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Light-harvesting II antenna trimers connect energetically the entire photosynthetic machinery — including both photosystems II and I

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ABSTRACT

In plant chloroplasts, the two photosystems (PSII and PSI) are enriched in different thylakoid domains and, according to the established view, are regarded as energetically segregated from each other. A specific fraction of the light harvesting complex II (LHCII) has been postulated to get phosphorylated by the STN7 kinase and subsequently to migrate from PSII to PSI as part of a process called 'state transition'. Nevertheless, the thylakoid membrane incorporates a large excess of LHCII not present in the isolatable PSII–LHCII and PSI–LHCII complexes. Moreover, LHCII phosphorylation is not limited to a specific LHCII pool and "state 2" condition, but is found in all thylakoid domains in any constant light condition. Here, using a targeted solubilization of pigment–protein complexes from different thylakoid domains, we demonstrate that even a minor detachment of LHCII leads to markedly increased fluorescence emission from LHCII and PSII both in grana core and non-appressed thylakoid membranes and the effect of the detergent to detach LHCII is enhanced in the absence of LHCII phosphorylation. These findings provide evidence that PSII and PSI are energy traps embedded in the same energetically connected LHCII lake. In the lake, PSI and LHCII are energetically connected even in the absence of LHCII phosphorylation, yet the phosphorylation enhances the interaction required for efficient energy transfer to PSI in the grana margin regions.

1. Introduction

Plants harvest solar energy by pigments embedded in thylakoid protein complexes, and convert it into chemical energy to sustain the growth and development. Photosynthetic light harvesting and water-splitting reactions are potentially highly vulnerable and in order to avoid photo-oxidative damage of the photosynthetic machinery all the photosynthetic energy transduction reactions need to function in balance in a highly coordinated manner. Paradoxically, the thylakoid membrane has evolved to overexcite photosystem II (PSII) as compared to photosystem I (PSI) and a steady-state phosphorylation of light harvesting complex II (LHCII) proteins in light by the STN7 kinase has evolved to correct the excitation imbalance [1–3]. This is contrary to traditional thinking that regulatory mechanisms are needed to correct the relative excitation imbalance only transiently, upon changes in light conditions, in a process called 'state transitions'. State transitions allow the re-adjustment in distribution of absorbed light quanta between PSII and PSI [4,5]. When PSII absorbs more light energy compared to PSI (e.g. red light), part of the excitation energy is redirected to PSI, detectable by a decrease of chlorophyll fluorescence yield (state 1 to state 2 transition). On the contrary, if PSI is over-excited compared to PSII (far-red light), part of excitation energy is redirected to PSII (state 2 to state 1 transition).

Initially, the state transition phenomenon was attributed to changes in grana stacking and energy spillover, which is the direct transfer of excitation energy from PSII to PSI [5,6]. Today, the established view to explain the state transition mechanisms is based on strict lateral heterogeneity of PSII and PSI as well as on exclusive migration of only the phosphorylated LHCII (P-LHCII) between appressed and non-appressed thylakoid regions [7,8]. It has been proposed that in the state 1 to state 2 transition, a portion of LHCII trimers or monomers gets phosphorylated and consequently migrates to PSI, located in grana margins and stroma lamellae, to balance the photosystem absorption cross sections [9,10]. The process has been described as reversible, involving dephosphorylation of LHCII and its return to grana into connection with PSII, in case of PSI over-excitation.

The state transition model explained above, however, completely fails to explain the physiological significance of steady state phosphorylation of LHCII proteins in all different thylakoid domains under any constant light condition [3,11,12]. The state transition

Abbreviations: Dig, digitonin; DM, n-dodecyl β-D-maltoside; lpCN-PAGE, large-pore clear-native polyacrylamide gel electrophoresis

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model also comprises an apparent obstacle for LHCII to move in a very crowded membrane environment [13,14]. Such high protein packing allows the excitation energy transfer between thylakoid pigment–protein complexes and is the structural basis for the so-called 'lake model', in which the PSII dimeric units are energetically connected to each other through LHCII (PSII connectivity) ([15,16]; see [17] for a review), thus improving the efficiency of light harvesting for PSII [14]. The concept of connectivity has been restricted to the PSII–LHCII complexes located in the grana and the strict lateral heterogeneity of the thylakoid membrane and photosystems has been thought to keep PSI energetically disconnected from PSII. It has, however, been proposed that there is a third thylakoid domain rich of LHCII and both PSII and PSI in grana margins [18–23]. This would imply the existence of a large contact area between PSII-enriched and PSI-enriched thylakoid regions, giving the possibility for PSII–PSI (or PSII–LHCII–PSI) contact and energy transfer. Indeed, it has been demonstrated that the phosphorylation of LHCII proteins increases the amount of PSI in grana margin region, which likely facilitates the excitation energy transfer to PSI from the pigment–protein complexes present in the area [24].

More recent biochemical studies have indicated that up to eight LHCII trimers can be bound to one PSII core dimer, instead of the commonly accepted number of four, implying the presence of "extra LHCII" [25–28] with yet unknown function and location. Moreover, after any solubilization of the thylakoid membrane, a large proportion of LHCII is released and exists as free trimers all over the thylakoid membrane instead of being bound to the different PSII–LHCII and LHCII–PSI–LHCI complexes [29].

In the present study, we aimed at reconstructing the pieces of the puzzle into a new model on the organization of the photosynthetic pigment–protein complexes and distribution of excitation energy in the thylakoid membrane. Our special focus is on the LHCII trimers that cannot be isolated together with PSII and PSI complexes and on whether the steady state protein phosphorylation affects the interactions of this "extra" LHCII antenna fraction. Selected depletion of pigment–protein complexes from the thylakoid membrane, followed by biochemical and biophysical analyses of energy distribution, was performed on wild-type (WT) *Arabidopsis thaliana* (hereafter *Arabidopsis*) plants and on the *stn7* mutant, which lacks the STN7 kinase and therefore is not capable of LHCII phosphorylation [1,30]. We assign the role for the LHCII antenna that does not form stable complexes with PSII or PSI as a common antenna for both PSII and PSI, with particular function in the grana margin regions. PSII and PSI reside in the same energetically connected antenna lake, where the energy spillover from PSII to PSI is controlled by lateral distribution of PSII and PSI in the lake. LHCII phosphorylation is not an absolute requirement for the intersystem energetic connectivity, but it strengthens the attraction forces between PSII–LHCII and PSI complexes in the grana margin regions.

2. Materials and methods

2.1. Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) and the *stn7* mutant [1] plants were grown in a mixture of soil and vermiculite (1:1) under constant moderate light (130 μmol photons m^{-2} s^{-1}), 8 hour photoperiod, in relative humidity of 60%. Light was provided by OSRAM PowerStar HQIT 400/D Metal Halide Lamps. Mature rosette leaves from 5–6 week old plants were used for experiments.

2.2. Thylakoid isolation and solubilization

Thylakoid membranes were always isolated from light-adapted leaves (four hours after the beginning of light period) as described in [31]. The chlorophyll content was determined according to [32].

For 2D lpCN electrophoresis, the thylakoids were solubilized substantially according to [29], with a final concentration of digitonin being 1% (w/v). The samples were solubilized with digitonin by gentle agitation for 8 min in darkness at room temperature. Subsequently, thylakoids were centrifuged (18 000 *g* at 4 °C, 25 min) and the supernatant, enriched in solubilized grana margins and stroma lamellae, was collected. The pellet, enriched in insolubilized grana cores, was resuspended into 25BTH20G buffer (25 mM Bis-Tris/HCl, pH 7.0, 20% (w/v) glycerol and 0.25 mg/ml Pefabloc) and further solubilized with 1% n-dodecyl β-D-maltoside (DM), diluted to the 25BTH20G buffer, for 2 min on ice. The insoluble material was removed by centrifugation at 18 000 *g* at 4 °C for 15 min, and equal volumes of the WT and *stn7* supernatants were loaded.

For sequential solubilization of thylakoid protein complexes by digitonin and DM, the isolated WT and *stn7* thylakoids were first resuspended to the storage buffer (50 mM Hepes/KOH pH 7.0, 100 mM sorbitol, 10 mM $MgCl_2$, 10 mM NaF). Detergents were then added to different aliquots to reach final concentrations of 0.2%, 0.5%, 1.0% and 1.5% (w/v). The digitonin solubilization was carried out as described above, the DM solubilization according to [29].

2.3. Gel electrophoresis, staining and immunoblotting

The lpCN and lpBN gels with an acrylamide gradient of 3.5–12.5% in the separation gel, as well as 1D- and 2D-SDS-PAGE were carried out as described in [29]. Molecular mass marker for lpBN was purchased from Invitrogen (Native Mark™ Unstained Protein Standard). Equal volumes of the WT and *stn7* supernatants were loaded. SYPRO® Ruby and ProQ® Diamond stainings were performed according to Invitrogen Molecular Probes™ instructions. Densitometry and immunoblotting were carried out according to [3]. The PsbE antibody was a kind gift from Dr. R.G. Herrmann. Other antibodies were purchased from Agrisera.

2.4. Chlorophyll fluorescence measurements

A specific solubilization of thylakoids was employed before the fluorescence measurements. Isolated thylakoids were first suspended in a storage buffer and then solubilized mildly by detergents (digitonin or DM) in order to detach the most exposed pigment–protein complexes from the integrity of the thylakoid membrane. Noteworthy, for the spectra shown in Figs. 6 and 7A the solubilized and insolubilized thylakoid fractions were not separated from each other but, instead, every sample containing both the detached (solubilized) and membrane-associated (insolubilized) protein complexes, was homogenized and frozen in liquid nitrogen at the final concentration of 40 μg chlorophyll (chl)/ml (50 μl volume). Fluorescence emission spectra were then measured from frozen suspension at 77 K or 253 K by using an Ocean Optics S2000 spectrometer. The samples were excited with blue light (480 nm wavelength). We estimated the possible alteration due to fluorescence self-absorption by recording the emission spectra at 10 and 40 μg chl/ml samples. Since the self-absorption effect was lower than the experimental error, we used the spectra from 40 μg chl/ml sample showing a higher signal/noise ratio for a more accurate peaks decomposition. The 253 K fluorescence emission spectra were measured on WT and *stn7* thylakoids equilibrated in a cooling bath (1 to 3 ratio of sodium chloride to ice). Before cooling, for every genotype 400 μl thylakoids (40 μg chl/ml) were solubilized. An aliquot of 100 μl was used to record the total thylakoid spectrum. The remaining sample was centrifuged; the supernatant was diluted to a final volume of 300 μl, the pellet was resuspended in storage buffer to a final volume of 300 μl. The approximate chl concentration in every sample was estimated based on Supplemental Fig. 3A.

3. Results

3.1. Distribution and phosphorylation of thylakoid pigment–protein complexes in grana cores, grana margins and stroma lamellae

The physiological role of strong steady-state LHCII protein phosphorylation prevailing under standard growth conditions has remained elusive [33] and so far research has mostly focused on state transitions and reversible LHCII protein phosphorylation, explained on the basis of strict lateral heterogeneity of the thylakoid membrane. Since research on thylakoid heterogeneity is almost exclusively based on investigations of dark acclimated leaves and illumination, in turn, has been suggested to induce migration of part of P-LHCII to stroma thylakoids, we first analyzed whether steady state illumination in the presence (WT) and absence (*stn7* mutant) of LHCII phosphorylation has so far uncharacterized consequences on the distribution of the pigment–protein complexes in the thylakoid membrane. Thylakoids were isolated four hours after the onset of illumination from WT and *stn7* mutant plants grown under moderate light (130 μmol photons m^{-2} s^{-1}), and subsequently solubilized by digitonin, a mild detergent that preferentially solubilizes protein complexes from the grana margins and stroma lamellae [29,34]. The "digitonin-insolubilized" fraction of thylakoids, mainly representing the grana core fraction, was isolated by centrifugation and the pellet was further solubilized by n-dodecyl β-D-maltoside (DM), which is able to efficiently solubilize also the grana cores [29]. The composition of thylakoid pigment–protein complexes in these two thylakoid fractions was analyzed by large-pore clear-native (lpCN)-PAGE [29], followed by SDS-PAGE separation of the protein subunits constituting each protein complex. The protein phosphorylation level was revealed by ProQ Diamond, a quantitative dye for phosphoproteins, and the relative amount of total proteins was subsequently estimated by staining the same gel with SYPRO Ruby, a quantitative dye for total proteins (Fig. 1). For quantitative purposes, we also performed densitometry analyses of both the ProQ and SYPRO stain images (Fig. 2).

Comparison of the pigment–protein complexes between samples from digitonin-solubilized thylakoids (grana margins and stroma lamellae) and DM-solubilized pellets (grana cores) reflected their very different origins. As expected, the digitonin-solubilized samples (Fig. 1B left panel) represented non-appressed thylakoid regions and were enriched in PSI that in WT appeared to be distributed mainly in four and in *stn7* in three distinct bands: i) a complex comprising PSI and LHCI; ii) PSI–LHCI bound to P–LHCII, i.e. the so-called 'state transition band' [35], which was absent in *stn7* and present in only a minor amount; iii) two very high molecular mass bands comprising PSI–LHCI and PSII–LHCII (Fig. 1). Digitonin also solubilized distinct PSII–LHCII complexes, which were enriched in a band showing an apparent molecular mass of approximately 1000 kDa (for the molecular mass estimation of thylakoid complexes, see Supplemental Fig. 1). At the lower molecular mass region in the gel, an intense spot corresponding to free LHCII trimers, composed almost exclusively of Lhcb1 and Lhcb2, was distinguished. As for the phosphorylation of the complexes, in the *stn7* mutant the phosphorylation level of LHCII was expectedly very low, whereas the PSII core proteins were more phosphorylated than in the WT control, particularly in the band composed of PSII–LHCII–PSI–LHCI (numbered as 2) (Fig. 2G). Although the molecular masses of the PSII–LHCII–PSI–LHCI bands (1) and (2) could not be accurately determined (Supplemental Fig. 1), it is evident that they are not just co-migrating aggregates of PSII–LHCII and PSI–LHCI complexes but indeed form a megacomplex comprising both photosystems and their LHC complexes.

After digitonin solubilization of grana margins and stroma lamellae, the subsequent pellet was finally solubilized with DM and as shown in Fig. 1B right panel, was mainly comprised the PSII core and LHCII complexes in different combinations. Also these samples revealed a considerably high amount of free LHCII trimers, composed almost exclusively of Lhcb1 and Lhcb2. In addition, the separated complexes included LHCII trimers composed of Lhcb1, Lhcb2 and Lhcb3 together with minor antennae CP24 and CP29, in the so-called "LHCII assembly complex" [36], which likely represents the previously characterized "Band 4" in [37] and [38]. Small amount of PSI–LHCI co-migrated in the native gel with PSII dimer-LHCII complex, as deduced from the molecular mass analysis of the complexes (Supplemental Fig. 1). The CP24 and CP29 proteins present in the four higher molecular mass PSII–LHCII bands increased their proportion with increasing mass of the complexes. Correspondingly, the ratio of LHCII trimers over PSII also increased in complexes with increasing molecular mass (Fig. 2B). This strongly indicated that these constituted PSII complexes with the addition of LHCII trimers. At the highest molecular mass region in the gel prepared from digitonin-insoluble membranes (Fig. 1B, grana core), we found other PSII–LHCII complexes whose intensity, however, showed a low reproducibility among several technical and biological replicates. This suggested that these complexes could be localized close to the grana margins, corresponding to the highest molecular mass bands found in digitonin-solubilized samples (Fig. 1B, grana margins and stroma lamellae). All the other complexes, on the contrary, were highly reproducible.

In the grana core (DM-solubilized pellets) fractions, the WT and *stn7* thylakoids showed similar LHCII amount and similar LHCII to PSII ratio (Fig. 2B and D and Supplemental Fig. 2C) as well as similar PSII to PSI ratio (Supplemental Fig. 2D). In the margin plus stroma lamellae (digitonin-solubilized) fraction, instead, the *stn7* mutant presented a higher LHCII/PSII ratio (Fig. 2C and Supplemental Fig. 2A) and a lower PSII/PSI ratio compared to WT (Supplemental Fig. 2B). Thylakoids were initially isolated from growth-light-acclimated leaves and an intriguing observation was that all LHCII trimer complexes showed phosphorylation, irrespectively of whether they resided in the WT grana core, grana margins or stroma lamellae fraction (Figs. 1 and 2E and F). In grana margins and stroma lamellae, LHCII bound to PSI exhibited the highest specific phosphorylation level (Fig. 2E). In grana core, the Lhcb1 and Lhcb2 proteins were located in seven different complexes and showed roughly similar phosphorylation levels (Fig. 2F). PSII was phosphorylated at the same extent in WT and *stn7* grana cores (Fig. 2H), while in grana margins and stroma lamellae the PSII core proteins were significantly more phosphorylated in the *stn7* mutant (Fig. 2G). In electrophoretic analyses on both appressed and non-appressed thylakoids, a noticeable amount of LHCII appeared to be not bound to PSII neither to PSI. In grana margins and stroma lamellae about half of Lhcb1 and Lhcb2 proteins were located in the free LHCII band (mean ± SD = 45 ± 6% in WT, 48 ± 3% in *stn7*). In grana cores the proportion of Lhcb1 and Lhcb2 in LHCII not associated to PSII or PSI (LHCII assembly and free LHCII bands) was even higher (66 ± 6% in WT, 64 ± 3% in *stn7*). Indeed, a large proportion of LHCII does not form stable complexes with either PSII or PSI. This antenna fraction, constituting roughly half of the light harvesting capacity of the thylakoid membrane, has so far been largely neglected in studies on the excitation energy distribution in the thylakoid membrane, even though it has been identified and denoted as 'extra LHCII' [25–28].

Intact thylakoids from light-acclimated leaves were next solubilized by using an increasing digitonin concentration in order to get further insights into the composition and function of the photosynthetic complexes located in enigmatic grana margin regions. It was assumed that digitonin has the easiest access to the most exposed stroma lamellae and with increasing digitonin concentration the solubilization proceeds deeper towards grana margins. For both genotypes, isolated thylakoids were divided into five aliquots, and every aliquot was subjected to solubilization by digitonin at a certain concentration (0, 0.2, 0.5, 1 and 1.5% w/v for the five aliquots, respectively). From every sample, the digitonin-solubilized proteins/protein complexes were isolated by centrifugation and analyzed with large pore blue native (lpBN)-PAGE to quantify the pigment–protein complexes released from the membrane by increasing digitonin concentration (Fig. 3). Digitonin concentration of 1 to 1.5% caused the solubilization of roughly 20–25% of all chl-binding complexes in the thylakoid membrane, in both WT and *stn7*

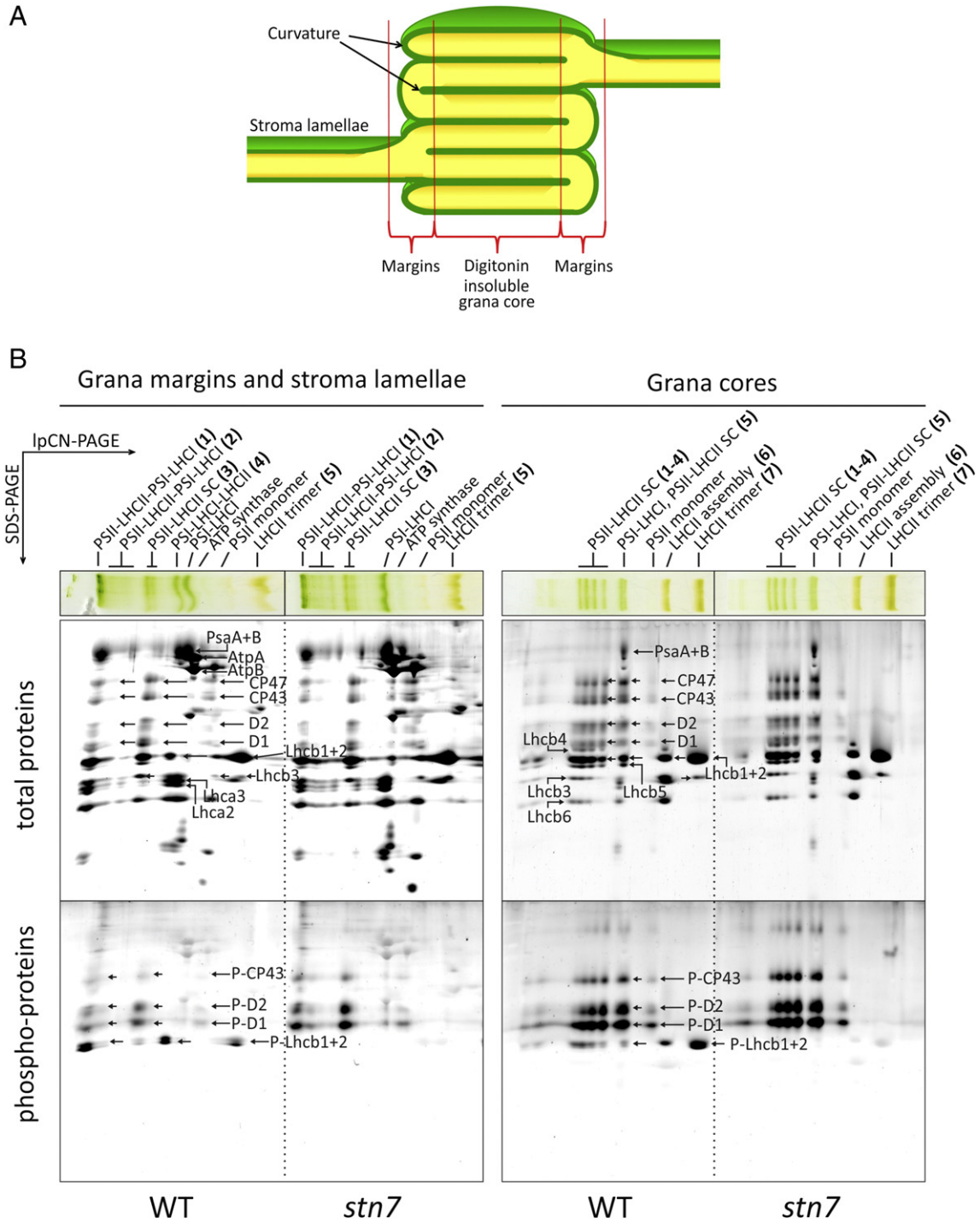

Fig. 1. Thylakoid membrane protein complexes in WT and *stn7* plants. (A) Scheme illustrating the isolated thylakoid fractions: grana margins and stroma lamellae were first solubilized by digitonin and grana cores subsequently by n-dodecyl β-D-maltoside (DM) solubilization. (B) Protein complexes from the two distinct thylakoid fractions were separated by large pore clear native electrophoresis (lpCN-PAGE). Protein subunits of each complex were subsequently separated in the second dimension by SDS-PAGE. Total proteins were visualized by SYPRO staining; phosphorylated proteins by ProQ Diamond staining. The nomenclature of protein complexes separated by lpBN-PAGE is based on [29], and the identification of the individual proteins is based on [36]. SC = supercomplex. Gels are representative examples of three biological replicates.

(Supplemental Fig. 3A). The chl a/b ratio of digitonin solubilized protein complexes, on the contrary, differed considerably: in WT ranging from about 8 to 6, in *stn7* samples from ~12 to ~7 (Supplemental Fig. 3B). In WT and *stn7*, a similar amount of free LHCII trimers was detached from thylakoids by digitonin treatment. As expected, *stn7* did not show the state transition band, while it showed a more intense PSI band compared to WT (Fig. 3A). PSII–LHCII supercomplexes appeared to be scarce in all digitonin-solubilized fractions. Anti-D1 immunoblotting of the same gel was used to analyze more accurately the relative amount of various PSII complexes released from the thylakoid membrane at certain digitonin concentration (Fig. 3B). PSII monomer was relatively abundant and showed roughly similar release from the thylakoid membrane by 0.5 to 1.5% digitonin concentration both in WT and *stn7* samples. The PSII dimers, on the contrary, released only weakly from both WT and *stn7* thylakoids by digitonin. PSII dimers that are bound to LHCII, the PSII–LHCII supercomplexes, are known to

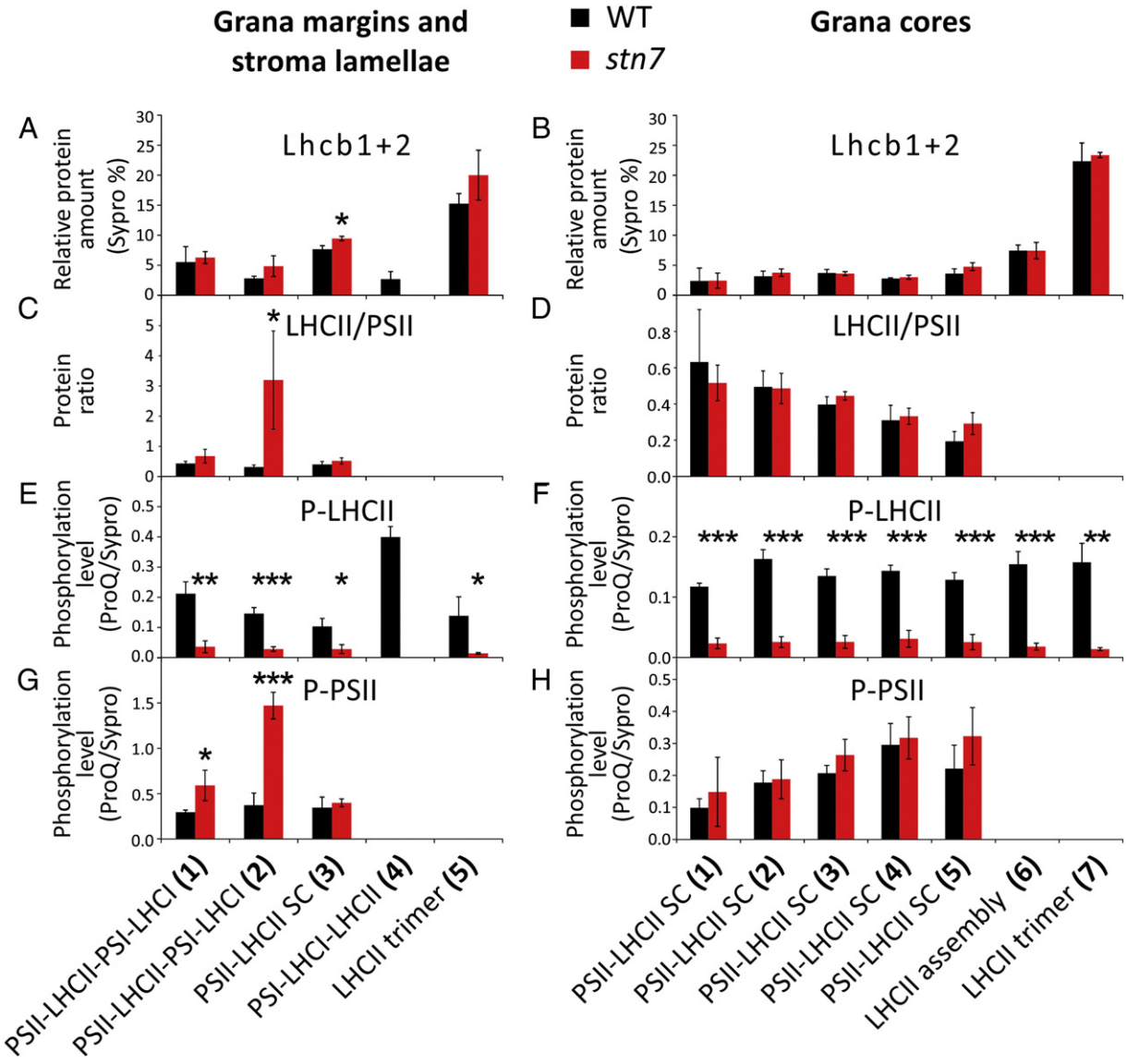

Fig. 2. Relative amount and phosphorylation levels of PSII and LHCII proteins in respective complexes from grana margins and stroma lamellae (A, C, E, G) and grana cores (B, D, F, H), isolated from WT and *stn7* plants. (A–B) Relative amounts of Lhcb1 and Lhcb2 proteins. (C–D) Ratio of LHCII (Lhcb1 and Lhcb2) proteins to PSII (D1, D2, CP43 and CP47) proteins. (E–F) Phosphorylation level of the LHCII proteins. (G–H) Phosphorylation level of the PSII proteins. In grana margins and stroma lamellae, LHCII bound to PSI exhibited the highest specific phosphorylation level. Values were obtained by densitometry of SYPRO and ProQ Diamond stained 2D-lpCN-PAGE gels (representatives of which are shown in Fig. 1B). The phosphorylation level was normalized to the protein amount (ProQ/SYPRO ratio). Data are means ± SD, n = 3; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test).

be enriched in grana cores [39,40]. Increase in digitonin concentration led to differential release of these supercomplexes from thylakoids, being much higher in WT than in *stn7*. Likewise, the PSII–LHCII–PSI–LHCI megacomplexes could be solubilized by digitonin, being more abundant in WT than in *stn7* (Fig. 3B). These results provide evidence that the supercomplexes become more exposed to stroma lamellae and grana margins by light-induced phosphorylation of LHCII proteins, and thereafter attach with the PSI–LHCI complexes.

To get further insights into the conclusion drawn above from Fig. 3, suggesting that STN7-induced phosphorylation of LHCII complexes opens up the grana structures, the amount and phosphorylation level of proteins in digitonin-solubilized fractions from WT and *stn7* thylakoids were further analyzed by SYPRO and ProQ staining of SDS-PAGE gels (Fig. 4). In both WT and *stn7* samples, the main proteins released from the thylakoid by digitonin solubilization clearly belonged to PSI, ATP synthase, LHCII and PSII complexes. From *stn7* thylakoids, even the highest digitonin concentration (1.5%) released much less of PSII proteins and considerably less of LHCII proteins than 0.5% digitonin solubilization released from WT thylakoids, thus confirming the results from lpBN-PAGE and explaining the higher chl a/b ratio of *stn7* fractions solubilized with digitonin (Supplemental Fig. 3B). It is noteworthy that most of the ATP synthase was solubilized by 0.2% digitonin, while only a small part of PSI and PSII proteins released from the membrane with 0.2% digitonin. Preferential solubilization of the entire ATP synthase enzyme was confirmed with immunodetection against the membrane embedded (CFo) subunit AtpF (Fig. 5). The same membrane was incubated with PsaB and PsbE antibodies to confirm the relative content of PSI and PSII proteins compared to ATP synthase in every digitonin-solubilized fraction.

The digitonin-insolubilized thylakoid fractions (i.e. the respective pellets) were also analyzed, and revealed a complementary protein pattern compared to digitonin solubilized fractions (Fig. 5). In particular, at 0.2% digitonin most of AtpF was released from the membrane and increasing the digitonin concentration did not have much further effect on the content of AtpF in corresponding WT or *stn7* pellets. Gradual increase in the release of PsaB from the thylakoid

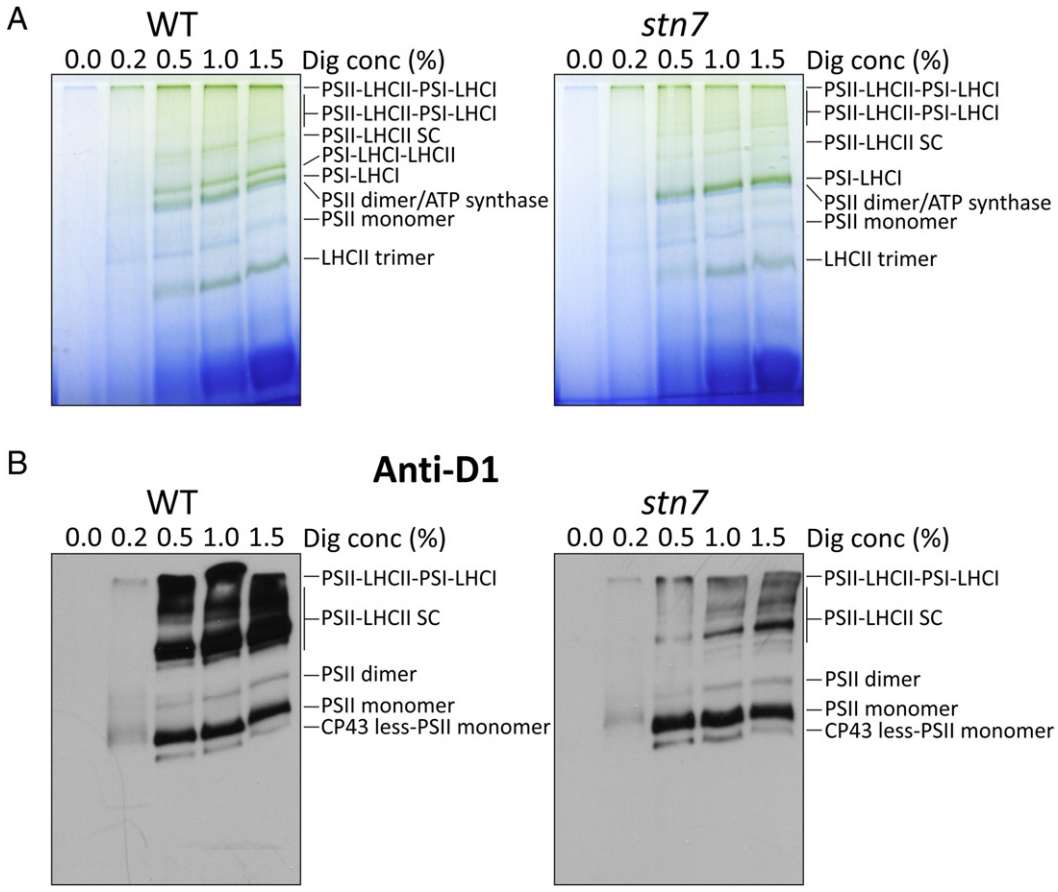

Fig. 3. Composition of the chlorophyll–protein complexes in digitonin solubilized thylakoids. Thylakoid membranes isolated from WT and *stn7* plants were partially solubilized by using increasing concentrations of digitonin (Dig conc, %) (w/v). (A) The solubilized fractions were separated and analyzed by lpBN-PAGE. (B) PSII content and distribution were revealed by anti-D1 immunoblotting carried out on the gel shown in (A). Gels are representative examples of three biological replicates.

membrane with increasing digitonin concentration, on the contrary, was reflected in gradual decrease of PsaB in the respective pellet fractions. However, part of the PsaB protein remained still bound to the membrane even at 1.5% digitonin concentration. The Cyt f subunit of cytochrome b_6f appeared to be localized mainly in non-appressed thylakoids (solubilized fractions), both in WT and *stn7*.

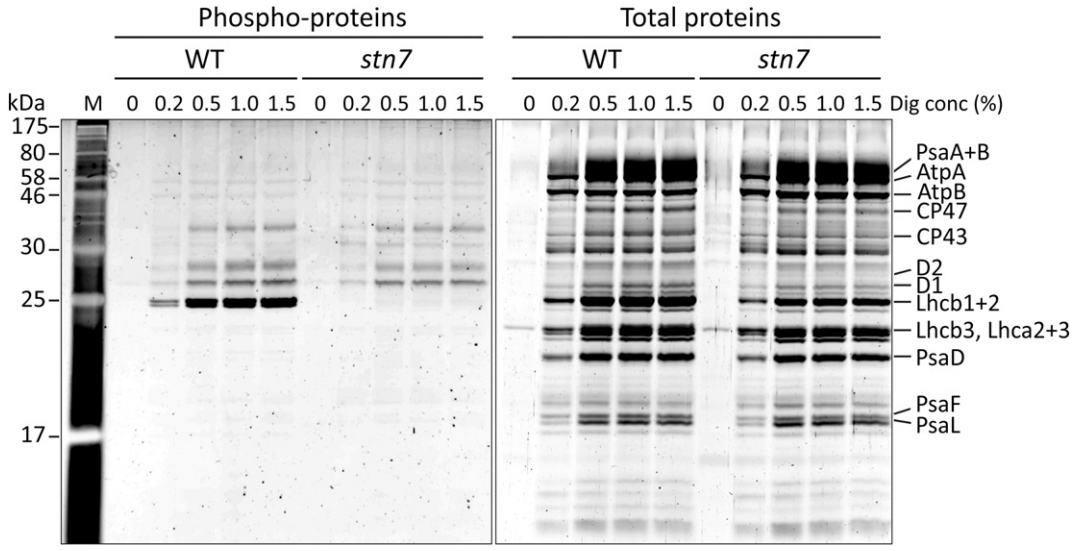

Fig. 4. Composition and the phosphorylation status of proteins released from the thylakoid membrane by solubilization with increasing digitonin concentration. Thylakoid membranes isolated from WT and *stn7* plants were partially solubilized by using increasing concentrations of digitonin (Dig conc, % w/v) for 10 min at room temperature. The solubilized fractions were separated and analyzed by SDS-PAGE. Phosphorylated proteins were revealed by ProQ Diamod staining, total proteins by SYPRO staining. Photosystem I proteins: PsaA, PsaB, PsaD, PsaF, PsaL. LHCI proteins: Lhca2, Lhca3. Photosystem II proteins: CP47, CP43, D2, D1. LHCII proteins: Lhcb1, Lhcb2, Lhcb3. ATP synthase proteins: AtpA, AtpB. M = molecular markers. Gels are representative examples of three biological replicates.

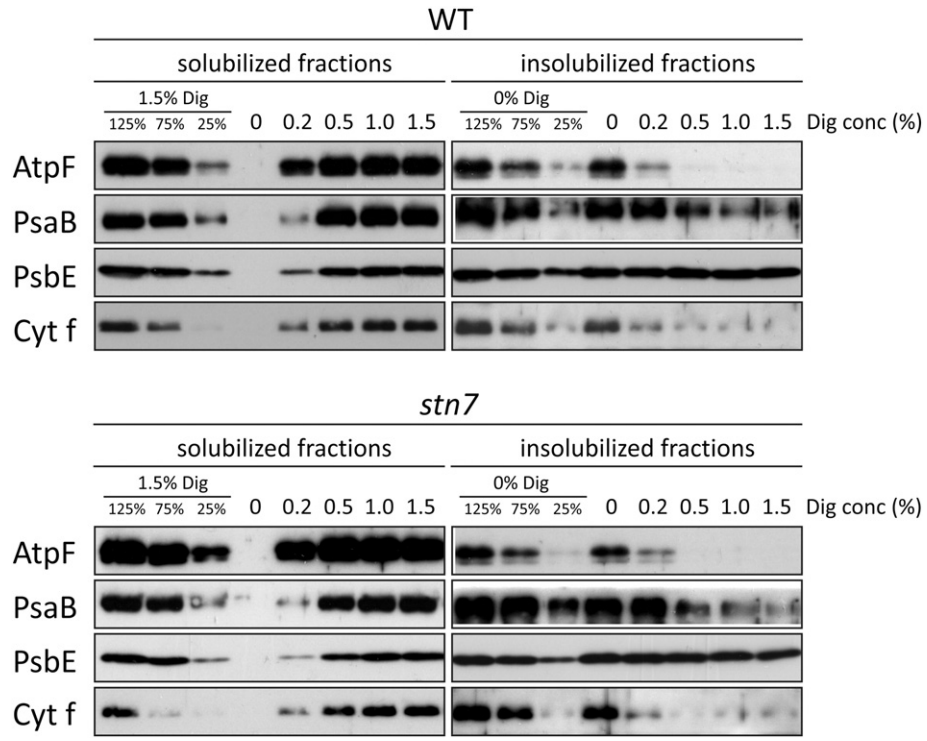

Fig. 5. Solubilization of the main photosynthetic protein complexes by increasing concentrations of digitonin (Dig conc, %) (w/v) from the thylakoid membranes isolated from WT and *stn7* plants. After partial solubilization, thylakoids were centrifuged and separated into supernatants (solubilized fractions) and pellets (insolubilized fractions). Protein amounts were revealed by immunoblotting with specific antibodies against ATP synthase (the membrane-spanning subunit AtpF), PSI (PsaB), PSII (PsbE) and cytochrome b_6f (Cyt f). The linearity of the signal was monitored by different quantities of the thylakoid fraction solubilized by 1.5% digitonin for solubilized fractions, and 0% digitonin for insolubilized fractions.

3.2. Detachment of LHCII antenna and PSI from the thylakoid enhances PSII excitation

In order to estimate the effect of chemical detachment of the LHCII complexes from grana margins and stroma lamellae on the distribution of excitation energy between the two photosystems, we recorded the 77 K chl fluorescence emission spectra from thylakoid membranes isolated from WT and *stn7* plants grown in standard light conditions. It is important to note that digitonin is a mild detergent that solubilizes the thylakoid membrane lipids but does not alter the pigment–protein interactions within any released chl–protein complex [41–43]. Before measurements, thylakoids were divided into five aliquots and partially solubilized by increasing digitonin concentration (from 0 to 1.5%) as described above. In this experiment, however, the solubilized and detached proteins and protein complexes were not separated from insolubilized material. Thus in every sample, both the solubilized and insolubilized protein complexes remained in the same tube, were mixed and eventually frozen in liquid nitrogen. Therefore, all the aliquots from one genotype contained exactly the same amount of photosystems and antennae. In this way, the differences observed in the 77 K spectra have to be attributed to changes in energetic connectivity between pigment–protein complexes, instead of changes in PSII/PSI or antennae/photosystems ratio. The absolute values of PSII and PSI fluorescence emission intensity were estimated at 695 nm and 733 nm, respectively, from non-solubilized thylakoids (0% digitonin) and from thylakoids solubilized by 1.5% digitonin (Fig. 6A). In both WT and *stn7* the PSII emission increased upon solubilization, while the PSI emission decreased. For a more detailed analysis, every 77 K chlorophyll fluorescence spectrum was decomposed into its main components (according to [44]): free LHCII trimers and monomers emit a peak with maximum at 680 nm (denoted as F680); PSII at F685 and F695; aggregated LHCII trimers at F700; PSI–LHCI at F720 and F735; Fv720 indicates a vibrational band attributed to PSI core (Fig. 6B). The area under each emission peak was calculated and used to estimate relative changes. By increasing digitonin concentration, the free LHCII emission peak increased in WT and *stn7* thylakoids, indicating a gradual solubilization of LHCII located in grana margins and stroma thylakoids (Fig. 6C). The PSII/PSI emission ratio increased concomitantly with the detachment of LHCII, although the *stn7* mutant thylakoids showed systematically higher ratios than WT (Fig. 6D) and the PSII/PSI emission ratio at 1 and 1.5% digitonin was significantly higher in *stn7* compared to WT ($P < 0.01$, $n = 6$).

The somewhat unexpected result, a clear increase in the amplitude of the PSII emission bands at F685 and F695 by the detachment of LHCII, PSII and PSI from the thylakoid membrane, called for further investigation. To this end, we measured fluorescence emission spectra from total thylakoids, and from digitonin-solubilized and -insolubilized fractions at the temperature of 253 K (Fig. 7). At this temperature, PSI centers can non-photochemically quench the excitation energy they trap [45,46]. The progressive detachment of LHCII by increasing the digitonin concentration provoked the increase of fluorescence emission from total thylakoids of WT and *stn7* at 680 and 740 nm wavelengths, attributed to LHCII [47], (Fig. 7A). This indicated that all or almost all of the extra LHCII detached by digitonin (revealed by lpCN-PAGE shown in Fig. 1) was energetically connected in intact thylakoids of both WT and *stn7*.

The digitonin solubilized and insolubilized thylakoid fractions were then separated, adjusted to the same volume and analyzed individually. As expected, in solubilized fractions of both WT and *stn7*, the absolute amount of fluorescence from LHCII increased in line with increasing proportion of solubilized thylakoids (Fig. 7B). Surprisingly, the absolute amount of LHCII fluorescence emission, together with PSII fluorescence, increased also in insolubilized thylakoids, representing grana cores, both in WT and *stn7*. The increase in digitonin concentration caused considerable release of LHCII from insolubilized fractions, yet at the same time an increase was recorded in the absolute amount of LHCII and PSII fluorescence emission from these fractions (after solubilization the main peak centered at 682 nm in both WT and *stn7*) (Fig. 7C). Intriguingly, the fluorescence increase in the insolubilized fraction was

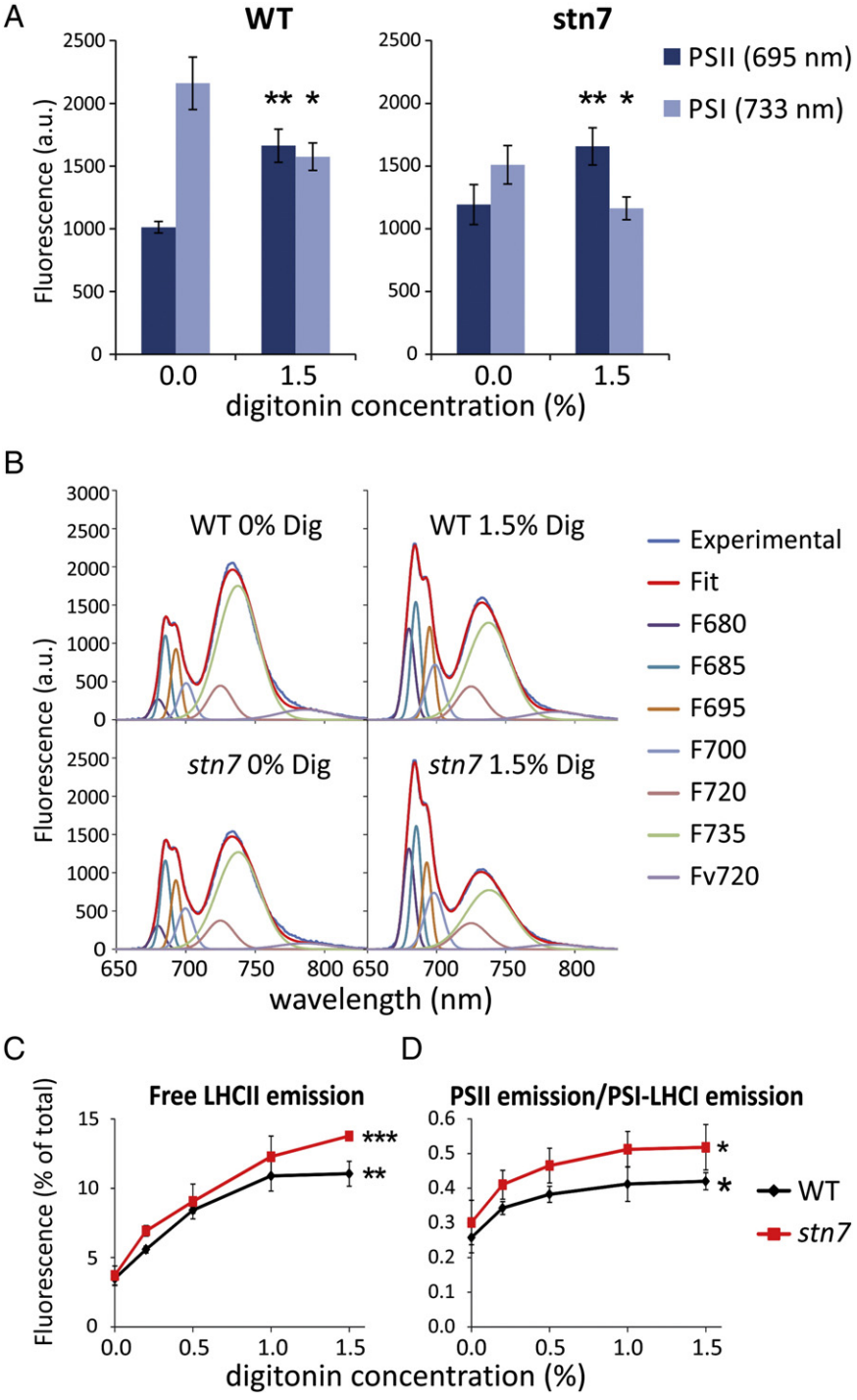

Fig. 6. Effect of digitonin treatment on distribution of excitation energy in the thylakoid membrane of WT and *stn7* plants, revealed by 77 K fluorescence emission spectra. Digitonin in different concentrations (%, w/v) was allowed to partially solubilize the thylakoid membrane; subsequently the suspension (including both solubilized and insolubilized fractions) was subjected to recording of the chlorophyll fluorescence emission spectra at 77 K. (A) Intensity of fluorescence emission from PSII and PSI (at 695 and 733 nm, respectively). Data are means ± SE, n = 12. (B) Emission spectra were decomposed into their components (see text for details). Peaks were denoted by the respective emission maxima wavelengths. Representative WT and *stn7* spectra only from non-solubilized (0% digitonin) and from 1.5% digitonin-solubilized thylakoids are shown. (C and D) The areas of single peaks shown in B were quantified and plotted against digitonin concentration: free LHCII (C), ratio of PSII emission over PSI–LHCI emission (D). Data are means ± SD, n = 3. Asterisks (*) indicate that the value of 1.5% digitonin-treated sample was significantly different than the value of the same sample that was not treated by digitonin: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test).

remarkably higher in *stn7* as compared to WT. It is worth noticing that the solubilized thylakoids from *stn7* exhibited a lower PSII/PSI ratio than WT (Figs. 1, 3, 4 and Supplemental Fig. 2B), while WT and *stn7* grana cores showed no significant difference in LHCII and PSII content (Figs. 1 and 2). These results collectively supported the idea of energetic connectivity between the appressed and non-appressed thylakoids via the margin region, and the occurrence of excitation energy quenching by PSI through LHCII connected to both PSII and PSI.

Besides digitonin treatment, in a different set of experiments the isolated WT and *stn7* thylakoids were directly solubilized by DM at different concentrations and subsequently subjected to biochemical and biophysical analyses. DM provoked significant LHCII solubilization already at the concentration of 0.5% (as shown in WT, Supplemental Fig. 4A). The 77 K fluorescence emission spectra revealed that the free LHCII peak increased with increasing DM concentration both in WT and *stn7* thylakoids, reaching about ten-fold higher values as compared

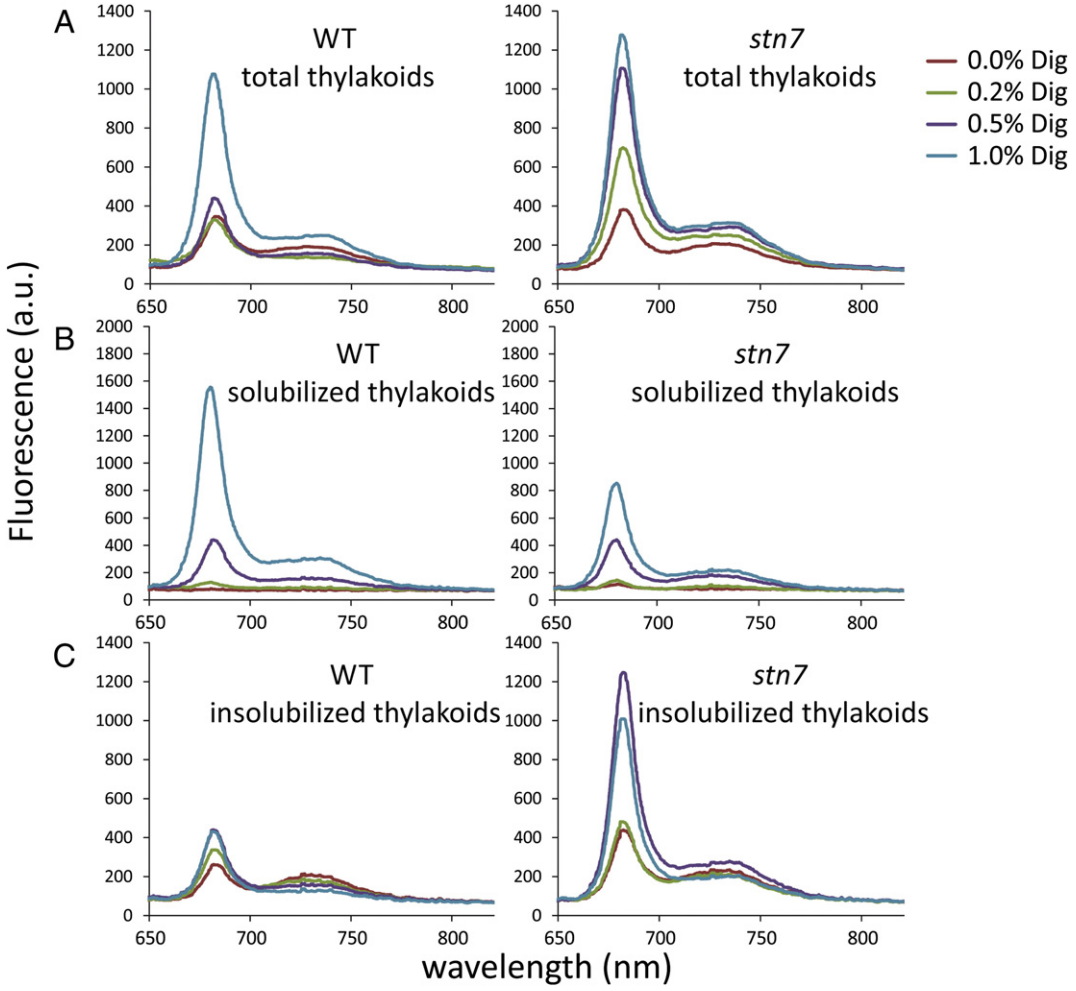

Fig. 7. 253 K fluorescence emission spectra from whole digitonin-treated WT and *stn7* thylakoids, and from separated solubilized and insolubilized fractions. (A) Total thylakoids (the chlorophyll concentration in every sample was 40 μg/ml). (B) Solubilized thylakoid fraction (the chlorophyll concentration increased from approximately 0 to 10 μg/ml upon increasing digitonin concentration). (C) Insolubilized thylakoid fraction (the chlorophyll concentration decreased from approximately 40 to 30 μg/ml upon increasing digitonin concentration; see the Materials and Methods section for details).

to values from intact thylakoids (0% DM) (Supplemental Fig. 4B and C). In WT, the PSII/PSI emission ratio increased and reached a plateau at 1% DM concentration, with a more than two-fold increase compared to the initial value. In *stn7* thylakoids the same parameter showed higher values than in WT in the range from 0 to 0.5% DM, while it reached the same value as that in WT at 1 and 1.5% DM (Supplemental Fig. 4C).

4. Discussion

Balanced light harvesting and electron transfer processes in the thylakoid membrane, from one hand provide energy in sustainable way for the growth and development of plants, and from the other hand guide plant acclimation to various abiotic and biotic stress conditions by participating in retrograde signaling cascades [48,49]. Therefore, in-depth understanding of the operation and regulation of light-harvesting and energy distribution processes in plant chloroplasts is of crucial importance in any studies of plant growth, development and stress acclimation.

In the present study we focus on the question how excitation energy is distributed from the LHCII trimers that do not form isolatable complexes either with PSII or PSI but consist of nearly half of total LHCII found in the thylakoid membrane. From the vast amount of literature in the field (see introduction), it is not clear whether (i) the two photosystems are both functionally and structurally separated from each other and only the so-called state transition mechanism, via LHCII protein phosphorylation, can modulate the relative antenna size of the two photosystems or whether (ii) PSII and PSI are to some extent energetically connected. (iii) Moreover, the functional role in excitation energy distribution of the LHCII fraction (>50% from total LHCII) that does not form stable complexes either with PSII or PSI (denoted as "extra LHCII") has remained completely unknown. The experiments presented in the Results section were designed to address these questions. Based on our results as well as on the rapidly expanding knowledge in the field, we present a model depicted in Fig. 8 that explains our current understanding of the distribution of excitation energy between the two photosystems, emphasizing the grana margins as a dynamic region for energy transfer between PSII and PSI.

As the first approach, we analyzed the distribution and phosphorylation pattern of the photosynthetic pigment–protein complexes present in the grana core (appressed membranes) and the grana margin plus stroma lamellae (non-appressed membranes) regions using thylakoids isolated from light-acclimated WT and *stn7* mutant plants. Importantly, the light-acclimated WT plants have steady state LHCII phosphorylation [3], while the *stn7* plants are practically devoid of LHCII phosphorylation. In the non-appressed thylakoid membranes (Figs. 1, 3, 4 and 5), the presence of megacomplexes comprising PSII–LHCII–PSI–LHCI was evident particularly in WT along with at least four smaller pigment–protein complexes: PSII–LHCII, LHCII trimers, PSI–LHCI, and only in WT the so-called "state transition" complex (PSI–LHCI bound to phosphorylated LHCII) [35]. The appressed membranes both in WT and

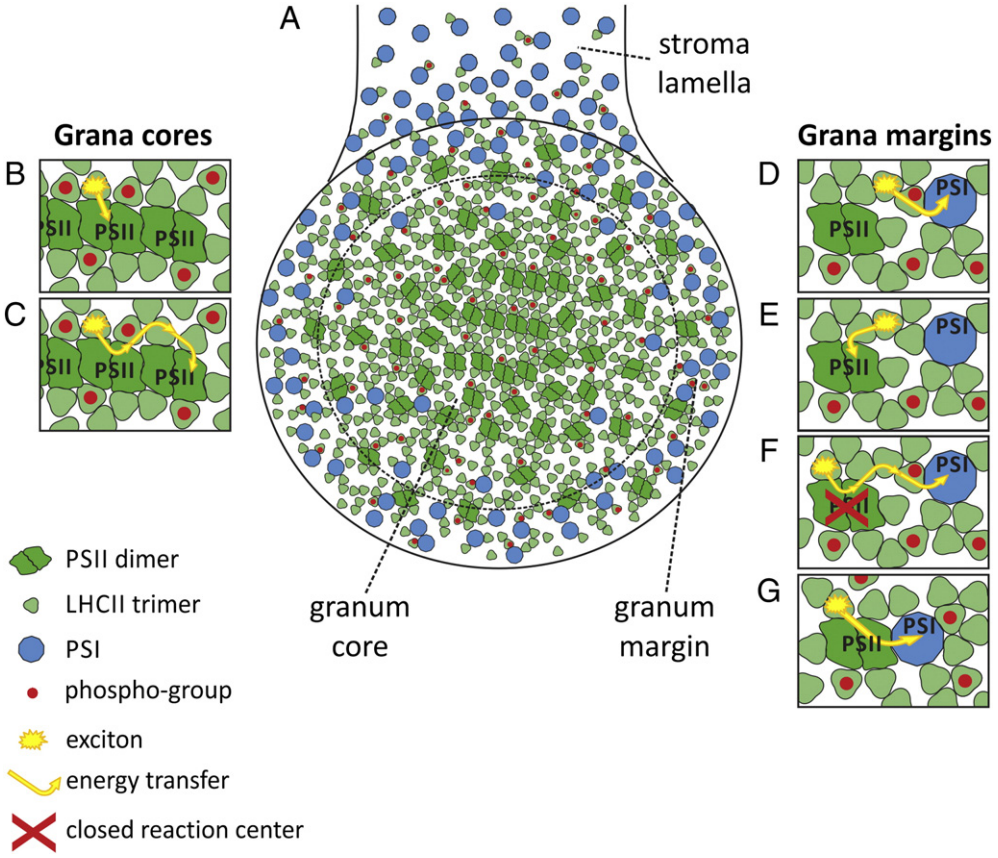

Fig. 8. Schematic representation of the distribution of photosystems and their antennae in the thylakoid membrane (A) and the possibilities of excitation energy flow in the PSII and LHCII rich grana core (B–C) and in the more exposed domains with high amount of PSI also (D–F). (A) PSII–LHCII complexes and the PSI complexes are enriched in the grana core and peripheral domains of thylakoid disks, respectively. Both PSII and PSI are present at peripheral areas of the grana disks. A considerable amount of LHCII trimers is interposed between the photosystems and all LHCII trimer populations are partially phosphorylated (for clarity, the phosphorylation of PSII is not shown). (B) Light is absorbed by an LHCII trimer, thus forming an excitation energy packet (exciton) that is transferred to an open PSII center. (C) Exciton moves from LHCII to a closed PSII and subsequently reaches an open PSII through LHCII connectivity. Towards the peripheral areas of the grana disks (D–F), the probability that exciton can move through LHCII connectivity to PSI increases. In the absence of LHCII phosphorylation the probability that exciton can enter to PSI is low. The LHCII phosphorylation, however, modulates the molecular interaction in the antenna lake to enhance energy trapping by PSI (D). (G) PSII and PSI can be in contact, allowing direct energy spillover from PSII to PSI.

stn7 were enriched in PSII–LHCII supercomplexes and LHCII trimers. The LHCII antenna is present in all other pigment–protein complexes except PSI–LHCI and PSII monomer complexes. Yet a remarkable portion of LHCII is present in gel electrophoresis analyses as free LHCII forms, not bound to either of the complexes (Figs. 1 and 2). The amount of LHCII that strongly binds to PSI and can be found in the state transition complex is very small as compared to the LHCII that does not bind either PSII or PSI and cannot alone explain the sufficient excitation energy supply of PSI.

Progressive solubilization of thylakoids by digitonin, i.e. a release of complexes from the thylakoid integrity, revealed more detailed information about interaction of the pigment–protein complexes. The ATP synthase enzymes (both extrinsic and membrane embedded subunits) almost entirely release from thylakoids by 0.2% digitonin (Figs. 4 and 5). With the same treatment, only a small amount of PSI, LHCII and PSII is released, indicating that ATP synthase resides in its own low-protein-density thylakoid domain that is very sensitive to digitonin solubilization. Indeed, higher digitonin concentrations are needed to release PSI, LHCII and PSII from the membrane. It is important to note that the increased digitonin concentration is able to detach increased amount of the PSI, PSII and LHCII complexes. The relative amounts of detached PSI, PSII and LHCII complexes, however, remain roughly the same with all digitonin concentrations. This may indicate that PSII and PSI are present in functional stoichiometry in the digitonin sensitive thylakoid domain and the actual electron transfer reactions take place in this particular domain, which is close to the ATP-synthase. Digitonin resistant grana core membranes, in turn, are highly enriched in PSII and LHCII with only a minor amount of PSI present.

An interesting finding was that the thylakoid domain solubilized by 0.5–1.0% digitonin and isolated from light-acclimated leaves (4 h after turning the lights on in the growth chamber) is far more enriched in PSII and LHCII in WT, representing steady-state phosphorylation of thylakoid proteins [2,3,11,33,50] than in *stn7* lacking LHCII phosphorylation (Figs. 3 and 4). Importantly, in WT the LHCII proteins (Lhcb1 and Lhcb2) are phosphorylated in all LHCII-containing complexes as well as in extra LHCII forms. This result indicates that LHCII phosphorylation (likely LHCII–PSI–LHCI complex) attracts PSII and LHCII out from the highly packed grana core towards the digitonin sensitive thylakoid domain. This is in line with our previous report showing that LHCII phosphorylation increases the amount of PSI in the PSII and LHCII rich thylakoid domain and that of PSII and LHCII in the PSI rich thylakoid domain [24].

In the data presented here, the grana core membranes, as expected, are highly enriched in PSII–LHCII supercomplexes both in WT (with all LHCII complexes similarly phosphorylated) and in *stn7* (lacking LHCII phosphorylation) (Figs. 1 and 2), thus implying that LHCII phosphorylation is not of special importance for supercomplex formation. In grana margins and stroma lamellae, the pigment–protein complexes and their phosphorylation patterns are more complicated and different between WT and *stn7*. As noted above, the steady state phosphorylation of LHCII (and PSII core) proteins in WT remarkably increased the presence of PSII and LHCII complexes in non-appressed thylakoid regions as compared to *stn7* (Fig. 3B, Supplemental Figs. 2B and 3B). Of

particular interest is the accumulation of mega- and supercomplexes in non-appressed membranes practically only in WT thylakoids (Fig. 3B). The state transition complex exhibited the highest phosphorylation level of LHCII proteins in WT (Fig. 2E), yet its relative amount is low (Fig. 1B left panel). On the other hand, in the absence of LHCII phosphorylation (the *stn7* mutant) and due to consequent redox imbalance [1,2] the PSII core proteins are more strongly phosphorylated (Fig. 2G) compared to WT. Although the mutual balance between PSII core and LHCII phosphorylation is a key factor for dynamics of thylakoid protein complexes [51], we focus here on LHCII phosphorylation, considered to be a more dominant component.

The results above indicate that under normal growth light conditions the LHCII proteins are phosphorylated and attached to protein complexes along all thylakoid regions, including the grana core, margins and the stroma lamellae. This is in fundamental disagreement with the traditional 'state transitions' theory, according to which only part of LHCII antenna is phosphorylated and when phosphorylated moves from grana to stroma lamellae into connection with PSI in order to balance the absorption cross section between the two photosystems [9,10]. Consequently, a new model is pertinently needed to accommodate the most recent results of several research groups in the field of LHCII protein phosphorylation and energy distribution between PSII and PSI (e.g. [11,12]).

Analysis of excitation energy distribution between PSII and PSI after partial solubilization of the thylakoid membrane provided interesting results. Digitonin-induced preferential detachment of LHCII and PSI (and a conspicuous amount also of PSII in WT) from non-appressed thylakoid membrane regions had a strong influence on excitation of PSII and LHCII in the appressed membranes, both in WT and *stn7* (Figs. 6 and 7). Indeed, the 77 K chl fluorescence emission spectra after solubilization of thylakoids (no separation of soluble and insoluble fractions) showed an increase in the absolute emission from LHCII and PSII and a concomitant decrease of emission from PSI (Fig. 6A and B). Moreover, the emission from LHCII positively correlated with enhanced PSII core fluorescence relative to PSI (Fig. 6C and D). Another surprising result was obtained when isolated digitonin-insoluble thylakoid fractions were analyzed (mainly appressed grana membranes) from both WT and *stn7*. These fractions demonstrated an increase in LHCII and PSII core absolute fluorescence emission despite the fact that gradually more and more of the chlorophyll–protein complexes were abolished from appressed grana membranes by increasing digitonin concentrations (Fig. 7). These observations provide further support to the fact that the LHCII lake (i.e. the extra LHCII not tightly bound to any PSII or PSI complex) is in vivo fully connected to photosystems and serves as a shared antenna for PSI and PSII, instead of complete energetic segregation of PSI and PSII. In case of full segregation of PSII and PSI, the depletion of energetically unconnected PSI should not affect the fluorescence from PSII, particularly in the *stn7* mutant without LHCII phosphorylation. Rather, in the absence of connectivity between PSII and PSI, the detachment of LHCII from PSII should decrease, not increase, the amount of fluorescence emitted by PSII.

Our results indicating that the detachment of PSI and LHCII from the thylakoid membrane strongly increases the fluorescence emission from PSII, both in WT and in *stn7*, provide strong evidence for structural and functional interaction between the two photosystems. Indeed, a noticeable amount of extra LHCII is present along the entire thylakoid membrane (Figs. 1 and 2) and most likely forms an energetically connected antenna lake embedding both photosystems (Figs. 6 and 7). In the peripheral areas of grana disks, i.e. the grana margin region, the PSII-enriched and PSI-enriched thylakoid regions overlap (Figs. 1 and 5) and there are no physical boundaries for energy transfer from PSII–LHCII complexes to PSI. The intersystem energy transfer could possibly occur directly, through direct interaction between PSII and PSI. However, LHCII trimers constitute a common antenna system where excitons can move before reaching PSII or PSI (Fig. 8). Consequently, it is highly likely that in grana margins the LHCII trimers energetically connect PSII and PSI, and can mediate energy transfer between PSII and PSI. Energy transferred to PSI either from LHCII or from PSII through LHCII, has a possibility to be quenched by PSI either photochemically or non-photochemically [45,46,52]. The high amount of extra LHCII [53], which makes the thylakoid membrane an energetically connected entity, implies that PSII and PSI compete for the excitation energy from the same antenna system. Since PSI is a more efficient energy trap than PSII [54], this configuration would favor PSI excitation. Consequently, the digitonin treatment that detaches the connection between PSII and PSI was shown to enhance PSII excitation (Figs. 6 and 7).

It is noteworthy that an increase of PSII excitation was observed also in *stn7* thylakoids. This indicates that the LHCII lake as such has a property to partially balance the excitation energy distribution between PSII and PSI, in a manner not completely dependent on LHCII phosphorylation. This conclusion is corroborated by the fact that the *stn7* mutant changes the LHCII content similar to WT upon long-term light acclimation [3]. Nevertheless, the *stn7* mutant changes also the photosystem stoichiometry, showing higher PSI content and higher LHCII/PSII ratio in grana margins and stroma lamellae compared to WT (Fig. 1 and Supplemental Fig. 2). Such configuration allows *stn7* to increase the energy transfer from LHCII to PSI in order to compensate for the lack of LHCII phosphorylation. To this regard, it is important to note that Zhang and Scheller [55] showed that also non-phosphorylated LHCII can bind to PSI, while LHCII phosphorylation increases the amount of LHCII bound to PSI.

Indeed, PSI–LHCI and PSI–LHCI–LHCII complexes co-solubilize from the thylakoid membrane together with a remarkable amount of PSII–LHCII supercomplexes and LHCII antenna, indicating that a large portion of PSI can directly interact with PSII–LHCII or LHCII (including extra LHCII) (Fig. 1). Our data provide evidence that all these complexes can be in contact and, consequently, transfer energy to each other. Therefore, we assign a role for the whole LHCII antenna lake functioning as a major LHCII antenna for both PSII and PSI. Importantly, there are two mechanisms that in concert control the energy distribution between PSII and PSI; firstly, the lateral distribution of PSII and PSI in the LHCII antenna lake and secondly, the phosphorylation of the thylakoid proteins. Such intersystem antenna connectivity provides maximal antenna size for both photosystems and flexibility to regulate the energy distribution without compromising the antenna size of either photosystem.

In our model we take into consideration only the protein complexes interaction and energy transfer along the plane of a single thylakoid membrane (lateral transfer). Nevertheless, it is worth noticing that energy transfer between opposing LHCII trimers located in adjacent thylakoid membranes (transversal transfer) has been proposed to contribute to the energy distribution [56]. In the latter energy transfer, the LHCII phosphorylation could potentially play a role in increasing the repulsion forces and decreasing the energy transfer between opposing LHCII trimers, thus constituting a further regulation level for energy distribution.

We are aware that the fluorescence data presented here are not definitive, and several diverse techniques will be needed to further investigate the excitation energy transfer in relation to connectivity between pigment–protein complexes. However, our biophysical results are consistent with the structural insights provided here and by other researchers, while they cannot be explained by the current functional models, and hence pave the way to reconsidering the interplay between thylakoid pigment–protein complexes. According to our new model (Fig. 8), LHCII constitutes a shared PSII–PSI antenna: similar as LHCII trimers allow the energy transfer between the PSII units, they also allow energy transfer between the PSII and PSI units in grana margins. Consequently, the established model of connected PSII units ('almost-lake') can be expanded to include also PSI. We define the whole antenna system, allowing energy transfer between the two photosystems, as the "LHCII connectivity model". This imposes an important difference to the canonical model that emphasizes PSII connectivity only. PSI is known to be a more efficient energy trap than PSII, and therefore the

connectivity model that includes also PSI comprises the possibility that PSI gets an excess of excitation energy. This problem is solved by partial lateral heterogeneity of PSII and PSI, and also by LHCII phosphorylation, which regulates the intermixing of PSII–LHCII and PSI complexes in the grana margin regions. Due to the structure of the thylakoid membrane, PSI is isolated from the major light harvesting antenna in a dark-adapted leaf [57]. Thus PSI would lack sufficient excitation energy upon exposure of plant to light, with consequent functional imbalance between PSII and PSI. Nevertheless, such imbalance is dynamically corrected in any changes of light conditions both by the connectivity through the LHCII lake and by the phosphorylation of LHCII trimers. Phosphorylation of LHCII increases the affinity between LHCII and PSI (demonstrated by the stability of PSI–LHCI–LHCII complex in digitonin-solubilized WT thylakoids) (Fig. 3A) and makes energy transfer from P–LHCII to PSI very efficient [58]. Moreover, the extra LHCII, not tightly bound to LHCII–PSI–LHCI, is in contact and exchanges energy with PSI–LHCI or with the LHCII trimer strongly bound to PSI. Therefore, the ultimate effect of LHCII phosphorylation is to enhance the energy transfer rate between PSII–LHCII, extra LHCII and LHCII–PSI–LHCI populations.

5. Conclusion

Based on biochemical and biophysical studies, we present a comprehensive model for the distribution of excitation energy in plant thylakoid membrane. This new model is in line with several recent reports on dynamics of the thylakoid membrane [24,59–65]. Nonetheless, it contradicts with the canonical state transition model [7,10], which is based on strict lateral heterogeneity of the thylakoid membrane with strict spatial segregation of PSI and PSII, and claims that the functional balance between PSII and PSI is provided by shuttling of only a minor phosphorylated LHCII pool between PSII and PSI. According to our results, PSII and PSI are energy traps embedded in the same “antenna lake” and are competing for excitation energy. LHCII protein phosphorylation is not an absolute requirement for the intersystem energetic connectivity occurring in the grana margin regions, but plays an important role in intensifying the interaction between the PSII–LHCII lake and PSI.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.03.004.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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