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Phosphorylation-dependent regulation of excitation energy distribution between the two photosystems in higher plants

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ABSTRACT

Phosphorylation-dependent movement of the light-harvesting complex II (LHCII) between photosystem II (PSII) and photosystem I (PSI) takes place in order to balance the function of the two photosystems. Traditionally, the phosphorylatable fraction of LHCII has been considered as the functional unit of this dynamic regulation. Here, a mechanical fractionation of the thylakoid membrane of *Spinacia oleracea* was performed from leaves both in the phosphorylated state (low light, LL) and in the dephosphorylated state (dark, D) in order to compare the phosphorylation-dependent protein movements with the excitation changes occurring in the two photosystems upon LHCII phosphorylation. Despite the fact that several LHCII proteins migrate to stroma lamellae when LHCII is phosphorylated, no increase occurs in the 77 K fluorescence emitted from PSI in this membrane fraction. On the contrary, such an increase in fluorescence occurs in the grana margin fraction, and the functionally important mobile unit is the PSI–LHCI complex. A new model for LHCII phosphorylation driven regulation of relative PSII/PSI excitation thus emphasises an increase in PSI absorption cross-section occurring in grana margins upon LHCII phosphorylation and resulting from the movement of PSI–LHCI complexes from stroma lamellae and subsequent co-operation with the P-LHCII antenna from the grana. The grana margins probably give a flexibility for regulation of linear and cyclic electron flow in plant chloroplasts.

1. Introduction

Photosynthetic pigment protein complexes are embedded in the thylakoid membrane of plant chloroplasts. These membranes are structurally complex, highly organized and dynamically regulated structures [1–3]. Stacked grana membranes are connected via non-appressed margins to the stroma membranes. Shade and sun plants differ in their chloroplast ultrastructure. Shade plants have evolved extensive granal stacking as an adaptation to low light conditions and, conversely, the sun plants have less appressed thylakoids [4]. Moreover, the thylakoid membrane organization can rapidly and reversibly respond to changes in light intensity and quality, and importantly, this regulation follows similar light dependent dynamics as the phosphorylation of LHCII [5]. In addition to this complex organization and behaviour, the two photosystems are laterally segregated in the thylakoid membrane: PSI–LHCI is located in non-appressed stroma regions whereas PSII–LHCII is predominantly located in the stacked grana core [6,7].

The PSII supercomplex is composed of a dimeric core complex of PSII, which is associated with two copies of each minor light-harvesting proteins, two strongly bound LHCII trimers and two or more less tightly bound trimers [8–10]. PSI is a monomeric complex in higher plants composed of 14 core proteins and 5 Lhca antenna proteins [11]. Light-harvesting antenna complexes in the thylakoid membrane absorb sunlight and transfer the excitation energy to PSII and PSI core complexes in order to drive electron transport. The light-harvesting antenna of PSII consists of at least six different chl-binding proteins Lhcb1-6 [12] and can serve both the PSII and PSI reaction centers via state transition [13; reviewed in Ref. 14]. LHCII, the major antenna complex of PSII, is formed of trimers [15] of three proteins; Lhcb1, Lhcb2 and Lhcb3 in different combinations.

On the stromal side of the thylakoid membrane, the N-terminal threonine residue of the Lhcb1 and Lhcb2 proteins is prone to reversible phosphorylation [16] regulated by the redox conditions in the thylakoid membrane and the surrounding stroma [17, reviewed in Ref. 18]. Lhcb phosphorylation is needed for reversible translocation of a fraction of LHCII between PSII and PSI. The reaction is catalysed by the STN7 kinase [19–22]. Three minor chl-binding proteins, Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24), are monomeric, and among these Lhcb4 is the only phosphoprotein in plants [23]. Phosphorylatable Lhcb1 and Lhcb2 are the most abundant antenna proteins and they are considered to form the antenna fraction (P-LHCII) moving between PSI and PSII. Dephosphorylated LHCII serves only PSII and when phosphorylated it is, at least partially, connected to the PSI core

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complex via the PsaH, PsaL and PsaO subunits, and indeed these minor PSI proteins are a prerequisite for the mobility of P-LHCII [24]. Studies on PSI mutants lacking one of the essential subunits for P-LHCII association with PSI have revealed a more complex nature of P-LHCII movement between the two reaction centers than was previously envisaged and which was simply based on the phosphorylation-induced movement of LHCII to the stroma thylakoids [25].

In this study, using a non-invasive mechanical membrane fractionation and two-phase partition method, we isolated five different fractions of the thylakoid membrane: the grana core, the grana margins, entire grana containing both the grana core and grana margins, the stroma lamellae, and deep stroma lamellae fraction called Y-100 [7,26]. These fractions were isolated both from thylakoids of dark acclimated plants (D) where LHCII is dephosphorylated and from plants after 2 h of low light acclimation (LL), earlier shown to induce maximal LHCII phosphorylation [27]. Obtained results prompted us to present a new model for LHCII phosphorylation-dependent regulation of excitation energy distribution between PSII and PSI.

2. Materials and methods

2.1. Growth of plants and fractionation of the thylakoid membrane

WT *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) and *stn7* mutant [22] plants were grown in phytotron under 120 μmol photons m^{-2} s^{-1}, 8 h photoperiod. Plants were exposed to light favoring the excitation of PSII (PSII light) and that of PSI (PSI light) to ensure the maximal phosphorylation and dephosphorylation of thylakoid proteins, respectively. A fluorescent tube (GroLux F58W/GROT8 Sylvania) covered with orange filter (Lee 105 filter, Lee Filters) served as PSII light and PSI light was obtained from halogen lamps (500 W) covered with an orange filter (Lee 105, Lee Filters) and a 'Median blue' filter (Roscolux # 83, Rosco Europe). Temperature was maintained at 23 °C by water-cooled glass chamber between the fluorescence tube and the plants.

Spinach plants (*Spinacia oleracea* L.) were grown hydroponically under cool white fluorescent lamps (Osram HQI-E400W/DV, Germany) at 20 °C with light/dark periods of 12 h and with the light intensity of 300 μmol photons m^{-2} s^{-1}. Before harvesting of the leaves, two month old plants were dark adapted for 24 h to fully dephosphorylate LHCII (D) or treated with 100 μmol photons m^{-2} s^{-1} for 2 h (LL), which induces the maximal phosphorylation of LHCII in spinach plants grown at 300 μmol photons m^{-2} s^{-1} [27]. Thylakoid fractionation was then performed according to Refs. [7,26]. All preparation procedures were made under weak green light at 4 °C, and the sample was kept on ice throughout the whole isolation process. All thylakoid fractions were prepared without any detergent to preserve the membrane as intact as possible. The membranes were mechanically broken by sonication and then submitted to an aqueous two-phase system to isolate the grana and stroma lamellae fractions as described in [7,28,29]. The grana fraction was further purified according to [7,30] to isolate the grana core fraction and the grana margin fraction, which does not contain end membranes. The Yeda press treatment of the thylakoid membrane followed by centrifugation steps according to [7,31,32] resulted in the Y-100 fraction, which is considered to be the most purified stroma lamellae fraction. The contents of chl a and b were determined according to Ref. [33].

2.2. SDS-PAGE, phosphoprotein staining and immunoblotting

Proteins of the thylakoid membrane and different membrane subfractions were separated by SDS-PAGE using 15% (w/v) acrylamide gels with 6 M urea [34]. Pro-Q Diamond Phosphoprotein Gel Stain and SYPRO® Ruby Protein Gel Stain (Molecular Probes) were used according to manufacture's instructions after determining the sample concentrations that give a linear response after staining. Membranes equivalent to 0.05 μg of chl a were loaded in gels to investigate the most abundant proteins and 0.8 μg of chl a was loaded to investigate the less abundant thylakoid proteins.

For immunoblotting, the amount of proteins loaded in the gels was tested for each antibody to give a linear immunoresponse. Accordingly, the protein amounts corresponding from 0.2 to 3.2 μg of chl were loaded in the gels, depending on the antibody used. The polypeptides were transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and the membrane was blocked with 5% (w/v) milk (Bio Rad) or with 5% fatty-acid free bovine serum albumin (Sigma-Aldrich) for P-thr antibody (New England Biolabs). Western blotting was performed with standard techniques using protein-specific antibodies purchased from Agrisera (Vännas, Sweden) (Lhcb1, Lhcb2, Lhcb3, CP26 (Lhcb5), CP24 (Lhcb6), PsaH, PsaL Lhca1, Lhca2, Lhca3, Lhca4, PsbS) and other antibodies as described previously [35,36]. Proteins were immunodetected using a Phototope-Star Chemiluminescent kit (New England Biolabs).

2.3. 77 K fluorescence emission and fluorescence excitation spectra

Thylakoid membranes and different thylakoid subfractions were diluted in buffer containing 50 mM Hepes/KOH, pH 7.5, 100 mM sorbitol, 10 mM $MgCl_2$, and 10 mM NaF to a chl concentration of 10 μg/ml. Then samples were immediately frozen and 77 K fluorescence emission spectra were recorded with a diode array spectrophotometer (S2000; Ocean Optics, Dunedin, FL, USA) equipped with a reflectance probe. To record the 77 K fluorescence emission curves the samples were excited with white light below 500 nm, defined by LS500S and LS700S filters (Corion Corp., Holliston, MA, USA). To obtain the 77 K fluorescence excitation spectra, the samples were excited with wavelengths from 400 to 540 nm with 5 nm steps by using f/3.4 Monochromator (Applied Photophysics, Surrey, U.K.). The emission between 600 and 800 nm was recorded (see Figs. 1 and 3 legends for details).

3. Results

3.1. Relation between LHCII phosphorylation and the relative excitation of PSI and PSII

Generally, different approaches have been taken to dephosphorylation or phosphorylation of the LHCII depending on the organism under study. In *Chlamydomonas*, light independent methods have been used most often [37], whereas in spinach plants the dark and low light treatments efficiently induce full dephosphorylation and phosphorylation of LHCII proteins, respectively [27]. On the contrary, for *Arabidopsis* the use of light qualities that selectively excite either PSI or PSII is the best choice. As shown in Fig. 1, the relative distribution of excitation energy between PSII and PSI is dependent on the phosphorylation of LHCII proteins. When LHCII is phosphorylated, the 77 K PSI/PSII (F733 nm/F685 nm) fluorescence ratio is higher compared to the dephosphorylated state (Fig. 1A). On the contrary, the *stn7* mutant, which cannot phosphorylate LHCII, keeps the relative excitation of PSI and PSII nearly constant during the whole 120 min treatment with the PSI and PSII lights (Fig. 1A). In fact, the *stn7* mutant is inert for even longer treatments with PSI, PSII or white light [22]. Under normal growth conditions the *stn7* mutant synthesizes more PSI–LHCI proteins compared to WT [22] in order to compensate the lack of energy donation from P-LHCII to PSI. This increases the 77 K F733/F685 ratio

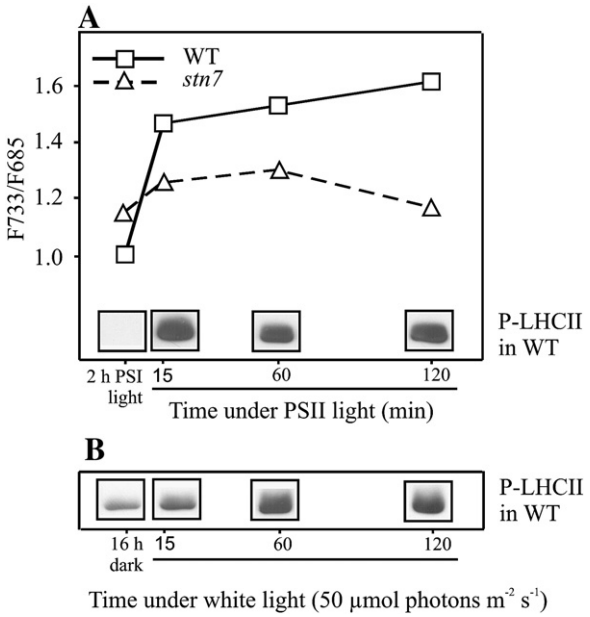

Fig. 1. Relationship between LHCII phosphorylation and 77 K fluorescence emission ratio (733 nm /685 nm) in WT and the *stn7* LHCII kinase mutant of *Arabidopsis*. A. WT and *stn7* plants were first treated for 120 min with light favoring PSI excitation (PSI light) and then transferred under light favoring PSII excitation (PSII light) for 15, 60 and 120 min. Phosphorylation level of LHCII proteins was detected by P-thr antibody (WT shown, no phosphorylation detected from *stn7*) and the amplitudes of 77 K fluorescence peaks (733 nm and 685 nm) were measured. All the 77 K samples were prepared in the presence of 10 mM NaF and diluted to 10 μg of chl/ml before the measurements. B. Kinetics of LHCII phosphorylation, as detected by P-thr antibody, in WT *Arabidopsis* upon illumination by low intensity of white light. Thylakoids were isolated after 16 dark treatment and after 15, 60 and 120 min illumination with white low light of 50 μmol photons m^{-2} s^{-1}. Thylakoids were isolated in the presence of 10 mM NaF.

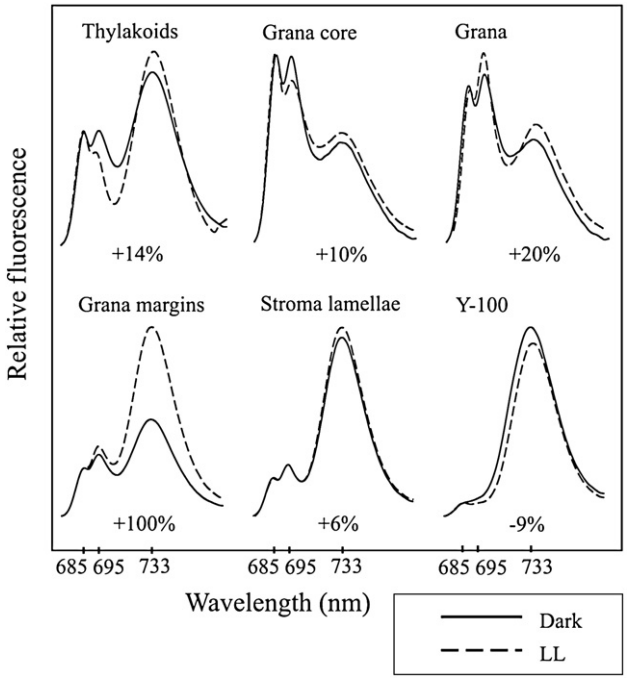

Fig. 2. 77 K fluorescence spectra of the thylakoid membrane and the membrane subfractions isolated from dark-treated (LHCII dephosphorylated, excitation of PSII favored) and LL-treated (LHCII phosphorylated, excitation of PSI favored) spinach leaves. Change in the amplitude of the relative PSI fluorescence (F733 nm/F685 nm, excitation ≤500 nm), representing the LL-induced phosphorylation-dependent state that favors PSI excitation, is shown as percentage values below the spectra. Dark treated leaves were harvested after 24 h of darkness and LL leaves after 2 h of illumination at 100 μmol photons m^{-2} s^{-1}. 77 K fluorescence spectra were normalized at 685 nm fluorescence. All the samples were prepared in the presence of 10 mM NaF and diluted to 10 μg of chl/ml before the measurements.

in *stn7* compared to WT under dephosphorylating conditions (Fig. 1A). These results indicate that LHCII phosphorylation is a prerequisite for dynamic redistribution of excitation energy between PSII and PSI in the timescales of minutes and hours, and noteworthy is not limited to minute time scales that are considered to represent the function of classical state transitions. Therefore, in this study, the state when excitation of PSI is favored is also called the *phosphorylated state* (state 2) and the state when PSII excitation is favored is called the *dephosphorylated* state (state 1).

In order to quantitatively analyze the LHCII phosphorylation-induced changes in relative excitation of PSI and PSII, in protein phosphorylation and in protein composition of the different domains of the thylakoid membrane, the method employed for thylakoid fractionation should not disturb the original, native organization and/or the interactions of the protein complexes in the membrane. The best available method is a non-invasive, detergent free mechanical fragmentation and two-phase partitioning technique to prepare different fractions of the thylakoid membrane [2,7,26,38–40] from leaves a priori treated to induce complete phosphorylation or dephosphorylation of LHCII. Because of serious technical difficulties, this gentle method could not be applied for *Arabidopsis* thylakoid fractionation and therefore spinach plants were used here to prepare the thylakoid subfractions. Plants were treated according to Rintamaki et al. [27] in order to completely dephosphorylate (dark treatment of plants) and to maximally phosphorylate (LL treatment of plants) LHCII, and consequently to reach the maximal excitation of PSII and PSI, respectively. It is also emphasized in Fig. 1B that as previously reported for spinach [27], also in *Arabidopsis* the maximal phosphorylation of LHCII at low light is not an extremely rapid phenomenon but rather occurs in the time scale of an hour.

3.2. 77 K fluorescence emission of dark and LL thylakoids and thylakoid subfractions

Transition from state 1 that prevails in darkness when LHCII is dephosphorylated to state 2 that prevails under LL when LHCII is phosphorylated increases the relative excitation of PSI [41]. This was clearly seen in intact thylakoids with a 14% increase in F733 nm/F685 nm emission ratio measured at 77 K (Fig. 2). The key question of this study was to define the origin of this change in relative excitations of PSII and PSI. The presupposition according to the traditional model for the relative PSI/PSII excitation was that the phosphorylated LHCII migrates from PSII-rich grana to PSI-rich stroma lamellae [42,43] thereby in phosphorylated state increasing the PSI fluorescence emitted from stromal thylakoids. However, our 77 K fluorescence results did not support this assumption (Fig. 2). Instead, the increase in 77 K fluorescence ratio caused by LHCII phosphorylation was localized mainly to the grana margin fraction of the thylakoid membrane. Indeed, this fraction showed the largest, nearly 100% increase in relative 77 K PSI/PSII fluorescence ratio, the entire grana showed 20% increase and even in the grana core 10% increase was recorded. On the contrary, the entire stroma lamellae showed only marginal 6% increase in PSI fluorescence. In the Y-100 fraction, in fact, the change in the F733 nm/F685 nm ratio was negative, practically meaning 9% decrease of PSI excitation in the Y-100 fraction. This result clearly implied that the LHCII phosphorylation-induced increase in relative excitation of PSI does not originate from the stromal PSI complexes, thus strongly implicating that the P-LHCII migration into contact with stromal PSI cannot be the underlying mechanism for regulation of relative PSI/PSII excitation.

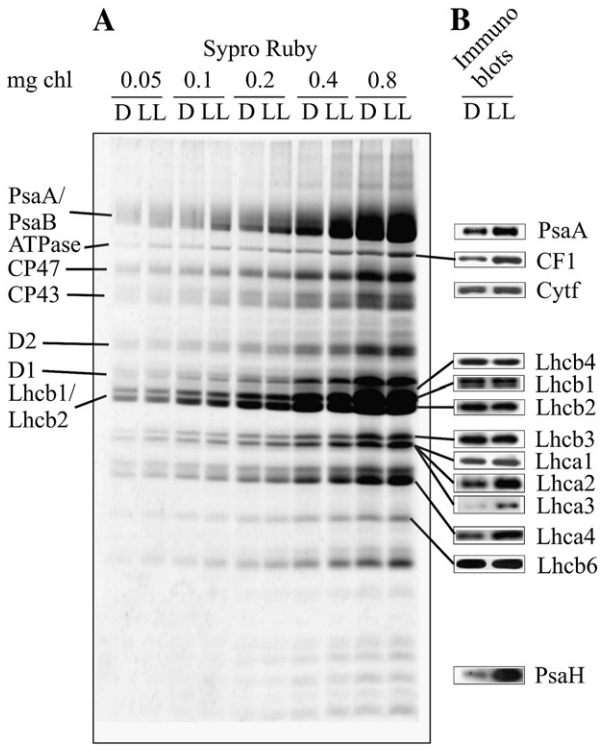

Fig. 3. Protein analysis of the grana margin fractions isolated from dark (LHCII dephosphorylated, excitation of PSII favored) and LL (LHCII phosphorylated, excitation of PSI favored) treated spinach leaves. A. SDS-PAGE stained with SYPRO® Ruby Protein Gel Stain. Chlorophyll amounts ranging from 0.05 μg to 0.8 μg were loaded to wells to reveal the changes in both the more and less abundant proteins. B. Immunoblot analysis of several proteins in the grana margins. Proteins were loaded according to the linearity tests with each antibody thus allowing the comparison of protein amounts in dephosphorylated state (D) and phosphorylated state (LL).

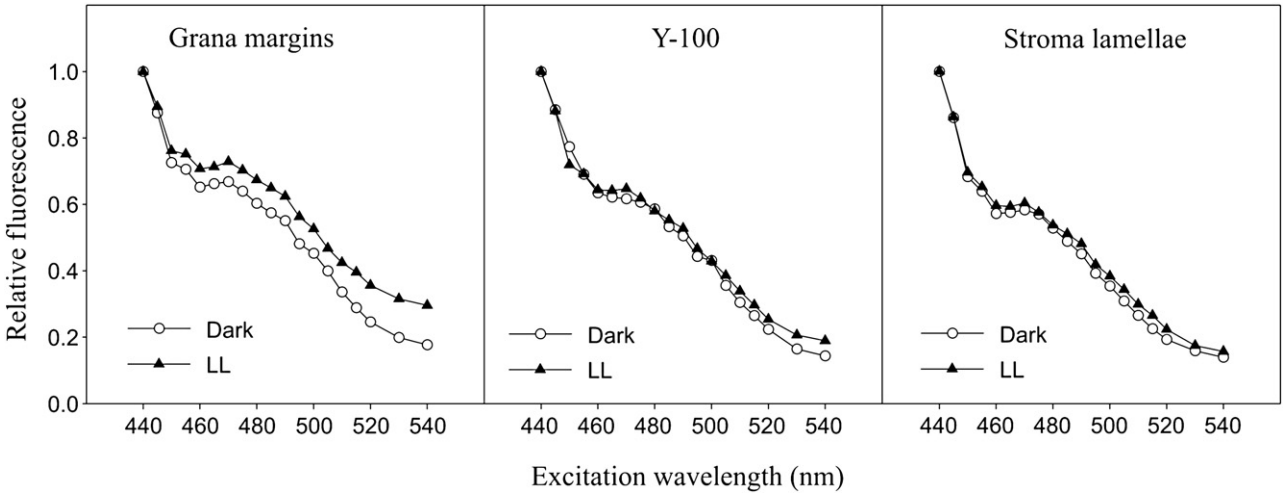

Fig. 4. 77 K fluorescence excitation spectra of PSI (detected at 733 nm) from grana margin, stroma lamellae and Y-100 subfractions of thylakoids isolated from dark (LHCII dephosphorylated, excitation of PSII favored) and from LL (LHCII phosphorylated, excitation of PSI favored) treated spinach leaves. All spectra have been normalized to the excitation by chl a at 440 nm. Peak at 480 nm represents the excitation of PSI by chl b and the excitation by longer wavelengths represents the excitation by carotenoids. Samples were treated as in Fig. 1.

3.3. Grana margins provide the flexibility to change the energy distribution between the two reaction centers

LHCII proteins are known to serve both the PSII and PSI reaction centers via state transitions, yet the mechanism remains elusive. The prevailing conception for explaining the mechanism for tuning the relative excitations of PSI and PSII is the phosphorylation-dependent movement of LHCII trimers between the reaction center core complexes PSI and PSII. Thus, the prominent increase in the 77 K F733 nm/F685 nm ratio in the grana margins (Fig. 2) was expected to result from an increased amount of P-LHCII in this membrane fraction. However, no clear increase in the amounts of phosphorylatable Lhcb1, Lhcb2 and Lhcb4 proteins could be detected in grana margins, judged either from quantitative protein staining (Sypro Ruby) or from immunoblotting (Fig. 3). Nor did the Lhcb3 and Lhcb6 proteins, the non-phosphorylatable LHCII proteins, accumulate in grana margins in LL (Fig. 3). In sharp contrast to LHCII proteins, the amounts of the PSI–LHCI complexes and the ATPase clearly increased in grana margins under LL as compared to the dark. Quantifications from three different gels revealed over 100% increase in the PSI core proteins PsaA and PsaH, in the LHCI proteins Lhca2 and Lhca3 and in the ATPase subunit CF1 and around 50% increase in the amounts of the LHCI proteins Lhca1 and Lhca4 in grana margins upon transition to state 2 (Fig. 3). Such movement of PSI–LHCI towards grana margins seems to be the mechanical basis of LHCII phosphorylation-induced regulation of excitation energy distribution between PSII and PSI. In phosphorylated state, the close contacts between PSI and PSII increased in grana margins, thus allowing PSI to trap more excitation energy from LHCII, and this apparently happened at the cost of PSII excitation. It has been calculated that the density of PSI in stroma lamella is about 400 particles/μm^2 and increases towards the grana margins up to 1600 particles/μm^2 [44]. If grana margins represents about 20% of the non-appressed thylakoid domains [30], it means that half of the PSI complexes locate in grana margins and the other half deeper in the stroma lamella. Thus under conditions where LHCII is phosphorylated and the amount of PSI has increased in grana margins, the majority of PSI can be under the influence of LHCII excitation.

In order to verify the origin of the increased PSI excitation in grana margins when LHCII is phosphorylated, the 77 K excitation spectrum

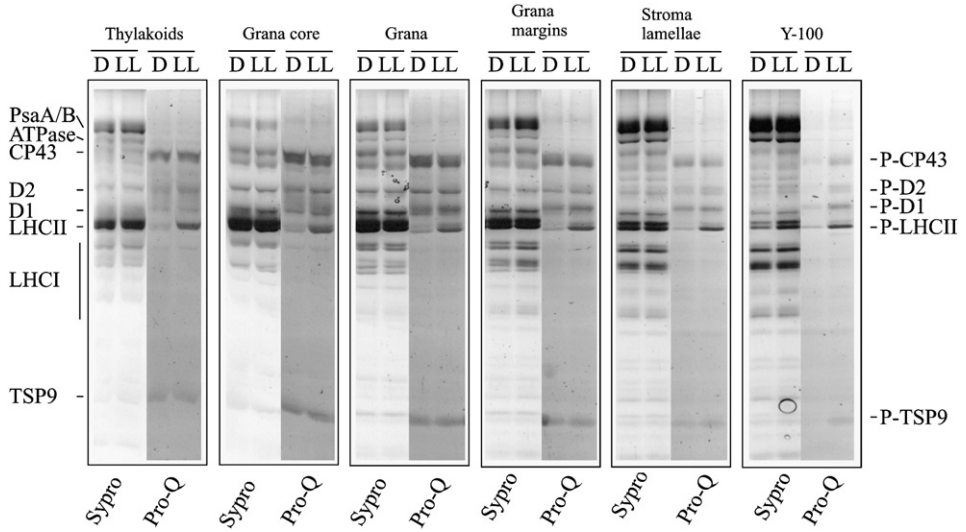

Fig. 5. Quantitative protein and phosphoprotein staining of thylakoid membrane subfractions isolated from dark (D) (LHCII dephosphorylated, excitation of PSII favored) and from LL (LHCII phosphorylated, excitation of PSI favored) treated spinach leaves. SYPRO® Ruby Protein Gel Stain and Pro-Q® Diamond Phosphoprotein Gel Stain stained SDS-PAGE gels from D and LL thylakoids and from the five isolated thylakoid subfractions. 1 μg of chl was loaded to each well.

of PSI was recorded from the grana margins, the stroma lamellae and the Y-100 fractions using excitation wavelengths from 400 nm to 540 nm. The intensity of PSI (733 nm) fluorescence emission when excited by chl a (440 nm) was used as a reference and set to be 1 in all fractions. It can be clearly seen from Fig. 4 that the excitation of PSI by 480 nm (chl b) and longer wavelengths (carotenoids), diagnostic for light absorption by LHCII trimers [45,46], had the strongest effect in the grana margins on the fluorescence emission of PSI, particularly when LHCII is phosphorylated. Thus, the absorption by chl b and carotenoids of LHCII in grana margins particularly contributed to the increase in the PSI (F733) fluorescence emission when LHCII was phosphorylated. In contrast, such contribution by chl b of LHCII was absent from the stroma lamellae and the Y-100 membrane fractions.

3.4. A global view of LHCII phosphorylation-induced protein movements in the thylakoid membrane

In general, a strong increase in the phosphorylation of Lhcb1 and Lhcb2 and a less extensive increase in the phosphorylation of the other thylakoid phosphoproteins CP43, D2, D1 and TSP9 were detected in all thylakoid subfractions upon shift of spinach plants from darkness to LL (Figs. 5 and 6). Still, the relative protein amounts of these phosphoproteins did not reveal drastic differences between dephosphorylated and phosphorylated state (Sypro Ruby lanes in Fig. 5), the Y-100 fraction being the only exception with a clear increase in Lhcb proteins (Fig. 6).

Despite the fact that the stroma lamellae and Y-100 fractions appeared not to tune the relative PSII/PSI excitation (Figs. 2 and 4), the most distinguished changes in various protein levels were seen in these fractions, especially in Y-100. Proteins of the major LHCII trimers, Lhcb1, Lhcb2 and Lhcb3, as well as the monomeric minor antenna proteins Lhcb4, Lhcb5 and Lhcb6 as well as the PsbS protein involved in NPQ [47] were transported to stroma lamellae as a response to LL illumination (LHCII phosphorylation). The amounts of the PSII core proteins also increased in the Y-100 fraction but less extensively. On the contrary, the amounts of the Cyt b/f (Fig. 6) complex proteins and the proteins of the ATPase (Fig. 5) remained rather constant. A special characteristic of the Y-100 fraction was also that the contents of the LHCII proteins correlated with their phosphorylation state (Fig. 6), indicating that the Lhcb1 and Lhcb2 proteins located in the Y-100 fraction are mainly phosphorylated whereas all other thylakoid subfractions host equally the phosphorylated and non-phosphorylated LHCII proteins.

Another thylakoid fraction showing changes in protein amounts was the grana margins fraction, where the Lhca proteins accumulated together with PSI core proteins (Figs. 3 and 6) after LHCII phosphorylation. Grana fractions, on the contrary, showed no distinct changes in protein amounts upon shift to phosphorylated state despite the fact that phosphorylated LHCII proteins clearly migrated to stroma lamellae. It is highly likely that partial de-stacking upon LHCII phosphorylation is not affecting the apparent stability of proteins located deeper in the grana partitions.

The chl a/b ratios of all thylakoid fractions are depicted at the bottom of Fig. 6. Changes in chl a/b ratios upon LHCII phosphorylation correlated with the migrations of chl a rich PSI–LHCI towards the grana and chl b rich LHCII proteins towards the stroma lamellae.

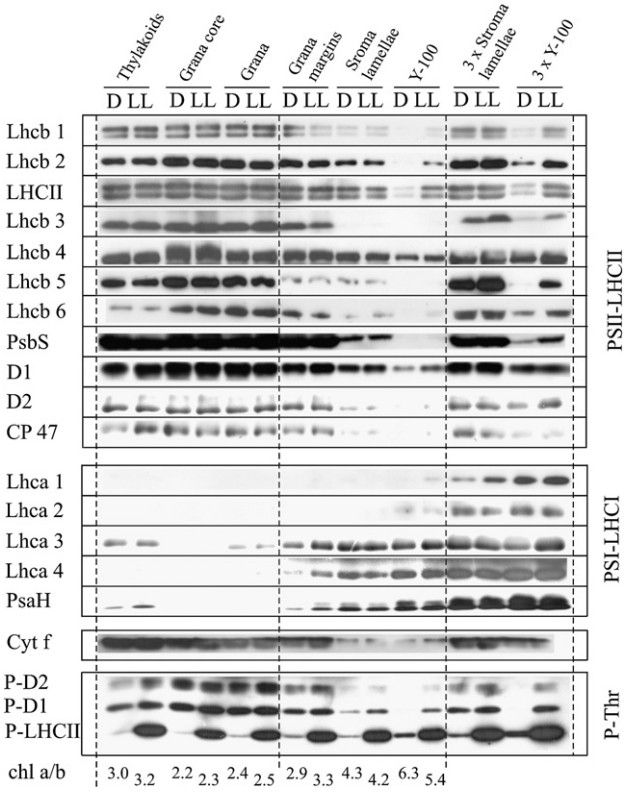

Fig. 6. Immunoblot demonstration of the abundance of the PSII and PSI proteins in the thylakoid membrane and in the five different membrane subfractions from dark (LHCII dephosphorylated, excitation of PSII favored) and from LL (LHCII phosphorylated, excitation of PSII favored) treated spinach leaves. From 0.2 to 3.2 μg of chl was loaded in the wells depending on the linearity test with each antibody. Chl a/b ratios of the thylakoid membrane and the membrane subfractions in dephosphorylated state (D) and in phosphorylated state (LL) are given below the immunoblots.

4. Discussion

The functional mechanism and biological significance behind the chloroplast capability to change the relative excitation of PSI and PSII have exercised the minds of researchers for decades. Mechanistically the phenomenon is based on ATP-dependent strong and reversible phosphorylation of the most abundant membrane proteins in the world, the Lhcb1 and Lhcb2 proteins.

4.1. LHCII phosphorylation, relative excitation of two photosystems and thylakoid lateral heterogeneity

According to the widely accepted view, the phosphorylated LHCII migrates from PSII in the appressed membranes to serve as a light-harvesting antenna for PSI in the stroma exposed membranes as a response to a change in light quality or quantity. Conversely, when LHCII becomes dephosphorylated, the excitation of PSII is again favored by the movement of LHCII back to the PSII [reviewed in Ref. 14]. It is well known that LHCII phosphorylation is a prerequisite for dynamic regulation of relative PSI/PSII excitation [19–22]. Although the movement of LHCII trimers from grana to stroma lamellae indeed occurs upon state 1 to state 2 transition [42,43] (Fig. 4), there is no unambiguous evidence supporting the movement of LHCII as a mechanism behind increased PSI excitation when LHCII is phosphorylated. In fact, the characterization of a mutant lacking the PsaH subunit of PSI gave an opposite signal: compared to WT, a stronger induction of LHCII phosphorylation was detected in PsaH mutant, yet P-LHCII stayed in contact with PSII [25]. Here we have used five different fractions of the thylakoid membrane isolated from leaves both in dephosphorylated state (D) and from phosphorylated state (LL) to clarify the mechanistic basis for LHCII phosphorylation-dependent distribution of excitation energy between PSI and PSII.

The 77 K fluorescence spectra of thylakoid subfractions in dephosphorylated state and in phosphorylated state showed that only the grana margins have a flexibility to regulate the relative excitation of PSI and PSII, not the stroma lamellae as was previously postulated [reviewed in Ref. 14]. This challenged the role of P-LHCII movement to stroma membranes as a sole mechanism for dynamic redistribution of excitation energy between the two reaction centers, although it is clear that LHCII phosphorylation is obligatory for this mechanism to

occur. We postulate that LHCII phosphorylation is required to create attractive forces between PSII–LHCII and PSI–LHCI supercomplexes, thus increasing the amount of loosely packed grana margins, enabling more PSI to migrate towards LHCII–PSII-rich grana and finally allowing PSI to capture energy from LHCII. Indeed, both the phosphorylation of LHCII and the dynamics of granal stacking are needed for regulation of the relative excitation of PSI and PSII. The biological structure/function relationship of the grana and stroma thylakoid

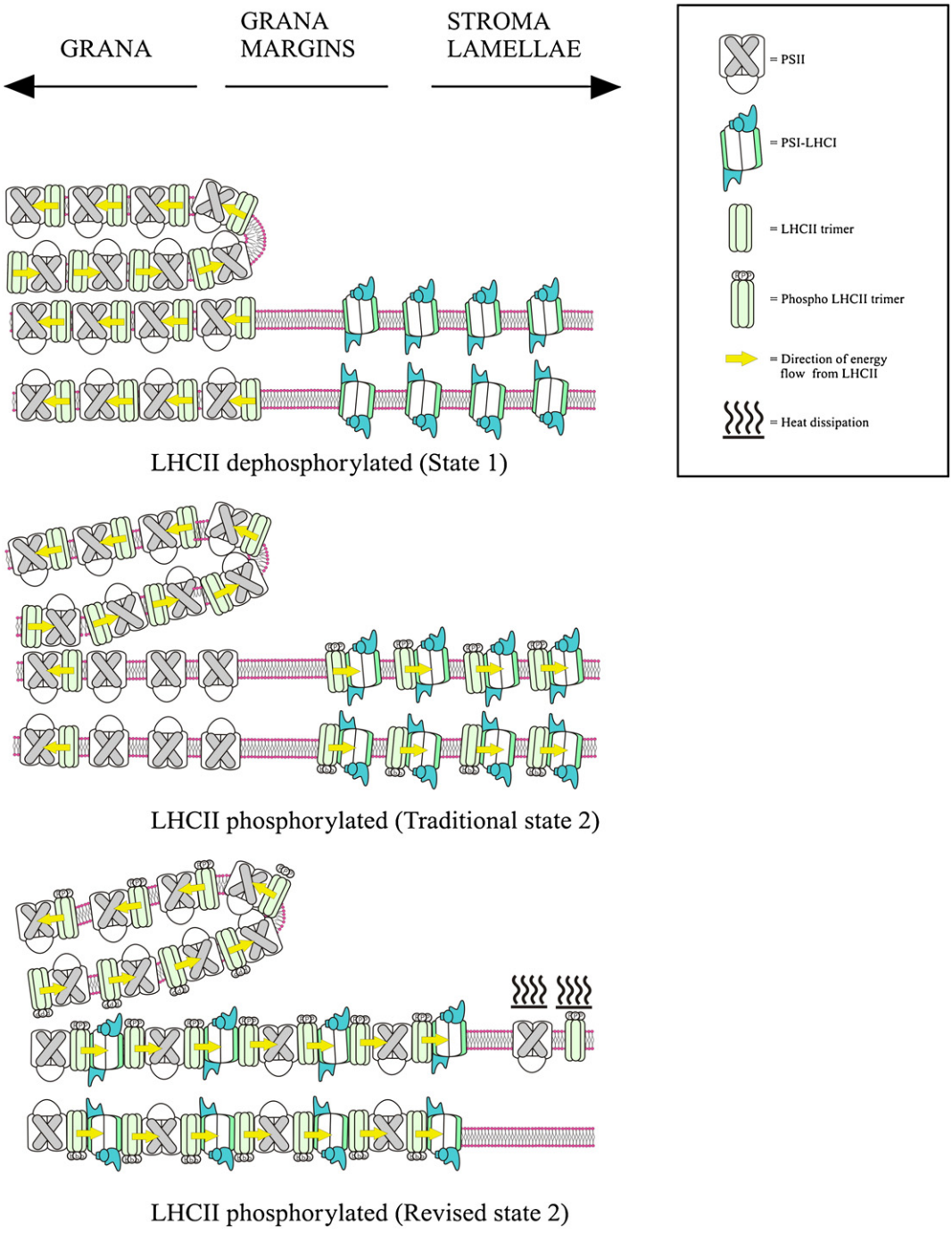

Fig. 7. Revised model for state 1 (LHCII dephosphorylated, excitation of PSII favored) to state 2 (LHCII phosphorylated, excitation of PSI favored) transition in plant chloroplast. State 1, redrawn according to Allen and Forsberg [14], is similar in both the traditional and revised state transition models; LHCII is dephosphorylated and thylakoid membranes are highly stacked. In traditional state 1 to state 2 transition model, LHCII migrates upon phosphorylation from PSII in grana to PSI in stroma lamellae, causing an increase in relative excitation of PSI. Present study, however, could not detect any changes in the relative excitation of PSI and PSII in the stroma lamellae despite a clear increase in the amount of P-LHCII proteins in this fraction. Moreover, the excitation spectra of PSI in the stroma lamellae remained unchanged despite the state transition. On the contrary, in grana margins the amount of PSI–LHCI increased in state 2 and concomitantly the relative fluorescence of PSI showed two-fold increase. Analysis of the PSI excitation spectra showed that this increase originated from wavelengths primarily absorbed by LHCII, and such an increase occurred only in the grana margin fraction. Based on these results and on the data known about the dynamics of granal stacking [5], we present an alternative hypothesis for the mechanism of the state transitional adjustment of the relative PSI/PSII excitation. During state 1–state 2 transition the phosphorylation of LHCII starts to attract PSI–LHCI complexes towards grana margins. Loosening of thylakoid appressions is a prerequisite for this to happen. The amount of PSI–LHCI in contact with P-LHCII increases thus allowing the flow of excitation energy from LHCII–PSII to PSI in grana margins. Upon transition to state 2, also a fraction P-LHCII–PSII proteins moves towards stroma lamellae by repulsion forces between P-LHCII-PSII complexes, and functions there in dissipation of absorbed energy. The underlying mechanism of state transitions is, however, the movement of LHCI–PSI towards PSII–LHCII in grana margins.

membranes has been under debate for nearly 70 years and many hypotheses have been postulated [2]. Probably the most popular theory is that the grana–stroma structure is evolved to increase the lateral heterogeneity of the thylakoid membrane to separate the slow PSII from the fast PSI in order to prevent energy spill over from PSII to PSI and thereby to maintain the linear electron flow functional [48].

Green alga, *Chlamydomonas reinhardtii* exhibits LHCII protein phosphorylation-dependent regulation of relative PSI/PSII excitation [19] but does not form regular grana stacks [49]. They can increase the excitation of PSI after LHCII phosphorylation much more compared to that of the higher plants [50] and the phosphorylated state induces extensive transition to cyclic electron flow [51]. However, in higher plants, there is no consensus of the role of LHCII phosphorylation in regulation of cyclic electron flow. This is because both the phosphorylation-dependent regulation of relative PSI/PSII excitation and cyclic electron flow are relatively minor phenomena in higher plants and their interrelationship has been hard to prove experimentally. We measured, however, a 100% increase in relative PSI excitation in grana margins in the phosphorylated state. In this thylakoid region the PSII-P-LHCII, PSI–LHCI and ATPase complexes are widely present (Fig. 3). Another special characteristic of increased PSI fluorescence in the grana margins was the fact that this was clearly excited by the wavelengths diagnostic for LHCII. Moreover, only in the grana margin fraction the excitation of PSI by wavelengths characteristic to chl b absorption increased after LHCII phosphorylation (Fig. 4). These results strongly suggest that the grana margins provide dynamic properties for the lateral segregation of photosystems and allow readjustments in the excitation of PSII and PSI (Fig. 2). Increased PSI excitation in grana margins resulted from the migration of PSI–LHCI from the stroma lamellae towards the granal membranes (Fig. 3), not from the movement of phospho-LHCII into contact with PSI in the stroma lamellae as has been previously postulated [reviewed in Ref. 14].

Under LL, LHCII was strongly phosphorylated in all the five thylakoid fractions, yet a clear difference in the contents of the Lhcb proteins between dephosphorylated state and phosphorylated state was observed only in the Y-100 fraction (Fig. 6). 77 K fluorescence spectra, however, give direct evidence that LHCII protein phosphorylation as such does not define the excitation increase of PSI in phosphorylated state. Characterization of the mutant lacking the PsaH subunit of PSI demonstrated that despite strong phosphorylation of LHCII it stayed in contact with PSII [25]. It is thus plausible that PsaH is required for the movement of the PSI complex towards grana margins. There is also compelling evidence that in state 2 the LHCII trimer really forms a complex with PSI [52], but the array of the proteins being in contact with PSI in state 2 seems to be wider than expected.

As mentioned above, in phosphorylated state the amounts of the Lhcb1, Lhcb2, Lhcb3 and PsbS proteins clearly increased in the Y-100 fraction. Also other LHCII proteins as well as the PSII core proteins behaved similarly, although to a lesser extent. During LHCII phosphorylation, there are thus protein migrations in two directions: movement of PSI–LHCI towards the grana by attractive electrostatic forces and/or by van der Waals forces, and the P-LHCII-PSII movement towards the stroma lamellae due to charge repulsion between adjacent P-LHCII proteins [reviewed in Ref. 14,53]. The "meeting point" of these complexes during their movement in the opposite directions is the margin fraction of the thylakoid membrane. Since the movable P-LHCII–PSII fraction neither lead to changes in 77 K PSI/PSII fluorescence emission ratios nor increased the 77 K fluorescence emission from the wavelengths diagnostic to free LHCII (680 nm) in stroma lamellae, the remaining option is that these P-LHCII–PSII units, segregated from the highly organized functional structure of grana and grana margins, dissipate the harvested excitation energy as heat, possibly with the help of co-migrating PsbS protein.

During evolution, the granal and stromal thylakoids have specialized into their own priority functions and the interface of these two functional regions, i.e. the grana margin, has preserved the capacity to regulate the excitation energy input from LHCII to PSI, which in turn is likely needed to regulate the photosynthetic production of NADPH and ATP. It is conceivable that the strict granal stacking has evolved in higher plants to make the thylakoid membrane structure stiffer, in order to reduce the capacity of LHCII phosphorylation to modulate the lateral heterogeneity of thylakoids. This probably has evolved in order to favor linear electron transport and the production of NADPH and to prevent extensive energy flow from LHCII to PSI and cyclic electron flow that produces only ATP. Optimization of the photosynthetic production of NADPH and ATP by regulation of relative excitation of PSI and PSII must be energetically advantageous compared to the catabolic production ATP via multistep biochemical pathways and transportation of the various molecules involved in the reactions. In algae and cyanobacteria, however, a less tight restriction of cyclic electron flow has remained, probably because it is indispensable to produce ATP and fuel the carbon concentrating mechanisms in order to pump enough CO_2 for their slow rubisco enzyme.

4.2. Revised mechanistic model for LHCII phosphorylation-dependent regulation of relative excitation of PSII and PSI

Results obtained from mechanical subfractionation of thylakoids with dephosphorylated LHCII and phosphorylated LHCII change the concept of extensive mobility of the P-LHCII between PSII and PSI as a functional basis for dynamic regulation of relative PSI/PSII excitation. Our revised model suggests that strong LHCII phosphorylation in grana appressions induces repulsion between adjacent P-LHCII–PSII complexes pushing them towards grana margins and stroma thylakoids (Fig. 7). When in grana margins, the P-LHCII–PSII complex in turn attracts the PSI–LHCI complex from stroma lamellae towards the grana margins. This leads to a decrease in lateral heterogeneity of the thylakoid membrane allowing the excitation energy flow from PSII–LHCII to PSI. The mechanical basis for increased PSI excitation could be a direct attachment of PSI to LHCII [52]. Dissociation of LHCII from PSII is, however, not needed, because due to slow donation of electrons from water to PSII and the capability of PSI to use excitation energy for cycling of electrons, PSI can function faster than PSII and capture excitation energy from the PSII–LHCII [48]. Granal stacking has probably evolved to structurally regulate the lateral heterogeneity in order to restrict the unwanted excitation energy flow from PSII–LHCII to PSI. However, the grana margins are still enough flexible to allow PSI to capture excitation energy from LHCII–PSII, but there the event is under tight and dynamic regulation. Thus, the dynamic nature of the grana margins plays an important role in this process. The evolutionary benefit of this strict regulation probably has been to optimize the NADPH/ATP production to serve maximal carbon assimilation. Granal stacking probably was a significant evolutionary step from aquatic algae to multi-cellular higher plants with an efficient rubisco enzyme.

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