Nucleus-Independent Control of the Rubisco Operon by the Plastid-Encoded Transcription Factor Ycf30 in the Red Alga *Cyanidioschyzon merolae*

Ayumi Minoda\(^2\)*, Andreas P.M. Weber, Kan Tanaka, and Shin-ya Miyagishima

Initiative Research Program, Advanced Science Institute, RIKEN, Wako, Saitama 351–0198, Japan (A.M., S.-y.M.); Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität, 40225 Duesseldorf, Germany (A.P.M.W.); and Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271–8510, Japan (K.T.)

Chloroplasts originated from a cyanobacterium, which was engulfed by a primitive eukaryotic host cell. During evolution, chloroplasts have largely lost their autonomy due to the loss of many genes from their own genomes. Consequently, expression of genes encoded in the plastid operon is mainly controlled by the factors transferred from the cytosol to chloroplasts. However, chloroplast genomes of glaucophytes and red algae have retained some transcription factors (hypothetical chloroplast open reading frame 27 to 30 [Ycf27–Ycf30]) that are absent from green algae and land plants. Here, we show that the red algal chloroplast up-regulates transcription of the Rubisco operon \(rbcL-S-cbbX\) via Ycf30 independently of nuclear control. Light-induced transcriptional activation of the Rubisco operon was observed in chloroplasts isolated from the red alga *Cyanidioschyzon merolae*. The activation was suppressed by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Furthermore, reduced NADP, ribulose-1,5-bisphosphate, and 3-phosphoglyceric acid triggered the up-regulation of Rubisco transcription in the dark, and the activation was dependent on Ycf30. Thus, red algal chloroplasts have retained a nucleus-independent transcriptional regulation of the Rubisco operon to respond to environmental changes. The autonomous system would have been necessary for the initial fixation of cyanobacterial photosynthesis in the ancient nonphotosynthetic eukaryotic host. It has remained functional in the red algal chloroplast over evolutionary time.

All present-day chloroplasts can be traced back to a single symbiotic association between a cyanobacterium and a mitochondriae eukaryote, called the primary endosymbiosis, which introduced photosynthesis into eukaryotes (Rodríguez-Ezpeleta et al., 2005; Deusch et al., 2008). Over time, many genes of the endosymbiont have been either lost or relocated to the nucleus. Consequently, chloroplasts almost lost their autonomy to proliferate and respond to environmental changes. Now, chloroplast biogenesis and homeostasis largely rely on cell signaling pathways of the host cell, which are composed of nucleus-encoded factors (host cell signaling pathways).

In autonomous bacteria including cyanobacteria, regulation of transcription is a major strategy to accommodate to environmental changes. However, chloroplasts have almost lost autonomous transcriptional regulation due to the loss of genes for regulatory factors, including transcription factors and sensory His kinases, from their own genomes. As a result, the expression of chloroplast genes in green algae and land plants is governed by nuclear factors at multiple steps after transcription (e.g. posttranscription, translation, and protein import steps; Bock, 2007). As an exception, it is known that the redox state of the plastoquinone pool controls the rate of transcription of chloroplast genes encoding reaction center apoproteins of photosystems (Plönnischmidt et al., 1999). In contrast, it appears that genes in red algal chloroplasts are still controlled largely at the transcriptional level.
Plastid Autonomous Transcriptional Control of Rubisco Operon

To examine the role of Ycf30 and the effects of environmental changes on chloroplast-encoded Rubisco transcription, we chose C. merolae (Matsuzaki et al., 2004). As a study organism, C. merolae has several advantages, such as the availability of chloroplast DNA microarray analyses and established techniques for cultivation and genetic manipulation (Minoda et al., 2004, 2005; Imamura et al., 2010). In this study, we have developed an experimental system in which transcriptional activation of the Rubisco operon can be evaluated in isolated and permeabilized chloroplasts. Our results show that the red algal chloroplasts, even when isolated, can detect internal physiological changes and activate transcription of the chloroplast Rubisco operon via Ycf30 independently of host cell signaling pathways.

In α-proteobacteria, CbbR regulates the expression of genes encoding CBB cycle enzymes including Rubisco (Tabita, 1999). On the other hand, cyanobacterial genomes encode several CbbR proteins that regulate distinct target genes (e.g. nitrate assimilation, adaptation to osmotic stress, and uptake of inorganic carbon; Maeda et al., 1998; Figge et al., 2001; Nishimura et al., 2008). Among several cyanobacterial CbbR proteins, RbcR is conserved in all cyanobacterial genomes and is most closely related to the chloroplast-encoded Ycf30 (Maier et al., 2000). Therefore, RbcR is a strong candidate for the regulator of Rubisco transcription. However, the function of RbcR or Ycf30 remains unknown, because the gene disruptants are lethal. LTTR has a conserved structure with an N-terminal helix-turn-helix motif for DNA binding and a C-terminal coinducer-binding domain. Coinducers (often a metabolite of the LTTR-regulated pathway) are important for the activation of transcription, as they make proper interaction between LTTR and RNA polymerase (Schell, 1993). In addition, studies using α-proteobacteria demonstrated that metabolites of the CBB cycle are coinducers of CbbR (Tabita, 1999). Given that Ycf30 (a CbbR ortholog) is still encoded in chloroplast genomes of glaucophytes and the red lineage of algae, Ycf30 might be a remnant of the autonomous transcriptional regulation in chloroplasts of these organisms. Therefore, elucidating the relationship between Ycf30 and Rubisco expression in response to environmental changes will give insight into chloroplast evolution.

Figure 1. Effects of the dark-light shift on the accumulation of rbcL and ycf30 transcripts in isolated chloroplasts. A, Gene maps of the Rubisco operon and ycf30 in the chloroplast genome. Gene designations are as follows: tmX, thiodoxin type M; rbcL, large subunit of Rubisco; rbcS, small subunit of Rubisco; cbbX, AAA+ family protein; ilvH, small subunit of acetolactate synthase; psbY, PSII subunit Y; ycf30, LysR-type transcription factor Ycf30; CysT, sulfate transport system permease protein CysT; CysW, sulfate transport system permease protein CysW; CrTR, β-carotene hydroxylase. Arrows a to c indicate the transcripts transcribed from the rbcL promoter. The sizes and positions of PCR products in B are indicated above each diagram of rbcL and ycf30, respectively. B, Intact chloroplasts isolated from dark-grown cells were transferred into the light (40 μM/2 s) and incubated at 42°C for 30 min under the light conditions without (−) or with inhibitors (DC, 10 μM DCMU; Rif, 250 μg mL−1 rifampicin). Total RNAs were extracted and used for quantitative RT-PCR analyses. For comparison, samples collected after 30 min of dark incubation at 42°C were also analyzed (Dark 30 min). Gene expression was normalized to the constitutive expression of rpl21 (Minoda et al., 2005). Values are averages of three experiments with SD values (vertical bars).
RESULTS

Transcription Activation of the Rubisco Operon in Isolated Chloroplasts

*rbcL* and *rbcS* genes encode the large and small subunits of Rubisco, respectively. In green algae and land plants, *rbcL* is encoded in the chloroplast genome and *rbcS* in the nuclear genome. In contrast, red algal *rbcLS* genes form the Rubisco operon, *rbcLS-cbbX*, together with *cbbX*, an AAA⁺ class protein in the chaperon-like ATPase family in the chloroplast genome (Bowien and Kusian, 2002; Fujita et al., 2008a; Fig. 1A).

When *C. merolae* cells were incubated for 16 h in the dark and transferred into light conditions (dark-light shift), an increase of three Rubisco transcripts was observed (Minoda et al., 2005). RNA gel-blot analyses using *rbcL* probe also gave information on the structure of the Rubisco operon: transcripts initiated from the *rbcL* upstream promoter partially terminated in *rbcS* and *cbbX* genes, but some continued to the 3’ end of *cbbX* (Minoda et al., 2005; mRNA a–c in Fig. 1A). Using primer extension mapping, a unique transcription initiation site was identified under dark and light conditions (Fujita et al., 2008b). Thus, transcription from the *rbcL* promoter was increased in response to the light.

To further investigate the effect of light on Rubisco transcription levels, isolated chloroplasts were subjected to a dark-light shift. Intact chloroplasts (greater than 90%) were isolated from dark-grown *C. merolae* cells (Supplemental Fig. S1) and then transferred into light conditions (40 μE m⁻² s⁻¹) at 42°C (optimal growth temperature for *C. merolae* cells). In these conditions, the dark-light shift resulted in increased levels of *rbcL* transcripts (Fig. 1B). This increase was blocked by the addition of 250 μg mL⁻¹ rifampicin, an inhibitor of bacterial-type RNA polymerases, suggesting that the increase in *rbcL* transcript levels is mainly caused by the up-regulation of transcription rather than the down-regulation of degradation (McClure and Cech, 1978). Therefore, the increase in the levels of *rbcL* transcripts is mainly due to the activation of Rubisco transcription. The increase in *rbcL* transcript levels was suppressed by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to isolated chloroplasts and intact cells (Fig. 1B; Supplemental Fig. S2). These results indicate that chloroplasts can autonomously activate Rubisco transcription in response to the activation of photosynthesis during the dark-light shift. In contrast to the increase in *rbcL* transcript levels, the levels of *ycf30* transcripts did not change upon the dark-light shift (Fig. 1B), which is consistent with results obtained using whole cells (Minoda et al., 2005). In addition, the level of Ycf30 protein remained stable in whole cells and intact chloroplasts during the dark-light shift (Supplemental Fig. S3).

To examine whether Ycf30 mediates transcription of the Rubisco operon, we developed a system using permeabilized chloroplasts. To obtain permeabilized chloroplasts, dark-grown cells were osmotically burst by dilution in a 10× volume of hypotonic buffer (5 mM HEPES-KOH, pH 7.6, and 5 mM KCl) and preincubated at 42°C for 2 min in the dark. After adding transcription substrates (0.5 mM each ATP, GTP, and CTP and 50 μM UTP containing 10 μCi of [α-³²P]UTP), the permeabilized chloroplasts were transferred into the light (40 μE m⁻² s⁻¹) and incubated at 42°C for 10 min. The level of [α-³²P]UTP-labeled *rbcL* transcripts increased after the dark-light shift in permeabilized chloroplasts, and this increase was inhibited by 63% ± 2.8% in the presence of 250 μg mL⁻¹ rifampicin (Fig. 2). These results confirmed that the increase in the level of

![Figure 2](https://www.plantphysiol.org/)

**Figure 2.** Light-induced transcription activation of the Rubisco operon via Ycf30 in permeabilized chloroplasts. Permeabilized chloroplasts prepared from dark-grown cells were preincubated at 42°C for 2 min in the dark. After adding transcription substrates (0.5 mM each ATP, GTP, and CTP and 50 μM [α-³²P]UTP), permeabilized chloroplasts were incubated at 42°C for 10 min under light conditions (40 μE m⁻² s⁻¹). Rifampicin (Rif; 250 μg mL⁻¹) was added just before the light. RNA was extracted and hybridized at 48°C for 48 h to single-strand DNA fragments dot blotted onto a nylon membrane. Signals were measured by liquid scintillation. Values are averages of four experiments with (vertical bars) and are plotted as percentages of transcription rate under light conditions.
rbcL transcripts largely rely on transcriptional activation of the Rubisco operon by the dark-light shift. Addition of anti-Ycf30 antibodies to permeabilized chloroplasts inhibited the increase in rbcL transcript levels by 46% ± 15%, whereas additions of anti-RbcL or anti-RbcS antibodies did not affect rbcL transcript levels. Like rbcL transcripts, the level of clpC transcripts increased after the dark-light shift in permeabilized chloroplasts, as was observed in whole cells (Minoda et al., 2005), but addition of antibodies did not affect the light-induced transcription of clpC. These results suggest that Ycf30 is specifically required for the activation of Rubisco transcription.

**Sequence-Specific Binding of Ycf30 to the Promoter Region of the Rubisco Operon**

To examine whether Ycf30 binds to the promoter region of the Rubisco operon, a 90-bp DNA fragment corresponding to the promoter region (Fig. 3A) was end labeled with 32P and used as a DNA probe in a gel retardation assay with a recombinant 6xHis-Ycf30 protein (Supplemental Fig. S4). The electrophoretic mobility shift assay (EMSA) showed a band corresponding to the DNA-protein complex, which increased in intensity with increasing concentration of the Ycf30 recombinant protein (Fig. 3B). Furthermore, binding of the radiolabeled probe was prevented by the addition of a 10-fold surplus of the unlabeled 90-bp probe DNA, while the band did not disappear after the addition of a 100-fold surplus of unlabeled nonspecific DNA (Fig. 3B). These results indicate that Ycf30 binds to the promoter region of the Rubisco operon specifically. Furthermore, compete-out experiments using double-stranded 30-bp oligonucleotides (oligo 1–oligo 3 in Fig. 3A) mapped the binding site to the region from −59 to −30 (Fig. 3C), although the addition of 30-bp oligonucleotides seemed to cause a variation in the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Sequence-specific binding of Ycf30 to the promoter region of the Rubisco operon. A. Sequence of the promoter region of the Rubisco operon in C. merolae. Transcription start site (Fujita et al., 2008b), translation initiation codon, and putative −10 box are indicated. Based on LTTR recognition sequence, three sequences (a–c) in the region from −59 to −30 are shown below the sequence. B. Gel retardation assay using Ycf30 recombinant protein (concentration indicated above each lane) and 32P-labeled 90-bp rbcL upstream sequence (DNA probe in A). Positions of free DNA (F) and DNA-protein complexes (C) are indicated. Competitors added were 10-fold excess (6.6 ng) of the unlabeled specific probe (S) or 100-fold excess (66 ng) of unlabeled nonspecific probes (NS; sonicated calf thymus DNA) on a weight basis. C. Recombinant protein (100 nM) and DNA probes were incubated in the absence (−) or presence of 10-fold or 100-fold molar excess unlabeled 30-bp competitor fragments (oligo 1–oligo 3 shown in A). D. Presumed Ycf30 recognition sequence. Identical nucleotides among sequences a to c found in the region from −30 to −59 (A) are shown as a consensus. E. Comparison of upstream sequences of rbcL genes in cyanobacteria and plastids of red algae and glaucophytes. Percentages at each position were obtained from boxed sequences in E. F. Putative Ycf30 recognition sequence among cyanobacteria and plastids of red algae and glaucophytes. Percentages at each position were obtained from boxed sequences in E.
band patterns of DNA-protein complexes by affecting the interactions (Rath et al., 2008). In this region, there are three candidates (a–c in Fig. 3A) for the LTTR recognition sequence, T-N11-A (Schell, 1993). All three oligonucleotides competed with the 90-bp probe in the binding to Ycf30 with only slight differences in binding affinity (Supplemental Fig. S5). This result indicates that the consensus among the three sequences (ATN7ANANAN) was sufficient for recognition by Ycf30 (Fig. 3D). In addition, this consensus sequence exists upstream of the rbcL gene in the chloroplasts of red algae, cryptophytes, haptophytes, diatoms, glaucophytes, and cyanobacteria (Fig. 3, E and F). Taken together, the above results suggest that Ycf30 binds to the rbcL upstream sequence, which is well conserved in cyanobacteria and chloroplasts.

Role of Ycf30 in the Autonomous Signaling Pathway of the C. merolae Chloroplast

Given that the Ycf30 protein levels remained constant during transcriptional activation of the Rubisco operon (Supplemental Fig. S2), it is likely that factors other than Ycf30 trigger the activation of Rubisco transcription via Ycf30. In α-proteobacteria and cyanobacteria, binding affinities of the CbbRs to the target DNA fragments are enhanced by NADP, NADPH, ADP, ATP, and metabolites derived from the CBB cycle (van Keulen et al., 1998; Dubbs et al., 2004; Nishimura et al., 2008). Therefore, these metabolites are candidates for coinducers of Ycf30 to enable autonomous transcriptional activation of the Rubisco operon in the chloroplast. In dark-incubated permeabilized chloroplasts, the level of [$\alpha$-32P]UTP-labeled rbcL transcripts increased by 10 min after the addition of NADPH, 3-phosphoglyceric acid (3-PGA), or ribulose-1,5-bisphosphate (RuBP) but not after the addition of NADP, ADP, ATP, or 2-phosphoglycolic acid (Fig. 4A). Transcriptional activation of the Rubisco operon by NADPH, 3-PGA, or RuBP was significantly inhibited by the addition of anti-Ycf30 antibodies, whereas it was not affected by the addition of anti-rbcL antibodies (Fig. 4B). These results suggest that NADPH, 3-PGA, and RuBP can trigger the activation of Rubisco transcription via Ycf30.

To further examine whether NADPH, 3-PGA, and RuBP affect the binding of Ycf30 to the rbcL promoter region, these metabolites were added into EMSA using the recombinant Ycf30 and 90-bp DNA probes (the same probe as in Fig. 3A). Gel retardation assays (performed as in Fig. 3B) were performed with [32P]-labeled DNA probe (1 nM) and Ycf30 proteins (100 nM) were incubated with or without RuBP or NADPH at the concentration indicated above each lane. Arrowheads indicate the positions of free DNA (F) and DNA-protein complexes (C1–C3).
between 0 μM NADPH and 0 μM RuBP is due to the difference in exposure time). Together, these results suggest that NADPH or RuBP directly enhances the binding of Ycf30 to the rbcL promoter region, thereby activating transcription of the Rubisco operon.

**DISCUSSION**

**Autonomous Transcriptional Activation of the Rubisco Operon via Ycf30 in Chloroplasts**

Many studies have shown that cellular signaling pathways of the host cell govern the expression of chloroplast genes (Bock, 2007). As a chloroplast autonomous transcriptional regulation, it is known that transcriptional control of psaA and psbA genes is by the redox state of the plastoquinone pool (Pflanzschmidt et al., 1999). In addition, chloroplast His kinase, HIK/CSK, is suggested as a possible redox sensor for the regulation (Puthiyaveetil and Allen, 2009). However, it is still unknown how the redox state is transmitted to up-regulate the transcription. Here, we show another autonomous regulatory system by the plastid-encoded transcription factor, Ycf30, in the red algal chloroplast as a relic of autonomous systems in cyanobacteria. During acclimation to environmental changes, this autonomous system activates the transcription of Rubisco in response to physiological changes inside the chloroplast.

In our analyses, the activation of Rubisco transcription occurred in isolated chloroplasts. The activation was dependent on Ycf30 and photosynthetic metabolites. These results suggest that chloroplast-encoded Ycf30 mediates the rapid up-regulation of Rubisco transcription in response to environmental changes without signals transmitted by host cell signaling pathways.

Based on our results, we suggest the following scheme for autonomous transcriptional regulation of the Rubisco operon in chloroplasts (Fig. 5). (1) A dark-light shift requires an increase in the amount of active Rubisco. (2) The activation of the CBB cycle leads to increased levels of RuBP, 3-PGA, or NADPH (photosynthetic metabolites) inside the chloroplast, as discussed below. (3) These metabolites directly increase the binding affinity of Ycf30 to the promoter of the Rubisco operon and/or change the conformation of the Ycf30-RNA polymerase complex. (4) Transcription of the Rubisco operon is activated. (5) Sufficient amounts of Rubisco are supplied for acclimation to the environmental changes.

Addition of DCMU to intact chloroplasts suppressed light-induced rbcL transcription (Fig. 1B). This could be explained by a decrease in the levels of signaling molecules for Ycf30, which could result from the inhibition of linear photosynthetic electron transport and its effects on the CBB cycle. In our analyses, NADPH, RuBP, and 3-PGA affected transcriptional activation of the Rubisco operon in C. merolae (Fig. 4, A and B).

Of the three molecules, RuBP is a substrate for Rubisco. The amount of RuBP was approximately 0.03 mM in cyanobacteria grown under photoautotrophic conditions, and it was almost the same when photosynthesis was partially suppressed after a shift to photomixotrophic conditions (Takahashi et al., 2008). On the other hand, the level of RuBP increased in response to light in both whole leaves and isolated chloroplasts of land plants (Sicher and Jensen, 1979; Salvucci et al., 1986; Arrivault et al., 2009). In addition, the amounts of RuBP in Arabidopsis (Arabidopsis thaliana) were reported as approximately 0.004 mM in the dark and approximately 0.12 mM in the light (Arrivault et al., 2009). The measured RuBP concentrations are consistent with those in our experimental conditions (Fig. 4C; Supplemental Fig. S6). These results indicate that RuBP is a candidate for a coinducer of Ycf30 in C. merolae.

In our analyses, Rubisco transcription was also activated by 3-PGA, produced by the carboxylase activities of Rubisco. 3-PGA is also supplied from recycling of 2-phosphoglycolic acid by the photosynthetic pathway. In cyanobacteria, the level of 3-PGA increases to approximately 1.3 mM under photoautotrophic conditions but decreases to approximately 0.13 mM after a shift to photomixotrophic conditions (Takahashi et al., 2008). In land plants, however, 3-PGA is exported from chloroplasts to the cytosol, and the level (0.14–0.2 mM) does not change significantly between dark and light conditions (Arrivault et al., 2009). As in cyanobacteria, in red algae the 3-PGA level would change due to a lack of the trans-
porter exporting 3-PGA from chloroplasts, unlike in land plants (Linka et al., 2008). The range of 3-PGA concentrations reported in cyanobacteria is consistent with that used in our experiments on *C. merolae* permeabilized chloroplasts (Fig. 3A; Supplemental Fig. S6). However, the change in Rubisco transcription was observed at lower concentrations (less than 0.1 mM) than those reported for cyanobacteria. Although further studies on red algae are required, it is possible that 3-PGA also acts as a coinducer of Ycf30.

NADPH also induced Ycf30-mediated Rubisco transcription, as reported for *Xanthobacter flavus* (van Keulen et al., 1998). However, in cyanobacteria and land plants, the NADPH:NADP ratio is maintained at a constant 1:1 (Takahashi et al., 2008; Arrivault et al., 2009), and the amount of NADPH shows little variation. In cyanobacteria, the amounts of NADPH are approximately 0.08 mM in photomixotrophic and approximately 0.14 mM in photoautotrophic conditions, and in land plants, the total amounts of NADP and NADPH are approximately 0.3 mM in dark and approximately 0.5 mM in light conditions (Takahashi et al., 2008; Arrivault et al., 2009). At present, it is unclear whether Ycf30 activity changes in response to the NADPH level of chloroplasts. Alternatively, NADPH might contribute to maintaining basal levels of Rubisco transcripts. Thus, Ycf30 could sense the rate of photosynthesis and carbon flow in the CBB cycle via the levels of RuBP and/or 3-PGA.

In our analyses, clpC transcription was also up-regulated in isolated chloroplasts by 10 min after the dark-light shift (Fig. 2). Unlike Rubisco transcription, the up-regulation of clpC transcription is not dependent on Ycf30. Therefore, there should be another as-yet-unknown mechanism that is responsible for chloroplast autonomous transcriptional control in response to light.

The Relationship between Ycf30-Mediated Autonomous Transcriptional Regulation and Chloroplast Evolution

Evolutionary studies have shown that Rubisco genes encoded in chloroplast genomes of red algae and organisms containing chloroplasts of red algal origin (except for some dinoflagellates) originated from α-proteobacteria (Delwiche and Palmer, 1996). It is suggested that a cyanobacteria-derived *rbcL*-cbbX operon was replaced by an α-proteobacterial *rbcL*-cbx operon by lateral gene transfer in a common ancestor of red algae. However, the *rbcL* operon and *ycf30* encoded in the chloroplast genome of glaucophytes are derived from cyanobacteria. The chloroplast-encoded *rbcL* gene and the nucleus-encoded *rbcS* gene in green algae and plants are also derived from cyanobacteria. Therefore, when the chloroplast was established in a common ancestor of Plantae, Ycf30 should have mediated the transcription of the Rubisco operon that originated from cyanobacteria. As revealed in this study, in red algal chloroplasts, a CbbR-type transcription factor and its characteristic consensus sequence in the upstream region of Rubisco genes is shared among α-proteobacteria, cyanobacteria, and chloroplasts (Fig. 3E). This similarity between cyanobacteria (and chloroplasts) and α-proteobacteria most likely enabled the acquisition of an α-proteobacterial Rubisco operon while retaining the autonomous transcriptional regulatory system in red algal chloroplasts.

Rubisco is a main electron acceptor for photosynthetic electron transport and a key player in central carbon assimilation. The Ycf30-mediated autonomous transcriptional regulatory system for the Rubisco operon in the chloroplast can rapidly optimize the photosynthetic machinery and sustain active photosynthesis independently of nuclear control. During evolution, Ycf30 was lost from the chloroplast genomes of green algae, land plants, and dinoflagellates that have chloroplasts of red algal origin. In green algae and land plants, the *rbcS* gene was transferred to the nuclear genome, and the gene expression of *rbcL* encoded in the chloroplast genome became mainly regulated at the posttranscriptional level (BERRY et al., 1990; Salvador et al., 1993; Shina et al., 1998). Some dinoflagellates have lost the Rubisco operon from the chloroplast genome and acquired a gene for form II Rubisco in the nuclear genome (Morse et al., 1995). Even though there has been evolutionary diversification, Ycf30-mediated autonomous transcriptional regulation of the Rubisco operon would have an essential part for the initial fixation of cyanobacteria-derived photosynthesis in nonphotosynthetic eukaryotic cells.

**MATERIALS AND METHODS**

**Algal Culture**

*Cyanidioschyzon merolae* 10D was grown in 50 mL of Allen’s medium in glass vessels at 42°C under continuous light (40 μE m⁻² s⁻¹) as described previously (Minoda et al., 2005). For dark-light shift experiments, cells at the mid-logarithmic phase (optical density at 750 nm = 0.6) were incubated in the dark for 16 h and transferred to the light condition (40 μE m⁻² s⁻¹). Just before the transfer, 10 μM DCMU was added to the medium.

**Chloroplast Isolation and Isolated Chloroplast Experiments**

Chloroplasts were isolated as described (Miya-gishima et al., 1999) except for that cells and chloroplasts were kept in the dark. For the isolation of chloroplasts, cells were suspended in the isolation buffer (20 mM HEPES-KOH, pH 7.6, 5 mM KCl, and 5 mM EGTA) containing 1 mM MgCl₂, 180 mM Suc, and 100 μg mL⁻¹ DNase I (DN-25; Sigma) and then lysed in a French pressure cell (Thermo Fisher Scientific) at 1,300 p.s.i. The lysate was incubated on ice for 1 h and applied on the top of a three-step gradient of 80%, 60%, and 40% Percoll (GE Healthcare) containing 330 mM Suc. After centrifugation for 1 h at 4,000 g in a swinging-bucket rotor, a fraction of intact chloroplasts was harvested from the interface between 60% and 80% Percoll gradients and washed twice with the isolation buffer containing 330 mM Suc and 5 mM EDTA. Finally, isolated chloroplasts were suspended in the incubation buffer (600 mM mannitol, 5 mM MES, pH 5.7, 10 mM KCl, 5 mM EGTA, 5 mM EDTA, and 25 μg mL⁻¹ bovine serum albumin [BSA]). Chloroplast intactness was estimated by phase-contrast microscopy (Supplemental Fig. S1). Isolated chloroplasts were kept at 4°C in the dark until use. To evaluate the light-induced transcription, isolated chloroplasts (1 mg mL⁻¹ total proteins) were incubated at 42°C for 10 min in the light (40 μE m⁻² s⁻¹) after 2 min of dark...
Permecinal Chloroplast Experiments

Isolated chloroplasts were sedimented by centrifugation for 3 min at 700g and osmotically burst by dilution in a 10x volume of hypertonic buffer (5 mM HEPES-KOH, pH 7.6, and 5 mM KC1). The transcription reaction was carried out in a 30-μL reaction mixture consisting of transcription buffer (5 mM HEPES-KOH, pH 7.6, 5 mM KC1, 10 units of RNase inhibitor, 25 μg/mL 1/2 BSA, 0.5 mM each ATP, GTP, and CTP, and 50 μM UTP containing 10 μCi of [α-32P]UTP [80 Ci mmol−1; MP Biomedical]) and permeabilized chloroplasts (3.5 mg mL−1 total proteins). To examine light-induced transcription, the reaction mixture without transcription substrates (0.5 mM each ATP, GTP, and CTP and 50 μM UTP containing 10 μCi of [α-32P]UTP) was preincubated at 42°C for 2 min in the dark. After the addition of transcription substrates, the reaction mixture was incubated at 42°C for 10 min in the light (40 μM m−2 s−1). Inhibitors and antibiotics (5 μg mL−1) were added just before the dark-light shift. To examine the effects of metabolites, transcription substrates were added after 2 min of dark preincubation, and the reaction mixture was incubated for a further 10 min in the dark. Metabolites (0.5 mM) and antibiotics (5 μg mL−1) were added at the onset of preincubation. After the reaction, RNA was extracted and hybridized at 48°C for 48 h in single-strand DNA fragments (0.4 μg) dot blotted onto a nylon membrane. For hybridizations, one-fifth of the total RNA was used for rbcL, and the rest was used for clec. The DNA blots were washed five times with 0.1× SSC and 0.1% SDS at 65°C for 15 min. Signals were measured by liquid scintillation (Beckman Coulter LS6500).

Real-Time Quantitative RT-PCR Analyses

The method for RNA extraction was described previously (Minoda et al., 2005). The primer sets used for the preparation of the probes are listed in Supplemental Table S1. RT reactions were performed using ReverTra Ace (Toyobo) with each primer set (Supplemental Table S1) and 0.2 μg of total RNA. Quantification was performed with the LightCycler instrument and the FastStart DNA MasterPLUS SYBR Green I kit (Roche) as described by Nikoh et al. (2010). For the detection of rbcL, rpl21, and ycf30 transcripts, PCRs were carried out in cycles of 94°C for 10 s, 52°C for 10 s, and 72°C for 6, 6, and 4 s, respectively. Results were analyzed using LightCycler software version 3.5 (Roche) and normalized to the expression of ribosomal protein gene, rpl21.

Preparation of Ycf30 Recombinant Protein

The coding sequence of ycf30 was amplified by PCR using the primers Y30RPf and Y30RPr (Supplemental Table S1) and total DNA of C. merolae as template. The PCR product, in which a Ndel recognition site had been created by site-directed mutagenesis, was cloned between the Ndel and BamHI sites of pET15b vector (Novagen). Expression in E. coli BL21 Codon Plus carrying the Ycf30 expression plasmid yielded the recombinant His-Ycf30 protein. For purification, cells were harvested via centrifugation and purified on nickel-nitrilotriacetic acid resin agarose under denaturing conditions, as suggested by the manufacturer (Qiagen). After the verification of a single protein band corresponding to the M, of Ycf30 by SDSPAGE, the purified protein was reactivated using dialysis with the reactivation buffer (100 mM HEPES, pH 8.0, 1 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], and 50% glycerol). Finally, the recombinant Ycf30 protein was recovered and stored at −20°C up to 1 month in the buffer.

Antibody Preparation and Immunoblot Analysis

The 6×His-Ycf30 recombinant protein prepared above was used to raise the polyclonal guinea pig antiserum against Ycf30, and the IgG was purified by passing them through the protein A-Sepharose CL4B column (GE Healthcare).

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