Functional architecture of higher plant photosystem II supercomplexes

Stefano Caffarri1,2,3,*, Roman Kouril4, Sami Kereiche4, Egbert J Boekema4 and Roberta Croce4,*

1Université Aix Marseille, Faculté des Sciences Luminy, Laboratoire de Génétique et Biophysique des Plantes, Marseille, France, 2CEA, DSV, iBEB, Marseille, France, 3CNRS, UMR Biologie Végétale et Microbiologie Environnementales, Marseille, France and 4Department of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh, Groningen, The Netherlands

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 C8S4M2 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 C8S4M2 at 12Å 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.

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; Yakushevska 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 (Yakushevska 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 C8S4M2 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.

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 (Roese 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) multigene 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; Yakushevska 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 (Yakushevska 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 C8S4M2 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.
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 s-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 LHCCI; (4) CP24/CP29/LHCCI (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 Lhc 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, LHCI-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 C2SM; B11, the C2SM2 supercomplex, the biggest one described so far for Arabidopsis; B12 contains mega-complexes of C2SM2 (Boekema et al, 1999b; Yakushevska 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, LHClI 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-
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 C2S2M2 supercomplex

To determine the supramolecular organization of the C2S2M2 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 C2S2M2 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 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 C2S2M2 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 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 Chls 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.

The EMBO Journal VOL 28 | NO 19 | 2009
©2009 European Molecular Biology Organization
our solubilization conditions. Similar amounts of Chls (0.5 mg) were loaded in each line, except for the B11/12 band (the clear gradient 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. 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 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 trimers (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.

Figure 2 SDS–PAGE analysis of the sucrose gradient fractions. 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 membranes prepared from three knock-out (KO) lines lacking Lhcb3, CP24 or CP26. Comparison of the band patterns in the gradients of Lhcb-deficient mutants and WT shows significant differences in the supercomplex compositions (Figure 5A). KoLhcb3 and koCP24 lines completely lack the largest supercomplexes (CnS2M and CnS2M2, 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.

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 Lhcb-deficient mutants and WT shows significant differences in the supercomplex compositions (Figure 5A). KoLhcb3 and koCP24 lines completely lack the largest supercomplexes (CnS2M and CnS2M2, 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
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 C2S and C2S2 particles, respectively (in the WT, C2M (B8) and C2SM (B9) complexes were also found). In koCP26, the significant increase of CP24 and Lhcb3 in the faint B8 and

Figure 4 Projection map of the C2S2M2 supercomplex. (A) Final projection map of the PSII C2S2M2 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 trimmer. (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 C2S2M (B10) and C2S2M2 (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 C2S2M and C2S2M2 complexes (B10 and B11), suggesting that most of the PSII in vivo in our grana preparation binds both trimer S and trimer M.
B9 fractions, as compared with the same bands in the WT, indicates an enrichment of complexes containing LHCII M-trimer (i.e. C₂M) and a strong reduction of C₂S and C₂S₂ 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 C₂S₂M₂ 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 supercomplexes was measured using an oxygen electrode. The results showed a significant increase in oxygen evolution in the npq4 mutant, indicating an enhanced photosynthetic activity.

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 9 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.
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–LHCl 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$_2$S$_2$M$_2$ 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$_2$S$_2$M$_2$)—implication for the energy transfer**

The structural organization and the orientation of the different light-harvesting proteins in the PSII C$_2$S$_2$M$_2$ 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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A μmol O₂ mg(Chl)⁻¹ hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>64.9 ± 5.9</td>
</tr>
<tr>
<td>B7</td>
<td>61.6 ± 6.3</td>
</tr>
<tr>
<td>B8</td>
<td>72.3 ± 1.3</td>
</tr>
<tr>
<td>B9</td>
<td>70.5 ± 3.8</td>
</tr>
<tr>
<td>B10</td>
<td>72.9 ± 2.9</td>
</tr>
<tr>
<td>B11</td>
<td>72.6 ± 3.4</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>53.1 ± 2.0</td>
</tr>
<tr>
<td>kolLhcb3</td>
<td>27.0 ± 1.3</td>
</tr>
<tr>
<td>koCP24</td>
<td>19.1 ± 1.8</td>
</tr>
<tr>
<td>koCP26</td>
<td>38.3 ± 1.3</td>
</tr>
</tbody>
</table>

(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 kolLhcb3 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).

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 LHCCI 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 (Yakushevskva et al., 2003). However, our results show that a complex consisting of a monomeric core, CP26 and one LHCCI 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 protein in mediating the association of trimer M with the PSII supercomplex. The reduction of Lhcb3 in koCP24 supports this hypothesis, as well as the model (Figure 7) suggesting that CP24 cannot transfer excitation energy directly to the core, 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.
trimer M in C2M is displaced compared with its position in the gradients of koCP24 and koLhcb3. On the other hand, core, as suggested by the absence of the CS/CP26 complex in presence of the pseudo-M, is less strongly associated to the 2008). It can also be concluded that trimer M has an influence et al pK (Figure 5B) large part of PSII is present in the gradient mild solubilization (Yakushevska PSII–LHCII supercomplexes could be found even on very stabilization of the S trimer is at least as high as that of CP29. complexes in the WT shows that the effect of CP26 on the association of trimer M 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 helixes 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 (Yakushevskia 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 (Yakushevskia 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 (Yakushevskia 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; Jannson 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; Tardd 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 ppm4 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 pH (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. PsbQ 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 PsbQ and PsbB 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 C2S2M2, 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 C2S2M2 supercomplex revealed 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 (TDNA insertion in the Lhcb5 gene, SALK_014869), koCP24 (TDNA 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 μmol m\(^{-2}\) 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, 15 mM NaCl, 2 mM MgCl\(_2\) and the protease inhibitors as before. This solution was centrifuged 10 min at 14000 g and pellet resuspended 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\(_2\), BFY 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% β-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 41000 rpm. Gradients were formed directly in the tube by freezing at −80 C and thawing at 4 C in a buffer solution containing 0.008% β-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% β-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% SDS–PAGE. First 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 B4. SDS-PAGE 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% SDS–PAGE. First 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 B4. SDS-PAGE 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% SDS–PAGE. First 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 B4.

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 Phillips CM120 electron microscope equipped with a LaB\(_6\) filament operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at either 80000 x (results shown in Figure 1) or 130000 x (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 GRIFFON 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 3σ criterion (Vanheel, 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 1RW7, respectively) were displayed using Pymol software _DeLano Scientific_, San Carlos, CA, USA). Truncated version and 2D projection map of LHCCI at 10 Å resolution was generated using routines from the EMAN package (Ludtke et al., 1999). For CP24 (Figures 4B and 7), the LHCCI monomeric structure depleted in the last 20 Å at the C-terminal was used, according to the sequence difference between these two antennas.

**Oxygen evolution**

O$_2$ production was measured in a Clark-type oxygen electrode system on BYB membranes concentrated 50 µg/ml (in Chls) in 0.4 M sorbitol, 15 mM NaCl, 5 mM MgCl$_2$, 10 mM Hepes KOH pH 7.5 at room temperature using 180 µmol m$^{-2}$ s$^{-1}$ white light. O$_2$ 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 CaCl$_2$ did not change the O$_2$ evolution both in membranes and supercomplexes.

## References


Betterle N, Bottalotti M, Zorzac S, de Bianchi S, Cazzaniga S, Dall’osto L, Morosinotto T, Bassi R (2009) Nearest-neighbor and dimeric photosystem II complexes from spinach and their relevance to the organisation of photosystem II. Currents in dimeric PSII complexes from spinach and their relevance to the organisation of photosystem II. _Z Naturforsch C_ 64C: 297–309


**Supplementary data**

Supplementary data are available at _The EMBO Journal_ Online (http://www.embojournal.org).

**Acknowledgements**

The authors thank Francesca Passarini for the generous gift of the seeds koCP24 and Luca Dall’Osto and Roberto Bassi for that of koCP26. This work is supported by the Council for Earth and Life sciences of the Nederlandse Organisatie voor Wetenschappelijk Onderzoek through a VIDI grant to RC. SC acknowledges support (visitor grant) from the ‘Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO)’ and the Partenariats Hubert Curien (Van Gogh project).

**Conflict of interest**

The authors declare that they have no conflict of interest.


