# Light regulates the gene expression of ribulosebisphosphate carboxylase at the levels of transcription and gene dosage in greening pea leaves

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Ribulosebisphosphate carboxylase, composed of large and small subunits, is induced by light in pea leaves. During induction, the synthesis rate of the two mRNAs and the gene dosage were measured. The relative rates of synthesis of the two mRNAs changed with the time of illumination, while the relative gene dosage changed only for the large subunit. The increase in the synthesis rate of the large subunit mRNA was shown to be at least partly due to an increase in gene dosage. These results indicate that the light induction of ribulosebisphosphate carboxylase in the pea is controlled at the levels of transcription and, for the large subunit, also of gene dosage.

*Ribulosebisphosphate carboxylase* *Light induction* *Gene expression* *Transcriptional regulation* *Gene-dosage regulation*

## 1. INTRODUCTION

A $CO_2$-fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), composed of large and small subunits, is localized in chloroplasts. The large subunit is encoded by chloroplast DNA [1], and the small subunit by nuclear DNA [2]. When an etiolated plant is exposed to light, the etioplasts in the pea leaves develop into chloroplasts with all the equipment for photosynthesis, including RuBisCO, the induction of which is controlled by the levels of mRNA coding for the two subunits [3–7]. At the same time, chloroplast DNA increases. In mature leaves, about 7000 copies of chloroplast DNA are found per cell [8], with 7000 copies of the large subunit gene [1,9]. About 10 copies of the small subunit gene are found in the nuclear DNA [10,11]. The significance of this gene dosage is not understood. We examined the relative changes of the two mRNAs and the relative changes of the two gene dosages in greening pea leaves and found that the gene dosage of the small subunit did not change, but the synthesis rate of its mRNA changed. On the other hand, the gene dosage of the large subunit changed, and transcription of the gene was proportional to the gene dosage. Thus, light regulates RuBisCO gene expression at the levels of transcription and gene dosage.

## 2. EXPERIMENTAL

### 2.1. *Growth of plants*

Pea seedlings (*Pisum sativum* var. Alaska) grown for 7 days in the dark were exposed to light (~13000 lx) for 0–7 days.

### 2.2. *Determination of the relative rate of synthesis of mRNA*

Pea seedlings illuminated for various lengths of time were pulse-labeled with [$^3$H]uridine. First, 5–20 $\mu$l of 1% Tween 80 solution per seedling was spread over all leaf surfaces 1.5 h before labeling. Then, 2–24 seedlings were labeled with 5–25 $\mu$l of a 1% Tween 80 solution of [$^3$H]uridine (10 $\mu$Ci/$\mu$l, 40 Ci/mmol, Radiochemical Centre) by spreading

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it over the pretreated leaf surfaces of intact plants 1.5 h before harvesting. Total RNA was extracted from these leaves as in [6]. LiCl precipitation was repeated 3 times to remove DNA; 2–4% of the applied radioactivity was incorporated into trichloroacetic acid-insoluble material. The specific activity of the labeled RNA was 3500–10000 cpm/$\mu$g measured with a Triton X-100-xylene scintillant. Labeled RNA was hybridized with immobilized DNA as described in fig.1 using a modification of the method in [12].

### 2.3. *Determination of gene dosage*

DNA was extracted from pea leaves illuminated for various lengths of time. Pea leaves (1.5 g) were quickly homogenized in a mortar with sand and with 3.3 ml of a solution containing 2.5 mM Tris–HCl (pH 7.4), 0.5 mM KCl, 4 mM NaCl, 500 mM EDTA, 0.5% (w/v) sarcosyl, and 330 $\mu$g proteinase K. The mixture was incubated at 50°C for 3 h, and extracted 3 times with an equal volume of phenol saturated with 10 mM Tris–HCl (pH 8) and 1 mM EDTA. The DNA fraction in the lower phase was dialyzed against 1 l dialysis buffer (50 mM Tris–HCl, pH 8), 10 mM EDTA and 10 mM NaCl) 3 times, and treated with 20 $\mu$g/ml of DNase-free RNase A and 10 units/ml of RNase T1 at 37°C for 3 h. The DNA fraction was extracted twice with phenol/chloroform (1:1, v/v), and precipitated with ethanol. After washing with 70% ethanol, the DNA was dissolved in 1.8 ml of 0.1 × SSC and then 0.2 ml of 3 M sodium acetate, 2 $\mu$l of 100 mM EDTA and 1.08 ml isopropanol were added to remove contaminating RNA. The precipitated DNA was washed with 70% ethanol and dissolved in a solution containing 10 mM Tris–HCl (pH 8) and 1 mM EDTA. From 1.5 g leaves 300–500 $\mu$g DNA was obtained. The deoxyribose content was determined as in [13] and confirmed that this preparation was pure DNA. The gene dosage in these preparations was determined as described in fig.2.

## 3. RESULTS AND DISCUSSION

For the determination of the synthesis rate of mRNA, a strongly pulse-labeled RNA is required. Because such an RNA is difficult to prepare from intact plants, the RNA is usually labeled in isolated nuclei [12,14]. In this experiment, however, labeling of both nuclear and chloroplast mRNA was required, and therefore we have developed a convenient way to do this in intact plants by spreading 1% Tween 80 over the leaf surfaces before labeling with [$^3$H]uridine. Using this method we obtained sufficient radioactivity incorporated into intact plants to measure the relative synthesis rate of the mRNAs by filter hybridization under DNA excess. However, we could not obtain highly labeled RNA from etiolated plants. Thus, in vivo pulse-labeled RNA extracted from illuminated leaves was hybridized with immobilized DNA probes specific for the large and small subunits. Tobacco large-subunit DNA (1.25 kbp *Bam*HI fragment) [15] and pea small subunit cDNA (0.75 kbp, *Eco*RI–*Bam*HI fragment of pGR 407) [16] were used as probes. To avoid competition of the labeled RNA with accumulated non-labeled mRNA, an excess of the DNA probes was immobilized on the filters. Hybridization was performed over a range where the hybridized counts were proportional to the total input counts. In this way, the transcription of nuclear and chloroplast mRNA could be observed for the first time in intact plants. The values are expressed as ppm of radioactivity incorporated into each mRNA with respect to the radioactivity incorporated into total RNA applied to the filters (fig.1). The relative rate of synthesis of both mRNAs increased with time of illumination up to 3 days, and then gradually decreased. These profiles are correlated with the induction curve of RuBisCO at the protein level (not shown). The results indicate that light coordinately controls the transcription of the two mRNAs and that control at the level of transcription is responsible for the light-regulated expression of the RuBisCO gene. The incorporation of label into the large subunit mRNA is about 10-times greater than that into the small subunit. If the sizes of the uridine pools in chloroplasts and nuclei were the same, this would indicate that the synthesis rate of the former is substantially larger than that of the latter.

The course of change of the gene dosage for the large and small subunits in illuminated pea leaves was examined by dot-hybridization. Spinach large subunit DNA (1.2 kbp, *Kpn*I fragment) [17] and pea small subunit cDNA (0.75 kbp, *Eco*RI–*Bam*HI fragment of pGR 407) [16] were labeled by nick-translation with [$\alpha$-$^{32}$P]dCTP and used as hybridization probes. Fig.2 indicates the effect of

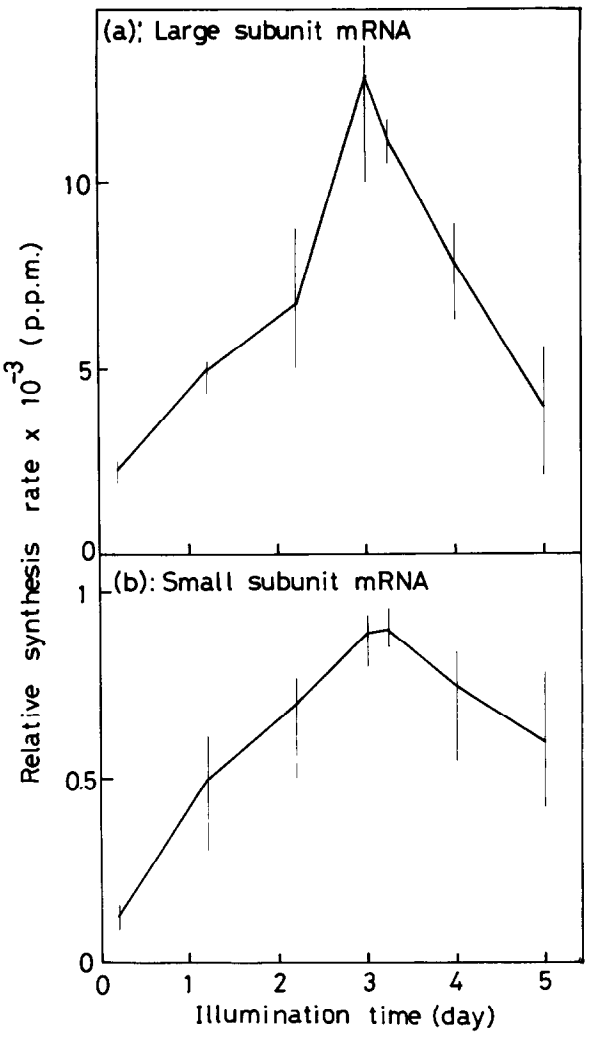


Fig.1. Effect of light on the relative rates of synthesis of large and small subunit mRNAs. In vivo pulse-labeled RNA was hybridized as follows. Control DNA (pBR 322), the pea small subunit cDNA fragment, and the tobacco large subunit DNA fragment were denatured as in [19], and an aliquot containing 1 $\mu$g or 1.7 $\mu$g DNA was spotted on a 25-mm-diameter nitrocellulose filter. The filters were dried, baked, washed and air-dried as in [19]. Prehybridization was performed overnight at 41°C in 50 $\mu$l of a solution containing 50% formamide, 40 mM Pipes–NaOH (pH 6.5), 0.5 M NaCl, 1 mM EDTA, 0.4% SDS, and 5 $\mu$g sheared *E. coli* rRNA. Three filters containing the same probe were hybridized in a bag with [$^3$H]RNA (0.5–1 × $10^6$ cpm/3 filters) in 150 $\mu$l of the above buffer. Three $\mu$g DNA fragment were used per hybridization experiment for RNA obtained after 0–2 days of illumination and 5 $\mu$g DNA for RNA obtained after 3–5 days of illumination. Carrier RNA (*E. coli* rRNA) was added to adjust the amount of RNA to 100 $\mu$g/filter. The hybridization was performed overnight at 41°C. The filters were washed at 60°C for 20 min 4 times with 2 × SSC, 0.1% SDS, and twice with 0.1 × SSC. Three filters were put into a vial and treated for 1 h with 250 $\mu$l of 40 mM NaOH. After neutralization with 100 $\mu$l of 0.1 M $CH_3COOH$, the radioactivity was determined in 8 ml of Triton X-100-xylene scintillant. The background hybridization values determined by hybridization to pBR322 were subtracted from the observed values. The efficiency of hybridization was measured by hybridizing [$^3$H]cRNA prepared as in [19] with each filter-bound DNA probe, and was found to be 29–36%. Corrections were made for the efficiency of hybridization but not for the size of the hybridization probe. The values are presented as ppm of total input RNA. Hybridizations were done in triplicate and data are presented as the mean ± SD.

light on the gene dosages for the large and small subunits. The small subunit gene dosage does not change with illumination time, in contrast to that of the large subunit. The individual dots in fig.2a were cut out, counted and plotted in fig.2d. The relative gene dosage of the large subunit increased with illumination time up to 3 days and then gradually decreased. To determine whether there is a specific amplification of the gene coding for the large subunit, we also examined the gene dosage of chloroplast 16 S rRNA by dot-hybridization using the same DNA sample. Pea chloroplast DNA has only one 16 S rRNA gene, located opposite to the large subunit gene on the gene map [9]. The liverwort 16 S rRNA gene (1.6 kbp, *Bam*HI–*Hin*dIII fragment) [18] was used as a probe. As shown in fig.2c,d, this gene dosage also increased with illumination time up to 3 days and then decreases, which implies that the increase of the large subunit gene is due to chloroplast DNA replication rather than to a specific amplification of the gene for the large subunit. The decrease after 4 days of illumination seems to be the result of leaf growth which is accompanied by a decrease of the amount of chloroplast DNA per cell [8].

The similarity between the two profiles in fig.1a and fig.2d indicates that the increase in the rate of synthesis of the large subunit is proportional to its gene dosage. Therefore, the expression of the large subunit is controlled at least partly at the level of

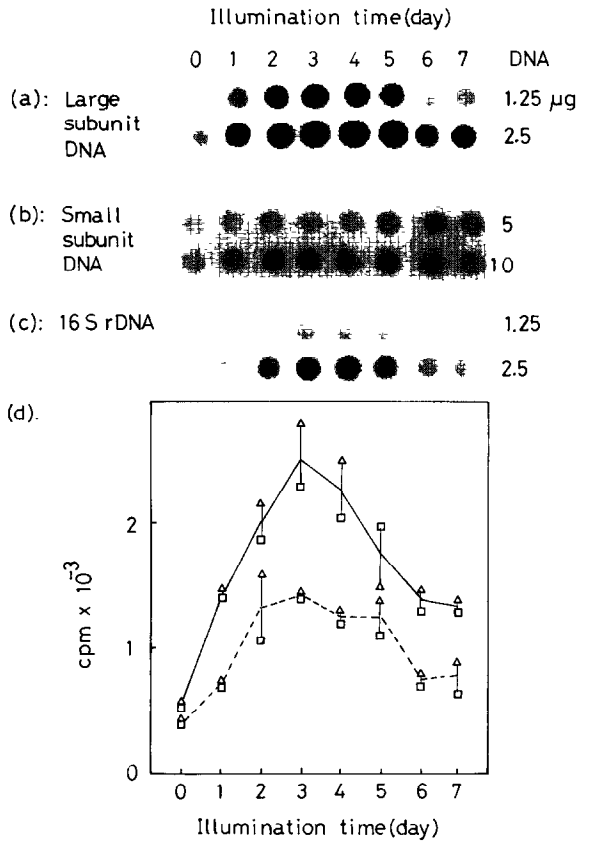


Fig.2. Effect of light on the relative gene dosages of the large (a) and small (b) subunits, and of chloroplast 16 S rRNA (c). DNA was sheared, denatured with 0.5 N NaOH at 100°C for 7 min, and immediately cooled; 0.9 vol. of 0.5 N HCl was added. After addition of an equal volume of 2 M $CH_3COONH_4$ [20], an aliquot of the DNA solution was applied on a membrane filter (Biodyne A, Pall) preequilibrated with 1 M $CH_3COONH_4$ using a water sucker and the Hybri-Dot system (Schleicher & Schüll). After baking at 80°C for 1 h, the membrane was prehybridized at 42°C for 18 h with a solution containing 50% formamide, 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin, 0.9 M NaCl, 6 mM EDTA, 50 mM phosphate buffer (pH 7.7), 0.2% SDS, and 100 μg/ml of sonicated and heat-denatured calf thymus DNA. The filters were hybridized to the pea small subunit cDNA fragment, the spinach large subunit DNA fragment or the chloroplast 16 S rDNA fragment from liverwort, labeled by nick-translation with [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol, Radiochemical Centre) to a specific activity of 3–6 × $10^7$ cpm/μg. After 18 h at 42°C, the membranes were washed with 2 × SSC, 0.1% SDS 4 times at room temperature for 5 min, and then twice with 0.2 × SSC, 0.1% SDS at 50°C for 15 min. The membranes were exposed to Fuji X-ray film with an intensifying screen for 16 h (a,c) or 10 days (b). The individual dots in (a) and (c) were cut out and counted in a Triton X-100-xylene scintillant. The radioactivity hybridized to 2.5 μg DNA was directly plotted (□), and that hybridized to 1.25 μg DNA was multiplied by 2 (△). (——) Large subunit gene, (– – –) 16 S rRNA gene.

gene dosage. On the other hand, the expression of the small subunit is controlled at the level of the transcription since the transcription rate changes (fig.1b) without change of the gene dosage (fig.2b). This result agrees with previous observations obtained with isolated nuclei [12,14]. Thus, light regulates RuBisCO gene expression at the levels of gene dosage and transcription.

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## REFERENCES

[1] Coen, D.M., Bedbrook, J.R., Bogorad, L. and Rich, A. (1977) Proc. Natl. Acad. Sci. USA 74, 5487–5491.
[2] Kawashima, N. and Wildman, S.G. (1972) Biochim. Biophys. Acta 262, 42–49.
[3] Tobin, E.M. and Suttie, J.L. (1980) Plant Physiol. 65, 641–647.
[4] Smith, S.M. and Ellis, R.J. (1981) J. Mol. Appl. Genet. 1, 127–137.
[5] Sasaki, Y., Ishiye, M., Sakihama, T. and Kamikubo, T. (1981) J. Biol. Chem. 256, 2315–2320.
[6] Shinozaki, K., Sasaki, Y., Sakihama, T. and Kamikubo, T. (1982) FEBS Lett. 144, 73–76.
[7] Sasaki, Y., Sakihama, T., Kamikubo, T. and Shinozaki, K. (1983) Eur. J. Biochem. 133, 617–620.
[8] Lamppa, G.K., Elliot, L.V. and Bendich, A.J. (1980) Planta 148, 437–443.

[9] Palmer, J.D. and Thompson, W.F. (1982) Cell 29, 537–550.
[10] Berry-Lowe, S.L., McKnight, J.D., Shah, D.M. and Meagher, R.B. (1982) J. Mol. Appl. Genet. 1, 483–498.
[11] Broglie, R., Coruzzi, G., Lamppa, G., Keith, B. and Chua, N.H. (1983) Bio/Technology 1, 55–61.
[12] Gallagher, T.F. and Ellis, R.J. (1982) EMBO J. 1, 1493–1498.
[13] Burton, K. (1956) Biochem. J. 62, 315–323.
[14] Silverthorne, J. and Tobin, E.M. (1984) Proc. Natl. Acad. Sci. USA, in press.
[15] Shinozaki, K. and Sugiura, M. (1982) Gene 20, 91–102.
[16] Bedbrook, J.R., Smith, S.M. and Ellis, R.J. (1980) Nature 287, 692–697.
[17] Zurawski, G., Perrot, B., Bottomley, W. and Whitfeld, P.R. (1981) Nucleic Acids Res. 9, 3251–3270.
[18] Ohyama, K., Yamano, Y., Fukuzawa, H., Komano, T., Yamagishi, H., Fujimoto, S. and Sugiura, M. (1983) Mol. Gen. Genet. 189, 1–9.
[19] McKnight, G.S. and Palmiter, R.D. (1979) J. Biol. Chem. 254, 9050–9058.
[20] Kafatos, F.C., Jones, C.W. and Efstratiadis, A. (1979) Nucleic Acids Res. 7, 1541–1552.