Functional architecture of higher plant photosystem II supercomplexes

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Photosystem II (PSII) is a large multiprotein complex, which catalyses water splitting and plastoquinone reduction necessary to transform sunlight into chemical energy. Detailed functional and structural studies of the complex from higher plants have been hampered by the impossibility to purify it to homogeneity. In this work, homogeneous preparations ranging from a newly identified particle composed by a monomeric core and antenna proteins to the largest C₂S₂M₂ supercomplex were isolated. Characterization by biochemical methods and single particle electron microscopy allowed to relate for the first time the supramolecular organization to the protein content. A projection map of C₂S₂M₂ at 12 Å resolution was obtained, which allowed determining the location and the orientation of the antenna proteins. Comparison of the supercomplexes obtained from WT and Lhcb-deficient plants reveals the importance of the individual subunits for the supramolecular organization. The functional implications of these findings are discussed and allow redefining previous suggestions on PSII energy transfer, assembly, photoinhibition, state transition and non-photochemical quenching.

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Introduction

Photosystem II (PSII) is a large pigment–protein supramolecular complex embedded in the thylakoid membrane of plants, algae and cyanobacteria, which splits water into oxygen, protons and electrons during the photosynthetic process (Barber, 2003). This complex thus provides the

energy and the oxygen, which sustain all life on earth. PSII is present mainly in dimeric form, each monomer consisting of at least 27–28 subunits organized in two moieties: the core complex and the antenna system (Dekker and Boekema, 2005). The core is composed of several proteins: (i) D1 and D2 containing the reaction centre P680 and all the cofactors of the electron transport chain; (ii) CP47 and CP43, which coordinate chlorophyll (Chl) *a* molecules and act as an inner antenna and (iii) several low molecular subunits whose role has not yet been fully understood (Shi and Schroder, 2004). The structure of the PSII core of cyanobacteria shows 35 Chl *a* molecules, 2 pheophytins and 12 molecules of β-carotene (Loll *et al.*, 2005; Guskov *et al.*, 2009). In higher plants, on the luminal side of the membrane, the products of the *psbO*, *psbP* and *psbQ* genes compose the oxygen evolving complex (OEC33, 23 and 17, respectively), which participates to the stabilization of the Mn cluster required for an efficient oxygen evolution. However, the exact role and locations of these subunits has not been fully clarified yet (Roose *et al.*, 2007). The peripheral antenna system has a primary role in light harvesting, transfer of excitation energy to the reaction centre and photosynthesis regulation through photoprotective mechanisms, which dissipate the excess of energy absorbed by the system as heat under stress conditions (non-photochemical quenching) (Schmid, 2008). It is composed of six different complexes, belonging to the Lhcb (light-harvesting complex) multigenic family (Jansson, 1999), which coordinate Chl *a*, Chl *b* and xanthophylls, in different ratios. The major antenna complex, LHCII, is organized in heterotrimers composed of the products of the *Lhcb1–3* genes (Caffarri *et al.*, 2004), while the three other subunits, CP29 (Lhcb4), CP24 (Lhcb6) and CP26 (Lhcb5) are present as monomers (Dainese and Bassi, 1991).

The supramolecular organization of PSII–LHCII has been studied by electron microscopy (EM) and single particle analysis on heterogeneous preparations obtained directly from mildly solubilized membranes or after a fast purification step, which allows enrichment of the high molecular weight complexes (Boekema *et al.*, 1999a; Yakushevskaya *et al.*, 2001). The location of the large core subunits was assigned by cross-linking experiments (Harrer *et al.*, 1998) and confirmed by EM on solubilized membranes of plants lacking individual antenna complexes (Yakushevskaya *et al.*, 2003). The larger supercomplex observed in *Arabidopsis thaliana* contains a dimeric core (C₂), two LHCII trimers (trimer S) strongly bound to the complex on the side of CP43 and CP26, and two more trimers, moderately bound (trimer M) in contact with CP29 and CP24. This complex is known as the C₂S₂M₂ supercomplex (Dekker and Boekema, 2005). A 3D reconstruction of a smaller supercomplex containing only one trimer per reaction centre and lacking CP24 (C₂S₂) was obtained by cryo-EM at 17 Å resolution (Nield *et al.*, 2000c; Nield and Barber, 2006). Although the overall organization of the system is known, the low resolution at which this structure is available does not allow to determine the exact position of the individual complexes,

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their respective orientations and the way in which they interact, thus hampering the possibility to understand the molecular details of the complex functioning of the system. This lack of information is mainly due to difficulties in obtaining a homogeneous and stable preparation of the supercomplex. This has not only restricted the possibility for detailed structural analysis but has also limited functional and spectroscopic studies to the level of non-homogeneous grana membrane preparations, which are enriched in PSII-LHCII (Broess *et al*, 2006; Veerman *et al*, 2007).

In this work, we present a protocol to obtain homogeneous preparations of the various types of PSII-LHCII supercomplexes. The possibility to relate the supercomplex organization to the protein content allows determining the role of the individual subunits in the overall organization. The functional implications of these findings for energy transfer, photoprotection, state transition and non-photochemical quenching are discussed.

Results

Isolation and characterization of the PSII supercomplexes

To obtain homogeneous preparations of PSII supercomplexes, we optimized the conditions for solubilization and fractionation of grana membranes. To this aim, the membranes were solubilized with a very low concentration of α -DM and the different complexes were separated on a dense sucrose gradient, which also contained a very low concentration of detergent to avoid destabilization of the complexes during ultracentrifugation. The detergent concentration was about 4–8 times less than what is normally used for a sucrose gradient, but still sufficient to maintain the complexes in solution. All the different steps of the procedure were performed in dim light and in the cold. This is a very important point as the temperature has a large effect on the stability of the complexes (Supplementary Figure S1). Using this procedure we were able to separate 12 distinct bands (B1–12) containing different PSII components and supercomplexes (Figure 1A). The upper bands (B1–5) have been well characterized in previous works (Caffarri *et al*, 2001) and correspond respectively to: (1) free pigments; (2) monomeric Lhcb proteins; (3) trimeric LHCII; (4) CP24/CP29/LHCII (M) trimer complex; (5) monomeric PSII core. The lower bands (B6–12), which from the apparent molecular weight should contain supercomplexes of increasing size, were further characterized by absorption spectroscopy, EM and SDS-PAGE. All fractions were stable in ice for at least 2 days as assessed by measuring the fluorescence emission (Supplementary Figure S1).

In Figure 1B, the absorption spectra of fractions 6–12 are shown. It is worth saying that the absorption spectra of the same bands from different preparations were identical, demonstrating the high reproducibility of the procedure. Chls *b*, bound to Lhc antenna proteins, show two main peaks around 470 and 650 nm, whereas Chls *a* are responsible for the absorption around 435 and 675 nm. The relative intensity of the absorption in the Chl *b* region increases from B7 to B12 suggesting that B7, almost lacking a Chl *b* contribution, corresponds to the dimeric PSII core (B5 being the monomeric core), whereas the fractions from B8 to B11 contain supercomplexes with increasing Lhc content (note that the spectrum of B12 is identical to that of B11). Unexpectedly B6,

which migrates between the monomeric and the dimeric PSII core, shows a strong Chl *b* absorption, indicating that it contains a complex enriched in Lhcb proteins.

To determine the structural organization of the supercomplexes, the fractions (B6–B12) were analysed by EM and single particle image analysis (Figure 1C): B6 contains almost exclusively a small supercomplex, never described before, consisting of monomeric PSII core, LHCII-S trimer and CP26; B7 contains the PSII dimeric core (C₂), as deduced from the absorption spectrum; B8, C₂S and very few C₂M supercomplexes (around 5%); B9, C₂S₂ and C₂SM particles; B10, mainly C₂S₂M; B11, the C₂S₂M₂ supercomplex, the biggest one described so far for *Arabidopsis*; B12 contains megacomplexes of C₂S₂M₂ (Boekema *et al*, 1999b; Yakushevskaya *et al*, 2001), which explains why this band has higher mobility than B11 in the gradient, but the same absorption spectrum (Figure 1A and B). Interestingly, the organization of the subunits in fraction B6 is identical to that in the dimeric complex (e.g. compare B6 with B8 in Figure 1C), which indicates that the monomerization does not influence the binding of CP26 and trimer S.

The exact protein composition of each band was determined by SDS-PAGE (Figure 2). The results are in line with the EM analysis, showing an increase of the ratio Lhcb/small-PSII-subunits and of CP24 content, which indicates increasing amounts of the M trimer (which requires CP24 for its binding; Kovacs *et al*, 2006), in bands from B8 to B11. In these fractions, the amount of CP29 and CP26 is identical, suggesting that these subunits are always bound in a 1:1 stoichiometry to the PSII core. B6 contains only core subunits, LHCII and CP26, lacking any trace of CP29. The analysis also reveals the distribution of the OEC proteins in the fractions: PsbO is present in all fractions containing PSII core but also in band 1, where it is not associated with the core. PsbP is detected in the fraction of monomeric PSII core (B5), in low amount in B6 and dimeric PSII core (B7) (where it is however not bound to the complexes, see discussion and Supplementary Figure S2) but is completely lacking in all PSII supercomplexes, in agreement with previous results (Hankamer *et al*, 1997). PsbQ is present in B6, absent in monomeric (B5) and dimeric PSII core (B7), but again present in increasing amounts (correlating with the Lhcb content) in supercomplexes from B8 to B11 (Figure 2).

The availability of two fractions containing either trimer M (B4) or trimer S (B6) allows determining the distribution of Lhcb1, Lhcb2 and Lhcb3 in the trimers. SDS-PAGE (Figure 2) and western blotting (Figure 3) show that Lhcb3 is present only in trimer M. Indeed the amount of Lhcb3 in B4 is very high, whereas this subunit is practically absent in B6 and B8 (note that this last band contains only very few C₂M particles). The opposite is true for Lhcb2, which is absent in B4 but present in B6 thus indicating that Lhcb2 is a specific component of trimer S. Lhcb1 is present in both trimers.

PsbS, the protein involved in the fast phase of non-photochemical quenching (Li *et al*, 2000), was present in fractions B5–B12, as confirmed by western blotting (Figure 3B). Considering that PsbS is a very hydrophobic protein (Dominici *et al*, 2002), the possibility of unspecific association or formation of aggregates with different sizes was investigated by loading equal volumes of all fractions including the intermediate fraction between B11 and B12 (B11/B12 band) on a gel. This fraction does not contain supercom-

Figure 1 Isolation and characterization of the PSII supercomplexes. (A) Sucrose gradient of solubilized membranes, showing 12 green bands. The content of each band is indicated on the basis of earlier work (Caffarri *et al*, 2001) (B1–B5) and this work (B6–B12). (B) Absorption spectra of bands 6–12. The spectra are normalized to the maximum in the red region. B11 and B12 are almost superimposed. The Chl b content, which is proportional to the antenna content, is deducible from the intensity of the bands at 470 and 650 nm. (C) EM analysis of the supercomplexes. The projections obtained for bands 6–12 are shown. B6 contains a newly identified supercomplex formed by a monomeric core, one LHCII S trimer and the minor antenna CP26. Contours representing the different complexes are superimposed. Also note that the position of the M trimer in the absence of trimer S (C2M in B8) is different. C, core; S, LHCII trimer strongly bound; M, LHCII trimer moderately bound (see text). The molecular weight of each particle, calculated on the basis of the protein content as determined by EM and SDS-PAGE, is also reported.

plexes and therefore should not contain any PsbS if its binding to the supercomplexes is specific. The western blot revealed the presence of similar amounts of PsbS (Figure 3B; Supplementary Figure S2) in all lower fractions, including B11/B12, thus suggesting that this protein is not stably associated with the supercomplexes.

Determination of the supramolecular structure of $C_2S_2M_2$ supercomplex

To determine the supramolecular organization of the $C_2S_2M_2$ supercomplex, fraction B11 was subjected to extensive single

particle EM analysis. In total, a set of 40 000 negatively stained single particle projections was analysed. Repeated alignment steps and classification revealed homogeneous classes of the $C_2S_2M_2$ projections in top-view orientation and few classes with projections in a slightly tilted position. A homogeneous class of about 13 000 top-view projections was summed in the final 2D projection map (Figure 4A; Supplementary Figure S3). The projection map was obtained at 12 Å resolution and it contains ample details to assign the orientation of the individual Lhcb complexes (Figure 4B). The unambiguous assignment of the M and S trimers in the EM

Figure 2 SDS-PAGE analysis of the sucrose gradient fractions. The protein composition of the 12 sucrose gradient fractions was analysed by loading similar amounts of total chlorophylls (3 μ g) on the gel (see Materials and methods for details on volumes). The identity of each Coomassie band is indicated. D1 and D2 are not visible in this gel system because they maintain a partial folding during migration, which makes them appearing diffused. Note that PsbP is better visible in lanes B1 and B2 (*). Note the presence of CP26 in B6 together with Lhcb1,2 and the increase of the Lhcb/ small-PSII-subunits ratio in the fractions from B8 to B12, according to the increased antenna size of the supercomplexes. PsbQ is absent in the fractions containing PSII core without antennas (B5 and B7).

Figure 3 Western blotting analyses of sucrose gradient fractions. Proteins co-migrating in SDS-PAGE were detected by western blotting. (A) Coomassie stained gel of sucrose gradient fractions. The B4 used was cleaned of contaminating B3 fraction (LHCII trimers) with a second sucrose gradient. Band B5 was highly contaminated by B4 due to the very low amount of PSII core in our solubilization conditions. Similar amounts of Chls (0.5 μ g) were loaded in each line, except for the B11/12 band (the clear gradient fraction between B11 and B12) where a similar volume as adjacent bands was loaded. (B) Western blot analyses using antibodies against PsbO, Lhcb2, Lhcb3 and PsbS. The absence of Lhcb2 in trimer M (B4) and of Lhcb3 in trimer S (B6 and B8) is clearly visible. PsbS is present in several fractions, including the fraction between B11 and B12 (B11/12) where a clear decrease in the PSII subunits is evident, but not for PsbS. This indicates that PsbS comigrates with the supercomplexes and it is not specifically bound to them.

projection was facilitated by the localization of corresponding densities in the calculated projection maps of the truncated X-ray high-resolution LHCII structure (Liu *et al*, 2004) (Figure 4C). A tripod-shaped stain-excluded area in the EM projection map of LHCII trimer (Figure 4B, pink lines) can also be recognized in the truncated model (Figure 4C). Further, pairs of high densities revealed in the EM projection map correspond well to the high-density spots of helix A and B, and to the strong density close to helix C (Figure 4B and C, green asterisks). The results indicate that M and S trimer are rotated by 18° with respect to each other.

Assignment of the minor antenna complexes (CP24, CP26 and CP29) was more difficult due to their less pronounced features in the EM projection map. The orientation of these complexes was determined under the assumption that the high densities of monomeric Lhcb in the EM projection map correspond to the pairs of densities observed in LHCII trimers (green asterisks) and a broad density near the end of helix A in the truncated projection of monomeric Lhcb (Figure 4B and C, pink asterisk). A different position of the monomeric Lhcb complexes, in particular a 180° rotation compared with the position in Figure 4, was considered in the modelling. It is noteworthy that neoxanthin protrudes sharply from one side from the monomeric Lhcb complexes (Figure 7, yellow) thus strongly constraining possible subunit orientations. Hence, at present the proposed model is by far the most likely arrangement of the antenna components, because it also brings them in positions close enough to allow fast energy transfer (see discussion). Although no standard criteria are available for fitting X-ray data into 2D EM maps, the resulting pseudo-atomic data can be considered to have a precision extending the resolution of the EM data at 12 Å, in the range of the size of an α helix or a chlorophyll molecule.

Isolation of supercomplexes from Lhcb- and PsbS-deficient mutants

To determine the role of the individual antennas in the architecture and in the stability of the PSII supercomplexes, we have used the solubilization procedure described before on grana membranes prepared from three knock-out (KO) lines lacking Lhcb3, CP24 or CP26. Comparison of the band patterns in the gradients of Lhc-deficient mutants and WT shows significant differences in the supercomplex compositions (Figure 5A). KoLhcb3 and koCP24 lines completely lack the largest supercomplexes (C_2S_2M and $C_2S_2M_2$, B10 and B11 in the WT) and the small complex LHCII(M)/CP24/CP29 (B4). Also, in the case of koCP26 no bands containing high molecular weight supercomplexes are visible and the amount of the fractions containing the smaller supercomplexes (B8 and B9) is extremely reduced. Furthermore, the amount of the PSII dimeric core band (B7) increases as well as that of the band containing LHCII trimers, indicating that supercomplexes lacking CP26 are more sensitive to detergent treatment than those of the WT. Interestingly, all mutants also lack band B6, the monomeric core/LHCII(S)/CP26 complex.

To determine the protein composition in both grana membranes and individual supercomplexes of the antenna-deficient lines, 1D and 2D SDS-PAGE analysis were performed (Figure 6). Unexpectedly, PsbQ was absent in the membranes of all three KO lines and Lhcb3 and CP24 mutants were additionally lacking PsbP (see Figure 6C). The absence of these subunits was also confirmed on stacked thylakoid

Figure 4 Projection map of the $C_2S_2M_2$ supercomplex. **(A)** Final projection map of the PSII $C_2S_2M_2$ supercomplex at 12 Å resolution. **(B)** Assignment of the subunits in the supercomplex by fitting the high-resolution structures of PSII core (Guskov *et al*, 2009) (subunits D1, D2, CP43, CP47 and extrinsic subunit PsbO are highlighted in blue, cyan, salmon, pink and yellow, respectively) and Lhcb (Liu *et al*, 2004) (trimeric LHCII and monomeric Lhcb in dark and light-green, respectively). Lhcb3 and the minor antennas, CP24, CP26 and CP29, are schematically depicted in dark green, light blue, magenta and orange contours, respectively. Green and pink asterisks indicate similar high densities of trimeric and monomeric LHCII, respectively. Tripod-shaped pink lines indicate a stain-excluded area of LHCII trimer. **(C)** Generated 2D projection maps of LHCII trimer and monomer from atomic model, truncated at 10 Å resolution. To allow comparison, corresponding densities of LHCII revealed in the EM projection map are indicated in the truncated 2D projection maps. Note that the fitting of the CP24 region with the LHCII monomeric structure leaves empty a large density next to helix C towards the outer part of the supercomplex. However, CP24 presents an extremely long helix C-helix A loop (28 amino acids more than LHCII) that would fit perfectly this region.

Figure 5 Sucrose gradient fractionation of solubilized grana membranes of WT, Lhcb-depleted lines and *npq4* mutant. **(A)** Supercomplexes were prepared from *Arabidopsis* lines lacking Lhcb3 (koLhcb3), CP24 (koCP24), CP26 (koCP26) and PsbS (*npq4*). Lhcb3 and CP24 mutants lack the small complex containing LHCII-CP24-CP29 (B4) and the high molecular weight supercomplexes corresponding to C_2S_2M (B10) and $C_2S_2M_2$ (B11). koCP26 is lacking almost completely the supercomplexes and shows an intense PSII dimeric core band (B7). All mutants also lack band 6 containing PSII monomeric core/LHCII S-trimer/CP26. The *npq4* mutant lacking the PsbS protein does not show any difference with respect to the WT. **(B)** Sucrose gradient of WT and *npq4* membranes solubilized at pH 5.5. Protonation of PsbS *in vitro* has no effect on the antenna binding to PSII core. Note also that the most abundant bands of supercomplexes correspond to C_2S_2M and $C_2S_2M_2$ complexes (B10 and B11), suggesting that most of the PSII *in vivo* in our grana preparation binds both trimer S and trimer M.

membranes indicating that this was not caused by the harsh grana preparation protocol (Supplementary Figure S4). However, western blotting analysis of the total protein content of the leaf shows the presence of these proteins, suggesting that they have been lost during the preparation of the thylakoid membrane (Supplementary Figure S4). PsbP was absent in the supercomplexes of koCP26 (B8 and B9)

(Figure 6B and D), indicating that this subunit is easily lost on purification as was observed for the WT. The content of CP24 in koLhcb3 was reduced, as was that of Lhcb3 in koCP24 suggesting that fractions B8 and B9 contain C_2S and C_2S_2 particles, respectively (in the WT, C_2M (B8) and C_2SM (B9) complexes were also found). In koCP26, the significant increase of CP24 and Lhcb3 in the faint B8 and

Figure 6 1D and 2D SDS-PAGE of grana membranes and supercomplexes of Lhcb-depleted lines. (A) Grana membranes of WT, koLhcb3, koCP24 and koCP26 mutants. Note that PsbQ is absent in the three mutants. (B) Supercomplexes (B8 and B9 of Figure 5) of the antenna mutants. (C) 2D SDS-PAGE separation of the PsbO-CP24 region of panel A, which allows a better investigation of the protein composition in the membrane of the mutants. Note the lack of PsbP in koLhcb3 and koCP24. (D) 2D SDS-PAGE separation of the PsbO-CP24 region of panel B allows highlighting the strong reduction of CP24 in the koLhcb3 supercomplexes, the lack of Lhcb3 in the koCP24 supercomplexes and the significant presence of Lhcb3 and CP24 proteins in the supercomplexes from koCP26.

B9 fractions, as compared with the same bands in the WT, indicates an enrichment of complexes containing LHCII M-trimer (i.e. C_2M) and a strong reduction of C_2S and C_2S_2 complexes.

To investigate the role of PsbS in the supercomplex organization, the *npq4* mutant lacking PsbS was analysed. We found that the absence of PsbS does not influence the supercomplex formation and stability, the gradient bands being identical to those of the WT (Figure 5). In addition, a high-resolution projection map of the $C_2S_2M_2$ supercomplex was obtained by single particle EM of the B11 fraction from

the *npq4* mutant. This projection is identical to that of the WT, thus excluding the possibility that PsbS is part of the supercomplex or strongly associated to it. This is in agreement with the SDS-PAGE analysis, which indicates that PsbS is not specifically associated with the purified supercomplexes in the WT (Figure 2), but it co-migrates with them (Figure 3).

Oxygen evolution

To test the photosynthetic activity of our preparations, oxygen evolution of the grana membranes and PSII supercom-

Figure 7 Model of the structure of photosystem II supercomplex $C_2S_2M_2$. The model has been assembled based on the projection map in Figure 4 using the crystal structures of the cyanobacterial PSII core (Guskov *et al*, 2009) (3BZ1 and 3BZ2) and LHCII trimer (Liu *et al*, 2004) (1RW1). For the monomeric antennas, the structure of a monomeric LHCII has been used. Proteins of the core, gold; LHCII, brown; CP24, blue; CP29, red; CP26, magenta; Chls *a*, cyan; Chls *b*, green; Neoxanthin, yellow (spheres); Lutein L1, orange; Lutein L2, dark-yellow. Chls 611 and 612 of all subunits are represented as cyan spheres. Chls 511 (in CP47), 479 and 486 (in CP43) of the core, which are the nearest-neighbour to the outer antenna are represented as blue spheres. Chl 603 of CP29 is also represented as blue spheres. For clarity the phytol chains of the Chls are omitted.

plexes of WT and *Lhcb*-deficient lines was measured (see Table I). Reduction in oxygen evolution was observed in the membranes of all three KO mutants, with values of 72% for *koCP26*, 50% for *koLhcb3* and 36% for *koCP24* as compared with the WT. The data also show that all supercomplexes, including the B6 fraction, evolve oxygen, which clearly indicates that all the preparations are active and can efficiently drive photosynthesis.

Discussion

During the past few years, a tremendous improvement in the knowledge of the organization of photosynthetic components was achieved, because the structures of most of the complexes of the thylakoid membrane could be obtained at atomic or near atomic level (Jordan *et al*, 2001; Stroebel *et al*, 2003; Liu *et al*, 2004; Loll *et al*, 2005; Yamashita *et al*, 2007; Guskov *et al*, 2009). At present, only the structural details of the PSII-LHCII supercomplexes remain partially obscure. This lack of information is primarily due to the impossibility of obtaining stable and homogeneous preparations of PSII-LHCII. In contrast to PSI, the interaction between the core and the outer antenna in PSII is extremely

weak and even mild solubilization leads to the disassembly of the supercomplexes (Caffarri *et al*, 2001). Moreover, the impossibility of obtaining stable and homogeneous preparations of PSII-LHCII has also prevented the study of the light harvesting and energy transfer processes in the system.

In this work, we were able to purify for the first time six homogeneous fractions of PSII-LHCII supercomplexes with increasing antenna sizes, ranging from PSII core to the large $C_2S_2M_2$ supercomplex, which are suitable for biochemical, structural and spectroscopic analysis. All fractions were analysed in detail, combining biochemical methods with single particle EM analysis. This allows, to our knowledge for the first time, to directly relate the presence/absence of individual subunits to the supramolecular organization of the complex and thus to get answers about their roles in the assembly and their positions in the supercomplexes.

12 Å resolution structure of PSII supercomplex ($C_2S_2M_2$)—implication for the energy transfer

The structural organization and the orientation of the different light-harvesting proteins in the PSII $C_2S_2M_2$ supercomplex were determined from a 12 Å resolution projection map obtained by single particle EM and image analysis

Table 1 Oxygen evolution measurements on sucrose gradient fractions and grana membranes of WT and antenna KO mutants

	A $\mu\text{mol O}_2 \text{ mg(Chl)}^{-1} \text{ hr}^{-1}$
(a)	
B6	64.9 $\pm$ 5.9
B7	61.6 $\pm$ 6.3
B8	72.3 $\pm$ 1.3
B9	70.5 $\pm$ 3.8
B10	72.9 $\pm$ 2.9
B11	72.6 $\pm$ 3.4
(b)	
WT	53.1 $\pm$ 2.0
koLhcb3	27.0 $\pm$ 1.3
koCP24	19.1 $\pm$ 1.8
koCP26	38.3 $\pm$ 1.3

(a) O₂ evolution measurements on gradient fractions from B6 to B11 show similar activity, including the monomeric CS complex (B6).

(b) Measurements on grana membranes from WT and antenna KO plants show a clear decrease in O₂ evolution activity in the three mutants, especially for koLhcb3 and koCP24 lacking both the PsbP and PsbQ subunits.

Difference between koLhcb3 and koCP24 could be due to the different membrane organization which influences the diffusion of the plastoquinone (de Bianchi *et al*, 2008).

(Figure 4). This allowed the reconstruction of a 3D pseudo-atomic model of the full PSII–LHCII supercomplex using the crystal structures of the LHCII trimer (Liu *et al*, 2004) and the recent refined structure of the cyanobacteria core (Guskov *et al*, 2009), which presents the full assignment of the small PSII subunits and an improved localization of the cofactors (Figure 7). For the minor antenna complexes, the structure of a monomeric unit of LHCII was used after modification according to the available biochemical data (e.g. pigment content) (Sandona *et al*, 1998; Caffarri *et al*, 2007). This allows the visualization of possible energy transfer pathways in the supercomplex. The model shows that trimer S is in direct contact with Chl 479 of the CP43 subunit to which it can transfer energy through Chls 611/612. These Chls represent the low-energy state in all Lhcb antenna complexes (Mozzo *et al*, 2008) and thus are the most populated ones at thermal equilibrium. CP26 can also transfer energy directly to the core, again through Chls 611/612, which are facing CP43 (Chl 486) in the structure. The nearest-neighbour Chls between CP26 and LHCII S are 604 and 605, which are a Chl *a* at high energy and a Chl *b* (Novoderezhkin *et al*, 2005), respectively, suggesting that there is small exchange of energy between the two subunits. The LHCII M trimer is in contact with CP24 and CP29, but not with the core. In the interaction region, all three complexes expose Chls 611/612, thus forming a pool of Chls *a* at low energy. The present model (Figure 7) suggests that CP24 cannot transfer excitation energy directly to the core, because there are no Chls of CP47 located near CP24. This indicates that the transfer from trimer M and CP24 to the core occurs through CP29. This makes CP29 a very suitable site for the regulation of the excitation energy flow and thus for playing a primary role in the non-photochemical-quenching process. In CP29, Chl 603 (a Chl *a* absorbing around 676 nm (Bassi *et al*, 1999), thus only slightly higher in energy than Chls 611/612) faces CP47 (Chl 511) to which it can transfer excitation energy. However, at the moment we cannot exclude that one of the small higher plant-specific subunits (PsbR or PsbW), which are absent in

the cyanobacteria structure, is accommodated near CP24 and binds Chls, thus allowing CP24 to directly transfer excitation energy to the core. Indeed, the EM projection indicates that there is room in this region for a small extra subunit.

The overall architecture of the antenna system indicates that excitation energy is transferred from the peripheral antenna to the core through specific pathways, which involve only Chl *a* molecules and in particular the low-energy forms. This has the effect of speeding up the energy transfer process, strongly decreasing the migration time and explaining the fast transfer observed in integer membranes (Broess *et al*, 2008). Interestingly, CP29, which is the link between the outer antenna and the core, has the lowest Chl *b* content of all Lhcb's (Sandona *et al*, 1998) and these Chls are located in a region rich in Chl *b* and neoxanthin, between trimers S, M and CP29, thus off the main highway from the antenna to the core.

A new complex shows that monomeric core can bind Lhcb antenna—implication for photoinhibition

In addition to the supercomplexes, a novel complex, composed of a monomeric core, one LHCII S-trimer and CP26 (CS/CP26) was isolated (band B6, Figure 1). No traces of CP29 were detected in this band. This finding is surprising, since so far the association of Lhcb antenna to the core was believed to be possible only for the dimeric conformation (Dekker and Boekema, 2005) and in the presence of CP29 as a docking protein (Yakushevskaya *et al*, 2003). However, our results show that a complex consisting of a monomeric core, CP26 and one LHCII trimer is stable enough to be purified in high yield. It is active in O₂ evolution, strongly suggesting that it could also be present in the membranes. One implication of this finding concerns the D1 protein degradation/repair cycle, a process that replaces the D1 subunit, which has a half-life of about 30 min (Godde *et al*, 1991). It is generally believed that the entire antenna system disassembles before monomerization of the PSII core complex (Adir *et al*, 2003). The newly found particle suggests that this is in principle not necessary: trimer S and CP26 can remain associated to the core, with the advantage of limiting the potentially dangerous presence of isolated antenna complexes in the grana membrane during the repairing cycle. Indeed, it is very likely that only one of the two D1 proteins present in the dimeric PSII is damaged, and needs to be replaced, at a time. The possibility to disassemble only the damaged moiety of the photosystem, leaving the 'healthy' core still in contact with its antenna, which has also a photoprotective function, helps to protect it from photodamage.

Assembly of the PSII–LHCII supercomplex: role of the Lhcb proteins

The analysis of Lhcb-depleted lines allows determining the hierarchy of the binding of the individual subunits within the PSII–LHCII supercomplex. The reduction of Lhcb3 in koCP24 and of CP24 in koLhcb3, suggests a mutual stabilization of these complexes. Furthermore, a complete absence in both mutants of complexes and supercomplexes containing trimer M indicates that CP24 and Lhcb3 have an important function in mediating the association of trimer M with the PSII complex. Indeed, the EM analysis of koCP24 membranes has shown that the supercomplexes of this mutant do not contain trimer M (Kovacs *et al*, 2006; de Bianchi *et al*, 2008). C₂S₂M₂ supercomplexes could be observed (Kereiche *et al*,

2007) in koLhcb3 membranes, but it was shown that the general packing of the trimers differed slightly from that of the WT. Our results indicate that the pseudo-M trimer (trimer M without Lhcb3) observed in the membrane of koLhcb3 (Kereiche *et al*, 2007) is only weakly interacting with the supercomplex as it can easily be lost on mild solubilization. It can be concluded that CP24 and Lhcb3 specifically mediate the binding of the M trimer to the supercomplex, suggesting that Lhcb3 is the monomeric unit facing CP24. This is also supported by sequence comparison of Lhcb3 with Lhcb1 and Lhcb2 (Caffarri *et al*, 2004), which reveals major differences at the end of the B helix, with the insertion of a Trp in Lhcb3, and at the beginning of the loop between helices B and C. This is exactly the domain of the LHCII monomeric unit facing CP24, as can be seen in the model (Figure 4).

CP24 and Lhcb3 are present only in higher plants (Alboresi *et al*, 2008) where they apparently evolved to increase the antenna size of PSII. Indeed in green algae only C₂S₂ complexes have been observed (Nield *et al*, 2000b; Iwai *et al*, 2008). It can also be concluded that trimer M has an influence on the assembly of trimer S that, in the absence of M or in the presence of the pseudo-M, is less strongly associated to the core, as suggested by the absence of the CS/CP26 complex in the gradients of koCP24 and koLhcb3. On the other hand, trimer M in C₂M is displaced compared with its position in the particles containing also trimer S (Figure 1), indicating that trimer S is influencing the location of trimer M.

In general, the data indicate that the stable connection of an antenna to the supercomplex requires interactions with two different partners. According to the model (Figure 4), association of trimer S to the core complex through CP43 is stabilized by interactions with CP26 and CP29. Although it has been shown that the S trimer is still connected to the supercomplex in the absence of CP26 (Yakushevskaya *et al*, 2003), our results indicate that its binding is far less stable (Figures 5 and 6). Moreover, the possibility to purify CS/CP26 complexes in the WT shows that the effect of CP26 on the stabilization of the S trimer is at least as high as that of CP29. Previous analysis of plants lacking CP29 showed that no PSII-LHCII supercomplexes could be found even on very mild solubilization (Yakushevskaya *et al*, 2003). Taken together, these data indicate a clear role of CP29 in the stability of the PSII-LHCII dimer. CP29, bound to one monomeric core, binds the S-trimer of the other monomeric core implicating a general stabilization of the supercomplex, while CP29 *per se* is not required for stable binding of trimer S.

Finally, it could be observed that on solubilization at low pH (Figure 5B) large part of PSII is present in the gradient as C₂S₂M or C₂S₂M₂ supercomplexes, while the amount of C₂S₂ is very low. As some M trimers clearly detached during the purification (presence of B4), it can be suggested that in the grana membranes of our plants most of the PSII is present as C₂S₂M₂ and not as C₂S₂. However, the detection in the gradient of an intense band of trimeric LHCII (B3) indicates the presence of additional LHCII complexes in the membranes. That fact that the crystalline arrays in *Arabidopsis* have a size compatible with C₂S₂M₂ complexes (Yakushevskaya *et al*, 2001) and that we were not able by very mild solubilization to purify complexes larger than those, supports the idea that LHCII-enriched regions exist in the grana membranes, as suggested earlier (Boekema *et al*, 2000).

State transitions: which trimer?

In agreement with earlier data (Dekker and Boekema, 2005), our results indicate that trimer M can easily be dissociated from the supercomplex. This makes it a good candidate for state transitions, during which some LHCII trimers migrate from grana to stroma lamellae where they associate with PSI (Allen, 1992; Kouril *et al*, 2005). However, it has been shown that Lhcb3 is not present in the stroma lamelle (Bassi *et al*, 1988; Jansson *et al*, 1997). Considering that Lhcb3 is a key component of trimer M and that, as shown here, this trimer has a structural role for the assembly of PSII together with CP24, it can be concluded that it is not responsible for state transitions. In agreement with earlier data, our results show that the association of trimer S with the PSII core is very strong and it is quite doubtful that it can easily detach from it. Moreover, biochemical and EM analysis of the membranes revealed the presence of at least one additional LHCII trimer for PSII monomeric core, probably located in regions enriched in LHCII trimers or loosely bound to the PSII (Boekema *et al*, 2000; Dekker and Boekema, 2005). We suggest that these trimers, instead of M or S associated with the supercomplexes, are involved in state transitions.

Non-photochemical quenching: PsbS is not stably associated to the supercomplexes

PsbS is key player in the process of non-photochemical quenching (Li *et al*, 2000), and it has been proposed to act in synergy with other proteins. Its localization would thus be important for understanding the quenching mechanism. Although several studies have addressed this point (Nield *et al*, 2000a; Thidholm *et al*, 2002; Teardo *et al*, 2007; Fey *et al*, 2008), the high propensity of this protein to form aggregates and to precipitate or to stick to other complexes (Dominici *et al*, 2002) has strongly complicated the interpretation of the results. Recently, it has been suggested that PsbS regulates the interactions between LHCII and PSII in the membranes (Kiss *et al*, 2008; Betterle *et al*, 2009). Our results show that the solubilization has an identical effect on WT and *npq4* mutant membranes (Figure 5A), also at low pH (Figure 5B) when PsbS is protonated and should facilitate the detaching of LHCII from the core *in vivo* (Kiss *et al*, 2008; Betterle *et al*, 2009). Our results thus suggest that the protonation of the two luminal glutamate residues (Li *et al*, 2004) is not sufficient to activate PsbS and regulate the interactions LHCII-core. Other factors such as a particular ion or the presence of a ΔpH (and not just a low pH as in the *in vitro* experiment) might be necessary for the activation of PsbS.

In addition, the results clearly show that PsbS is not located in or stably associated with the supercomplexes, indicating that either it has a transient binding to them or it is located in the LHCII-enriched membrane regions as suggested earlier (Dekker and Boekema, 2005). However, we cannot exclude that PsbS is located in between two adjacent photosystems and that its binding is not strong enough to survive the purification.

Oxygen evolution: the OEC subunits organization depends on the antenna system

PsbO was present in all fractions containing PSII core indicating a strong binding to the monomeric core, as observed earlier (Hankamer *et al*, 1997; Nield *et al*, 2000c). PsbQ was present only in Lhcb-containing supercomplexes, in amounts

that are roughly proportional to the antenna size. In all these fractions, PsbP was absent, which indicates, in contrast to previous data (Berthold *et al*, 1981), that the binding of PsbQ does not require PsbP. Instead, it requires the peripheral antenna system, or at least the domain composed of CP26/LHCII(S). This is confirmed by the absence of PsbQ in the membranes of all three Lhcb mutants, indicating that it can be stably associated with the supercomplex only when the outer antenna is perfectly assembled. PsbP was found in the membrane of koCP26, while it was absent in koCP24 and koLhcb3, indicating that the domain formed by CP29/CP24/trimer M is needed for the assembly of this subunit. These results suggest that the localization of the OEC subunits in the 3D reconstruction of PSII-LHCII (Nield and Barber, 2006) needs to be revised. In addition, the oxygen evolution measurements on isolated membranes, where these subunits are not present, show lower values for koLhcb3 and koCP24 and partially also for koCP26 as compared with the WT, thus confirming that PsbP and PsbQ have an important function in the PSII activity.

Conclusions

In this work, homogeneous and stable PSII supercomplexes with different antenna sizes were isolated. A full gallery of complexes, from the core to the largest $C_2S_2M_2$, was characterized by EM and biochemical methods, which allows relating for the first time their protein content to the supramolecular organization. A new complex containing a monomeric core, a trimeric LHCII(S) and CP26 was isolated, showing that the antenna proteins can stably bind to the monomeric core, in contrast to the current opinion. A projection map at 12 Å resolution of the $C_2S_2M_2$ supercomplex reveals the positions and the orientations of the antenna complexes and allows to suggest the main pathways of excitation energy transfer from the antenna to the core. Comparison of the supercomplexes obtained from WT and Lhcb-deficient plants allowed determining the hierarchy of the assembly and the role of the individual subunits in the supramolecular organization. The binding of the M trimer depends on interactions between CP24 and Lhcb3, which are proposed to face each other in the supercomplex. CP26 has a strong effect on the stable binding of trimer S, whereas CP29 is mainly involved in the stabilization of the PSII dimer. PsbS has not been found associated to the supercomplexes and its presence does not influence the interactions between core and outer antenna leading to the conclusion that it is located either peripherally or in the LHCII-enriched domains. The data also indicate that the stable binding of PsbQ to the supercomplex requires trimer S, but not PsbP, in contrast to the current view.

Moreover, we show that stable supercomplexes can be obtained, which can be further used for structural and functional analysis, opening the way to a full comprehension of the largest photosynthetic complex.

Materials and methods

PSII supercomplexes preparation

PSII-enriched membranes (grana membranes) were prepared from WT Col0 plants and the following mutants (Columbia ecotype): *npq4.1* (Li *et al*, 2000), koLhcb3 (SALK_051600), koCP26 (T-DNA insertion in the Lhcb5 gene, SALK_014869), koCP24 (T-DNA insertion in the Lhcb6 gene SALK_077953). Results on koCP24

were confirmed (not shown) also on an independent mutant in Landsberg erecta ecotype (Arabidopsis Gene Trap line GT6248). Plants were grown under $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light (8 h/day) at 21°C. Membranes were prepared according to Berthold *et al* (1981) with few modifications. In particular, Arabidopsis leaves were shortly grinded in a solution (B1) containing 20 mM Tricine KOH pH 7.8, 0.4 M NaCl, 2 mM MgCl_2 and the protease inhibitors 0.2 mM benzamidine, 1 mM ϵ -aminocaproic acid. Solution was centrifuged 10 min at 1400 g and pellet resuspended in a solution (B2) containing 20 mM Tricine KOH pH 7.8, 0.15 M NaCl, 5 mM MgCl_2 and protease inhibitors as before. This solution was centrifuged 10 min at 4000 g, pellet resuspend in 20 mM Hepes 7.5, 15 mM NaCl, 5 mM MgCl_2 (solution B3) and centrifuged again 10 min at 6000 g. Pellet was finally resuspend in a small volume of B3. Chlorophyll concentration was adjusted to 2.5 mg/ml and then PSII membranes were prepared by solubilizing stacked thylakoids at 2.1 mg/ml final concentration with 3/16 volumes of 20% Triton X100 (w/v), 15 mM NaCl, 5 mM MgCl_2 for 20 min on ice and soft agitation. To remove non-solubilized material, a 5 min-centrifugation at 3500 g was done. Supernatant was then centrifuged for 30 min at 40 000 g, pellet washed once with solution B3 to remove excess detergent and then centrifuged as before. Finally, membranes were resuspended in a small volume of 20 mM Hepes 7.5, 0.4 M sorbitol, 15 mM NaCl, 5 mM MgCl_2 . BBY membranes can be frozen in liquid nitrogen and stored at -80°C . The entire preparation was done in cold condition.

For the PSII supercomplex preparations, 150 μg of membranes (in Chls) were washed once with 5 mM EDTA, 10 mM Hepes pH 7.5, then with 10 mM Hepes pH 7.5 and finally solubilized at 0.5 mg/ml by adding an equal volume of 0.6% α -DM in 10 mM Hepes 7.5 and vortexing for a few seconds. The solubilized samples were centrifuged at 12 000 g for 10 min to eliminate unsolubilized material and then fractionated by ultracentrifugation on a sucrose or maltose gradient in a SW41 rotor, for 14–16 h at 4°C at 41 000 rpm. Gradients were formed directly in the tube by freezing at -80°C and thawing at 4°C a 0.65 M sugar solution containing 0.008% α -DM and 10 mM Hepes pH 7.5. For low pH preparations (Figure 5B), 10 mM MES pH 5.5 (instead of Hepes), 100 μg of membranes and a gradient at 0.01% α -DM were used. Maltose was used for EM experiments for better particle resolution in negative staining of EM samples, thus avoiding a dialysis step to remove the excess of sugar. Band separation and absorption spectra were identical using maltose or sucrose. Keeping the samples at 4°C during the entire preparation (i.e. solubilization in cold conditions, gradient loading in the cold room) was essential to improve significantly the yield of high molecular weight supercomplexes (see also Supplementary Figure S1).

SDS-PAGE

1D electrophoresis was performed using the Tris-Tricine system (Schagger, 2006) at 14.5% acrylamide concentration. Second dimension was realized as in Laemmli (1970) using a 14% acrylamide concentration. Different volumes of each band were loaded on the gel in Figure 2. The values normalized to 1 for band B3 (corresponding to 13 μl) are: B1, 31; B2, 5; B3, 1; B4, 2; B5, 23; B6, 11; B7, 16; B8, 11; B9, 13; B10, 19; B11, 31; B12, 31.

Spectroscopy

Absorption spectra were recorded using a Cary4000 (Varian Inc.). When dilution was necessary, the same solution as for the gradients was used.

Electron microscopy

Samples were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. EM was performed on a Philips CM120 electron microscope equipped with a LaB₆ filament operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at either 80 000 $\times$ (results shown in Figure 1) or 130 000 $\times$ (results shown in Figure 4) magnification at a pixel size (after binning the images) of 3.75 Å and 2.3 Å, respectively, at the specimen level with GRACE software for semi-automated specimen selection and data acquisition (Oostergetel *et al*, 1998). Single particle analysis was performed using GRIP software including multireference and non-reference alignments, multivariate statistical analysis, and classification, as in Boekema *et al* (1999a). Resolution was measured using Fourier-ring correlation and the 3 σ criterion (Vanheer, 1987). X-ray structures of the PSII core (Loll *et al*, 2005) and LHCII complex (Liu *et al*, 2004) (the

PDB accession numbers 2AXT and 1RWT, respectively) were displayed using Pymol software *DeLano Scientific*, San Carlos, CA, USA). Truncated version and 2D projection map of LHCII at 10 Å resolution was generated using routines from the EMAN package (Ludtke *et al*, 1999). For CP24 (Figures 4B and 7), the LHCII monomeric structure depleted in the last 20 AA at the C-terminal was used, according to the sequence difference between these two antennas.

Oxygen evolution

O₂ production was measured in a Clark-type oxygen electrode system on BBY membranes concentrated 50 µg/ml (in Chls) in 0.4 M sorbitol, 15 mM NaCl, 5 mM MgCl₂, 10 mM Hepes KOH pH 7.5 at room temperature using 180 µmol m⁻² s⁻¹ white light. O₂ production in supercomplexes was measured on the sucrose fractions diluted at 5 µg/ml (in Chls) at 12°C. Ferricyanide 0.5 mM and DCBQ 0.5 mM were added as electron acceptors. Addition of 5 mM CaCl₂ did not change the O₂ evolution both in membranes and supercomplexes.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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