca. In addition, the polysome-*RbcL* complex was slightly heavier in OX-mRca than that in WT (Fig. 4), an indication that translation is also enhanced in OX-mRca. From these observations, Rubisco biosynthesis steps from transcription to the translation are not considered to be a major factor affecting Rubisco content in Rca transgenic rice. In this study, ^{35}S-labeled RbcL was negatively correlated with Rca expression levels from the early period (Fig. 5). Therefore, the Rubisco biosynthesis step, especially, post-translational modification of RbcL, is a candidate point that may affect Rubisco content. After translation, RbcL undergoes folding and assembly to become hexadecameric L_8S_8 Rubisco. Assembly of RbcL and RbcS into the functional L_8S_8 Rubisco requires the assistance of many folding and assembly factors. Among these factors, the knockouts of RAF1, RAF2, BSD2 and Cpn60α1 lead to significant decreases in Rubisco content (Brutnell et al. 1999; Feiz et al. 2012; Feiz et al. 2014; Kim et al. 2013), suggesting that these are essential for Rubisco biosynthesis. Therefore, if Rca reduces the expression of these genes or interferes the action of these factors, Rubisco content would decrease. However, the transcript levels of RAF1, RAF2, BSD2 and Cpn60α1 in transgenic lines were similar to that of WT (Fig. S5), indicating that Rca does not affect the expression of these genes. Recently, it was found that Rca docks onto and interacts with a limited region of Rubisco over one active site at a time (Bhat et al. 2017). After folding, the RbcL forms an antiparallel dimer and then assembles into an octamer assisted by RAF1 or RbcX (Hauser et al. 2015). Finally, binding of RbcS displaces RAF1 or RbcX and completes assembly of the hexadecameric holoenzyme. The catalytic site is located at the interface of the dimer. Therefore, Rca might interact with the assembly intermediate of Rubisco after the formation of RbcL dimers and prevent subsequent assembly processes, including interaction with the assembly chaperone. This hypothesis, namely, the inhibition of Rubisco biosynthesis due to post-translational mechanisms by Rca, can explain the results obtained in this study. However, it remains a matter of speculation that needs to be verified.

There have been many reports showing marked changes in Rubisco content compared to the other photosynthetic proteins. For example, under elevated CO_2 conditions, the decrease in Rubisco content was remarkable among the photosynthetic proteins (Long et al. 2004). Treatments of high C/N, abscisic acid and rain predominantly reduced the content of Rubisco (Ishibashi et al. 1996; Kato et al. 2005; Fukayama et al. 2010). In Rubisco knockdown rice, the decrease in Rubisco content during senescence was notably slower compared to the other photosynthetic enzymes (Ishizuka et al. 2004). In addition, changes in sink-strength by fruit removal induces the formation of an insoluble form of Rubisco in soybean (Crafts-Brandner et al. 1991). Considering these observations, it is likely that Rubisco content is controlled by distinct mechanisms and more strictly so than the other photosynthetic enzymes in plants. Thus, it is possible that some important mechanism contributing to the strict regulation of Rubisco content in plants remain unknown.

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Legends to Figures

Fig. 1 Expression of Rca and Rubisco in transgenic rice.

a Expression of Rca analyzed by immunoblotting. Soluble proteins (6.4 μg) of 4th leaf blade of 4.5 leaf stage seedlings were separated by 10% SDS-PAGE. The large isoform of rice Rca (OsRcaI), small isoform of rice Rca (OsRcaII) and maize Rca (ZmRca) were detected by immunoblotting using anti-rice Rca antibodies. **b** Band intensity of Rca in leaf blades at different positions. The 4th to 7th leaf blades at the 6.5 leaf stage were used for immunoblotting blot analysis as above. The band intensity of Rca was determined using NIH-image software. **c** Rubisco content in leaf blades at different positions. The same samples as in Fig. 1b were used for the determination of Rubisco content by the ^{14}C-CABP method. Different letters indicate significant differences among genotypes by Tukey-Kramer test ($P<0.05$). WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Fig. 2 Expression of Calvin-Benson-Bassham cycle enzymes in transgenic rice.

a Expression of Calvin-Benson-Bassham cycle enzymes analyzed by immunoblotting. Soluble proteins (6.4 μg) of 3-5th leaf blades at the 4.5 leaf stage were separated by 12% SDS-PAGE and subjected to immunoblot analysis using antibodies raised against chloroplastic fructose-1,6-bisphosphate aldolase (FBA), sedoheptulose-1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRK). **b** Band intensity of Calvin-Benson-Bassham cycle enzymes analyzed by immunoblotting. Band intensity of Calvin-Benson-Bassham cycle enzymes in immunoblotting were determined using Image Lab software (Bio-Rad). Data represent the means ± SD of four replicates. There were no significant differences among genotypes by Tukey-Kramer test ($P<0.05$). WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Fig. 3 Expression of *RbcS* and *RbcL* in transgenic rice.

The expression of *OsRbcS2*, *OsRbcS3*, *OsRbcS4*, *OsRbcS5* and *OsRbcL* in the 4th to 7th leaf blades (the 7th leaf is the youngest developing leaf) at the 6.5 leaf stage were analyzed by qRT-PCR using gene specific primers (Supplemental Table 1). Expression data were normalized to *Actin.* Data represent the means ± SD of four replicates. Asterisks indicate significant differences between WT and transgenic lines using Student’s t test

(**P*<0.05, ***P*<0.01). WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Fig. 4 Polysome loading profile of *OsRbcL* and *OsRbcS2* in transgenic rice after fractionation by sucrose gradient centrifugation.

The 7th leaf blade at the 7.5 leaf stage was used for the experiment. The mRNA levels of *OsRbcL*, *OsRbcS2* and *Actin* were determined by qRT-PCR and expressed as percentages of the sum of all fractions. WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Fig. 5 Turnover of RbcL analyzed by ^{35}S pulse-chase labeling of rice leaf blades.

a SDS-PAGE of ^{35}S-labeled soluble protein in leaf blade. The 4th leaf blades at the 4.5 leaf stage were treated with ^{35}S-Met for 1 h and harvested at 1, 3 and 7 days after ^{35}S-labeling. Soluble proteins were separated by 12% SDS-PAGE and stained with Coomassie blue. **b** Ratio of ^{35}S-labeling of RbcL to soluble protein. RbcL bands of SDS-PAGE were cut out from the gel. ^{35}S in RbcL bands and total soluble protein were measured by liquid scintillation counting. WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Fig. 6 Blue Native-PAGE analysis of Rubisco.

The 4th leaf blades at the 4.5 leaf stage were treated with 1.85% formaldehyde for 0-80 min to crosslink Rubisco with interacting proteins. Soluble proteins (1.0 μg) were separated by 3-12% Native-PAGE. Soluble proteins were stained with Coomassie blue (upper panel). Rubisco was detected by immunoblotting using anti-rice Rubisco antibodies (lower panel). Positions of Rubisco and putative RbcL-chaperonin complex are shown. WT, non-transgenic rice; OX-mRca, transgenic rice overexpressing maize Rca; KD-Rca, Rca antisense transgenic rice.

Colour figure

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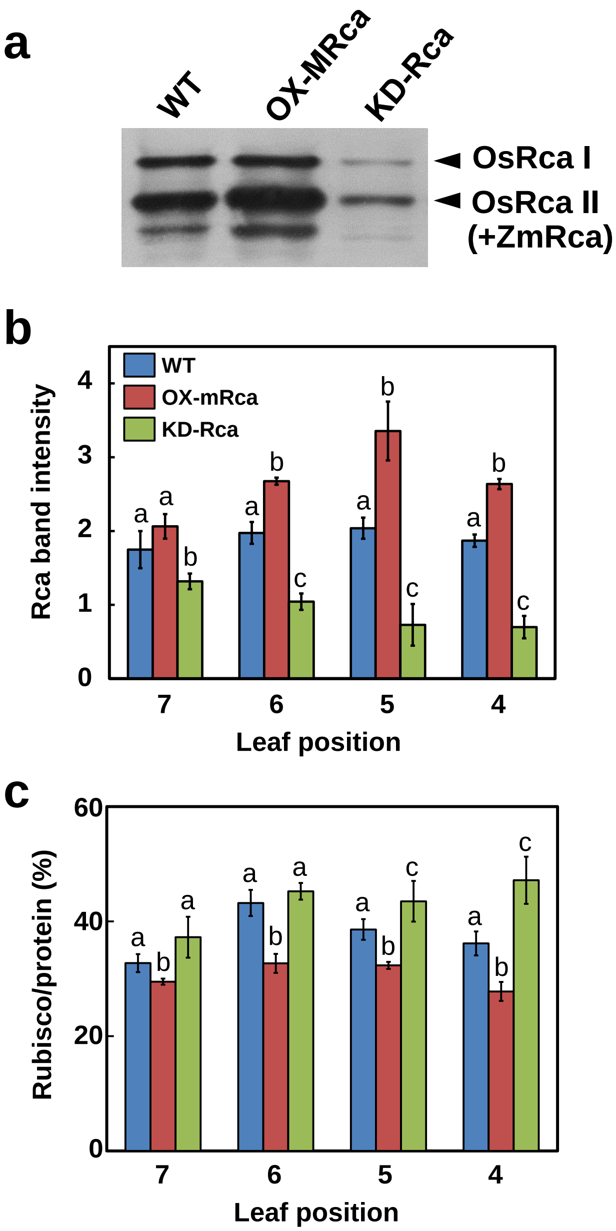
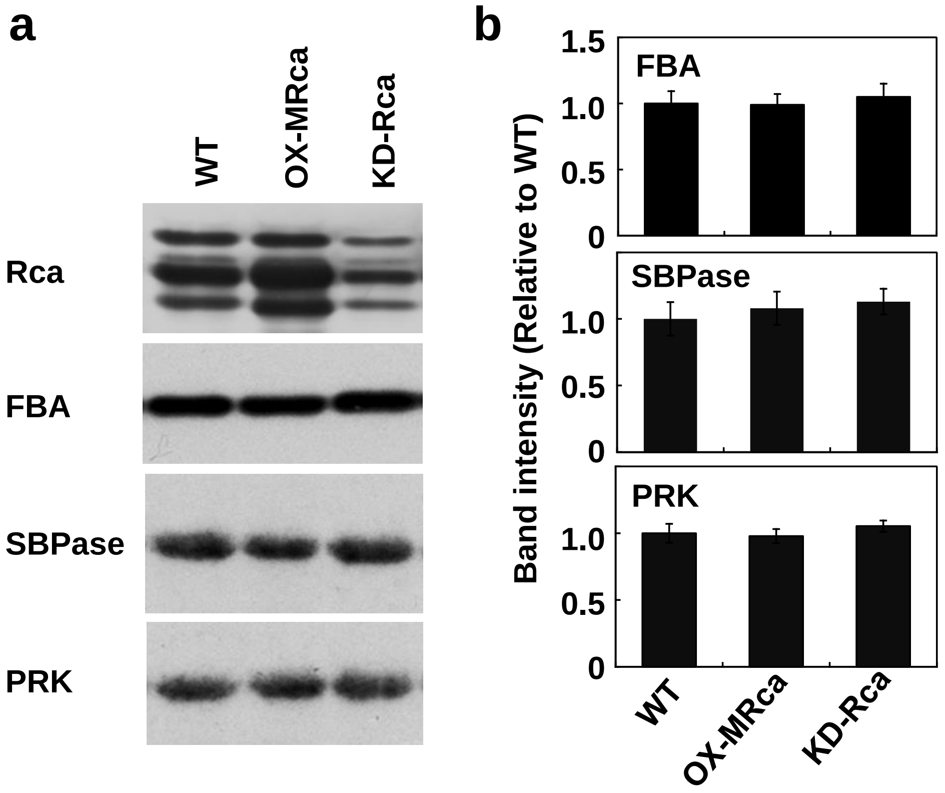


Fig.1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OX-MRca
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b
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SBPase
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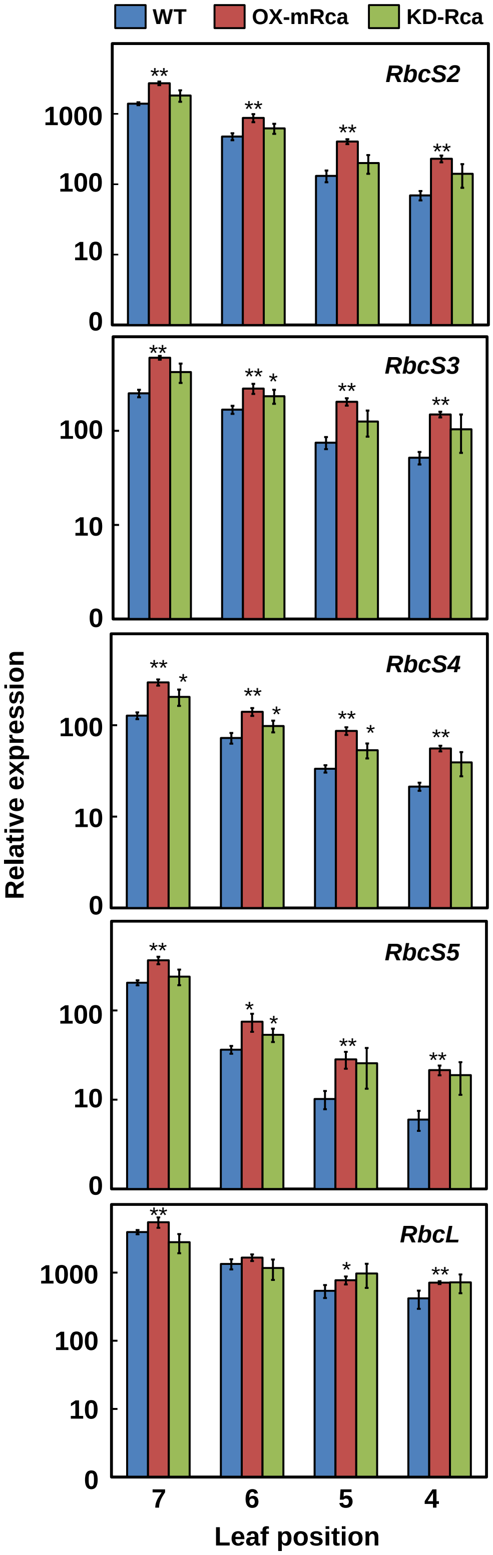
Band intensity (Relative to WT)
WT
OX-MRca
KD-Rca

Fig.2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RbcS4
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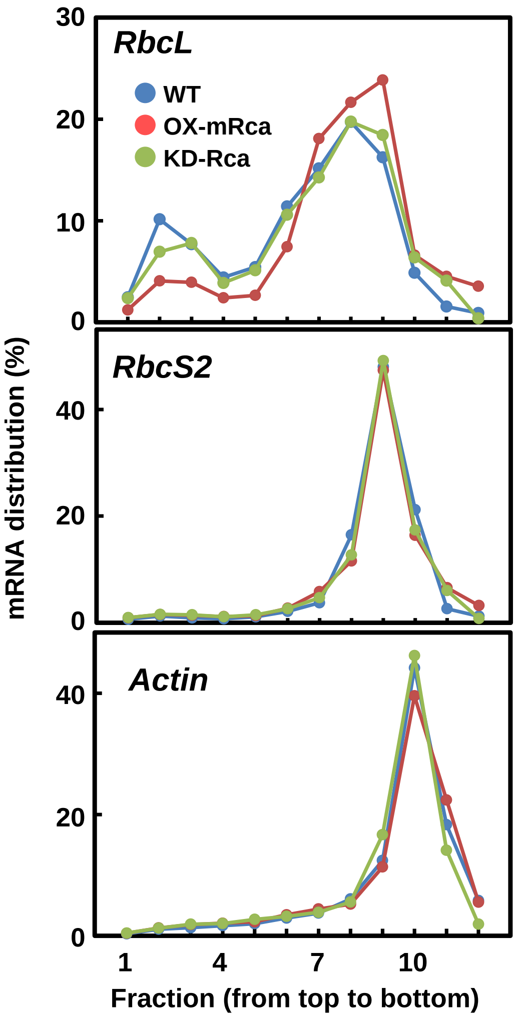
4
Leaf position

Fig.3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20
10
0
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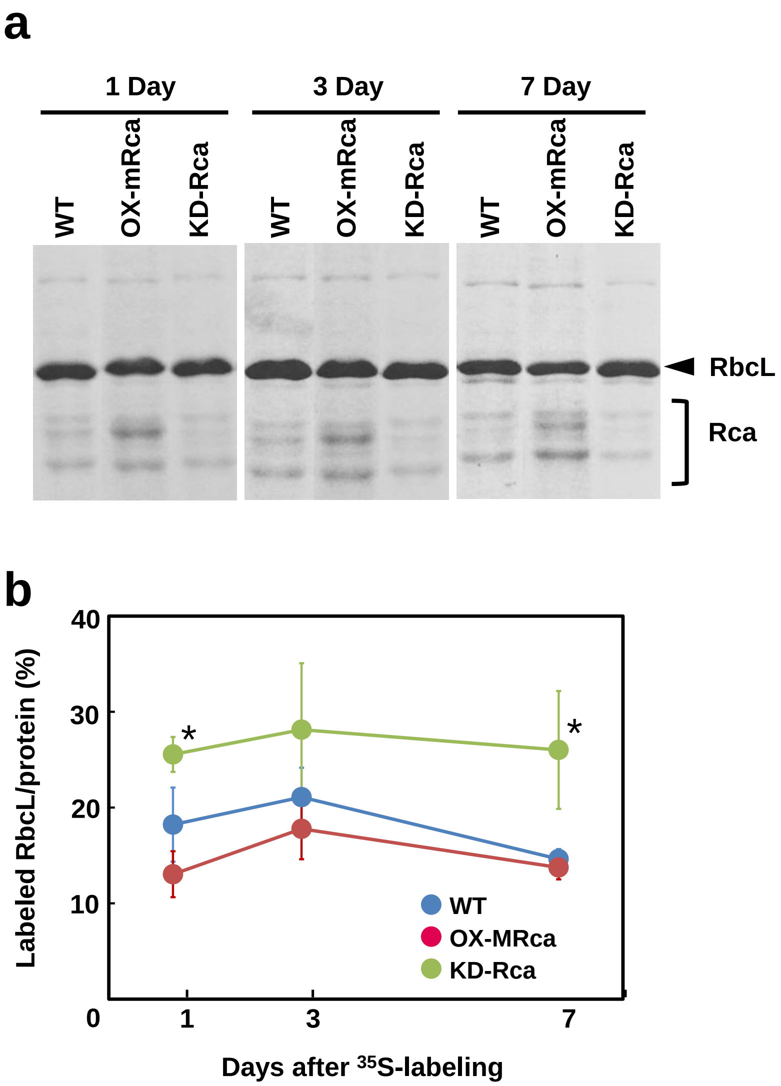
1
4
7
10
mRNA distribution (%)
Fraction (from top to bottom)

Fig.4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WT
OX-mRca
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WT
OX-mRca
KD-Rca
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OX-mRca
KD-Rca
RbcL
Rca
b
Labeled RbcL/protein (%)
40
30
20
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Days after ³⁵S-labeling
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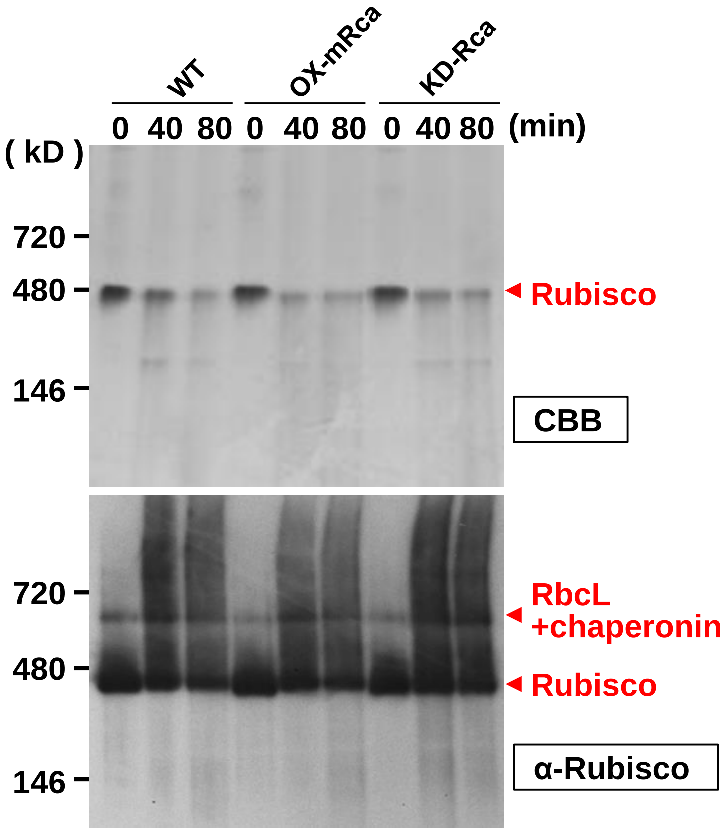
WT
OX-MRca
KD-Rca

Fig.5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146
Rubisco
CBB
720
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146
RbcL
+chaperonin
Rubisco
α-Rubisco

Fig.6

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