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The specific localizations of phosphorylated Lhcb1 and Lhcb2 isoforms reveal the role of Lhcb2 in the formation of the PSI-LHCII supercomplex in *Arabidopsis* during state transitions

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ABSTRACT

State transitions are an important photosynthetic short-term response that maintains the excitation balance between photosystems I (PSI) and II (PSII). In plants, when PSII is preferentially excited, LHCII, the main heterotrimeric light harvesting complex of PSII, is phosphorylated by the STN7 kinase, detaches from PSII and moves to PSI to equilibrate the relative absorption of the two photosystems (State II). When PSI is preferentially excited LHCII is dephosphorylated by the PPH1 (TAP38) phosphatase, and returns to PSII (State I). Phosphorylation of LHCII that remain bound to PSII has also been observed. Although the kinetics of LHCII phosphorylation are well known from a qualitative standpoint, the absolute phosphorylation levels of LHCII (and its isoforms) bound to PSI and PSII have been little studied. In this work we thoroughly investigated the phosphorylation level of the Lhcb1 and Lhcb2 isoforms that compose LHCII in PSI-LHCII and PSII-LHCII supercomplexes purified from WT and state transition mutants of *Arabidopsis thaliana*. We found that, at most, 40% of the monomers that make up PSI-bound LHCII trimers are phosphorylated. Phosphorylation was much lower in PSII-bound LHCII trimers reaching only 15–20%. Dephosphorylation assays using a recombinant PPH1 phosphatase allowed us to investigate the role of the two isoforms during state transitions. Our results strongly suggest that a single phosphorylated Lhcb2 is sufficient for the formation of the PSI-LHCII supercomplex. These results are a step towards a refined model of the state transition phenomenon and a better understanding of the short-term response to changes in light conditions in plants.

1. Introduction

Oxygenic photosynthesis is driven by two photosystems: photosystem II (PSII) and photosystem I (PSI). By using light energy and working in series, PSII and PSI are able to oxidase H_2O molecules and feed an electron transport chain that ultimately reduces $NADP^+$ into NADPH. Concomitantly, an electrochemical potential across the photosynthetic membrane is created and this is used by the ATP synthase to phosphorylate ADP into ATP with Pi. NADPH and ATP, energetically rich chemical compounds, are used to reduce CO_2 to create the organic matter that finally sustains almost all life on earth. In green eukaryotic organisms the photosystems are located within the thylakoid membranes inside the chloroplasts and are organized in pigment-protein supercomplexes. Both photosystems are organized in two moieties: a core complex, mainly involved in the photochemical reactions, and an antenna system that is composed of the light harvesting complexes (Lhc), a large family of homologous membrane proteins [1]. PSII is usually found as a dimer surrounded by Lhcb antennas, while PSI is found as a monomer with Lhca antennas on one side of the complex [2–6].

In plants and green algae the main light harvesting complex of PSII (LHCII) is a heterotrimer. In higher plants, the heterotrimer consists of three specific Lhcb isoforms: Lhcb1, Lhcb2 and Lhcb3 [1,7]. In plant thylakoids under non-saturating light, up to four LHCII trimers per monomeric PSII core are present [8,9]. The position of two of the LHCII trimers within the PSII supercomplex is well defined: the S-LHCII trimer is strongly bound to PSII next to the core subunit CP43 and the monomeric Lhcb5 (CP26); the M-LHCII trimer is moderately bound to PSII and is connected to the photosystem *via* the monomeric antennas Lhcb4 (CP29) and Lhcb6 (CP24) [4,10]. The two other trimers, usually called L-LHCII, are loosely bound and indeed they have not yet been purified attached to PSII [10,11].

The absorption properties of the two photosystems differ due to different pigment contents, PSII being much richer in chlorophylls *b* (Chls) than PSI. In addition the different pigment-pigment and pigment-protein environments modulate the Chl absorption properties, and in

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particular create the very low energy forms of Chl found in PSI [12,13] (see [6] for a review). These different absorption properties can cause an imbalanced light absorption between the two photosystems.

Due to rapidly fluctuating environmental conditions (light intensity, light quality, temperature), photosynthesis therefore requires extremely sophisticated regulatory mechanisms to maintain efficiency under low light and to avoid photo-oxidative stress under high light [14]. The state transition is one of the fastest regulatory systems, and is necessary for balancing the electron flux into the two photosystems under non saturating light [15–19]. State transitions allow the redistribution of excitation energy between PSII and PSI on the time scale of a few minutes. This is achieved by the redistribution of a mobile LHCII trimer that can bind either to PSII (State I) or PSI (State II). Recent investigations suggest that in addition to the movement of LHCII, entire photosystems may also be relocated during the state transition. This allows direct energy transfer from PSII to PSI under light that preferentially excite PSII [20].

Despite uncertainty regarding the precise mechanism of the state transition, it is clear that the phosphorylation of LHCII is essential to promote the transition from State I to State II. Phosphorylation occurs on the third threonine at the N-terminal of the Lhcb1 and/or Lhcb2 isoforms, while Lhcb3 does not contain a phosphorylable threonine.

In plants, when PSII is preferentially excited, LHCII is phosphorylated by the STN7 kinase [21,22] and about 20–25% of the total trimers moves and attaches to PSI (State II) [23]. The mobile trimers mainly belong to the pool of loosely bound L-LHCII trimers [24] which move towards the grana margins and unstacked region of thylakoids where PSI is located. On the contrary, when PSI is preferentially excited, LHCII is dephosphorylated by the PPH1 phosphatase (also called TAP38) and migrates back to PSII (State I) [25–27].

Recent evidence highlights the fact that the non-mobile LHCII trimers (LHCII that remain bound to PSII) can also be phosphorylated [20,28]. Through a genetic approach and phosphorylation kinetic analysis using antibodies against P-Lhcb1–2 proteins, it has been recently suggested that the phosphorylation of Lhcb1 and Lhcb2 could play complementary roles in state transitions [29,30].

In this work we performed a thorough investigation into the phosphorylation levels of the Lhcb1 and Lhcb2 isoforms in PSI-LHCII and PSII-LHCII supercomplexes purified from *Arabidopsis thaliana* plants in State II conditions and from the *pph1* mutant lacking the PPH1 phosphatase for LHCII [25,26]. Dephosphorylation assays using a recombinant PPH1 phosphatase allowed us to investigate the role of the two isoforms in the state transition phenomenon, and support a major role for Lhcb2 in the formation of the PSI-LHCII supercomplex.

2. Materials and methods

2.1. Membranes preparation and State II induction

Thylakoids and BBY membranes were prepared as described in [11] from *A. thaliana* Col0 WT, *pph1* (*tap38*) [25,26] and *stn7* mutants [21], grown at 120 μmol m^{-2} s^{-1} at 21 °C, short day. For thylakoid preparation, the protocol was stopped before the Triton solubilization. Membranes were stored at −80 °C in 20 mM Hepes 7.5, 0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$.

State II in WT plants was induced on chloroplasts in solution B1 (after grinding of the leaves and filtration) in presence of 10 mM sodium fluoride (NaF), by incubation in moderate light (about 100 μmol m^{-2} s^{-1}) with shaking for 30 min.

2.2. Isolation of supercomplexes

PSI-LHCII supercomplexes were isolated as previously described in [24]. In short, after thylakoid solubilization, a first sucrose gradient allows the separation of PSI-LHCII from PSI (Fig. 1). The PSI-LHCII is then concentrated/diluted/concentrated and loaded on a second gradient for a better purification. During the procedure, we do not detect any degradation of the PSI-LHCII supercomplex thanks to its high stability in our working conditions [24].

PSII supercomplexes were isolated from thylakoid membranes using a similar protocol, but the solubilization was done without $MgCl_2$ and in presence of 1 mM EDTA to destack thylakoids. PSII supercomplexes from BBY membranes were isolated with a similar protocol, without EDTA and with a final detergent concentration of 0.5% digitonin/0.2% α-dodecylmaltoside (α-DDM).

All fractionations were performed on sucrose gradients obtained by freezing and thawing a solution of 0.35 M sucrose, 10 mM Hepes-KOH pH 7.5 and 0.015% digitonin. Ultracentifugation was done in a SW60 rotor, at 60,000 RPM and 4 °C, for 3 h, or in a SW32 rotor at 32,000 RPM and 4 °C for 17 h (for gradients with 0.5 M sucrose).

For LHCII trimers isolation from PSI-LHCII and PSII supercomplexes, particles were loaded on a second gradient (0.35 M sucrose, 10 mM Hepes-KOH pH 7.5 and 0.05% α-DDM) after incubation in the dark at room temperature for 30 min with 0.3% α-DDM (for separation of all LHCII trimers) or for 15 min with 0.15% α-DDM (for fractionation of M and S trimers).

2.3. SDS-PAGE, Phos-tag and immunoblotting

SDS-PAGE was performed as in [31], with a 12% concentration of acrylamide/bis-acrylamide with a 37:1 ratio and in presence of 6 M urea. Phosphorylated Lhcb was separated using similar gels with 20 μM Phos-tag™ (Wako Pure Chemical Industries) and 80 μM $MnCl_2$. Gels were loaded with 0.1 to 0.3 μg of chlorophylls of LHCII, depending on the gels, and stained with Sypro Ruby.

Immunoblots were performed with Agrisera antibodies (for Lhcb1 and Lhcb2 antibodies, lot 0806 and 0512, respectively) and chemiluminescent detection using a Fusion FX7 detection system (Vilbert–Lourmat).

Quantification was performed by densitometry. Error bars correspond to standard error of the mean, with n = 7 (PSI-LHCII WT or *pph1*, respectively), n = 10 (PSII total phosphorylation), n = 5 (ratio LHCII trimer M/S) and n = 6 (ratio Thylakoid/BBY phosphorylation and P-Lhcb2 dephosphorylation, respectively).

2.4. Production of recombinant PPH1 phosphatase

The cDNA sequence coding for PPH1 (At4g27800.1) minus the transfer peptide and the transmembrane domain (final cloned sequence was from Ser42 to Val362) and with addition of a C-terminal 6xHis-tag, was cloned into a pETMHis plasmid, derived from pET28a + (Novagen) and introduced in *Escherichia coli* Rosetta 2 by electroporation. Expression was induced with 1 mM IPTG at room temperature overnight. Recombinant protein was purified using a Ni-NTA column (Qiagen). Protein presence and purity was controlled by SDS-PAGE and immunoblot using a PPH1 antibody [26] (Supplemental Fig. S1) kindly provided by Roberto Barbato. The absence of unspecific phosphatase activity was also checked (Supplemental Fig. S1).

2.5. In vitro dephosphorylation tests

For dephosphorylation tests, about 1 μg of phosphatase was added to 45 μL of supercomplexes containing 0.1 μg LHCII chlorophyll, in a buffer containing 100 mM Hepes pH 7.8, 5 mM $MgCl_2$, 5 mM DTT and 0.05% digitonin, with or without 0.05% α-DDM (see Results section). The mixture was incubated 1 h at room temperature, then loaded onto an SDS-PAGE Phos-tag gel and blotted as described above.

For dephosphorylation tests in the fluorimeter to detect disassembly of the PSI-LHCII supercomplex, a similar reaction was performed in 500 μL with PSI-LHCII at OD 0.1 (in the red peak) with a protocol similar to that described in [24]. The sample was excited at 475 nm and emission measured at the maximum emission peak of LHCII (680 nm).

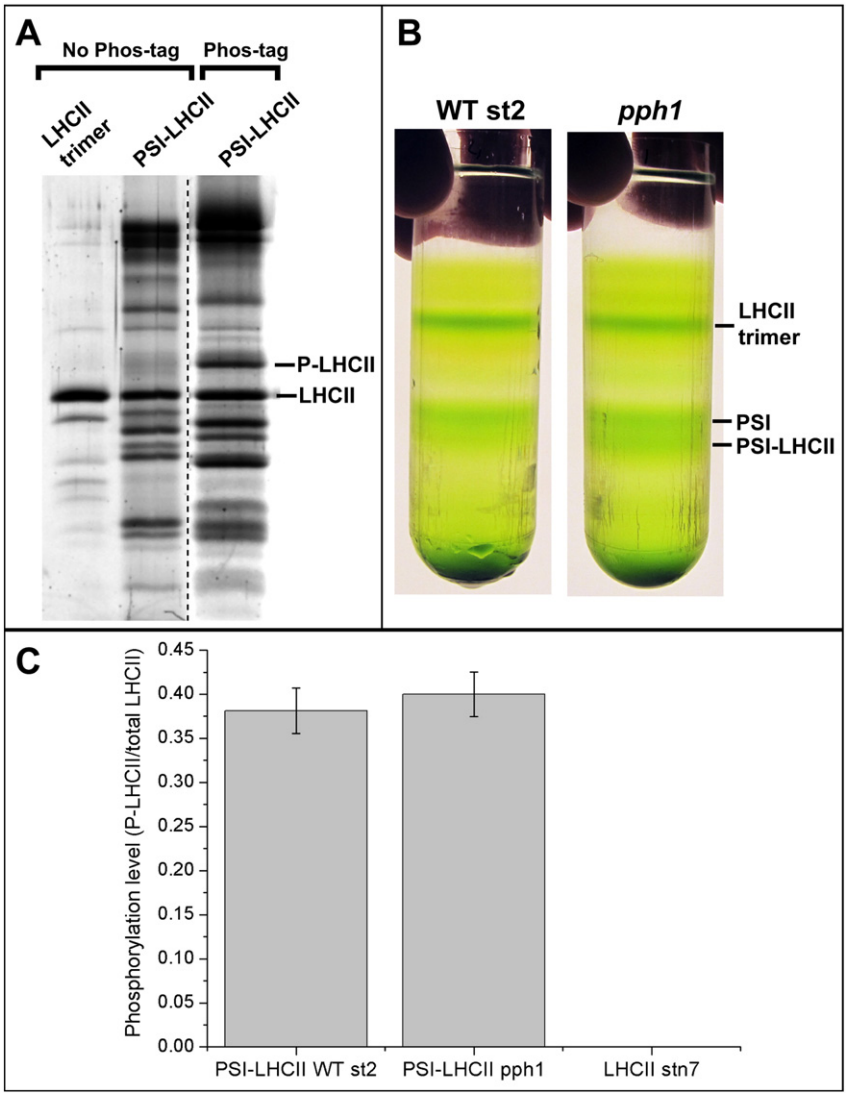

Fig. 1. Separation of P-Lhcb1–2 from non-phosphorylated Lhcb1–2 and analysis of phosphorylation in the PSI-LHCII supercomplex. A) Migration of purified PSI-LHCII by SDS-PAGE with Phos-tag (right) and without (left). A band corresponding to P-LHCII is visible only in the presence of Phos-tag. A trimeric LHCII fraction (as in panel B) is used as control for LHCII position in the non-Phos-tag gel. B) Purification of PSI-LHCII supercomplexes by sucrose gradient ultracentrifugation of solubilized thylakoids from WT in partial State II (left) and from the *pph1* mutant (right). Thylakoids from *pph1* plants, which have a maximum level of LHCII phosphorylation due to the absence of the PPH1 phosphatase, showed a higher content of PSI-LHCII supercomplexes than WT thylakoids, as assessed by the relative intensity of the PSI and PSI-LHCII bands. C) Densitometry analysis of the Phos-tag gels of PSI-LHCII fractions from WT and *pph1* plants of panel B revealed that the phosphorylation level of LHCII in the supercomplex was around 40% (ratio P-LHCII/total LHCII) and similar in both samples (WT st2, n = 7; *pph1*, n = 5). Trimeric LHCII from the *stn7* mutant was used as a control.

3. Results

3.1. Detection of phosphorylated Lhcb by Phos-tag SDS-PAGE

P-LHCII has been previously detected in several different ways: using anti-P-Threonine antibodies, using the recently developed P-Lhcb1 and P-Lhcb2 antibodies [29], and staining using ProQ following SDS-PAGE [32]. The recently developed Phos-Tag molecule [33] can slow down the migration of phosphoproteins when incorporated into a denaturing polyacrylamide gel. It should be noted that, besides LHCII, several thylakoid proteins can be phosphorylated, especially in PSII and in PSI [34,35]. For this latter complex, phosphorylation has already been detected in PsaD, PsaE, PsaF, PsaL, PsaN and PsaP (see [36] for a review), which might also be detected in this way.

We found that Phos-Tag SDS-PAGE could effectively separate P-LHCII from the non-phosphorylated LHCII. The use of antibodies does not allow the absolute quantification of the phosphorylation level, and with ProQ diamond this quantification is not straightforward. However, staining with Coomassie or Sypro, which interact primarily with basic amino acids and do not interact with P-threonine, allows the direct comparison of P-LHCII with non-P-LHCII. First we optimized the separation of P-LHCII from non-P-LHCII by using the purified PSI-LHCII complex isolated from *Arabidopsis* plants in State II [24]. As visible in Fig. 1A the separation of the two bands is very clear and allows a direct measurement of LHCII phosphorylation by densitometry. The P-LHCII band appears only in the presence of Phos-tag (Fig. 1A) and its identity was further verified by performing a 2D SDS-PAGE (Supplemental Fig. S2) and immunoblot analyses (as in Fig. S3, Fig. 3 and Fig. 5).

We found that the ratio between P-LHCII and total LHCII in the PSI-LHCII complex was ~40%, indicating that, on average, slightly more than one monomer per trimer is phosphorylated. We tried to detect the amount of phosphorylation of the pool of Lhcb1 and Lhcb2 isoforms using antibodies against total Lhcb1 and total Lchb2 (see details in Materials and methods). Unfortunately our antibodies mainly recognized the phosphorylated form of LHCII (Supplemental Fig. S3). Indeed a significant quantity of non-P-LHCII is clearly present in the PSI-LHCII supercomplex, and at least one of the two antibodies does not have

the same cross-reactivity towards the two forms of LHCII (phosphorylated and non-phosphorylated) (Supplemental Fig. S3).

3.2. Quantification of Lhcb1–2 phosphorylation in PSI-LHCII from plants at different degrees of state transition

The state transition is a phenomenon that starts rapidly (within a few seconds) after a light switch promoting the transition from State I to State II and takes several minutes to complete. It is therefore interesting to check the levels of LHCII phosphorylation in samples that have undergone different degrees of state transition. Using the *pph1* mutant that lacks the PPH1 phosphatase it is possible to purify samples where PSI-LHCII formation and LHCII phosphorylation should be at their maximum. As expected (Fig. 1B), the relative quantity of PSI-LHCII and PSI complex is lower in gradients of thylakoids from WT plants in partial State II as compared with thylakoid gradients from the *pph1* mutant. The purified PSI-LHCII complexes from both plants were separated by SDS-PAGE to check the level of phosphorylation of the mobile LHCII trimer attached to PSI. Surprisingly, the phosphorylation of LHCII was near 40% and practically the same in the WT and *pph1* samples (Fig. 1C).

This result indicates that the phosphorylation of one monomer of the trimer is sufficient for LHCII movement and binding to PSI. Our finding that the two other monomers are not or are only a little phosphorylated was unexpected. However, this cannot be explained by a rapid dephosphorylation of these monomers during the preparation of the membranes and of the supercomplex: for the WT a phosphatase inhibitor was used (NaF) and for the *pph1* mutant there is no phosphatase present to dephosphorylate LHCII. Thus we can explain the results in two ways: 1) once phosphorylated, the mobile LHCII rapidly moves to PSI and the STN7 kinase has no longer has access to this trimer; and 2) the remaining non-phosphorylated Lhcb1–2 isoforms composing the mobile trimer are not phosphorylable. In principle all Lhcb2 isoforms have the same N-terminal, and so no difference in phosphorylation susceptibility is expected. However, among the different Lhcb1 isoforms, Lhcb1.4 lacks a phosphorylable threonine at the N-terminal (3rd position). In a previous work [24], we found that this isoform is enriched (together with Lhcb1.5) in mobile trimers. However, even though we cannot determine the absolute content of this isoform, it is unlikely that the two non-phosphorylated monomers are mainly Lhcb1.4 [24]. Thus we suggest that the LHCII attached to PSI is probably less accessible to the kinase, or that it is difficult for STN7 to access the other sites after the first has been phosphorylated.

3.3. Lhcb1 and Lhcb2 are phosphorylated in both S- and M-LHCII trimers bound to PSII

Recent investigations indicate that LHCII trimers that remain bound to PSII are phosphorylable [20,28]. As for PSI-LHCII, we investigated the level of LHCII phosphorylation in PSII supercomplexes. To this end, we prepared PSII supercomplexes with different antenna size similarly as in [11] both from thylakoid membranes and from BBY membranes (the grana core, which contains almost exclusively PSII and no other photosynthetic supercomplex). PSII from thylakoid membranes can provide information on the average phosphorylation level of LHCII bound to PSII in the whole membranes (PSII in the grana core + in the grana margins). On the other hand PSII from BBY membranes should provide a picture of the LHCII phosphorylation in the most internal part of the grana.

Three bands of PSII supercomplexes are visible in a sucrose gradient from solubilized thylakoid membranes. Starting from the top of the gradient (Fig. 2A), they contain mainly the $C_2S_2 + C_2SM$, C_2S_2M and $C_2S_2M_2$ complexes (B9, B10 and B11 bands respectively using the nomenclature in [11]), where C2 indicates the presence of a dimeric core and S and M the presence of S-LHCII and M-LHCII trimers. By treating PSII particles with the detergent α-dodecylmaltoside (α-DDM), it is possible to detach LHCII trimers from PSII [11]. By loading onto a second sucrose gradient it is then possible to isolate the detached trimers. If high α-DDM concentrations are used all LHCII trimers (S and M) form a single band on the second gradient (B3 as in [11]), while at low α-DDM concentrations the detached LHCII form a band of trimeric LHCII (B3) enriched in S-trimers and a band containing an Lhcb assembly (M-LHCII-CP29-CP24, B4 in [11]) (Fig. 2C–D). In this way we can estimate with a good approximation the phosphorylation level of the two kinds of trimers bound to PSII (S- and M-LHCII).

As shown in Fig. 2B, phosphorylation of total LHCII bound to PSII is much lower than that of LHCII to PSI, being between less than 20% of the total LHCII in the smallest PSII supercomplex and decreasing to less than 15% in the largest $C_2S_2M_2$ complex. No differences were found in PSII from WT in State II and *pph1* mutants (not shown).

As the antenna size of PSII increases there is an increase in the ratio of M-LHCII to S-LHCII trimers. We found that the phosphorylation level decreases as the antenna size increases (Fig. 2B), suggesting that M-trimers are less phosphorylated than S-trimers.

Indeed when we compared the P-LHCII level in M-trimers dissociated from PSII and isolated from the B4 band with the P-LHCII level in the B3 band (enriched in S-trimers, but containing also some M-LHCII) (Fig. 2C–D), we found that the phosphorylation level in M-LHCII is, at most, ~74% ± 2.4% of the phosphorylation level in S-LHCII, confirming that S-LHCII are more phosphorylated than M-LHCII.

The lower phosphorylation of M-LHCII can be explained in different ways: 1) in M-LHCII the Lhcb3 isoform is present in a stoichiometry of one monomer per trimer, and Lhcb3 does not contain the phosphorylable threonine (indeed the N-terminal is quite different) [7]; 2) M-LHCII is particularly enriched in the Lhcb1.4 isoform (and it almost does not contain Lhcb2) [11,24] and, as discussed above, Lhcb1.4 possesses an N-terminal that is probably not phosphorylable; and 3) fewer monomers are accessible to the kinase in M-LHCII compared with S-LHCII. In support of the third possibility it is clear from the structural model of the PSII-LHCII supercomplex [11] that only one Lhcb1–2 monomer from the M-LHCII trimer would be well exposed to the exterior of PSII.

Finally, we compared the phosphorylation level of LHCII in PSII from thylakoids and from BBY membranes. The results clearly show that S + M-LHCII trimers from the whole membranes were more phosphorylated than the same kinds of LHCII located in the grana core: the ratio of the phosphorylation levels in thylakoids with respect to BBY (prepared from the same thylakoids) was 2.77 (±0.283 SE; n = 6). WT plants in an intermediary State II showed a lower PSII-LHCII phosphorylation than *pph1* plants, although the ratio of the phosphorylation levels between thylakoids and BBY was the same. Our findings are different from recent results from Grieco et al.[20] who found that LHCII phosphorylation was similar in the grana margins and the grana core. These differences are probably due to the different strategies used to prepare the grana core membranes: triton solubilization of stacked thylakoids in our case vs. digitonin solubilization in [20]. Triton solubilization allows the isolation of purer grana membranes than those that can be obtained with digitonin. This is demonstrated by the absence of PSI in BBY prepared with triton [11,37], and the presence of significant amounts of PSI in grana cores membranes prepared with digitonin [20].

3.4. P-Lhcb2 is the most important subunit for PSI-LHCII assembly

In order to better investigate the binding of LHCII to PSI under State II conditions, we performed dephosphorylation assays *in vitro* using a recombinant PPH1 enzyme in a soluble form without the native C-terminal transmembrane helix (see Materials and methods for details).

Using anti-P-Lhcb1 and anti-P-Lhcb2 antibodies we compared the phosphorylation level in the mobile LHCII before and after treatment with the PPH1 phosphatase. As visible in Fig. 3A, we obtained an unexpected result: while P-Lhcb1 is almost totally dephosphorylated *in vitro*, Lhcb2 in the intact PSI-LHCII supercomplex is little dephosphorylated. On average, 76.2% (±5.2% SE, n = 6) of P-Lhcb2 remained

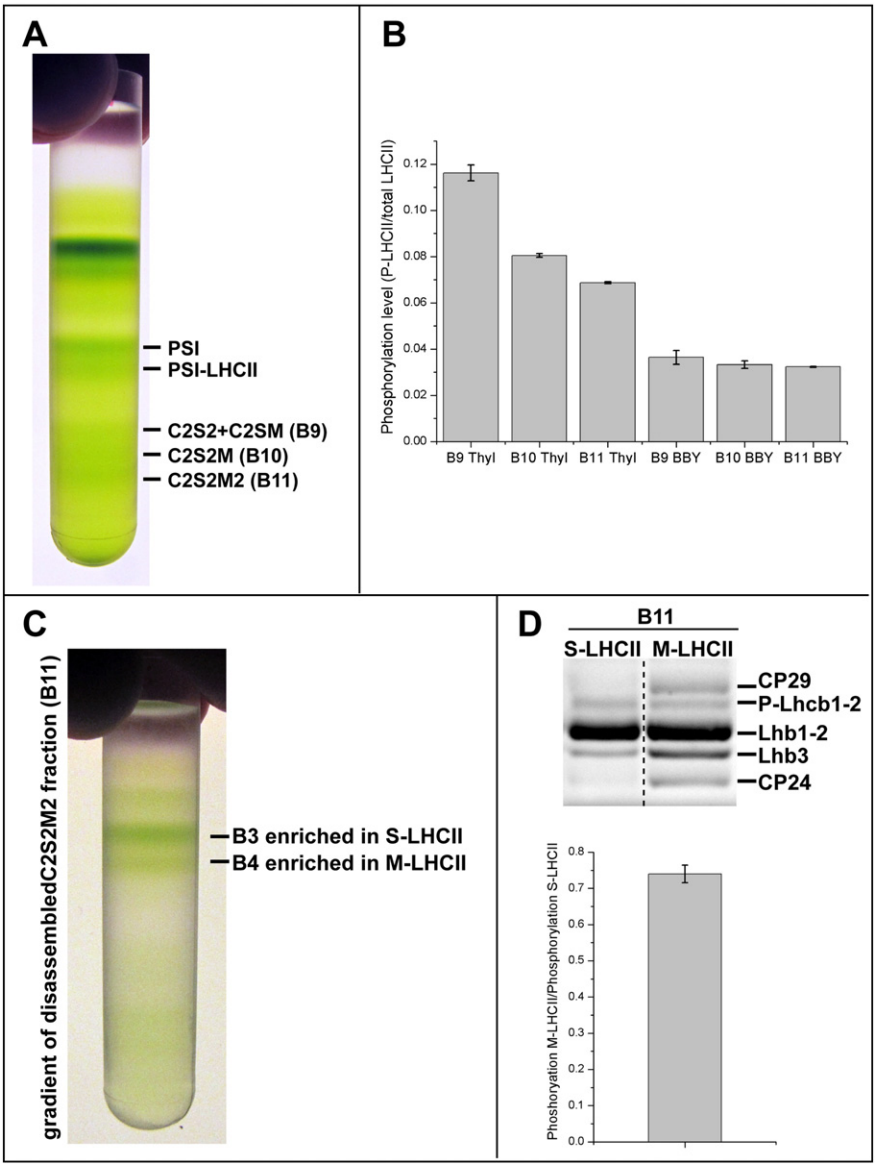

Fig. 2. Analysis of the LHCII phosphorylation level in PSII-LHCII supercomplexes. A) Example of purification of PSII-LHCII supercomplexes by sucrose gradient ultracentrifugation of solubilized thylakoids from WT plants in State II. Three bands were harvested, corresponding to $C_2S_2 + C_2SM$ (B9), C_2S_2M (B10) and $C_2S_2M_2$ particles (B11) [11]. B) Overall LHCII phosphorylation levels in PSII particles purified from thylakoids (as in panel A) as assessed by Phos-tag gels on purified LHCII trimers after detachment from PSII and purification on a second gradient. Larger particles containing higher amounts of M-LHCII are less phosphorylated. C) Detachment of LHCII from PSII by a short α-DDM treatment allows the recovery of a fraction enriched in S-LHCII (B3) and a fraction enriched in M-LHCII associated to CP29 and CP24 (B4) on a second gradient [11]. D) Phos-tag gels of LHCII fractions from panel C indicate that the phosphorylation level of M-LHCII is at most ~74% (±2.4% SE, n = 5) of the phosphorylation level in S-LHCII.

phosphorylated after the treatment. However, if LHCII is dissociated from PSI with a soft treatment in α-DDM [24], the efficiency of Lhcb2 dephosphorylation is much higher: only 14.4% ± 3.5% of the initial P-Lhcb2 remained phosphorylated.

The *in vitro* activity of LHCII dephosphorylation of recombinant PPH1 proteins has been tested also by other groups [26,27]. Furthermore, the same recombinant protein we used for our *in vitro* assays is active enough to complement the *pph1* mutant *in vivo* (Supplemental Fig. S4). However, it is possible that there is a change in the activity of the recombinant PPH1 due to the different environment *in vitro* (particles are in detergents and not in membranes). This partial activity was useful for our purposes, since it allowed us to show a difference in the accessibility of P-Lhcb1 and P-Lhcb2 for dephosphorylation. The latter seems to be somehow protected *in vitro* from PPH1 activity in the PSI-LHCII supercomplex, and it is only dephosphorylated after separation from the supercomplex. This result strongly suggests the Lhcb2 is the isoform that makes the most important contact for PSI-LHCII assembly.

Moreover, when we compared the intensity of the P-LHCII and non-phosphorylated LHCII in a Phos-tag gel of PSI-LHCII before and after phosphatase treatment the P-LHCII band intensity remains almost unchanged despite the almost complete dephosphorylation of P-Lhcb1 (Fig. 3B). Considering that P-Lhcb2 is little dephosphorylated in this condition (Fig. 3A), this strongly indicates that the majority of P-LHCII in PSI-LHCII is represented by phosphorylated Lhcb2 proteins.

Since about 40% of the trimer is initially phosphorylated (Fig. 1C) and, as indicated above, ~76% remains phosphorylated after incubation with the PPH1 phosphatase, we estimate that we still have roughly 30% of all Lhcb in a phosphorylated state after the treatment. This is an average of one monomer per trimer. To determine if this minor dephosphorylation has an impact on the integrity of the PSI-LHCII supercomplex, we measured the fluorescence emission of the complex during a dephosphorylation assay. As shown in Supplemental Fig. S5, no increase in fluorescence was observed during incubation with the recombinant PPH1. However, after the addition of a small quantity of α-DDM to

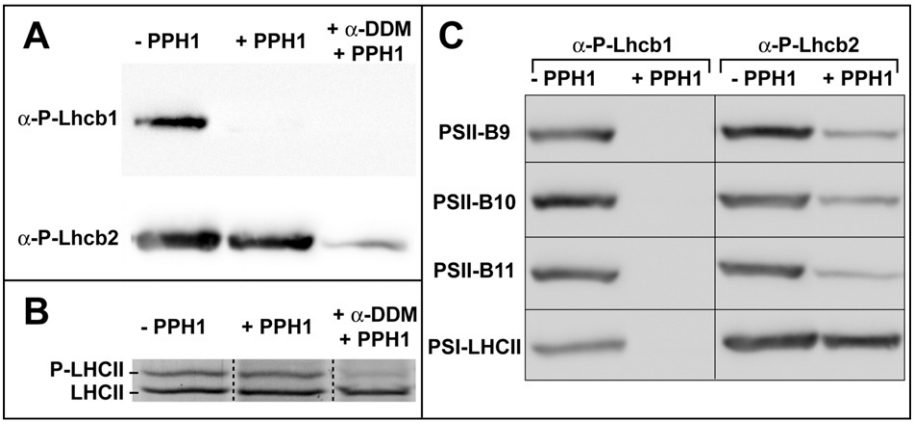

Fig. 3. *In vitro* dephosphorylation assays. A) Intact PSI-LHCII particles were incubated with recombinant PPH1 phosphatase ("+ PPH1" lane), loaded on a Phos-tag gel and proteins transferred on a nitrocellulose membrane. Immunoblot analysis with anti-P-Lhcb1 and anti-P-Lhcb2 antibodies revealed that Lhcb1 was almost completely dephosphorylated *in vitro* when bound to PSI, while 76.2% (±5.2% SE, n = 6) of P-Lhcb2 remained in a phosphorylated state. When P-Lhcb2 is detached from PSI by a soft treatment with α-DDM, P-Lhcb2 is also efficiently dephosphorylated *in vitro*. B) Sypro-stained Phos-tag gels of PSI-LHCII particles before ("-PPH1" lane) and after ("+ PPH1" lane) incubation with recombinant PPH1 showed that the intensity of the P-LHCII band is little changed after the incubation, despite a full dephosphorylation of P-Lhcb1. This suggests that the P-LHCII band contains mainly P-Lhcb2 isoforms. C) Similar experiments as in panel A on PSII supercomplexes (B9 to B11 as in Fig. 2) showed that dephosphorylation of P-Lhcb1 is very efficient in intact PSII-LHCII particles (similarly as in PSI-LHCII). In the case of Lhcb2, in PSII-LHCII supercomplexes this isoform is much more dephosphorylated than in the PSI-LHCII supercomplex.

disassemble the supercomplex the fluorescence increased about tenfold, as expected due to the much higher excited-state average lifetime of free LHCII compared to the excited-state average lifetime of PSI-LHCII [24]. This result suggests that one monomer per trimer is sufficient for maintaining the association of the PSI-LHCII supercomplex *in vitro*.

When similar dephosphorylation assays were performed on purified PSII-LHCII particles (Fig. 3C) the P-Lhcb1 isoforms were dephosphorylated as easily as in the PSI-LHCII supercomplex. Interestingly, the P-Lhcb2 isoforms were also more easily dephosphorylated and reached 20.7% (±3.6% SE, n = 6) of the initial phosphorylation level. This result supports our finding that Lhcb2 is protected when bound to the PSI in the PSI-LHCII supercomplex, but much less so in PSII-LHCII supercomplexes.

3.5. Lhcb1–2 phosphorylation in PSII in grana cores, in grana margins and in the stn7 mutant

Considering the importance of P-Lhcb2 in the PSI-LHCII supercomplex, we next investigated the phosphorylation of Lhcb1 and Lhcb2 isoforms in PSII-LHCII using antibodies against P-Lhcb1 and P-Lhcb2 [29].

In particular we compared PSII-LHCII particles purified from entire thylakoids and BBY membranes in WT and *pph1* plants. For the same concentration of total LHCII (phosphorylated and non-phosphorylated), we found a sharp decrease in Lhcb2 phosphorylation in the grana core compared to the grana margins for both genotypes (Fig. 4). This is in agreement with results using Phos-tag gels showing an overall lower LHCII phosphorylation in grana cores than in grana margins. Surprisingly, however, Lhcb1 phosphorylation was about the same in all membranes (Fig. 4). This shows that the lower phosphorylation of the BBY membranes compared to the entire thylakoids is due to a decrease in Lhcb2 phosphorylation.

To further investigate Lhcb1 and Lhcb2 phosphorylation, we checked LHCII phosphorylation attributable to STN8, a kinase mainly involved in the phosphorylation of PSII core subunits [34]. To this aim we used the *stn7* mutant lacking the kinase for LHCII [21], in which phosphorylation of LHCII is only possible by STN8, as shown previously by Leoni et al. [29] using a similar approach on total thylakoid LHCII. We did not detect a P-Lhcb band on Sypro-stained Phos-tag gels for LHCII bound to PSII and total LHCII (Fig. 5), indicating a very low level of LHCII phosphorylation in *stn7* plants. No signal was detected in P-Lhcb2 immunoblots. However, a very faint signal was detected using the anti-P-Lhcb1 antibody (Fig. 5), implying that the PSII kinase STN8 can only phosphorylate Lhcb1 in growth chamber conditions, even if this is to a very low extent.

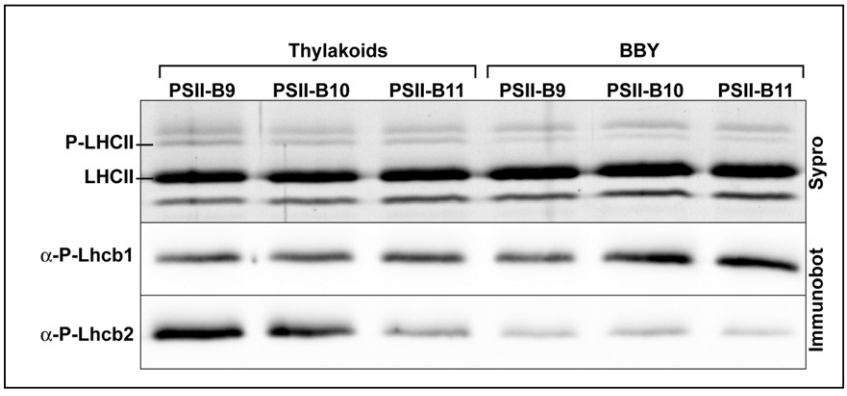

Fig. 4. PSII phosphorylation in grana cores. LHCII trimers from PSII supercomplexes from either entire thylakoids or BBY membranes (purified from the same thylakoids) were separated by Phos-tag SDS-PAGE and stained with Sypro Ruby or transferred onto a nitrocellulose membrane. Overall, LHCII bound to PSII (bands B9 to B11 as in Fig. 2) from BBY were less phosphorylated than from thylakoids. Immunoblot analysis with anti-P-Lhcb1 antibodies revealed a similar Lhcb1 phosphorylation in both membrane preparations. Immunoblotting with anti-P-Lhcb2 antibodies, however, showed a lower Lhcb2 phosphorylation in BBY compared to thylakoids: the P-Lhcb2 signal from BBY is about 25% (±2.6% SE; n = 6) of the signal from thylakoids.

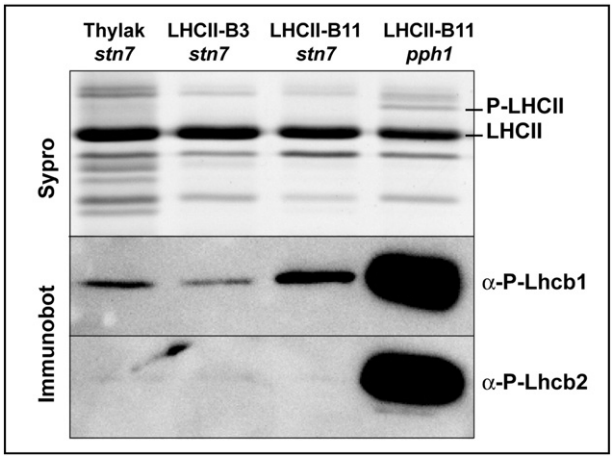

Fig. 5. LHCII phosphorylation in the *stn7* mutant. Thylakoids membranes from the *stn7* mutant, trimeric LHCII purified from either entire membranes (B3) or from PSII (B11 band) from the same mutant and from *pph1* plants were separated by Phos-tag SDS-PAGE. In the Sypro stained gel, P-LHCII was visible only in the *pph1* sample. Immunoblot against P-Lhcb1 and P-Lhcb2 showed detectable phosphorylation only for the Lhcb1 isoforms.

Together, these results suggest that phosphorylation of Lhcb1 could have a role in the function of the PSII-LHCII supercomplex, while phosphorylation of Lhcb2 is important for the assembly of the PSI-LHCII supercomplex during state transitions.

4. Discussion

In this study we investigated the phosphorylation level of LHCII bound to PSI and to PSII in plants in State II conditions. The state transition is an important regulatory mechanism that is necessary for balancing the energy distribution between PSII and PSI under fluctuating light conditions [38]. It has been known for a long time that phosphorylation of LHCII is necessary for transition from State I to State II [23,39], but it is only recently that this phenomenon has been investigated more deeply [20,29,30]. One of the questions still open regards the absolute phosphorylation status of the Lhcb1 and Lhcb2 isoforms bound to PSI or to PSII. So far the use of anti-P-Threonine antibodies or the recently developed anti-P-Lhcb1 and anti-P-Lhcb2 antibodies [30] has only allowed relative measurements of phosphorylation.

4.1. LHCII phosphorylation in the PSI-LHCII supercomplex: the role of Lhcb2

By using the Phos-tag molecule, we were able to separate P-LHCII from non-phosphorylated LHCII by SDS-PAGE and quantify the intensity of each band by staining with Sypro Ruby. In this way we found that the mobile LHCII trimers in the PSI-LHCII supercomplex contain on average just more than one phosphorylated monomer in three in WT plants in an incomplete State II condition. The same ratio was also found for PSI-LHCII isolated from the *pph1* mutant that is blocked at a maximal level of phosphorylation because it lacks the PPH1 LHCII phosphatase [25,26]. By performing dephosphorylation experiments using a recombinant PPH1 phosphatase, we found that almost all the phosphorylated LHCII signal in the PSI-LHCII supercomplex was due to phosphorylation of the Lhcb2 isoforms (Fig. 3A–B).

During *in vitro* experiments on the intact PSI-LHCII we were able to reduce the phosphorylation level of the trimer down to ~30% at most. This strongly suggests that one monomer per trimer remains phosphorylated due to protection of the phosphorylated N-terminal from our recombinant PPH1. Under *in vivo* conditions PPH1 is anchored to the thylakoids by a transmembrane helix at the C-Terminal. Our recombinant PPH1 lacks the transmembrane helix for solubility. However, when the recombinant soluble PPH1 is expressed *in vivo* it is still able to complement *pph1* mutant plants, indicating that the N-terminal of P-Lhcb2 is accessible for desphosphorylation (Supplemental Fig. S4). The different activities of our recombinant enzyme under *in vitro* and *in vivo* conditions could be due to the different environments: PSI-LHCII particles in detergent *in vitro* and inserted in the membranes *in vivo*. It is also interesting to observe that the anchorage of PPH1 to the thylakoid membrane is not necessary for its activity *in vivo*.

Thus our results strongly suggest that i) one phosphorylated monomer is sufficient for the formation of the PSI-LHCII complex; ii) this monomer is an Lhcb2 isoform; iii) the phosphate at the N-terminal is involved in the binding between LHCII and PSI, and iv) it is very likely that Lhcb2 is located next to the subunit PsaH. PsaH has been shown to be important for the formation of the PSI-LHCII supercomplex [40,41]. Furthermore, according to a previously proposed structural model of the PSI-LHCII supercomplex [24], the monomer next to PsaH would be the only one in the trimer with the N-terminal well in contact with PSI (Fig. 6).

Recent results obtained using a genetic approach highlighted the importance of Lhcb2 in state transitions [30]. Our biochemical results support this proposition and build an understanding of the biochemical mechanism underlying the formation of the PSI-LHCII supercomplex.

Interestingly, a new X-ray structure of the pea PSI [42] shows a quite remarkable difference in the positions of PsaH and PsaK (which are close to the LHCII binding site, see Fig. 6) with respect to a previous structure [43]. Even if these are not the only differences between the two structures, this fact may suggest that these subunits have some flexibility and they could undergo a conformational change and interact with Lhcb2 during the formation of the PSI-LHCII supercomplex. To

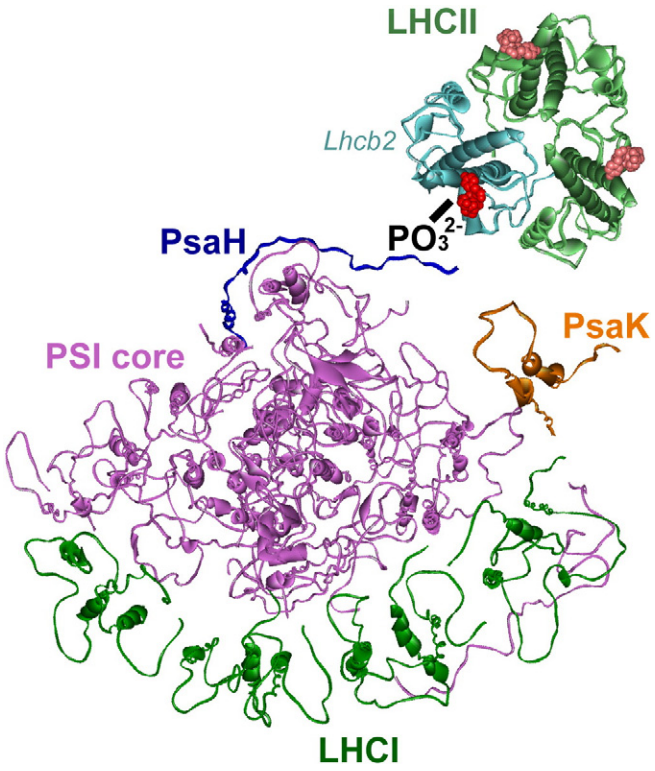

Fig. 6. Structural model of PSI-LHCII showing the probable position of the phosphorylated Lhcb2 monomer. The N-terminal of the three monomers composing LHCII are indicated as red space filling models from Ser14 to Tyr17 (note that the first residues, including the phosphorylable Thr3, are lacking in the crystal structure of LHCII [59]). The only N-terminal that could make a contact with PSI is proposed to belong to P-Lhcb2 (in cyan) and should be indispensable for the formation of the PSI-LHCII supercomplex. The positions of PsaH (in blue) and PsaK (in orange), which are also necessary for the formation of the PSI-LHCII supercomplex, are indicated. The PSI-LHCII structural model is the same as in [24], which was based on electron microscopy density map of the PSI-LHCII particles [24] and the crystal structure of PSI [43].

solve this question more structural studies on PSI and PSI-LHCII will be necessary.

It is also interesting to note that the Lhcb1 and Lhcb2 isoforms are only clearly distinguishable in vascular plants (which are often referred as higher plants), something which is not the case in non-vascular plants such as the bryophyte *Physcomitrella patens* [44] or in green algae like *Chlamydomonas reinhardtii* [45]. In both these cases the LHCII trimers are composed of Lhcbm monomers. In *Chlamydomonas* certain aspects of the state transition phenomenon are different from plants, for example the state transition involves a much greater proportion of the LHCII trimers and also includes monomeric antennas [46–48]. In addition two LHCII trimers bind to PSI in State II [49]. Almost a billion years of separate evolution and adaptation to different environmental niches is likely to be behind these differences between the state transitions in green algae and land plants. On the contrary, the moss *Physcomitrella* is nearer to *Arabidopsis* in both habitat and evolution (although about 400 million years still separate the two species [50]). Indeed, state transitions in *Physcomitrella* are more similar to those in *Arabidopsis* [51] compared to those in *Chlamydomonas*. Therefore we analysed the Lhcbm isoforms of *Physcomitrella* [44] to determine whether any are homologous to Lhcb2 at the N-terminal. We found that in *Physcomitrella* Lhcbm02 (Supplemental Fig. S6) the first 7 residues of the N-terminal (which contains the phosphorylable threonine) are identical to those in the Lhcb2 isoforms of *Arabidopsis*. It is therefore possible that, in moss, Lhcbm02 isoform could play the role of Lhcb2 in *Arabidopsis*. Lhcbm02 was named because it is the second most abundant Lhcbm isoform in a *Physcomitrella* EST database after Lhcbm01 [44]. However, we note that other specific residues that characterize the Lhcb2 isoforms in higher plants [1] are not conserved.

4.2. LHCII phosphorylation in PSII supercomplexes

We also investigated the phosphorylation level of LHCII bound to PSII. Two kinds of trimers can be purified in PSII particles: S-LHCII and M-LHCII, which differ in position and composition [4,11,24]. As previously shown [20,28], LHCII trimers bound to PSII are phosphorylable, but little information was available about the level of phosphorylation and whether differences between the two kinds of trimers were present.

We found that the phosphorylation level of M- and S-LHCII is lower than in the mobile LHCII bound to PSI. For LHCII bound to PSII, our results show that at most about one monomer in six is phosphorylated, in agreement with recent result by Grieco et al. [20]. Moreover, we found that M-LHCII is less phosphorylated than S-LHCII, likely due to the presence of the non-phosphorylable monomer Lhcb3 and Lhcb1.4 in M-LHCII, and also possibly due to the lower accessibility M-LHCII monomers to the STN7 kinase.

In the case of M- and S-LHCII, we found that both P-Lhcb1 and P-Lhcb2 are easily dephosphorylated *in vitro* using the recombinant PPH1 phosphatase. This suggests that both monomers are well exposed.

In our experiments we could not detect the phosphorylation level of the loosely bound L-LHCII trimers. However recent investigations [20] leaded to the proposition that also these trimers are phosphorylated and largely relocalised at the grana margins in State II conditions.

Moreover we found that LHCII phosphorylation in PSII-LHCII was lower in the grana core as compared to total PSII in thylakoids (grana cores + grana margins), indicating that LHCII is more phosphorylated at the grana margins. This result differs from the findings of Grieco et al. [20], who detected a similar PSII-LHCII phosphorylation in the stroma lamellae and grana margins compared to the grana cores. This difference can probably be explained by the different methods used for the purification of the grana cores: we isolated BBY membranes, which contain almost exclusively PSII supercomplexes [11] as a model for grana cores, whereas Grieco et al. [20] used pelleted membranes left after a soft solubilization of the thylakoids with digitonin, where a significant amount of PSI was still present.

The lower phosphorylation that we detected in the grana core could be explained by the lower abundance of the STN7 kinase in this region. It has been suggested that STN7 could be bound to Cytochrome b_6f [22,52], which is absent from grana core preparations. However, we note that the precise localisation of Cyt b_6f is a question that has not been completely resolved, since it has been proposed to be evenly distributed *in vivo* but lost during grana preparation [4,53]. The higher concentration of STN7 at the grana margins could explain the higher phosphorylation of LHCII in this region. However, mobility of membrane complexes in the thylakoid membranes, despite the high protein/lipid ratio [54], should be fast enough for homogeneous redistribution of the complexes [55], at least on a several minute time-scale as required for state transitions. Therefore it is also likely that the higher phosphorylation of LHCII at the grana margins is due to a specific localisation of PSII supercomplexes containing P-LHCII. We propose that phosphorylation of LHCII associated with PSII could be a factor necessary for changing the conformation of the grana, which become less stacked at the margins, as detected by different microscopy techniques [56]. This change in conformation would be necessary for the achievement of a full State II.

It should also be noted that we could not detect any P-Lhcb2 in our *stn7* thylakoid membranes, implying that only Lhcb1 had been phosphorylated by the PSII kinase, STN8. This result supports the idea of an independent and specific role for P-Lhcb1. Furthermore, we found that P-Lhcb1 is evenly distributed in PSII-LHCII in the thylakoids, whereas P-Lhcb2 is present in higher quantity in the grana margins compared to the grana cores. P-Lhcb1 could therefore have a role in PSII organization within grana membranes, but further investigations are necessary to fully elucidate its specific role.

In short, phosphorylation of LHCII would promote movement of a particular pool of mobile LHCII trimers that are able to bind to PSI thanks to the presence of a P-Lhcb2 monomer. This trimer would transfer energy to PSI very efficiently [24]. Phosphorylation of Lhcb1 and Lhcb2 in M- and S-LHCII bound to PSII (and in L-LHCII) would promote partial destacking of the grana and movement of PSII-LHCII towards the margins, thus allowing a mixing of the photosystems and an energetic connection between PSII and PSI. Since PSI possesses lower energy Chls, under State II conditions it could receive energy from PSII by a spillover phenomenon, which has been detected *in vivo* [57].

Thus we agree with similar conclusions recently proposed by other groups [20,57]. However, we believe that evidences for the formation of stable PSI-LHCII-PSII-LHCII megacomplexes, as it has been proposed by Grieco et al. and Yokono et al. [20,57], are still lacking. In these reports a PSI- and PSII-containing high MW band in native gels is proposed to contain such megacomplexes. Nevertheless, a direct proof of their existence, such as by electron microscopy, is currently missing. The top band in the native gels assigned to such megacomplexes however has a much higher MW than the one calculated by the authors [20,57]. In both these works, despite the use of a protein ladder, the MW ladder used as reference on the gel does not seem to be correct for membrane supercomplexes (maybe due to different biochemical properties of the photosynthetic membrane complexes and the proteins used as standards). In both papers the largest PSII-LHCII supercomplex ($C_2S_2M_2$), whose ~1500 kDa MW is easily calculated from the protein and cofactor content ([11] and Supplementary Table S1), migrates much lower in the gel than the 1048 kDa MW standard: in [20], $C_2S_2M_2$ particles are the “band 1” in the grana core lane of supplementary Fig. 1C; in [57], $C_2S_2M_2$ particles are the “w2” band in Fig. 2. In the same gels the 1236 kDa band of the ladder migrates almost at the top of the gel, much higher than the 1500 kDa $C_2S_2M_2$ band. In short, considering that the 1500 kDa $C_2S_2M_2$ band is quite low in the gel and that there is a rapid increase of the MW approaching to the top of the gel, the highest green band, which has been proposed to be a PSI-PSII megacomplex of about 2400 kDa, clearly has a much higher MW. In our opinion this band corresponds rather to aggregated material, whose composition can somehow differ after digitonin solubilization

of thylakoids in State I and State II due to the different structure and composition of the grana margins [20,56]. A similar argumentation was already proposed in a previous work [24].

In conclusion, phosphorylation of LHCII (L, M, and S) in State II conditions would promote partial destacking of the grana, movement of "free" LHCII and PSII-LHCII complexes towards the grana margins, mixing with PSI, and energy transfer from LHCII/PSII complexes to PSI to balance photosystem absorption. A pool of LHCII can strongly bind to PSI to form the PSI-LHCII supercomplex and this would require the phosphorylation of Lhcb2. Probably the simple spillover from PSII to PSI is not efficient enough for balancing light absorption between photosystems, thus the formation of the PSI-LHCII supercomplex would be required to further increase PSI antenna size (by about 20%), since in this case LHCII to PSI energy transfer is very efficient [24]. It is worth noting that under State II conditions, at least half of the PSI forms a supercomplex with LHCII ([24] and Fig. 1B). However the formation of this stable PSI-LHCII supercomplex mediated by P-Lhcb2 may even have a function under saturating light conditions, when in principle kinase activity is down regulated [58] and state transitions are less (or not) important. Indeed, the existence of the complex under such conditions has recently been demonstrated [28]. In such conditions LHCII would function as a stable and intimate part of the PSI antenna system.

5. Conclusion

In this study, we show that phosphorylated Lhcb1 and Lhcb2 have different localizations and roles in *Arabidopsis*. Using Phos-tag gels, we were able to separate phosphorylated LHCII from its non-phosphorylated form, which permitted the absolute quantification of LHCII phosphorylation in diverse supercomplexes and membrane types. We found that in State II LHCII phosphorylation is higher in PSI-LHCII (around 40%) than in PSII-LHCII supercomplexes (around 15% in the $C_2S_2M_2$ supercomplex purified from thylakoids and even lower in complexes purified from grana core membranes). Dephosphorylation assays with a recombinant PPH1 phosphatase allowed us to show that P-Lhcb2 is protected in the PSI-LHCII supercomplex, but not P-Lhcb1. This is different from PSII-LHCII where both isoforms are easily dephosphorylated. We also found that both isoforms have different localizations in the thylakoid membranes: P-Lhcb2 constitutes the major part of phosphorylated LHCII in PSI-LHCII, but its phosphorylation decreases in the grana cores compared to whole thylakoids membranes. On the contrary, Lhcb1 phosphorylation was similar in PSII supercomplexes from entire thylakoids and grana core membranes.

We thus propose that a single P-Lhcb2 monomer form the most important contact with PSI, likely through the PsaH subunit, for the assembly of the PSI-LHCII supercomplex during state transitions. Further investigations will be needed to uncover the precise role of P-Lhcb1, which, considering its localization, probably affects PSII dynamics. These results are thus a step towards a refined model of the state transition phenomenon and a better understanding of the short-term response to changes in light conditions in plants.

Transparency document.

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.09.005.

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