

Significance of molecular crowding in grana membranes of higher plants for light harvesting by photosystem II

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Abstract Significance of molecular crowding in grana thylakoids of higher plants on photosystem II function was studied by ‘titrating’ the naturally high protein density by fusing unilamellar liposomes of the native lipid mixture with isolated grana membranes (BBY). The incorporation of lipids was monitored by equilibrium density gradient centrifugation and two-dimensional thin layer chromatography. The excitonic coupling between light-harvesting (LHC) II and photosystem (PS) II was analysed by chlorophyll *a* fluorescence spectroscopy. The fluorescence parameters Fv/Fm and Fo clearly depend on the protein density indicating the importance of molecular crowding for establishing an efficient excitonic protein network. In addition the strong dependency of Fo on the protein density reveals weak interactions between LHCII complexes which could be important for dynamic adjustment of the photosynthetic apparatus in higher plants.

Keywords Grana membranes · Lipids · Liposome · Molecular crowding · PSII

Abbreviations

Chl	Chlorophyll
DGDG	Digalactosyldiacylglycerol
Fo	Prompt chlorophyll α fluorescence level (QA oxidised)

Fm	Maximal chlorophyll α fluorescence level (QA reduced)
Fv	Variable chlorophyll α fluorescence level HII, hexagonal phase II of MGDG
L	Lipid
LHC	Light harvesting complex
LUV	Large unilamellar vesicle
MGDG	Monogalactosyldiacylglycerol
PG	Phosphatidylglycerol
PS	Photosystem
SQDG	Sulfoquinovosyldiacylglycerol
TLC	Thin layer chromatography

Introduction

Under optimal conditions the photosynthetic machinery of vascular plants localised in the thylakoid membrane of chloroplasts transforms light energy into ATP and NADPH with a very high efficiency (Blankenship 2002). This is mainly realised by coupling of photochemical traps in PSI and PSII with an extended light harvesting network. The antenna system of PSII is subdivided into minor light harvesting complexes II and major LHCII (Jansson 1999). In grana thylakoids PSII is associated with three minor (monomeric) LHCII and about four trimeric major LHCII leading to a functional antenna size of about 230 chlorophylls (Hankamer et al. 1997). Furthermore, several PSII units are excitonically coupled probably by a network of many trimeric LHCII (connectivity) which could be a strategy to maintain optimal light utilisation in low light. To keep the energy transfer efficiency high in this extended light harvesting system ultrafast migration rates of the exciton are required. Otherwise the excited state would be deactivated by competing fluorescence. High transfer rates

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are guaranteed by short separation distances and optimal orientation of neighbouring chlorophylls (Blankenship 2002). This may be one reason why grana membranes are crowded by LHCII and PSII complexes (Kirchhoff et al. 2004) because dense packing ensures an intimate contact between LHCII. On the other hand, dense packing in membranes is known to hinder lateral diffusion processes (Saxton 1989). For thylakoid membranes of sessile vascular plants, this is of high physiological relevance because dynamic responses of the photosynthetic machinery are a strategy to acclimate on changing environmental conditions. Thus, there seems to be a conflict between efficient light harvesting ensured by high protein packing in grana on the one hand and high flexibility on the other hand needed for regulation, turnover and biogenesis, which is impaired by molecular crowding. In this study, we analyse the interrelationship between molecular crowding and light harvesting by PSII. Therefore, a preparation protocol was established for a controlled “dilution” of the protein density in isolated grana thylakoids.

Material and methods

Preparation of grana core membranes

BBY membranes were isolated (according to Schiller and Dau 2000) with some modifications: The grinding buffer contained 0.4 M sucrose, 25 mM HEPES (pH 7.5), 1 mM EDTA, 15 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂ as well as freshly added BSA (2 g/l) and ascorbate (1 g/l). Triton incubation was performed in 1 M betaine, 25 mM Mes (pH 6.2), 15 mM NaCl, 10 mM MgCl₂ and 5 mM CaCl₂. For the preparation of grana cores 10% (w:w) Triton X-100 (diluted in 25 mM Mes (pH 6.2), 15 mM NaCl, 10 mM MgCl₂ and 5 mM CaCl₂) were added in a stoichiometry of 1:1 (v:v) to the thylakoid suspension (chlorophyll concentration of 4 mg/ml). After 2 min of incubation, the starch was pelleted by a 2 min, 1,800×g centrifugation step. The supernatant was spun for 20 min at 48,000×g. The pellet was washed once in cryo-buffer (1 M betaine, 25 mM Mes (pH 6.2), 15 mM NaCl, 5 mM MgCl₂ and 5 mM CaCl₂), centrifuged for another 12 min and the final pellet resuspended in 200 µl cryo-buffer. The BBY-membranes were aliquoted, shock-frozen in liquid nitrogen and stored at -20°C until use.

Preparation of large unilamellar liposomes

The lipid composition of grana membranes was derived from Duchêne and Siegenthaler (2000). Large unilamellar liposomes (LUV) consisting of this native mixture were

prepared on the basis of Webb and Green (1989). After 30 min of solvent removal under vacuum, the lipids (Avanti Polar Lipids Inc., Alabaster, AL, USA) were dispersed at 5 mg/ml in 10 mM HEPES (pH 7.6) by vigorous vortexing and 30 s in a sonication bath. Ten extrusions through a 0.2 µm SPI-Pore™ (SPI supplies, West Chester, PA, USA) filter followed by ten extrusions through a 0.1 µm filter were performed to produce unilamellar vesicles of an average diameter of ~60 nm (determined by dynamic light scattering).

Fusion of BBY membranes with LUVs

The liposomes were mixed (w:w) with BBY membranes (100 mM total chlorophyll) to give the desired lipid concentration in 10 mM HEPES (pH 7.6) and 0.3 mM MgCl₂. The incorporation was accomplished by 30 s tip-sonication with 20 W (Branson Sonic Power Company, model B-12) on ice followed by a freeze-thaw cycle. The fusion products were thawed slowly on ice to ensure a constant and maximal incorporation.

Equilibrium density step gradient ultracentrifugation

The incorporation of lipids was monitored by equilibrium density gradient centrifugation. The gradient matrix Ficoll®400 (Serva, Heidelberg, Germany) was dissolved in HEPES 10 mM (pH 7.6) with 0.3 mM MgCl₂. The gradient was underlayered as follows: 0.5 ml 10% Ficoll, 0.5 ml 20% Ficoll, 0.5 ml 30% Ficoll and 0.2 ml 40% Ficoll. After sample loading the gradient was spun for 16 h at 4°C at 225,000×g.

Determination of lipid composition and quantification of lipid: chlorophyll stoichiometry in fusion products

As the fusion sample might still contain free liposomes which would falsify the quantification these had to be removed. Therefore lipid quantification was performed with the green gradient bands harvested from the Ficoll density gradient, carefully avoiding the upper band containing unfused liposomes. The harvested bands of approximately 500 µl were mixed with 1 ml isopropanol in a 25” Sovirell glass, incubated at 90°C in a water bath for 10 min and cooled down to RT under running water. 5.25 ml of chloroform:methanol (2:1) were added. After vortexing, the addition of 1.575 ml 0.9% KCl and another vortexing, the mixture was centrifuged for 5 min at 1,300×g at 4°C. The lower non-polar phase was quantitatively harvested and its volume determined. The chlorophyll content of a 20%

aliquot was measured according to (Porra et al. 1989) after evaporating the organic solvent under N_2 gas and addition of 80% acetone. The organic solvents of the remaining non-polar phase were also evaporated under N_2 gas. The resulting lipid film was dissolved in small amounts of chloroform and quantitatively spotted in the lower left corner of a pre-coated TLC plate Adamant UV 254 (Macherey-Nagel, Düren, Germany). A mixture of monogalactosyldiacylglycerol (MGDG) and phosphatidylglycerol (PG) (2.5 nmol each) and a mixture of sulphoquinovosyldiacylglycerol (SQDG) and digalactosyldiacylglycerol (DGDG) (2.5 nmol each) were spotted next to each other in the down right corner of the plate as lipid standards for the run in the first dimension. As lipid standards for the run in the second dimension, a mixture of MGDG and DGDG and a mixture of SQDG and PG were spotted in the bottom left corner of the plate which was previously tilted at a 90° angle counterclockwise to the first dimension. The two-dimensional run was carried out according to (Christie and Dobson 1999). The developed and dried plate was dipped in a bath containing 10% copper sulphate and 7.5% phosphoric acid (Gellermann et al. 2006) and heated (170°C) for about 7 min. After scanning the plate the resulting 8-bit gray scale image was analysed with the image processing software Optimas (Media Cybernetics, Silver Spring, MD, USA). The lipid content (in mol) of a particular lipid spot was deduced by comparing its staining intensity with the staining intensity of the corresponding standard, both being corrected against the background.

Steady-state fluorescence

All fluorescence parameters were measured with a chlorophyll concentration of $3\ \mu\text{g}/\text{ml}$ in stacking buffer (10 mM Mes (pH 6.5), 40 mM KCl, 7 mM MgCl_2). For F_0 measurements, the sample was oxidised by adding $50\ \mu\text{M}$ potassium ferricyanide. After 5 min of dark adaptation the prompt fluorescence was determined. Reduction of the sample for F_{max} measurements was achieved by 1 min incubation with 10 mM sodium-dithionite. All fluorescence parameters were normalised to the relative chlorophyll content derived from measurements of the Q_y band chlorophyll absorption. Additionally an average light-leak was subtracted which was measured after 1 min incubation with $3.5\ \text{mM}$ of the fluorescence quencher juglone.

Results

Characterisation of chimeric membranes

For studying the impact of molecular crowding on the photochemical efficiency of PSII a preparation protocol for

fusing isolated BBY membranes with lipid-liposomes was established. Successful fusion of the two membranes is demonstrated by Ficoll step gradient ultracentrifugation (Fig. 1). Unfused BBY membranes run at the interface between 30% and 40% Ficoll (Fig. 1, upper photo, left tube) whereas free liposomes are localised at 0% Ficoll. Increasing the liposome/BBY ratio in the fusion batch (expressed as mol lipid/mol chlorophyll) leads to a gradual decrease in the density of the resulting chimeric membranes. This is a clear indication for the fusion of both membranes. Note that free liposomes are still visible in parallel to fused BBY membranes (in the same tube) indicating that unfused liposomes were separated by this method.

Harvested density gradient bands were used for lipid analysis by 2D-TLC (Fig. 1, bottom). The lipid content of each individual lipid class was estimated by comparing the staining intensity of a lipid spot with the staining intensity of its corresponding reference lipid which ran in parallel on the same TLC plate (see Fig. 1). The relative distribution of lipid classes deduced from this analysis is shown in Fig. 2. Despite a very small decrease in MGDG fraction and an increase in PG and SQDG, the distribution is very similar to the control BBY membranes. From this, it follows that in chimeric membranes, the lipid content is increased without significant changes in the distribution of lipid classes. Figure 3 shows the correlation between the total lipid content deduced from 2D-TLC analysis as a function of the (additional) lipid/chlorophyll ratio used in the fusion batch. To distinguish the lipid/chlorophyll ratio used in the fusion batch and the measured ratio deduced from 2D-TLC we name the first $L/\text{chl}(\text{theor})$ and the second $L/\text{chl}(\text{meas})$ (Fig. 3). The $L/\text{chl}(\text{meas})$ of about 1.3 (mol/mol) for unfused BBY membranes is significantly lower than that determined for thylakoid membranes (1.95) isolated from spinach plants grown under the same conditions (Kirchhoff et al. 2002). This is expected because the protein density in grana membranes is higher compared to whole thylakoids.

Influence of lipid addition to grana membranes on the quantum efficiency of PSII

The influence of the addition of lipids to BBY membranes on the functionality of PSII was analysed by stationary chlorophyll *a* fluorescence measurements (Fig. 4). For both the F_v/F_m ratio (maximal quantum yield for PSII photochemistry) as well as the F_0 fluorescence level, a strong dependency on the L/chl ratio is apparent. Increasing the L/chl leads to a decrease in F_v/F_m and an increase in F_0 . Note that the F_v/F_m of about 0.8 for unfused BBY is similar to what is expected for intact PSII complexes (0.83)

Fig. 1 Visualisation of the incorporation of lipids into BBY membranes. The upper photo shows Ficoll density step gradients (0–40%). L, free (unfused) liposomes; BBY, BBY grana membranes. The numbers at the top of each tube give the ratio of lipid/chlorophyll (L/chl(theo) in mol/mol) used in the fusion batch. The photos in the lower part show 2D-TLC plates. (a) 2D-TLC of lipids isolated from control membranes; (b) 2D-TLC of lipids isolated from fusion products with L/chl(theo) of 11. The numbers indicate different lipid classes: 1, MGDG; 2, DGDG; 3, SQDG; 4, PG

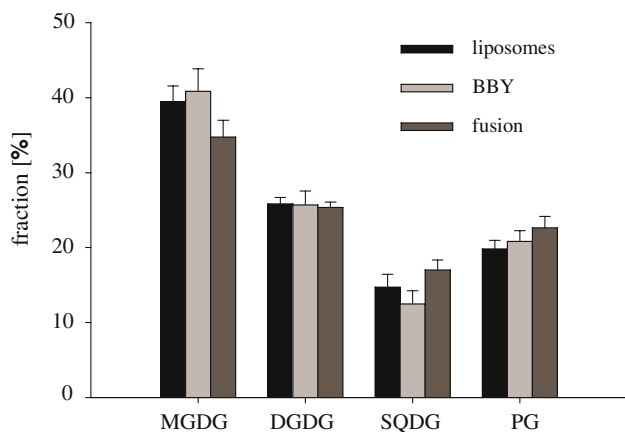
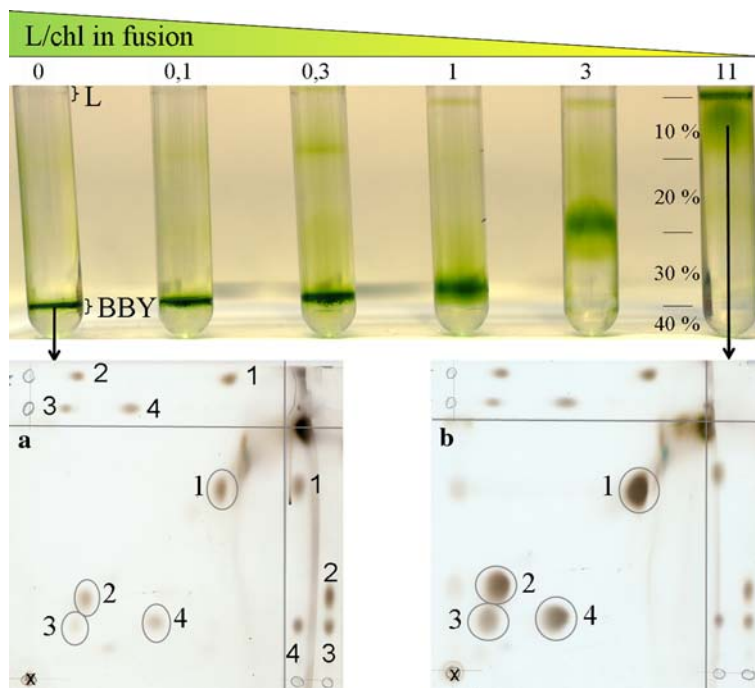


Fig. 2 Distribution of the four major lipid classes in liposomes, BBY control membranes and fusion products. The data were deduced from 2D-TLC analysis (see Fig. 1) and the mean of 2–5 determinations with standard deviation

(Krause and Weis 1991) indicating no significant change in PSII photochemistry of unfused BBY membranes potentially caused by the fusion procedure. The simplest explanation for the increase in F_0 in the fusion products is a detachment of LHCII antenna complexes from PSII. If LHCII are functionally coupled to PSII, F_0 is low due to efficient quenching by oxidised QA (photochemistry). However, if LHCII are uncoupled, their absorbed light energy cannot be trapped by PSII photochemistry leading to an increased fluorescence yield. Increase of F_0 is accompanied by a decrease in quantum efficiency of PSII photochemistry (F_v/F_m , Fig. 4). Plotting both parameters

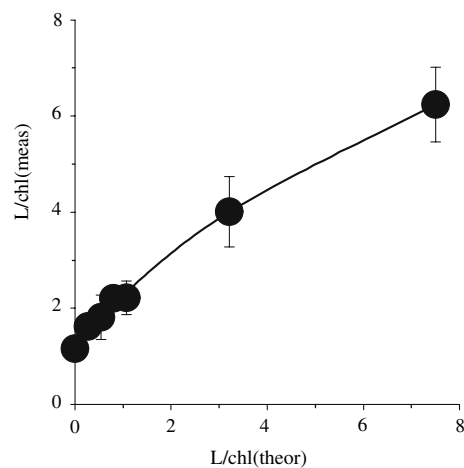


Fig. 3 L/chl(theo) plotted against the measured L/chl(meas) derived from 2D-TLC both in [mol/mol]

against each other reveals an almost linear correlation (Fig. 5). Thus, the decrease in PSII quantum efficiency in grana membranes with higher L/chl ratio could be caused by detachment of LHCII from PSII complexes.

Discussion

Chimeric membranes with a reduced protein density

Density gradient ultracentrifugation analysis gave clear evidence that the fusion of BBY membranes with

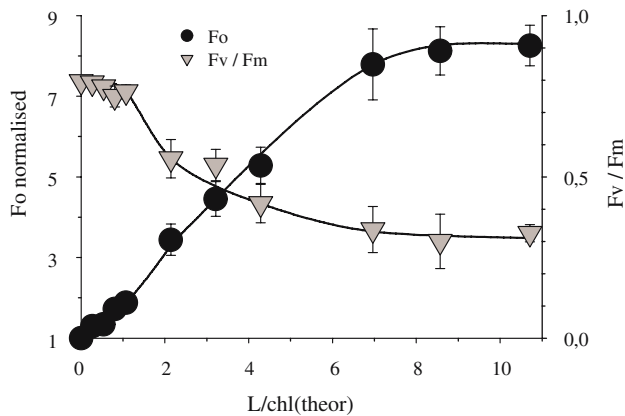


Fig. 4 Dependency of the chlorophyll *a* fluorescence parameters (F_o and F_v/F_m) on $L/\text{chl}(\text{theo})$ in chimeric membranes. Bars indicate standard deviations

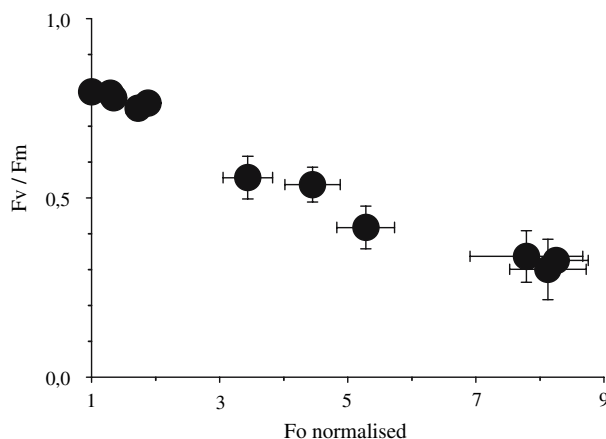


Fig. 5 Correlation between the prompt fluorescence F_o and the quantum yield of PSII photochemistry F_v/F_m . The data were taken from Fig. 4. Bars indicate standard deviations. Note the almost linear correlation between both parameters

unilamellar liposomes generated a new chimeric membrane consisting of both membranes. This is supported by the fact that in addition, functional PSII parameters (F_o , F_v/F_m) change significantly indicating an alteration in the molecular arrangement in chimeric membranes. The almost linear correlation between $L/\text{chl}(\text{theo})$ and $L/\text{chl}(\text{meas})$ (Fig. 3) indicates a high tendency for fusion of the two membranes. Interestingly, we found that the efficiency for fusing BBY membranes with phosphatidylcholine liposomes depend on fatty acid length (unpublished results). The highest efficiency was measured for PC species with C16 and C18 fatty acids which corresponds to the length of native lipids. Obviously hydrophobic matching plays a key role for efficient fusion. Furthermore, due to the fact that the size of the liposomes (about 60 nm in diameter) is much smaller than the BBY membranes (diameter of about 500 nm) the protein density can be altered in very small steps by changing the liposome/BBY ratio. This is seen in

Fig. 3. Already low $L/\text{chl}(\text{theo})$ values lead to a significant increase in $L/\text{chl}(\text{meas})$. In addition, the constancy of the lipid class distribution (Fig. 2) gives evidence that it is possible to generate membranes with higher lipid/protein ratios without significantly changing the lipid composition. Thus, the fusion procedure presented in this study is a valuable tool for manipulating the protein density in grana membranes. It has to be pointed out that freeze-fracture EM data show no HII phase formation of the lipid MGDG in our liposomes (not shown). This is in agreement with earlier studies (Webb and Green 1989) and indicates that HII phase formation can be suppressed by the addition of bilayer lipids.

Significance of molecular protein crowding on PSII function

As mentioned above the simplest explanation for the decrease in the PSII quantum efficiency for photochemistry induced by incorporating lipids into grana membranes is a detachment of LHCII complexes from PSII. This is indicative for a dispersion of a well-coupled excitonic LHCII-PSII network by lipids. Most likely addition of lipids leads to a dilution of the protein density causing a separation of LHCII and PSII and a decreased excitonic coupling. Obviously high protein packing in grana membranes is necessary to ensure a high PSII quantum efficiency. Dilution leads to a malfunction of light harvesting of PSII and points to the physiological significance of molecular crowding. On the other hand, the strong dependency of F_o on $L/\text{chl}(\text{meas})$ shows that protein interactions between LHCII and/or PSII are weak. If strong interactions are apparent, it is expected that no dispersion occurs at low $L/\text{chl}(\text{meas})$ because attractive forces would avoid dispersion. This is in contrast to our observation. A picture can be drawn in which weak interacting LHCII form an efficient excitonic network which is enabled by molecular crowding. Weak interactions could be important for regulatory adjustments, biogenesis and turnover of the photosynthetic machinery, because they require lateral protein traffic between stacked and unstacked thylakoid regions. This is an important difference to e.g. photosynthetic bacteria which seem to have a more rigid light harvesting network (Blankenship 2002). Probably a highly flexible protein organisation is necessary for dynamic acclimation in sessile land plants.

Figure 4 demonstrates that F_v/F_m decreases to a minimum of about 0.3 and not to zero. Most likely, this lower threshold value corresponds to a more stable PSII core complex which cannot be disrupted by lipid addition. Following this interpretation implies a hierarchy of protein-protein interactions in the PSII antenna system: weakly

interacting LHCII were dissociated by increasing the lipid content whereas stronger interactions withstood solubilisation.

A closer look on the dependency of Fv/Fm on the L/chl(meas) value reveals that up to a value of 1 Fv/Fm remains constantly high (Fig. 4). This gives evidence for a certain stability of the light harvesting network associated with PSII: Although additional lipids tend to disrupt this network, attractive forces withstand dispersion up to this threshold value. It is interesting to estimate the protein area fraction at this threshold value. This can be done on the basis of the following assumptions:

- (i) incorporated lipids form bilayers (no HII phases) and lead to a homogenous protein dilution.
- (ii) the mean lipid area is 0.54 nm^2 (Kirchhoff et al. 2002).
- (iii) the mean protein/chl ratio is 1.2 nm^2 (Kirchhoff et al. 2002).

The protein area fraction is then given by:

$$\frac{1.2 \text{ nm}^2 \text{ protein}}{1.2 \text{ nm}^2 \text{ protein} + 0.54 \text{ nm}^2 \text{ lipid} \times (\text{L}/\text{chl}(\text{meas}))/2}$$

L/chl(meas) was divided by 2 due to bilayer organisation. This equation predicts a protein area fraction of 78% for unfused control membranes (L/chl(meas) = 1.2, Fig. 3) and of 66% for a L/chl(meas) of 2.3. The latter value corresponds to the L/chl(theo) of 1 for which the Fv/Fm remains almost unchanged (Fig. 3). This estimation indicates that protein packing ensures efficient light harvesting by PSII in higher plant grana thylakoids only if the protein area fraction is not lower than two thirds.

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References

- Blankenship RE (2002) Molecular mechanisms of photosynthesis. Blackwell Science
- Christie WW, Dobson G (1999) Thin-layer chromatography re-visited. *Lipid Technol* 11:64–66
- Duchêne S, Siegenthaler P-A (2000) Do glycerolipids display lateral heterogeneity in the thylakoid membrane? *Lipids* 35(7): 739–744
- Gellermann GP, Appel TR, Davies P, Dieckmann S (2006) Paired helical filaments contain small amounts of cholesterol, phosphatidylcholine and sphingolipids. *Biol Chem* 387:1267–1274
- Hankamer B, Barber J, Boekema EJ (1997) Structure and membrane organization of photosystem II in green plants. *Annu Rev Plant Physiol Plant Mol Biol* 48:641–671
- Jansson S (1999) A guide to the Lhc genes and their relatives in arabidopsis. *Trends in Plant Sci* 4:236–240
- Kirchhoff H, Mukherjee U, Galla H-J (2002) Molecular architecture of the thylakoid membrane: Lipid diffusion space for plastoquinone. *Biochemistry* 41:4272–4282
- Kirchhoff H, Tremmel I, Haase W, Kubitscheck U (2004) Supramolecular photosystem II organization in grana thylakoid membranes: Evidence for a structured arrangement. *Biochemistry* 43:9204–9213
- Krause GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. *Annu Rev Plant Physiol Plant Mol Biol* 42:313–349
- Porra RJ, Thompson WA, Kriedelmann PE (1989) Determination of accurate extractions and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentrations of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975:384–394
- Saxton MJ (1989) Lateral diffusion in an archipelago. Distance dependence of the diffusion coefficient. *Biophys J* 56:615–622
- Schiller H, Dau H (2000) Preparation protocols for high-activity photosystem II membrane particles of green algae and higher plants, pH dependence of oxygen evolution and comparison of S₂-state multiline signal by X-band EPR spectroscopy. *J Photochem Photobiol* 55:138–144
- Webb MS, Green BR (1989) Permeability properties of large unilamellar vesicles of thylakoid lipids. *Biochim Biophys Acta* 984:41–49