Diurnal Fluctuations in the Content and Functional Properties of the Light Harvesting Chlorophyll a/b Complex in Thylakoid Membranes

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Diurnal Fluctuations in the Content and Functional Properties of the Light Harvesting Chlorophyll *a/b* Complex in Thylakoid Membranes[1]

Correlation with the Diurnal Rhythm of the mRNA Level

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ABSTRACT

Diurnal fluctuations were observed in the content and some structural and functional properties of the light-harvesting chlorophyll (Chl) *a/b* pigment-protein complex of photosystem II (LHCII) in young developing wheat (*Triticum aestivum*) leaves grown under 16 hours light/8 hours dark illumination regime. The fluctuations could be correlated with the diurnal oscillation in the level of mRNA for LHCII. The most pronounced changes occurred in the basal segments of the leaves. They were weaker or hardly discernible in the middle and tip segments. As judged from the diurnal variations of the Chl-*a*/Chl-*b* molar ratio, the LHCII content of the thylakoid membranes peaked around 2 pm. This can be accounted for by the cumulative effect of the elevated level of mRNA in the morning and early afternoon. In the basal segment, the extent of the fluctuation in the LHCII content was approximately 25%, as determined from gel electrophoresis ("green gels"). The amplitude of the principal bands of the circular dichroism (CD) spectra of isolated chloroplasts paralleled the changes in the LHCII content. Our circular dichroism data suggest that the newly synthesized LHCII complexes are incorporated into the existing helically organized macrodomains of the pigment-protein complexes or themselves form such macrodomains in the thylakoid membranes. Chl-*a* fluorescence induction kinetics also showed diurnal variations especially in the basal segments of the leaves. This most likely indicates fluctuations in the ability of membranes to undergo "state transitions." These observations suggest a physiological role of diurnal rhythm of mRNA for LHCII in young developing leaves.

The light-harvesting Chl *a/b* pigment-protein complex of PSII (LHCII[3]) is the predominant integral protein complex of the thylakoid membranes in green algae and higher plants. It makes up about half of the total protein and pigment content of the thylakoid membranes and serves as a light-harvesting molecular antenna (32). LHCII is of paramount importance in maintaining the characteristic granal stacking of thylakoids. As indicated by recent macroscopic and microscopic CD and circular differential scattering measurements LHCII is assembled into large helically organized elastic macroarrays in the granum (8, 9, 11, 14).

LHCII plays an important role both in short- and long-term adaptational processes (1). Short-term regulation, such as "state transition" (7), involves protein kinases and phosphatases which catalyze the phosphorylation and dephosphorylation of the LHCII which in turn affect the mobility of antenna complexes and their association with PSI and PSII (4, 5). Long-term acclimation processes, *e.g.* sun and shade adaptation, are governed by light-dependent regulation of the gene expression (1).

LHCII is encoded by nuclear gene families (32). Regulation of LHCII gene expression by light has been studied extensively (17, 10, 27, 29). There are reported diurnal and circadian fluctuations of the LHCII mRNAs for several plant species such as petunia (28) tomato (26), tobacco (24), and wheat (22). It has been shown that mRNA for LHCII in wheat is predominantly accumulated in the cells in the basal part of the leaf (18).

The precursor forms of LHCII are synthesized on cytoplasmic ribosomes. This step is followed by transport into the chloroplast, processing of the polypeptide, pigment synthesis and incorporation of the pigment-protein complex into the thylakoid membrane. The regulatory steps involved in these processes are largely unknown.

Recent results obtained in maize mesophyll and bundle sheath cells showed that the deficiency of the bundle sheath chloroplasts in LHCII is mainly due to differences in the active transport systems of the two types of chloroplasts. Level of mRNA was high in both types of cells and could not be correlated with the gross differences in the LHCII content (30). Limiting factor can also be the Chl biosynthesis. During greening, the LHCII protein levels in thylakoid membranes

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[3] Abbreviations: LHCII, light-harvesting Chl *a/b* pigment-protein complex of PSII; CD, circular dichroism; DDM, *n*-dodecyl-β-D-maltoside.

were not related to the level of mRNA for LHCII, but were correlated with the Chl content (20). On the other hand, studies in developing leaves suggested that the LHCII mRNA synthesis is apparently adapted to the needs of the leaves (21). These examples illustrate that the physiological importance of the factors regulating at the level of mRNA for LHCII cannot be assessed without investigating the incorporation and the functional properties of LHCII in the thylakoid membranes.

In the present study we show correlation between the diurnal cycle of the level of mRNA in the cytosol and the accumulation of LHCII in the thylakoid membranes. Macroorganization of LHCII and the ability of membranes to undergo state 1-state 2 transition also showed large fluctuations which could be correlated with the oscillation of the mRNA level for LHCII.

MATERIALS AND METHODS

Plant Material

Wheat seedlings (*Triticum aestivum,* cv GK Sågvåri) were grown in a phytotron under the diurnal regime of 16 h light (from 6 AM till 10 PM) and 8 h dark (from 10 PM till 6 AM). The continuous white light of 1000 μmol/m^{2}s^{-1} was provided by fluorescent lamps. Leaves were cut above the mesocotyl intersection. The coleoptide and the inner second leaf were removed. Because wheat leaves at the early stages of development have a basal meristem which produces a gradient of cellular and plastid development from the base to the tip of the leaf (6), the leaves were sectioned into three parts: 1.5–1.5 cm long segments from the base and the tip and the residual middle section of about 2 to 4 cm. These segments were investigated separately in all measurements.

Chloroplasts were isolated from the base, middle, and tip segments following the procedure of Webber *et al.* (31). For all experiments the suspension and assay medium consisted of 50 mM Hepes, pH 7.6, 10 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose.

Isolation and Analysis of RNA

RNA was isolated from leaf segments using aurin tricarboxylate as an RNAase inhibitor and the analysis of the Cab-1 RNA level was performed by 5′ nuclease S_1 protection assays according to Nagy *et al.* (22).

Pigment Content

Pigments were extracted from the leaf segments with 80% acetone. Total Chl content per fresh weight and Chl-*a*/Chl-*b* ratio in leaf segments and isolated chloroplasts were determined by the method of Arnon (3). The Chl-*a*/Chl-*b* molar ratio was also determined in thylakoid membranes isolated from different segments of the leaves. No marked difference was seen in data from leaf segments and membranes.

Electrophoresis

Chl-protein complexes from the thylakoid membranes were resolved by gel electrophoresis following the procedure described in (2), except, that the thylakoids were solubilized with DDM instead of SDS. Thylakoids isolated from the tip and middle segments of 8 days old leaves were solubilized at a final DDM/Chl weight ratio of 7.5:1. To obtain a good resolution, in the base this ratio had to be increased to 10:1. After centrifugation at 12,000*g* for 5 min, the solubilized membranes were immediately applied to gels, which were prerun at 15 mA for 20 min. The gels were run on an LKB Midget electrophoresis system at 4°C for 30 to 45 min and scanned at 650 and 670 nm with a Beckman DU-8B spectrophotometer. The electrophoretic bands were analyzed in terms of relative accumulation of LHCII, a method similar to that reported by Larsson *et. al* (19). In our analysis, however, LHCII content was quantified relative to CP1+CP1a bands, instead of CPa. This latter band was poorly resolved in some of our samples especially in the base thylakoid membranes. (Further analysis of the protein complexes by reelectrophoresis is in progress and will be reported elsewhere.)

CD

The CD analysis of isolated thylakoid membranes was carried out in a JASCO 40C spectropolarimeter, the optical bandwidth was adjusted to 4 nm. Chl (a + b) content of the samples was adjusted to 20 μg/ml. The sample in a standard optical cell of 1 cm path-length was placed close to the photomultiplier window (distance 15 mm). The CD intensities are given in units of ellipticity (m^0).

Chl-a Fluorescence Kinetics

Induction kinetics of Chl fluorescence in leaves was followed in a cylindrical type of phosphoroscope. After harvesting, the leaves were kept in the dark at 4°C for about 2 h. Base, middle or tip segments of the leaf were mounted between two plexiglass frames and placed diagonally in a 1 cm standard glass cuvette. The 485 nm (4 nm half-bandwidth) measuring light was selected from the beam of a 35 W tungsten lamp with an SPM2 (Carl Zeiss, Jena, FRG) grating monochromator. The photomultiplier tube was protected

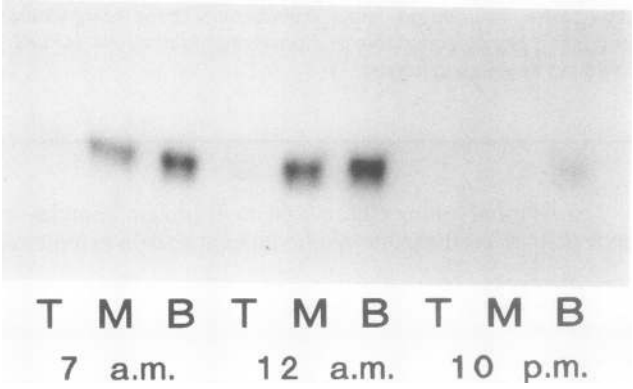

Figure 1. The level of Cab-1 mRNA in the morning (7 a.m.), noon (12 p.m.) and evening (10 p.m.) in the basal (B), middle (M) and tip (T) segments of 7-d-old wheat leaves. Each sample contained 20 μg RNA. (For other conditions see "Materials and Methods.")

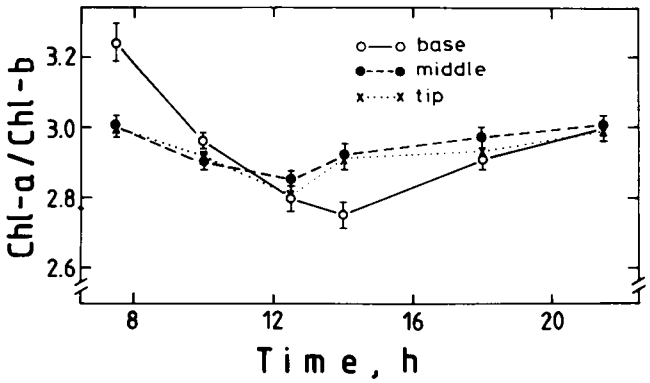

Figure 2. Diurnal fluctuation of the Chl-*a*/Chl-*b* ratio in the basal, middle and tip segments of 6-d-old wheat leaves. Mean values and standard errors were obtained from six independent experiments.

against stray measuring light by a Corning CS 2–58 filter. The actinic light was provided by a 650 W tungsten lamp and filtered by a heat filter and red or far red cut-off glass filters (Schott RG 1 and RG 9, respectively) and entered the sample compartment at 90° with respect to the measuring beam. During the time period when the slit on the cylinder opened for the actinic beam the path from the measuring beam and the photomultiplier were closed and vice versa.

After a 10-min dark adaptation in the sample holder at room temperature the leaf was illuminated with red actinic light for 10 min to reach the steady state fluorescence level (state 2). Then far-red light (light 1) was applied and a new steady state level of the Chl fluorescence (state 1) was reached. After 10 min, light 1 was switched off and light 2 was turned on and the fluorescence yield changes characteristic of state 1-state 2 transition were recorded on a chart recorder.

RESULTS AND DISCUSSION

Diurnal Oscillation of mRNA Level

Figure 1 shows a dramatic diurnal oscillation for the Cab-1 mRNA level in the basal and middle segments of the leaves. The level of Cab-1 mRNA is highest between 10 AM and noon

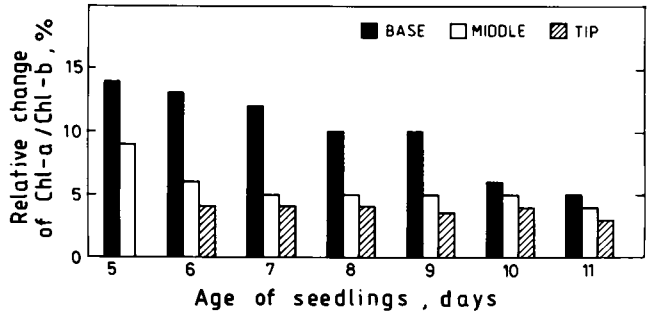

Figure 3. Relative changes of the Chl-*a*/Chl-*b* ratios in the base, middle and tip segments of wheat leaves during the ageing of the seedlings. The relative changes were calculated between the values determined at 7 a.m. and 2 p.m., respectively. Mean values were obtained from five independent experiments.

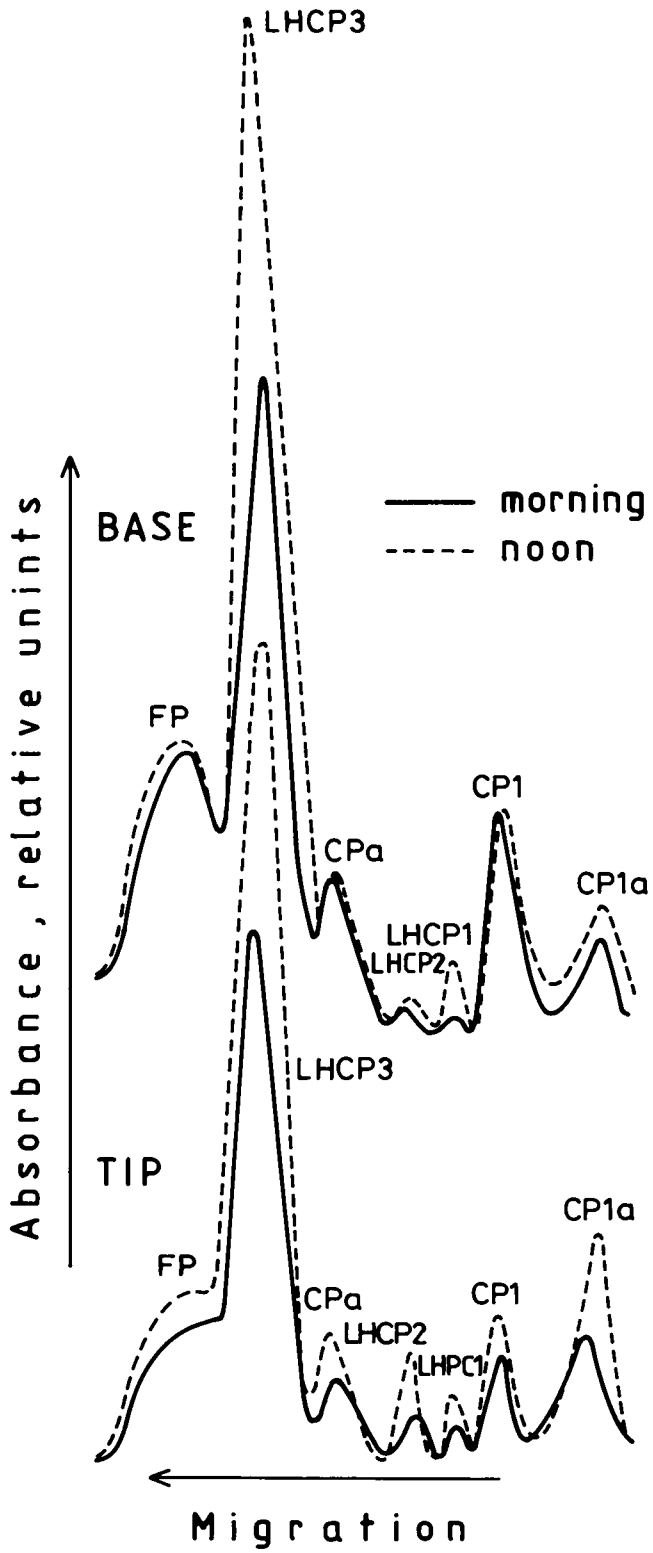

Figure 4. Gel scans at 650 nm of the pigment-protein complexes resolved by gel-electrophoresis of thylakoid membranes isolated from the base and tip segments of 8-d-old wheat leaves, morning, 6 a.m., noon, 2 p.m.

Table I. *Variations in Amount of LHCII (LHCP1 + LHCP3) Gel Electrophoresis Bands Relative to PSI Bands (CP1 + CP1a)*

Thylakoid membranes were isolated in the morning (6 AM), at noon (2 PM) and in the evening (10 PM). Determination of Chl-*a*/Chl-*b* and gel electrophoresis were performed as described in the "Materials and Methods." The corresponding values of the Chl-*a*/Chl-*b* ratios are given in parentheses. Data represent mean values obtained from three independent experiments. (All morning values of (LHCP1 + LHCP3)/(CP1 + CP1a) were taken as 100%.)

Membrane	(LHCP1 + LHCP3)/(CP1 + CP1a)		
	Morning	Noon	Evening
		%	
Base	100 (2.99)	125.1 (2.67)	110.3 (2.74)
Middle	100 (2.78)	102.0 (2.66)	105.2 (2.78)
Tip	100 (2.73)	112.1 (2.62)	105.3 (2.69)

and then steadily declines toward the end of the day (23). Confirming previous findings of Lamppa *et al.* (18) no detectable amount of Cab-1 gene was found in the tip section of the leaves. Similar results were obtained by Northern hybridization experiments indicating that the Cab gene family as a whole follows the same expression pattern in young developing wheat seedlings (data not shown).

Chl-a/Chl-b Molar Ratios

LHCII contains about half of the Chl (a + b) and almost the total Chl-*b* content of mature thylakoid membranes. The Chl-*a*/Chl-*b* molar ratio in the LHCII from various higher plants is about 1.2, whereas in all other pigment-protein complexes the value of this ratio is much higher (15). Hence, gross changes in the LHCII content must be reflected in the Chl-*a*/Chl-*b* molar ratio and vice versa, a substantial decrease in the value of Chl-*a*/Chl-*b* must be linked to the accumulation of the LHCII. The Chl-*a*/Chl-*b* ratio of this complex is constant even in reconstituted systems (25). The Chl-*a*/Chl-*b* molar ratio thus serves as a simple, sensitive, semiquantitative indicator of the LHCII content of membranes, which is especially useful when a large number of samples are to be analyzed.

Young wheat leaves from their base to the tip exhibit a gradual increase in the Chl content per unit area and a decrease in the Chl-*a*/Chl-*b* ratio (31). The Chl-*a*/Chl-*b* ratio changes throughout the day in all parts of the leaf. However, similarly to the oscillations in the mRNA levels, the largest changes are observed in the basal part of the leaf. The middle and tip segments show much smaller diurnal oscillations (Fig. 2).

The maximum and minimum values in the Chl-*a*/Chl-*b* ratio were reached in the early morning and early afternoon, respectively. This can be correlated with the oscillation pattern of mRNA (Fig. 1) by taking into account that a gradual increase in the LHCII content is a cumulative effect of the elevated level of mRNA for LHCII. A decrease in the Chl-*a*/Chl-*b* from 3.1 to 2.7, typical in the basal segment of young leaves can be estimated to correspond to a 38% increase in the LHCII content relative to that in the morning. (It was presumed that the changes are solely due to differences in LHCII content and that at a (Chl-*a*/Chl-*b*) = 2.7 one-third of Chl-*a* was bound to LHCII. Different presumptions lead to slightly different figures but do not change the conclusion that the observed decrease in the Chl-*a*/Chl-*b* ratio originate from a substantial accumulation of LHCII in the thylakoid membranes.) A decrease in the Chl-*a*/Chl-*b* ratio from 3.1 to 2.8 in pea thylakoid membranes was shown to originate from a 20% increase in LHCII (LHCP1 + LHCP3) green gel bands (19).

In an unreported experiment it was verified that significant decreases in the Chl-*a*/Chl-*b* ratio were accompanied with an increase in the Chl content per fresh weight which indicates *de novo* synthesis of LHCII.

When the relative changes were plotted against the age of leaves it became evident that oscillation of the LHCII content gradually dampened with the ageing of leaves (Fig. 3). This is most likely the consequence of the gradual decrease of mRNA level during the development of leaves (F Nagy, unpublished results).

We note that the correlation between mRNA levels and LHCII content is less pronounced in the older tissue of the middle segment. In these tissues the diurnal cycle of the LHCII content is minimal while mRNA levels show characteristic

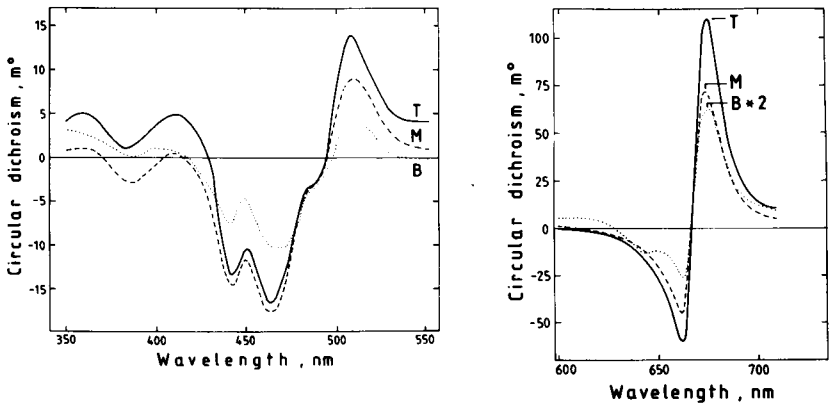

Figure 5. CD spectra of chloroplasts isolated in the morning from the basal (B), middle (M) and tip (T) segments of 6-d-old wheat leaves.

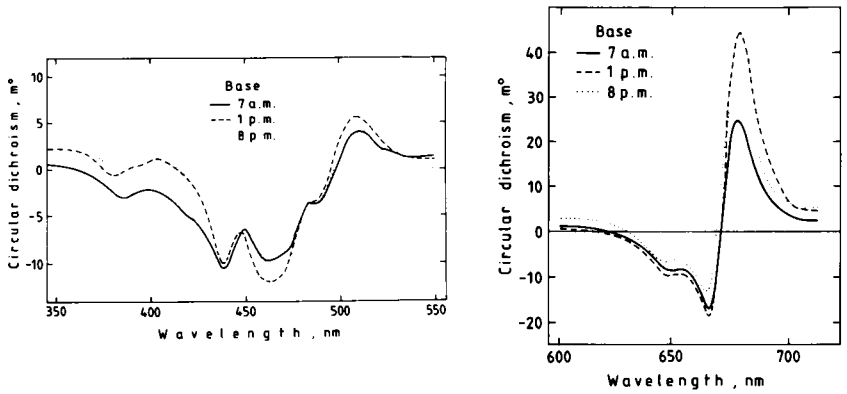

Figure 6. CD spectra of chloroplasts isolated from the basal segment of 6 day-old wheat leaves in the morning (7 a.m.), early afternoon (1 p.m.), and evening (8 p.m.).

fluctuation. We explain this observation by the relatively large size of the middle segments (2–4 cm) and as a consequence by the presence of both young and older cells in the samples. High transcription rates of Cab genes in the young cells result in the diurnal changes of mRNA levels (with no or little contribution to mRNA levels from older cells) whereas the transcriptionally less active older cells with more Chl probably dominate the LHCII content data.

Nonetheless, as a trend, the oscillatory pattern of the mRNA level and the Chl-*a*/Chl-*b* ratio could be correlated with each other in all experiments. The only exception was that no oscillation of the Chl-*a*/Chl-*b* ratio was observed when the leaves were kept in the dark, clearly due to the lack of Chl synthesis in the dark. Steady-state level of mRNA continues to oscillate for several days in the dark (23).

Pigment-Protein Complexes

The diurnal fluctuation in the Chl-*a*/Chl-*b* ratios was reflected in variations in the relative amount of LHCII complexes. As shown in Figure 4 and Table I, the amount of LHCII (LHCP1 + LHCP3) accumulated in the base between the morning and noon relative to the PSI complexes (CP1 + CP1a). Much smaller diurnal variations are in seen the middle and tip sections. The reproducibility was good in the different batches, *e.g.* in the base in three independent experiments the Chl-*a*/Chl-*b* ratio decreased from 2.98 to 3.03 in the morning to 2.63 to 2.67 at noon. In solubilized base membranes (LHCP1 + LHCP3)/(CP1 + CP1a) values at noon ranged between 122 and 133%, when compared to the morning values normalized to 100%. (This normalization had to be introduced due to substantial differences in the solubilization of membranes isolated from different segments of the leaves.)

CD

CD spectra of chloroplasts isolated from the base, middle and tip segments of the leaves resemble each other (Fig. 5). They all exhibit the characteristic split "big" CD signal of mature granal chloroplasts (*cf.* ref. 13) which has been shown to originate from the helically organized macrodomains of the pigment protein complexes (9, 11–14). Thus, the presence of this signal in the base, middle and tip thylakoid membranes shows that qualitatively the macroorganization of the pigment protein complexes is very similar in all samples. However, the amplitude of the main bands in the basal chloroplasts was significantly weaker than in the chloroplasts isolated from the middle and tip sections. This can most likely be accounted for by a decreased size of the macrodomains (*cf.* refs 16, 11), which appears to correlate the LHCII-deficiency of the base chloroplasts in the morning (see below).

Inspection of electron micrographs taken from different parts of the leaves in the morning, at noon and in the evening, respectively did not reveal major changes in the membrane organization (Zs Tóth, unpublished results).

As shown in Figure 6, the amplitude of the CD signal in the basal thylakoid membranes shows a diurnal oscillation which is correlated to the accumulation of LHCII. These changes, similarly to the oscillation in the LHCII content and mRNA level (Figs. 1 and 2), are less readily recognizable in the middle and tip chloroplasts (Table II). It is to be emphasized that the CD signal is normalized to the Chl content of

Table II. *Diurnal Fluctuations of Amplitude of Two Major CD Bands of Chloroplasts*

Chloroplasts were isolated in the morning and afternoon (around 7 AM and 2 PM, respectively) from the basal, middle, and tip segments of 6-d-old wheat leaves. The Chl content of the suspension was adjusted to 20 μg/mL. Mean values and standard errors were obtained from five independent experiments. The amplitudes are given in units of ellipticity (m^0).

Segment	Morning		Afternoon	
	CD (673 nm)	CD (506 nm)	CD (673 nm)	CD (506 nm)
Base	38.7 ± 3.9	5.5 ± 0.8	48.6 ± 3.3	8.8 ± 1.3
Middle	93.4 ± 5.8	12.3 ± 1.2	97.4 ± 5.5	11.4 ± 1.2
Tip	140.0 ± 14.5	13.4 ± 1.3	131.0 ± 11.8	12.9 ± 0.5

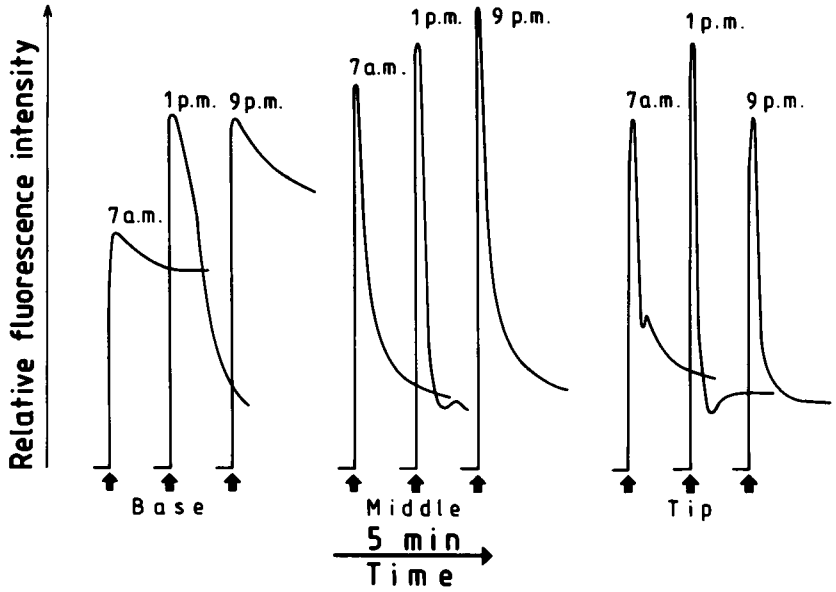

Figure 7. Fluorescence induction kinetics upon illumination of segments of 6-d-old leaves with intense PSII light, following a preillumination with PSI light. (For details of the protocol see "Materials and Methods.")

the suspension. Thus the increase cannot be explained by accumulation of Chl molecules in the membrane. Relative enrichment of membranes in LHCII *per se* does not explain the changes because the CD signal of the nonaggregated or low aggregation level form of LHCII is very weak compared to the signal in chloroplasts or LHCII-aggregates (9, 13).

The anomalous big CD signal "accumulates" together with the LHCII in the thylakoid (8) and can also be generated upon the macroaggregation of this isolated pigment protein complex (11, 13). The big CD signal of chloroplasts has been shown to originate from helically organized macrodomains of the pigment-protein complexes (9, 11, 14). Thus, the fluctuations in the amplitude of the principal CD bands can be accounted for by changes in the macroorganization of the pigment-protein complexes. Specifically, the increase of the CD signal in the basal chloroplasts between the early morning and noon suggests that the newly synthesized LHCII complexes are incorporated into the existing helically organized macrodomains or they themselves form such macrodomains in the thylakoid membrane.

Chl-a Fluorescence Induction Kinetics

The ability of chloroplasts to dynamically regulate the distribution of excitation energy between the two photosystems, *i.e.* to perform a "state transitions," can be determined by monitoring the Chl-a fluorescence yield induction kinetics in leaves exposed to illumination preferentially exciting PSII and PSI.

Figure 7 shows typical induction kinetics upon illumination with light 2 following a prolonged illumination by light 1. In the base of young leaves harvested in the morning, the ability of state 1-state 2 transition is lacking. Similar observation has earlier been made by Webber *et al.* (31) when comparing the ability of "state transitions" in the base, middle and tip segments of 4 and 8 d old wheat leaves. Figure 7 shows that the ability of basal segment to undergo state 1-state 2 transition exhibits a diurnal fluctuation. It is very weak in the morning, dramatically increased in the afternoon and reversed again in the evening hours. The changes in the middle and tip segments appear far less dramatic. This type of fluorescence yield changes are thought to be correlated with the reversible phosphorylation of LHCII and its lateral diffusion in the membrane (4, 5). Indeed, the observed fluorescence yield changes appear to correlate with the diurnal fluctuations linked to LHCII (mRNA level, Chl-*a*/Chl-*b* ratios, LHCII content and CD amplitudes). Although a non-casual coincidence of these data can not be excluded, our fluorescence yield data strongly suggest that the diurnal fluctuations affect the functional properties of LHCII, specifically the ability of membranes to undergo "state transitions."

CONCLUSION

Diurnal fluctuation of the steady-state level of mRNA for LHCII genes has been studied extensively. To our knowledge, however, the investigations were not extended toward the thylakoid membranes. Our data strongly suggest a close correlation between the fluctuation of mRNA level in the cytosol and the content and function of LHCII in the thylakoid membranes. Hence, our data may indicate the physiological importance of the regulation of the gene expression. The regulatory factors controlling the diurnal oscillations and its physiological role must be explored by further experiments.

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