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Stability of the Apoproteins of Light-Harvesting Complex I and II during Biogenesis of Thylakoids in the Chlorophyll *b*-less Barley Mutant *Chlorina f2*[1]

Susanne Preiss* and J. Philip Thornber

Department of Biology, University of California, Los Angeles, California, 90095–1606

Transcription and translation of *Lhc* (*cab*) genes have been compared in the *chlorina f2* mutant of barley (*Hordeum vulgare*) and its wild type to study the effect of chlorophyll *b*'s absence on the regulation of assembly of the light-harvesting complexes (LHC). All tested genes were transcribed and the amount of their respective mRNAs increased rhythmically upon illumination of etiolated mutant plants. The synthesis of individual LHC apoproteins also had a rhythmic pattern when total leaf protein extracts were examined, whereas they increased gradually in the thylakoid. Only some LHC pigment-proteins present in wild-type thylakoids were found in mature mutant membranes. Thus, only the 25-kD (type 3) apoprotein of the three apoproteins of the major LHC IIb complex survived. The amount of the minor LHC II pigment-proteins was considerably reduced but not to zero. Photosystem I had some of the two LHC Ia apoproteins but had little of those of LHC Ib. This was reflected in a shift of the 77-K emission maximum of whole leaves from 741 to 732 nm. It is concluded that the two largest LHC IIb and the LHC Ib apoproteins need chlorophyll *b* for stable integration into the membrane and that posttranslational regulation plays a major role in LHC assembly.

The thylakoid membrane of higher plants contains pigmented multiprotein complexes that absorb and transform light energy into chemical energy. The two major complexes are PSI and PSII, each of which can be fractionated into several smaller pigment-protein complexes. A photosystem is composed of a CC containing the photochemical reaction center and a core antenna and an LHC, which serves as an auxiliary antenna system. The LHCs absorb photons and transfer the energy via the core antenna to the reaction centers where charge separation takes place. The essential pigment co-factors of the CCs are Chl *a* and β-carotene, whereas the LHCs contain all of the Chl *b* in the plastid in addition to Chl *a* and xanthophylls. Higher plants contain at least nine different light-harvesting pigment-proteins: four associated with LHC I (Preiss et al., 1993) and five associated with LHC II, each with a distinct pigment composition (summarized by Thornber et al., 1993). Their apoproteins are encoded by a nuclear multigene (*cab*) family, now termed *Lhc* genes (Green et al., 1991; Jansson et al., 1992).

Mutants of higher plants that are deficient or lack Chl *b* are excellent tools to study the impact of Chl *b* on the assembly of the LH pigment-protein complexes (for review, see Somerville, 1986). One of the most investigated is a Chl *b*-less (*chlorina f2*) mutant of barley (*Hordeum vulgare*). Earlier work showed that this mutant lacked the major light-harvesting pigment-protein complex of PSII, now termed LHC IIb; however, the mutant had normal electron transport rates for PSI and PSII (Boardman and Highkin, 1966; Thornber and Highkin, 1974; Waldron and Anderson, 1979). Bellemare et al. (1982) confirmed that this mutant lacked or had greatly decreased amounts of several polypeptides in the size range of the major LHC II apoproteins. However, the mRNA for those polypeptides was translatable in the mutant, and in vitro translation studies showed a polypeptide pattern indistinguishable from that obtained from wild-type mRNA. Ryrie (1983) proposed that some functional LHC apoproteins remain in the mutant.

After it was discovered that more than one LH pigment-protein is present in wild-type thylakoids, the question arose as to whether every LHC pigment-protein was affected in the same way as the major LHC II component (LHC IIb) by the lack of Chl *b*. In wild-type plants, minor LHC II pigment-proteins have been described and are believed to be located closer to CC II than the much more abundant LHC IIb trimers (White and Green, 1988; Dainese and Bassi, 1991; Peter and Thornber, 1991; Harrison and Melis, 1992). Each of the minor LHC complexes is generally seen as a monomeric pigment-protein complex upon solubilization of thylakoid membranes, which is in contrast to the trimeric LHC IIb complex (Peter and Thornber, 1991; Dreyfuss and Thornber, 1994a; Kühlbrandt et al., 1994). Of these minor LHC components, CP 29 (LHC IIa) apoprotein in barley *chlorina f2* membranes was detected by White and Green (1988), who stated that more than one LHC I polypeptide was present. A 25-kD LHC II polypeptide was present in substantial amounts. Studies of this mutant by Melis and co-workers (Harrison and Melis, 1992; Harrison et al., 1993) showed that the sizes of its PSI and PSII antennae were reduced by 20 and 80%, respectively. They

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* Corresponding author; e-mail ibb9tho@mvs.oac.ucla.edu; fax 1-310-206-4386.

Abbreviations: CC, core complex; LHC, light-harvesting complex.

concluded that the greater decrease in the PSII unit size results in the synthesis of more CC II to maintain a balanced linear electron transport. Their studies showed a 24-kD LHC II subunit present at an equal copy number per PSII unit in mutant and wild-type barley, whereas PSI showed a partial loss of a 25-kD polypeptide; all other LHC apoproteins were greatly decreased. Peter and Thornber (1991) found that this mutant had a smaller amount of LHC IIa (CP 29) but none of the LHC IIb apoproteins of 28 and 27 kD, whereas the LHC IIc (CP 26) apoproteins and the smallest and least prevalent 25-kD subunit of the LHC IIb (type 3) were relatively abundant. They were unable to distinguish whether a prominent 21-kD protein band was the apoprotein of LHC I, LHC IId, or both.

Thus, the lack of Chl *b* affects individual LHCs differently with regard to their presence in mature tissue, which is perhaps surprising considering that their apoproteins have similar sizes and a high degree of homology in their primary structures. However, direct comparison of mature plants does not yield a complete picture of LHC assembly and its regulation. Thus, a study of which LHC pigment-proteins or apoproteins are present at different stages of biogenesis should reveal more. One way to examine light-induced LHC biogenesis is to expose etiolated seedlings, which have very small amounts of *Lhc* mRNAs and are devoid of the Chl-containing photosynthetic complexes, to continuous illumination. Using this greening system we have studied the synthesis of *Lhc* mRNAs and their translation in the absence of Chl *b*.

MATERIALS AND METHODS

Growth of Plant Material and Preparation of Total Leaf Extracts and Thylakoid Membranes

Wild-type barley (*Hordeum vulgare*, var Prato) and *chlorina f2* seeds were soaked overnight, and seedlings were grown in vermiculite in a greenhouse at 25°C for 5 d under a regimen of 15 h of light and 9 h of darkness to obtain mature plant tissue. Etiolated *chlorina f2* seedlings for use in the biogenesis experiments were grown in a growth chamber in complete darkness at 25°C for 5 d and were subsequently exposed to continuous illumination (100 μE m^{-2} s^{-1}) for times specified in "Results." Total leaf extracts were prepared as described by Dehesh et al. (1986). Leaf tissue was frozen and ground in liquid N_2 immediately after illumination, and all soluble as well as membrane-bound proteins were extracted and heated at 50°C for 15 min in a denaturing buffer (Peter et al., 1991). Thylakoid membranes were prepared as described by Peter and Thornber (1991) in the presence of protease inhibitors (5 mM aminocaproic acid, 1 mM benzamidine, 10 mM PMSF) in the grinding and wash buffers on ice to reduce any proteolytic degradation during the isolation. An equal weight of leaf tissue was used for all samples. Tissue was obtained from a 2-cm middle section of leaves. This region was chosen because of its homogeneity in chloroplast number and stage of development, since plastids in barley display a developmental gradient from the base to the tip of the leaf (Leech, 1984).

Electrophoretic Separation of the Pigment-Proteins and Western Blot Analysis

Thylakoid membranes obtained from 20 g of leaf tissue were suspended in 1.5 mL of storage buffer. A thylakoid aliquot (20 μL) was solubilized by adding 4 μL of 10% detergent stock (4.5% decyl-maltoside, 4.5% octyl-thioglucoside, 1% SDS), and the pigment-proteins were fractionated by nondenaturing Deriphat-PAGE. For the comparison of one sample with another, gel lanes were loaded on an approximately equal protein basis (15 μg) and not on an equal Chl basis, as is more usual (Peter et al., 1991), because the amount of Chl per unit of thylakoid is very limited during early greening and, furthermore, it increases during plastid development. Proteins for western blotting were separated by fully denaturing SDS-PAGE (Peter and Thornber, 1991) and transferred to nitrocellulose membranes (Costar, Cambridge, MA) according to the method of Towbin et al. (1979). The antisera used were kind gifts from Dr. R. John Ellis (anti-LHC I, P2; Williams and Ellis, 1986), Dr. Sylvia Darr (anti-LHC IIb MLH 9, anti-LHC MLH 12, anti-LHC IIb MLH 1; Darr et al., 1986), and Dr. Gunilla Høyer-Hansen (anti-LHC Ia CMp; Hoyer-Hansen et al., 1988). Alkaline phosphatase-conjugated antibodies were used as secondary antibodies at dilutions specified by the manufacturer (Sigma), and the color reagents 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were used to detect binding.

RNA Preparation and Northern Hybridization

Preparation of total RNA from barley seedlings was carried out by the method of Kirby and Cook (1967). For northern blotting 4 μg of total RNA were separated on 1.2% agarose gels and transferred to Nytran (Schleicher & Schuell) overnight. Hybridizations and washes were performed as described by Greenberg and Bender (1991).

Fluorescence Spectroscopy

Fluorescence emission spectra of mutant or wild-type barley leaves were recorded at 77 K with an Aminco (Silver Spring, MD) SPF 500 spectrophotometer. The excitation wavelength was 436 nm. Middle leaf sections of 2 cm were analyzed. The spectra of the mature samples were normalized at their long-wavelength maximum, and those of the greening mutant were normalized at their emission peak at 688 nm.

Estimation of Pigment Content of Leaves and Thylakoids

Chl was estimated in 80% acetone using the equations of Ziegler and Egle (1965). Carotenoids were estimated as described by Lee and Thornber (1995).

Reagents and chemicals were purchased from Sigma unless otherwise mentioned. Acrylamide was obtained from Kodak. The glycosidic surfactants were from Calbiochem (La Jolla, CA); Deriphat-160 (*N*-lauryl-β-iminodipropionate) was obtained from Henkel Corp. (Hoboken, NJ).

RESULTS

Biogenesis of Pigmented Photosynthetic Complexes

Thylakoid membranes of 5-d-old etiolated *chlorina f2* barley seedlings were exposed to continuous light for 0 to 24 h, and samples of mature tissue of this mutant and wild-type barley were isolated and fractionated by nondenaturing Deriphat-PAGE to examine which of the pigmented photosynthetic complexes they contained (Fig. 1). As expected from previous studies, there was a considerable difference between the two mature samples (Fig. 1). The PSI complex of the mutant was more blue-green and of slightly greater mobility (i.e. smaller) than the PSI holocomplex (CC I plus LHC I) of the wild type. The LHC IIb oligomer was missing in the mutant. A faint orange-brown band in the mutant, migrating in the size range of the LHC IIb trimer in wild-type barley, contained the PSII reaction center constituents (Peter and Thornber, 1991) and migrated close to a readily visible blue-green band containing CP 47 and CP 43 of the CC II antenna (Fig. 1). Below this band the mutant thylakoids had a yellow-green band that had the same migration rate as the LHC pigment-protein monomers in the wild type. Subsequent studies enabled us to identify the light-harvesting components that remained in the mutant and migrated in this band (see below). The free pigment band seemed to be relatively stronger in the mutant, which implies that those pigment-proteins that are synthesized might be less stable to detergent treatments. Although gentler solubilization conditions are available for mature wild-type thylakoids (Peter and Thornber, 1991; Dreyfuss and Thornber, 1994a, 1994b), the solubilization condition chosen to obtain the fractionations shown in Figure 1 is the optimum for the developing *chlorina f2* thylakoids. A sufficient solubilization of samples between 0 and 4 h could not otherwise be obtained (data not shown).

The nondenaturing PAGE pattern of green bands obtained from plastids greened for 4 to 24 h (Fig. 1) showed a gradual increase of the pigmented complexes during this period. A faint blue-green band in the PSI region appeared by 8 h, some monomeric pigment-proteins became visible at 12 h, and the band of CP 47 and CP 43 also appeared at this time. Later, the intensity of these bands became stronger, indicating a light-dependent accumulation. However, none of the monomeric LHCs in the mutant accumulated into oligomeric complexes as observed for LHC IIb and LHC I in wild-type barley (Dreyfuss and Thornber, 1994a, 1994b).

Expression of LHC I and LHC II Genes

LHC IIa-1, *pcab-2* (IIb), *LHC IIc*, *LHC I-20*, and *LHC I-21* cDNA clones (Chitnis et al., 1988; Morishige and Thornber, 1992a, 1992b; Anandan et al., 1993) were used to study the expression of their respective mRNAs during greening of 5-d-old etiolated mutant seedlings. The accumulation patterns of the steady-state mRNA levels for LHC IIa, IIb, IIc, and the two LHC Ibs of 20 and 21 kD (*Lhcb 4*, *Lhcb 1*, *Lhcb 5*, *Lhca 1*, and *Lhca 4*) are shown in Figure 2. The ethidium bromide stain shows equal loading of total RNA for each time point. The *Lhca* and *Lhcb* mRNAs began to accumulate after 1 to 2 h of illumination. Clearly seen is the light induction of the messages and the rhythmic accumulation, displaying maxima at 6 and 24 h. The steady-state levels of the mRNAs increased with longer exposure to light. This has been observed previously for the steady-state mRNA levels during greening of etiolated wild-type barley seedlings (Morishige and Thornber, 1992a; Anandan et al., 1993).

Accumulation of the LHC Apoproteins

One-dimensional SDS-PAGE analysis (Fig. 3) shows the polypeptide pattern of total leaf extract (Fig. 3A) and thylakoid membrane (Fig. 3B) samples used in Figure 1. Each loading was derived from an equal amount of leaf tissue, and the gels were either stained for protein or electrophoretically transferred for western blot analysis (Fig. 4). The LHC apoproteins are labeled according to their cross-reactivity with antibodies (Fig. 4; Table I). The total leaf extracts (Fig. 3A) show a remarkably consistent pattern, indicating that no substantial change occurred in the composition of the translated leaf proteins throughout the biogenesis time course. Differences were, however, observed when a subfraction of the leaf extract, the thylakoid membrane, was examined during the same period (Fig. 3B). The amount of the subunit I of PSI (*psa* A and *psa* B gene products) and of the 33-kD oxygen-enhancer protein

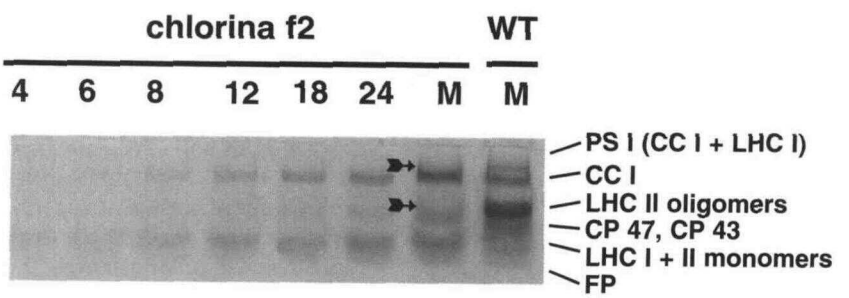

Figure 1. Fractionation of barley *chlorina f2* thylakoid pigment-proteins. Deriphat-PAGE of decyl-maltoside/octyl-thioglucoside/SDS-solubilized thylakoid membranes of 5-d-old etiolated seedlings that had been exposed to continuous light for 0 to 24 h and of solubilized thylakoid membranes from mature tissue of the mutant (M) and wild-type barley (WT) is shown. Each lane is loaded with 24 μL of solubilized thylakoids (15 μg of protein) (see "Materials and Methods" for further details). Arrows point out the major differences. FP, Free pigment band.

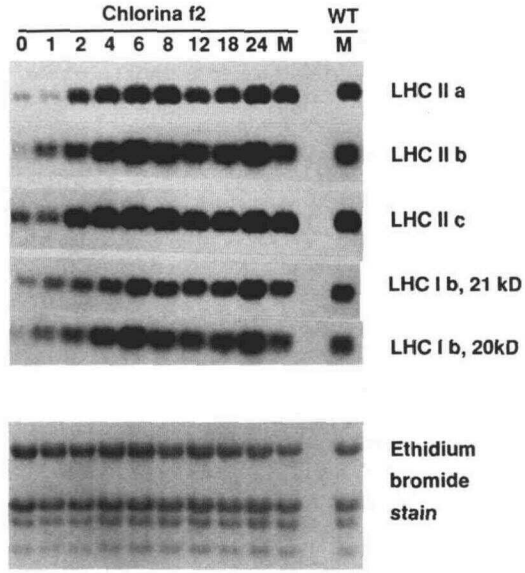

Figure 2. Northern blot analysis of total barley RNA probed with *Lhc* genes. The accumulation of the steady-state mRNA levels for LHC IIa, IIb, IIc, and the two LHC Ib's of 20 and 21 kD (gene products of *Lhcb 4, Lhcb 1, Lhcb 5, Lhca 1,* and *Lhca 4,* respectively) after exposure of etiolated *chlorina f2* seedlings to illumination (0–24 h) is shown. The mRNA levels of mature *chlorina f2* and of wild-type barley, grown for 5 d in the greenhouse, are shown in lanes labeled M. Equal amounts of RNA (4 μg) were loaded per sample as shown by ethidium bromide staining.

(OEE1) increased, reflecting an accumulation of CC I and CC II, respectively, as observed in Figure 1. A comparison of mature wild-type and mutant membranes showed a dramatic difference in polypeptide content in the 27- to 29-kD range, where the major LHC IIb polypeptides are to be found in the wild type, and in the 21- to 22-kD area, where the LHC Ib polypeptides are found. The lack or drastic depletion of these polypeptides seen in Figure 3B is indicated by arrows in Figure 5.

To identify more precisely which LHC polypeptides were depleted and which were stably integrated into the mutant's membrane, immunoblot analysis of the gels in Figure 3 was performed (Fig. 4). The cross-reactivity of the antibodies used are listed in Table I. Leaf tissue was frozen and ground in liquid N_2 immediately after illumination to prepare total leaf extracts. These preparations yielded sharper bands in western analysis than those from isolated thylakoid membranes. The data obtained (Fig. 4) show that all of the LHC apoproteins were translated and detectable in the total leaf extracts of etiolated mutant plants after 2 to 4 h in continuous light. Their amounts oscillated during the greening period with maxima occurring at 8 and 24 h (Fig. 4A); the 12- and 18-h times show a significantly lower level of all detectable LHC apoproteins. This oscillation was reflected in the steady-state mRNA levels (Fig. 2), except that the first maximum occurred 2 h earlier than for the proteins, indicating a lag between apoprotein and mRNA accumulation (Apel and Kloppstech, 1978). It is clear that some LHC apoproteins in the mature *chlorina f2* leaf tissue were present at almost the same level as in mature wild-type barley, whereas others were considerably decreased (see below for more details). This situation is very different from that in wild-type barley. The LHC apoproteins in wild-type barley total leaf extracts increased continuously during a 0- to 36-h time course, reaching a maximum after 24 h and remaining at this level upon further maturation; no decrease or pronounced oscillation was observed (Morishige and Thornber, 1992a; Anandan et al., 1993).

Accumulation of the Minor LHCs

The accumulation pattern for the minor LHC apoproteins in the mutant's thylakoid membranes (Fig. 4B) differed from that seen in its total leaf extracts (Fig. 4A). The MLH 12 antibody (Table I) detected their presence after 2 to 4 h of illumination in total leaf extracts, and thereafter they showed a rhythmic pattern of accumulation with maxima at 8 h and 24 h (Fig. 4A). In the mutant thylakoid membranes, as opposed to total leaf extracts, no oscillation, but rather a slow, gradual increase of the minor LHC apoproteins, occurred during the time course of greening (Fig. 4B). The CMp antibody was used to explore the accumulation pattern for the minor LHC IIa 31-kD apoprotein (CP 29 type 2, *Lhcb4*) and the LHC Ia 21.5-kD apoprotein (type 2, *Lhca2*) because of their weak reaction with the MLH 12 antibody. Both polypeptides reached their maximum amount at 24 h in greening mutant membranes. The MLH 12 antibody showed a substantial amount of the 29-kD LHC IIc (CP 29 type 1, *Lhcb5*) and the 26.5-kD LHC IIc' (seen as a strong band between 24 and 29 kD) in the mature mutant's thylakoids; the latter was identified as a novel LHC II protein by Morishige and Thornber (1994) (Fig. 4B). The 20-kD LHC IId subunit can also be detected in total leaf extracts with this antibody (Fig. 4A).

Accumulation of the LHC IIb Apoproteins

The MLH 9 blot of total leaf extracts of the mutant (Fig. 4A) showed the appearance of the LHC IIb apoproteins during greening, with its two largest apoproteins appearing at 2 h and the smallest 25-kD subunit being detectable at 4 h after the start of illumination. The apoproteins continued to accumulate, with maxima at 8 and 24 h. However, in the mature *chlorina f2* leaf tissue only the 25-kD LHC IIb apoprotein remained at a relatively abundant level. The other LHC IIb polypeptides were essentially undetectable. In the MLH 9 blot of the mutant thylakoid membranes (Fig. 4B), an oscillating pattern slightly different from that seen in the total leaf extracts was observed. The first maximum was sometimes not detectable or occurred at 12 h, and LHC IIb apoproteins could first be detected after 4 to 6 h of illumination rather than at 2 to 4 h as observed for the total leaf extract. They reached a maximum amount at 24 h, but in the mature thylakoid sample the larger LHC IIb apoproteins (types 1 and 2) were missing or greatly decreased, and only its 25-kD (type 3) subunit remained. The most drastic effect of the lack of Chl *b* on a single LHC apoprotein was demonstrated by the blots using the MLH 1 antibody, which reacted specifically with the largest LHC IIb (28 kD, type 1) apoprotein (Fig. 4). The data show that this apopro-

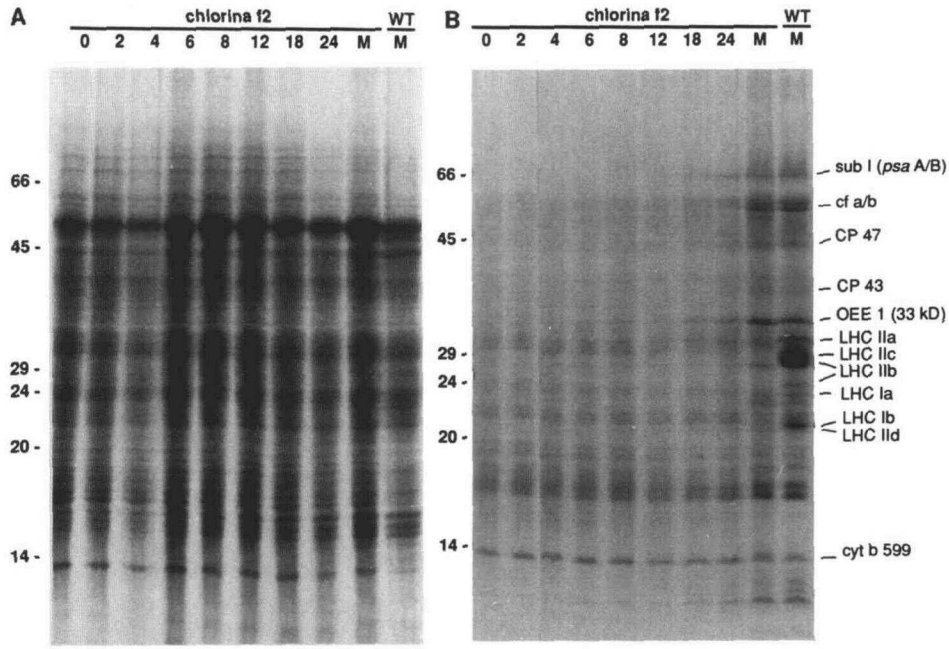

Figure 3. A, SDS-PAGE of detergent-solubilized total leaf extracts of the greening series (0–24 h). In each lane an equal amount of leaf tissue (25 μg of protein) was loaded. B, SDS-PAGE of solubilized thylakoid membranes of the same series. Gels are stained with Coomassie blue. The location of LHC I and LHC II apoproteins are marked, in addition to some CC I and CC II polypeptides. Molecular masses (kD) are marked at left. OEE1, Oxygen-enhancer protein 1; M, mature *chlorina f2*; WT, wild type.

tein was translated and detectable in the total leaf extracts but only at the 8- and 24-h maxima of the steady-state mRNA levels (Fig. 4A). At all other times this apoprotein was dramatically reduced in quantity, and it was not detected in the mutant's thylakoid membranes at any stage of greening (Fig. 4B). The antibody showed a strong signal with the respective wild-type control samples. Thus, although this apoprotein is translated in the mutant, it was also rapidly degraded and not stably integrated into the mature thylakoid membrane in the absence of Chl *b*.

Accumulation of the LHC I Apoproteins

Western blots using the P2 antibody showed that a situation similar to that for LHC IIb pertains for the larger LHC Ia 24-kD polypeptide and the two 20- and 21-kD LHC Ib polypeptides (Fig. 4). There was a greater decrease of the LHC Ib than the LHC Ia apoproteins in the mature mutant total leaf extracts, as well as in thylakoid membranes (Fig. 4). The early maximum of the LHC I apoproteins seen at 8 h in total leaf extracts was not reflected in the equivalent studies using isolated thylakoid membranes, and a delay of 2 to 4 h occurred before the apoproteins were detectable in the thylakoid membranes (Fig. 4). This delay could be due to a slower maturation of the import machinery or to a rapid degradation of the apoproteins before or after their insertion into the membrane (see "Discussion").

Two-Dimensional Analysis of the Subunits of the Pigmented LHCs

Two-dimensional analysis (Peter et al., 1991) of the proteins of mature wild-type barley and mutant thylakoid membranes is shown in Figure 5. The membranes were first separated by nondenaturing Deriphat-PAGE (see Fig. 1) and the resulting gel strip was subjected to SDS-PAGE to obtain a fingerprint of the composition of each pigmented band. Such a separation showed the subunit composition of the pigmented complexes, and in addition, their relative positions to each other. The positions of CC II polypeptides (OEE 1, D1 and D2, CP 47 and CP 43) as well as those of CC I subunits were unaffected in the mutant membranes. Some LHC apoproteins were still detectable, especially LHC IIa, the 25-kD (type 3) LHC IIb, LHC IIc/c', LHC Ia, LHC Ic, and small amounts of LHC Ib as previously shown by western blot analysis (Fig. 4). However, differences were observed in the position in which these LHC I and LHC II components migrate. In the mutant, there were no subunits of LHC IIb in the region corresponding to the trimeric form of LHC IIb in wild type. Those LHC Ia or Ib subunits that are stably synthesized were not retained with CC I to form a PSI holocomplex as in wild type. Thus, the corresponding holocomplex in the mutant migrates in a position very similar to that of the wild-type CC I complex (cf. Fig. 1). Arrows in Figure 5 point out these major differences.

77-K Fluorescence

The data above (Figs. 4 and 5) show that in the mutant the amount of the two LHC Ib apoproteins was greatly decreased and that of the LHC Ia subunits were decreased to a lesser extent. Because together these two complexes yield the longest wavelength fluorescence at 77 K (Bassi and Simpson, 1987), it was reasoned that low-temperature fluorescence of the greening mutant leaves should reflect

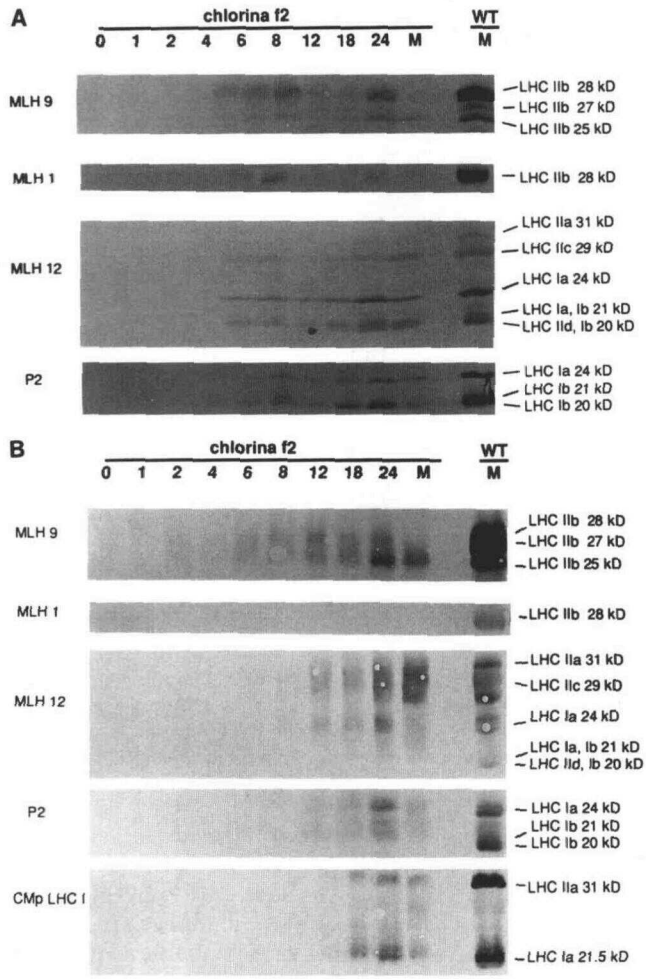

Figure 4. Western blot analysis of SDS-PAGE gels of total leaf extracts (A) and thylakoid membranes (B) of the greening series (see Fig. 3). The cross-reactivity of the antibodies used are listed in Table I. LHC I and LHC II apoproteins are labeled. M, Mature *chlorina f2*; WT, wild type.

their reduced content (Fig. 6). Our data show a long-wavelength emission shoulder at 722 nm during the early greening, presumably arising from CC I (Nechushtai et al., 1986). Starting at 4 h of greening, a gradual increase of the emission maximum centered at 732 nm occurs. It is clear that the 741-nm emission in the wild type is shifted to 732 nm in the mutant, indicating a lack or reduction of the longest wavelength emitters in the mature mutant leaves. Although there have been reports that LHC Ia fluoresces at 680 nm and LHC Ib at 735 nm at 77 K (Bassi and Simpson, 1987), our data indicate that LHC Ia might contribute most of the mutant's longer wavelength emission because it forms the bulk of the remaining LHC I components in *chlorina f2*. Supporting evidence that LHC Ia has a wavelength emission longer than 680 nm comes from a study of the *chlorina-104* barley mutant, which can be grown so that it is depleted in the 21.5-kD LHC Ia subunit (*Lhca* 2) when it displays a greatly reduced emission at 730 nm (Knoetzel and Simpson, 1991). The 688-nm peak in the samples reflects the presence of the PSII core antenna. The ratio of the longer (732 or 741 nm, respectively) to the shorter wavelength emission maxima (688 nm) is greater in the mutant concomitantly with reduced PSII antennae (mainly LHC IIb).

Table I. *Cross-reactivity of antibodies used in these studies*

Antibody	Specificity	Reference
MLH 9	25-, 27-, 28-kD LHC IIb	Darr et al., 1986
MLH 1	28-kD LHC IIb	Darr et al., 1986
MLH 12	Minor LHC IIs and LHC Is, but no reaction with LHC IIb	Darr et al., 1986
P2	24-kD LHC Ia, 20- and 21-kD LHC Ib	Williams and Ellis, 1986
CMp LHC I	21.5-kD LHC Ia, 31-kD LHC IIa	Høyer-Hansen et al., 1988

DISCUSSION

The mutation in *chlorina f2* of barley is most likely a single nuclear gene mutation, homozygous for a recessive gene controlling the accumulation of Chl *b* (Leverenz et al., 1992). The mutant's ability to synthesize Chl *a* and carote-

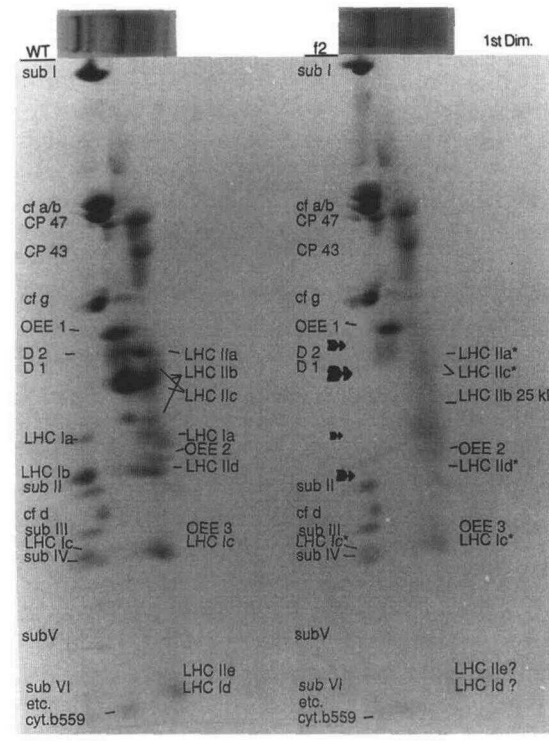

Figure 5. Two-dimensional PAGE of mature wild-type (WT) and *chlorina f2* membranes. Equal amounts of leaf tissue were loaded for electrophoresis in the first Deriphat-PAGE dimension (1st Dim.). Entire excised lanes or each excised pigmented band from Deriphat-PAGE was incubated in denaturing buffer (4% SDS, 5 mM EDTA, 75 mM Tris, pH 6.8, 200 mM DTT) for 15 min and then applied to the top of a SDS-PAGE gel after 90° rotation from the direction of electrophoresis in the Deriphat-PAGE. PSI and PSII subunits are marked. Arrows point out the major differences.

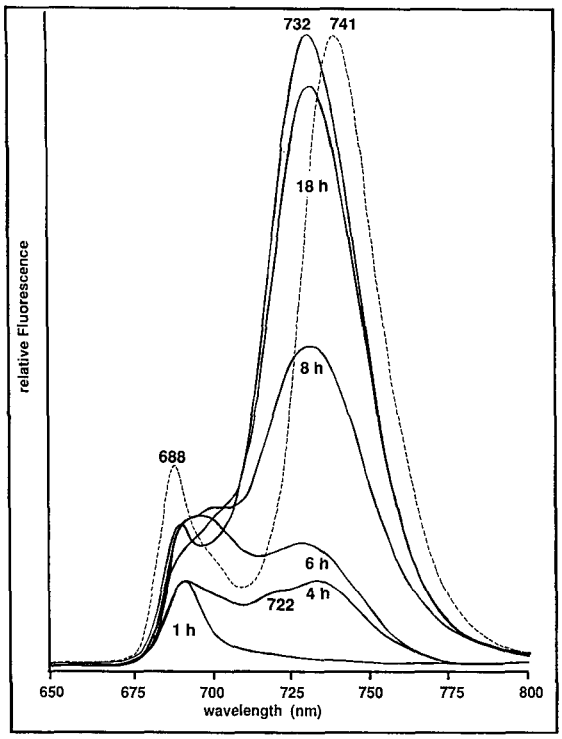

Figure 6. Fluorescence emission spectra at 77 K of mid-leaf sections from greening *chlorina f2* seedlings and of mature *chlorina f2* and wild-type barley plants. The emission maxima are indicated. The spectra of 1 and 4 h as well as 6 to 18 h were normalized to their 688-nm emission. The spectra of mature mutant leaves and wild-type barley (– – – –) were normalized to their long-wavelength emission maxima.

noids is not affected, and therefore, it has intact CC I and CC II units; furthermore, it has only a 10% lower quantum yield for PSII photochemistry but a higher sensitivity to photoinhibition (Leverenz et al., 1992). We studied this mutant to examine how a complete lack of Chl *b* alters the assembly of LHC I and LHC II, which of their respective apoproteins were present during biogenesis, and at which level regulation takes place.

All barley *Lhca* (LHC I) and *Lhcb* (LHC II) genes are transcribed and accumulate very soon after exposure of etiolated *chlorina f2* seedlings to light (Fig. 2). The various *Lhc* (*cab*) gene transcripts accumulated during a 24-h time course in a distinctive rhythmic pattern with maxima at 6 and 24 h. Previously observed 24-h periodic patterns of the *Lhc* (*cab*) gene mRNA accumulation, triggered by various light-dark treatments, have been attributed to a circadian clock controlling their expression (for a review, see Piechulla, 1993). Our etiolated mutant seedlings were not exposed to any light-dark cycles that would trigger a precise 24-h periodicity. Nevertheless, it seems likely that the accumulation pattern we observed reflects this transcriptional control mechanism (Fig. 2). Comparing the light-induced accumulation of the messages in the mutant with equivalent mRNAs in wild-type barley (Morishige and Thornber, 1992a; Anandan et al., 1993) shows very similar patterns. Despite the similarity shown for each of the clones we tested, there is a recent report that the *Lhca2* gene in wild-type *Arabidopsis* has a slightly different expression pattern that results in a significantly lower steady-state mRNA level (Zhang et al., 1994). This may also have an impact on the presence of its gene product, the 21.5-kD LHC Ia apoprotein, in the mutant.

The LHC I and LHC II apoprotein accumulation in total leaf extracts of the mutant during the period of greening shows that all apoproteins are translated and that their accumulation displays a rhythmic pattern similar to their mRNA levels in the mutant. This rhythmic pattern contrasts strongly with wild-type barley (total leaf extracts), in which a gradual increase of the apoproteins occurs during greening, reaching a maximum in mature samples (Morishige and Thornber, 1992a; Anandan et al., 1993). The mutant also differs markedly from the wild type in the abundance of each of the multiple LHC polypeptides if their polypeptide content at the 24-h time point is compared with that of the mature stage (Fig. 4). The lack of Chl *b* results in the complete absence of two, but not all, of the different LHC IIb apoproteins. The larger 28- and 27-kD subunits of LHC IIb can be detected only during the early stages of greening and are completely absent in the mature mutant. In contrast, the amount of the smallest 25-kD (type 3) LHC IIb subunit appears to be at or close to the wild-type level in the mutant (Fig. 4) (Peter and Thornber, 1991; Harrison and Melis, 1992). This 25-kD polypeptide is contained only in a multimeric subcomplex composed of LHC IIa, LHC IId, and a trimeric LHC IIb unit, which has two copies of the 28-kD subunits and one of the 25-kD subunit. The complex is thought to funnel light energy between the bulk LHC IIb (composed of the 28- and 27-kD polypeptides) and CC II (Dainese and Bassi, 1991; Peter and Thornber, 1991). The absence of the 28- and the 27-kD LHC IIb subunits in the mature mutant explains why no trimeric or higher oligomeric form of LHC IIb is present (Fig. 1).

Newly synthesized LHC I apoproteins appear in wild-type barley thylakoids primarily as monomeric pigment-protein complexes and form trimeric complexes during later stages of greening, which assemble with CC I to form a PSI holocomplex (Dreyfuss and Thornber, 1994b). The 77-K fluorescence emission maximum shifts from 722 to 741 nm and increases relative to the 688-nm emission during this transition. In the mutant both LHC Ib apoproteins are essentially absent, whereas the two LHC Ia subunits occur in slightly decreased amounts (Fig. 4). These changes in LHC I composition are reflected in the 77-K fluorescence spectra (Fig. 6), which have a long-wavelength maximum at 732 nm rather than at 741 nm as in wild-type barley. The PSI antenna size in *chlorina f2* is not as greatly decreased as that of PSII. A total of 150 Chl *a* molecules per PSI unit was calculated; 95 Chl *a* molecules are thought to be associated with CC I (Ghiradi et al., 1986; Harrison et al., 1993). Thus, a substantial number (55) of Chl *a* molecules have to be associated with the remaining LHC I units, mostly LHC Ia apoproteins according to our western blot analysis.

The effect of the lack of Chl *b* on the minor LHC IIs varies. Our data (Fig. 4) show that those minor LHC ap-

oproteins that are stably synthesized most likely contribute, together with the 25-kD LHC IIb subunit, to the pigmented monomeric LHC band (Fig. 1). Substantial amounts of the LHC IIa (CP 29) apoprotein were detected in the mature mutant membranes (Fig. 4B). Because this pigment-protein complex has the highest Chl *a/b* ratio of all LHC II complexes (Peter and Thornber, 1991; Morishige and Thornber, 1992b), it is perhaps a good candidate to survive in the absence of Chl *b* in the mutant. Substantial amounts of the 29-kD LHC IIc and 26.5-kD IIc' and the 20-kD LHC IId subunits are also present (Fig. 4). An interesting question is how the available Chl *a* molecules are distributed among the LHC II pigment-proteins. Of those 50 Chl *a* molecules remaining in PSII of the mutant, 37 of them are said to be associated with the core and 13 with the remaining LHC II polypeptides (Ghiradi et al., 1986; Harrison et al., 1993). Because Harrison et al. (1993) found only one copy of the LHC IIa apoprotein but three copies of the 25-kD LHC IIb apoprotein per three PSII reaction centers, they concluded that the latter binds most of the auxiliary Chl *a* molecules and is the first in the hierarchy of assembly; therefore, it must be the most proximal to the core. Thirteen Chl *a* molecules seem too few for a 1:1:1 ratio of a LHC IIb (type 3):LHC IIa:LHC IIc apoprotein that we propose occurs in the mutant, because all monomeric LHC units are likely to have at least seven to eight Chl *a* molecules per monomer (Lee and Thornber, 1995). Differences in growth conditions (particularly light intensity) may account for the low Chl *a* number and for the extent of stably synthesized LHC pigment-proteins in the mutant. We are currently studying the pigment content of the mutant thylakoid membranes during greening and have found major differences in xanthophyll content depending on the light intensity during biogenesis (S. Preiss and J.P. Thornber, unpublished data). Under identical growth conditions the mutant has, based on the Chl *a* level, a much lower neoxanthin level but higher violaxanthin and β-carotene levels than the wild type, reflecting the greater reduction in LHC II than in LHC I pigment-proteins; LHC I components lack neoxanthin. Another interesting question is whether Chl *a* can replace Chl *b* in the LH protein frame work and, therefore, stabilize the mutant's monomeric LHC complexes; but if so, why does such a replacement not work for LHC IIb and LHC Ib? Such a substitution could be important for stable assembly during early stages of greening (cf. Ito et al., 1993, and Tanaka et al., 1991).

In this study it is clearly seen that which LHC proteins are present depends on the stage of the mutant's chloroplast development. The LHC apoproteins accumulate during the early stages of greening in total leaf protein extracts but cannot be detected in protein extracts of mutant thylakoid membranes before 10 to 12 h of greening (Fig. 4). This could be the consequence of delayed maturation or an impaired import machinery, which could result in accumulation of soluble LHC proteins in the cytosol or stroma. Another possibility could be the rapid degradation of some of the proteins inserted into the membrane by specific proteases because of a lack of pigment co-factors at these early stages. We are left with the intriguing puzzle of why LHC pigment proteins differ in their turnover rate in the absence of Chl *b*. This applies particularly to the 25- (type 3) and the 28- and 27- kD LHC IIb subunits (types 1 and 2), all three of which show a high sequence similarity (80%) and highly conserved Chl-binding sites (Schwartz et al., 1991). One reason could be the absence of the first 12 amino acids in the type 3 subunit that are found in both types 1 and 2. This N-terminal region has multiple Arg and Lys residues, which could serve as destabilizing residues in a ubiquitin-dependent proteolysis according to the N-end rule (for review, see Varshavsky, 1992). In barley, ubiquitin molecules are encoded by a small multigene family (Callis and Viersta, 1989). We did a heterologous hybridization using a ubiquitin clone from *Arabidopsis* as a probe on total mRNA of the mutant greening series (data not shown). It showed slightly elevated ubiquitin mRNA levels during the first 2 h of greening and afterward a constitutive transcription level. In addition to the possibility of ubiquitin-dependent degradation, specific proteases could be involved (Hoober and Hughes, 1992). Such proteases could degrade those polypeptides that are not sufficiently stabilized by pigments and thereby have an important regulatory role during biogenesis of the photosynthetic apparatus. It has been proposed that LHC apoproteins can be degraded when new CC units are required and Chl synthesis is limited (Shimada et al., 1990; Tanaka et al., 1991). Although this could apply during early greening in the barley mutant, in the mature stage Chl *a* is available and yet the two largest LHC IIb and the LHC Ib apoproteins are turned over, indicating that they almost certainly need Chl *b* for stabilization (cf. Kim et al., 1994).

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