Effects of α-amanitin on coordination of two mRNAs of ribulose-bisphosphate carboxylase in greening pea leaves

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The effects of α-amanitin on light induction of the two mRNAs of ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) in greening pea leaves were examined. Induction of the nuclear-coded small-subunit mRNA but not of the chloroplast-coded large-subunit mRNA was significantly reduced by α-amanitin, indicating that the steady-state level of the large-subunit mRNA is not directly affected by that of the small subunit. This suggests that there is no direct link between nuclear transcription and light induction of the large-subunit mRNA. The synthesis of RuBisCO was also slowed in proportion to the small-subunit mRNA level, and there was not a large excess of the unassociated large subunit polypeptide. The synthesis of the two subunits is probably coordinated. Thus, the expression of the large subunit is partly controlled at the post-transcriptional level.

Ribulose-bisphosphate carboxylase (Pea seedling) mRNA level Light induction Post-transcriptional regulation Coordination α-Amanitin

1. INTRODUCTION

Ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) is composed of two subunits, a chloroplast-coded large subunit and a nuclear-coded small subunit. The two subunits are synthesized in different cell compartments, but almost stoichiometric amounts of the polypeptides are found in pea leaves [1]. This enzyme is induced by light, and the induction is primarily controlled at the level of transcription in greening pea leaves [2–4]. Changes in the two subunit mRNA levels are roughly coordinated, as if the steady-state levels of the two respond to each other during greening [1]. To see whether induction of the small-subunit mRNA affects the level of the large-subunit mRNA, and to determine whether light-induced transcription of the other nuclear-coded genes is needed for induction of the large-subunit mRNA, the effects of α-amanitin, an inhibitor of eukaryotic mRNA synthesis, on the two mRNA levels were examined in greening pea leaves. The relative rate of synthesis of RuBisCO protein and the extent of labelling of the unassociated subunits were also measured to identify the events of regulation when α-amanitin was present.

2. EXPERIMENTAL

2.1. *Plant growth and α-amanitin treatment*

Pea seedlings (*Pisum sativum* var. Alaska) were grown in darkness for 7 days at 20°C in a Biotron and continuously illuminated for various times with a white fluorescent lamp of about 13000 lx. An α-amanitin solution (5 μg/ml) was continuously supplied by transpiration via cotton threads sewn through the stalk at a flow rate of about 21 μg/h. Manipulations in darkness were done under a green safety light. As the control, water was used instead of α-amanitin solution.

2.2. *Measurement of steady-state levels of mRNA*

Total RNA was extracted from the frozen apical buds of 3–5 seedlings treated with α-amanitin by the method in [1]. Northern analysis and dot-blot analysis were done as reported [1]. The probe used

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to detect the large-subunit mRNA was tobacco large-subunit DNA (1.25 kbp *Bam*HI fragment) provided by Dr K. Shinozaki [5], and that for the small-subunit mRNA was pea small-subunit cDNA of pGR 407 (0.7 kbp *Eco*RI-*Bam*HI fragment) provided by Dr S.M. Smith [6]. The amounts of each mRNA relative to total RNA were compared.

2.3. *Measurement of the rate of RuBisCO synthesis and extent of labelling of unassociated subunits*

Apical buds of pea seedlings were pulse-labeled for 1.5 h before harvesting as described in [1]. The labelled protein was extracted and immunoprecipitated by anti-RuBisCO IgG as in [1]. The immunoprecipitates were electrophoresed and the radioactivity of the large and small subunits measured. The relative synthesis rate of RuBisCO was calculated from the radioactivity of the large and small subunits divided by that in the total soluble protein measured by the method of Bollum [7] as described in [1]. The extent of labelling of the unassociated subunits was measured basically as in [1] using specific IgG against the large- and small-subunit polypeptides.

3. RESULTS AND DISCUSSION

Pea seedlings grown in darkness for 7 days were continuously illuminated with white light to induce the two mRNAs of RuBisCO. To stop the induction of small-subunit mRNA, two experiments were designed. First, to determine the effects of α-amanitin at the onset of illumination, α-amanitin solution was provided continuously starting 3 h before the onset of the illumination, and the course of the induction of RuBisCO and mRNA was followed. Second, to investigate the effects of α-amanitin during light induction, the inhibitor was provided continuously starting 24 h after the illumination started. α-Amanitin selectively inhibits in vivo poly(A)$^+$ RNA synthesis in plant cells [8]. In the presence of this drug, the seedlings grew normally under the conditions tested, but growth was gradually reduced compared to the control. The total RNA content per cell is almost constant during greening, and the amount of total soluble protein per cell does not change greatly (not shown). Therefore, the changes in mRNA of the total RNA and in RuBisCO synthesis of total soluble protein can be taken to be values for each cell and compared.

Total RNA was extracted from the apical buds of treated plants and used for the measurement of the two mRNA levels. Northern analysis (fig.1) showed that hybridizable RNA with large- or small-subunit probes were the mRNAs in question because their molecular masses corresponded to the reported values (19 S for the large- [9] and 11 S for the small-subunit mRNA [10]). One example of dot-blot analysis is shown in fig.1. Each dot was scanned using a densitometer. The relative value was calculated in the concentration range in which the measured area is in proportion to the dotted RNA, and is expressed relative to the value at 24 h of illumination.

As shown in fig.1, the light-induced increase in the small-subunit mRNA was reduced by uptake of α-amanitin before the onset of induction. Light induction of the large-subunit mRNA was not immediately reduced compared to the control. α-Amanitin provided during greening inhibited the induction of the small-subunit mRNA but not that of the large one. These results indicate that the steady-state level of the large-subunit mRNA is not directly affected by that of the small-subunit mRNA, and suggest that light induction of the large-subunit mRNA does not require the light-induced transcription of the other nuclear-coded genes. The large-subunit mRNA is probably synthesized irrespective of nuclear transcription, although it is shown that the two subunit polypeptides are coordinately expressed [1]. The gradual inhibition of the large-subunit mRNA compared to the control may arise from the disturbance of normal growth by blockage of nuclear transcription. The reduction of the induction of the small-subunit mRNA by α-amanitin supports previous observations that light induction of this small subunit mRNA is regulated at the level of transcription [2–4].

The effects of α-amanitin on the rate of RuBisCO synthesis was examined by pulse labelling with [^{35}S]methionine, followed by immunoprecipitation and electrophoretic analysis. The extent of labelling of the unassociated large and small subunits was also measured for the experiment in which α-amanitin was provided during induction, the results being shown in fig.2. The incorporation of [^{35}S]methionine into total soluble

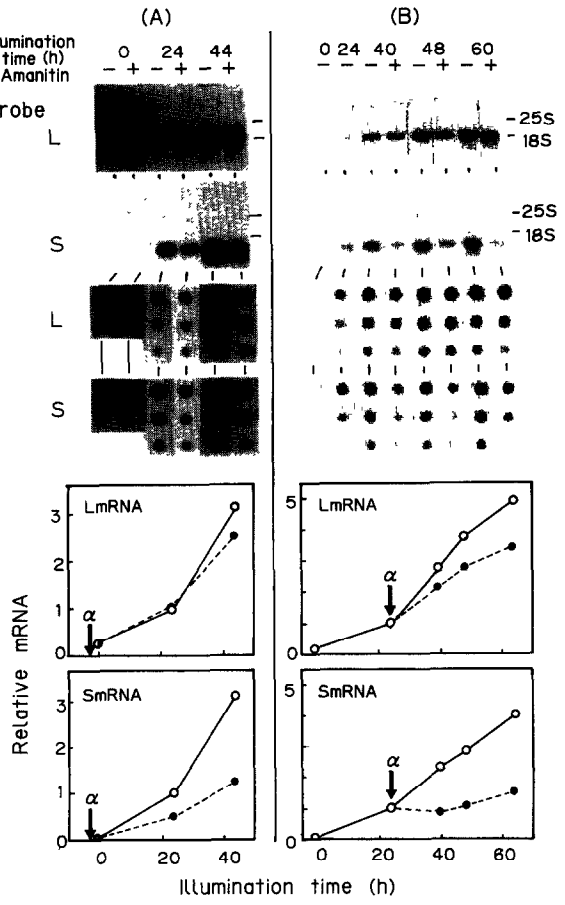

Fig.1. Effects of α-amanitin on induction of the two mRNAs of RuBisCO. Etiolated seedlings (7 days old) were continuously illuminated with white light. α-Amanitin solution (5 μg/ml) was continuously provided starting 3 h before the onset of illumination (A) and starting 24 h after illumination began (B). Total RNA (3 μg) was glyoxylated, electrophoresed, and transferred to a nylon membrane (Biodyne A, Pall). The membrane was baked, heated, prehybridized, and hybridized as described in [1]. The probes used are described in section 2. The specific activity of the nick-translated DNA was 0.5–1 × 10^8 cpm/μg and the probe concentration in hybridization buffer about 0.02 μg/ml. Exposure time was 7–72 h. 0.25–1 μg glyoxalated total RNA was dot-blotted, baked, heated, prehybridized, and hybridized as in [1]. The conditions for hybridization were the same as those of Northern [1]. Dots were measured using a densitometer (Shimadzu CS 930). The mean of three analyses was calculated and expressed relative to that after 24 h illumination without α-amanitin treatment. (○) Control, (●) α-amanitin; the time at which α-amanitin was provided is indicated by an arrow.

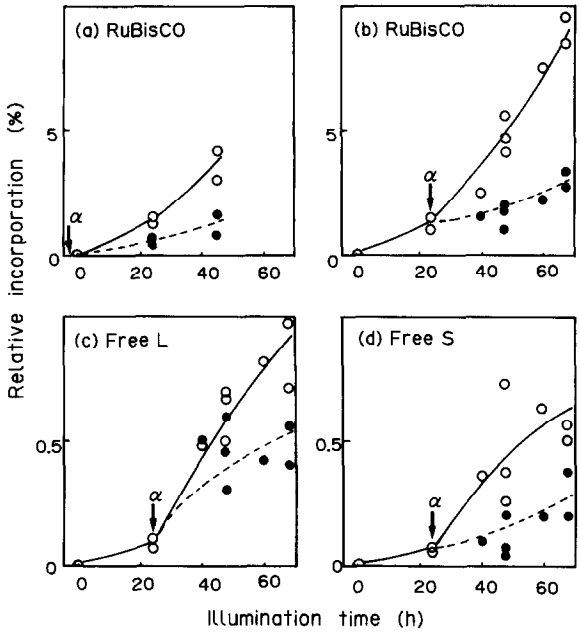

Fig.2. Effects of α-amanitin on synthesis of RuBisCO and the extent of labelling of unassociated subunits. Apical buds of pea seedlings were pulse labelled for 1.5 h with [^{35}S]methionine as described in section 2. The labeled protein was extracted and the synthesis rate of RuBisCO and the extents of labelling of the two subunits were measured as in [1]. Incorporation was expressed relative to the total soluble proteins. (a) Relative rate of RuBisCO synthesis when α-amanitin was provided before the start of illumination; (b) relative rate of RuBisCO synthesis when α-amanitin was provided during light induction; (c) relative incorporation into unassociated large subunits when α-amanitin was provided during light induction; (d) relative incorporation into unassociated small subunits when α-amanitin was provided during light induction. (○) Control, (●) α-amanitin; the time at which α-amanitin was provided is indicated by the arrow.

protein was not greatly affected by α-amanitin (not shown). The increase in rate of RuBisCO synthesis was inhibited by α-amanitin and correspondence was close between the rate of RuBisCO synthesis and the level of small-subunit mRNA. The pool size of methionine in the cytoplasm and chloroplast did not change in the presence or absence of α-amanitin because the ratio of radioactivity of the large and small subunits of the immunoprecipitates found using anti-RuBisCO IgG was the same in the presence and absence of

α-amanitin during greening. Hence, the slowness of RuBisCO synthesis in the presence of α-amanitin is ascribable to the low level of the small-subunit mRNA.

The steady state level of the large-subunit mRNA was about the same in the presence and absence of α-amanitin (fig.1), and post-transcriptional control of the expression of the large subunit gene is possible. As shown in fig.2, unassociated (free) large and small subunits were always present, but there was not a great excess of the large subunit compared to the level of the small subunit or the control value. The amount of the large subunit in the insoluble protein fraction was measured and found to be negligibly small (not shown). Thus, overproduction of the large-subunit polypeptide was not found. Probably the synthesis of the large subunit is regulated at the level of translation under these conditions, although rapid degradation of unassociated large subunits not detected by pulse labelling is possible as shown in isolated chloroplast [11,12]. There may be translational control of the large subunit for *Volvox* [13] and pea ([14]; Sasaki, Y., in preparation) as well.

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REFERENCES

[1] Sasaki, Y., Tomoda, Y., Tomi, H., Kamikubo, T. and Shinozaki, K. (1985) Eur. J. Biochem. 152, 179–186.
[2] Gallagher, T.F. and Ellis, R.J. (1982) EMBO J. 1, 1493–1498.
[3] Sasaki, Y., Tomoda, Y. and Kamikubo, T. (1984) FEBS Lett. 173, 31–35.
[4] Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.H. (1985) Nature 315, 200–204.
[5] Shinozaki, K. and Sugiura, M. (1982) Gene 20, 91–102.
[6] Bedbrook, J.R., Smith, S.M. and Ellis, R.J. (1980) Nature 287, 692–697.
[7] Bollum, F.J. (1959) J. Biol. Chem. 234, 2733–2734.
[8] Jendrisak, J. (1980) J. Biol. Chem. 255, 8529–8533.
[9] Sano, H., Spaeth, E. and Burton, W.G. (1979) Eur. J. Biochem. 93, 173–180.
[10] Cashmore, A.R. (1979) Cell 17, 383–388.
[11] Liu, X.Q. and Jagendorf, A.T. (1984) FEBS Lett. 166, 248–252.
[12] Malek, L., Bogorad, L., Ayers, A.R. and Goldberg, A.L. (1984) FEBS Lett. 166, 253–257.
[13] Kirk, M.M. and Kirk, D.L. (1985) Cell 41, 419–428.
[14] Inamine, G., Nash, B., Weissbach, H. and Brot, N. (1985) Proc. Natl. Acad. Sci. USA 82, 5690–5694.