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CALCIUM-INDUCED FORMATION OF CHLOROPHYLL *b* AND LIGHT-HARVESTING CHLOROPHYLL *a*/*b*-PROTEIN COMPLEX IN CUCUMBER COTYLEDONS IN THE DARK

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Dark-grown cucumber seedlings were exposed to intermittent light (2 min light and 98 min dark) and then cotyledons were incubated with 50 mM $CaCl_2$ in the dark. Chlorophyll (Chl) *a* was selectively accumulated under intermittent light and Chl *b* was accumulated during the subsequent dark incubation with $CaCl_2$. The change in chlorophyll-protein complexes during Chl *b* accumulation induced by $CaCl_2$ in the dark was investigated by SDS-polyacrylamide gel electrophoresis. Chlorophyll-protein complex I and free chlorophyll were major chlorophyll-containing bands of the cotyledons intermittently illuminated 10 times. When these cotyledons were incubated with $CaCl_2$ in the dark, the light-harvesting Chl *a*/*b*-protein complex was formed. When the number of intermittent illumination periods was extended to 55, small amounts of Chl *b* and light-harvesting Chl *a*/*b*-protein complex were recognized at the end of intermittent light treatment, and these two pigments were further increased during the subsequent incubation of the cotyledons with $CaCl_2$ in the dark compared to water controls.

Introduction

Chlorophyll accumulation is regulated by many factors such as light [1], temperature [2], hormones [3] and age of the tissues [4]. Chl *b* is synthesized from Chl *a* under continuous light and is eventually found in chlorophyll-protein complexes, such as LHCP [5] and another Chl *a*/*b*-containing protein, CP 29 [6]. The conversion of Chl *a* to Chl *b*, as well as the synthesis of Chl *a*, does not occur in the dark [7], and under intermittent light conditions only Chl *a* is synthesized [8].

Recently, much information about chlorophyll-protein complexes has been obtained using SDS-polyacrylamide gel electrophoresis; there are many reports about the relationship between chlorophyll accumulation and the formation of chlorophyll-protein complexes. When etiolated tissues are exposed to intermittent light Chl *a* is selectively accumulated and LHCP is absent [9]. When such tissues are transferred to continuous light, Chl *b* and LHCP begin to appear [9]. The Chl *b*-less chlorina mutant of barley also lacks LHCP [10]. Genge et al. [11] investigated the chlorophyll-protein complexes of plant tissues having varying Chl *a*/*b* ratios by changing illumination conditions for different species. The amount of pigment-protein complex II was correlated with the Chl *b* content. However, gymnosperms which can synthesize Chl *a* and *b* in the dark can also form LHCP as well as CP I in the dark [12]. These observations indicate that Chl *b* is necessary for the formation of LHCP and in angiosperm LHCP cannot be formed in the dark because of the absence of Chl *b*.

Abbreviations: Chl, chlorophyll; LHCP, light-harvesting Chl *a*/*b*-protein complex; CP, chlorophyll-protein complex.

We have reported that in the early phase of greening of cucumber cotyledons, there exists unstable chlorophyll [13], and that when greening cotyledons exposed to short periods of illumination (4 h) or intermittent light (10 periods) are subsequently incubated with Ca^{2+} in the dark, Chl *b* accumulates [14]. In the present paper, we report that LHCP was formed in intermittent light-treated cotyledons during the subsequent dark incubation with Ca^{2+} without any net synthesis of chlorophyll.

Materials and Methods

Plant materials. Cucumber seeds (*Cucumis sativus* L. cv. Aonagajibai) were soaked in distilled water and then germinated on moist vermiculite in the dark at 28°C. After 4 days of germination, seedlings were illuminated with continuous or intermittent (2 min light followed by 98 min dark) white light at an intensity of 5000 lx at 28°C. Cotyledons were excised without hypocotyl hook and groups of 20 cotyledons were incubated on a 5.5 cm filter paper (Toyo No. 2) wetted with 1.6 ml of water or 50 mM $CaCl_2$ in the dark at 28°C as described previously [14]. Cotyledons were sampled at various times and analyzed for Chl *a* and *b*. For the study with SDS-polyacrylamide gel electrophoresis, after 24 h of dark incubation with water or 50 mM $CaCl_2$, about 1400 cotyledons were washed with distilled water and the chloroplasts were isolated immediately.

Chlorophyll determination. Chlorophyll was determined by the hydroxylamine method [15] as described previously [14]. Chlorophyll content was expressed as $\mu g/g$ fresh weight.

Chloroplast isolation. Washed cotyledons were homogenized for 6 s at high speed in a blender in a grinding medium containing 0.5 M sucrose, 0.05 M Na^+-Tricine (pH 8.0) and 5 mM EDTA. The homogenate was passed through a layer of miracloth and centrifuged for 15 min at $2000 \times g$. The pellet was washed with 5 mM EDTA (pH 8.0) and centrifuged for 15 min at $2000 \times g$ (washed membranes).

SDS-polyacrylamide gel electrophoresis. Washed membranes of chloroplasts from fully greened cotyledons (3 days illumination) were dissolved in 0.3 M Tris-HCl (pH 8.8), 10% glycerol and 1% SDS at an SDS/chlorophyll weight ratio of 10–20. Washed membranes of chloroplasts from cotyledons illuminated with intermittent light or 4 h of continuous light were dissolved in 0.05 M Tris-HCl (pH 8.8) and 1% SDS and centrifuged at $20000 \times g$ for 30 min to remove insoluble matter. The green supernatant was made up to 0.3 M Tris-HCl (pH 8.8), 10% glycerol and 1% SDS at an SDS/chlorophyll weight ratio of 200 (4 h of continuous illumination and 10 periods of intermittent light) or 40 (55 periods of intermittent light). Electrophoresis was done in a cold room according to the method of Anderson et al. [16] as modified by Akoyunoglou [17]. After electrophoresis the gel was scanned at 675 nm with a gel scanner. The absorption spectrum of the pigments banding on the gel was directly measured with incident light through a slit permitting a spectral bandwidth of 10 nm in a Hitachi 556 spectrophotometer at room temperature.

Results

When the etiolated seedlings were exposed to 10 periods of intermittent light Chl *a* was selectively accumulated (Table I, Fig. 1). During subsequent dark incubation of these cotyledons with water, Chl *a* decreased slightly and Chl *b* did not appear. However, when the samples were treated with 50 mM $CaCl_2$ in the dark, Chl *a* began to decrease and Chl *b* accumulated; the Chl *a*/*b* ratio was 7 after 24 h incubation (Table I, Fig. 1). The accumulation of Chl *b* during the incubation with $CaCl_2$ stopped within 24 h; no further accumulation of Chl *b* was observed when the incubation period was prolonged. These results suggest that a part of Chl *a* was converted to Chl *b* in the dark in the presence of $CaCl_2$. The decrease in Chl *a* was larger than the increase in Chl *b* during the dark incubation with $CaCl_2$ after 10 periods of intermittent light, resulting in a net loss of total chlorophyll (Table I).

When thylakoid membranes from cotyledons illuminated for 3 days (fully greened) were examined by SDS-polyacrylamide gel electrophoresis, seven chlorophyll-containing bands were resolved, with the chlorophyll-protein complex Ia (CP Ia) appearing as a shoulder of the chlorophyll-protein complex I (CP I) band under

TABLE I

CHLOROPHYLL CONTENT BEFORE OR AFTER DARK INCUBATION OF THE ILLUMINATED COTYLEDONS

Etiolated cucumber seedlings were exposed to continuous light or intermittent light (2 min light and 98 min dark). Subsequently, cotyledons were excised and incubated with water or $CaCl_2$ for 24 h in the dark. The chlorophyll content was measured after illumination (initial) or dark incubation. Each value represents the mean of four samples ± S.D.

Light regime	Dark incubation	Chlorophyll (μg/g fresh wt.)		Chl *a*/*b*
		Chl *a*	Chl *b*	
4 h illumination	Initial	79.6 ± 3.9	23.6 ± 1.3	3.4
72 h illumination	Initial	1505 ± 73	403 ± 26	3.7
10 periods of intermittent light	Initial	56.6 ± 3.5	0	–
	Water	50.5 ± 3.5	0	–
	50 mM $CaCl_2$	40.5 ± 3.1	5.7 ± 1.4	7.1
55 periods of intermittent light	Initial	334 ± 13	20 ± 3	17
	Water	346 ± 2	22 ± 1	16
	50 mM $CaCl_2$	338 ± 5	30 ± 3	11

these conditions (Fig. 2A). When the SDS/chlorophyll ratio was lowred, CP Ia became a more prominent peak with a concomitant decrease in the CP I band.

4 h after the onset of continuous illumination (early greening), the lag phase of chlorophyll formation was over and chlorophyll was being actively synthesized. Now, only three chlorophyll-containing bands, CP I, LHCP and free chlorophyll were prominent with several minor bands recognizable (Fig. 2B). The relative amount of the free chlorophyll band in the electrophoretogram of pigment complexes from cotyledons in the early phase of greening was larger than that from fully greened cotyledons.

Changes in the chlorophyll-protein complexes accompanied by Chl *b* formation induced by $CaCl_2$ in the dark were investigated by SDS-polyacrylamide gel electrophoresis. Fig. 3A shows the electrophoretic pattern of chlorophyll-protein complexes from cotyledons exposed to 10 periods

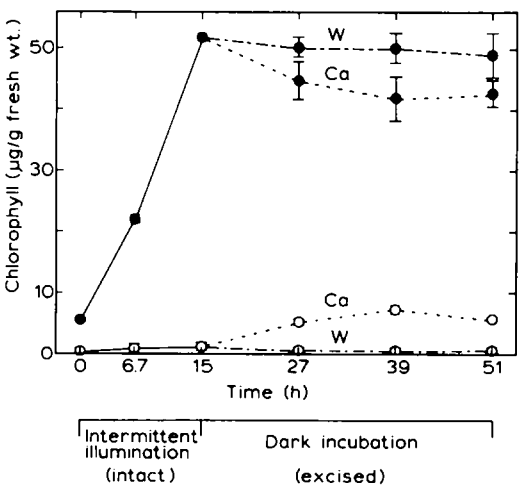

Fig. 1. Time course of chlorophyll accumulation during intermittent light and dark incubation. Cucumber seedlings were exposed to 10 periods of intermittent light (————). Excised cotyledons were incubated in the dark with water (— · —) (W) or 50 mM $CaCl_2$ (- - - - -) (Ca). (●) Chl *a*, (○) Chl *b*. Each plot represents the mean of four samples. Vertical bars indicate the standard error of the mean.

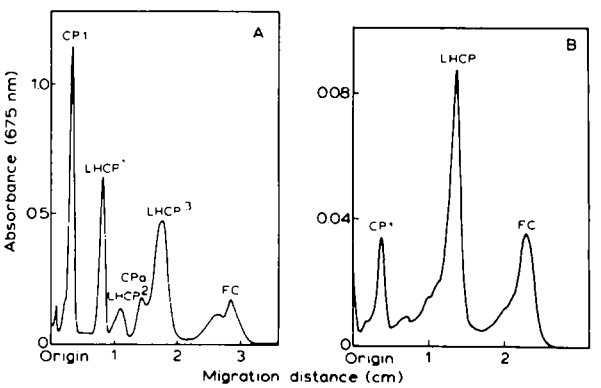

Fig. 2. Densitometer tracing (675 nm) of chlorophyll-protein complexes isolated by SDS-polyacrylamide gel electrophoresis of chloroplast thylakoids from cotyledons of 3 day (A) and 4 h (B) illuminated seedlings. FC, free chlorophyll.

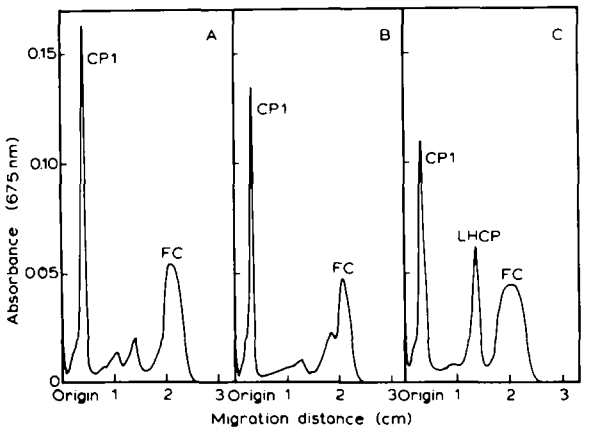

Fig. 3. Densitometer tracing (675 nm) of chlorophyll-protein complexes isolated by SDS-polyacrylamide gel electrophoresis of chloroplast thylakoids from cotyledons exposed to 10 periods of intermittent light. (A) Immediately after the end of illumination, (B) after 24 h dark incubation with water following intermittent light, (C) after 24 h dark incubation with 50 mM $CaCl_2$ following intermittent light. FC, free chlorophyll.

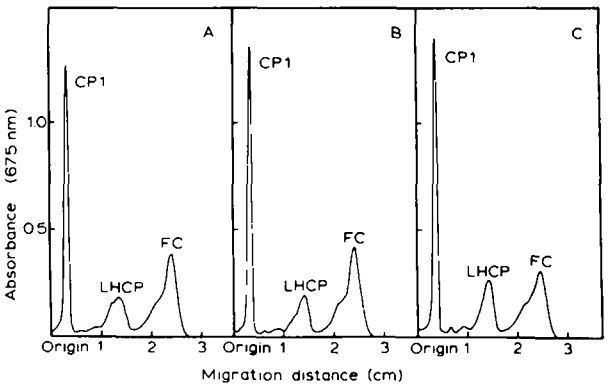

Fig. 4. Densitometer tracing (675 nm) of chlorophyll-protein complexes isolated by SDS-polyacrylamide gel electrophoresis of chloroplast thylakoids from cotyledons exposed to 55 periods of intermittent light. A–C are the same as in Fig. 4 except for the number of intermittent light periods.

of intermittent light. There are two major bands, CP I and free chlorophyll, and two minor bands, one of which was located at the position of LHCP; no Chl *b* accumulated under these conditions (Table I, Fig. 1). When the cotyledons which had been exposed to 10 periods of intermittent light were incubated with water, the main bands of CP I and free chlorophyll remained unchanged (Fig. 3B). However, a remarkable change of chlorophyll-protein complexes was observed when the cotyledons were incubated with $CaCl_2$ after intermittent light; a large amount of LHCP was formed concomitant with Chl *b* accumulation (Figs. 1, 3C, Table I). Chl *b* which accumulated in the dark by $CaCl_2$ treatment could be incorporated into LHCP. This indicates that if Chl *b* was synthesized, LHCP was formed even in darkness. In cotyledons treated with 10 periods of intermittent light as well as in samples illuminated for 4 h, the free chlorophyll band was large compared to that in fully greened cotyledons.

When the cotyledons were exposed to 55 periods of intermittent light, a small amount of Chl *b* accumulated; the Chl *a*/*b* ratio was 17. The CP I, CPa, LHCP and free chlorophyll bands were observed in chloroplasts from these cotyledons (Fig. 4A). When these cotyledons were incubated with water in the dark, no noticeable changes were observed in either the amount of chlorophyll, Chl *a*/*b* ratio (Table I), or the pattern of chlorophyll-protein complexes (Fig. 4B). However, when these

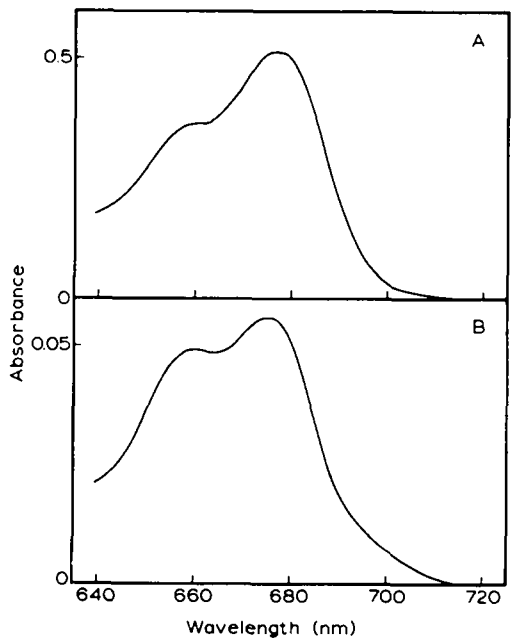

Fig. 5. Absorption spectra of LHCP isolated by SDS-polyacrylamide gel electrophoresis. (A) LHCP from cotyledons of 3 day illuminated seedlings (same sample as for Fig. 2A), (B) LHCP from cotyledons incubated with 50 mM $CaCl_2$ in the dark after 10 periods of intermittent light (same sample as for Fig. 3C).

cotyledons were incubated with $CaCl_2$, Chl *b* accumulation was induced in the dark and the Chl *a/b* ratio was 11 (Table I). Among the chlorophyll-protein complexes, LHCP was increased by $CaCl_2$ treatment (Fig. 4C). However, the changes in the Chl *a/b* ratio and LHCP content for cotyledons treated with 55 periods of intermittent light were not as drastic as those for 10 periods of intermittent light. These results indicate that there was a distinct relationship between Chl *b* accumulation and LHCP formation in the dark.

The absorbance spectrum of LCHP of fully greened cotyledons and cotyledons treated with $CaCl_2$ in the dark after 10 periods of intermittent light were compared. The existence of Chl *b* was evident from the peak at about 655 nm in the latter, as well as in the former (Fig. 5).

Discussion

The formation of LHCP is regulated by light at different levels. mRNA for the apoprotein of LHCP was induced by light [18–20] and the turnover of the apoprotein was regulated by light [21,22]. Synthesis of Chl *b* in angiosperms requires continuous illumination [1], and Chl *b* and LHCP are not formed under intermittent light [9]. However, gymnosperms which can synthesize Chl *b* in the dark can also form LHCP in the dark. Lewandowska and Öquist [12] reported that dark-grown seedlings of pine could accumulate Chl *a* and *b* and form LHCP in the dark, though the Chl *b/a* ratio and the relative amount of LHCP of dark-grown seedlings were lower than those of seedlings illuminated for longer periods. The Chl *b*-less barely mutant lacks LHCP [10]. These observations indicate the close relationship between Chl *b* accumulation and LHCP formation. Why can angiosperms not form LHCP in the dark? There may be two possible explanations: (i) Owing to the lack of synthesis of Chl *b*, one of the major pigments of LHCP, this chlorophyll-protein complex cannot be formed in the dark or under intermittent light. (ii) Because the formation of LHCP itself needs continuous illumination, LHCP cannot be formed in the dark even if Chl *b* is synthesized. Recently, we reported that Chl *b* synthesis was induced by $CaCl_2$ in the dark. Our present results show that LHCP can be formed in complete darkness if Chl *b* is synthesized; thus, LHCP formation does not necessarily need continuous illumination.

As for the action of $CaCl_2$, there remain two possibilities that $CaCl_2$ induces only Chl *b* which then binds to preexisting LHCP apoprotein, or that $CaCl_2$ induces both Chl *b* and LHCP apoprotein formation if both are absent in intermittent light-treated cotyledons. However, Akoyunoglou et al. [23] reported that the protein moiety of LHCP preexisted in etioplasts of bean leaves and that under intermittent light the Complex II-to-Complex I protein ratio did not change considerably but Chl *a* selectively bound to Complex I protein.

We confirmed earlier results [23] that most of the Chl *a* formed in intermittent light was bound to CP I; however the amounts of free chlorophyll are very high in the early stages of greening (Refs. 12 and 23 and Figs. 2–4), making it difficult to estimate exactly how much of the Chl *a* is bound in vivo because the origin of the free chlorophyll is not well understood at present.

We demonstrated that when the cotyledons which had been exposed to 10 periods of intermittent light were incubated with $CaCl_2$ in the dark, Chl *b* and LHCP were induced. Under these conditions, there may be four kinds of Chl *a* accumulated under intermittent light: (i) Chl *a* which will be converted to Chl *b*; (ii) Chl *a* which will be incorporated into LHCP; (iii) Chl *a* which will be incorporated into CP I; and (iv) Chl *a* which is bound to CP I. When the number of periods of intermittent light was prolonged from 10 to 55, the relative amount of Chl *b* and LHCP newly induced by $CaCl_2$ in the dark decreased (Table I and Figs. 3 and 4). This may indicate that a large part of Chl *a* was organized under an extended time of intermittent treatment and the amount of type-i and -ii Chl *a* decreased. However, the possibility cannot be excluded that type-iv Chl *a* bound to CP I might be incorporated into LHCP or converted into Chl *b* and that Chl *a* which will change as types i–iii might be part of the same Chl *a* population.

There have been several reports about how and where Chl *a* is converted to Chl *b*. Shlyk [24] mentioned that Chl *a* and *b* were synthesized in a multi-enzyme system of a biosynthetic center and

termed newly synthesized chlorophyll, 'young chlorophyll', which is easily converted to Chl *b*. We reported that in the early phase of greening there exists unstable chlorophyll which is converted to Chl *b* in the dark by $CaCl_2$ treatment [14]. Argyroudi-Akoyunoglou and Castorinis [17] showed, in their experiments tracing labeled chlorophyll during intermittent light and subsequent continuous illumination, that Chl *a* once bound to apoproteins of chlorophyll-protein complexes under intermittent light was not exchanged freely for Chl *a* or *b* which was synthesized after tansfer to continuous illumination, and that the latter chlorophylls bound primarily to LHCP. As LHCP is a final acceptor of Chl *b*, the study of LHCP formation is essential for an understanding of chlorophyll metabolism and its regulation. Recently, Fradkin et al. [25] using digitonin-polyacrylamide gel electrophoresis have demonstrated the presence of a submembrane fraction enriched in Chl *b* but without photochemical activity, which they considered the most intensely developing area in the metabolically heterogeneous chloroplast membrane system. We succeeded in inducing Chl *b* formation under the condition where new chlorophyll is not synthesized. We showed that induced Chl *b* was finally incorporated into LHCP but could not yet clarify the origin of the Chl *b*, namely, which Chl *a* was converted to Chl *b*, because of the difficulty that there was a large amount of free chlorophyll that might be released from some protein during solubilization or electrophoresis.

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