

In Vitro Synthesis of Chlorophyll A in the Dark Triggers Accumulation of Chlorophyll A Apoproteins in Barley Etioplasts*

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An *in vitro* translation system using lysed etioplasts was developed to test if the accumulation of plastid-encoded chlorophyll *a* apoproteins is dependent on the *de novo* synthesis of chlorophyll *a*. The P700 apoproteins, CP47 and CP43, were not radiolabeled in pulse-chase translation assays employing lysed etioplasts in the absence of added chlorophyll precursors. When chlorophyllide *a* plus phytolpyrophosphate were added to lysed etioplast translation assays in the dark, chlorophyll *a* was synthesized and radiolabeled P700 apoproteins, CP47 and CP43, and a protein which comigrates with D₁ accumulated. Chlorophyllide *a* or phytolpyrophosphate added separately to the translation assay in darkness did not induce chlorophyll *a* formation or chlorophyll *a* apoprotein accumulation. Chlorophyll *a* formation and chlorophyll *a* apoprotein accumulation were also induced in the lysed etioplast translation system by the photoreduction of protochlorophyllide to chlorophyllide *a* in the presence of exogenous phytolpyrophosphate. Accumulation of radiolabeled CP47 was detectable when very low levels of chlorophyll *a* were synthesized *de novo* (<0.01 nmol/10⁷ plastids), and radiolabel increased linearly with increasing *de novo* chlorophyll *a* formation. Higher levels of *de novo* synthesized chlorophyll *a* were required prior to detection of radiolabel incorporation into the P700 apoproteins and CP43 (>0.01 nmol/10⁷ plastids). Radiolabel incorporation into the P700 apoproteins, CP47 and CP43, saturated at a chlorophyll *a* concentration which corresponds to 50% of the etioplast protochlorophyllide content (0.06 nmol of chlorophyll *a*/10⁷ plastids).

In monocotyledons such as barley, the early phase of primary leaf and plastid development proceeds uninhibited in the absence of light (Mullet, 1988; Baumgartner *et al.*, 1989). When seedlings are grown in the dark, proplastids of the developing leaf differentiate into etioplasts (Robertson and Laetsch, 1974). During this developmental phase numerous changes occur including increase of plastid number/cell, volume/plastid (Henningsen and Boynton, 1969; Robertson and

Laetsch, 1974; Baumgartner *et al.* 1989), and of expression of most nuclear (Klein and Mullet, 1987) and plastid genes encoding plastid polypeptides. However, etioplasts do not synthesize chlorophyll (Chl)¹ (Castelfranco and Beale, 1983) and fail to accumulate the nuclear-encoded chlorophyll *a/b*-binding antennae proteins (Apel, 1979; Bennet, 1981; Bennet *et al.*, 1984) and plastid-encoded Chl *a*-binding proteins (Herrmann *et al.*, 1985; Klein and Mullet, 1986; Kreuz *et al.*, 1986; Sutton *et al.* 1987; Gamble *et al.* 1989). Upon illumination of dark-grown plants, plastid development is controlled by at least three photoreceptors: phytochrome, a blue light receptor and the protochlorophyllide:NADPH reductase holochrome (Hooper, 1984). Although transcription of some nuclear genes encoding chloroplast polypeptides is controlled by phytochrome and the blue light photoreceptor (Apel, 1979; Mösiniger *et al.*, 1988;), other steps in chloroplast development are dependent on photoreduction of protochlorophyllide (PChlide) via the protochlorophyllide-oxidoreductase holochrome complex (Apel *et al.*, 1980) and subsequent esterification of chlorophyllide *a* (Chlide *a*) to Chl *a* via chlorophyll synthetase (Rüdiger *et al.*, 1980). The conversion of PChlide to Chl *a* leads to disintegration of the prolamellar body and its dispersal into the primary lamellar layers (Henningsen and Stummann, 1982). Chl formation is paralleled by accumulation of the plastid-encoded Chl *a*-binding apoproteins (Klein and Mullet, 1986) and the assembly of the photosynthetic apparatus.

Plastid DNA encodes at least six Chl *a* apoproteins including the proteins encoded by *psaA* (P700 apoprotein), *psaB* (P700 apoprotein), *psbA* (D₁), *psbB* (CP47), *psbC* (CP43), and *psbD* (D₂) (Ohya *et al.*, 1986). Although transcripts for Chl *a*-binding proteins are present in etioplasts from dark-grown seedlings (Herrmann *et al.*, 1985; Klein and Mullet, 1986; Kreuz *et al.*, 1986), only D₂ accumulates radiolabel when plants or isolated plastids are fed [³⁵S]methionine (Klein *et al.*, 1988a). However, illumination of 4.5-day-old dark-grown barley for 1 min induces accumulation of the other Chl *a*-binding proteins in subsequent translation assays (Klein *et al.*, 1988a). The increase in Chl *a* apoprotein accumulation occurs without changes in transcript levels, indicating that Chl *a* apoprotein accumulation is regulated translationally or posttranslationally at this stage of plastid development (Klein

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¹ The abbreviations used are: Chl, chlorophyll; PChlide, protochlorophyllide; Chlide *a*, chlorophyllide *a*; Chl *a*, chlorophyll *a*; PhPP, phytolpyrophosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; P700, CP47, CP43, D₁, and D₂ are protein products of plastid genes *psaA/psaB*, *psbB*, *psbC*, *psbA*, and *psbD*, respectively; W, watt; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PS I (and II), photosystem I (and II).

and Mullet, 1986). Studies using the Chl-deficient mutant *xan j⁶⁴*, which accumulates Chlide in the light but only low levels of Chl *a*, showed reduced accumulation of radiolabeled Chl *a* apoproteins upon illumination (Klein *et al.*, 1988a). This result suggested that not only the light-dependent conversion of PChlide to Chlide *a* but also the formation of Chl *a* from Chlide *a* is necessary for Chl *a* apoprotein accumulation. In this study we tested the influence of Chl *a* synthesis on Chl *a* apoprotein accumulation in the absence of light. We developed an *in vitro* system using isolated etioplasts that is capable of both translation and Chl *a* synthesis. To exclude the influence of light, Chl *a* was synthesized in the dark from soluble exogenously added Chlide *a* and phytylpyrophosphate (PhPP). The results show that *de novo* synthesis of Chl *a* is necessary and sufficient to trigger accumulation of the P700 apoproteins, CP47 and CP43.

MATERIALS AND METHODS

Plant Growth—Barley (*Hordeum vulgare* L. var. Alexis) seeds were planted in vermiculite and watered with half-strength Hoagland's nutrient solution. Seedlings were grown for 4.25 days at 26 °C in a light-tight growth chamber located in a darkroom. At this stage of development, seedlings were 5–6-cm tall. All manipulations of dark-grown seedlings were performed in dim green safe light (7×10^{-4} W/m²) which was unable to photoconvert measurable amounts of PChlide.

Plastid Isolation—After 4.25 days etiolated primary leaves were cut 1 cm above the seed and immediately placed in ice water. Water was drained and tissue surface sterilized for 3 min (Klein and Mullet, 1986). All manipulations during the isolation procedure were performed on ice in a light tight dark-room in the presence of a dim green safe light.

Synthesis of Chl *a* Apoproteins—Optimum conditions for protein and Chl *a* synthesis in lysed etioplasts are obtained in a buffer mixture (volume: 75 μ l) containing 40 mM Hepes/KOH (pH 8.0), 2.0 mM ATP, 0.2 mM GTP, 7 mM magnesium acetate (pH 7.0), 118 mM potassium acetate (pH 7.0), 10 mM dithiothreitol, 100 μ M of each amino acid (minus methionine), and 80 μ Ci of [³⁵S]methionine (specific activity > 800 Ci/mmol, Amersham & Buchler, Braunschweig, Federal Republic of Germany). Optimized protein synthesis mixtures contained 1.375×10^7 plastids and were incubated for 15 min in the dark at 26 °C. At that time, unlabeled methionine (9 mM final concentration) was added to allow chain elongation of incomplete nascent polypeptides but terminate further incorporation of [³⁵S]methionine (the chase period was 15 min). Synthesis of Chl *a* in lysed etioplasts was either achieved in the dark by exogenous substrates or through illumination with red light (>550 nm) of various fluences. For dark synthesis of Chl *a* translation assays were supplemented with 0.4 mM PhPP and various amounts of Chl *a* as specified under results. Chlide *a* was kept in diethyl ether under N₂ at -20 °C. Chlide *a* as substrate for Chl *a* synthesis was transferred to 50 mM Hepes/KOH (pH 8.0). To allow solubilization of Chlide *a* in Hepes solution, diethyl ether was evaporated under N₂ and Chlide *a* successively brought into solution by vigorous mixing. The solution was micro-fuged for 5 min and the supernatant saved. An aliquot of the clear supernatant containing solubilized Chlide *a* was extracted in 80% acetone and the concentration determined by spectroscopy. For synthesis of Chl *a* in the light, 0.25 mM NADPH and 0.4 mM PhPP were added. To analyze Chl *a* and Chl *a* apoprotein accumulation in parallel, assays were divided after the 15-min labeling period. An aliquot of 61.3 μ l was directly frozen in liquid N₂ and kept at -20 °C for later Chl *a* analysis, another 13.7- μ l aliquot was held at 26 °C and polypeptides chased for an additional 15 min. For analysis of radiolabeled Chl *a* apoproteins the plastid fraction corresponding to 2.55×10^6 plastids were fractionated into membrane and soluble polypeptides. Measurements of trichloroacetic acid-insoluble radioactivity were obtained and samples subsequently electrophoresed and fluorographed (Mullet *et al.*, 1986).

Immunoprecipitation of Radiolabeled Chl *a* Apoproteins—Chl *a* apoproteins radiolabeled in lysed etioplast translation assays were immunoprecipitated (Mullet *et al.*, 1990) and immunoprecipitated proteins separated on SDS gels and detected by fluorography as described above.

Pigment Analysis—For quantitation of PChlide *a*, Chlide *a*, and

Chl *a* the plastid fraction corresponding to 1.12×10^7 plastids was microcentrifuged for 5 min and the supernatant discarded. The plastid pellet was resuspended in 20 μ l of 50 mM Hepes/KOH (pH 8.0) and pigments extracted with 80 μ l of cold 100% acetone. Samples were kept on ice for 15 min, microcentrifuged for 3 min, and the acetone extract removed to a new tube. Acetone extracts were frozen in liquid nitrogen and 70- μ l aliquots taken for reversed phase HPLC analysis (Schwartz *et al.*, 1981). Acetone extracts were chromatographed onto a 4.6×150 -mm 1.5-nm C-8 reversed phase column and a 4.6×25 -mm guard column at a flow rate of 1.0 ml/min. Pigments were eluted with a gradient, adapted to curve 7, (Waters Associates, Model 660) consisting of solvent A, methanol/water (75/25 v/v) at time 0 and solvent B, ethyl acetate (100%) at time 10 min. For detection of pigments a diode array spectrophotometer (Hewlett Packard No. 8451) equipped with a 7- μ l flow cell was coupled to the column. Pigments were measured every 4 s at eight wavelengths (410, 430, 624, 644, 662, 664, and 666 nm) and spectra analyzed by three-dimensional computing. Pigments were identified and quantitated using isolated (PChlide, Chlide *a* and purchased (Chl *a* and Chl *b*; Sigma) standards as described above.

Isolation of Authentic Pigments—PChlide and Chlide *a* were isolated from 7-day-old dark-grown oat (*Avena sativum* L.) seedlings or after brief illumination of seedlings with white light (Griffiths, 1978). Chl *a* and Chl *b* were purchased from Sigma. Purity of Chls were tested on RP8-HPTLC-F₂₅₄S plates (Merck, Darmstadt), (Schoch *et al.*, 1984) and Mg²⁺-free derivatives of PChlide and Chlide *a* tested on HPTLC-silicagel 60 H (Merck, Darmstadt) (Endo *et al.*, 1982).

Synthesis of PhPP—PhPP was synthesized from phytol and triethylammoniumphosphate and retained as tetraammoniumsulfate salt as described previously (Joo *et al.*, 1973; Widmaier *et al.*, 1980).

Irradiation Conditions—Energy fluence rates were measured using a radiometrical detector (United Detector Technology, Inc.) coupled to a light meter. To obtain red radiation, white light was passed through a OG 550 filter (Schott, Mainz, West Germany) which eliminates all light below 550 nm. Different red light fluence rates were achieved by varying the distance between the lysed etioplasts and the light source. Irradiation was done for 15 s. Fluence rates for individual experiments are given in figure legends.

Quantitation of [³⁵S]Methionine Incorporation into Chl *a* Apoproteins—For quantitation of radiolabeled Chl *a* apoproteins, translation products were localized on the gel by fluorography. Bands were excised from the gel and gel slices dissolved in 24% perchloric acid, 20% H₂O₂ at 60 °C in closed scintillation vials overnight. Radioactivity was measured by liquid scintillation counting.

RESULTS

In Vitro System for Synthesis of Chl *a* and Chl *a*-binding Proteins in the Dark—Light-induced accumulation of the Chl *a* apoproteins could be due to light-dependent photoreduction of PChlide to Chlide *a* or esterification of Chlide *a* with PhPP to produce Chl *a*. As a first step toward distinguishing between these possibilities, we developed a lysed etioplast translation system (see "Materials and Methods"). Incorporation of [³⁵S]methionine was initiated by the addition of intact plastids to the hypotonic reaction mixture. Addition of pancreatic RNase A (1 μ g/ml) reduced [³⁵S]methionine incorporation 86%, indicating that the plastid envelope had been disrupted. Incorporation of [³⁵S]methionine was proportional to plastid number between 1.2×10^7 and 4.8×10^7 plastids/reaction. Above this range [³⁵S]methionine incorporation was no longer proportional to the plastid number eventually leveling off. Reaction conditions were also optimized for ATP, GTP and potassium concentration. At a constant magnesium concentration of 7 mM, [³⁵S]methionine incorporation exhibited a strong ATP optimum of 2 mM. At ATP concentrations below 1 mM or above 4 mM, [³⁵S]methionine incorporation declined nearly 9-fold. The addition of 0.2 mM GTP stimulated [³⁵S]methionine incorporation 2-fold, whereas increasing GTP concentrations to 2 mM did not further increase incorporation. Similarly, a broad potassium optimum between 115 and 140 mM was observed. Potassium concentrations above or below this range reduced [³⁵S]methionine incorporation. Under these optimized conditions the addition of other compo-

nents including bovine serum albumin, RNase inhibitors (*i.e.* RNasin), and spermidine did not increase incorporation of [³⁵S]methionine. Incorporation of [³⁵S]methionine into protein in the lysed plastid system exceeded incorporation in isolated intact plastids by 50%. In addition, most of the radiolabel incorporation occurred in the first 10 min after assay initiation. This indicated that radiolabel incorporation was probably due to elongation of previously initiated polypeptides. The ability of the lysed etioplasts to photoreduce endogenous PChlide was tested next. Illumination of isolated lysed etioplasts showed that endogenous PChlide could be photoreduced to Chlide *a* with an efficiency of up to 70%, but only very low amounts of Chl *a* (≤ 5 pmol/ 10^7 plastids) were synthesized. The addition of PhPP to the assay activated the *in vitro* system to synthesize Chl *a* in the light. The yield of Chl *a* from endogenous Chlide *a* and exogenously added PhPP was 65–90% (Fig. 1). In the absence of exogenous PhPP the translation pattern of lysed etioplasts was not affected by the photoreduction of endogenous PChlide (Fig. 2, lane 5). However, when supplemented with PhPP, the P700 apoproteins, CP47 and CP43, identified previously by their migration on SDS-PAGE (Klein and Mullet, 1986) accumulated in the membrane fraction of lysed etioplasts (Fig. 2, lane 6). To separate the influence of light and Chl *a* synthesis on accumulation of Chl *a* apoproteins, Chl *a* was synthesized in the dark by the following approach. The addition of exogenous Chlide *a* and exogenous PhPP to the translation system in the dark reveals that neither PhPP nor Chlide *a* alone alter the translation pattern (Fig. 2, lanes 1–3). However, when both Chlide *a* and PhPP were added to the translation system, Chl *a* was synthesized and accumulation of radiolabel in Chl *a* apoproteins selectively induced (Fig. 2, lane 4). We next tested whether Chl *a* added directly to the translation system would stimulate Chl *a* apoprotein radiolabeling. [³⁵S]Methionine incorporation in lysed etioplasts was not altered by addition of various concentrations of Chl *a*, and no accumulation of radiolabeled Chl *a* apoproteins was detected (data not shown). The results suggest that Chl *a* synthesized from soluble precursor molecules can induce the accumulation of several Chl *a*-binding proteins of PS I and PS II, but exogenous Chl *a* is unable to induce a similar response.

Identification of Radiolabeled Chl *a* Apoproteins—To verify the identity of the induced etioplast translation products,

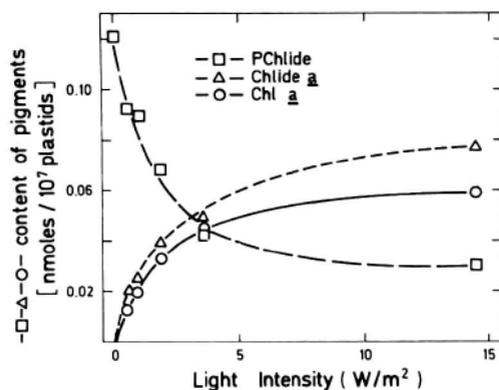


FIG. 1. Photoreduction of PChlide and synthesis of Chl *a* in lysed barley etioplasts. Etioplasts, isolated from dark-grown barley leaves, were lysed, illuminated with increasing light intensities (0, 0.56, 0.98, 1.816, 3.632, and 14.528 W/m²) for 15 s, and incubated without PhPP for analysis of PChlide and Chlide *a* and with PhPP (0.4 mM) for analysis of Chl *a* in the dark. Etioplasts corresponding to 1.12×10^7 plastids were separated into membrane and soluble fractions and membranes extracted with 80% (*v/v*) ice-cold acetone. Pigments were detected as described under "Materials and Methods."

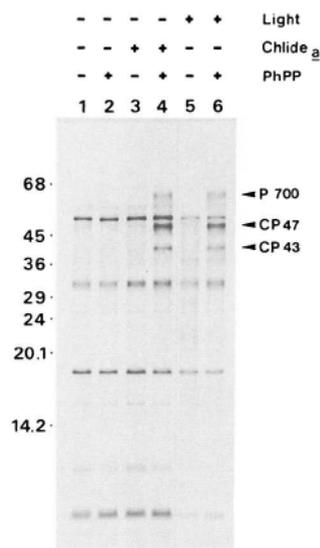


FIG. 2. *In vitro* induction of Chl *a* apoprotein synthesis. Etioplasts were lysed and incubated without or with PhPP either in the dark or after brief illumination. Incubations performed in the dark were run either without or with Chlide *a*. Following the labeling period, etioplasts were fractionated into membrane and soluble polypeptides and membrane fractions equivalent to 8×10^6 etioplasts separated on a 12.5% SDS-PAGE gel containing 4 M urea. The gel was fixed, fluorographed, and exposed to x-ray film for either 12 h (lanes 1–4) or 24 h (lanes 5 and 6). Lanes 1–6 show a fluorogram of membrane polypeptides synthesized by lysed etioplasts. Lane 1, no additions; lane 2 addition of 0.4 mM PhPP; lane 3, addition of 5.3 μ M Chlide *a*; lane 4, addition of 0.4 mM PhPP and 5.3 μ M Chlide *a*; lane 5, irradiation with 29 W/m², no PhPP added; lane 6, irradiation with 29 W/m² and 0.4 mM PhPP added. Chl *a* values were 0.6, 1, 2, 180, ≤ 5 , and 63 pmole/ 10^7 plastids for lanes 1–6, respectively. Numbers to the left indicate mobility of standards (kilodaltons).

radiolabeled polypeptides synthesized in the lysed translation system were immunoprecipitated with either poly-specific antibodies directed against the 65/68-kDa P700 Chl *a*-binding proteins which comprise the PS I reaction center, (Herrmann *et al.*, 1983) or with monospecific antibodies directed against the 47- and 43-kDa Chl *a*-binding PS II polypeptides (CP47 and CP43)² (Fig. 3). Translation assays supplemented with Chlide *a* and PhPP were carried out in the dark and then separated into membrane and soluble protein fractions. The membrane proteins were solubilized, immunoprecipitated, and labeled protein precipitates separated by SDS-PAGE (Fig. 3, lanes 2–4). The immunoprecipitated translation products were compared with translation profiles of lysed etioplasts under inductive (Fig. 3, lane 5) or noninductive conditions (Fig. 3, lane 1) for Chl *a* biosynthesis. We could clearly identify the P700 apoproteins, CP47 and CP43, as polypeptides induced by *in vitro* synthesis of Chl *a* in the dark.

Influence of Chl *a* on Lysed Etioplast Translation—To quantify the influence of Chl *a* synthesis on the appearance of Chl *a*-binding proteins in more detail, we synthesized increasing amounts of Chl *a* either by increasing the light intensity during a 15-s light pulse or by increasing the concentration of exogenous Chlide *a* added during translation in the dark. The amount of Chl *a* and Chl *a* apoproteins synthesized was then quantitated and compared (Fig. 4A, lanes 1–8, and Fig. 4B). CP47 synthesis could be readily detected at very low Chl *a* concentrations (≤ 0.01 nmol/ 10^7 plastids) and radiolabel incorporation increased linearly with increasing Chl *a*. Higher Chl *a* levels were required to detect radiolabel accumulation

² Oswald, A., Streubel, M., Ljungberg, U., Hermans, J., Eskins, K., and Westhoff, P. (1990) *Eur. J. Biochem.*, in press.

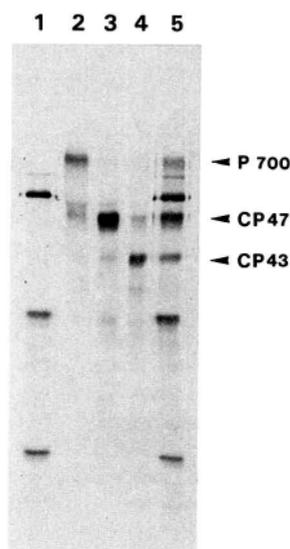


FIG. 3. Immunoprecipitation of radiolabeled Chl *a* apoproteins. Etioplasts were lysed and incubated without (lane 1) or with (lane 5) 5.3 μM Chlide *a* and 0.4 mM PhPP during translation assays in the dark. Following the labeling period, etioplasts incubated with Chlide *a* and PhPP were fractionated into membrane and soluble polypeptides, and membrane fractions were immunoprecipitated. Equal amounts of radioactivity were loaded on a 12.5% SDS-PAGE gel, and the gel was fixed, fluorographed, and exposed to x-ray film for 12 h. Lanes 1 and 5, membrane polypeptides synthesized by lysed etioplasts in the dark and in the dark after addition of PhPP and Chlide *a*, respectively; lane 2, labeled PS I-Chl *a* proteins immunoprecipitated with a polyspecific antibody raised against P700 apoproteins; lane 3, labeled PS II Chl *a* proteins immunoprecipitated with a monospecific antibody raised against protein products of *psbB* genes; lane 4, labeled PS II-Chl *a* proteins immunoprecipitated with a monospecific antibody raised against protein products of *psbC* genes.

in the P700 apoproteins and CP43 (Fig. 4A, lane 1–7 and Fig. 4B). The P700 apoproteins were induced sharply at a Chl *a* concentration corresponding to 10–20% of the endogenous PChlide pool (Fig. 4A, lanes 1–7, and Fig. 4B). Radiolabel incorporation into the P700 apoproteins, CP47 and CP43, saturated at a similar Chl *a* concentration which corresponded to conversion of about 50% of endogenous PChlide to Chl *a* (0.55 nmol of Chl *a*/nmol of PChlide) (Fig. 4A, lane 7, and Fig. 4B). Synthesis of D₁ (32-kDa polypeptide) was also induced during light-stimulated *in vitro* Chl *a* synthesis, but in the gels utilized, D₁ is not well resolved from D₂, which is synthesized in the dark in barley etioplasts (Fig. 4A, lanes 1–8). To investigate the influence of higher Chl *a* concentrations on translation in the absence of light, Chl *a* was synthesized from increasing amounts of exogenously added Chlide *a* and PhPP. Quantitative and qualitative analysis of Chl *a* and Chl *a* apoprotein synthesis reveal that Chl *a* apoprotein radiolabeling was saturated at 0.55 nmol of Chl *a*/nmol of PChlide (Fig. 5A, lane 5, and Fig. 5B). The P700 apoproteins, CP47 and CP43, accumulate in the same ratios when Chl *a* is synthesized in the dark as in illuminated plastids. Chl *a* formation in the dark in the presence of Chlide *a* plus PhPP does not plateau in contrast to the light-dependent formation of Chl *a* (Figs. 5B and 4B). Within the concentration range of Chlide *a* used (0.3–7.8 μM) Chl-synthetase could not be saturated (Fig. 5B). The Chl *a* produced *in vitro* is associated with the membrane fraction and is assumed to be solubilized in the membrane lipid phase.

DISCUSSION

Chloroplast and thylakoid membrane development requires a highly regulated and time-resolved coordination of the

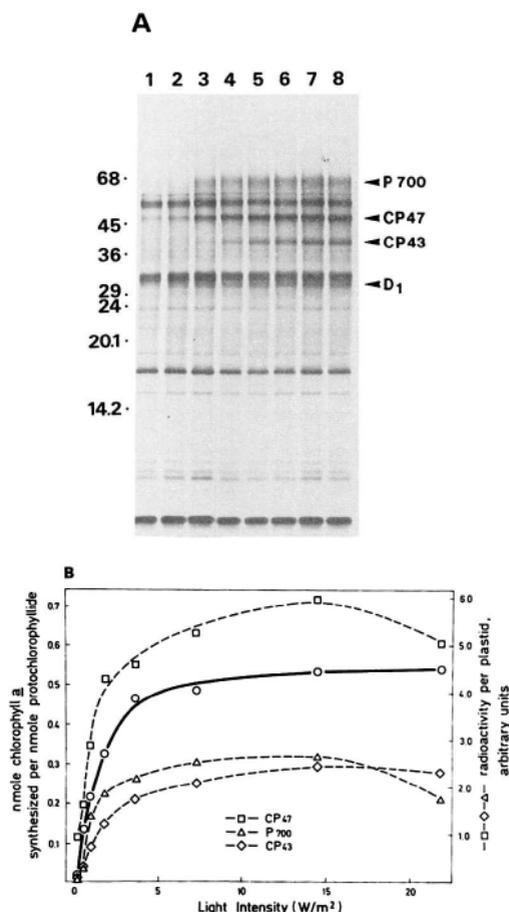


FIG. 4. Accumulation of radiolabeled Chl *a* apoproteins after photoconversion of endogenous PChlide by increasing light intensities. A, fluorogram of membrane polypeptides synthesized by lysed etioplasts. Membrane fractions corresponding to 4×10^6 etioplasts were loaded on 12.5% SDS-PAGE gels containing 4 M urea. Gels were fixed, fluorographed, and exposed to x-ray film for 36 h. Lane 1 (control), irradiation with 14.53 W/m^2 for 15 s, no PhPP added; lanes 2–8, addition of 0.4 mM PhPP and irradiation with 0.56, 0.91, 1.82, 3.63, 7.26, 14.53, and 21.80 W/m^2 for 15 s, respectively. Numbers to the left indicate mobility of standards (kilodaltons). B, quantification of [³⁵S]methionine incorporation into Chl *a* apoproteins and Chl *a* synthesized. [³⁵S]Methionine incorporation into Chl *a* apoproteins was measured by liquid scintillation counting of excised gel slices (Fig. 4A, lanes 2–8). Amounts of Chl *a* synthesized from Chlide *a* were determined by HPLC. Lysed etioplasts contained 0.10–0.13 nmol of PChlide/ 10^7 plastids.

expression of nuclear and plastid genes. A key requirement in the control of developmental processes in higher plants is fulfilled by light which acts as an environmental control factor regulating the transcription of some nuclear genes which encode plastid localized proteins (e.g. Chl *a/b*-binding proteins of the light-harvesting complex, NADPH-protochlorophyllide oxidoreductase, and the small subunit of ribulose-1,5-bisphosphate carboxylase) (Mösinger *et al.*, 1985; Silverthorne and Tobin, 1984; Gallagher and Ellis, 1982; Berry-Lowe and Meagher, 1985). A second level of light regulation involves the coordination of apoprotein and cofactor (e.g. chlorophyll, carotenoid, heme, quinone, iron and manganese) accumulation during chloroplast biogenesis. In this regard the appearance of certain chloroplast-encoded Chl *a*-binding proteins (e.g. P700, CP47, CP43, and D₁) was shown to depend on light and to be correlated with Chl *a* synthesis during plastid biogenesis (Klein and Mullet, 1986). Studies to elucidate the molecular mechanism involved were hampered by the fact that the influence of light on Chl *a* apoprotein synthesis could

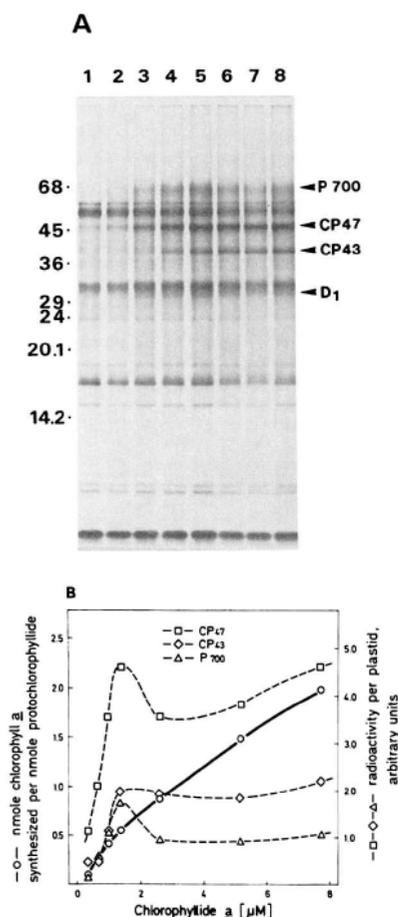


FIG. 5. Accumulation of radiolabeled Chl *a* apoproteins during dark synthesis of Chl *a* from increasing amounts of exogenously added Chlide *a*. *A*, fluorogram of membrane polypeptides synthesized by lysed etioplasts in the dark. Membrane fractions corresponding to 4×10^5 etioplasts were loaded on 12.5% SDS-PAGE gels containing 4 M urea. Gels were fixed, fluorographed, and exposed to x-ray film for 36 h. Lane 1 (control), addition of 1.28 μM Chlide *a*, no PhPP added; lanes 2–8, addition of 0.4 mM PhPP and 0.23, 0.64, 0.96, 1.28, 2.57, 5.14, and 7.72 μM Chlide *a*, respectively. Numbers to the left indicate mobility of standards (kilodaltons). *B*, quantification of [^{35}S]methionine incorporation into Chl *a* apoproteins and Chl *a* synthesized. [^{35}S]Methionine incorporation into Chl *a* apoproteins was measured by liquid scintillation counting of excised gel slices (Fig. 5A, lanes 2–8). Amounts of Chl *a* synthesized from Chlide *a* were determined by HPLC. Lysed etioplasts contained 0.10–0.13 nmol of PChlide/ 10^7 plastids.

not be separated from light-dependent synthesis of Chlide *a* and Chl *a* which occur in parallel during illumination. In this paper we were able to separate the potential elicitor activities of PChlide, Chlide *a*, Chl *a*, and PhPP from the influence of light. It is clear from the data presented that the synthesis of Chl *a* from Chlide *a* and PhPP in the dark triggers the accumulation of the P700 apoproteins, CP47, CP43, and probably D₁. Therefore, light-induced accumulation of the Chl *a* apoproteins *in vivo* requires photoreduction of PChlide to Chlide *a* which in the presence of PhPP can be converted to Chl *a* by Chl synthetase (Rüdiger *et al.*, 1980). The presence of PhPP in the lysed etioplast translation system is essential for activation of Chl *a* apoprotein synthesis. Five-day-old dark-grown barley seedlings contain PhPP but no geranylgeranylpyrophosphate (Liljenberg, 1977), whereas soluble extracts from 7-day-old dark-grown oat (*Avena sativa* L.) seedlings contain geranylgeranylpyrophosphate and PhPP in a ratio of 7.5:1 (Benz *et al.*, 1983). In both cases total tetrapren-

ylpyrophosphate to PChlide ratio is about 1. Upon isolation of intact etioplasts, however, PhPP limited Chl *a* synthesis from endogenous Chlide *a* as indicated by the fact that Chl *a* synthesis increased 12.6-fold when assays were supplemented with 0.4 mM PhPP (Fig. 2). As was demonstrated for etioplasts from 7-day-old dark-grown oat (*Avena sativa* L.) leaves, isolation of intact etioplasts leads to a rapid loss of geranylgeranylpyrophosphate (Benz *et al.*, 1981). Chl synthetase might thus be a central enzyme regulating Chl *a* apoprotein translation.

Several mechanisms could account for the ability of Chl *a* to induce Chl *a* apoprotein accumulation. One mechanism could involve ribosome or nascent apoprotein binding to receptors on the thylakoid membrane in the absence of Chl *a*, leading to a block of translation (Jensen *et al.*, 1986; Mullet *et al.*, 1987). If this binding was mediated via nascent polypeptide chains, then synthesis of Chl *a* and binding to the nascent chain could release the translational block. Several groups have reported that transcripts for membrane proteins are already loaded with polysomes in the dark and are associated with the thylakoid membrane (Yamamoto *et al.*, 1980; Klein *et al.*, 1988b; Friemann and Hachtel, 1988). As transcript levels for Chl *a* apoproteins and polysome profiles of membrane-associated Chl *a* apoproteins do not change upon illumination of 4.5-day-old dark-grown barley leaves, it can be suggested that if regulation of translation occurs, it operates at a late step in translation elongation (Klein *et al.*, 1988b). Several reports from elongation of eukaryotic mRNA indicate how this mechanism of plastid translation regulation could operate. The best known example is the translation of secretory proteins which are blocked in elongation by the signal recognition particle. Translation is only achieved when appropriate endoplasmic reticulum components are added back to the system (Walter and Blobel, 1981a; Walter and Blobel, 1981b; Walter *et al.*, 1981). Therefore, the lack of radiolabel incorporation into Chl *a* apoproteins in the absence of Chl *a* could indicate that chain elongation is blocked as a result of binding of the nascent polypeptide chain to a membrane-bound or membrane-associated receptor. Artificial loosening of this apoprotein-receptor linkage (*e.g.* with 2% polyoxytridecylether) could lead to increased radiolabel incorporation into Chl *a* apoproteins (Klein *et al.*, 1988b). However, this artificial stimulation of apoprotein synthesis is less specific than *in vitro* stimulation of Chl *a* apoprotein translation by Chl *a* (Fig. 2). Interestingly, exogenous Chl *a* added to the translation assays did not elicit the appearance of the Chl *a* apoproteins. The exact reasons for this are unknown so far. Chl *a* synthesized by Chl synthetase could be selectively delivered to the Chl *a* apoproteins by a channel inaccessible to Chl *a*. Alternatively Chl synthetase could function as a membrane receptor or operate in close proximity to a putative receptor. Exogenously added Chl *a* might therefore be unable to exert an effect on the nascent apoprotein-receptor linkage and release the translational block. Finally we cannot exclude the possibility that the absence of a stimulatory effect of exogenous Chl *a* is due to our inability to directly deliver Chl *a* to its site of action.

Alternatively the induction of Chl *a* apoprotein accumulation by Chl *a* could involve the binding of Chl *a* to the Chl *a* apoproteins which stabilizes these proteins. Stabilization could involve a change in apoprotein conformation or apoprotein insertion into the thylakoid membranes thereby rendering the proteins protease inaccessible. It is also possible that Chl *a* synthesis directly inhibits the proposed protease allowing the accumulation of radiolabeled Chl *a* apoproteins. We have found recently that CP43 is radiolabeled when etioplasts

are pulse-labeled for 5 min. Furthermore, the radiolabeled CP43 was very unstable in etioplasts compared with chloroplasts (Mullet *et al.*, 1990). Translation intermediates of D₁ up to 23 kDa in size were also detected in pulse-labeled etioplasts, although no full-length D₁ accumulated. These results indicate that CP43 and D₁ are translated in etioplasts but require Chl *a* binding cotranslationally or shortly after apoprotein formation to allow apoprotein accumulation. In a similar way Chl *a* and Chl *b* are believed to increase the stability of the nuclear-encoded Chl *a/b* apoproteins (Apel, 1979; Bennet, 1981). Increased plastocyanin turnover is also observed in the absence of copper binding (Merchant and Bogorad, 1986). Furthermore, abnormal or truncated proteins are rapidly degraded within plastids (Liu and Jagendorf, 1986).

Etioplasts from 4.25-day-old barley plants contain 0.10–0.13 nmol of PChlide/10⁷ plastids. We routinely obtained 50–70% photoconversion of PChlide to Chlide *a*, which resulted in a final Chl *a* concentration of 0.063 nmol/10⁷ plastids (Fig. 1). This amount of Chl *a* synthesized *in situ* is optimal for initial accumulation of the Chl *a* apoproteins. Using exogenously supplied Chlide *a* and PhPP, much higher amounts of Chl *a* can be synthesized by the etioplasts (Fig. 5B), but this does not result in increased amounts of radiolabeled P700 apoproteins CP47 or CP43 (Fig. 5A). The amount of radiolabel accumulation in the Chl *a* apoproteins probably represents elongation of previously initiated proteins. Radiolabel incorporation continues for only 10 min in lysed plastids, suggesting that reinitiation of translation is low in this system. From the results we conclude that the amount of Chl *a* limits the initial accumulation of the P700 apoproteins, CP47 and CP43, unless Chl *a* synthesis exceeds 0.55 nmol of Chl *a*/nmol of PChlide. At low Chl *a* concentration, radiolabeled CP47 is detected, but not the P700 apoproteins or CP43. This could indicate that CP47 has higher affinity for Chl *a* than CP43 or the P700 apoproteins. Alternatively, accumulation of CP47 may require the binding of fewer Chl *a* molecules for stabilization. CP43 and the P700 apoproteins required higher levels of Chl *a* for accumulation. This could indicate that most of the Chl *a*-binding sites in these apoproteins need to be filled before these proteins will accumulate. Future studies will be required to define the nature of the Chl *a*-binding sites and dynamics of Chl *a* and apoprotein association.

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