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Structural variability of plant photosystem II megacomplexes in thylakoid membranes

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SUMMARY

Plant photosystem II (PSII) is organized into large supercomplexes with variable levels of membrane-bound light-harvesting proteins (LHCIIs). The largest stable form of the PSII supercomplex involves four LHCII trimers, which are specifically connected to the PSII core dimer via monomeric antenna proteins. The PSII supercomplexes can further interact in the thylakoid membrane, forming PSII megacomplexes. So far, only megacomplexes consisting of two PSII supercomplexes associated in parallel have been observed. Here we show that the forms of PSII megacomplexes can be much more variable. We performed single particle electron microscopy (EM) analysis of PSII megacomplexes isolated from *Arabidopsis thaliana* using clear-native polyacrylamide gel electrophoresis. Extensive image analysis of a large data set revealed that besides the known PSII megacomplexes, there are distinct groups of megacomplexes with non-parallel association of supercomplexes. In some of them, we have found additional LHCII trimers, which appear to stabilize the non-parallel assemblies. We also performed EM analysis of the PSII supercomplexes on the level of whole grana membranes and successfully identified several types of megacomplexes, including those with non-parallel supercomplexes, which strongly supports their natural origin. Our data demonstrate a remarkable ability of plant PSII to form various larger assemblies, which may control photochemical usage of absorbed light energy in plants in a changing environment.

Keywords: clear native polyacrylamide electrophoresis, *Arabidopsis thaliana*, photosystem II, megacomplex, single particle electron microscopy, grana membrane.

INTRODUCTION

Photosystem II (PSII) is one of the key protein complexes involved in light reactions of photosynthesis. It is embedded in thylakoid membranes of cyanobacteria, algae and higher plants, where it uses captured light energy for splitting water molecules. In cooperation with other protein complexes such as photosystem I (PSI) and cytochrome b_6f complex, it participates in the production of energetically rich molecules of ATP and NADPH, which drive reactions of CO_2 assimilation.

Plant PSII consists of a dimeric core complex (C_2) and a variable number of light-harvesting proteins (Lhcb1–Lhcb6), which form light-harvesting complex II (LHCII). The major part of the plant LHCII is represented by LHCII trimers, which consist of three Lhcb proteins (Lhcb1–Lhcb3), and which are associated with C_2 via monomeric antenna proteins Lhcb4 (also called CP29), Lhcb5 (CP26) and Lhcb6 (CP24). According to the strength of their binding to C_2, the LHCII trimers were designated as ‘S’ and ‘M’ (strongly and moderately bound LHCII, respectively; Dekker and Boekema, 2005; Kouřil *et al.*, 2012). Occasionally, C_2 can also associate with the ‘L’ (loosely bound) trimers (Boekema *et al.*, 1999a). Single-particle electron microscopy (EM) analysis of PSII in various land plant species indicates that the $C_2S_2M_2$ supercomplex is the largest stable form of the PSII supercomplex. In this supercomplex, C_2 associates with four LHCII trimers: two of them are strongly bound (S trimers) at the side of Lhcb5, and two of them are moderately bound (M trimers) via Lhcb4 and Lhcb6 (Boekema *et al.*, 1995; Caffarri *et al.*, 2009). A recent finding has revealed that the composition and architecture of the $C_2S_2M_2$ supercomplex is not conserved through all land plant species. Two land plant groups lack Lhcb3

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(a constituent of the M trimer) and Lhcb6 proteins: the pine family (Pinaceae) and Gnetales. Apart from as yet unspecified physiological consequences, the absence of these proteins results in a structural modification of the $C_2S_2M_2$ supercomplex. This modified supercomplex is unique among land plants (Kouřil *et al.*, 2016) and resembles its counterpart in green alga *Chlamydomonas reinhardtii* (Tokutsu *et al.*, 2012; Drop *et al.*, 2014).

Despite progress in the specification of the positions of Lhcb proteins in PSII supercomplexes, there are still some Lhcb proteins with unclear localization. Biochemical analysis indicates that in the thylakoid membrane, up to eight LHCII trimers can be present per C_2 (Peter and Thornber, 1991; van Oort *et al.*, 2010; Kouřil *et al.*, 2013); however, the binding capacity of C_2 is limited to six LHCII trimers (including the L trimers). The remaining LHCIIs have so far been considered to be 'free' in the thylakoid membrane.

Besides a demand for the improvement of structural information about the PSII supercomplexes, the investigation of their organization in thylakoid membranes is also highly relevant. Considering that the excitation energy transfer between pigment-protein complexes strongly depends on their mutual distances, the interactions and connectivity between adjacent PSII complexes in the thylakoid membrane are very important for the regulation and optimization of their photochemical yield (e.g. van Oort *et al.*, 2010; Amarnath *et al.*, 2016). Most of the EM studies suggest that the organization of PSII supercomplexes in the thylakoid membrane is random (Dekker and Boekema, 2005; Kouřil *et al.*, 2012); however, in some cases a preference for a parallel association of PSII supercomplexes into megacomplexes was observed both on the level of isolated protein complexes (see Dekker and Boekema, 2005) and in isolated grana membranes (Kirchhoff *et al.*, 2008). The mutual interaction between two parallel PSII supercomplexes involves C_2, the M trimers, and the minor antenna proteins Lhcb5 and Lhcb6. S trimers and the Lhcb4 protein were also shown to be able to mediate the interaction between supercomplexes, but only in the case of the smaller C_2S_2 supercomplexes (Boekema *et al.*, 1999a,b; Yakushevska *et al.*, 2001a). The megacomplexes can further associate into various semi-crystalline arrays, which have been often observed in grana thylakoid membranes (Boekema *et al.*, 1999a,b, 2000; Yakushevska *et al.*, 2001a,b; Kirchhoff *et al.*, 2007; Daum *et al.*, 2010; Kouřil *et al.*, 2013). A mechanism controlling the formation of the megacomplexes and semi-crystalline arrays, as well as their functional relevance, is still not fully understood; however, there is increasing evidence that these structures, in analogy with respiratory megacomplexes in mitochondria (see e.g. Dudkina *et al.*, 2010 for review), are important for the regulation and optimization of photosynthetic processes and small protein traffic (for reviews, see e.g. Kouřil *et al.*, 2012; Kirchhoff, 2013; Tietz *et al.*, 2015), and may also contribute to grana formation (Daum *et al.*, 2010).

In this work, we have revealed a remarkable ability of PSII supercomplexes from *Arabidopsis thaliana* to form variable types of megacomplexes. Apart from the known parallel association of two PSII supercomplexes, we have also found variable associations between two non-parallel PSII supercomplexes. In some megacomplexes, novel binding positions for additional LHCII trimers (including the LHCII trimers so far considered to be 'free') were revealed at the sides of the S and M trimers. Importantly, we have also found some of these megacomplexes on the level of grana membranes, which evidences their natural origin. We propose that a dynamic formation of different types of PSII megacomplexes can optimize photochemical utilization of absorbed light energy under variable environmental conditions.

RESULTS

Separation of PSII megacomplexes using CN-PAGE

The PSII supercomplexes and megacomplexes can be separated from gently solubilized thylakoid membranes by ultracentrifugation using sucrose gradient (Caffarri *et al.*, 2009) or by clear/blue-native polyacrylamide gel electrophoresis (CN/BN-PAGE; e.g. Järvi *et al.*, 2011). The advantage of CN/BN-PAGE is that it provides well-focused protein zones. In order to preserve the integrity and to maximize the yield of PSII megacomplexes, a mild detergent such as *n*-dodecyl α-D-maltoside is often used. We solubilized thylakoid membranes from *A. thaliana* leaves using this detergent and modified the gradient of the resolving gel in order to achieve the optimal resolution of pigment-protein complexes of the highest molecular weight. Figure 1(a) shows that a combination of these approaches ensured a clear separation of PSII- and PSI-containing supercomplexes and PSII megacomplexes at the expense of the small protein complexes/proteins, such as trimeric or monomeric LHCII (see the band at the bottom part of the gel).

To clarify the band assignment, we measured chlorophyll fluorescence from the whole gel at room temperature (22 °C) using a gel imager (Figure 1a). As the quantum yield of PSII fluorescence at room temperature is much higher than the quantum yield of PSI fluorescence, this measurement enabled us to identify both types of photosystems. Using this approach, PSI supercomplexes (PSI core with LHCI) were identified in a relatively dense band with undetectable fluorescence (see Figure 1a). In native electrophoresis of pigment-protein complexes from thylakoid membranes (BN-PAGE and CN-PAGE), the PSII core dimer migrates close to the PSI supercomplex because it has similar molecular weight (e.g. Lípová *et al.*, 2010; Järvi *et al.*, 2011). In our gel, the PSII core dimer is represented

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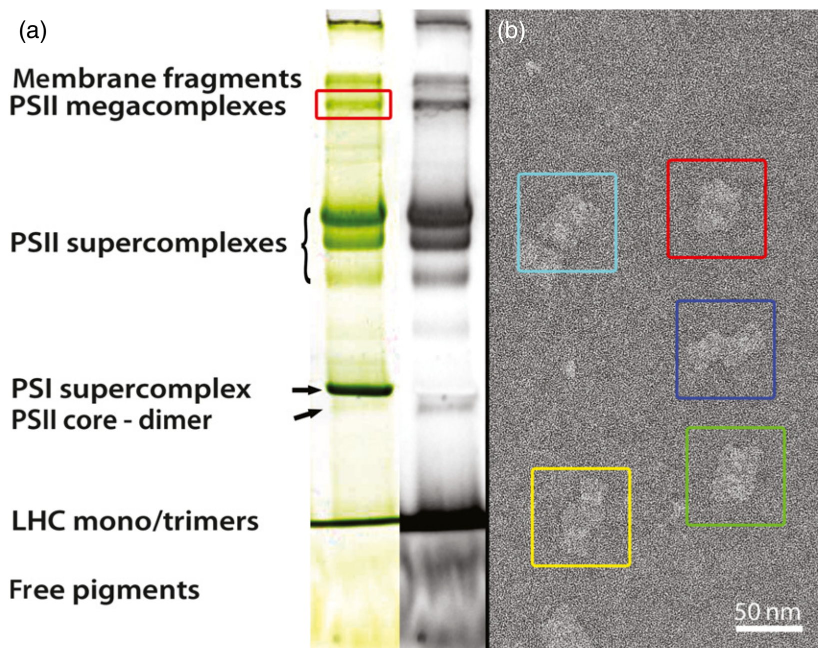

Figure 1. Separation and imaging of *Arabidopsis thaliana* pigment-protein complexes. (a) Clear-native polyacrylamide gel electrophoresis (CN-PAGE) separation of pigment-protein complexes from thylakoid membranes solubilized by *n*-dodecyl α-D-maltoside. The red frame indicates the band with megacomplexes subjected to elution and subsequent single-particle electron microscopy analysis. The black and white image represents the chlorophyll fluorescence emission detected from the same gel. The fluorescence signal was detected through a bandpass filter (690–720 nm); the excitation wavelength was 460 nm. (b) Part of the electron micrograph of a negatively stained specimen with photosystem II (PSII) megacomplexes. The colour frames highlight different forms of PSII megacomplexes.

by a very faint green band, which can be observed just below the PSI supercomplex band and which has high chlorophyll fluorescence yield. The fluorescence imaging of the gel further revealed that the green bands above the PSI supercomplex band are highly fluorescent, i.e. that they contain PSII. Based on the analogy with many papers dealing with native electrophoresis of chlorophyll-containing proteins from thylakoids (e.g. Järvi *et al.*, 2011; Albanese *et al.*, 2016), we designated the group of bands above the PSI supercomplex band as PSII supercomplexes and PSII megacomplexes.

It is clearly visible that the yield of isolated PSII megacomplexes is much smaller compared with the yield of supercomplexes. The lower yields of PSII megacomplexes can be caused either by lower stability during the isolation procedure (both solubilization and separation by CN-PAGE) or by a lower abundance in the thylakoid membrane. In order to characterize the structure and the composition of the separated megacomplexes, we excised the corresponding green band from the gel, extracted the pigment-protein megacomplexes by spontaneous elution and performed their detailed structural characterization by single-particle EM and image analysis.

PSII megacomplexes with specifically associated supercomplexes

Figure 1(b) shows an electron micrograph of a negatively stained specimen, where several PSII megacomplexes of different shape can be distinguished. Image processing of a large number of projections (about 50 000) selected from almost 12 000 micrographs revealed the presence of 13 different types of megacomplexes. Each megacomplex consisted of two PSII supercomplexes. Based on the mutual position of individual PSII supercomplexes, the PSII megacomplexes could be divided into two groups. Whereas the first group represents the majority of megacomplexes (about 80% of the data set), with the PSII supercomplexes associated in parallel (Figure 2a–f), the second group (about 20% of the data set) represents PSII supercomplexes interacting in a non-parallel manner (Figure 2g–m). In order to reveal the architecture of individual megacomplexes in detail, the EM projection maps were fitted with the pseudo-atomic X-ray model of the PSII supercomplex (Caffarri *et al.*, 2009). It is obvious that most of the megacomplexes are formed by two copies of the complete $C_2S_2M_2$ supercomplex (Figure 3), with the exception of one megacomplex that lacks one M trimer (Figure 3l). Interestingly, the detailed image analysis revealed the presence of additional LHCII trimers in some of the megacomplexes (Figure 3e,f,g,k). These LHCII trimers are not regular constituents of PSII supercomplexes and so far have been assumed to be ‘free’ in the thylakoid membrane. Our results indicate that these trimers can interact with PSII supercomplexes at as yet uncharacterized binding sites.

Electron microscopy of grana membranes

In order to investigate the physiological relevance of the PSII megacomplexes separated using CN-PAGE, we also searched for megacomplexes on the level of isolated grana membranes. Figure 4(a) shows an example of an electron micrograph of the grana membrane with resolved densities of PSII complexes. Projections of individual PSII complexes were selected and processed by image analysis. If there are any specific interactions between some of these

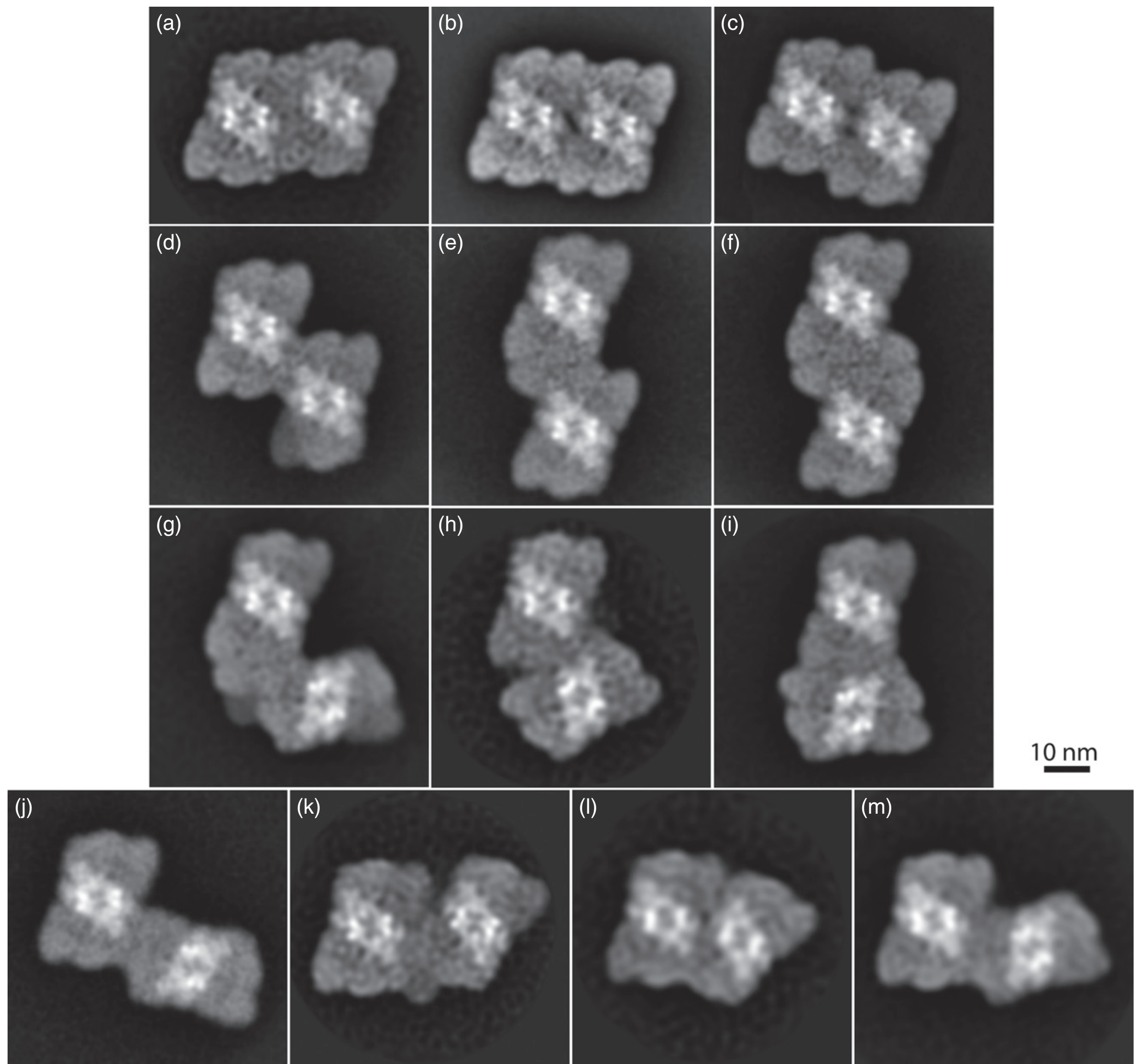

Figure 2. Structural characterization of photosystem II (PSII) megacomplexes: (a–f) represent the megacomplexes with parallel orientation of PSII supercomplexes, whereas (g–m) represent the megacomplexes formed by two supercomplexes associated in non-parallel manner. The total sums of particles contributing to the final images: (a) 1637, 4%; (b) 8411, 22%; (c) 16 928, 45%; (d) 2105, 6%; (e) 378, 1%; (f) 779, 2%; (g) 1640, 4%; (h) 418, 1%; (i) 2789, 7%; (j) 506, 1%; (k) 582, 2%; (l) 488, 1%; (m) 1082 (3%). The percentages indicate the relative abundance of the particular form of PSII megacomplex.

neighbouring PSII complexes in the grana membrane, they should be revealed as distinct classes after image processing. Indeed, image analysis revealed five specific classes with resolved densities of pairs of PSII core complexes (Figure 4b–f). Based on their mutual distance and orientation, we were able to link these pairs to the corresponding class averages of PSII megacomplexes separated using CN-PAGE (Figure 4g–k). Using this approach, the PSII megacomplexes with both parallel and non-parallel association of PSII supercomplexes were identified in the granal thylakoid membrane, with the parallel associations being about two times more abundant than the non-parallel associations. This result provides evidence that the PSII megacomplexes separated using CN-PAGE represent native PSII structures appearing in thylakoid membranes.

DISCUSSION

Structural studies of plant PSII revealed its remarkable ability to form variable types of PSII supercomplexes, consisting of PSII core and Lhcb proteins. Moreover, the proximity of PSII supercomplexes in the grana membrane enables the formation of larger assemblies, i.e. PSII

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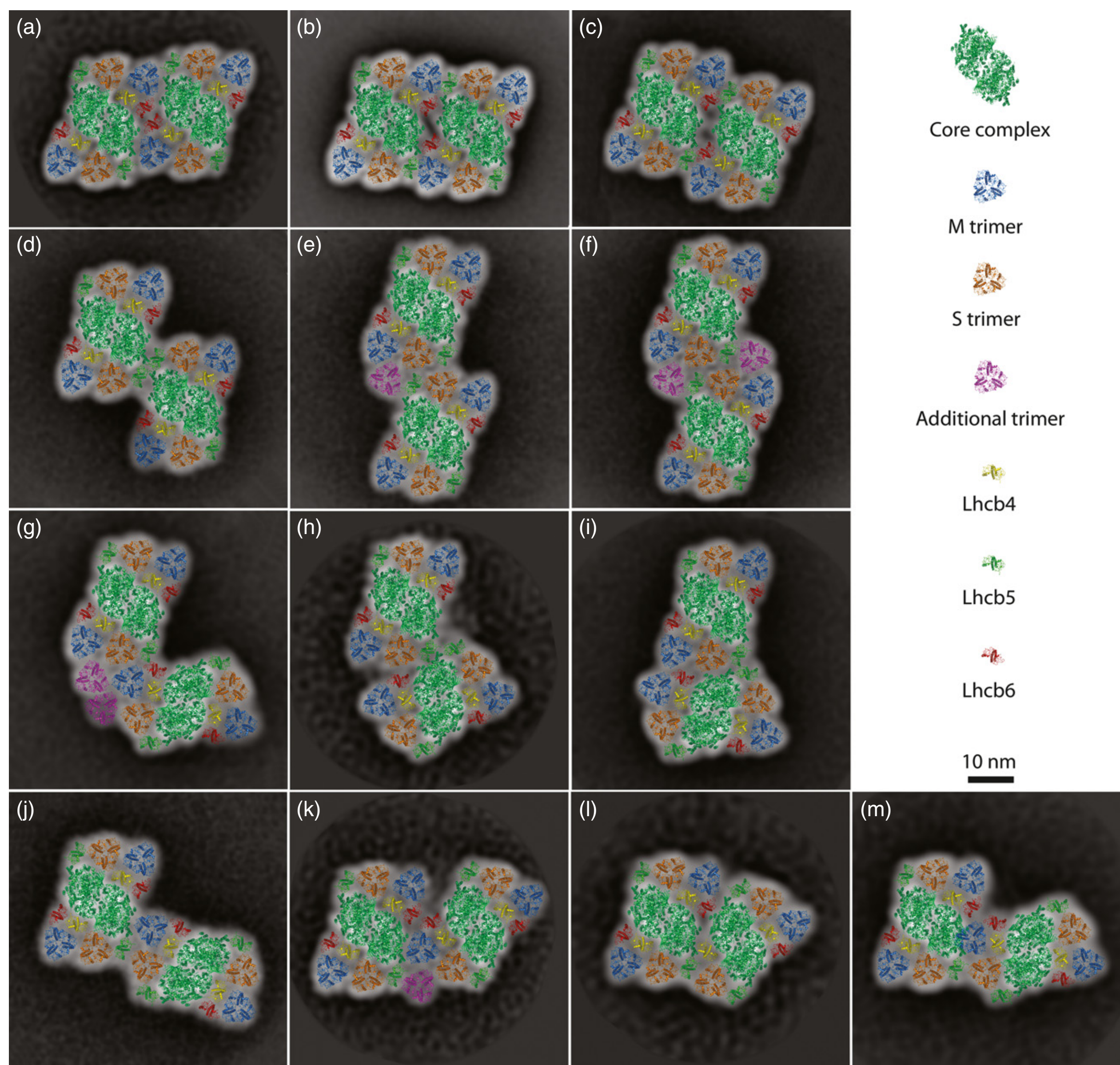

Figure 3. Structural models of the PSII megacomplexes shown in Figure 2. (a–m) Photosystem II (PSII) megacomplexes fitted with the proposed PSII crystalline structure, as published by Caffarri *et al.* (2009). Individual PSII subunits are color-coded in the following manner: pale green, core complex; blue, M trimer; orange, S trimer; magenta, additional LHCII trimers; yellow, Lhcb4; green, Lhcb5; red, Lhcb6.

megacomplexes or even structures of higher order (see Dekker and Boekema, 2005; Kouřil *et al.*, 2012 for reviews). Assembly/disassembly of PSII supercomplexes or megacomplexes modulates the antenna size of PSII, which was found to have an influence on the overall photochemical yield (e.g. Amarnath *et al.*, 2016). These changes of higher PSII organization can represent one of the responses of plants to dynamic changes of environmental conditions, such as light intensity (Ballottari *et al.*, 2007; Kouřil *et al.*, 2013). A recent theoretical study indicates that the excitation can move diffusively through the antenna proteins within a radius of about 50 nm until it reaches the reaction center (Amarnath *et al.*, 2016). As the dimensions of the PSII supercomplex $C_2S_2M_2$ are 20 nm × 33 nm, the excitation can thus be shared within the whole megacomplex formed by two supercomplexes.

Our structural analysis of PSII megacomplexes separated using CN-PAGE revealed that a majority of them is formed by the parallel association of two PSII supercomplexes (Figures 2a–f and 3a–f). The reason for their abundance can be their higher structural stability when compared with the megacomplexes formed by the non-parallel association of PSII supercomplexes. Alternatively, it could reflect the fact that the megacomplexes with PSII

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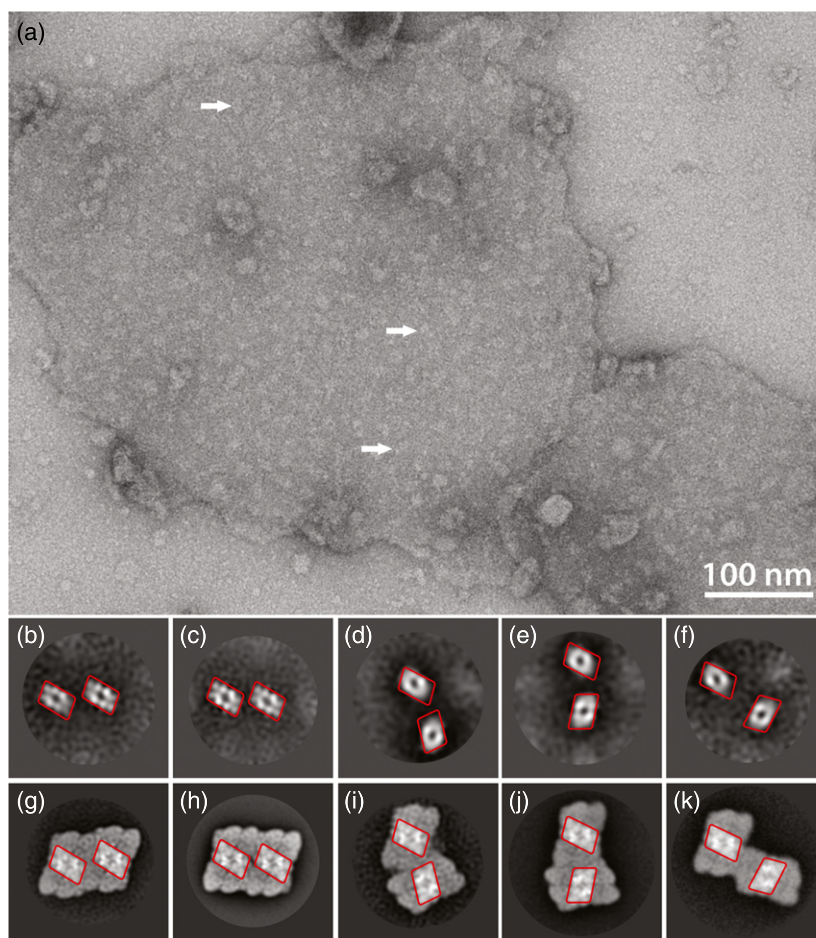

Figure 4. Photosystem II (PSII) megacomplexes found within an intact thylakoid membrane. (a) An example of an electron micrograph of negatively stained thylakoid membrane isolated from *Arabidopsis thaliana*, with densities corresponding to the PSII core complex indicated by white arrows. (b–f) PSII megacomplexes found within the thylakoid membrane (the number of summed projections was 1838, 2620, 940, 682 and 825, respectively). (g–k) PSII megacomplex analogs found in the sample separated by clear-native polyacrylamide gel electrophoresis (CN-PAGE). (g–k) Megacomplexes (a), (b), (h), (i) and (j), respectively, from Figure 2. The red frames surround core complexes of individual PSII supercomplexes and highlight that the megacomplexes found in the thylakoid membrane match with those obtained using CN-PAGE.

supercomplexes associated in parallel originate from solubilized semi-crystalline arrays, which appear occasionally in grana membranes (Boekema *et al.*, 1999a,b, 2000; Yakushevska *et al.*, 2001a,b; Kirchhoff *et al.*, 2007; Daum *et al.*, 2010; Kouřil *et al.*, 2013).

In the most abundant megacomplexes, PSII supercomplexes interact in parallel via core complexes, M trimers, and Lhcb5 and Lhcb6 proteins (Figure 3a–c). Obviously, the involvement of all these components in the interaction increases the overall stability of megacomplexes, resulting in their relatively high abundance; however, it seems that the interaction between Lhcb5 and the core complex alone is strong enough for the formation of the ‘parallel’ PSII megacomplex (Figure 3d). Moreover, novel types of PSII megacomplexes that consist of the parallel supercomplexes and additional LHCII trimers were revealed (Figure 3e,f). The additional LHCII trimers seem to be indispensable for the stability of these megacomplexes, as no analogous PSII megacomplexes lacking these additional trimers were detected.

In addition to the parallel association of the PSII supercomplexes into megacomplexes, the PSII megacomplexes with non-parallel orientation of supercomplexes were detected for the first time (Figure 3g–m). As in the previous case, the supercomplex interactions within these megacomplexes are mediated by core complexes, S and M trimers, Lhcb5 and Lhcb6 proteins, and additional LHCII trimers, although not all components are always involved in the megacomplex formation. As a result of the asymmetric structure, these megacomplexes lack the possibility to form an arrangement similar to two-dimensional crystals.

Another interesting question that can be at least partially answered by our structural study is which subunits are, in general, essential for the formation of PSII megacomplexes. Their identification will help to understand a regulatory mechanism controlling the formation and dissociation of these megacomplexes. We propose that the contribution of Lhcb5 to PSII megacomplex formation is the most significant, as it participates to some extent in the formation of all types of PSII megacomplexes, even in those where Lhcb6 and the M trimer are not involved (Figure 3d–f).

In the grana membrane, most of the PSII supercomplexes seem to be randomly organized (Figure 4; see also Kouřil *et al.*, 2013); however, the observed variability in the architecture of the PSII megacomplexes separated using

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CN-PAGE indicates that what originally looked like complete randomness can at least partially be explained by the abundance of specific megacomplex forms. Image analysis of PSII supercomplexes within the grana membrane revealed specific associations of PSII supercomplexes (both parallel and non-parallel interactions), which nicely corresponds with the structures of PSII megacomplexes isolated using CN-PAGE (Figure 4). In light of these results, we realize that the positions of interacting PSII supercomplexes that we observed previously in the cryo-tomogram of the grana membranes (Kouřil *et al*., 2011) do not have to be random, but can indeed be specific.

Taken together, the two sets of characterized PSII megacomplexes (with parallel and non-parallel arrangements of PSII supercomplexes) indicate that there are more LHCII trimers bound in specific positions to PSII than has been considered previously (Dekker and Boekema, 2005). This fact reduces the pool of 'free' LHCII trimers and supports the idea of a more defined packing of all PSII-related components in the grana membrane. The packing of PSII supercomplexes with 'free' LHCII trimers can be important for the regulation of effective PSII antenna size. The dynamic formation/disintegration of PSII megacomplexes can efficiently manage the utilization of absorbed light energy by PSII supercomplexes, as it enables the contact points between PSII reaction centers and adjacent antenna proteins to change. Nevertheless, the physiological significance and potential benefit of the formation of PSII megacomplexes under varying environmental conditions remains to be elucidated.

EXPERIMENTAL PROCEDURES

Plant material and sample preparation

Arabidopsis thaliana plants were grown in a growth chamber at 21°C, with an 8-h light/16-h dark photoperiod, at an irradiance of 100 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (400–700 nm). Thylakoid membranes were isolated from 8-week-old plants using the protocol described by Dau *et al.* (1995). The chlorophyll content in the final thylakoid membrane suspension was determined by a pigment extraction into 80% acetone (Lichtenthaler, 1987). Thylakoid membranes with 10 μg of chlorophylls were solubilized with *n*-dodecyl α-D-maltoside using a detergent: chlorophyll mass ratio of 20, and supplemented with sample buffer (50 mM HEPES, pH 7.2, 400 mM sucrose, 5 mM $MgCl_2$, 15 mM NaCl, 10% glycerol) to a final volume of 30 μl. Non-solubilized membranes were removed by a short centrifugation (22 000 ***g***, 4°C). After centrifugation, the supernatant was immediately loaded onto a polyacrylamide gel with 4–8% gradient resolving gel and 4% stacking gel (Wittig *et al*., 2007). The electrophoretic separation was conducted in a Bio-Rad Mini protean tetra cell system (Bio-Rad, http://www.bio-rad.com), starting with a constant current of 4 mA for 15 min and then continuing with a constant current of 7 mA until the front reached the bottom of the resolving gel. The CN-PAGE gel was analyzed using a gel scanner Amersham Imager 600RGB (GE HealthCare Life Sciences, http://www.gelifesciences.com). To visualize all the bands, the gel was scanned in transmission mode using white light illumination. The black and white image of the same gel was acquired in fluorescent mode to identify PSI- and PSII-containing bands. The excitation wavelength was 460 nm and the fluorescence signal was detected through a bandpass filter (690–720 nm). Subsequent elution of protein complexes from the gel and the preparation of the specimen for EM analysis was performed according to the procedure described by Kouřil *et al.* (2014).

Grana membranes were obtained by the solubilization of thylakoid membranes using digitonin (0.5 mg of chlorophylls per ml, 0.5% digitonin in buffer; 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$). Incubation (20 min at 4°C while slowly stirred) was followed by centrifugation in an Eppendorf table centrifuge (5 min, 12 000 ***g***, 4°C). The pellet with the non-solubilized grana thylakoid membranes was used for EM analysis.

Electron microscopy and image processing

Electron microscopy was performed on a Tecnai G2 20 Twin electron microscope (FEI, http://www.fei.com) equipped with a LaB_6 cathode, operated at 200 kV. Images were recorded with an UltraScan 4000 UHS CCD camera (Gatan, http://www.gatan.com), either at 130 000× magnification (in the case of isolated PSII megacomplexes) or at 80 000× magnification (in the case of grana membranes), with a pixel size of 0.224 and 0.375 nm, respectively, at the specimen level after binning the images to 2048 × 2048 pixels. GRACE (Oostergetel *et al*., 1998) was used for the semi-automated acquisition of about 12 000 images, from which a data set of about 50 000 single-particle projections of PSII megacomplexes separated by CN-PAGE was obtained. Single-particle image analysis (see e.g. Boekema *et al*., 2009) was performed using GRIP and RELION (Scheres, 2012). Image analysis revealed that about 75% of the projections from the data set could be assigned to one of the distinct classes. The remaining 25% of the data set represented projections of the PSI-NDH supercomplex (Kouřil *et al*., 2014), which co-migrated with the PSII megacomplexes during CN-PAGE separation, and projections of other unassigned particles. In the case of grana membranes, about 800 images were recorded and about 20 000 projections of PSII particles were manually selected. Image analysis using RELION revealed that about 35% of the projections from the data set could be resolved into five specific classes, for which we were able to reliably determine the mutual orientation of the PSII core complexes. The remaining 65% of the projections represented classes where the orientation could not be determined, either because of a low signal to noise ratio (i.e. a small number of particles) or because of non-specific interactions between the adjacent PSII complexes.

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