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PROTEIN SYNTHESIS IN ISOLATED ETIOPLASTS AFTER LIGHT STIMULATION

V. J. HEARING*
Department of Biology, Catholic University of America, Washington, D.C.

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Key Word Index—*Zea mays*; Gramineae; etioplasts; protein synthesis; light stimulation; lamellar proteins; cycloheximide.

Abstract—Grains of *Zea mays* were germinated in the dark for 5 days. Etioplasts were then isolated in the dark and exposed to light in the presence of labelled amino acids and various inhibitors. After periods of incorporation, either in the dark or light, proteins were isolated and then examined with the aid of polyacrylamide gel electrophoresis. The highest specific activity of incorporation was found in the lamellar protein fraction. The use of inhibitors enabled the specific products of etioplast incorporation to be identified on the gels. Analyses of radioactivity in protein bands indicate that the plastid is capable of responding to light *in vitro* in at least two ways: (1) by an increase in the rate of protein synthesis; and (2) by a reproducible control of the various proteins synthesized either in the dark or light, which resulted in the 'turning off' of some proteins synthesized in the dark, and the subsequent initiation of the synthesis of others, in response to light. The results presented in this study indicate that the plastid *in vitro* is capable of a rather complex response mechanism when subjected to environmental change, such as light stimulation. This suggests that the plastid is capable of a great degree of autonomy, at least when necessary, and is possibly more independent of nuclear control than heretofore suggested in the literature.

INTRODUCTION

OUR KNOWLEDGE of the metabolic patterns of cellular organelles has increased logarithmically over the past 15 years. Nuclei, mitochondria, chloroplasts and other organelles have been shown capable of protein, DNA and RNA synthesis, both *in vivo* and *in vitro*. The degree of interplay between these systems remains unknown for the large part, although it must be very precisely controlled, in view of the delicate balance necessary to maintain life within each cell. The synthesis of protein by chloroplasts has been shown to be DNA- and RNA-dependent. The 70*S* ribosome, when isolated from the chloroplast, is capable of mediating protein synthesis when supplied with the necessary substrates, and is about twenty times more active in amino acid incorporation *in vitro* than its cytoplasmic analog, the 80*S* ribosome.[1,2] Although the mechanism of protein synthesis in the chloroplast appears to resemble that of the cytoplasm very closely, there are several important differences, including varied inhibition characteristics with several translational inhibitors.[3,4]

It is known that many biochemical changes, as well as morphological ones, take place in dark grown (etiolated) plants when they are exposed to light, the most obvious being the

* Present address: Dermatology Branch, Bldg 10 Rm 12 N 254, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

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immediate synthesis of chlorophyll and the extensive rearrangement of membranes within the chloroplast.[5–9] On the molecular level, a marked stimulation of protein,[10–12] RNA[13,14] and lipid[15,16] biosynthesis is observed immediately after exposure of etiolated plants to light. Whether the isolated etioplast is capable of exerting the response observed *in vivo* is not known: thus the degree of control that chloroplast or nuclear DNA exerts over this response mechanism cannot be evaluated. It is the purpose of this investigation to determine if etioplasts are capable of responding to light stimulation *in vitro*, since their ability or inability to respond might provide an indication of the degree of chloroplast autonomy within the cell.

RESULTS AND DISCUSSION

Characterization of Etioplast Amino Acid Incorporation in vitro

Table 1 records the inhibition characteristics of the system under study. Chloramphenicol, a specific inhibitor of the 70*S* ribosome, inhibits this system by 94·7%. Cycloheximide, which blocks amino acid incorporation by 80*S* ribosomes, can be seen to decrease incorporation by only 3·5%. This evidence eliminates cytoplasmic ribosomes from consideration as major contributing factors to observed incorporation. However, mitochondria and bacteria, which contain 70*S* ribosomes and which are also capable of amino acid incorporation, still remain to be considered. Triton X100, which at this concentration is known to solubilize plastids only,[17] and which has no effect on mitochondria or bacteria,[18] was then used to solubilize the etioplast pellet for 10 min. All remaining insoluble material was immediately centrifuged at 100 000 *g* for 15 min and resuspended in Medium 199 and

TABLE 1. INHIBITION CHARACTERISTICS OF ETIOPLAST AMINO ACID INCORPORATION IN THE PRESENCE OF LIGHT

No.	Inhibitor	Concentration (mg/ml)	^{3}H(cpm)	% Inhibition
1	Control	0·000	28 369	0·0
2	Chloramphenicol	0·300	1 493	94·7
3	Cycloheximide	0·025	27 361	3·5
4	Triton-X 100 pellet	10·000	704	97·5

Etioplast suspensions were incubated with 50 μCi ^{3}H-leucine for 60 min in the presence of light and inhibitors as shown. Proteins were precipitated with TCA and collected on a Millipore filter, washed and counted as described in Methods. Results shown are those of one experiment. Comparable data were obtained from each of five similar experiments.

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incorporation measured as described. Only 3% of the observed incorporating activity of controls remained, indicating that no more than 3–4% of the observed activity under these conditions can be ascribed to contaminants. All other incorporation experiments routinely included cycloheximide (25 μg/ml) to reduce activity by any contaminating 80*S* ribosomes.

Stimulation of Incorporation by Light in vitro

To determine the effect of light upon incorporating activity of the etioplast, etioplasts were resuspended in approximately 20 ml of Medium 199, labelled amino acid was added, as noted in Table 2, and equal portions, about 2 ml, were distributed in tubes. After 30 min of incubation in the light, amino acid incorporation is increased 10·0% when compared to controls in the dark. After 60 min in the light, a 14·5% stimulation is observed over the dark controls.

TABLE 2. STIMULATION OF AMINO ACID INCORPORATION BY ETIOPLASTS *in vitro* BY LIGHT

No.	Time (min)	^{3}H(cpm)	% Stimulation
1 Dark	30	55 249	—
2 Light	30	60 804	10·0
3 Dark	60	163 101	—
4 Light	60	186 734	14·5

Etioplast suspensions were incubated with 50 μCi ^{3}H-leucine, after which proteins were precipitated with TCA, collected on a Millipore filter and washed as described in the Experimental. Results shown are those of one experiment. Comparable data were obtained from each of five similar experiments.

Light Stimulated Amino Acid Incorporation in Protein Fractions

Table 3 records the specific activities of various protein fractions isolated after 60 min incubation in the presence of light. The total protein fraction (tube 1) had a specific activity of 25 200 cpm/mg protein. Fraction I protein seems to be synthesized to a limited extent, while structural protein is very poorly labelled, if at all. Membrane and lamellar protein

TABLE 3. SPECIFIC INCORPORATION OF AMINO ACIDS INTO PROTEIN FRACTIONS BY ETIOPLASTS

No.	Fraction	^{3}H(cpm/ml)	^{3}H sp. act. (cpm/mg)
1	Acid-insoluble	169 800	25 200
2	Fraction I	2240	2950
3	Structural protein	1450	1020
4	Membrane protein	23 100	6080
5	Lamellar protein	93 400	25 800

Seven tubes with approximately 5 ml etioplast suspension in each were incubated with 50 μCi ^{3}H-leucine each, for 60 min in the presence of light. Specific activity of each isolated protein fraction was then measured. Results presented are those of one experiment. Comparable data were obtained from each of two other similar experiments.

fractions have relatively higher specific activities, as would be expected in light of the great amount of membrane formation in the light stimulated etioplast *in vivo*.

Polyacrylamide Gel Electrophoresis of Total Etioplast Protein

The ability of the etioplasts to incorporate amino acids into protein and the response of this incorporation to light stimulation having been established, the question remained as to whether the *m*RNA synthesized and/or already present in the plastid was translated randomly, or whether there was some degree of control exercised by the etioplast over the polypeptide synthesis. Total etioplast protein can be resolved into 20 reproducible bands

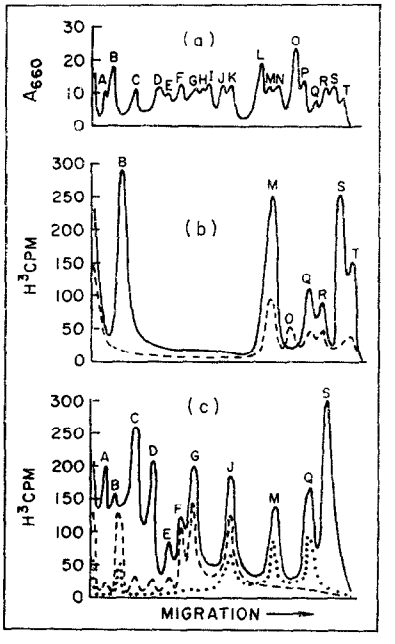

FIG. 1. THE DISTRIBUTION OF PROTEIN BANDS OF MAIZE ETIOPLASTS AFTER SEPARATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS (pH 8·3), AND THE DISTRIBUTION OF RADIOACTIVITY INCORPORATED INTO THESE BANDS.

Etioplast suspensions were incubated with 100 μCi ^{3}H-leucine. The reaction was stopped and proteins precipitated by the addition of TCA. The proteins were then washed, solubilized in buffer, and subjected to electrophoresis. Approximately 50 μg of protein were layered on each gel column. 1 (a) Densitometer tracing of banding pattern of total protein preparation. 1 (b) Distribution of radioactivity in total protein preparation incubated with labelled leucine in the dark. – – – 60 min, ——— 180 min. 1 (c) Distribution of radioactivity of total protein preparations synthesized by etioplasts *in vitro* in the presence of light. – – – 60 min, ——— 180 min, · · · · 180 min, with chloramphenicol, 300 μg/ml.

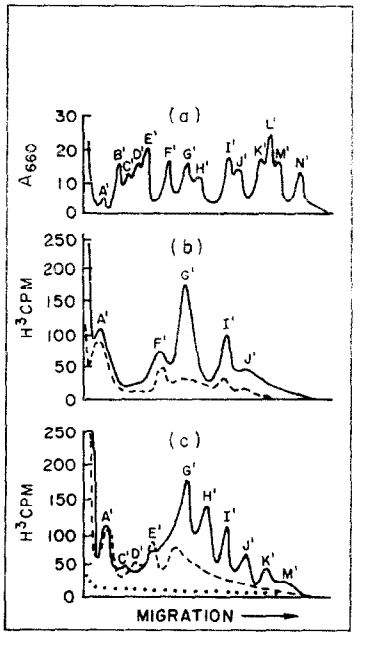

FIG. 2. THE DISTRIBUTION OF PROTEIN BANDS FROM LAMELLAR PROTEIN PREPARATIONS SEPARATED BY POLYACRYLAMIDE GEL ELECTROPHORESIS (pH 3·1), AND THE DISTRIBUTION OF RADIOACTIVITY INCORPORATED INTO THESE BANDS.

Etioplast suspensions were incubated with 50 μCi ^{3}H-leucine. The reaction was stopped and proteins precipitated by the addition of TCA. Protein samples were then isolated, washed, solubilized in buffer and subjected to electrophoresis. Approximately 50 μg of protein were layered on each gel column. 2 (a) Densitometer tracing of the banding pattern obtained from lamellar protein preparations of maize etioplasts. 2 (b) Distribution of radioactivity in lamellar proteins incubated with labelled leucine in the dark. – – – 60 min, ——— 180 min. 2 (c) Distribution of radioactivity of lamellar proteins as synthesized by etioplasts *in vitro* during light stimulation. – – – 60 min, ——— 180 min, · · · · · 180 min, with chloramphenicol, 300 μg/ml.

under alkaline conditions (Fig. 1a). There are four bands labelled in the presence of chloramphenicol, notably bands B,J,M and Q (Fig. 1c), and thus these are eliminated from consideration as products of the 70*S* etioplast ribosome. As judged from Figs. 1b and c, there are basically four classes of proteins represented: (1) unlabelled proteins (H,I,K,L,N,P); (2) proteins labelled in the dark and not in the light (O,R,T); (3) proteins labelled in the dark and the light (B,M,Q,S); (4) proteins labelled in the light and not in the dark (A,C,D,E,F,G). It seems very apparent from these results that etioplasts are capable of responding to light stimulation *in vitro* in several ways, including the 'turning off' of the synthesis of some proteins produced in the dark, and the initiation of the synthesis of others. The fact that all these bands are represented in dark grown etioplast fractions indicates that the etioplast has reserves of most, if not all, the proteins required for initial light adaptation.

Polyacrylamide Gel Electrophoresis of Etioplast Lamellar Protein

The chloramphenicol control shows that no labelled amino acids are incorporated into light stimulated etioplast lamellar protein fractions by 80*S* ribosomes (Fig. 2c). In the dark (Fig. 2b), there are five bands labelled (A′,F′,G′,I′,J′). When light is used to stimulate incorporation, label can be found in four bands after 60 min incorporation (A′,E′,G′,C′) and in ten bands after 180 min of light stimulation *in vitro* (A′,D′,E′,F′,G′,-H′,I′,J′,K′,M′). Proteins in bands A′, G′ and I′ are produced in similar quantities both in the light and the dark, while production of band F′ is decreased in the light. It appears then, that production of at least one protein (F′) is diminished in response to light *in vitro* whereas production of several other proteins (D′,E′,H′,K′,M′) is initiated.

CONCLUSION

The results presented in this study indicate that the etioplast is capable of responding to light *in vitro* in at least two ways: (1) by an increase in the overall rate of protein synthesis; and (2) by a reproducible control of the nature of the proteins synthesized in the dark or light, resulting in the turning off of some proteins synthesized, and subsequent initiation of others. Implicit in these results is the presence of an independent mechanism of regulation of protein synthesis within the plastid as an entity. The level on which this regulation is accomplished is unknown, but, in view of the extensively complex nature of the entire process described herein, it would seem reasonable to assume that more than one simple level of control is present.

EXPERIMENTAL

Isolation of etioplasts. Grains of *Zea mays* (Eureka ensilage), obtained from commercial suppliers, were germinated in the dark for 5 days. The shoots were then excised and minced in a Virtis homogenizer for 15 sec in 2 vols. of STM (40 mM sucrose, 50 mM Tris, 10 mM KCl, 10 mM $MgCl_2$, 4 mM 2-mercapto-ethanol, pH 7·9). The suspension was filtered through 4 layers of cheesecloth and then 4 layers of facial tissue. The resulting filtrate was centrifuged at 500 *g* for 5 min to sediment most of the contaminating nuclei and any whole cells which remained. The supernatant was poured off and the etioplasts sedimented by centrifugation at 4000 *g* for 10 min. The pellet was then resuspended in STM, filtered through facial tissue, the contaminants sedimented at 500 *g* for 5 min, and the supernatant centrifuged at 4000 *g* for 10 min to yield the 'washed etioplast pellet'. All isolation procedures were carried out at 4° in the dark, or with dim green light illumination.[19]

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Amino acid incorporation and assay. The washed etioplast pellet was resuspended in Medium 199,[20] which was supplemented with excess ATP. Except where noted, cycloheximide (25 μg/ml) was routinely added at this point to suppress activity by any contaminating 80*S* ribosomes. This suspension was then divided into equal portions, with labelled amino acids added as specified in the table legends. Test samples were then removed to a room illuminated with fluorescent lights (*ca.* 800 lx), while others were left in the dark. A complete system, minus etioplasts was used as a control in all experiments. At the end of the desired incubation periods, incorporation was stopped by the addition of 2 vols. of 10% (w:v) TCA containing 100× excess unlabelled leucine. The mixtures were kept overnight at 4° to precipitate the protein. The precipitate thus collected was treated in one of two ways. (1) Protein samples for electrophoresis were washed several times with cold 10% TCA, once with hot (90°) 10% TCA, once with EtOH, finally with acetone, air dried and stored frozen until used. Separation procedures are listed below. (2) For assays of incorporation, the procedure described by Mans and Novelli[21] was employed. The filter, after washings, was placed in a scintillation vial and covered with 0·5 ml 100% formamide. After 30 min, 10 ml of scintillation fluid (0·9% PPO, 0·01% POPOP (w:v) in 2-methoxyethanol–toluene (1:2)) were added. This mixture solubilized both the Millipore filter and the protein layered on it. The vials were then counted in a Packard Tri-Carb Scintillation Spectrometer, Model 3375.

Polyacrylamide gel electrophoresis. Protein samples were separated under both acidic and basic conditions on disc columns. The method of Takayama *et al.*[22] was followed for the acidic (pH 3) gel system. A modification of the procedure described by Peacock and Dingman[23] was used for preparing the basic (pH 8) gels, which consisted of adding 1% sodium dodecyl sulfate. Samples were concentrated by a prerun 0·5 mA/tube for 1 hr, before adjusting the current to 3 mA/tube for 4 hr at 20°. The gels were stained in 1% fast green FCF (w:v) in 7·5% HOAc (v:v) then destained in 7·5% HOAc alone. The gels were scanned at 660 nm with a Gilford Spectrometer, Model 2000, equipped with a linear transport scanner, then sliced into 1 mm pieces and dissolved overnight in 0·5 ml 30% H_2O_2 (v:v) at 50°. 10 ml of scintillation fluid were added, the vials cooled overnight and counted.

Preparation of protein fractions. After etioplasts were allowed to incorporate ^{3}H-leucine (250 μCi) for 60 min in the presence of light, the suspension was divided into 5 equal parts and protein fractions were collected as follows. Total protein was prepared by precipitation with 10% TCA, washing twice with cold 10% TCA, once with hot 10% TCA, once in EtOH, air drying and storing frozen until used. The method described by Wilson and McCalla[24] was followed for the isolation of fraction I protein. The procedure described by Eyton and Ohad[25] was followed for the isolation of membrane proteins. Structural protein was isolated according to the method described by Criddle and Park,[26] while the method of Lockshin and Burris[27] was used to isolate lamellar protein. Protein concentrations were determined by the method of Lowry *et al.*[28] and by UV absorption.

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