

The Effect of Cd on Chlorophyll and Light-Harvesting Complex II Biosynthesis in Greening Plants

Leto Tziveleka^a, Athanassios Kaldis^a, Attila Hegedüs^b, Judit Kissimon^b, Anastasia Prombona^a, Gabor Horváth^b and Joan Argyroudi-Akoyunoglou^{a,*}

^a Institute of Biology, NCSR "Demokritos", 15310 Aghia Paraskevi, Attiki, Greece.
Fax: (301) 6511767. E-mail: yako@mail.demokritos.gr

^b Department of Plant Physiology, University of Horticulture and Food Industry,
P. O. Box 53, H-1518 Budapest, Hungary

* Author for correspondence and reprint requests

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Cadmium, Chlorophyll, Light-Harvesting Complex II, Thylakoid-Bound Protease, Transcription

The effect of Cd on chlorophyll (Chl) as well as on light-harvesting complex II (LHCII) accumulation, has been examined during the early stages of development in etiolated *Phaseolus vulgaris* leaves exposed to intermittent light-dark cycles. We found that at the Cd concentrations studied, both Chl and LHCII accumulation were drastically reduced, although the LDS-solubilized total leaf protein level remained unaffected. However, on the basis of total chlorophyll present, the amount of stabilized LHCII was similar in both Cd-treated and non-treated samples. Additionally, the thylakoid-bound protease known to degrade LHCII, was found to be inhibited by Cd treatment both *in vivo* and *in vitro*. Finally, Northern hybridization analysis indicated that Cd affects LHCII accumulation by reducing drastically the steady-state level of *Lhcb* transcripts.

Introduction

Cd administration to plants during the greening process has been found to reduce drastically Chl accumulation (Stobart *et al.*, 1985; Padmaja *et al.*, 1990; Horvath *et al.*, 1996). This was considered to reflect the Cd involvement in the biosynthesis route of Chl (Stobart *et al.*, 1985; Padmaja *et al.*, 1990), but also in the process of Chl to protein integration/binding during formation of pigment-protein complexes (Horvath *et al.*, 1996). Steps like the light-dependent synthesis of δ -ALA and the formation of the ternary POR-NADPH-PChl(ide) complex (Stobart *et al.*, 1985), or the activity of ALA dehydratase (Padmaja *et al.*, 1990) have been reported to be inhibited by Cd. Similarly, Cd has been shown to inhibit oligomerization

of LHCII by inhibiting the synthesis of *trans*- Δ^3 -hexadecenoic acid (Krupa, 1988), which is needed for the process (Tremolieres *et al.*, 1981). Lately, studies of leaf low-temperature fluorescence emission suggested that Cd acts mainly by disturbing the integration of Chl into stable pigment-protein complexes of the photosynthetic membrane (Horvath *et al.*, 1996).

In the present study we tried to see whether and how LHCII appearance and stabilization during its biosynthesis at the early stages of greening might be affected by the administration of Cd to etiolated plants during the very early stages of their exposure to light. We worked with etiolated plants exposed to intermittent light-dark cycles, which are known to accumulate selectively Chl *a* and to stabilize LHCII in barely detectable levels (Argyroudi-Akoyunoglou and Akoyunoglou, 1970; Argyroudi-Akoyunoglou and Akoyunoglou, 1979; Tzinis *et al.*, 1987; Tziveleka and Argyroudi-Akoyunoglou, 1998); in these leaves we found that the immunodetectable level of LHCII is reduced, that this reduction follows the reduction in the level of Chl in a parallel way, and that the main effect of Cd administration on the biosynthesis/assembly of LHCII is exerted at the transcriptional

Abbreviations: δ -ALA, δ -aminolevulinic acid; Chl, chlorophyll; pCMB, p-chloromercuribenzoate; DEPC, diethylpyrocarbonate; DMSO, dimethylsulfoxide; ImL, intermittent light; LDS, lithium dodecylsulfate; LHCII, light-harvesting pigment-protein complex serving photosystem II; PChl, protochlorophyll; POR, protochlorophyllide oxidoreductase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Tricine, N-tris(hydroxymethyl)-methylglycine; Tris, tris(hydroxymethyl)-aminomethane; TX-100, Triton X-100.

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and not at the post/translational level (stabilization).

Materials and Methods

Phaseolus vulgaris plants (var. Red Kidney) were grown in the dark in a Conviron S10H growth chamber (22 °C, 80% humidity). Cotyledons were harvested from six-day-old etiolated leaves. One cotyledon was removed, and the leaves attached to the remaining cotyledon were placed on wet filter paper in covered petri dishes. Afterwards they were immersed for 1 min in 0.5, 1.0 or 2.0 mM $\text{CdCl}_2 \cdot \text{H}_2\text{O}$ 6 h before, just upon and again every 24 h after exposure to intermittent light (2 min white light every 98 min dark in cycles; $38 \mu\text{mol}/\text{m}^2 \cdot \text{sec}$). Control samples were immersed in distilled deionized water.

Chlorophylls and PChl(ide) were extracted in 80% acetone and determined spectrophotometrically according to Mackinney (1941) and Anderson and Boardman (1964), respectively. For total leaf protein extraction, leaves (0.2–0.5 g fresh weight) were harvested and kept in liquid nitrogen; they were solubilized in 50 mM Na_2CO_3 , 12% sucrose, 50 mM DTT and 2% LDS at a ratio of leaf to LDS buffer equal to 1/10 (w/v). The extracts were incubated in a boiling water bath for 10 min, and the solubilized total leaf proteins were recovered in the supernatant after a 10 min centrifugation at high speed in an Eppendorf centrifuge. Samples were kept at –20 °C. The protein content was determined by the method of Lowry *et al.* (1951). Analysis by SDS-PAGE, Western blotting and immunodetection of the LHCII apoprotein were as previously described (Laemmli, 1970; Towbin *et al.*, 1979; Blake *et al.*, 1984); 100 μg total LDS-solubilized protein were applied per slot. The immunostain was quantified densitometrically in a Scan Pack II densitometer (Biometra).

Thylakoids were isolated as described earlier (Argyroudi-Akoyunoglou *et al.*, 1982) and were washed twice with 50 mM Tricine, pH 7.3. For solubilization, thylakoids were incubated at 4 °C for 1 h under constant stirring with 1% TX-100 [TX-100/protein=1 (w/w)] (Anastassiou and Argyroudi-Akoyunoglou, 1995). The solubilized protein was recovered in the supernatant after centrifugation at $10,000 \times g \times 10 \text{ min}$.

Proteolytic activity against endogenous LHCII was assessed after Western blotting of assay mix-

tures prior to and after incubation at 37 °C. Assay mixtures contained sample protein at 1 $\mu\text{g}/\mu\text{l}$ in 40 mM Tris-HCl, pH 8.6. The reduction of the LHCII level was estimated following immunodetection on Western blots and densitometric evaluation of the area under the LHCII peak. Primary thylakoids were used without solubilization, while mature thylakoids were solubilized in TX-100 and the final TX-100 concentration in the assay was 0.2% (Tziveleka and Argyroudi-Akoyunoglou, 1998).

For the isolation of total RNA, 0.5 g fresh weight leaves were harvested and immediately frozen in liquid nitrogen. The leaves were ground to powder under liquid nitrogen with sand in a mortar. The powder was added to 5 ml extraction mixture immediately thereafter and the samples were homogenized by vigorous shaking on a vortex (10 times, 30 sec bursts). The extraction mixture contained 2 ml phenol – 3 ml buffer (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 50 mM EGTA; 4.8% p-aminosalicylic acid, Na-salt and 0.8% triisopropyl-naphthalene sulphonic acid, Na-salt) (Sofianopoulou and Scazzocchio, 1989). 2 ml CHCl_3 : isoamyl alcohol (24:1 v/v) were added and after phase separation, the RNA was precipitated from the supernatant by addition of LiCl at 2 M final concentration. The RNA was washed at room temperature with 3 M Na-acetate, pH 5.2, then with cold 70% ethanol, and dissolved in DEPC-treated water [0.1% (v/v)]. The concentration was determined photometrically and the RNA was then denatured in glyoxal/DMSO and separated by 1.2% agarose electrophoresis. For Northern hybridization at 39 °C in 50% formamide, *Lhcb1* cDNA from pea (690 bp fragment) was labeled with [α - ^{32}P]dCTP by random primer reaction (Sambrook *et al.*, 1989). For densitometric quantitation of mRNA levels, the autoradiograms were scanned as above. As a loading control of total RNA applied on slots the 16S ribosomal RNA was used, after staining of the transfer membrane with methylene blue.

Results and Discussion

Figure 1 and Table I show the effect of Cd administration on the Chl a cumulation in young primary leaves of *Phaseolus vulgaris* during their exposure to intermittent light-dark cycles. These plants, in accordance to earlier findings, accumu-

Table I. The effect of Cd treatment on leaf Chl *a*, Chl *b* and PChl, and on the ratio of immunodetectable LHCII level to that of the total Chl / g F W present in etiolated bean leaves exposed to 28 light-dark cycles. Values represent the mean \pm SE ($n=3$).

Cd [mM]	Chl <i>a</i> [μ g/g F W]	Chl <i>b</i> [μ g/g F W]	Chl <i>a/b</i>	PChl [μ g/g F W]	LHCII*·(Chl/g F W) ⁻¹
0.0	228.1 \pm 24.7	5.5 \pm 0.6	41.5	11.8	5.8
0.5	156.0 \pm 17.6	5.8 \pm 0.4	26.9	12.3	5.8
1.0	107.5 \pm 9.4	6.1 \pm 0.4	17.6	8.1	6.3
2.0	85.7 \pm 2.3	6.2 \pm 0.2	13.8	10.2	–

6-day-old etiolated bean leaves attached to one cotyledon were immersed in water (control) or Cd solutions for one min, once 6 h prior to exposure to intermittent light, once just before exposure to intermittent light and once 24 h after exposure to intermittent light. Sampling of leaves was immediately after the last flash of light.

* Immunodetectable LHCII; arbitrary units of the area under the LHCII peak obtained in densitometric scans of immunoblots. For immunodetection 100 μ g total LDS-solubilized protein was applied per slot.

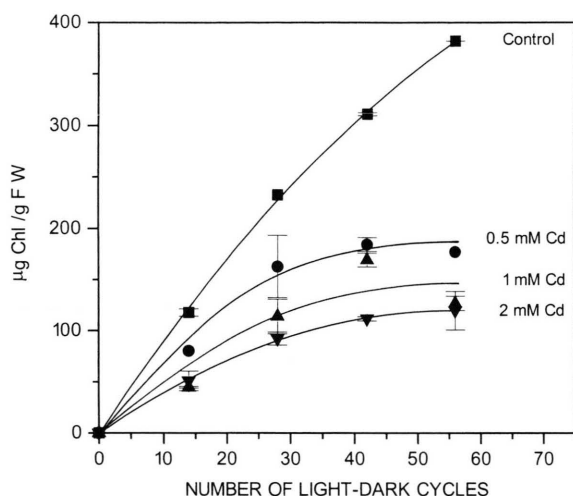


Fig. 1. The effect of Cd administration on the capacity of etiolated bean leaves to accumulate Chl in intermittent light-dark cycles. Values represent the mean \pm SD ($n=3$).

late selectively Chl *a* (Argyroudi-Akoyunoglou and Akoyunoglou, 1970), exhibiting high Chl *a/b* ratio (see Table I). Cd administration reduced mainly the level of accumulated Chl *a*, but the amount of Chl *b* / g F W remained more or less unaffected. In these experiments sampling of leaves was immediately after the last flash of light. The amount of Chl accumulated, therefore, shows in essence the total amount of PChl(ide) regenerated during the dark phase of the light-dark cycles, in addition to that phototransformed during the light phase of the cycles. This amount, as suggested from Fig. 1 and Table I, is drastically reduced by Cd treatment. This may reflect an effect of Cd on PChl(ide) accumulation, regeneration and/or pho-

totransformation. The amount of PChl(ide) in these leaves, shows the level of the non-photo-transformable PChl(ide), since sampling was immediately after the last flash. The data in Table I suggests that PChl(ide) phototransformation in intermittent light plants is reduced by Cd treatment; as deduced from the values listed, the ratio of the remaining PChl(ide) following the flash to the Chl present in the respective sample is about two fold greater in Cd-treated leaves than in controls.

Figure 2 compares the level of accumulated Chl during exposure of control or Cd-treated etiolated leaves to 28 light-dark cycles with the level of the LDS-solubilized total leaf protein and of the immunodetectable LHCII present in these extracts. In contrast to the drastic effect of Cd on Chl accumulation, no effect was found on the level of LDS-

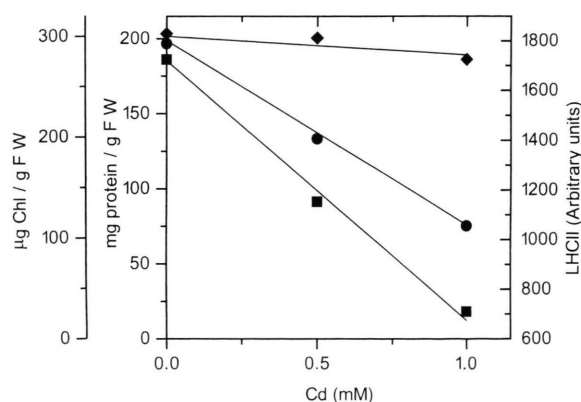


Fig. 2. The effect of Cd on the amount of LDS-solubilized total leaf protein (◆), the level of immunodetectable LHCII (●), and the accumulation of Chl (■) in 6-day-old etiolated bean leaves exposed to intermittent light for 28 light-dark cycles.

solubilized total leaf protein. However, the level of the immunodetectable LHCII is reduced in Cd-treated leaves; this reduction follows more or less the reduction observed in Chl accumulation. Similar results were obtained with etiolated leaves exposed to 14, 42 or 56 light-dark cycles. It should be noted here that in ImL plants, despite the presence of *Lhcb* mRNA (Viro and Kloppstech, 1982) only minute amounts of LHCII can be accumulated, which can only be observed by immunodetection (not by Coomassie staining, nor after non-denaturing SDS-PAGE as a Chl-protein complex) (Argyroudi-Akoyunoglou and Akoyunoglou, 1979); this probably reflects the insufficient Chl binding on the LHCII apoprotein, due to the limited amount of Chl present in these plants (about 1/7th of that in etiolated leaves of the same age exposed to continuous light, Argyroudi-Akoyunoglou and Akoyunoglou, 1970). As known, Chl binding on the LHCII apoprotein and formation of the pigment-protein complex is the mechanism which rescues the protein from proteolytic attack (Tzinis *et al.*, 1987; Tziveleka and Argyroudi-Akoyunoglou, 1998). In Table I comparison is made of the ratio in the level of the immunodetectable LHCII (present in 100 μ g LDS-solubilized total leaf protein) to that of the Chl present in the leaves (per g F W); as shown, this ratio in Cd-treated leaves is almost equal to that found in

control leaves. Thus, the reduction in LHCII level follows closely the reduction in Chl level, not allowing us to conclude whether this reduction arises from insufficient Chl or LHCII availability in Cd-treated leaves.

The question therefore arose whether the reduced LHCII level is due to increased proteolytic activity or to reduced gene expression. To see whether the Cd-induced reduction in LHCII level may arise from activation of the thylakoid-bound protease specific for LHCII, we tried to assess the level of protease activity in the primary thylakoids obtained from ImL plant leaves, as well as *in vitro* in TX-100-solubilized mature thylakoid protein. Figure 3A shows the effect of Cd pretreatment on the proteolytic activity of primary thylakoids against their endogenous LHCII. Cd administration resulted in the reduction of the proteolytic activity. In this figure representative results obtained from etiolated plants exposed to 28 light-dark cycles are shown. Similar results were obtained with primary thylakoids isolated from leaves exposed to 42 or 56 light-dark cycles. Similarly, in *in vitro* assays of the proteolytic activity of TX-100-solubilized thylakoids, where Cd was added in the assay mixtures, a drastic inhibitory effect of Cd was observed. Figure 3B shows results obtained with thylakoids isolated from 6-day-old etiolated plants exposed to continuous light for 4

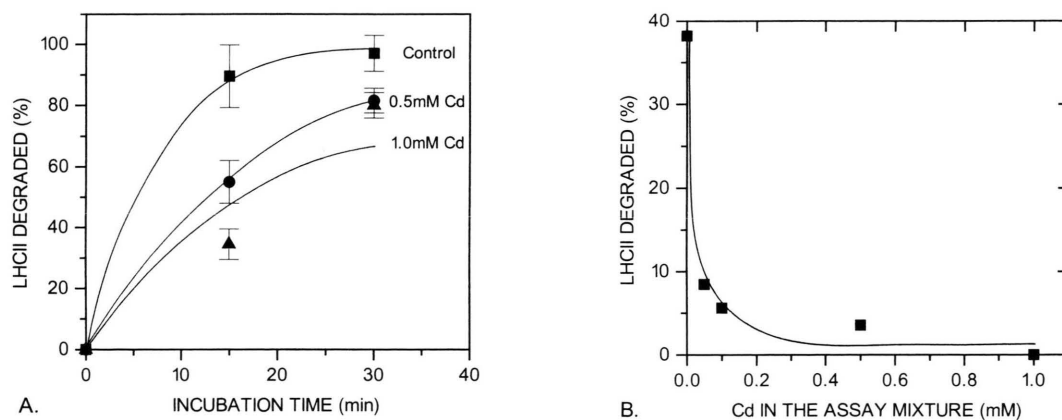


Fig. 3. The thylakoid-bound proteolytic activity as affected by Cd administration *in vivo* and *in vitro*. (A): 6-day-old etiolated bean plants with or without Cd treatment were exposed to 28 light-dark cycles. Primary thylakoids were isolated and their proteolytic activity was assessed against their endogenous LHCII, by immunodetection of the LHCII remaining after incubation at 37 °C. 20 μ g primary thylakoid protein were applied per slot. Values represent the mean \pm SD ($n=3$). (B) TX-100 solubilized thylakoids obtained from 6-day-old etiolated plants exposed to continuous light for 4 days were used for the proteolytic assay. After addition of Cd, the assay mixtures were incubated at 37 °C for 60 min. The degradation of LHCII was assessed by the reduction of immunostain on the immunoblots. 2 μ g thylakoid protein were applied per slot.

days. In this case, the proteolysis at 0.05 mM Cd is inhibited by about 75%. These results, therefore, clearly suggest that the reduction induced by Cd treatment in LHCII level cannot be accounted for by the activation of the thylakoid bound protease against LHCII. In addition, the inhibition of the proteolytic activity by Cd further suggests that the protease involved is an SH-enzyme, as also earlier suggested by the inhibition exerted on the proteolytic activity by p-CMB (Anastassiou and Argyroudi-Akoyunoglou, 1995).

We then tried to assess whether the reduction in LHCII level results from altered gene expression, caused by Cd. Northern blot analyses performed with total RNA samples isolated from control and Cd-treated intermittent light leaves demonstrated that *Lhcb* transcription is negatively affected by Cd. Figure 4 shows results obtained from autoradiograms after hybridization with *Lhcb1* cDNA. No transcripts were detectable in leaves of the 6-day-old etiolated plants kept in the dark for two additional days (Fig. 4A, DARK). In contrast, the leaves of 6-day-old etiolated plants exposed for two additional days to intermittent light (28 cycles (Fig. 4A, CONTROL) showed a clearly detectable

Lhcb transcript level (albeit 35% of that observed in a green control exposed to continuous light for 2 days, i.e. at equal developmental stage, GREEN). The RNA in Cd-treated leaves, however, was barely detectable after hybridization with the *Lhcb* cDNA. It should be noted that the control leaves were immersed in water just as the Cd-treated leaves were immersed in Cd solutions. Figure 4B shows in addition the effect of Cd treatment on *Lhcb* transcript level in plants exposed to a series of light-dark cycles. Here also the Cd-induced reduction in the steady-state level of transcripts is obvious, which becomes more severe upon repeated Cd administration. It should be noted that Cd was administered every 14 light-dark cycles (i.e. every 24 h).

The latter results taken together with those on Chl accumulation suggest that Cd affects in a parallel way the transcription of *Lhcb* and the accumulation of Chl. In addition, our data suggest that the reduction in LHCII level in Cd-treated leaves is most probably due to reduced *Lhcb* expression rather than to increased proteolysis of the LHCII apoprotein.

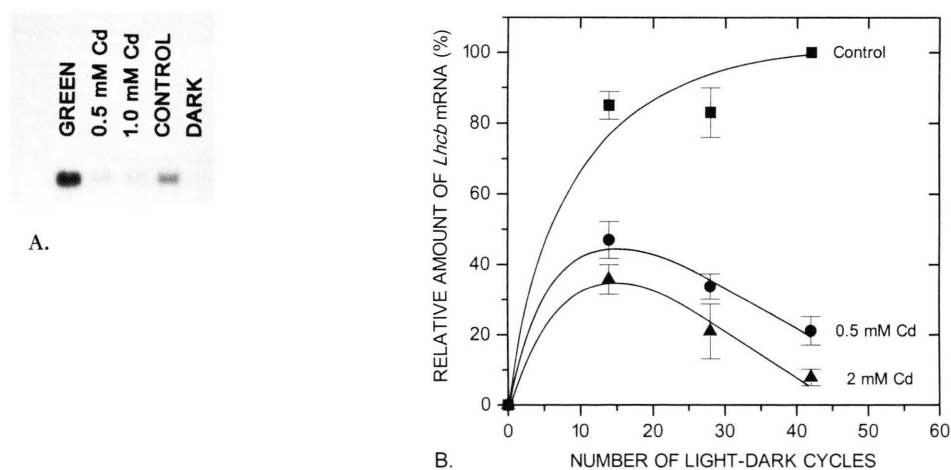


Fig. 4. The accumulation of *Lhcb* mRNA in intermittent light leaves as affected by Cd treatment. 6-day-old etiolated bean leaves attached to one cotyledon were immersed in Cd prior to and after exposure to intermittent light (see Materials and methods). Total leaf RNA was hybridized to *Lhcb1* cDNA. (A): Autoradiogram of a hybridized membrane obtained with 10 μ g total RNA isolated from leaves of 6-day-old etiolated plants either exposed to 28 light-dark cycles (Control, Cd-treated) or to 48 h continuous light (green) or kept in the dark for 48 h (Dark). (B): Quantitation of the data in autoradiograms of hybridized membranes blotted with 20 or 40 μ g total RNA from Cd-treated or control leaves exposed to 14, 28 and 42 light-dark cycles. Quantitation is based on the area under the *Lhcb* peak as obtained by scanning of the autoradiogram spots. Values represent the mean \pm SD ($n=4$) and are plotted as percentage of a control sample obtained from leaves exposed to 42 light-dark cycles.

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