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During State 1 to State 2 Transition in *Arabidopsis thaliana*, the Photosystem II Supercomplex Gets Phosphorylated but Does Not Disassemble*

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Background: State transition balances the excitation pressure between the two photosystems of plants.
Results: The organization of photosystem II supercomplexes and megacomplexes is the same in state 1 and state 2.
Conclusion: Phosphorylation is not sufficient to induce the disassembly of the supercomplexes.
Significance: This work helps to understand how plants optimize light harvesting under ever changing light conditions.

Plants are exposed to continuous changes in light quality and quantity that challenge the performance of the photosynthetic apparatus and have evolved a series of mechanisms to face this challenge. In this work, we have studied state transitions, the process that redistributes the excitation pressure between photosystems I and II (PSI/PSII) by the reversible association of LHCII, the major antenna complex of higher plants, with either one of them upon phosphorylation/dephosphorylation. By combining biochemical analysis and electron microscopy, we have studied the effect of state transitions on the composition and organization of photosystem II in *Arabidopsis thaliana*. Two LHCII trimers (called trimers M and S) are part of the PSI supercomplex, whereas up to two more are loosely associated with PSII in state 1 in higher plants (called “extra” trimers). Here, we show that the LHCII from the extra pool migrates to PSI in state 2, thus leaving the PSI supercomplex and the semicrystalline PSII arrays intact. In state 2, not only is the mobile PSI phosphorylated, but also the LHCII in the PSII supercomplexes. This demonstrates that PSI phosphorylation is not sufficient for disconnecting LHCII trimers S and M from PSII and for their migration to PSI.

Oxygenic photosynthetic organisms harvest light via two large pigment-protein assemblies called photosystems I (PSI) and II (PSII). The two photosystems, connected through the plastoquinol pool and the cytochrome b/f complex, work in series to transform light energy into chemical energy (1). Both photosystems have a specific absorption spectrum. In plants and green algae, PSII is rich in chlorophyll b (absorption maxima ~475 nm and 650 nm) which is coordinated by light-harvesting complexes, whereas PSI is rich in chlorophyll a (absorption maxima ~440 nm and 680 nm). In addition, PSI has chlorophylls that can harvest light at wavelengths longer than 700 nm (2). For optimal performance of linear electron transport, the excitation pressure on the two photosystems needs to be balanced. In natural fluctuating light environments, a mechanism known as state transitions serves to redistribute the excitation energy between PSI and PSII (3, 4). In plants and algae, this is achieved by the migration of part of the major light harvesting complex (LHCII) between PSI and PSII. State 1 (St1) is traditionally defined as the condition in which PSI is preferentially excited and all LHCII is associated with PSII. When light conditions change, favoring PSII excitation (St2), a mobile pool of LHCII moves from PSI and associates with PSII (5). This process is triggered by phosphorylation of LHCII by the Stn7 kinase, which is activated upon oxidative reduction of the plastoquinol pool (6, 7). Conversely, in state 2 (St2), preferential excitation of PSI oxidizes the plastoquinol pool inactivating the Stn7 kinase. The constitutively active LHCII phosphatase, (TAP38/PPH1 (8, 9)), dephosphorylates the mobile LHCII, which moves to PSI. Recently, we showed that LHCII is associated with both photosystems in most natural light conditions, and it disconnects from PSI only upon far red illumination or high light stress, this is contrary to the common assumption that LHCII is only associated to PSI as short term response (10). If exposed to different light qualities for a long time, plants can adjust their PSI to PSII stoichiometry (11–15). Instead, long term changes in light intensity lead to changes in the antenna size of both PSI and PSII by regulating the level of the major light harvesting complex (LHCII), which functions as an antenna for both photosystems (10).

PSII is composed of a core complex, where the primary photochemistry takes place, and a peripheral antenna system that is encoded by the *Lhcb1–6* genes (16). LHCII is present as a trimer composed of a combination of the *Lhcb1–3* gene products. A threonine in the N terminus of Lhcb1 and Lhcb2 is the

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2 The abbreviations used are: PSI, photosystem I; α/β-DM, α- or β-n-dodecyl-D-maltoside; St1, state 1; St2, state 2; LHCII, major light-harvesting complex of Photosystem II.
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The largest PSII supercomplex observed in Arabidopsis thaliana contains one copy of each minor complex and two LHCII trimers per core (C), which are indicated as S (strongly bound) and M (moderately bound), forming the C₃S₂M₃ supercomplex. Several PSII supercomplexes can associate to form mega-complexes (18). Depending on light growth conditions, up to two more LHCII trimers per PSII core can be present in the thylakoids (18-20), but their physical interaction with the PSII supercomplex is rather weak or possibly absent in the sense that part of these complexes are probably not in direct contact with the core, although they are still able to transfer energy to it via other antennas (21). We call these trimers extra LHCII (10). The grana membrane in St1 contains the PSII supercomplexes (mainly in the C₃S₂M₃ configuration) and extra LHCII that are located in between the supercomplexes but probably also at the grana margins (19).

Regarding the mobile LHCII involved in state transitions, it is known that the association of LHCII with PSI occurs on the PsH and PsL side (22) and involves one LHCII trimer (23). However, less is known about the association of this mobile LHCII with PSII in St1. Although several models have been proposed of how state transitions influence PSII organization, the identity of the trimer that moves from PSII to PSI is still under debate. The involvement of trimer M (and even S) has been suggested in various models (24, 25), but it is controversially discussed in literature. On the one hand, Lhc6, which is a component of trimer M (26, 27), was not found in the stroma lamellae in St2 (28) or in the PSI-LHCII supercomplex (10, 29). On the other hand, upon St1 → St2 transition an increased fraction of C₃S₂Mₛ and a decreased fraction of C₃S₂Mₜ complexes was observed, suggesting that trimer M goes from PSI to PSII (23). This was further supported by the finding that the rate of state transitions was enhanced in CP24 and Lhc6 KO mutants, where trimer M is not (KO CP24) or less (KO Lhc6) stably associated with the PSII supercomplex (30, 31). More recently, it was proposed that remodeling of PSII supercomplexes is required for state transitions (24), as has been suggested before for the green alga Chlamydomonas reinhardtii (32), whereas Tikkanen et al. (33) propose that both PSI and PSII move to the grana margins in St2 where they share LHCII. In this study, we combine biochemical and electron microscopy analysis to investigate the effect of state transitions on the PSII supercomplex organization and to determine which LHCII trimer(s) is/are involved in this process.

EXPERIMENTAL PROCEDURES

Plant Material—A. thaliana (Columbia) WT plants were grown at 100 μEinstein/m²/s, 70% humidity, 22 °C, and 8 h/16 h day/night regime (Plant Climatics Percival Growth Chamber, Model AR-36L, Germany). Only leaves fully exposed to the light were used for thylakoid isolation.

Plant Treatment and Thylakoid Isolation—Leaves were harvested either directly after overnight dark adaptation (St1) or after 50 min of 20 μE/m²/s white light treatment (St2). Harvested leaves were directly transferred to ice water. Thylakoid membranes were prepared according to Ref. 34 with the addition of 10 mM of NaF to all buffers, to inhibit phosphatase activity. The membrane was resuspended in 20 mM Hepes, pH 7.5, 0.4 mM sorbitol, 15 mM NaCl, 5 mM MgCl₂, and 10 mM NaF, quickly frozen in liquid N₂, and stored at 193 K until use. The chlorophyll a/b ratio (measured as described (35)) was the same for St1 and St2 thylakoids, indicating that no changes in PSI/PSII ratio had occurred during the 50 min of light treatment.

77 K Fluorescence Measurements—The 77 K emission spectra were measured in 66% (w/v) glycerol, 10 mM Hepes, pH 7.5, 5 mM MgCl₂, 15 mM NaCl, and 10 mM NaF with a Fluorolog (Jobin-Yvon) with 3.5-nm bandwidth in excitation and 2-nm bandwidth in emission. The OD₅₆₀ of the sample was <0.1, and the diameter of the circular cuvette was 4 mm.

PAGE—First or second dimension denaturing PAGE was performed with the Tris-Tricine system (36) at a 14.5% acrylamide concentration. Blue native PAGE was performed as described in Ref. 37, with the 25BTH2O buffer and an acrylamide/bisacrylamide ratio of 32:1 in both stacking (3.5%) and resolving (4 to 14%) gel. The final chlorophyll concentration was 0.5 mg/ml, and the final detergent concentration was 1% α- or β-n-dodecyl-β-maltoside (α/β-DM) or 1% digitonin/0.1% α-DM as used in Ref. 29. It should be noted that the very small amount of α-DM added to digitonin does not have any effect in the solubilization of the membrane but only helps the solubilization of digitonin (29). The cathode buffer was supplemented with 0.02% Coomassie Blue G (Serva). Second dimension PAGE was performed as described in Ref. 37. Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) was used as described in the user manual with fluorescence detection, except that the samples were not desalted and delipidated. We confirmed that the signal was linear with the protein concentration. Gels were stained with 0.025% Coomassie Blue G in 10% acetic acid as described in Ref. 36. The Coomassie and Pro-Q Diamond stained gels were imaged with ImageQuant LAS-4000 (GE Healthcare).

EM and Image Analysis—Thylakoid membranes were solubilized in 20 mM Bis-Tris buffer, pH 6.5, 5 mM MgCl₂ using digitonin (0.5 mg of chlorophylls/ml, 0.5% digitonin) for 20 min at 4 °C with slow stirring, followed by centrifugation in an Eppendorf table centrifuge for 10 min. The pellet, which contained the non-solubilized grana thylakoid membranes, was washed one more time using the same buffer, centrifuged for 5 min, and then used for EM analysis. Specimens were prepared by negative staining with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed (34) with 80,000× magnification and semi-automated data acquisition (38). Sub-areas (256 × 256 pixels) of PSII arrays selected from individual electron micrographs were analyzed separately. To determine a density of PSII complexes in a membrane area, mid-mass positions of the PSII core complexes were marked manually using Groningen Image Processing software (GRIP).

The overlay of the C₃S₂M₂ projection maps on the micrographs of the grana membranes with randomly distributed PSII complexes was based on a cross-correlation alignment using GRIP software. To enhance a contrast of PSII complexes and to
facilitate their localization in the grana membrane, high frequency noise was filtered out.

RESULTS

State Transitions—In this study, the plants were brought to St1 by dark adaptation, whereas St2 was induced by 50 min of low intensity white light treatment, conditions that are physiological for the plant. To check whether this treatment was successful, the level of LHCII phosphorylation in the thylakoids was evaluated by Pro-Q Diamond Phosphorylation gel stain (Fig. 1A). As expected, LHCII was far less phosphorylated after dark adaptation (St1) than after light adaptation (St2). Furthermore, the 77K emission spectrum shows enhanced 735 nm (PSI 77K fluorescence) emission in St2 thylakoids compared with those in St1, confirming that St1 and St2 were successfully induced.

Influence of State Transition on the Organization of PSII Supercomplexes—To compare the organization of PSII supercomplexes in St1 and St2, the thylakoid membranes in the two states were solubilized with β-DM or with the milder α-DM and loaded on a blue native gel. Freshly prepared thylakoids where used for this experiment to avoid freeze/thaw cycles that might affect membrane and supercomplex solubilization. PSII supercomplexes were present in equal amount in St1 and St2 for both solubilization conditions, indicating that the amount of the supercomplexes in intact St1 and St2 membranes is the same. This idea is further supported by using a very mild solubilization condition (1% digitonin, 0.1% α-DM, Fig. 2) that solubilizes only the grana margins, which is the region where the largest difference in the supercomplexes organization between St1 and St2 can be expected. Also, in this case, the amount of supercomplexes were identical in St1 and St2, indicating that not even the supercomplexes in the grana margin disassemble during state transitions. It should be added that when not using fresh thylakoids, the amount of supercomplexes decreases in St2 as compared with St1, especially upon treatment with the β-DM (data not shown), suggesting that the supercomplexes in St2 are less stable than those in St1.

Organization of PSII Supercomplexes in the Membrane—The organization of PSII supercomplexes in the grana membrane was further investigated by EM. The analysis reveals pairs of grana membranes where the typical features of PSII core complex are observed (Fig. 3). The major part (>90%) of the inspected micrographs (555 for St1 and 480 for St2) showed a random organization of PSII supercomplexes in the membrane (Fig. 3, A and D), whereas the other fraction (~7% in St1 and 9%
in St2) showed a semi-crystalline organization (Fig. 3, B and E). The very similar amount of crystalline arrays in the two states is not in agreement with the recent model of Dietzel et al. (24), which suggests that disassembly of semi-crystalline PSII arrays is a prerequisite for the St1 → St2 transition.

To identify the type of PSII supercomplexes present in the semi-crystalline arrays in the two states, image analysis of sub-areas of 2D arrays followed by fitting of the projection map to an x-ray pseudo-atomic model of the PSII supercomplex (34) was performed. In both St1 and St2, all supercomplexes in the 2D arrays were of the C₅S₅M₂ type (Fig. 3C, F).

To investigate the effect of state transitions on the organization of PSII in the random regions, the PSII density in the random area was determined. The white spot in the micrographs represent PSII core complexes which protrude out of the membrane. About 1200 PSII particles were manually selected from 8 micrographs for both St1 and St2. The average PSII density was 1549 ± 128 particles/μm² (data are presented as mean ± S.D., n = 8 micrographs) in St1 and 1601 ± 146 particles/μm² in St2, meaning that the PSII density does not significantly change during state transitions (there is no statistically significant difference at p = 0.05 level determined using the Student’s t test), whereas differences were observed with the same method when the amount of LHClI changes (19). To give an idea of the possible organization of the supercomplexes in the membrane, the position of the PSII complexes in St1 and St2 membranes were highlighted by overlaying the projection map with the C₅S₅M₂ supercomplex (see Methods, Fig. 3, B and D, respectively) (34). The pattern is very similar in both St1 and St2 indicating (i) a very similar supercomplex distribution and (ii) that the large majority of PSII complexes can be present in the form of the C₅S₅M₂ supercomplex, in agreement with previous results obtained with cryo-electron microscopy (39). We stress here that it was not possible to investigate the PSII density in the grana margins. A change in the PSII density could occur in this region. In conclusion, the molecular and supramolecular organization of PSII supercomplexes does not change during state transitions; neither the occurrence/composition of the crystalline arrays (from which the megacomplexes originate) nor the grana region with randomly oriented supercomplexes were altered.

Phosphorylation State of PSII Supercomplexes—To clarify which LHClI complexes are phosphorylated in the different states, the thylakoid membranes of plants in St1 and St2 were separated by blue native PAGE, and their protein content was analyzed in the second dimension by SDS-PAGE. The phosphorylated proteins were stained with Pro-Q Diamond stain, after which a Coomassie stain was used to visualize the total protein content (Fig. 4). The PSI-LHClI complex is not present in this gel, as it disassembles into PSI and LHClI trimers upon α-DM solubilization. The analysis shows that Lhcb1,2 present in the St2 LHClI trimers are phosphorylated. Indeed, it has been shown previously that the LHClI polypeptides associated with PSI are phosphorylated (10, 29) in agreement with the standard model that LHClI moves to PSI when phosphorylated and to PSII upon dephosphorylation (8, 9, 40, 41, 42). However, in contradiction with the general model, the gel shows that intact PSII supercomplexes are also phosphorylated in St2, confirming recent results of Grieco et al. (43). Thus, phosphorylated LHClI in the PSII supercomplex does not move to PSI, which means that the standard state transitions model needs to be revised.

DISCUSSION

The Mobile Pool of LHClI Involved in State Transitions Consists of the Extra (Non-PSII Supercomplex) LHClI—In A. thaliana two LHClI trimers (S and M) per monomeric PSII core are located in the PSII supercomplex (18, 34), whereas up to two extra LHClIs per PSII monomer are present in the thylakoid membrane (19, 20). Our investigation of their role in state transitions shows that the involvement of trimer M and S can be excluded: the absence of trimer S would lead to the full disassembly of PSII supercomplexes, which does not occur as we demonstrate here. Mobility of trimer M can also be excluded, as Lhcb3, a marker for this trimer, is not a component of the mobile LHClI fraction (10, 29). These findings lead to the conclusion that the extra LHClIs are the mobile antenna, which can migrate between PSI and PSII, and are already present near the grana-margins in St1, such that transfer into the stroma lamellae (St2) only needs a short migration distance. This might explain why the PSII density inside the PSI grana does not change upon state transitions (Fig. 3) (44).

The migration of the extra LHClIs has clear advantages for the plant. In the PSII supercomplex, the short distance between the trimers and the PSII core allows for fast excitation-energy transfer and thus high PSII operating efficiency (45). The extra LHClIs are located more distantly from the PSII core, which leads to slower, and thus less efficient, excitation-energy migration to the core (10). Thus, evolution has “chosen” to send the least efficient LHClI antenna to PSI, whereas the efficient PSII supercomplex remains fully functional.
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St1 and St2. The LHClII that migrates to the stroma lamellae in St2 is a subpopulation of extra LHClII, which is probably located close to the grana margin in St1, as suggested by the absence of changes in the PSII density in the grana in the two conditions. In St2, not only the mobile LHClII complexes are phosphorylated but also the LHClII trimers, which are associated with the core to form the PSII supercomplexes. This indicates that phosphorylation is not sufficient to induce the movement of LHClII from grana to stroma as suggested previously (22). Moreover, the phosphorylation is not sufficient to detach the LHClII trimers from the PSII supercomplex. The population of extra LHClII is weakly connected with PSII supercomplexes as indicated by the fact that they transfer energy relatively slowly to the reaction center of PSII in St1 (21) and that it has thus far not been possible to purify a PSII supercomplex from plants with these trimers attached (34). It is therefore likely that this connection is sufficiently weak to be broken by phosphorylation. At the same time, it can be expected that phosphorylated LHClII has a higher affinity for PSI, such that the effect is synergetic. The presence of the migrating LHClII in the grana margins also means that the LHClII do not have to migrate very far during state transitions, thus limiting the possible danger caused by the presence of “free” pigment-protein complexes in the membrane. In addition, we have recently shown that in basically all natural light conditions for plants, a part of the mobile LHClII population is associated with PSI (10). It can thus be concluded that the association of LHClII with PSI has evolved from a short term response as observed in green algae, where it involves up to 80% of the LHClII population (46) and has a large effect on the PSI organization (32), to a long term response in plants, where LHClII is an antenna of PSI in most light conditions.

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Phosphorylated LHClII Does Not Have to Move to PSI—According to the accepted model of state transitions, the phosphorylation of LHClII induces its dissociation from PSII and its movement/association to PSI. Only in mutants lacking PSI (46) or the LHClII anchor point in PSI (22) was it observed that phosphorylated LHClII could not dock to PSI and stayed in the grana, supporting the “molecular recognition model” (22, 47). However, this observation has several possible explanations: (i) phosphorylation involves only the extra LHClII, which in the absence of PSI cannot move to PSI; (ii) phosphorylation involves also trimer M and S (the trimers that are part of the supercomplex) and induces their dissociation from the core, although in the absence of PSI, they remain in the grana; (iii) trimer M and S get phosphorylated but do not dissociate from the core. Here we show that both the LHClII of the PSII supercomplex as well as the extra trimers get phosphorylated upon St1 to St2 transition but that phosphorylated trimer M and S remain associated with the PSI core. And this in WT plants, when the docking side on PSI is available. This is in agreement with previous results (48), which showed that there are at least two pools of LHClII that can be phosphorylated, of which only one moves to the stroma lamellae. Our data clearly indicate that the association of trimers S and M with the PSI core is too strong to be broken by phosphorylation in the membrane. However, phosphorylation can be instrumental in breaking the weak interactions between the PSII supercomplex and the extra LHClII trimers, thus allowing the latter to move to PSI.

Remodeling of PSII Supercomplexes and Megacomplexes Is Not Required for State Transitions—In this work, we have investigated several important aspects of state transition in higher plants at the level of supercomplexes, looking at their composition and organization. However, note that other mechanisms, such as thylakoid membrane reorganization, might also occur during state transitions. The results of this study are summarized in Fig. 5: the composition and organization of the PSI supercomplexes does not show significant differences between
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