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KINETIC STUDIES ON CHLOROPLASTIC RIBOSOMAL RNA AND CHLOROPHYLL DEVELOPMENT DURING GREENING OF PEA BUDS

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Abstract—The developmental kinetics of cytoplasmic and chloroplastic ribosomal RNA (r-RNA) and chlorophyll in pea buds upon transfer from dark to light were investigated. A relationship was found between the light-dependent development of chloroplastic r-RNA and chlorophyll while cytoplasmic r-RNA showed no direct relationship to these two. Chloroplastic r-RNA showed no lag period in its rate of formation while chlorophyll showed a lag of 24 hr after initiation of the light. Inhibition studies with cycloheximide and chloramphenicol showed a parallel inhibition between chloroplastic r-RNA and chlorophyll. The constant relationship between these two components in the developing pea buds and the appearance of chloroplastic r-RNA before chlorophyll leads to the conclusion that there is a direct dependence of the production of chlorophyll on the amount and appearance of chloroplastic r-RNA. This supports the hypothesis that the enzymatic or structural proteins involved in chlorophyll production are formed on the chloroplast ribosomes.

INTRODUCTION

THE OCCURRENCE of DNA and RNA within chloroplasts is now well known[1] and has given rise to the hypothesis that the chloroplast is a semi-autonomous self-duplicating organelle. Moreover, the chloroplastic DNA and RNA appears to be different from that existing in the cytoplasm of the same cell. Loening and Ingle[2] found that the ribosomal RNA from French bean leaves contained four components with a size equivalent to sedimentation coefficients of 25S, 23S, 18S and 16S. The 23S and 16S components only occurred in green tissues and their amount was independent of the 25S and 18S components. It was also observed that RNA prepared from chloroplasts isolated from bean leaves showed an eight-fold enrichment of the 23S and 16S components. Consideration of these facts led them to conclusively associate the 23S and 16S components with chloroplastic RNA.

The development of chlorophyll is light dependent and requires a lag period during which the conversion of protochlorophyllide to chlorophyll occurs followed by a period of rapid chlorophyll synthesis.[3] Various inhibitors of RNA and protein synthesis are found to prevent chlorophyll synthesis and these results have been interpreted as a requirement for either enzymes[3] or structural protein[4] for chlorophyll formation.

Not only is the formation of chlorophyll light-dependent, but so is that of chloroplastic r-RNA. Ingle[5] found that cotyledons of both light- and dark-grown radish seedlings accumulate chloroplastic r-RNA but the amount of chloroplastic 4-RNA relative to cytoplasmic r-RNA varied according to the conditions of illumination, there being much more of the

[1] J. T. O. KIRK and R. A. E. TILNEY BASSETT, *The Plastids*, Freeman, London (1967).
[2] U. E. LOENING and J. INGLE, *Nature* **215**, 363 (1967).
[3] M. GASSMAN and L. BOGORAD, *Plant Physiol.* **42**, 774 (1967).
[4] J. T. O. KIRK, *Planta* **78**, 200 (1968).
[5] J. INGLE, *Plant Physiol.* **43**, 1850 (1968).

chloroplastic component in the light-germinated seedlings. The development of chloroplastic r-RNA in the light-grown seedlings was found to be selectively inhibited by chloramphenicol[5,6] while other inhibitors were found to inhibit both chloroplastic and cytoplasmic r-RNA.

In these experiments we set out to examine whether there was any relationship between the developmental kinetics of chlorophyll and chloroplastic r-RNA.

RESULTS

Kinetics of r-RNA and Chlorophyll Development Upon Transfer From Dark to Light

The rate of formation of r-RNA and chlorophyll in pea buds following transfer of the intact pea plants from darkness to constant illumination is shown in Fig. 1. Almost no chloroplastic RNA was found in the pea buds that had been in the dark for up to 7 days.

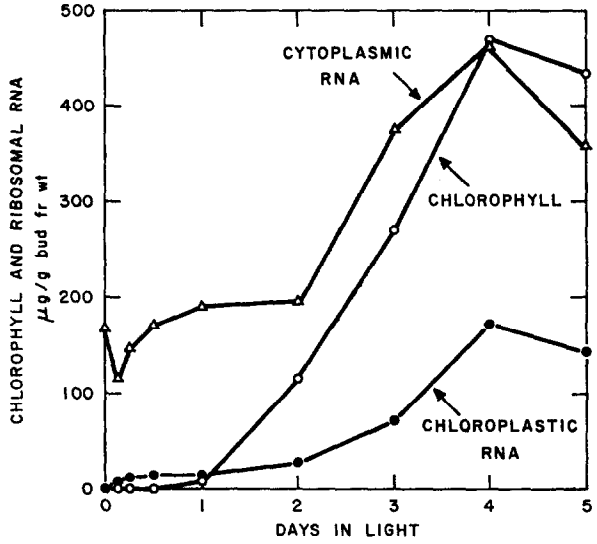

FIG. 1. THE KINETICS OF DEVELOPMENT OF CYTOPLASMIC AND CHLOROPLASTIC r-RNA AND CHLOROPHYLL IN PEA BUDS FOLLOWING TRANSFER FROM DARK TO LIGHT.

Chloroplastic r-RNA was detected after 3 hr illumination, thereafter increasing slowly to 24 hr followed by an exponential increase up to 96 hr. While the chloroplastic RNA component was developing, the amount of cytoplasmic RNA showed little change for the first 48 hr. As the buds expanded from the second day onwards resulting from the exposure to light both the amounts of cytoplasmic and chloroplastic RNA increased, but at different rates—95 and 114 per cent respectively from day 2 to 3 and 22·5 and 140 per cent respectively from day 3 to day 4. The development of chlorophyll showed a lag of about 24 hr and then a linear increase up to 96 hr.

The Influence of Inhibitors on the Development of r-RNA and Chlorophyll Upon Transfer From Darkness to Light

In the first experiment chloramphenicol at 0·1, 0·3, and 1·0 g/l and cycloheximide at 0·05 and 0·1 g/l were applied to the rooting medium of the pea plants and the experiment

[6] R. J. ELLIS, *Science* **163**, 447 (1969).

TABLE 1. THE INFLUENCE OF CHLORAMPHENICOL AND CYCLOHEXIMIDE TREATMENTS ON THE SIZE, CHLOROPHYLL, CYTOPLASMIC AND CHLOROPLASTIC r-RNA CONTENT IN PEA BUDS AFTER 48 hr IN CONSTANT LIGHT. THE INHIBITORS WERE WATERED ONTO THE ROOTING MEDIUM OF THE PEA PLANTS (0·22 ml per cm^3 OF ROOTING MEDIUM) IN THE DARK, 24 hr BEFORE THE LIGHT TREATMENT

Inhibitor	(g/l)	Bud weight (g)	% inhibition of bud weight	Chlorophyll (μg/g f/wt.)	% inhibition of chlorophyll	Cyt. RNA (μg/g f/wt.)	% inhibition Cyt. RNA	Chl. RNA (μg/g f/wt.)	% inhibition Chl. RNA
None	—	0·11	—	277	—	377	—	96	—
Chloramphenicol	0·1	0·12	−9	208	25	302	20	49	49
Chloramphenicol	0·3	0·11	0	138	50	336	11	36	62
Chloramphenicol	1·0	0·09	18	57	79	280	26	21	78
Cycloheximide	0·05	0·07	36	250	10	168	55	30	69
	0·1	0·05	55	114	59	221	41	25	74

terminated after 48 hr in the light (Table 1). The inhibitors were clearly absorbed by the roots with chloramphenicol producing an inhibition of greening of the buds in the light and cycloheximide considerably reducing bud size. Chloramphenicol at 1·0 g/l produced the greatest amount of inhibition of the chlorophyll and chloroplastic r-RNA, 79·5 and 78 per cent respectively, but only a 25 per cent inhibition of the cytoplasmic RNA component. The cycloheximide inhibited the chloroplastic RNA and chlorophyll to a lesser extent but had a greater effect on the growth of the plant, reducing bud size from 0·11 g/bud (no inhibitor) to 0·07 g/bud and 0·05 g/bud at concentrations of 0·05 g/l and 0·1 g/l respectively. Chloramphenicol produced no significant change in bud size.

In the second experiment the kinetics of the light-induced development of chlorophyll and chloroplastic RNA were examined in the presence of chloroamphenicol at 0·3 g/l and 1·0 g/l, which showed increasing inhibition of both these components with increasing time (Fig. 2).

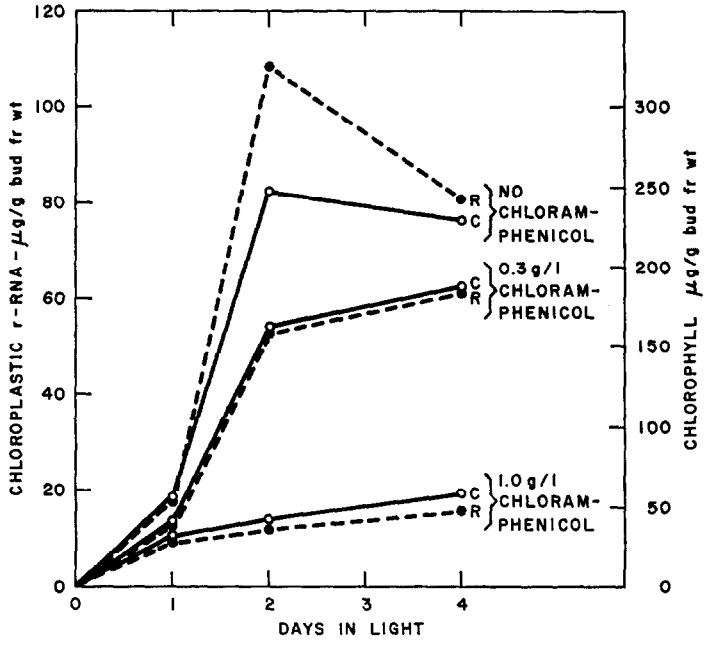

FIG. 2. THE INFLUENCE OF CHLORAMPHENICOL ON THE DEVELOPMENT OF CHLOROPLASTIC r-RNA (R) AND CHLOROPHYLL (C) IN PEA BUDS FOLLOWING TRANSFER FROM DARK TO LIGHT.

Comparison of the Amounts of Chlorophyll and Chloroplastic r-RNA Induced by Light

No consistent relationship was found between the content of cytoplasmic r-RNA and either chloroplasmic r-RNA or chlorophyll. When the bud contents of chloroplastic r-RNA and chlorophyll are compared at any time during their light-induced development, there is a clear, almost linear, relationship between the two (Fig. 3). This applies whether or not their development was reduced by the presence of chloramphenicol. The difference between the two lines in Fig. 3 is that the experiments were carried out using different batches of pea seed.

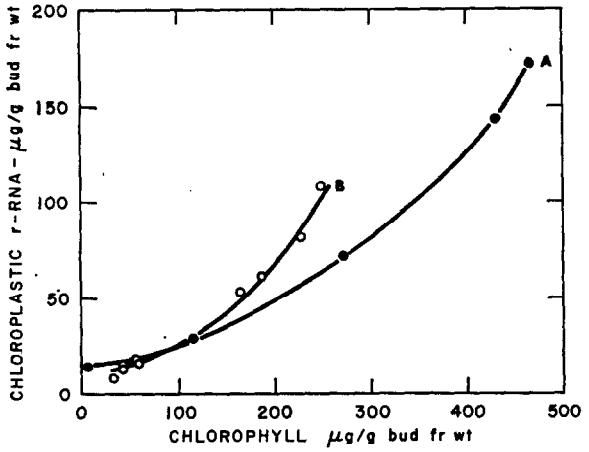

FIG. 3. THE RELATIONSHIP BETWEEN THE CONTENT OF CHLOROPHYLL AND CHLOROPLASTIC r-RNA IN PEA BUDS AT VARIOUS TIMES FOLLOWING TRANSFER FROM DARK TO LIGHT.

A—kinetic study without inhibitors. B—kinetic study in the presence of varying concentrations (0, 0·3, 1 g/l) of chloramphenicol (A and B were from different batches of seed).

DISCUSSION

Both chlorophyll and chloroplastic r-RNA were increased by light as has been previously demonstrated.[3,5] The cytoplasmic r-RNA, however, showed no net increase with exposure to light but rather only as a result of growth, with the enlarging of the buds in the light. Chloroplastic r-RNA showed no measurable lag period upon initiation of light treatment though no chlorophyll was detectable until 24 hr, giving a considerable lag period for the development of chlorophyll after chloroplastic r-RNA. This may well indicate the need for a specific amount of chloroplastic RNA before chlorophyll can be produced or a required time for the chloroplastic DNA and RNA to code for and form the enzymatic or structural proteins involved in chlorophyll formation. Following this lag period the accumulation of both chlorophyll and chloroplastic RNA seemed to parallel each other while there was less relationship between the amount of cytoplasmic RNA and either chlorophyll of chloroplastic RNA.

The inhibition studies using chloramphenicol and cycloheximide further established the relationship between chlorophyll and chloroplastic r-RNA. The cycloheximide seemed to be more effective in inhibiting the growth of the tissue (as measured by the bud size) whereas the chloramphenicol had virtually no effect on growth. Cycloheximide had a greater inhibitory effect on the cytoplasmic RNA than did chloramphenicol but these inhibitions showed little relation to the amount of chlorophyll inhibited. In the case of chloroplastic RNA the inhibitors were equally effective and thus indicated a greater relative selectivity for chloramphenicol. Chloramphenicol was also more effective in inhibiting chlorophyll development.

The overall results on the development of both types of RNA investigated and the influence of inhibitors match fairly closely those of Ingle[5] using radish cotyledons. No chloroplastic r-RNA was, however, found in the pea buds prior to placing in the light while it was found in the radish cotyledons, and the actual levels of RNA inhibition produced by the two inhibitors differ slightly. These discrepancies are probably due to the differences between the

species used, the cotyledons of radish forming the first photosynthetic surface in that epigeal germinating species, while pea has hypogeal germination, the buds remaining very small until placed in the light. For the inhibitor treatments the radish cotyledons were floated on the inhibitor solutions, while in these experiments the inhibitors were applied to the roots of intact seedlings allowing the plants to retain their integrity and the connection between the nutrient source and the developing buds.

The demonstration of a direct relationship between the amount of chloroplastic r-RNA and chlorophyll and the appearance of chloroplastic r-RNA slightly before that of chlorophyll indicate that the enzymatic or structural proteins involved in chlorophyll formation are manufactured within the chloroplast itself, on the chloroplast ribosomes, and probably not transported from the cytoplasm surrounding the chloroplast. This indicates that the chloroplast possesses at least a certain degree of autonomy and supports the hypothesis that chloroplasts were once independent organisms that became symbiotically incorporated into the cell.

EXPERIMENTAL

Plant Material and Treatments

Peas, *Pisum sativum* va. Alaska, were sown in vermiculate in plastic containers and were maintained at 23° in the dark until 1 day after they were 9 cm high. They were then transferred to constant light of 6300 $\mu W/cm^2$ at 23°. When inhibitors were used they were applied to the vermiculate in which the peas were growing (in 110 ml/500 cm^3 of vermiculite) 24 hr before moving into the light and again 72 hr later. After varying times of light with and without inhibitors, 5 g fresh wt. of buds per sample were taken, cut at the third node which was the node just below the bud. The tissue was stored at −20° until analysis.

Chloramphenicol and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Missouri.

RNA Extraction

RNA was extracted from 5 g of buds per treatment at 0° according to Loening and Ingle's[2] method A. Precautions were taken to prevent breakdown of the chloroplastic r-RNA as pointed out by Ingle.[7]

RNA Analysis and Estimation

The various r-RNA fractions were separated by polyacrylamide gel electrophoresis techniques, essentially as those described in Loening and Ingle,[2] using purified acrylamide and methylenebisacrylamide.[8] Plexiglass tubes of 6·5 mm dia. were sealed at one end with a piece of Parafilm and filled to a depth of 9 cm with a solution of 2·4% acrylamide. Following gel polymerization, the Parafilm seals were replaced with wet sections of dialysis tubing held in place by small rubber bands. The gels were then placed in the electrophoresis tank and allowed to equilibrate for 45 min. at 5 mA/tube.

Each sample was then dissolved in 1 ml of running buffer[2] containing 10% sucrose. A portion was taken to determine its purity and amount present by spectrophotometry. All samples showed a high degree of purity with a 260/280 ratio of 2·0–2·8. 10 or 20 μl were then loaded on the electrophoretic gels at a concentration of 2 $\mu g/\mu l$. The electrophoresis was run at 5 mA/tube for 4·5 hr at room temperature after which time the gels were removed and scanned at 260 nm on a Gilford recording spectrophotometer. Excellent separations of the r-RNAs were obtained similar to those previously published.[2,5] The RNA content was determined from the respective peak areas taking 1 absorbancy unit to equal 43·5 μg RNA.

Chlorophyll Extraction and Estimation

5 g of tissue was placed in 50 ml of 80% acetone for 10 min and then ground in an Omnimixer for 1 min; the slurry was then left for 6–8 hr in darkness. The mixture was centrifuged and the absorption of the supernatant recorded at 645 and 663 nm from which the chlorophyll content was calculated.[9]

Acknowledgements—Supported in part by a U.S. Public Health Service grant to Professor A. W. Galston.

[7] J. INGLE, *Plant Physiol.* **43**, 1448 (1968).
[8] U. E. LOENING, *Biochem. J.* **102**, 251 (1967).
[9] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).