Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 248-255

AN EFFICIENT AND PROLONGED IN VITRO TRANSLATIONAL SYSTEM FROM ISOLATED CUCUMBER ETIOPLASTS

H. DANIELL*[1], M. KRISHNAN, U. UMA BAI AND A. GNANAM[2]

*Department of Biochemistry and Biophysics,
Washington State University, Pullman, WA 99164
School of Biological Sciences,
Madurai Kamaraj University, MADURAI 625021, INDIA

Received January 10, 1986

Etioplasts were isolated from dark grown cucumber cotyledons pretreated with kinetin and gibberellic acid. When incubated in a cofactor enriched medium these etioplasts incorporated [^{35}S] methionine into a hot trichloroacetic acid-insoluble fraction; this incorporation was linear for 8 h of incubation and was inhibited by chloramphenicol but not by cycloheximide. Over the same time period, the etioplasts showed continued linear synthesis of the chlorophyll precursors protochlorophyllide, Mg-protoporphyrin and protoporphyrin IX. Analysis of products of in vitro protein synthesis by etioplasts and cotyledons showed the thylakoid membrane polypeptide profiles to be identical. Continued incorporation of [^{35}S] methionine into the large subunit of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) for 8 h has been confirmed further by immunoprecipitation with anti-spinach RuBisCO. This competent in vitro translation system should be useful for future studies of chloroplast protein synthesis and gene expression.

Isolated chloroplasts have been used to identify products of chloroplast protein synthesis as well as to investigate post-translational protein transport, processing, assembly and insertion into membranes. Though in vitro translation of chloroplast mRNA in E. coli, rabbit reticulocyte and wheat germ extracts has been demonstrated, heterologous systems differ from chloroplasts in regard to specific features of the transcription and translation machinery, as well as recognition of regulatory and processing signals present on chloroplast genes (1). As a result, translation of some chloroplast transcripts may be inaccurate in such systems (1, 2). The usefulness of transcription-translation systems for chloroplast gene expression and regulation is dependent upon their competence.

[1]All correspondence and reprint requests should be addressed to Dr. H. Daniell, Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164, USA.
[2] Presently Vice-Chancellor, Bharathidasan University, Tiruchirapalli 620001, India.

0006-291X/86 $1.50

However, light driven chloroplast protein synthesis is rapid during the first 5 min of illumination and then declines, stopping altogether by 15 to 30 min (3, 4).

We have demonstrated recently that pretreatment of etiolated cotyledons with hormones induces excess accumulation of prothylakoid proteins. Etioplasts thus enriched in precursors of proteins and incubated in a cofactor enriched medium were capable of synthesizing protochlorophyllide (5) and chlorophyll (6) at rates higher than the highest rates observable in greening tissues in vivo (7). Etioplasts developed in vitro showed extensive grana formation (8), development of photosystem I activity coupled to phosphorylation (9) and development of O_2 evolution concomittant with the synthesis of all the required polypeptides (10). The progress made in this in vitro system has been reviewed recently (11, 12). We report here our observations that these enriched etioplasts show continued linear pigment biosynthesis and incorporation of labeled amino acid into a hot trichloroacetic acid-insoluble fraction for 8 h. Analysis of products of protein synthesis by etioplasts and cotyledons showed the thylakoid membrane and soluble protein profiles to be identical. The results have been further confirmed by immunoprecipitation with ribulose bisphosphate carboxylase/ oxygenase antiserum.

MATERIALS AND METHODS

Cucumber (Cucumis sativus L.) seeds were germinated in moist vermiculite at 28°C in the dark. Cotyledons were excised with hypocotyl hooks and pretreated in the dark with 0.5 mM kinetin and 2 mM gibberellic acid for 20 h. Plastids were isolated as reported earlier (5) and purified by discontinuous sucrose density gradient centrifugation. The tetrapyrrole biosynthetic capabilities of the isolated etioplasts were determined by assaying, simultaneously, various sections of the chlorophyll biosynthetic pathway following incubation of the etioplasts for different durations (13). Light driven in vitro protein synthesis was carried out by incubation of the etioplasts with [^{35}S] methionine (20 μCi) at 27°C on a metabolic shaker, irradiated with cool white fluorescent light of 30 $\mu E.M^{-2}.S^{-1}$ intensity. Aliquots transferred at indicated time intervals to Whatman No. 3 filter paper discs were processed by the method of Bollum (14). In order to minimize bacterial contamination, cotyledons were surface sterilized before plastid isolation; sterile media and glassware were used throughout the experiments. For studying the process of greening in vivo, cotyledons pretreated with hormones were infiltrated with [^{35}S] methionine for 20 min and were exposed to cool white fluorescent light of 70 $\mu E.M^{2}.S^{-1}$ for different durations. Protein was estimated according to Lowry et al. (15). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described in (16) and fluorography as in (17). Soluble proteins were either resolved by SDS-PAGE directly or

following immunoprecipitation with anti-spinach ribulose bisphosphate carboxylase/ oxygenase (RuBisCO) serum conjugated to protein A-Sepharose.

RESULTS AND DISCUSSION

Incubation of etioplasts in a cofactor enriched medium resulted in the immediate commencement of protochlorophyllide (Pchlide) and Mg-protoporphyrin [MP(E)] synthesis. While the synthesis and accumulation of Pchlide and MP(E) continued to increase for up to 8 h of incubation, significant increase in the synthesis of protoporphyrin $\overline{IX}$ (Proto) was observed only after 6 h of incubation (Fig. 1). Continued synthesis of the Pchlide and MP(E) reflects the maintenance of the intactness of the isolated etioplasts during several hours of incubation because these enzymatic reactions are highly sensitive to structural disorganization (18). This is further documented by our electron microscopic studies of etioplasts during incubation (8). Drastic increase in the level of Proto after 6 h of incubation and decline in the level of MP(E) (Fig. 1) indicates that ATP has become limiting. An absolute requirement of ATP for the insertion of Mg into Proto is known (7).

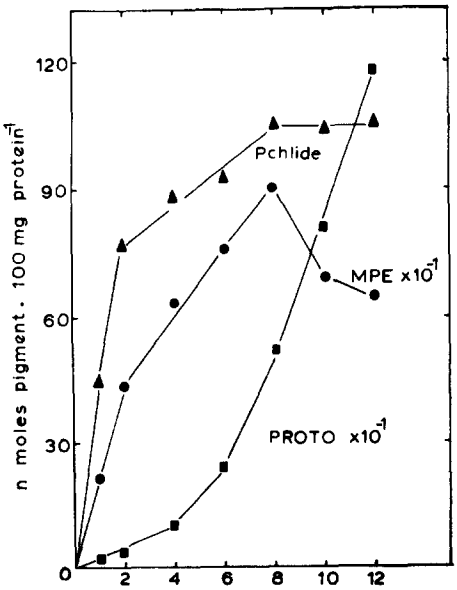

Fig. 1. Tetrapyrrole biosynthetic capabilities of the etioplasts. The plastids were incubated in the dark for different durations. The values plotted here indicate net synthesis of pigments (total synthesis minus 0 h values).

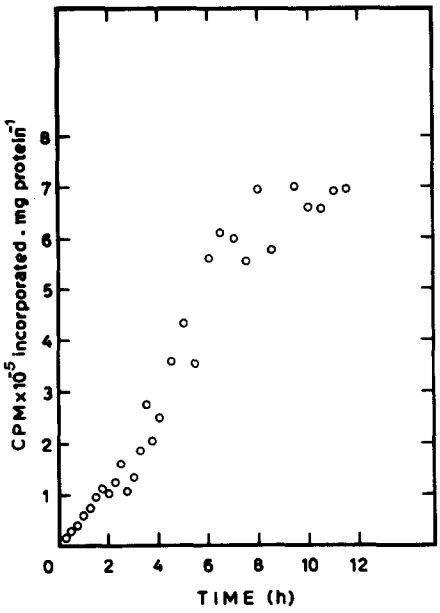

Fig. 2. Time course of labeled amino acid incorporation by etioplasts incubated with [^{35}S] methionine at 27°C in the light.

Prolonged synthesis of the various pigment pools for up to 8 h of incubation (Fig. 1) prompted us to examine the protein synthetic efficiency of this cell-free system. Incorporation of [^{35}S] methionine into hot trichloroacetic acid-insoluble fraction by isolated etioplasts was linear for 8 h of incubation and thereafter remained stable (Fig 2) in parallel with the observed pigment biosynthesis (Fig. 1). Prolonged protein synthesis observed here may be due to several reasons. We did not observe prolonged protein synthesis when etioplasts isolated from these cotyledons were incubated in buffer without added cofactors (19). It is known that etioplasts use ATP and not light energy to drive protein synthesis (4). In addition to the role of ATP, another important factor contributing to sustained in vitro protein synthesis may be the improved incubation conditions that stabilize plastid structure. Although earlier workers reported the rapid distruption of etioplasts upon illumination (20) etioplasts under our experimental conditions showed a very high degree of structural preservation (8).

Cycloheximide, an inhibitor of protein synthesis in eukaryotic cytoplasm, had no effect on the level of protein synthesis (Fig. 3) indicating that the

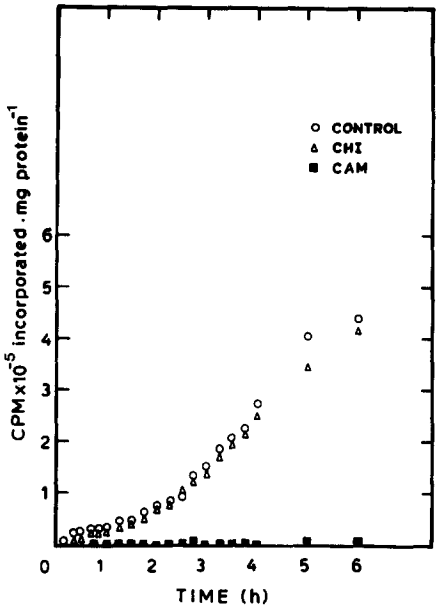

Fig. 3. Effect of inhibitors on in vitro protein synthesis by etioplasts. Etioplasts were preincubated with cycloheximide (100 µg/ml) or chloramphenicol (50 µg/ml) for 5 min at 27°C after which the reaction was initiated by the addition of labeled amino acid.

etioplast system under study was not contaminated with active cytoplasmic 80 S ribosomes. On the other hand, D-threo chloramphenicol completely inhibited in vitro protein synthesis by isolated etioplasts (Fig. 3). Synthesis of the large subunit of RuBisCO encoded by the chloroplast genome but not the small subunit of RuBisCO (Fig. 5B) encoded by the nuclear genome further confirms the lack of cytoplasmic contamination.

Figure 4A shows an autoradiograph of thylakoid membrane polypeptides labeled with [^{35}S] methionine during greening in cotyledons. Similar polypeptide profiles were observed in etioplasts developed in vitro (Fig. 4B). However, the 32 kDa protein was labeled more intensely in etioplasts developed in vitro (Fig. 4B) than in vivo (Fig. 4A). This polypeptide is a widely known major product of in vitro chloroplast protein synthesis (4). Analysis of [^{35}S] labeled soluble proteins from etioplasts incubated in vitro showed continued incorporation of [^{35}S] methionine into various polypeptides up to 8 h (Fig. 5A). One major product is the large subunit of RuBisCO. The identity of this protein was further confirmed by immunoprecipitation with anti-spinach RuBisCO (Fig. 5B).

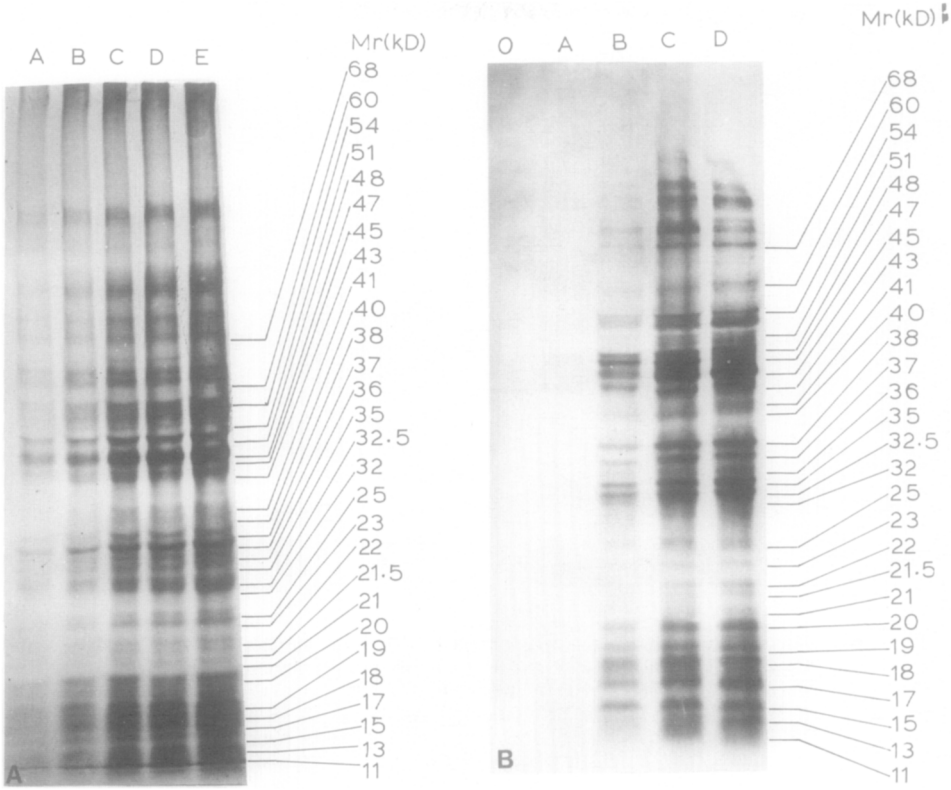

Fig. 4A. Autoradiograph of thylakoid membrane polypeptides labeled during A) 0 h; B) 2 h; C) 4 h; D) 6 h; E) 8 h of greening cotyledons. The polypeptides were resolved in a 7.5-15% gradient SDS polyacrylamide gel. 4B. Thylakoid membrane polypeptides labeled during O) 0 h; A) 2 h; B) 4 h; C) 6 h; D) 8 h of incubation of isolated etioplasts.

Bacterial contamination of plastid preparations has been previously tested by solubilizing the preparation in 2% Triton X-100 at the end of incubation, and measuring the radioactivity present in a 10,000 x g pellet (21). Such an analysis performed in etioplasts revealed a slight increase in radioactivity associated with the pellet fraction as incubation progressed (Fig. 6). It should be pointed out here that a similar procedure has been used to isolate photosystem II particles from chloroplasts (22) and so the incorporation observed in the pellet fraction may also be due to incorporation into these particles. Contribution of contaminating bacteria to the soluble protein fraction can be ruled out because the etioplasts were lysed by freeze thawing and soluble proteins were obtained after discarding the high speed centrifugation

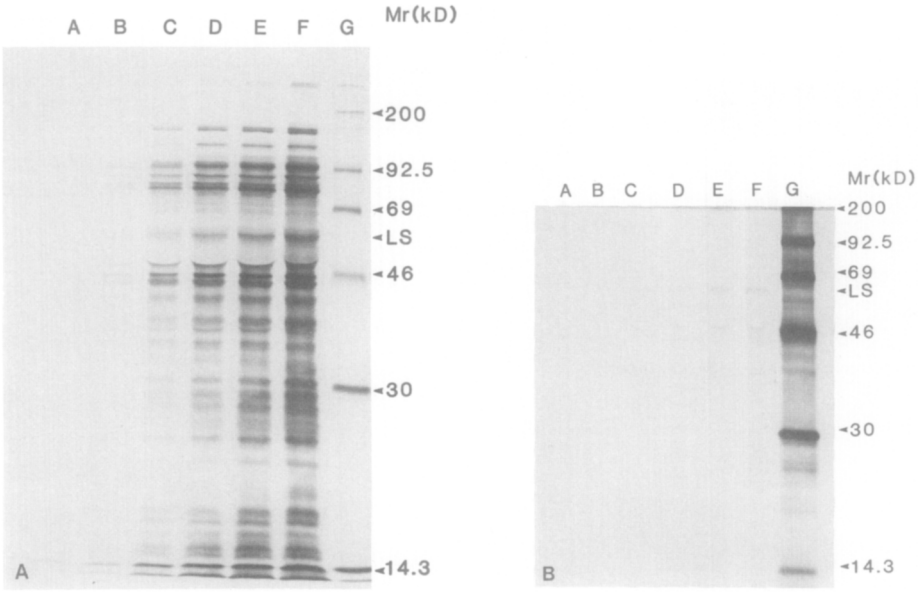

Fig. 5A. Autoradiograph of soluble proteins labeled during A) 0 h; B) 1 h; C) 2 h; D) 4 h; E) 6 h and F) 8 h of incubation of isolated etioplasts. The soluble proteins (50 µg/slot) were resolved in a SDS polyacrylamide gel polymerized from 12.5% acrylamide. ^{14}C-methylated protein standards (G) contain myosin (200 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). 5B. Immunoprecipitation of RuBisCO.

pellet. The contaminating bacteria, if any, should be present in the high-speed pellet because they are not lysed by a single freeze/thaw cycle (23).

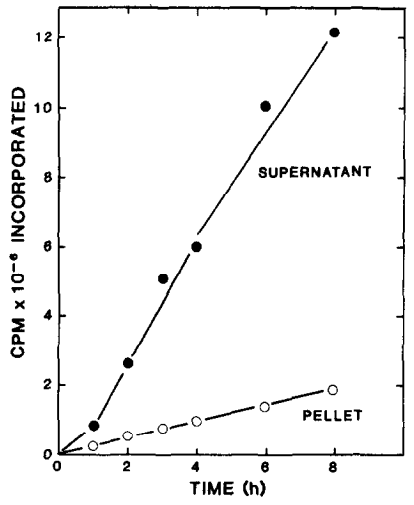

Fig. 6. Purity of the isolated etioplasts. At the end of incubation the plastid preparations were solublilized in 2% Triton X-100, centrifuged at 10,000 x g for 10 min. Incorporation of labeled amino acid was analyzed in each assay in the supernatant and the pellet.

ACKNOWLEDGMENTS

We thank Professor B.A. McFadden for providing facilities to conduct experiments on soluble protein and immunoprecipitation, Jose Torres Ruiz for the gift of RuBisCO antiserum from spinach and Dr. M.J. Conder for critical reading of the manuscript. This work was supported by research grant PL-480 (USDA) grant no. FG-IN-577 (IN-SEA-172).

REFERENCES

1. Bard, J., Bourque, P.N., Hilderbrand, M., and Zaitlin, D. (1985) Proc. Natl. Acad. Sci. USA 82, 3983-3987.
2. Bottomley, W., and Whitfeld, P.R. (1979) Eur. J. Biochem. 43, 31-39.
3. Blair, G.E., and Ellis, R.J. (1973) Biochim. Biophys. Acta 319, 223-234.
4. Ellis, R.J. (1981) Ann. Rev. Plant Physiol. 32, 111-137.
5. Daniell, H., and Rebeiz, C.A. (1982) Biochem. Biophys. Res. Commun. 104, 837-843.
6. Daniell, H., and Rebeiz, C.A. (1982) Biochem. Biophys. Res. Commun. 106, 466-470.
7. Daniell, H., and Rebeiz, C.A. (1984) Biotechnology and Bioengineering 26, 481-487.
8. Rebeiz, C.A., Montazer-Zouhoor, A., and Daniell, H. (1984) in Chloroplast Development: Structure, Function and Regulation of the Photosynthetic Apparatus (Ohad, I., and Klein, S., eds.) pp. 225-235, The Weizmann Science Press of Israel, Israel.
9. Daniell, H., Ramanujam, P., Krishnan, M., Gnanam, A., and Rebeiz, C.A. (1983) Biochem. Biophys. Res. Commun. 111, 740-749.
10. Daniell, H., Sarojini, G., Krishnan, M., and Gnanam, A. (1985) submitted for publication.
11. Rebeiz, C.A., Daniell, H., and Mattheis, J.R. (1982) in Biotechnology in Energy Production and Conservation (Scott, C.D., ed.) Vol. 12, pp. 413-439, John Wiley and Sons, New York.
12. Rebeiz, C.A., Wu. S.M., Kuhadja, M., and Daniell, H. (1983) Mol. Cell. Biochem. 57, 97-125.
13. Rebeiz, C.A., Mattheis, J.R., Smith, B.B., Rebeiz, C.C., and Dayton, D.F. (1975) Arch. Biochem. Biophys. 171, 549-567.
14. Bollum, F.J. (1960) J. Biol. Chem. 235, 2399-2403.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Chua, N.H. (1980) Methods in Enzymology 69, 434-446.
17. Laskey, R.A., and Mills, A.D. (1975) Eur. J. Biochem. 56, 335-341.
18. Smith, B.B., Rebeiz, C.A. (1979) Plant Physiol. 63, 227-231.
19. Uma Bai, U., Daniell, H., and Gnanam, A. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.) Vol. IV, pp. 681-684, D.W. Junk Publishers, The Hague.
20. Wellburn, F.A.M., and Wellburn, A.R. (1971) J. Cell. Sci. 9, 271-287.
21. Ellis, R.J. (1977) Biochim. Biophys. Acta 463, 185-215.
22. Daniell, H., Krishnan, M., Ranganathan, M., and Gnanam, A. (1984) Biochem. Biophys. Res. Commun. 125, 988-995.
23. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) in Molecular Cloning, a laboratory manual, pp. 61-62, Cold Spring Harbor, New York.