# Structure-function correlation during the etioplast-chloroplast transition in cucumber cotyledonary leaves

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## Abstract

We studied the development of chloroplasts from etioplasts in the cotyledonary leaves of 4-d-old dark-grown cucumber (*Cucumis sativus*) seedlings after irradiation (20 μmol $m^{-2}$ $s^{-1}$). Upon irradiation, the triggering of chlorophyll (Chl) synthesis and accumulation showed a relatively short lag phase. The irradiation of etiolated seedlings initiated the synthesis of apoproteins of pigment-protein complexes. While Chl-protein 2 (CP2) was detected at 6 h after irradiation, CP1 only after 29 h. The appearance and accumulation of some of the apoproteins were monitored by Western-blotting. LHC2 apoprotein was detected after a 6 h-irradiation. The amounts of D1 protein of photosystem (PS) 2 and PsaA/B protein of PS1 were quantitated by ELISA. Further, the thylakoid membrane function during this time period in terms of PS1- and PS2-mediated electron transfer activity and intersystem electron pool size were analyzed. While PS1 activity was detected after 4 h, PS2-mediated $O_2$ evolution was detected only after a 17 h-irradiation. $F_v/F_m$ value of Chl *a* fluorescence

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*Received* 24 August 1998, *accepted* 26 November 1998.

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*Abbreviations*: ATP, adenosine triphosphate; Chl, chlorophyll; CP, chlorophyll-pigment protein complex; Cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ELISA, enzyme-linked immunosorbent assay; $F_0$, minimal fluorescence; $F_m$, maximal fluorescence; $F_v$, variable fluorescence; LDS, lithium dodecyl sulphate; LHC, light-harvesting complex; LHCP, light-harvesting complex protein; MSP, manganese stabilizing protein; NADP, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; SDS, sodium dodecyl sulphate; TMPD($H_2$), tetramethyl *p*-phenylenediamine (reduced); Tricine, N-tris(hydroxymethyl)methyl-glycine; Tris, Tris(hydroxymethyl)aminomethane.

*Acknowledgements*: The present study was supported by the grant DAE-BRNS No. 4/3/95-R&D-II to PM. We gratefully acknowledge Prof. A.K. Mattoo, U.S. Department of Agriculture, Beltsville, U.S.A. for antibodies against D1, Prof. M. Ikeuchi, Department of Biology, University of Tokyo, Japan for antibodies against D2, Prof. I. Enami, Department of Biology, University of Tokyo for antibodies against PsaA/B, Prof. T. Kuwabara, Institute of Biological Sciences, University of Tsukuba, Japan for antibodies against manganese stabilizing protein, and Prof. Janet Meehl, Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, U.S.A. for antibodies against LHC2. We also thank Dr. S. Kar, Centre for Biotechnology, JNU for his help in the ELISA studies.

measurements indicated that the photochemical efficiency of these leaves reached its maximum after 29 h of irradiation. The intersystem pool size of cotyledonary leaves was equivalent to that of the control cotyledonary leaves grown for 25 h under continuous irradiation. Thus etioplasts develop into fully functional chloroplasts after approximately 25 h when 4 d-dark grown cucumber seedlings are continuously moderately irradiated. The development of photosynthetic electron transport chain seems to be limited in time at the level of PS2, possibly at the donor side.

*Additional key words*: chlorophyll fluorescence; intersystem pool size; light-harvesting complexes; photosystems 1 and 2; pigment-protein complexes; reaction centre proteins.

## Introduction

Photons are the major factor which regulates plastid biogenesis both from proplastids and etioplasts (Wellburn 1982, Jenkins *et al.* 1983, Akoyunoglou and Argyroudi-Akoyunoglou 1986). While some of the thylakoid electron transport chain components such as cytochromes (Cyt) *b* and *f*, the Rieske Fe-S protein, β-subunit of ATP synthase, ferredoxin, ferredoxin-NADP-oxidoreductase, and the extrinsic polypeptides of the oxygen evolving system are synthesized in the dark (Boardman 1981, Takabe *et al.* 1986, Willey and Grey 1988, Kusnetsov *et al.* 1994, Denev and Minkov 1997), synthesis of chlorophyll (Chl) and Chl-binding proteins such as P700A, P700B, D1, D2, CP43, and CP47 requires radiant energy (Vierling and Alberte 1983, Klein and Mullet 1986, Shinozaki *et al.* 1986, Mullet 1988).

Thus, development of fully functional photosynthetic membranes requires assembly of light-induced and pre-existing components (Nyitrai 1997). Though the differentiation of chloroplasts from etioplasts is different from their development from proplastids (Mohr 1984, 1986), this system has been used extensively for its ease and speed of operation (Link 1991). Different aspects of the chloroplast development from etioplasts have been reported (Kyle and Zalik 1982, Tanaka and Tsuji 1985, Morisey *et al.* 1989, Shibasaka *et al.* 1993, Dreyfuss and Thornber 1994, Sigrist and Staehelin 1994). However, there seem to be species-specific differences. Many of the studies so far have dealt with selected aspects of PS2 formation and there is need for studying the complex structure-function relationship during greening of etioplasts.

Cotyledonary leaves have often been used as a model system to study the process of greening. Since many plastid-encoded genes are regulated post-transcriptionally after irradiation of the etioplasts (Viro and Kloppstech 1982, Kusnetsov *et al.* 1994), we studied the accumulation of protein components and pigment-protein complexes of the thylakoid membranes in cotyledonary leaves of etiolated seedlings of cucumber (*Cucumis sativus*) at fairly low irradiance (20 μmol $m^{-2}$ $s^{-1}$) so that the events during this transition could be precisely characterised and quantified. A correlation between structure and photochemical function of the thylakoid development after irradiation was established.

## Materials and methods

Cucumber (*Cucumis sativus* L. cv. Poinsette) seeds were germinated and grown on moist cotton in the dark at 25 °C. The 4-d-old seedlings were then continuously irradiated by "white light" (20 μmol $m^{-2}$ $s^{-1}$) at the same temperature.

The etioplasts and photosynthetic membranes at various stages of greening were isolated using a method similar to that of Nakatani and Barber (1977). The leaves were homogenised in ice-chilled isolation medium containing 0.4 M sorbitol, 15 mM Tricine (pH 7.8), and 5 mM NaCl. The homogenate was filtered through four layers of mira cloth and centrifuged at 2 100×*g* for 5 min. The pellet was washed in buffer containing 10 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$, and finally resuspended in the same buffer to which 0.1 M sorbitol was added.

Chl *a* and *b* were estimated according to Porra *et al.* (1989) with 100 % dimethylformamide as solvent. Protein in the isolated membranes was estimated according to Markwell *et al.* (1981) with a final concentration of 0.7 % (m/v) sodium dodecyl sulphate (SDS). Pigment-protein complexes were analyzed on a 10 % (0.33 % cross-linker) non-denaturing polyacrylamide gel according to the method of Thornber and Highkin (1974) with modifications. Lithium dodecyl sulphate (LDS) was used in the running buffer (0.1 %, m/v) and sample buffer (2 %, m/v) instead of SDS. Samples were prepared for electrophoresis by suspending the thylakoids at 4 °C for 20 min and electrophoresed at 4 °C. Polypeptides were resolved on a 12.5 % (2.7 % cross-linker) SDS-denaturing gel according to Laemmli (1970). 15 μg of protein was loaded into each well. The gels were stained with Coomassie brilliant blue.

For immunological detection of specific thylakoid membrane proteins, they were separated on a 12.5 % acrylamide gel containing 2 M urea. The sample buffer contained 6 M urea, 1.0 % (m/v) SDS, and 2.5 % (v/v) 2-mercaptoethanol in 62.5 mM Tris-HCl buffer, pH 6.8. Prior to electrophoresis, the membranes were incubated in the sample buffer for 30 min at room temperature. The separated polypeptides were then transferred onto nitrocellulose membrane electrophoretically. Specific proteins on the Western-blots were detected using anti-rabbit IgG-alkaline phosphatase conjugate according to Towbin *et al.* (1979). Reaction centre proteins of PS2 (D2) and PS1 (PsaA/B) were quantified by ELISA by coating 100 μg membrane protein onto the plates, and developing the colour of the reaction after incubation with anti-rabbit IgG-horse radish peroxidase. 30 % $H_2O_2$ and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid in 100 mM phosphate-citrate buffer, pH 5.0 were used as substrates for peroxidase, and the activity was determined spectrophotometrically at 405 nm.

Electron transport activities of the membranes were measured as $O_2$ evolution (PS2) or consumption (PS1) using an $O_2$-electrode assembly from *Hansatech*, England (model *DW2*) at 25 °C essentially as described by Vani *et al.* (1996). Membranes were suspended in a buffer containing 0.1 M sorbitol, 10 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$. For the assay of PS2 activity, *p*-benzoquinone (1 mM) was used as electron acceptor. PS1 activity was measured with reduced tetramethyl *p*-phenylenediamine ($TMPDH_2$, 100 μM) as electron donor and methylviologen (50 μM) as electron acceptor. For PS1 assay, the assay buffer

contained 10 mM sodium azide and 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

Chl *a* fluorescence induction curves were recorded on a pulse-amplitude modulated (PAM) fluorimeter (*Walz*, Germany) as described by Tiwari and Mohanty (1996). The P700 reduction capacity of the photosynthetic electron transport chain was measured with the instrument used for Chl *a* fluorescence fitted with an emitter-detector unit (*ED 800T*) which monitors the absorbance changes caused by $P700^+$ radical around 830 nm (Schreiber *et al.* 1988) as described in Tiwari and Mohanty (1996).

## Results

**Chl accumulation**: The accumulation of Chl *a*, *b*, and (*a*+*b*) in cucumber cotyledonary leaves on continuous "white light" was completed in about 30 h (Fig. 1). While Chl *a* synthesis did not show a significant lag, Chl *b* synthesis occurred after 4 h. The Chl *a*/*b* ratio dropped in the first 6 h of greening but was almost constant later (Fig. 1, *inset*). Depending on plant species and experimental conditions, a lag period of 2-8 h has been observed in Chl biosynthesis by Mohanty and Mohanty (1988), Ohashi *et al.* (1989), Kauoa and Laval-Martin (1995), and others.

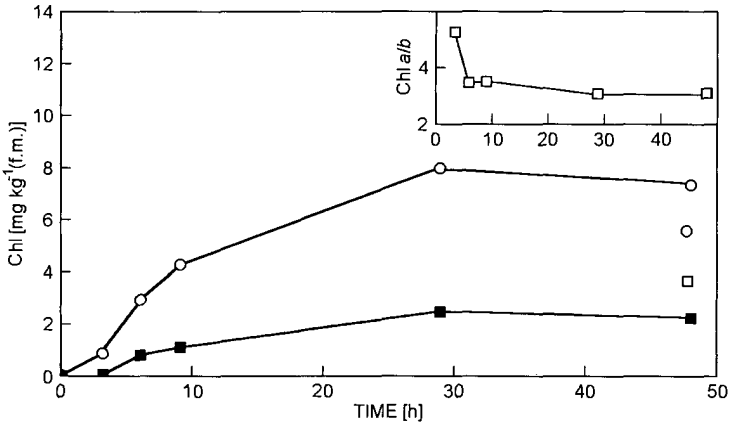


Fig. 1. Synthesis of chlorophyll (Chl) *a* (○) and *b* (■) in 4-d-old etiolated cucumber cotyledons exposed to irradiance of 20 μmol $m^{-2}$ $s^{-1}$. The amount of Chl was estimated at various times after irradiation. *Inset*: the ratio of Chl *a*/*b* during greening. Means of three independent experiments; the variation within the samples is less than 5 %.

**Thylakoid membrane proteins**: The photosynthetic electron transport system consists of two major light-harvesting supramolecular pigment-protein complexes LHC1 and LHC2, and the Cyt $b_6/f$ complex. These pigment-protein complexes are not present in etioplasts but are synthesized during light-induced greening of the etioplasts. The temporal sequence of synthesis and assembly of the apoproteins of these pigment-protein complexes is important in the development of photochemical function and it

has not been well documented in cotyledonary leaves. Thus, we monitored the synthesis of some of these apoproteins, CP1 and CP2, both by SDS-polyacrylamide gel electrophoresis and Western-blotting using specific antibodies. There was a gradual loss of some unidentified proteins (Fig. 2*B*) while the contents of CP1 and CP2 increased during irradiation (Fig. 2*A*). While CP2, derived from LHC2 (Thornber and Highkin 1974), could be detected on the non-denaturing gel as early as after 6 h, CP1 could only be detected at 29 h (Fig. 2*B*). The protein corresponding to CP1 was detected on Coomassie-blue stained green gel only at 11 h, but that of CP2 already at 6 h (Fig. 2*B*).

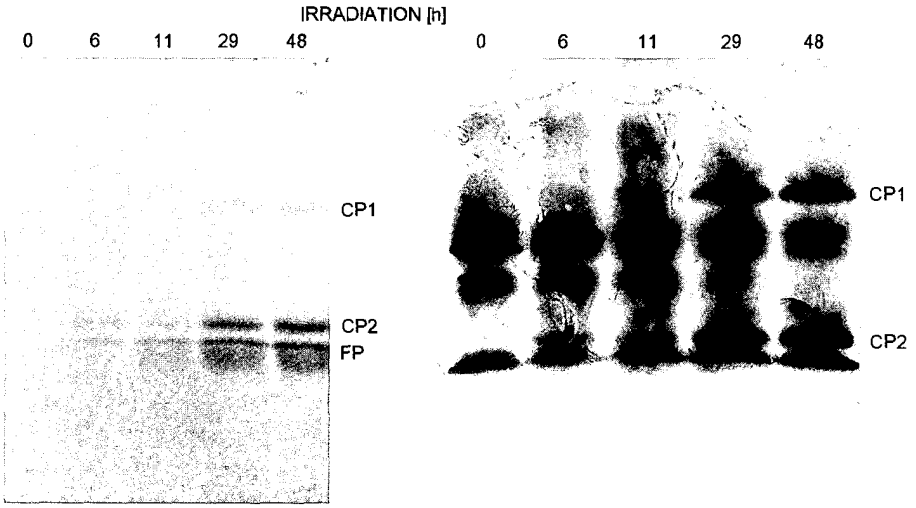


Fig. 2. Analysis of pigment-protein complexes by electrophoresis on 10 % (0.33 % C) polyacrylamide gel with mild detergent treatment (LDS) of thylakoid membranes of cucumber cotyledonary leaves at different stages of greening upon irradiation of 4-d-old etiolated seedlings for 6 to 48 h. Each lane was loaded with 50 μg protein. CP1, CP2, and free pigment (FP) are denoted. *A*: Unstained green gel, *B*: Coomassie stained green-gel.

The synthesis of apoproteins of pigment-protein complexes was also analyzed by SDS-PAGE (Fig. 3). The apoproteins of PS1 reaction centre (67 kDa), Chl *a* antenna of PS2 (47 and 43 kDa), the PS2 reaction centre (32 kDa), and the multiple forms of the LHC2 (26-29 kDa) appeared and their contents gradually increased in a time-dependent manner on irradiation.

An analysis of synthesis of different proteins in the photosynthetic electron transport system during etioplast differentiation upon irradiation was further carried out employing Western-blots. Fig. 4 shows such an analysis using antibodies against D1, LHC2, and manganese-stabilizing protein (MSP). An analysis of these Western-blots shows that D1 and LHC2 apoproteins were not detected in etioplasts. All the three proteins showed an increased synthesis during transition to chloroplasts. However, the rate of synthesis of these proteins was different. While appearance of the LHC2 apoprotein in Western-blots was conspicuous by 6 h, D1 showed only faint bands at this time, in comparison to 29 and 48 h samples (Fig. 4). MSP, in

contrast, could be detected even in etioplasts, the intensity increasing with longer irradiation as observed by Hashimoto *et al.* (1993) in leaves of barley seedlings.

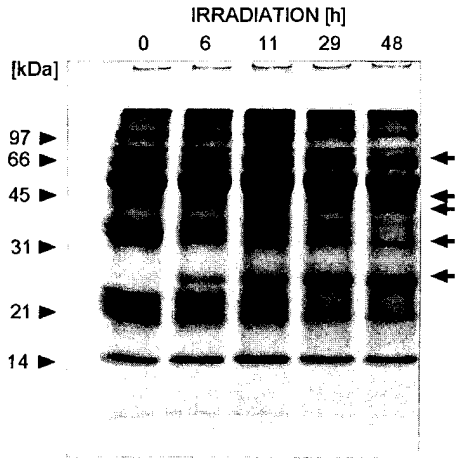


Fig. 3. Coomassie blue stained polypeptide analysis of thylakoid membrane proteins of cucumber cotyledonary leaves at different stages of greening upon 6-48 h irradiation of 4-d-old seedlings. The polypeptides were resolved on a 12.5 % (2.7 % C) SDS-denaturing polyacrylamide gel. The positions of standard molecular mass markers are shown on the left. *Arrows* indicate the polypeptides whose quantity has increased on irradiation.

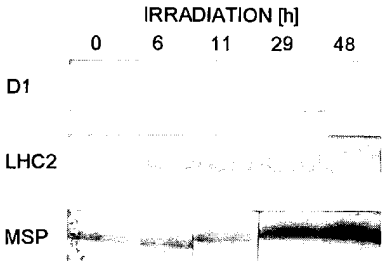


Fig. 4. Western-blot analysis of different thylakoid membranes. Proteins were resolved on a 12.5 % (2.7 % C) SDS-PAGE and transferred onto nitrocellulose membranes. Antibodies against D1, LHC2, and manganese stabilising proteins were used to analyse accumulation of the proteins during 6-48 h greening of cucumber cotyledonary leaves with alkaline phosphatase conjugated anti-rabbit IgG.

Two of the reaction centre proteins, one each of PS2 and PS1 (D2 and PsaA/B, respectively), were quantified using ELISA: their contents gradually increased on irradiation of the etiolated seedlings as reflected in the horse-radish peroxidase activity measured as $A_{405}$ (Fig. 5). Contents of both these proteins showed a rapid increase up to 12 h of irradiation. During this time, the proteins attained almost 90 % of their steady-state value. The presence of PsaA/B protein in etioplasts before irradiation of the seedlings was also observed in Western-blots (values not presented). Our results also correspond with the observations of Paproth and Hauska

(1986) with the P700 apoprotein in spinach, of Radunz *et al.* (1986) with *Phaseolus vulgaris*, and of Schantz (1985) with the Chl-binding proteins in *Euglena*.

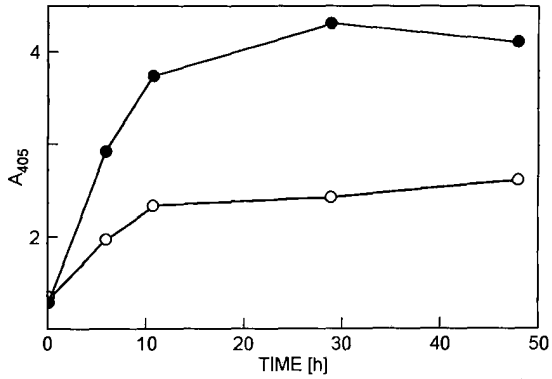


Fig. 5. ELISA test of D2 (○) and PsaA/B (●) of thylakoid membranes during greening of cucumber cotyledonary leaves. The proteins were analyzed with specific antibodies and horse-radish peroxidase conjugated anti-rabbit IgG. Means of three independent experiments; the variation within the samples is less than 5 %.

Table 1. Photosystem (PS) 1 and 2 activities [mmol $kg^{-1}$(Chl) $s^{-1}$] of the thylakoids from 4-d-old dark grown cucumber cotyledonary leaves during greening. The activities were assayed with an oxygen electrode polarographically either as $O_2$ evolution (PS2) or consumption (PS1). The reaction mixture contained 10 μg chlorophyll (Chl) in 10 mM Tricine buffer (pH 7.8), 0.1 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$. The electron donors for PS1 and PS2 were 100 μM reduced tetramethyl *p*-phenylenediamine ($TMPDH_2$) and water, respectively, while the acceptors were 50 μM methylviologen and 1 μM *p*-benzoquinone, respectively. For the assay of PS1 activity 10 mM sodium azide and 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were also added to the reaction mixture.

| Duration of greening [h] | PS2 | PS1 |
|---|---|---|
| 0 | 0 | 0 |
| 4 | 0 | 283.0 |
| 6 | 0 | 187.0 |
| 17 | 11.9 | 93.6 |
| 20 | 10.3 | 92.0 |
| 26 | 10.0 | 98.3 |
| 42 | 20.0 | 91.4 |
| 48 | 12.8 | 97.5 |

**PS1 and PS2 activities** were assayed polarographically during the development of etioplasts into chloroplasts in the cucumber cotyledonary leaves. Usually, PS1 activity is detected ahead of PS2 activity (Wellburn and Hampp 1979). Our results with cucumber cotyledonary leaves are in agreement with these observations: PS1 activity measured as electron transfer from $TMPDH_2$ to methylviologen was found as early as after 4 h while the PS2-catalyzed $O_2$ evolution with *p*-benzoquinone as acceptor was detected only at 17 h of greening (Table 1). Hence our results agree

with the observation of Bradbeer (1981) that PS1 activity is detected even before massive accumulation of Chl. The PS2 activity increased to 20 mmol $kg^{-1}$(Chl) $s^{-1}$ by 42 h and decreased to 12.8 mmol $kg^{-1}$(Chl) $s^{-1}$ at 48 h (Table 1). The PS1 activity, on the other hand, was highest at 4 h (Table 1) followed by a decline during further irradiation till 17 h. A similar decrease in PS1 activity was observed by Ohashi *et al.* (1989) in greening barley seedlings. Akoyunoglou (1981) has observed that while initially very high PS1 activity decreases and levels off during greening, the initially low PS2 activity increases and only after reaching a plateau decreases. Our results with *Cucumis* cotyledonary leaves showed a similar trend.

**Fluorescence measurements**: To analyze the development of electron transfer linked primary charge separation in PS2 reaction centres in the photosynthetic membranes during the transition, we measured fast Chl *a* fluorescence transient rise. In response to actinic radiation, Chl *a* fluorescence rises from minimum ($F_0$) to maximum ($F_m$) fluorescence. The kinetics and the extent of this rise, termed rise of variable fluorescence ($F_v$), is correlated to the redox state of primary acceptor of PS2 (Butler 1978). In our experiments, a 6 h irradiation induced substantial value of $F_0$ which during further 5 h irradiation attained its maximum (Table 2). Also $F_m$ increased significantly as the etiolated plants were irradiated. From 6 to 29 h of irradiation, the $F_0$ increased only by 25 %, but the $F_v$ showed a dramatic increase of 450 %. In the etiolated seedlings irradiated for 6 h, $F_0$ accounted for 60 % of the total Chl *a* fluorescence ($F_m$). The rest 40 % emerged as $F_v$. However, after 29 h irradiation, contribution of $F_0$ was reduced to 25 %. The ratio of $F_v/F_m$, a measure of the ability of PS2 photochemical efficiency (Björkman 1987, Joshi and Mohanty 1995) at 6 h was 0.400 and increased to 0.761 after 29 h (Table 2).

Table 2. Development of photosystem 2 (PS2) photochemical function as monitored in terms of chlorophyll (Chl) *a* fluorescence during the greening of 4-d-old etiolated cucumber cotyledonary leaves on irradiation. Fast Chl *a* fluorescence transients were recorded at different times during greening.

| Duration of greening [h] | $F_0$ | $F_m$ | $F_v = F_m - F_0$ | $F_v/F_m$ |
|---|---|---|---|---|
| 6 | 0.012 | 0.020 | 0.008 | 0.400 |
| 11 | 0.015 | 0.037 | 0.022 | 0.595 |
| 29 | 0.015 | 0.059 | 0.044 | 0.746 |
| 48 | 0.016 | 0.067 | 0.051 | 0.761 |

**Reduction of P700**: PS1 was oxidized by far-red radiation and this radiation was kept on during the entire time of measurement. Under such conditions, P700 absorbance was 0. A multiple turnover flash subsequent to a single turnover flash reduced all P700 molecules which were reoxidized by the continuous far-red background radiation (Schreiber *et al.* 1988). We analyzed the area under the curve of P700 reduction and its subsequent oxidation by far-red radiation and used it as a measure of capacity of photosynthetic electron transport chain to reduce P700. In the developing chloroplasts the extent of P700 reduction is affected by the intersystem pool size as

well as by PS2 activity. In response to irradiation, P700 reduction capacity gradually increased (Fig. 6). Etiolated plants irradiated for 25 h attained an efficiency of P700 reduction equivalent to control cotyledonary leaves exposed to continuous irradiation from germination (Fig. 6 and *inset*). Intersystem transport pool complexes are present in the etioplasts (Boardman 1981, Takabe *et al.* 1986, Willey and Grey 1988).

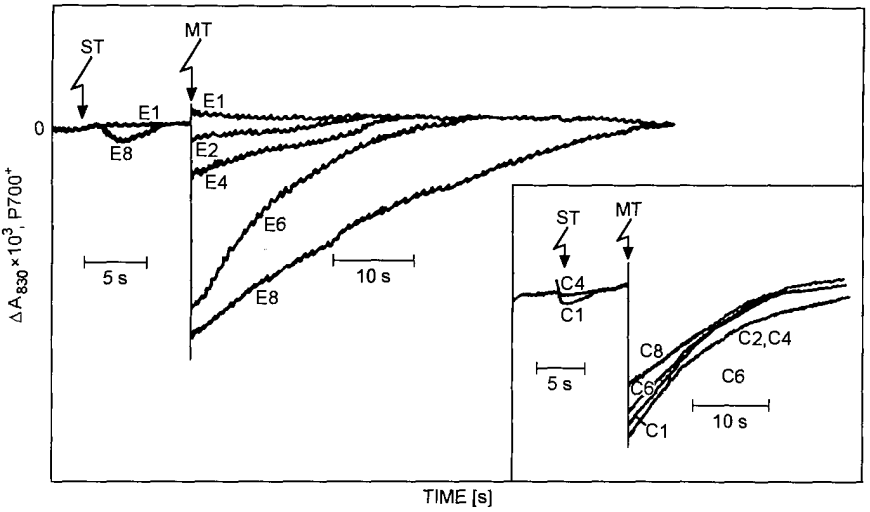


Fig. 6. Development of intersystem pool size in the etiolated cucumber cotyledonary leaves on irradiation. The pool size was determined by the measuring irradiation induced 830 nm absorbance change. 5 min dark adapted cotyledonary leaves were irradiated with far-red radiation (approximately 100 μmol $m^{-2}$ $s^{-1}$) for 1 min which was kept switched on during the entire measurement. The kinetic response to single turnover (ST) and multiple turnover (MT) flashes are shown. The duration of ST and MT flashes was 6 μs and 50 ms, respectively. *Inset*: similar measurements in seedlings grown under continuous irradiation of 20 μmol $m^{-2}$ $s^{-1}$ from the beginning. E1, E2, E4, E6, and E8 (*main panel*) are measurements at 0, 6, 11, 29, and 48 h of greening, respectively, and C1, C2, C4, C6, and C8 (*inset*) are the corresponding curves with the seedlings under continuous irradiation from the beginning.

## Discussion

Some components of the thylakoid membranes are synthesized in the etioplasts while some others require radiant energy for synthesis. We found that CP1 and CP2 were synthesized in response to irradiation (Fig. 2*A*). Similar observations have been reported on LHC2 apoproteins during chloroplast development from etioplasts (Shimada *et al.* 1990, Mysliwa-Kurdziel *et al.* 1997). Tavladoraki *et al.* (1986) observed a cessation of Chl-binding protein synthesis when the duration of etiolation was long, and they were reinduced only after irradiation. Since the appearance of CP1 and CP2 apoproteins (Fig. 2*A*) also correlated with the synthesis of Chl (Fig. 1) we believe that these apoproteins, similar to Chl, are synthesized in response to irradiation.

Detection of CP1 at 29 h (Fig. 2*B*) while Chl *a* biosynthesis was instantaneous on irradiation of etiolated seedlings (Fig. 1) probably reflects the stoichiometry of Chl *a* to CP1 apoprotein and the sensitivity of detection of complexes on the gel. This seems true since we could detect PS1 activity as early as after 4 h (Table 1). On the other hand, the appearance of CP2, the Chl *a/b*-protein complex derived from LHC2 (Markwell 1986) and the LHC2 apoprotein (Fig. 4) was concomitant with Chl *b* biosynthesis (Fig. 1). In barley seedlings, Tanaka and Tsuji (1985) have detected CP1 as early as 45-60 min afer irradiation while LHC accumulation accompanied Chl *b* synthesis after 2.5 h. This is contrary to our results detecting CP2 earlier than CP1 on the gel.

Our results on appearance of PS1 activity before massive accumulation of Chl agree with the observations of Bradbeer (1981). However, PS2 activity could not be detected polarographically before the detection of LHCP by Boardman (1981), Tanaka and Tsuji (1985), Burkey (1986), and Ohashi *et al.* (1989). The absence of PS2-catalyzed $O_2$ evolution can possibly be attributed to low amounts of D1 and D2 reaction centre proteins of PS2 (Figs. 4 and 5, respectively). The detection of PS1 activity earlier than PS2 activity (Table 1) shows that non-cyclic electron transport is limited by the development of PS2 activity.

A decrease in PS2 (Ohashi *et al.* 1989, Tanaka *et al.* 1991) and PS1 (Akoyunoglou 1981) activities after reaching a maximal value during chloroplast development was also observed earlier. Kauoa and Laval-Martin (1995) suggest that the Chl accumulating in the later stages of greening may correspond to either PS1 and PS2 antenna Chls or inactive PS2 centres which are unable to support $O_2$ evolution. However, further analysis is needed to see whether the drop in activity is due to inactive PS2 centres.

Chl *a* fluorescence analysis is a sensitive method to estimate the functional aspects of PS2. Since $F_0$ emanates from LHC2 (Telfer *et al.* 1984), a significant contribution of $F_0$ to $F_m$ after 6 h of irradiation of etiolated seedlings (Table 2) indicated that the chloroplasts were enriched with LHC2. The reduction in contribution of $F_0$ to $F_m$ after 29 h of irradiation (Table 2) reflects the development of effective coupling of LHC2 to the PS2 reaction centre and the ability of the reaction centre to reduce $Q_A$ resulting in higher levels of $F_v$. A steady increase in $F_v$ in response to irradiation showed a gradual enhancement of PS2-dependent photochemistry which resulted in 90 % increase in $F_v/F_m$ from 6 to 29 h (Table 2). This enhancement in $F_v/F_m$ also correlates with PS2 reaction centre proteins and PS2 activity (Figs. 4 and 5). The ratio of $F_v/F_m$ at 6 h of irradiation (0.4, Table 2) suggests that the functional development of PS2 may be limited at the donor side because PS2 catalyzed $O_2$ evolution could be detected only later (Table 1). According to Wellburn and Hampp (1979), water oxidation capacity of PS2 is acquired late; it is probably due to the requirement for quanta to activate manganese (Tamura *et al.* 1997).

The development of PS1 activity preceded the development of activity of PS2 even though the pigment-protein complexes of PS1 (CP1) could only be resolved at a much later stage. Chl *a* fluorescence measurements and polarography showed that the fully functional activity of PS2 developed after about 25 h of exposure of etioplasts to low irradiance (20 $\mu$mol $m^{-2}$ $s^{-1}$). Non-cyclic electron transport attained maximum

activity during a similar time span. The time-dependent appearance of PS2 reaction centre proteins was well correlated with the enhancement of PS2 electron transport activity and with the extent of P700 reduction (Fig. 6), suggesting that PS2 assembly is the limiting step during the etioplast to chloroplast transition.

In summary, our study is a detailed analysis of the development of major pigment-protein complexes of photosynthetic apparatus during conversion of etioplasts into chloroplasts in cucumber cotyledonary leaves which are used as a model system. We also established temporal correlation between structure and function by quantifying PS2 and PS1 activities, and intersystem pool size. We suggest that sequential development of photofunction is limited at PS2 and this limitation may have arisen from the donor side of PS2 centres. Further work is necessary to characterize the nature of limitation at PS2.

## References

Akoyunoglou, G.: Assembly of functional components in chloroplast photosynthetic membranes. - In: Akoyunoglou, G. (ed.): Photosynthesis. Vol. V. Pp. 353-366. Balaban International Science Services, Philadelphia 1981.

Akoyunoglou, G., Argyroudi-Akoyunoglou, J.: Post-translational regulation of chloroplast differentiation. - In: Akoyunoglou, G., Senger, H. (ed.): Regulation of Chloroplast Differentiation. Pp. 571-582. A.R. Liss, New York 1986.

Björkman, O.: Low temperature chlorophyll fluorescence in leaves and its relationship to photon yield of photosynthesis in photoinhibition. - In: Kyle, D.J., Osmond, C.B., Arntzen, C.J. (ed.): Photoinhibition. Pp. 123-144. Elsevier, Amsterdam - New York - Oxford 1987.

Boardman, N.K.: Thylakoid membrane formation in the higher plant chloroplast. - In: Akoyunoglou, G. (ed.): Photosynthesis. Vol. V. Pp. 325-339. Balaban International Science Services, Philadelphia 1981.

Bradbeer, J.W.: Development of photosynthetic function during chloroplast biogenesis. - In: Hatch, M.D., Boardman, N.K. (ed.): The Biochemistry of Plants. Vol. 8. A Comprehensive Treatise. Pp. 424-467. Academic Press, New York - London - Toronto - Sydney - San Francisco 1981.

Burkey, K.O.: Chlorophyll-protein complex composition and photochemical activity in developing chloroplasts from greening barley seedlings. - Photosynth. Res. **10**: 37-49, 1986.

Butler, W.L.: Energy distribution in the photochemical apparatus of photosynthesis. - Annu. Rev. Plant Physiol. **29**: 345-378, 1978.

Denev, I.D., Minkov, I.N.: Use of fluorescence probes 1-aniline-8-naphthalene sulfonate and pyrene for studying the localisation of proteins in inner membranes from wheat etioplasts. - Photosynthetica **33**: 303-312, 1997.

Dreyfuss, B.W., Thornber, J.P.: Assembly of the light-harvesting complexes (LHCs) of photosystem II. Monomeric LHC IIb complexes are intermediates in the formation of oligomeric LHC IIb complexes. - Plant Physiol. **106**: 829-839, 1994.

Ellis, R.J.: Chloroplast proteins: Synthesis, transport, and assembly. - Annu. Rev. Plant Physiol. **32**: 111-137, 1981.

Hashimoto, A., Akasaka, T., Yamamoto, Y.: Characteristics of the assembly of the 33 kDa oxygen-evolving complex protein in the etioplasts and the developing chloroplasts of barley seedlings. - Biochim. biophys. Acta **1183**: 397-407, 1993.

Jenkins, G.I., Hartley, M.R., Bennett, J.: Photoregulation of chloroplast development: transcriptional, translational and posttranscriptional controls? - Philos. Trans. roy. Soc. London B **303**: 419-431, 1983.

Joshi, M.K., Mohanty, P.: Probing photosynthetic performance by chlorophyll *a* fluorescence. Analysis and interpretation of fluorescence parameters. - J. sci. ind. Res. **54**: 155-174, 1995.

Kaoua, M.E., Laval-Martin, D.L.: Evolution of PS IIα and PS IIβ centers during the greening of *Euglena gracilis* Z: Correlations with changes in lipid content. - Photosynth. Res. **43**: 155-163, 1995.

Klein, R.R., Mullet, J.E.: Regulation of chloroplast-encoded chlorophyll-binding protein translation during higher plant chloroplast biogenesis. - J. biol. Chem. **261**: 11138- 11145, 1986.

Kusnetsov, V.V., Oelmuller, R., Sarwat, M.I., Porfirova, S.A., Cherepneva, G.N., Hermann, R.G., Kulaeva, O.N.: Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons without notable effect on steady-state mRNA levels. Specific protein response to light/phytohormone interaction. - Planta **194**: 318-327, 1994.

Kyle, D.J., Zalik, S.: Development of photochemical activity in relation to pigment and membrane protein accumulation in chloroplasts of barley and its virescent mutant. - Plant Physiol. **69**: 1392-1400, 1982.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **277**: 680-685, 1970.

Link, G.: Photoregulated development of chloroplasts. - In: Bogorad, L., Vasil, I.K. (ed.): The Photosynthetic Apparatus: Molecular Biology and Operation. Pp. 365-394. Academic Press, San Diego - New York - Boston - London - Sydney - Tokyo - Toronto 1991.

Markwell, J.: Electrophoretic analysis of photosynthetic pigment-protein complexes. - In: Hipkins, M.F., Baker, N.R. (ed.): Photosynthesis: Energy Transduction - A Practical Approach. Pp. 27-49. IRL Press, Oxford 1986.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E., Bieber, L.L.: Protein determination in membrane and lipoprotein samples: Manual and automated procedures. - In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 72. Pp. 296-303. Academic Press, New York - London 1981.

Mohanty, N., Mohanty, P.: After-effect of elevated temperature on the development of photosynthetic characteristics in wheat seedlings. - J. Plant Physiol. **133**: 235-239, 1988.

Mohr, H.: Phytochrome and chloroplast development. - In: Baker, N.R., Barber, J. (ed.): Chloroplast Biogenesis. Pp. 305-347. Elsevier, Amsterdam 1984.

Mohr, H.: Control by light of plastidogenesis as part of a control system. - In: Akoyunoglou, G., Senger, H. (ed.): Regulation of Chloroplast Differentiation. Pp. 623-634. A.R. Liss, New York 1986.

Morisey, P.J., Glick, R.E., Melis, A.: Supramolecular assembly and function of subunits associated with the chlorophyll *a-b* light harvesting complex II (LHC II) in soybean chloroplasts. - Plant Cell Physiol. **30**: 335-344, 1989.

Mullet, J.E.: Chloroplast development and gene expression. - Annu. Rev. Plant Physiol. Plant mol. Biol. **39**: 475-502, 1988.

Mysliwa-Kurdziel, B., Barthelemy, X., Strzalka, K., Franck, F.: The early stages of photosystem II assembly monitored by measurements of fluorescence life time, fluorescence induction and isoelectric focusing of chlorophyll-proteins in barley etiochloroplasts. - Plant Cell Physiol. **38**: 1187-1196, 1997.

Nakatani, H.Y., Barber, J.: An improved method for isolating chloroplasts retaining their outer membranes. - Biochim. biophys. Acta **461**: 510-512, 1977.

Nyitrai, P.: Development of functional thylakoid membranes: Regulation by light and hormones. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 391-406. Marcel Dekker, New York - Basel - Hong Kong 1997.

Ohashi, K., Tanaka, A., Tsuji, H.: Formation of the photosynthetic electron transport system during the early phase of greening in barley leaves. - Plant Physiol. **91**: 409-414, 1989.

Paproth, B., Hauska, G.: Thylakoid proteins in seeds and etiolated plants of spinach. - In: Akoyunoglou, G., Senger, H. (ed.): Regulation of Chloroplast Differentiation. Pp. 193-196. A.R. Liss, New York 1986.

Porra, R.J., Thompson, W.A., Kriedemann, P.E.: Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents. Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. - Biochim. biophys. Acta **975**: 384-394, 1989.

Radunz, A., Schmid, G.H., Bertrand, M., Dujardin, E.: Comparative serological studies on some proteins of etioplasts and chloroplasts of *Phaseolus vulgaris* var. *commodore* L. - In: Akoyunoglou, G., Senger, H. (ed.): Regulation of Chloroplast Differentiation. Pp. 197-203. A.R. Liss, New York 1986.

Schantz, R.: Mapping of the chloroplast genes coding for the chlorophyll *a* binding proteins in *Euglena gracilis*. - Plant Sci. **40**: 43-49, 1985.

Schiff, J.A., Schwartzbach, S.D.: Photocontrol of chloroplast development in *Euglena*. - In: Buetow, D.E. (ed.): The Biology of *Euglena*. Vol. III. Pp. 313-352. Academic Press, New York 1982.

Schreiber, U., Klughammer, C., Neubauer, C.: Measuring P700 absorbance changes around 830 nm with a new type of pulse modulation system. - Z. Naturforsch. **43c**: 686-698, 1988.

Shibasaka, M., Tanaka, A., Tsuji, H.: Changes in spectral properties of chlorophyll during greening of barley leaves. - Photochem. Photobiol. **58**: 432-437, 1993.

Shimada, Y., Tanaka, A., Tanaka, Y., Takabe, T., Takabe, T., Tsuji, H.: Formation of chlorophyll-protein complexes during greening. 1. Distribution of newly synthesized chlorophyll among apoproteins. - Plant Cell Physiol. **31**: 639-647, 1990.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., Sugiura, M.: The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. - EMBO J. **5**: 2043-2049, 1986.

Sigrist, M., Staehelin, L.A.: Appearance of type 1, 2, and 3 light-harvesting complex II and light-harvesting complex I proteins during light-induced greening of barley (*Hordeum vulgare*) etioplasts. - Plant Physiol. **104**: 135-145, 1994.

Takabe, T., Takabe, T., Akazawa, T.: Biosynthesis of P700-chlorophyll *a* protein complex, plastocyanin and cytochrome *b/f* complex.- Plant Physiol. **81**: 60-66,1986.

Tamura, N., Kuwabara, M., Sasaki, Y., Watanabe, K., Oku, T.: Redox dependence for photoligation of manganese to the apo-water-oxidizing complex in chloroplasts and photosystem II membranes. - Biochemistry **36**: 6171-6177, 1997.

Tanaka, A., Tsuji, H.: Appearance of chlorophyll-protein complexes in greening barley seedlings. - Plant Cell Physiol. **26**: 893-902, 1985.

Tanaka, A., Yamamoto, Y., Tsuji, H.: Formation of chlorophyll-protein complexes during greening. 2. Redistribution of chlorophyll among apoproteins. - Plant Cell Physiol. **32**: 195-204, 1991.

Tavladoraki, P., Akoyunoglou, G., Bitsch, A., Meyer, G., Kloppstech, K.: Age and phytochrome-induced changes at the level of the translatable mRNA coding for the LHC-II apoprotein of *Phaseolus vulgaris* leaves. - In: Akoyunoglou, G., Senger, H. (ed.): Regulation of Chloroplast Differentiation. Pp. 559-564. A.R. Liss, New York 1986.

Telfer, A., Bottin, H., Barber, J., Mathis, P.: The effect of magnesium and phosphorylation of light-harvesting chlorophyll *a/b*-protein on the yield of P700-photooxidation in pea chloroplasts. - Biochim. biophys. Acta **764**: 324-330, 1984.

Thornber, J.P., Highkin, H.R.: Composition of the photosynthetic apparatus of normal barley leaves and a mutant lacking chlorophyll *b*. - Eur. J. Biochem. **41**: 109- 116, 1974.

Tiwari, S., Mohanty, P.: Cobalt induced changes in photosystem activity in *Synechocystis* PCC 6803. Alterations in energy distribution and stoichiometry. - Photosynth. Res. **50**: 243-256, 1996.

Towbin, H., Staehelin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. - Proc. nat. Acad. Sci. USA **76**: 4350-4354, 1979.

Vani, B., Saradhi, P.P., Mohanty, P.: Effect of short-term heat treatment of rice seedlings on sensitivity of thylakoid membranes to photoinhibition. - Biol. Plant. **38**: 501-509, 1996.

Vierling, E., Alberte, R.S.: Regulation of synthesis of the photosystem I reaction center. - J. Cell Biol. **97**: 1806-1814, 1983.

Viro, M., Kloppstech, K.: Expression of genes for plastid membrane proteins in barley under intermittent light conditions. - Planta **154**: 18-23, 1982.

Wellburn, A.R.: Bioenergetic and ultrastructural changes associated with chloroplast development. - Int. Rev. Cytol. **80**: 133-191, 1982.

Wellburn, A.R., Hampp, R.: Appearance of photochemical function in prothylakoids during plastid development. - Biochim. biophys. Acta **547**: 380-397, 1979.

Willey, D.L., Gray, J.C.: Synthesis and assembly of the cytochrome *b-f* complex in higher plants. - Photosynth. Res. **17**: 125-144, 1988.