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Light-Induced Biogenesis of Chlorophyll–Protein Complexes in Developing Wheat Thylakoids

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Abstract—It is shown that the apoproteins of core complexes (CC) I and II, the α- and β-subunits of CF_1 ATP-synthase complexes, are present in seedlings grown under intermittent light (IML). The levels of light-harvesting complex (LHC) apoproteins in the 30- to 18-kD region increase rapidly upon exposure to continuous light (CL). The newly synthesized LHC apoproteins appear to be present predominantly in monomeric forms that later assemble into higher-order oligomeric forms. During the early stages of greening of wheat seedlings, polypeptides in the 20.5-19 and 17.5-15.5 kD regions (so-called "early light-induced proteins" (ELIP)) are observed, but they disappear fully after 6 h. As greening proceeds, the 727-nm band in low-temperature fluorescence spectra (77 K) gradually shifts to longer wavelength (740-742 nm), which clearly demonstrates the light-driven biogenesis of LHC I and its assembly with CC I.

Key words: greening, fluorescence, early light-induced proteins, plastids, light-harvesting complex, wheat

Photosystem (PS) I and II of higher plant thylakoid membranes include core complex (CC) containing the photochemical reaction center and chlorophyll (Chl) *a*/*b*-containing light-harvesting complex (LHC), the function of which is to absorb photons and to transfer excitation energy to the reaction center [1].

Developing chloroplasts are useful for studying plastid biogenesis; in them, the stages of sequential structural changes can be followed [2]. On investigation of the transition of etioplast to chloroplast during illumination of plants that were grown in full darkness, it is possible to follow the synthesis of separate components of the photosynthetic membrane (proteins, lipids, pigments, etc.) and their assembly and development in detail. Some phenomena in developing etioplasts can be controlled by light intensity and quality and by duration of light exposition. Thus, etioplasts are a standard model for studying the biogenesis of the photosynthetic apparatus of higher plants [3].

Growth of etiolated plants in intermittent light arrests plastid development at the stage of the protochloroplast, which synthesizes Chl *a* and carotenoids selectively and are essentially devoid of Chl *b* [4]. These protochloroplasts have a complete electron transfer chain and functional CC I and CC II components. In spite of the presence of substantial amounts of LHC mRNAs, there is little accumulation of LHC apoproteins, particularly of the LHC II apoproteins, in IML-grown leaves [5]. In plants exposed to intermittent light, or in plants transferred to darkness after brief preexposure to continuous light (i.e., under conditions where Chl synthesis is stopped), preaccumulated LHC II apoproteins are degraded because of rapid turnover of the apoproteins in the absence of stabilization by the photosynthetic pigments, while the reaction center components continue to be synthesized [6]. Thus, when Chl accumulation is low, LHC apoproteins cannot form Chl–protein complexes [7-10].

The purpose of the present work was to examine the assembly of chlorophyll–protein complexes of the chloroplast thylakoid membrane in greening wheat seedlings. In particularly, we studied the cooperation between synthesis and accumulation of chlorophyll–protein complex proteins and their native spectral properties for more precise determination of the *in vivo* status of the assembly and formation of these complexes in developing plastids.

MATERIALS AND METHODS

A hard winter variety of wheat (Barakatli-95) was used for the study. Wheat (*Triticum durum* L.) seeds were germinated and grown in complete darkness in a growth chamber at 23 ± 2°C and 50-60% relative humidity. Five-six-day-old etiolated seedlings were subjected to intermit-

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tent white light flashes (2 min light, $I = 40$ μE·sec^{-1}·m^{-2}; 118 min darkness) for three days. Then they were transferred to continuous light (irradiance, $I = 125$ μE·sec^{-1}·m^{-2}, "white" luminescent lamps LB-40 W; Razno, Russia) and exposed to various periods (0, 2, 6, 12, 36, and 48 h; 0-h is samples after the intermittent light cycle). Mature green control plants were germinated and grown in a phytotron for 7-8 days.

Leaves were mixed with chilled grinding buffer (0.4 M sucrose, 10 mM Tricine-NaOH, pH 7.6, 10 mM $MgCl_2$) and homogenized for 5 sec at full power in a MPW-302 blender (Mechanika Precyzyja, Poland). Chloroplast isolation and thylakoid membrane precipitation were carried out according to Peter and Thornber [11]. The chloroplast suspension was centrifuged at 3,000*g* for 2 min at 4°C in a K-24 centrifuge (VEB MLW Medizintechnik Leipzig, Germany; rotor radius $r = 8$ cm) and the chloroplast pellet was resuspended in lysis buffer (10 mM Tricine-NaOH, pH 7.6, 2 mM Na-EDTA) with a glass homogenizer. The homogenate was centrifuged at 27,000*g* (Beckman L-90K ultracentrifuge, USA; rotor radius $r = 16$ cm) for 5 min at 4°C, and the precipitate of whole thylakoids were resuspended with small volume of grinding buffer. Materials of thylakoids were used immediately or frozen in liquid N_2 and stored at 77 K. The chlorophyll concentration was determined spectrophotometrically in 80% acetone [12].

The polypeptide composition of samples was analyzed by gradient (10-25%) SDS-PAGE in slab gels (1 × 160 × 180 mm) according to Laemmli [13] as described in [14]. After electrophoresis the gels were stained for 30 min by a solution of 0.04% (w/v) Coomassie Brilliant Blue G-250 prepared in 3.5% perchloric acid ($HClO_4$). The gels were scanned using an ULTROSCAN 2202 Densitometer (LKB, Sweden) with a 633-nm laser as the light source. When necessary, the gels are dried in a SLAB GEL Dryer 2003 (LKB, Sweden).

Fluorescence spectra at 77 K were measured using a Hitachi-557 double-beam spectrophotometer (Japan) and a Hitachi-850 spectrophotometer as described previously [15]. Fluorescence emission spectra were corrected for the spectral sensitivity of the spectrofluorimeter using rhodamine B. The samples are immersed in liquid N_2 during measurements.

RESULTS AND DISCUSSION

The fully formed degree of photosynthetic membranes of greening etiolated seedlings can be characterized by completion of pigment accumulation. As seen from Fig. 1, chloroplasts isolated from the wheat seedlings grown in intermittent light contain very small amounts of pigments on a fresh weight basis, and Chl *a* is

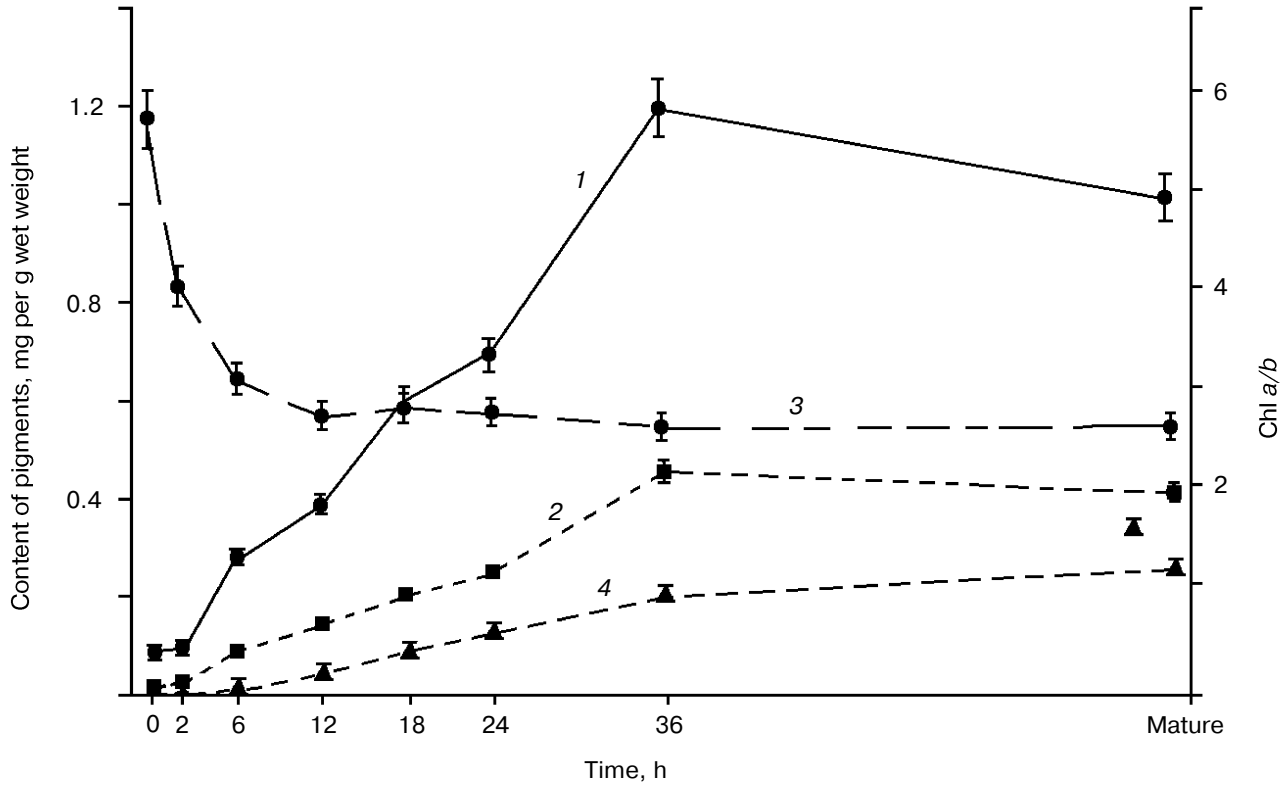

Fig. 1. Pigment content of IML-grown wheat seedlings exposed to continuous light for the indicated times: *1*) Chl *a*; *2*) Chl *b*; *3*) Chl *a*/Chl *b*; *4*) carotenoids.

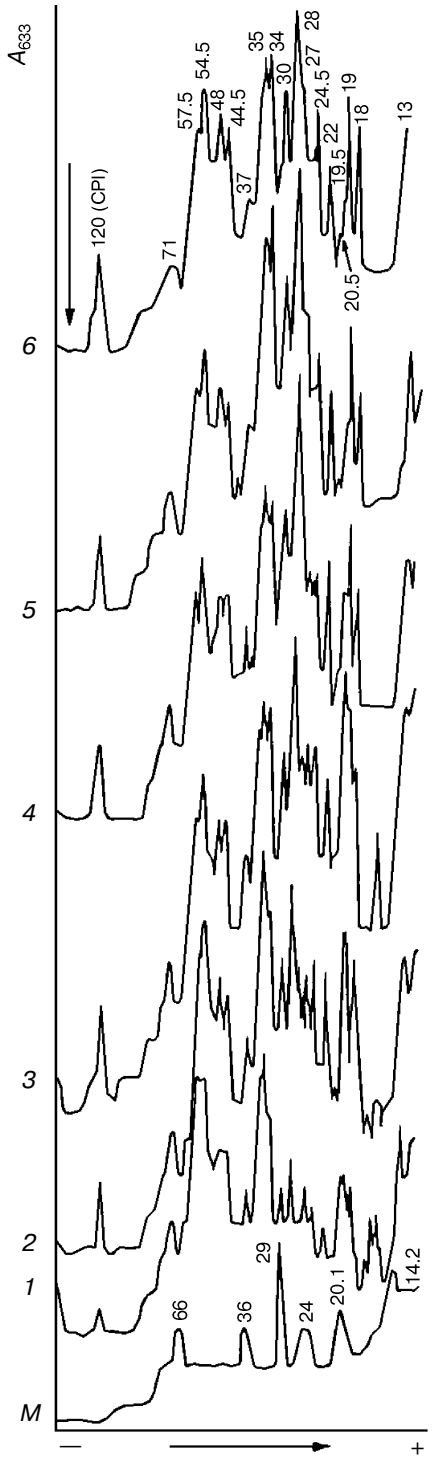

Fig. 2. Density patterns from gradient (10-25%) SDS-PAGE analysis of thylakoid membranes of IML-grown seedlings exposed to continuous illumination for 0 to 36 h: *1*) 0; *2*) 2; *3*) 6; *4*) 12; *5*) 36; *6*) mature greenhouse-grown seedlings. Standard proteins are shown in lane M (kD): 66 (bovine serum albumin), 36 (glyceraldehyde-3-phosphate dehydrogenase), 29 (carbonic anhydrase), 24 (trypsinogen), 20.1 (trypsin inhibitor), 14.2 (lactalbumin). Electrophoresis was carried out in Tris-glycine buffer, pH 8.3, at 4°C for 16 h.

the dominant pigment in these samples. The pigment content increases slowly upon initial exposure to continuous light; after 12 h of illumination, both Chl *a* and Chl *b* appear to accumulate in the steady manner. The Chl *a/b* ratio reaches that of mature chloroplasts (Chl *a/b* ~ 3), which does not change upon further illumination. According to the data of some investigators, the Chl *b* content forming in intermittent light is dependent on the periodicity and duration of the light cycle [16].

The optical density of high-resolution gradient electrophoregrams in 10-25% polyacrylamide gel is shown in Fig. 2. Because significant changes are not observed in the region of PS I core (CP (chlorophyll–protein) I), CP I apoprotein, α- and β-subunits of CF_1 ATP-synthase complex, and core antenna of PS II (CP 47 and CP 43), our main attention was concentrated on the polypeptides of antenna complexes of PS I and II (LHC I and LHC II, respectively) in the 30-18 kD region. As shown in Fig. 2, in 0-h samples there are some bands with low intensity in this region; at 2-h exposure to continuous light the number and intensity of the bands tend to increase, clearly showing the formation of monomeric forms (Fig. 2 and the table). Then, upon illumination to mature chloroplasts, the number of bands on electrophoregram decreases, but the intensity of the main proteins of antenna complexes of the photosystems increases. Hence, it was concluded that the monomeric forms of light-harvesting chlorophyll *a/b*-proteins aggregate to form higher-order oligomeric forms. Thus, it can be suggested that this stage is the highest of LHC assembly in developing chloroplasts.

We focused our interest on the polypeptides found in SDS-PAGE in the 20.5-19 and 17.5-15.5 kD regions (Fig. 2). In samples from IML-grown plants (0-h samples), they form some bands. After 2 h of illumination with CL the number of bands related to these polypeptides decreases. At the same time, the band in the ~15-kD region increases. It is of interest to note that the synthesis of this protein reaches a maximum level in 6 h, and it almost disappears a few hours later on continuous light. According to contemporary literature data, this has been called early light-inducible protein (ELIP) [17-19]. It seems that ELIP fulfils some function in plastid development, and in the further growth and development of chloroplasts it is not necessary and perhaps is subjected to proteolysis by enzymes. The nature and function of ELIP is still not completely known, but involvement in pigment synthesis in plastids or in PS II repair after photoinhibition has been suggested [17-19]. It has also been suggested that ELIPs are transient chlorophyll-binding proteins not involved in light-harvesting but functioning as scavengers for chlorophyll molecules during turnover of pigment-binding proteins [20]. On the other hand, they seem to have a special role in photoprotection.

According to the fluorescence spectra (77 K) presented in Fig. 3, chloroplasts isolated from IML-grown leaves

Light-harvesting complex proteins in the region 30-18 kD

Time, h	Molecular mass of proteins, kD							
	29	28	27	25	24.5	22	19	18
0	+	+	—	+	trace	+	+	trace
2	+	+	trace	+	+	+	+	+
6	+	+	+	+	+	+	+	+
12*	+	+	+	—	+	+	+	+

* After 12 h of illumination no significant changes in the composition of key light-harvesting complex polypeptides are observed.

(0-h samples) display their main emission band at 687 nm and a small maximum at 727 nm characteristic of the presence of PS II and CC I, respectively [21]. After 2 h in CL, the 727-nm band, corresponding to core complex of PS I, gradually shifts to longer wavelengths (736-738 nm) and at the same time increases in intensity relative to the 687-nm emission band. After further illumination in seedlings transferred to continuous light, this band is at 740 nm, which clearly demonstrates the presence of completely synthesized PS I units. Thus, it was found that the fluorescence spectra of chloroplasts isolated from 12-h greened seedlings are very similar to those of mature chloroplasts in terms of the corresponding maxima. The appearance of long-wavelength fluorescence emission at 740-742 nm during greening suggests that the peripheral antennae of PS I is synthesized when these plants are exposed to continuous illumination [8, 22, 23]. The addition of antennae to PS I may occur either by the addition of Chl to preexisting protein sites or by the synthesis and insertion of new chlorophyll–protein complexes. The changes in the proportion of the long wavelength emission band probably explain the decreased presence of core complex I polypeptides in the thylakoid membrane, because core complex I is utilized in the formation of PS I [8]. The data on the appearance of the LHC I and LHC II subunits during light-driven biogenesis of the plastid are correlated with the appearance of the long-wavelength fluorescence band at 77 K, which is characteristic for LHC I biogenesis and its assembly with core complex I.

Then, low-temperature derivative absorption spectra of samples were monitored to follow the formation and state of native forms of pigments in developing plastids and to explain the nature of the long-wavelength fluorescence.

Figure 4 shows the absorption spectra (A) and their fourth derivatives (A^{IV}) at 77 K of chloroplasts from the seedlings exposed to intermittent light and for continuous-light seedlings. In chloroplasts for IML seedlings, bands of Chl *a* appeared at 660, 665 (shoulder), 669, 676, 682, 690, 696, 704, and 712 nm and a small maximum of Chl *b* at 648 nm. On transfer of the seedlings from intermittent to continuous illumination, in the A^{IV} spectrum (77 K) the intensity of the Chl *b* band at 648 nm increases considerably, this clearly corresponding to the synthe-

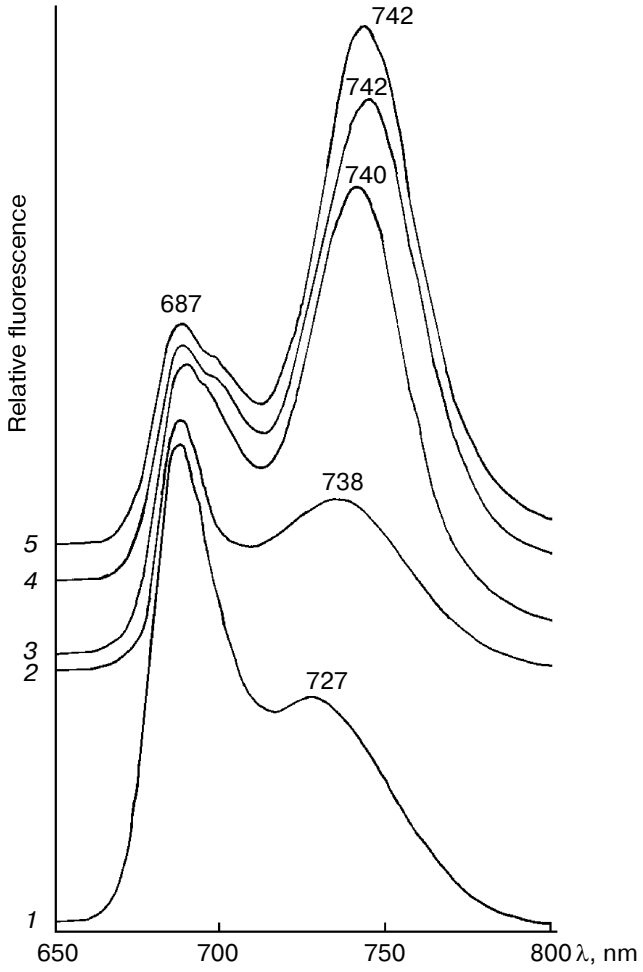

Fig. 3. Fluorescence emission spectra at 77 K obtained from chloroplasts of IML-grown wheat seedlings exposed to various periods of constant illumination: *1*) 0; *2*) 2; *3*) 6; *4*) 12 h; *5*) mature light-grown seedlings. Spectra were normalized to equivalent emission at 687 nm.

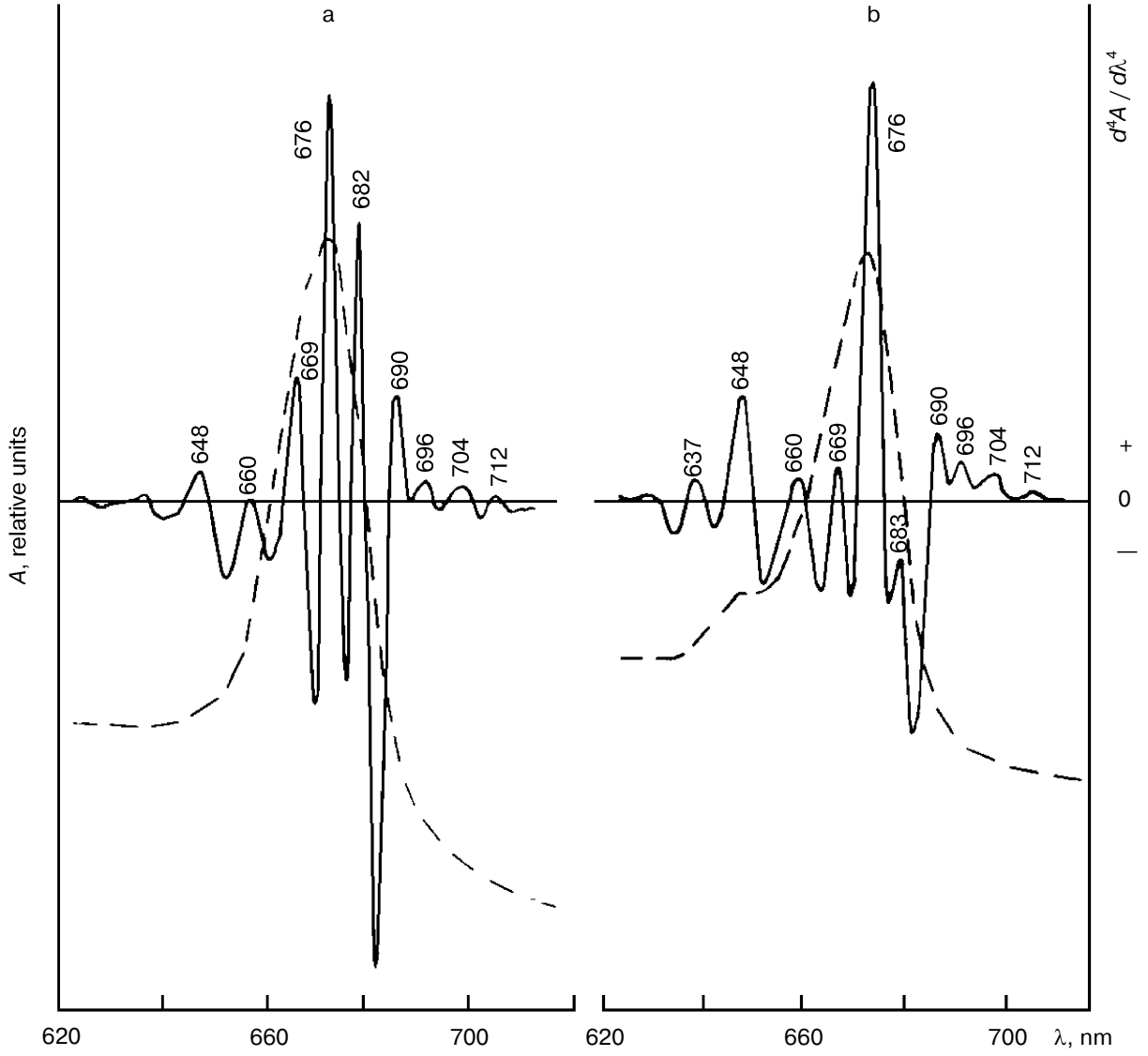

Fig. 4. Absorption spectra (dashed curves) and fourth derivatives of the absorption spectra (solid curves) at 77 K of chloroplasts of wheat seedlings grown in intermittent light (a) and after transfer to continuous light (b). The differentiation intervals are $\Delta\lambda = 7$ and 6 nm, respectively.

sis under these conditions of light-harvesting complexes LHC I and LHC II [21]. At the same time, a sharp decrease in the band intensity at 682 nm relative to the first spectrum occurs. The Chl *a* form at 710-712 nm is known to be responsible for the long-wavelength fluorescence band of chloroplasts at 740 nm [24]. It is important to note that the long-wavelength Chl *a* forms are seen in the fourth derivative absorption spectra at 77 K of chloroplasts from IML-grown wheat seedlings (Fig. 4). Therefore, the shift to shorter wavelength with the large decrease in the intensity of the long-wavelength fluorescence band of the chloroplasts in IML cannot be explained by the absence of the longest wavelength Chl *a* forms. According to our results, the IML-grown chloroplasts most probably lack energy transfer from the light-harvesting complex to the Chl *a* form at 710-712 nm and, therefore, this form fluoresces weakly. Thus, on switching the seedlings from intermittent light to continuous light a sharp increase in the fluorescence intensity at 740 nm occurs (Fig. 3), this being due to restoration of energy transfer to the Chl *a* form at 710-712 nm from LHC I synthesized during continuous light [15].

These results can be interpreted within the framework the concept of Tzinas and Akoyunoglou [25] according to which there is a competition of the polypeptides of reaction center and light-harvesting complex for the newly synthesized Chl *a* molecules. In the absence or lack of Chl *b* (and consequently LHC I and LHC II), newly synthe-

sized Chl *a* molecules are taken up to a greater degree by the polypeptides of reaction centers. In seedlings transferred from intermittent to continuous light, the newly synthesized Chl *a* molecules are taken up by the apoproteins of light-harvesting complexes, and the fraction of Chl *a* in reaction centers and, consequently, the relative content of chlorophyll–protein complexes, decreases. The affinity of Chl *a* for reaction center polypeptides I and II is supposed to be higher than for apoLHCs.

The data presented in this work allow indicated that during development of the plastid newly synthesized LHC apoproteins appear in the thylakoid membranes primarily as monomeric pigmented complexes that later assemble into higher-order oligomeric forms. The appearance of the oligomeric forms of LHC is delayed in comparison to the accumulation of the monomeric LHC II and minor LHC I apoproteins and the decrease of the Chl *a/b* ratio. This delay may be due to a sequential pathway of assembly and may explain the longer time to form the monomer, or it may be due to a requirement of other factors for the assembly of the monomeric LHC into its oligomeric forms.

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