Investigation of the Lateral Light-induced Migration of Photosystem II Light-harvesting Proteins by Nano-high Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry*

Anna M. Timperio and Lello Zolla†‡
From the Department of Environmental Science, University of Tuscia, 01100 Viterbo, Italy

This study reports a detailed analysis of the light-induced lateral migration of the photosystem II (PSII) antennae between appressed and non-appressed thylakoid membranes. The relative PSII antennae that migrated to stroma lamellae were readily established on the basis of peak areas of the separated stroma proteins in the ultraviolet chromatograms. Phosphorylation was predicted by intact molecular mass measurements, and this was confirmed by immunoblotting. When thylakoid membrane and chloroplasts were illuminated at 100 μE m⁻²s⁻¹, light-harvesting complex type II (Lhcb2) was the first PSII antenna to migrate, preferentially in phosphorylated form. However, the amount of Lhcb2 that migrated decreased after the first 20 min when the total amount of the three different Lhcb1 isoforms (1.1, 1.2, and 1.3) reached maximum. Lhcb1.1 was always found in the unphosphorylated form and migrated later than the other two isoforms, although the latter were also found to have low levels of phosphorylation. At the same time, major antennae on the grana were not found to be phosphorylated, whereas Lhcb4 showed a significant increase in molecular mass. At higher light intensity Lhcb2 migration was negligible, whereas migration of Lhcb1 isoforms was little changed, increasing in irradiated chloroplasts. Because there was no significant phosphorylation at high light intensity, and yet pigments were found to have significantly increased on the stroma lamellae, it may be that pigments play a role in migration and that, in fact, there is no direct correlation between phosphorylation and migration. We hypothesize that the Lhcb1 isoforms expressed by the multigene families play a role in plant adaptation.

The main constituents of higher plants for the harvesting of solar energy and the light-driven electron transport of photosynthesis are contained in two main complexes of the thylakoid membranes: photosystem I and photosystem II (PSI and PSII, respectively). Photosystem I is localized in the stroma lamellae and peripheral membranes of the grana, whereas Photosystem II is localized mainly in grana appressions. Each photosystem is composed of several chlorophyll-protein complexes, most of which function as antennae that capture visible light (1). These are called light-harvesting chlorophyll a/b pigment proteins (LHCl and LHCl II, respectively, or Lhca and Lhcb). The major light-harvesting proteins of PSII have been designated Lhcb1, Lhcb2, and Lhcb3 (1, 2) on the basis of the nomenclature for the genes encoding these proteins (3), whereas other less abundant proteins have been called minor antenna proteins and designated Lhcb4, Lhcb5, and Lhcb6. LHCl II can form Lhcb1 homotrimers and Lhcb1/2 heterotrimers, which are believed to be a mobile complex (4, 5). Recently it has been demonstrated that Lhcb1 antenna proteins can exist in several very similar isoforms (6). Because specific isoforms are not well conserved between species, it is not clear whether they are redundant rather than having specific functions. The PSI antenna proteins are made up of four different chlorophyll/carotenoid binding polypeptides (Lhca1–4) that are organized into dimers (7, 8).

To optimize photosynthetic performance and to avoid damage when exposed to excess light, plants must balance the excitation of the two photosystems (9–11). When photosystem II is favored, a mobile pool of light-harvesting complex II moves from photosystem II to photosystem I. The dissociation of a mobile subpopulation of the phospho-LHCl II from PSII and its subsequent lateral migration from the appressed grana membranes to the PSI containing stroma-exposed membranes (12) are thought to be important for balancing the excitation energy, protection against photodestruction of PSI, and aclimation of the photosynthetic apparatus in plants (12). This short term and reversible redistribution is known as a state transition. It is associated with changes in the phosphorylation of light-harvesting complex II, but the regulation is complex. Two models have been proposed to explain the movement of LHCl II. According to one model, alteration in the surface charge upon phosphorylation leads to structural changes of the thylakoid membrane and results in the movement of phospho-LHCl II away from the grana stacks (13–15). The addition of negatively charged phosphate groups to the LHCl II complex causes it to dissociate from PSI and favors its migration out of the appressed regions of the grana and into the stroma lamellae, where PSI centers are located (16). This indicates that the correlation between LHCl II phosphorylation and state transition is complex. According to another model, the net movement of LHCl II toward PSI in State 2 is caused by PSI having a higher affinity for unphosphorylated LHCl II and PSI a higher affinity for phospho-LHCl II; therefore, movement of phospho-LHCl II is a question of molecular recognition (11). Although the models differ in the way of explaining state transitions, they both involve phosphorylation of LHCl II as a prerequisite for the initiation of State 1-State 2 transitions.

The purpose of the present work was to better characterize...
and quantify which type of chlorophyll proteins, comprising the isoforms, are involved in the migration and consequently in the regulation of energy distribution. For this purpose both spinach chloroplasts and thylakoid membrane were subjected to varying light stress over different time periods. Stroma and grana lamellae were then isolated using digitonin, and their protein composition was analyzed by nano-HPLC coupled with mass spectrometry. Through this technique it has been possible to identify and quantify which type of Lhc1 isomer is involved in the migration and whether it exists in the phosphorylated form. Differences were observed at low and high light intensity.

**Experimental Procedures**

**Chemicals—**Reagent-grade sodium chloride, magnesium chloride, sorbitol, N-tris(hydroxymethyl)methylglycine (Tricine), tris(hydroxymethyl)aminomethane (Tris), natrium fluoride, triﬂuoroacetic acid, methanol, ethanol, formamide, as well as HPLC-grade water and acetonitrile, were obtained from Carlo Erba (Milan, Italy). Digitonin was obtained from Sigma, acrylamide, N,N’-methylene-bis-acrylamide, and all other reagents for SDS-PAGE were purchased from Bio-Rad.

**Plant Material and Growth Conditions—**Hydropnic cultures of spinach (Spinacia oleracea L.) were grown in a greenhouse at a photon flux density of 400 μmol m⁻² s⁻¹ with 10% light/14% dark hours at 25 °C. Whole leaves were used in the experiments.

**Isolation of Intact Chloroplasts—**Well-developed spinach leaves were used to isolate intact chloroplasts using the method of Walker et al. (17) with minor modifications. Briefly, chloroplast isolation procedures were carried out at 4 °C. Spinach leaves (10 g), grown under normal light conditions and harvested in the middle of the photoperiod, were chopped using a blender in 50 ml of extraction buffer (0.3 mM sorbitol, 50 mM HEPES/ KOH, pH 7.9, 5 mM MgCl₂, 2 mM isocitrate). The homogenates were then filtered through 100-mm meshes, followed by centrifugation at 1006 × g for 3 min. To enrich for chloroplasts, the pellets were resuspended in 2 ml of extraction buffer and precipitated again at 10006 × g for 7 min. Chloroplasts were further purified by isopycnic centrifugation using 50% Percoll gradients (18).

**Isolation of Chloroplast Thylakoids—**Chloroplast thylakoid membranes (PSII membranes) were isolated from spinach leaves according to the method of Berthold (19) with the following modifications. Leaves were powdered in liquid nitrogen and subsequently homogenized in an ice-cold 20 ml Tricine pH 7.8 buffer containing 0.3 μl sucrose and 5.0 mM magnesium chloride (B1 buffer). The homogenization was followed by filtration through one layer of Miracloth (Calbiochem) centrifuged at 4,000 × g for 10 min at 4 °C. Pellets were suspended in B1 buffer and centrifuged as above. This second pellet was resuspended in 20 ml Tricine pH 7.8 buffer containing 70 mM sucrose and 5.0 mM magnesium chloride (B2 buffer) and centrifuged at 4,500 × g for 10 min. Pellets containing the thylakoid membranes were then resuspended in 50 mM MESS pH 6.3 buffer containing 5 mM chloride and 5 mM magnesium chloride (B3 buffer) at 2.0 mg of chlorophyll/ml. The concentration of chlorophyll was determined according to the method described by Porra (20).

**Light Treatments of the Chloroplast and Thylakoid Membranes—**Migraion of PSII antenna were induced in chloroplast thylakoid membranes and in chloroplasts, at 0.2 mg of chlorophyll/ml, by exposure to 100, 500, and 1000 μE m⁻² s⁻¹ from a metal halide HQI-T 250W daylight lamp that served as a light source in a temperature-controlled glass cuvette at 4 °C with gentle stirring. Chloroplasts were gently resuspended in an incubation buffer containing 330 mM sorbitol, 20 mM Tricine (pH 8.6), 6.6 mM MgCl₂, 1 mM Na₂HPO₄, whereas isolated thylakoids were resuspended in 20 mM Tricine (pH 8.6), 100 mM sorbitol, 5 mM MgCl₂, 1 mM ATP, and both were irradiated as described above.

**Separation of Grana/STroma Lamellae—**Thylakoid membranes and chloroplasts were resuspended to a concentration of 200 μg of Chl/ml and 55 μg of Chl/ml, respectively, in 20 mM Tricine (pH 7.8), 0.1 mM sorbitol (0.3 μl for chloroplast), 10 mM NaCl, 5 mM MgCl₂, 10 mM NaF. Recrystallized digitonin (1% in water) was added to the stirred membranes to give a final concentration of 0.4%. The 2-min detergent treatment was terminated by a 10-fold dilution of the sample with resuspension buffer at 0 °C. Differential centrifugation according to Anderson and Boardman (21) yielded pellets following 1,000 × g for 10 min, 10,000 × g for 30 min, 40,000 × g for 30 min, and 144,000 × g for 60 min. The centrifuged fractions were injected directly onto the column without any further sample pretreatment.

**Nano-High Performance Liquid Chromatography and Electrospray Mass Spectrometry—**Liquid chromatography was carried out at 200
For silver staining, gels were fixed in 50% (v/v) methanol-water and 10% (v/v) ethanol-water solutions, stained with 0.1% (w/v) silver nitrate-water solution, and developed in 3.5% (w/v) aqueous sodium carbonate containing 0.05% (v/v) formamide.

Detection of Thylakoid Phosphoproteins by Polyclonal Thr(P) Antibody—Following electrophoresis, the polypeptides were transferred to an Immobilon-P membrane (Millipore), which was blocked with 1% bovine serum albumin (fatty acid; Sigma). Phosphoproteins were immunodetected using an Immun-Lite assay kit (Bio-Rad). Three different commercial antibodies to phosphothreonine were tested: rabbit polyclonal antibody and monoclonal antibody (Zymed Laboratories Inc. and mouse monoclonal anti-phosphothreonine (Sigma).

Pigment Determination by High Performance Liquid Chromatography—Thylakoid membrane or chloroplasts were frozen in liquid N₂ immediately after sampling. Pigments were extracted from samples under low and high light by grinding in liquid N₂ followed by grinding in degassed 100% acetone at 0–4 °C. On occasion a small amount of NaHCO₃ (≈0.1 g g⁻¹ plant material) was added to plant material before extraction to ensure no acidification of the extract occurred; however, this had no influence on carotenoid composition. The pigment extracts were filtered through a 0.2-mm membrane filter and applied immediately to an HPLC column. Pigment composition was determined by HPLC by using a Waters Nova-Pak C18 radial compression column according to Johnson et al. (23). Additionally a Spherisorb ODS-1 column (5-mm particle size, 250 × 4.6 mm ID) was used based on the method by Gilmore and Yamamoto (24). Pigment concentrations were calculated from the respective peak areas at 440 nm. Before injection, however, samples were normalized on the basis of chlorophyll a and b concentration evaluated by spectrophotometric absorption.

RESULTS

To investigate qualitatively and quantitatively which type of PSII antenna protein is involved in light-induced migration and over what time period this occurs, spinach thylakoid membrane or chloroplasts were illuminated at different light intensities. The analysis consisted of separating proteins by nanoreversed-phase liquid chromatography (nano-RP-HPLC), interfaced to mass spectrometry (MS) with an electrospray (ESI) ion source (nano-RP-HPLC ES-MS) to identify them.

Both thylakoids and chloroplasts were irradiated at different light intensities in the presence of the phosphatase inhibitor NaF, in order to block dephosphorylation during sample ma-

![Reconstructed ion chromatogram of the antenna proteins in stroma fraction by using nano-RP-HPLC (conditions as in Fig. 1). Insets A–C show the ESI spectra and the relative deconvolution analysis of mass spectra. The column was interfaced with an ion trap mass spectrometer Esquire 3000 plus, 200 nl/min; detection, ESI-MS; scan 500–2000 atomic mass units; injection volume, 5 μl.](image)
Manipulation, and then assayed for phosphorylation capacity (25). The actual distribution of the various forms of PSII complexes in the thylakoid membrane was obtained using digitonin, which causes specific and rapid (<3 min) cleavage of the thylakoid membrane (26) into grana and stroma lamellae. Recent studies that used this technique found that grana membranes were strongly enriched in PSII dimers and PSI-LHCCI supercomplexes, whereas only a small amount of PSI antenna proteins and traces of PSI complexes were present (26). In the stroma thylakoids, the PSI complexes as well as the various free LHCCI subassemblies were in the minority, whereas the PSI complexes and ATP synthase were the dominating membrane protein complexes (27, 28). The stroma lamellae from leaves kept in darkness overnight were found to contain a small amount of PSII Lhcb1 antennae, estimated as 10% of the Lhca3 present; the latter was chosen as an internal reference (26), whereas Lhcb2 levels were negligible. Thus the heavier grana membranes were easily and quickly separated from the lighter stroma membranes, which were recovered from the supernatant. Grana and stroma lamellae were separately injected onto a capillary C4 column and chromatographed using a linear water-acetonitrile elution gradient in trifluoroacetic acid. Because antenna proteins from PSI have different elution times than PSII antennae, it was easy to determine the relative amount of each. Further confirmation of protein identity in each UV trace was obtained by deconvolution analysis of each reconstruction ion current chromatogram peak (26). This method allowed rapid separation and identification of stroma and grana lamellae proteins with few manipulations, while the relative distributions of the chlorophyll-protein complexes derived from appressed (grana) and non-appressed (stroma) regions, before and after illumination of leaves, were determined from the area underlying each chromatographic peak.

Antennae Migration upon Illumination of Thylakoid Membranes

Illumination at 100 \( \mu E \, m^{-2} \, s^{-1} \) — In a pilot investigation thylakoids were irradiated at 100 \( \mu E \, m^{-2} \, s^{-1} \) for 20 min in the presence of ATP 0.4 m at 4 C to obtain the maxima antenna migration, in accordance with work reported by Rintamaki (25). Fig. 1 compares the chromatogram recorded by nano-HPLC at 280 nm after injection of stroma proteins extracted from control or light-stressed thylakoids. A significant increase was recorded in peaks corresponding to Lhcb1 (Lhcb1.1, Lhcb1.2, and Lhcb1.3, respectively) after illumination. Although leaves kept in darkness overnight contained a small amount of Lhcb1 (~10%) (26), the amount increased significantly upon illumination. Interestingly, a new peak appeared near Lhca3, which deconvolution analysis (see later) showed to be PSI Lhcb2. It is worth emphasizing that it was barely detected in stroma lamellae kept in darkness overnight.

Fig. 2 shows the reconstructed ion current that was recorded simultaneously with the UV trace upon injection of the stroma membrane fraction. As previously shown (26, 27), most of the UV peaks had a corresponding peak in the reconstructed ion chromatograms, so it was possible to identify each protein by deconvolution analysis of ESI spectra and determine whether it had undergone any chemical modifications. Deconvolution analysis of reconstruction ion current (RIC) spectra corresponding to Lhcb2 (Fig. 2C, inset) showed that two proteins were present with a difference in molecular mass of 80 Da (24,760 and 24,839, respectively). These are probably the phosphorylated and unphosphorylated forms of Lhcb2, because the difference in molecular mass corresponds to a single phosphorylation. Interestingly, with deconvolution analysis of ESI spectra it is possible to estimate approximately the amount of phosphorylated protein present as a percentage of the total protein by comparing the intensity of deconvolution of each protein. In the case of Lhcb2, it was ~70% phosphorylated (see Fig. 2C, inset), suggesting that most of this protein migrated in this form. Regarding the Lhcb1, reversed phase of Lhcb1 differentiated two peaks, the first one containing the Lhcb1.1 isomeric form and the second the isomers Lhcb1.2 and Lhcb1.3, respectively (29). Deconvolution analysis of reconstruction ion current spectra revealed that Lhcb1.1 (Fig. 2A, inset) was not phosphorylated upon illumination, whereas both Lhcb1.2 and Lhcb1.3 had phosphorylated forms (Fig. 2B, inset), displaying the characteristic increase in molecular mass of 80 Da (Lhcb1.2: 24,936.7 and 25,019; Lhcb1.3: 25,006 and 25,083). However, in contrast to Lhcb2, the phosphorylated form of both Lhcb1.2 and Lhcb1.3 is <10% of the total protein (see Fig. 2B, inset).

To confirm that these 80-Da mass increases actually represent protein phosphorylation, the stroma proteins were run on semi-preparative RP-HPLC in a butyl silica column to separate the main peaks, which were collected, lyophilized, and probed with phosphothreonine antibody. The latter was performed by dissolving fractions containing the major antennae Lhcb2, or Lhcb1.1, or Lhcb1.2 and Lhcb1.3, and Lhcb4 in 120 mM TRIS/HCl pH 8.45 buffer containing 5 μl urea, and 4% (w/v) SDS, which were then analyzed by SDS-PAGE using according to the method reported by Schagger (22) (Fig. 3A). Following electrophoresis, the gels were either silver stained or transferred to nitrocellulose and incubated with a phosphothreonine antibody (Fig. 3B). As expected, the antibody did not bind to Lhcb1.1, whereas a signal was detected for Lhcb2 and the two isomers Lhcb1.2 and Lhcb1.3; therefore, measuring differences in intact molecular mass appears to be a reliable way of identifying proteins and their post-translational phosphorylation. This analysis also confirmed previous observations of relative phospho-protein concentrations derived from immunoblots of total thylakoid membrane (30) and intact molecular mass measurements in the present study, because the Lhcb2 (70% phosphorylated) band...
stained much more intensely with Coomassie than the Lhcb1.2 and Lhcb1.3 bands (10%). Only traces of PSII Lhcb3 were found on illuminated stroma lamellae, and no minor antenna proteins have ever been detected.

We then went back to the grana lamellae, which were present in the bottom of centrifuged thylakoid after digitonin treatment, and analyzed them as above. Comparison of HPLC chromatograms from control or stressed grana did not reveal any significant differences in terms of peak number and relative stoichiometry (data not shown). This was partly because the sample was strongly diluted before injection onto a column, so it was difficult to differentiate a small decrease in the total amount of major antennae that migrated, and also because very little of the major antennae migrate anyway. Moreover, in stressed grana the deconvolution analysis of ESI spectra showed that only the minor antenna Lhcb4 or CP29 (Fig. 4D, inset) had an increased (≈35% of total protein) molecular mass of 110 Da, suggesting the presence of post-translational modifications on the apoprotein. Immunoblotting of isolated Lhcb4 (see Fig. 3B) revealed the presence of phosphorylation, indicating that the apoprotein at this light illumination contains only one phosphorylation and some other post-translational modification(s).

Time Course of Antennae Migration—To collect information about the time course of antennae migration, stroma lamellae from thylakoid membrane illuminated at 100 μE m⁻²s⁻¹ were analyzed after different periods of illumination. Fig. 5A shows the chromatograms recorded, whereas Fig. 5B summarizes the evaluation of the percentage of migration of a given substance that has been performed using the abundance of the corresponding chromatographic peaks. However, the absolute values of the abundance cannot be directly compared, because of the different response of the detector in different runs. Therefore, the absolute abundance has been scaled by a normalizing factor obtained by comparing the absolute abundance in different runs of PSI Lhca2 whose concentration remains practically unchanged after the “stressing” procedure. It can be seen that after the onset of illumination Lhcb2 and all Lhcb1 isoforms started to migrate. Lhcb2 reached its maximum concentration after 20 min and then the amount of protein decreased, whereas all Lhcb1 isoforms continued to migrate, reaching a concentration plateau after 30 min. Unfortunately, in this investigation it was not possible to evaluate whether the decrease in Lhcb2 after the first 20-min illumination period was because of its degradation or because it moved back to the grana, which may explain why there was no significant information from the concomitant analysis of grana. Interestingly, at low light intensity the phosphorylatable Lhcb1.2 and Lhcb1.3 accumulated more quickly than the unphosphorylatable Lhcb1.1, which required a significantly longer illumination time to migrate.
Antennae Migration at Different Light Intensities—To investigate how the intensity of light can influence antennae migration, we analyzed both stroma and grana lamellae from thylakoid membranes illuminated at different light intensities. Fig. 6A shows the chromatograms recorded after 20 min when the maxima migration occurred, under illumination at 100, 500, and 1000 µE m⁻²s⁻¹. Fig. 6B shows the relative amounts of Lhcb1, Lhcb1.2, Lhcb1.3, and Lhcb2 migrated. It can be observed that Lhcb2 migrated significantly at low light intensity (100 µE m⁻²s⁻¹), whereas its concentration had decreased at 500 and was even less at 1000 µE m⁻²s⁻¹. However, very little (~2–3%) of the Lhcb2 found at this high intensity was phosphorylated (data not shown). Similarly, Lhcb1 isoforms decreased as light intensity increased but not as dramatically as...
Lhcb2, indicating that at high light intensity Lhcb1 isoforms are the predominant PSII antennae involved. At this high light intensity the Lhcb4 was still post-translationally modified on the grana lamellae, though it was less than 10%.

**Antennae Migration upon Illumination of Chloroplasts at Different Light Intensities**—To investigate whether the thylakoid membrane reacts to light in the same way in vivo as it does in isolation, we extended our study to chloroplasts. Thus, chloroplasts from dark-adapted mature spinach leaves were irradiated at different intensities for 1 h, and the relative amounts of PSI major antennae present on stroma lamellae were determined using the method reported above. Fig. 7 shows the migration of PSI major antennae upon chloroplast illumination. At low light intensity, accumulation of Lhcb2, Lhcb1.1, and Lhcb1.2 and Lhcb1.3 on stroma lamellae is roughly similar to levels observed in illuminated thylakoid membranes. At high intensity Lhcb2 decreases, whereas Lhcb1 after a mild decrease at 500 µE m⁻² s⁻¹ accumulates again at 1000 µE m⁻² s⁻¹. Thus, in sharp contrast to that observed in thylakoids, high light intensity causes Lhcb1 to migrate. Moreover, at low intensity both Lhcb2 and Lhcb1 are phosphorylated, whereas at high light intensity the phosphorylation is negligible for both (<5%). Interestingly, at high light intensity the non-phosphorylatable isoform Lhcb1.1 is the most predominant Lhcb1 antenna on stroma lamellae of illuminated chloroplasts, suggesting that some stromal components, rather than a membrane compound, are involved in this high intensity light-induced migration process. Thus, a different process than phosphorylation is more active in intact chloroplasts.

Bearing this in mind, it is interesting that upon illumination of thylakoid membrane at 1000 µE m⁻² s⁻¹ the total pigments (neoxanthin, lutein, violaxanthin) on stroma lamellae, determined by C18 column, increase significantly with respect to native protein, is a good indication of the presence of the increased molecular mass of 80 Da, or multiples thereof with respect to native protein. Hence, the increased pigment concentration either, indicating that the extra pigment is not produced in the stroma lamellae itself but probably occurs as a consequence of Lhcb1 migration. Illumination of chloroplasts at 1000 µE m⁻² s⁻¹ resulted in an increase of xanthophyll cycle pigments, and the reversible conversion of the carotenoid violaxanthin (V) via antheraxanthin (A) to zeaxanthin (Z) (Fig. 8B).

**DISCUSSION**

Mobile LHCII is well known to be implicated in the regulation of excitation energy distribution between appressed and non-appressed thylakoid membranes during the dynamic mechanism that enables plants to respond rapidly to changes in illumination (state transitions). However, detailed information on the time course and which type of Lhcb1 isoform is involved and to what extent have never been investigated. In this report, lateral distribution of the PSII major chlorophyll-protein antennae has been analyzed by nano-RP-HPLC, interfaced to mass spectrometry with an ESI ion source. With this technique it has been possible to obtain the separation and simultaneous identification of the different major PSII antennae, including the different Lhcb1 isoforms, allowing one to analyze differential PSII antennae migration as a function of time and light intensity. The investigation was carried out using digitonin as a detergent, so that the grana stacks could be quickly separated from the stroma lamellae and then the proteins of each rapidly separated and identified using reversed-phase column (26). The advantage of this analytical method is that PSI antenna proteins elute as well resolved peaks with highly repeatable retention times (31) and the PSII antenna proteins have different elution times (32). Simple and rapid identification of each antenna on stroma lamellae was possible from molecular mass measurements because of the different molecular masses occurring between PSI and PSII antennae (26). Furthermore, the intact molecular mass measurements also provided reliable indications on the presence or absence of post-transcriptional modification, like phosphorylation that was confirmed by immunoblotting analysis performed on isolated antennae. The increased molecular mass of 80 Da, or multiples thereof with respect to native protein, is a good indication of the presence of a phosphoryl group on the protein and represents a potential...
Lhcb1.2 and Lhcb1.3 increase more than the Lhcb1.1 isoforms. The phosphorylable isomeric forms are not phosphorylated at low light, where dephosphorylation is reduced. When Lhcb2 is used as the major PSII antenna protein to be phosphorylated (29). Lhcb2 migrated preferentially in its phosphorylated form, thus indicating that a superimposed regulatory mechanism from the antenna phosphorylation seems to come into play, causing the PSII antennae to migrate from grana to stroma lamellae at increasing light intensity. In another report (33), the in vivo studies yielded contrasting results. Stroma lamellae coming from stressed thylakoid at 1000 μE m⁻² s⁻¹ had a higher level of pigments that come from the migrated PSII Lhcb1 antennae, corroborating the hypothesis that at higher light intensity the pigments have a major role both in Lhcb1 migration and in the energy balance. Moreover, in chloroplast, the prevalent antenna found was the Lhcb1.1 isomer, which as stated above does not contain any phosphorylation site. In fact, Lhcb1.1, 1.2, 1.3 share almost identical amino terminal sequences in which only one Ser is mutated to Thr in the third amino acid from the amino terminal end (29). The absence of a threonine enables post-translational modification by phosphorylation, which may be important for migration at low light intensity. Thus, it may be speculated that the higher accumulation of Lhcb1.1 upon illumination of chloroplast with respect to thylakoids is evidence that this isomeric Lhcb1 form is the favorite target.

It is not yet known whether pigments have a differential affinity for different Lhcb1 isoforms as well as for Lhcb2. This work is under way in our laboratory.

No Correlation between Phosphorylation and Migration and Changes in the Association-Dissociation State of LHCII—In our study, carried out both in vitro and in vivo, no direct correlation was found between migration and LHCII phosphorylation. Table I summarizes the percentage of PSII antennae migration observed at different intensities of light. At low light intensity Lhcb2 migrated preferentially in its phosphorylated form, whereas most of the Lhcb1 proteins migrated with little or no phosphorylation. At high intensity light, migrated LHCII proteins contained a higher pigment concentration and a low phosphorylation. Consequently, the phosphorylation was not involved in the energy balance but the pigments seemed to have a major role, especially in intact chloroplast where the xanthophyll cycle is more active. Thus the LHCII migration during state transitions cannot be explained sufficiently by different affinities of phosphorylated and unphosphorylated LHCII for PSI but is likely to involve structural changes in thylakoid organization. Probably, according to Zhang et al. (34), the phos-

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**TABLE I**

| Source       | Photon flux density (μE m⁻² s⁻¹) | Antenna type | Migration | Phosphorylation | Ratio of pigment/ chlorophyll a and b |
|--------------|---------------------------------|--------------|-----------|----------------|--------------------------------------|
|              |                                 |              |           |                | Neoxanthin, Xanthophyll, Lutein       |
| Thylakoid    | Control                         | Lhcb 1.1     | 5         | 0              | 0.03, 0.13, 0, 0.41                   |
|              |                                 | Lhcb 1.2; 1.3| 5         | 0              |                                      |
|              |                                 | Lhcb 2       | 0         | 0              |                                      |
| 100          |                                 | Lhcb 1.1     | 26        | 0              | 0.13, 0.20, 0, 0.52                   |
|              |                                 | Lhcb 1.2; 1.3| 23        | 10             |                                      |
|              |                                 | Lhcb 2       | 11        | 70             |                                      |
| 1000         |                                 | Lhcb 1.1     | 17        | 0              | 0.53, 0.56, 0, 0.68                   |
|              |                                 | Lhcb 1.2; 1.3| 11        | 0              |                                      |
|              |                                 | Lhcb 2       | 2         | 3              |                                      |
| Chloroplast  | Control                         | Lhcb 1.1     | 5         | 0              | 0.08                                 |
|              |                                 | Lhcb 1.2; 1.3| 5         | 0              |                                      |
| 1000         |                                 | Lhcb 2       | 0         | 0              | 0.09, 0, 0, 0.11                      |
|              |                                 | Lhcb 1.1     | 22.2      | 0              | 0.02, 0.15, 0.02, 0.03, 0.23          |
|              |                                 | Lhcb 1.2; 1.3| 16        | 0              |                                      |
|              |                                 | Lhcb 2       | 6.15      | 0              |                                      |

Pigment content of stroma lamellae from thylakoid and chloroplast stressed at different intensities of light

Pigments were extracted with acetone and separated by HPLC. Pigments were calculated from the respective areas at 440 nm as the quotients of Chl a and b. Estimations of migration and phosphorylation percentages are reported under “Results.”

| Source       | Photon flux density (μE m⁻² s⁻¹) | Antenna type | Migration | Phosphorylation | Ratio of pigment/ chlorophyll a and b |
|--------------|---------------------------------|--------------|-----------|----------------|--------------------------------------|
|              |                                 |              |           |                | Neoxanthin, Xanthophyll, Lutein       |
| Thylakoid    | Control                         | Lhcb 1.1     | 5         | 0              | 0.03, 0.13, 0, 0.41                   |
|              |                                 | Lhcb 1.2; 1.3| 5         | 0              |                                      |
|              |                                 | Lhcb 2       | 0         | 0              |                                      |
| 100          |                                 | Lhcb 1.1     | 26        | 0              | 0.13, 0.20, 0, 0.52                   |
|              |                                 | Lhcb 1.2; 1.3| 23        | 10             |                                      |
|              |                                 | Lhcb 2       | 11        | 70             |                                      |
| 1000         |                                 | Lhcb 1.1     | 17        | 0              | 0.53, 0.56, 0, 0.68                   |
|              |                                 | Lhcb 1.2; 1.3| 11        | 0              |                                      |
|              |                                 | Lhcb 2       | 2         | 3              |                                      |
| Chloroplast  | Control                         | Lhcb 1.1     | 5         | 0              | 0.08                                 |
|              |                                 | Lhcb 1.2; 1.3| 5         | 0              |                                      |
| 1000         |                                 | Lhcb 2       | 0         | 0              | 0.09, 0, 0, 0.11                      |
|              |                                 | Lhcb 1.1     | 22.2      | 0              | 0.02, 0.15, 0.02, 0.03, 0.23          |
|              |                                 | Lhcb 1.2; 1.3| 16        | 0              |                                      |
|              |                                 | Lhcb 2       | 6.15      | 0              |                                      |
pho-LHCII seems to show higher affinity for PSI because the phosphorylation causes structural changes in the thylakoid membranes, which promote movement of LHCII, in both its phosphorylated and unphosphorylated form.

Regarding the association-dissociation state of LHCII, it is already known that the LHCII proteins, Lhcb1, Lhcb2 and Lhcb3, form homo- or heterotrimers (35). Studies by electron microscopy and image analysis have revealed the presence of supercomplexes consisting of trimeric LHCII in three different types of binding position (35). It may be that the different trimers, which form super- and mega-aggregates, consist of heterotrimers (Lhcb2/Lhcb1) or homotrimers containing different isoform Lhcb1 proteins. In a previous report (6) we assumed that the differences in hydrophobicity present in the first part of the amino terminus of the three Lhcb1 subpopulations play an important role in the interaction of different Lhcb1 isoforms to form supramolecular aggregates. Thus, the differences in primary structure can provide a modulation of the physiological effect of LHCII proteins, which have distinct topological locations within the PSII supramolecular complexes.

Changes in the association-dissociation state of LHCII are thought to part of the regulatory mechanisms that optimize light-harvesting function under differing light conditions (36, 37). In particular, trimeric LHCII quenches more slowly and to a lesser extent than monomeric antennae (38). Light-induced monomerization of LHCII trimers has also been reported (39), although the physiological role of this is uncertain. Therefore, it is not surprising that monomerization of trimers may be induced either by phosphorylation or by a change in pigment contents. Heterotrimers or different homotrimers dissociate differently depending on the presence in the heterotrimer of Lhcb2, which is phosphorylated more rapidly, or Lhcb1 isoforms, which may or may not be phosphorylated. Migration also takes place without phosphorylation, and if Lhcb1 isomers have a different affinity toward pigments dissociation of trimers and consequent migration may be affected as a result.

An apparent functional difference among different isoforms may provide an understanding of the biological significance of different trimerizations, and the different migration behaviors of the three Lhcb1 isoforms reported in this study may be at the root of a possible explanation for the existence and maintenance of the numerous multigene families in the photosystem apparatus and provide an answer to the question of why several genes encode isoforms of the same polypeptides. When plants adapt to different light intensities some genes may overexpress; consequently, the amount of some heterotrimers or homotrimers could increase with respect to others in order to better adapt the plant to the new environmental conditions.

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