Plant Biotechnology 22(2), 145–149 (2005)

Short Communication

Synthesis of Rubisco gene products is upregulated by increasing the copy number of *rbcL* gene in *Chlamydomonas* chloroplast genome, without increased accumulation of the two Rubisco subunits

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Received February 21, 2005; accepted April 21, 2005 (Edited by M. Umeda)

Abstract In an attempt to increase the amount of Rubisco in a green alga, the *Chlamydomonas reinhardtii rbcL* gene connected to the *aadA* cassette was integrated into chloroplast inverted repeats in this organism to generate a transformant with three copies of *rbcL* (3L). Synthesis and accumulation of the *rbcL* transcript in 3L were 2.3 and 1.4 times those of the control, respectively. Although synthesis of the large subunit (LSU) in 3L was 2.6 times that in the control, accumulation of the LSU was only 1.1 times that in the control. Synthesis of *rbcS* transcript and the small subunit (SSU) in 3L was also upregulated to 1.7 and 1.3 times those in the control, respectively, while their accumulation was unaffected. These results suggest that an increase in *rbcL* gene number upregulates transcription of *rbcL* from chloroplast genomes and that of *rbcS* from a nuclear genome, but does not affect the accumulation of LSU and SSU.

Key words: *Chlamydomonas reinhardtii*, chloroplast gene transformation, *rbcL*, *rbcS*, Rubisco.

Carbon fixation due to photosynthesis starts with assimilation of CO_2 by Rubisco. The properties of this enzyme determine the photosynthetic efficiency and ultimately the productivity of plants (Woodraw and Berry 1988). Modification of Rubisco is expected to be one of the most prospective strategies to create a plant with higher photosynthetic ability (Spreitzer 1998; Uemura et al. 2000; Spreitzer and Salvucci 2002). While the enzymatic rate of eukaryotic Rubisco is slowest (Mann 1999), the number of Rubisco molecules is the most abundant protein in the cells of the photosynthetic organisms (Gutteridge and Keys 1985).

In a green alga *Chlamydomonas reinhardtii*, two Rubisco genes, large and small subunit genes, are split into chloroplast (Dron et al. 1982) and nucleus (Goldschmidt-Clermont and Rahire 1986) genomes. There have been reports on the reduced expression of *rbcL* (Mishkind and Schmidt 1983; Schmidt and Mishkind 1983; Spreitzer et al. 1985; Hong and Spreitzer 1994; Thow et al. 1994; Spreitzer et al. 1995), and *rbcS* (Khrebtukova and Spreitzer 1996) in this organism, but there have been no reports on the expression of the Rubico gene more than the wild-type level. Since the chloroplast inverted repeat region (Ohyama et al. 1986; Shinozaki et al. 1986) is duplicated on each of 80 chloroplast genomes in a *Chlamydomonas* chloroplast (Kuroiwa and Nakamura 1986; Gillham 1994), modification of this region might result in one of the most drastic changes in the expression of related genes. In the present study, we introduced a *Chlamydomonas rbcL* gene into the inverted repeat region of this organism, generated a three-*rbcL* *Chlamydomonas*, and characterized the expression of Rubisco genes in this transformant.

Chlamydomonas reinhardtii, 137c, *mt*+, was used as a transformation host in this study. Solidified tris-acetate-

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Abbreviations: LSU, large subunit; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit.

phosphate (TAP) medium (Gorman and Levine 1965) containing 50 μg ml^{-1} spectinomycin was used for transformation and Southern hybridization of cells. For characterization of Rubisco gene products, cells were cultured in liquid high salt minimal (HSM) medium (Sueoka 1960) containing spectinomycin in Erlenmyer flasks set on shakers at 25°C under approximately 30 μE^{-2} S^{-1} illumination with a 16-h photoperiod. Cultured cells grew to a stationary phase 1.5 weeks after inoculation, and gene expression analysis was performed using cells harvested at an early logarithmic growth phase, 4 days after the inoculation. Chloroplast transformation was performed using a particle delivery system (Boynton et al. 1988), PDS-1000/He (Bio-Rad, Hercules, CA). The 1 μm-gold particles were coated with 0.25 μg of a plasmid, and used for bombarding 1×10^8 host cells on TAP agar plates containing spectinomycin. For DNA integration between host chloroplast genome and transformation plasmids, the 5.5-kb *Eco*RI/*Xho*I region (pBSEX in Figure 1A) in the *Chlamydomonas* chloroplast R24 fragment (Rochaix and Malnoe 1978; Harris 1989) was used. Into a single *Bam*HI site in this region, the *aadA* cassette (Goldschmidt-Clermont 1991) alone (pC-EX), or the *Chlamydomonas rbcL* gene connected to the *aadA* cassette (pL-EX), was inserted to generate a transformation plasmid. The above-mentioned insert of pC-EX was a 1.9-kb *Eco*RV/*Sma*I fragment from pUC-atpX-AAD (Goldschmidt-Clermont 1991), while that of pL-EX was a 4.1-kb fragment consisting of a 2.7-kb *Hpa*I fragment from R15 (Rochaix and Malnoe 1978; Harris 1989) and a 1.4-kb *Hpa*I/*Sma*I fragment from the pUC-atpX-AAD. Spectinomycin-resistant colonies that appeared three weeks after the bombardments, were restreaked on fresh plates two more times. Spectinomycin-resistant colonies (*mt*+) were crossed with wild-type *mt*− cells, and the *mt*+ progenies were isolated. Transformant progenies were kept on TAP agar plates containing spectinomycin. To check the integration of plasmid fragments into chloroplast genomes in the transformants, we performed Southern blot analysis (Figure 1B). The *rbcL* ORF probe (probe B1 in Figure 1A) detected a 3.2-kb band in DNAs from the wild-type (host) cells, and in a pC-EX transformant candidate (a control), while this probe hybridized with a 3.0-kb band as well as the 3.2-kb band in DNA from a pL-EX transformant candidate (3L). In 3L, the intensity of the 3.0-kb band was much stronger than that of the 3.2-kb. This stoichiometry of the bands suggested that 3L had two exogenous *rbcL* genes and an endogenous one. To check the homoplasmicity in the transformant chloroplast genomes, we performed Southern blot analysis using probe B2 as shown in Figure 1A. This probe detected a 4.1-kb band in the wild-type, a 2.6-kb band in the control, and a 2.7-kb band in 3L (Figure 1B).

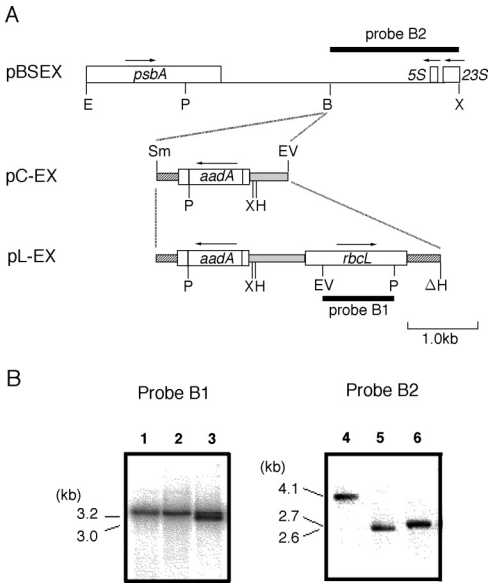

Figure 1. Introduction of a *Chlamydomonas rbcL* gene into inverted repeats in the chloroplast genome. A, Physical maps of plasmids used. The 5.5-kb *Chlamydomonas* chloroplast inverted repeat region was cloned into a pBluescript vector whose MCS *Bam*HI site was disrupted (pBSEX). A *aadA* cassette only (pL-EX), or *Chlamydomonas rbcL* gene connected to the *aadA* cassette (pC-EX) was inserted into a *Bam*HI site in insert of pBSEX. Shown are *Bam*HI (B), *Eco*RI (E), *Eco*RV (EV), *Hpa*I (H), *Pst*I (P), *Sma*I (Sm), *Xho*I (X), 5S rDNA (5S), and 23S rDNA (23S). Arrows and closed boxes indicate gene transcription directions and probe regions used in panel B, respectively. Scale bar is indicated at the bottom. B, Southern blot analysis of transformants obtained with bombardment of pC-EX, or pL-EX. In the left panel, DNA was digested with PstI and hybridized with probe B1. Lanes1, 2 and 3 are of wild-type, transformant progenies from pC-EX and pL-EX, respectively. In the right panel, DNA was treated with *Pst*I/*Xho*I and then hybridized with probe B2. Lanes 4, 5 and 6 are of wild-type, transformant progenies from pC-EX and pL-EX, respectively.

After extended exposure of this autoradiogram, the 4.1-kb band was not detected in 3L, nor in the control (data not shown), which suggested that chloroplast genomes in these transformants were homoplasmic.

Rubisco gene transcript synthesis was characterized by slot blot analysis (Edington and Hightower 1990) of transcripts pulse-labeled with [$^{32}PO_4$] (Pace et al. 1970) for 20 min. Accumulation of the transcripts was analyzed by Northern blot analysis of total RNA using [^{32}P]-labeled probes. Plasmids specific for *C. reinhardtii rbcS* gene family (Goldschmidt-Clermont and Rahire 1986), *rbcL* gene (Dron et al. 1982), and ribosomal DNA were used for hybridization. Quantification of Rubisco gene transcripts was analyzed by radioactivity measurements using FLA-3000 (Fujifilm, Tokyo, Japan). For characterization of Rubisco subunit protein synthesis, cells were pulse-labeled with [^{35}S] methionine (Mori et al. 1981) for 5 min,chased with non-labeled methionine for 1 h, and the proteins were subjected to SDS-PAGE followed by autoradiography. Accumulation of the subunit protein was analyzed by SDS-PAGE followed by

CBB staining, and by Western blot analysis using anti-spinach Rubisco holoenzyme antibodies diluted 1 : 12000. Quantification of Rubisco subunit proteins was analyzed by densitometry of autoradiograms and PVDF membranes using FASIII equipped with Quantity One (Toyobo, Osaka, Japan).

The *rbcL* transcript synthesis in 3L, which was detected by the pulse-labeling experiment, was 2.3 times that in the control (Figure 2A). This was in contrast to 16S rRNA synthesis in 3L, which was only 1.1 times that in the control. The transcript synthesis of *rbcS* in 3L was also 1.7 times that in the control. Then, we examined the accumulation of these transcripts. In Northern hybridization, 16SrRNA amount in 3L was equal to that of the control (Figure 2B). The amount of *rbcL* transcript accumulated in 3L was 1.4 times that in the control, while that of *rbcS* was almost the same level as in the control.

Figure 2C shows the synthesis of Rubisco subunit proteins revealed by pulse-labeling. Relative LSU synthesis within 1 h in 3L was 2.6 times that in the control (Figure 2C). Relative SSU synthesis in 3L was 1.3 times that of the control. To examine Rubisco subunit accumulation, we subjected soluble protein extracted from each culture to SDS PAGE followed by CBB staining. The accumulation of both LSU and SSU in 3L was detected in amounts equal to those in the control and wild-type (data not shown). Then, in immunoblots with anti-Rubisco holoenzyme antibody, we examined the relative subunit band intensity of 3L to that in the control (Figure 2D). By quantitative analysis of immuno-stained Rubisco subunits, both LSU and SSU in 3L detected in almost the same levels as those in the control.

We have introduced two *rbcL* genes into the chloroplast genome to produce the transformant 3L. The amounts of *rbcL* transcript (Figure 2A) and LSU (Figure 2C) synthesized in 3L were 2.3 and 2.6 times those in the control, respectively. To our knowledge, this is the first report to show increase of *rbcL* gene product synthesis in a transgenic *Chlamydomonas*. Large and small subunit genes, which are split into chloroplast and nuclear genomes, act together in gene expression. Downregulated expression of *rbcS* was observed in *rbcL*-mutants and cells treated with prokaryotic inhibitor (Schmidt and Mishkind 1983; Spreizer et al. 1985; Thow et al. 1994), and lowered *rbcL* expression was reported in a *rbcS*-deleted strain (Khrebtsukova and Spreitzer 1996). In 3L, synthesis of *rbcS* and SSU was 1.7 and 1.3 times that in the control, respectively (Figures 2A, 2C). The present study shows that the increase in *rbcL* gene number upregulated the synthesis of *rbcS*. These results suggest that intra-genome crosstalk of Rubisco genes may function in both positive and negative regulation. In contrast, *rbcS* transcript and SSU were accumulated in levels almost equal to that in the control (Figures 2B, 2C)

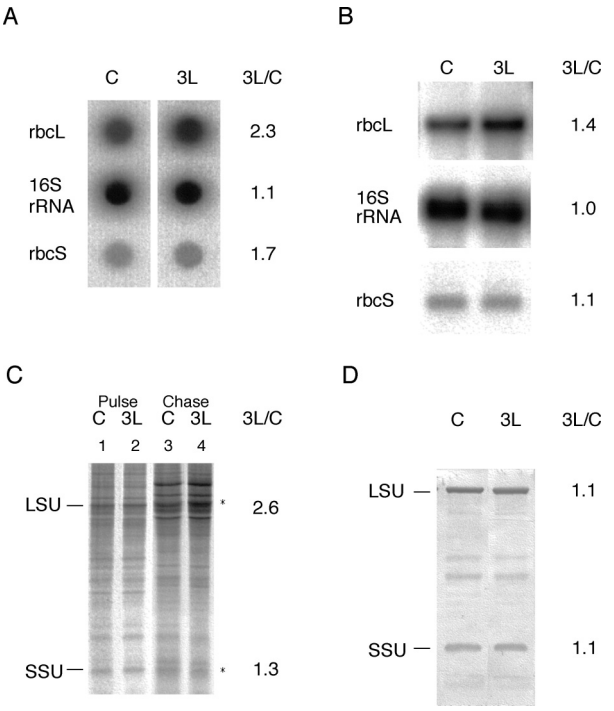

Figure 2. Syntheses and accumulations of Rubisco gene products in 3L transformant as compared with those in the control. A, Slot blot hybridization of *in vivo* pulse-labeled transcripts in 3L or control. RNAs isolated from ^{32}P-orthophosphate labeled cells of 3L (3L) or control (C) were hybridized with a plasmid of either *rbcL*, *16SrDNA* or *rbcS* on the same membrane. Relative radio activities in 3L transcripts to those in the control are shown at the right. B, Northern blot hybridization of transcripts in 3L or control using a ^{32}P-dCTP labeled probe of either *rbcL*, *16SrDNA* or *rbcS*. Relative radioactivities in 3L transcripts are shown as in panel A. C, The *in vivo* pulse labeling of total cell proteins in 3L or in the control. Cells were labeled with [^{35}S]methionine for 5 min (lanes 1, 2) and chased with non-labeled methionine for 1 h (lanes 3, 4). The proteins were extracted, and equal amounts of radioactivity were subjected to SDS/PAGE followed by fluorography. Lanes 1, 3 are from the control, 2 and 4 from 3L, respectively. Strips of the gel were subjected to CBB staining and western blot analysis, and Rubisco subunits (*) in the corresponding autoradiogram were recognized. In both strains, the subunit amounts in the chase were divided by those in the pulse, and the relative 3L values to those of the control are shown as in panel A. D, Western blot analysis using soluble proteins from 3L (3L), or the control (C). Equal amounts of proteins extracted from the two strains were subjected to SDS PAGE and Western blot analysis using antiserum against Rubisco holoenzyme. Relative intensity of subunit bands in 3L to those in the control were measured using a quantification software. In panels A to D, experiments were performed two times and gene product amount measurement was performed two times to obtain an average in each experiment. Mean values of relative 3L amounts of either Rubisco product are shown in a representative photo.

and the wild-type (data not shown), and accumulation of LSU in 3L was only 1.1 times that in the control (Figures 2B, 2D). These results suggest that the accumulation of LSU and SSU in 3L soluble proteins would remain at levels equal to those in the wild-type, and keep a 1 : 1 molecule ratio. A previous report showed that particular surfaces of SSU and LSS in the holoenzyme contribute to holoenzyme thermal stability (Chen et al. 1988; Chen

et al. 1993; Du and Spreitzer 2000), suggesting that LSU and SSU would be required at equal moles for proper conformation of Rubisco. In 3L, SSU accumulation kept at an endogenous level might retract over-synthesized *rbcL* gene products from accumulation. A model of plant Rubisco holoenzyme assembly has been proposed (Roy 1989). In this model, the LSU monomer associates with Rubisco subunit binding proteins, and an octamer of LSU was formed with the consumption of ATP, and the LSU octamer associates with SSUs. Increase of SSU, enhancement of chaperonin activity, or more ATP supply in 3L might increase Rubisco accumulation.

Acknowledgments

We thank Drs. Michel Goldschmidt-Clermont, University of Geneva, for providing us plasmids, pR15, pR24 and pUC-atpX-AAD, Hiroshi Yamamoto, RITE, for preparing anti-Rubisco holoenzyme antibody, and Soichi Nakamura, University of Ryukyus, for donating us a wild type *mt*− stock. We also thank Drs. Yuichiro Takahashi, Okayama University, Ko Kato, and Akira Isogai, Nara Institute of Science and Technology for encouragement throughout this study. This work was partly supported by the Petroleum Energy Center (PEC) subsidized from the Ministry of Economy, Trade and Industry of Japan, and was partly performed as one of the technology development projects of the "Green Biotechnology Program" in New Energy and Industrial Technology Development Organization (NEDO).

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