PHOTOSYNTHETICA 39 (4): 521-527, 2001

Development of chloroplast in dark-grown wheat seedlings irradiated at elevated temperature

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Abstract

Elevated temperature inhibited the accumulation of chlorophyll and photosynthetic proteins, and the development of photochemical activity, however, carotenoids continued to accumulate. Signal transduction pathway involved in protochlorophyllide oxidoreductase was unaffected by elevated temperature of 38 °C. Two-dimensional gel electrophoresis of stroma proteins showed similar patterns in the dark-grown seedlings and seedlings irradiated at elevated temperature, although some low molecular mass proteins accumulated at 38 °C. In contrast, seedlings irradiated at 25 °C showed complex pattern of proteins. Hence the development of chloroplast and its associated functions during irradiation of etiolated seedlings are inhibited by elevated temperature.

Additional key words: carotenoids; chlorophyll; nucleic acids; photosystem 2; proteins; *Triticum aestivum*.

Introduction

Chloroplasts are derived from small non-differentiated proplastids, which are inherited maternally by the plant zygote (Mullet 1988). In dark-grown seedlings, proplastids differentiate into etioplasts. Both proplastids and etioplastids can develop into functional chloroplasts. The principal effector in this process is radiant energy (Tobin and Silverthorne 1985). During the irradiation-induced biogenesis of chloroplast, there is rapid accumulation of Chl, photosynthetic membranes and its associated proteins, activation of plastid transcription, increase in plastid DNA copy number, plastid transcript abundance, and plastid number per cell (Baumgartner *et al.* 1989, Gruissem 1989).

During irradiation-induced chloroplast development, the most dramatic impact is on the Chl synthesis (Mullet 1988). Dark-grown plants do not accumulate Chl, although its precursor protochlorophyllide (Pchlide) accumulates. Pchlide forms a ternary complex with NADPH and protochlorophyllide oxidoreductase (POR), enzyme that reduces Pchlide to chlorophyllide (Chlide) (Reinbothe and Reinbothe 1996). This ternary complex is photolabile and as soon as plants are irradiated, Pchlide is reduced to Chlide that is subsequently esterified with geranylgeranyl pyrophosphate in a light-independent step to form Chl (Mullet *et al.* 1990).

Besides accumulation of Chl, the content of some chloroplast proteins can increase more than 10 000-fold during irradiation-induced chloroplast development. Although transcription and post-transcription controls can influence accumulation of these proteins, the largest factor affecting this process is protein synthesis (Mayfield *et al.* 1995). Several studies have suggested that irradiation-dependent accumulation of proteins may be controlled by non-translated region of the gene that could provide the binding sites for the proteins encoded by nuclear genes (Danon and Mayfield 1991, Mayfield *et al.* 1995).

Received 3 October 2000, *accepted* 4 October 2001.
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Abbreviations: Car, carotenoids; Chl, chlorophyll; Chlide, chlorophyllide; CHAPS, 3,3-cholamidopropyldimethylammonia-1-propanesulfonate; cDNA, chloroplast DNA; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; IEF, isoelectric focussing; PAGE, polyacrylamide gel electrophoresis; Pchlide, protochlorophyllide; POR, protochlorophyllide oxidoreductase; PS, photosystem; SDS, sodium dodecyl sulphate.

Acknowledgments: We thank Dr. A. Mattoo for his kind gift of D1 antibody, Dr. N. Mishra for CP43 antibodies, and Dr. B.C. Tripathy for CF1α and POR antibodies and for POR cDNA. Antibodies and cDNA of POR were generous gift from Dr. W.T. Griffiths to Dr. B.C. Tripathy. AKS is thankful to UGC for the senior research fellowship.

In some other cases, accumulation of chloroplast proteins was linked to accumulation of its co-factor. For example, the accumulation of D1, D2, CP43, CP47, and P700 apoproteins depends on the presence of Chl. Chl is probably required either to stabilise the nascent or mature polypeptide chain or to release a translation arrest. In absence of its co-factors, however, the polypeptides are either degraded or synthesis is pre-maturely terminated due to translation arrest (Gruissem 1989, Mullet *et al.* 1990).

The efficient transformation of etioplast to chloroplast during irradiation of dark-grown seedlings requires the co-ordination between chloroplast and nucleus. This co-ordination under optimum conditions, *i.e.*, normal irradiance and ambient temperature, leads to acquisition of plastid functions in a time dependent manner. Any changes in the conditions may delay or stop the chloroplast development. For example, accumulation of a number of thylakoid proteins is dependent on the accumulation of Chl. Therefore any condition which delays or stops the Chl accumulation would be expected to interfere with the chloroplast development.

In the present investigation, we studied the effect of elevated temperature on the irradiation-induced chloroplast development from etioplast in the dark-grown seedlings. Elevated temperature inhibits the functional integrity of photosynthetic complexes, particularly the photosystem 2 (PS2) complex (Berry and Björkman 1980), loss of Chl, and development of non-ribosome plastid (Feierabend and Berberich 1991). Different parameters including accumulation of pigments, proteins, and total plastid DNA and RNA were determined to compare the chloroplast development at normal and elevated temperatures.

Materials and methods

Plants: Wheat seeds (*Triticum aestivum* L. cv. HD 2329, Indian Agricultural Research Institute, Pusa, New Delhi, India) were pre-soaked in distilled water for 12 h and germinated on moist germination paper in dark at 25 °C in a controlled environmental chamber. 5-d-old dark-grown seedlings were irradiated with 50 μmol(photon) m^{-2} s^{-1} in a growth chamber maintained at either 25 or 38 °C.

Intact plastids from the wheat seedlings were isolated on *Percoll* step gradients in complete darkness. Green dim safe light was used whenever necessary. Seedlings were chopped in small pieces with the help of scissors and grounded in the isolation buffer consisting of 0.33 M sorbitol, 2 mM EDTA, and 50 mM HEPES, pH 7.6. The slurry was passed through eight layer of cheese cloth and centrifuged at 2 000×*g* for 10 min at 4 °C. The resultant pellet was softly dissolved in the isolation buffer and was layered on a *Percoll* step gradient (upper layer 30 %, lower layer 65 %). The gradient consisted of 1 % (m/v) *Ficoll*, 1 % (m/v) bovine serum albumin, and 3 % (m/v) of *PEG-6000* in *Percoll*. After layering the crude chloroplast preparation on *Percoll* gradient, it was centrifuged at 9 000×*g* for 10 min at 4 °C. The band at the interface of 65-30 % was collected, diluted with isolation buffer, and centrifuged at 2 000×*g* for 10 min at 4 °C. Plastids were lysed by dissolving the pellets in autoclaved distilled water and pippetting up-down several times. The thylakoid and stroma fractions were collected after centrifugation at 10 000×*g* for 10 min at 4 °C. Thylakoid membranes were washed once and pellet was dissolved in the isolation buffer. Both the thylakoid membranes and stroma fractions were stored at –80 °C.

Photosynthetic electron transport activity was measured as described in Singh and Singhal (1999).

Photosynthetic pigments were extracted from leaves in 80 % (v/v) aqueous acetone and the contents of Chl and carotenoids (Car) were determined spectrophotometrically as described in Arnon (1949).

2-D gel electrophoresis was carried out essentially as described by Hochstrasser *et al.* (1988). Gel mixture for the IEF gels contained 10 g of urea, 6.5 cm^3 of deionised water, 3 cm^3 of acrylamide/bis-acrylamide (30 % T : 0.8 % C), 1 cm^3 of detergent mixture (10 % *Nonidet P-40* and 30 % m/v CHAPS), 0.8 cm^3 of 3.5-10.0 and 0.2 cm^3 of 5-7 ampholines. The polymerisation was initiated by adding 20 mm^3 of TEMED and 40 mm^3 of a 10 % (m/v) solution of ammonium persulphate.

Samples for the IEF gel were prepared by adding sample buffer to the dried protein samples. The sample buffer consisted of 0.1 g of DTT, 0.4 g of CHAPS, 5.4 g of urea, 0.5 cm^3 of 3.5-10.0 and 0.1 cm^3 of 5-7 ampholines, and 6.5 cm^3 of H_2O. 100 μg of stroma proteins was used for a single IEF gel. The anolyte and catholyte contained 6 mM phosphoric acid and 20 mM NaOH, respectively. All the samples were very gently overlaid with the catholyte to the top of the capillary tubes. The isoelectric focusing was performed at room temperature with a constant voltage of 200 V for 2 h, followed by 500 V for 5 h, and then 800 V for 16 h.

After the end of run, tubes were extruded from the glass tube and either stored at –20 °C or treated with 5 cm^3 of transfer solution [4 cm^3 of 10 % (m/v) SDS, 2 cm^3 of 0.5 M Tris-HCl, pH 6.8, 8 cm^3 of water and

bromophenol blue] for 5 min. These tubes were then immediately used for the analysis in second dimension on 12.5 % SDS-PAGE.

SDS-PAGE and Western blot: Thylakoid and soluble proteins were electrophoresed on 12.5 % SDS-PAGE essentially as described by Laemmli (1970), in the presence of 6 M urea. Proteins were transferred on nitrocellulose membrane using a *Bio-Rad* transfer apparatus in buffer consisting of 25 mM Tris, 192 mM glycine, and 20 % methanol. Immunodecoration of blot was carried as described in Singh and Singhal (1999).

DNA probe preparation: The cDNA clone of POR gene was kind gift of Dr. W.T. Griffiths. The cDNA clone was isolated, restriction digested and purified as described in Sambrook *et al.* (1989). DNA fragment was radio-labelled using Random primer labelling kit (*Promega Co.*).

RNA isolation and Northern blotting: Total RNA from wheat seedlings was isolated as described by Choniczynski and Sacchi (1987). RNA concentration was determined spectrophotometrically at 260 nm using an extinction coefficient of 25 kg m^{-3}. Equal amount of RNA (10 μg) was used for Northern blotting. Total RNA was denatured and fractionated on 1.2 % agarose-formaldehyde gels and blotted to nylon membrane by the capillary transfer method as described in Sambrook *et al.* (1989). All pre-hybridisation and hybridisation reactions were carried out as described in Sambrook *et al.* (1989). Washing was carried out twice in buffer containing 2× SSC, 0.1 % SDS for 5 min followed by washing twice in buffer containing 0.1× SSC, 0.1 % SDS for 10 min at 65 °C.

Plastid DNA and RNA: Plastid number was determined by counting on a haemacytometer. Total plastid DNA from 10^8 plastids was isolated as described by Singh *et al.* (1995). Total plastid RNA was isolated as described by Choniczynski and Sacchi (1987). Total plastid DNA and RNA preparations were treated with RNAse for 30 min at 37 °C and RNAse-free DNAse for 30 min at 25 °C, respectively, and precipitated with ethanol. The concentrations of DNA and RNA were measured at 260 nm.

Results

Pigments: Irradiation of dark-grown seedlings with "white light" (50 μmol m^{-2} s^{-1}) at 25 °C lead to the accumulation of Chl, and subsequently the Chl *a/b* ratio decreased (Fig. 1). The rapid decline of Chl *a/b* is a common indicator of chloroplast development (Kyle and Zalik 1982). However, when the dark-grown seedlings were irradiated at 38 °C, Chl failed to accumulate, and there was no change in the Chl *a/b* ratio as compared to control. In contrast to this, Car continued to accumulate at 38 °C although the rate of increase was less than in seedlings irradiated at 25 °C (Fig. 1).

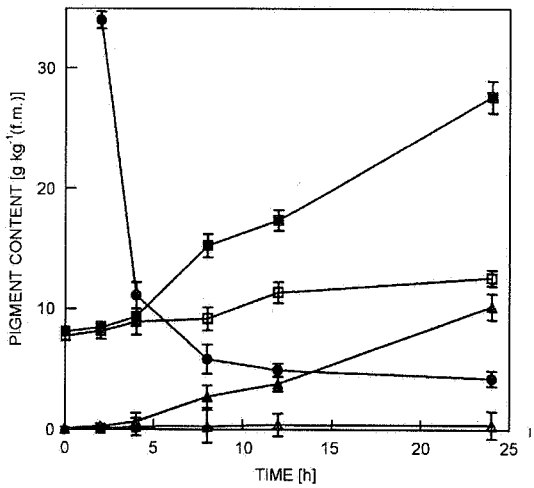

Fig. 1. Effect of high temperature on total chlorophyll, Chl (*triangles*) and carotenoid, Car (*squares*) accumulation and Chl *a/b* ratio (*circles*). *Closed and open symbols* represent samples from 25 and 38 °C, respectively. Means of five independent experiments.

Photochemical activity: Electron transport through PS2 in dark-grown seedlings irradiated at 25 and 38 °C was measured following DCIP photoreduction in the presence of DPC. No oxygen evolving activity was detected within first 4 h of irradiation at either temperature (Fig. 2). Further irradiation of seedlings lead to development of oxygen evolving capacity at 25 °C. However, no PS2 activity

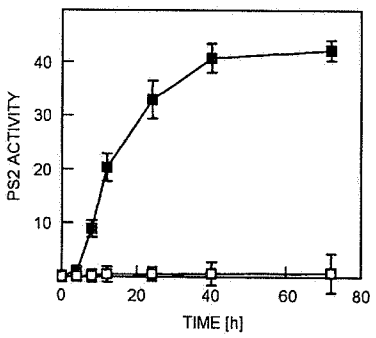

Fig. 2. Development of photosystem 2, PS2 activity during irradiation of dark-grown seedlings at 25 °C (*closed squares*) and at 38 °C (*open squares*). PS2 activity was determined following DCIP photo-reduction in the presence of DPC. PS2 activity [163 mmol($DCIPH_2$) kg^{-1}(Chl) s^{-1}] measured as DCIP photo-reduction in the presence of DPC in 7-d-old light-grown plants was normalised at 100 %.

could be detected at 38 °C even after prolonged irradiation. Absence of PS2 activity at 38 °C was expected as similar treatment of dark-grown seedlings failed to accumulate Chl.

Total plastid DNA and RNA contents remained the same during irradiation of dark-grown seedlings either at 25 or 38 °C as compared to dark-grown seedlings. The similar contents of plastid DNA and RNA in dark-grown seedlings and seedlings irradiated at 25 °C are in accordance with the results of Baumgartner *et al.* (1989). In monocots such as barley and wheat, developmental process leads to the activation of plastid transcription, increase in DNA copy number, plastid RNA and plastid number per cell even in the absence of irradiation.

POR: Chl synthesis in higher plants is regulated by POR that requires photons for the reduction of Pchlide. The content of POR protein drastically decreases during irradiation of dark-grown seedlings. Besides, transcription of POR gene is negatively regulated by irradiation (Reinbothe and Reinbothe 1996). Irradiation of dark-grown seedlings either at 25 or 38 °C lead to the loss of POR protein as compared to control (Fig. 3*A*). Moreover, the transcript level of POR also decreased in the seedlings irradiated at 38 °C similar to the seedlings irradiated at 25 °C (Fig. 3*B*).

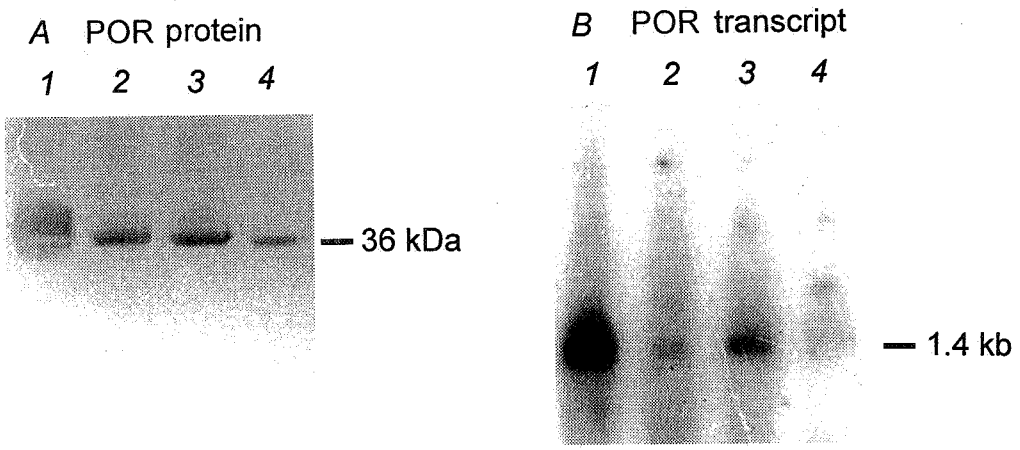

Fig. 3. Effect of elevated temperature on expression of POR. *A*: Thylakoid proteins were electrophoresed on 12.5 % SDS-PAGE, electro-blotted on nitrocellulose and immuno-decorated with anti-POR antibodies. Thylakoid membranes were isolated from dark-grown seedlings (lane *1*), seedlings irradiated at 25 °C for 8 h (lane *2*), or irradiated at 38 °C for 4 h (lane *3*) or 8 h (lane *4*). *B*: Total RNA from seedlings was isolated, fractionated on denaturing agarose gel, and transferred to nylon membrane as described in Materials and methods. Blot was probed with radio-labelled cDNA clone of POR gene. Lane descriptions are the same as in *A*.

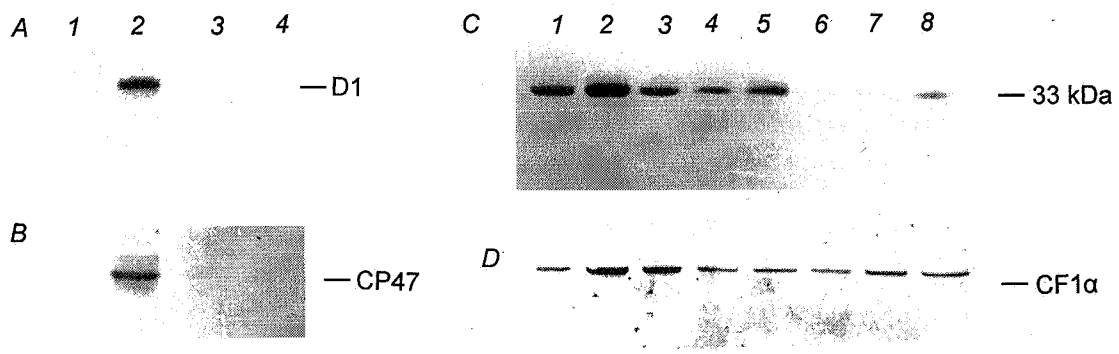

Fig. 4. Effect of elevated temperature on the accumulation of thylakoid proteins. Lanes *1-4* are the same as described in Fig. 1 and lanes *4-8* are the corresponding stroma fractions. Blots were immunodecorated with antibodies raised against (*A*) D1, (*B*) CP47, (*C*) 33 kDa, and (*D*) CF1α.

Thylakoid proteins: Effect of elevated temperature on thylakoid proteins such as D1, CP47, 33 kDa, and CF1α during irradiation-induced chloroplast development was studied using specific polyclonal antibodies against these proteins. D1 and CP47 accumulated only in light-grown plants whereas 33 kDa and CF1α are present in dark-grown plants. None of the PS2 proteins like D1 (Fig. 4*A*) and CP47 (Fig. 4*B*) encoded by cDNA were present in the dark-grown seedlings. Irradiation of dark-grown seedlings at 25 °C lead to the accumulation of these proteins. However, these proteins failed to accumulate when dark-grown seedlings were irradiated at 38 °C.

The 33 kDa protein was present in dark-grown seedlings and was distributed equally in the thylakoid membranes and soluble fractions (Fig. 4*C*). Irradiation, however, led to the increase of 33 kDa protein in the membrane fractions and content in the soluble fraction decreased as compared to control. In contrast, irradiation of dark-grown seedlings at 38 °C lead to the loss of 33 kDa protein in both fractions as compared to control. Similar to the accumulation of 33 kDa protein, CF1α subunit was also present in both fractions and irradiation caused an increase in the content of CF1α in the membrane fractions (Fig. 4*D*). Irradiation of dark-grown seedlings at 38 °C lead to the loss of CF1α protein in both fractions, however, the loss was slower as compared to the 33 kDa protein.

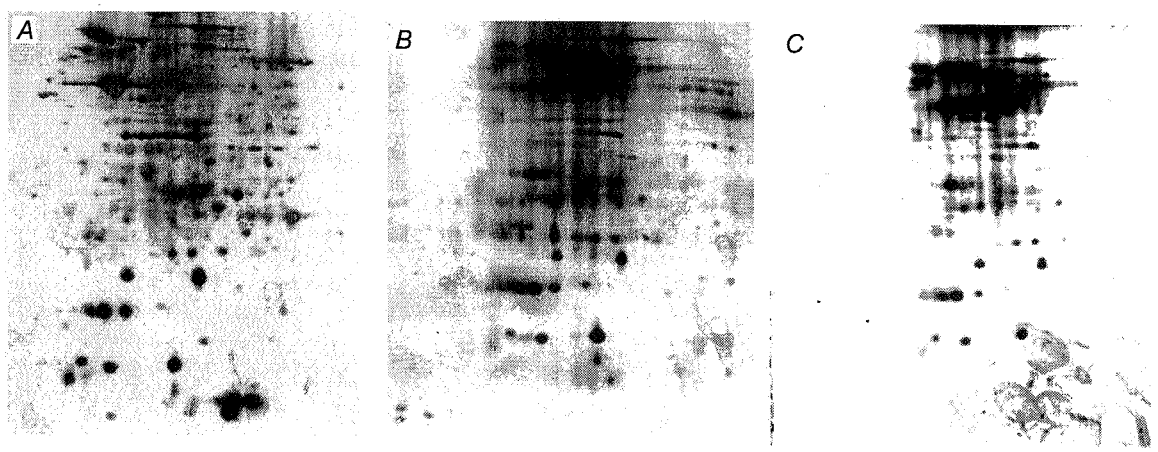

Fig. 5. 2-D gel electrophoresis of stroma proteins from seedlings isolated and separated on 2-D gel as described in Materials and methods. Stroma fractions of seedlings irradiated at 25 °C for 8 h (*A*) or at 38 °C for 8 h (*B*). or of dark-grown seedlings (*C*).

Stroma proteins: Effect of elevated temperature on the stroma proteins was studied using 2-D gel electrophoresis. Protein patterns were similar in dark-grown seedlings and seedlings iradiated at 38 °C (Fig. 5). However, some low molecular mass proteins specifically appeared in seedlings irradiated at 38 °C. Identity of these proteins remains unknown. In contrast, the protein patterns of seedlings irradiated at 25 °C showed the appearance of a number of proteins. It is evident that a number of proteins required for the plastid functions fail to accumulate during irradiation of etiolated seedlings at elevated temperature.

Discussion

We examined the effect of elevated temperature on irradiation-induced chloroplast development in dark-grown seedlings. The development at ambient temperature leads to the accumulation of Chl, photosynthetic membranes and its associated proteins, and activation of plastid transcription (Mullet 1988, Gruissem 1989). However, irradiation of dark-grown seedlings at 38 °C inhibited the accumulation of Chl and thylakoid proteins, and development of photochemical activity whereas the contents of total plastid DNA and RNA were not affected. Failure to accumulate Chl at elevated temperature could be explained in two ways: (*1*) the irradiation-induced reduction of Pchlide is inhibited, and (*2*) the Chlide or the synthesized Chl is bleached during irradiation of dark-grown seedlings at elevated temperature. Our results showed that signal transduction pathway involved in the regulation of POR transcript and protein by irradiation is functional at elevated temperature. Therefore we suggest that the photochemistry related to POR involved in the Chl synthesis is similar at both the temperatures. According to Tewari

and Tripathy (1998) the function of POR is relatively unaffected by high temperature. We propose that failure of Chl accumulation during irradiation of dark-grown seedlings at 38 °C is due to Chl bleaching. Chl is bleached in plants and isolated systems due to high irradiance or high temperature (Mishra *et al.* 1994, Thomas and Ortiz 1995).

Unlike Chl, Car continued to accumulate at 38 °C although the rate was less as compared to seedlings irradiated at 25 °C. This was surprising since, as with Chl, elevated temperature would also lead to Car bleaching. Car degrade in the photosynthetic apparatus in adverse conditions (Mishra *et al.* 1994). The increased content of Car therefore may have resulted due to activation of protective systems during elevated temperature treatment. Car are important in photoprotection of PS2 during adverse conditions (Demmig-Adams and Adams 1992).

Elevated temperature also inhibited the accumulation of D1 and CP47 during irradiation of dark-grown seedlings. Chl is required for the stability of these proteins (Mullet *et al.* 1990). Therefore absence of these proteins in the elevated temperature-treated seedlings could be due to absence of Chl. However, involvement of other factors such as increased turnover of proteins, loss of plastid translational activity, or loss of transcripts could also account for the absence of these proteins at elevated temperature. Elevated temperature affects also the translation machinery (Feierabend and Berberich 1991). Moreover, transcript levels of a number of plastid genes coding for photosynthetic proteins are affected by elevated temperature (Singh and Singhal 1999). Involvement of these factors in the loss of proteins comes from observation that elevated temperature also induces loss of 33 kDa and CF1α proteins. These proteins do not require Chl and accumulate in the dark-grown seedlings.

A number of nuclear coded proteins are required for various plastid functions including photosynthesis, transcription, transcript stability, and translation activation (Rochaix 1992, Mayfield *et al.* 1995). Besides, some nuclear genes are required for chloroplast differentiation (Reiter *et al.* 1994, Zhang *et al.* 1994). Two-dimensional gel analysis of stroma proteins from dark-grown seedlings and seedlings irradiated either at 25 or 38 °C revealed that a number of proteins which accumulate during irradiation of dark-grown seedlings at normal temperature are not present in the dark-grown seedlings or in seedlings irradiated at 38 °C. At present we have no evidence to show that the loss of various plastid functions at elevated temperature results from the loss of these proteins. However, given the fact that a number of nuclear and plastid-coded proteins are required for chloroplast development, we speculate that these proteins are required for the chloroplast development during irradiation of dark-grown seedlings.

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