Chapter 11

Oxygenic Photosynthesis — Light Reactions within the Frame of Thylakoid Architecture and Evolution

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Linear electron transfer chain in the thylakoid membrane of oxygenic photosynthetic organisms is rather similar from cyanobacteria to higher plants. On the contrary, the light harvesting systems and various regulation mechanisms of energy distribution and electron transfer routes show distinct evolution. Development of chlorophyll *b*-containing light harvesting systems in plants and complex regulatory networks of energy and electron transfer reactions led to the development of distinct lateral heterogeneity of the thylakoid membrane. Light-induced dynamics in lateral heterogeneity of higher plant thylakoid membrane allows fluent photosynthetic electron transfer and equal light harvesting capacity as well as efficient photoprotection of both photosystems in response to changes in the light environment. On the contrary, the fluency of electron flow in cyanobacteria thylakoid membrane is largely dependent upon a broad range of electron valves that have gradually disappeared during evolution of plant chloroplasts. After a great breakthrough in demonstration of the lateral heterogeneity of the thylakoid membrane in higher plant chloroplasts in 1980, our knowledge on light-induced dynamics of such a heterogeneity has slowly evolved in parallel with the development of isolation and characterization methods of thylakoid subdomains.

1. Introduction

Thylakoid membrane-embedded high molecular mass pigment-protein complexes photosystem I (PSI) and photosystem II (PSII) together with their light harvesting antenna systems collect the light energy and use it to drive the photosynthetic electron transfer reactions. In 1960, Hill and Bendall published their revolutionary concept of the Z-scheme demonstrating the serial trans-thylakoid electron transfer reactions, which constitute the linear electron transfer (LET) chain [Hill and Bendall, 1960]. Thereafter, our knowledge on the electron transfer reactions and their regulation has increased remarkably. The electrons originally derived from water to replace the electron hole in PSII can be directed to various routes in order to balance the function of the two photosystems in relation to each other and to eventually provide electrons to the carbon fixing and other reductive reactions. The diversity of regulation mechanisms of the photosynthetic light reactions show distinct evolution among oxygenic photosynthetic organisms, from cyanobacteria to various algal species and continuing in the land from mosses to lycophytes and gymnosperms, and eventually to the angiosperms.

2. Thylakoid Membrane Heterogeneity — From Cyanobacteria to Higher Plants

LET chain in the thylakoid membrane is composed of similar components in all oxygenic organisms; the water splitting PSII, the plastoquinone (PQ) pool, cytochrome (Cyt) b_6f complex, plastocyanin and PSI (Figure 1). Conversely, the light harvesting systems and regulation of light reactions show distinct changes during evolution of photosynthetic organisms. Cyanobacteria, the progenitors of plastids, typically use the soluble phycobilisomes (PBS) as light harvesting antennae of PSII, lack the thylakoid membrane stacking and show only modest lateral heterogeneity of the protein complexes [Sherman *et al.*, 1994]. Further, cyanobacteria mainly rely upon alternative electron transfer pathways and electron valves to protect the photosynthetic apparatus against damage upon abrupt, short-term alterations in the light environment. Among others, the flavodiiron (Flv) proteins are important players in the network of alternative electron transfer pathways of cyanobacteria. The photoprotective function of the *flv4-2 operon*, encoding the Flv2, Sll0218 and Flv4 proteins, is limited to β-cyanobacteria, whilst the Flv1 and Flv3 proteins function as an electron valve catalyzing the water–water cycle and protecting PSI under fluctuating light conditions in a much broader range of photosynthetic organisms [Allahverdiyeva *et al.*, 2013; Bersanini *et al.*, 2017; Ermakova *et al.*, 2014; Gerotto *et al.*, 2016] including also eukaryotes green algae, mosses, lycophytes and gymnosperms but, importantly, missing angiosperms.

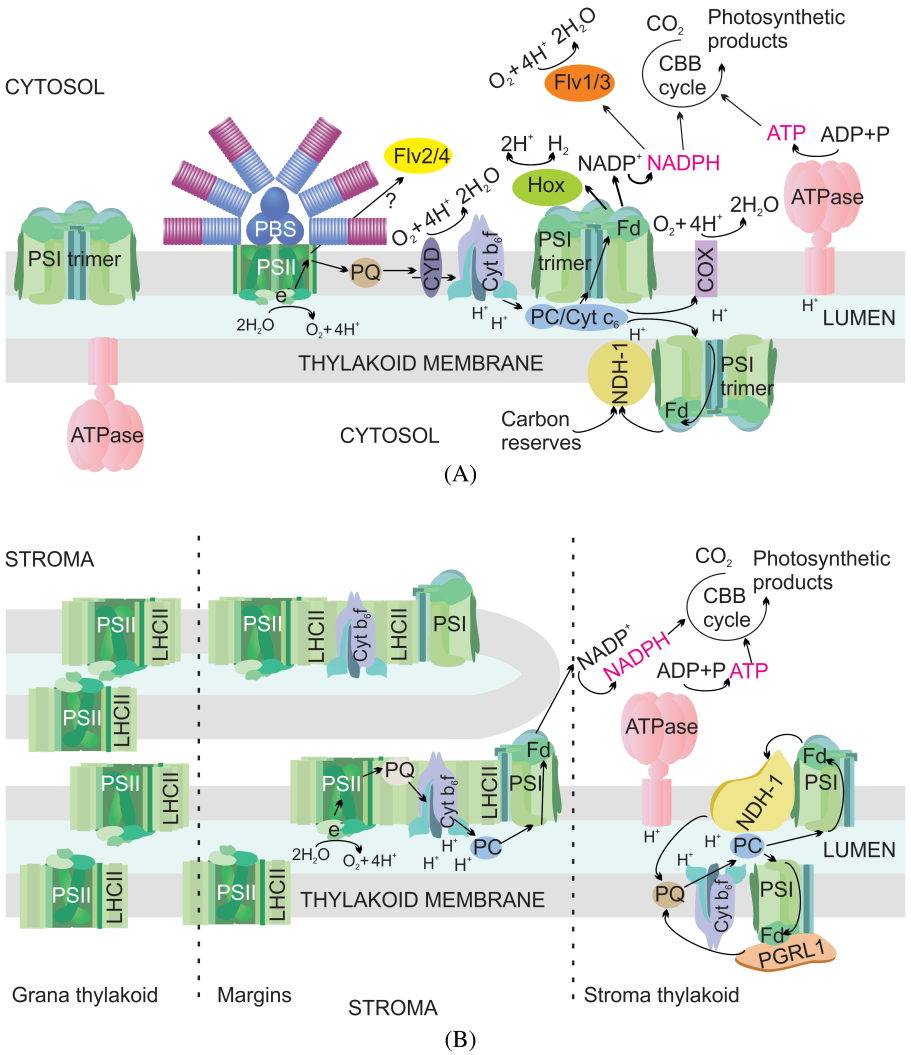

Figure 1. Schemes showing the thylakoid membrane organization, photosynthetic protein complexes, the light harvesting systems and electron transfer routes in cyanobacteria (A) and higher plants (B). While the connectivity between the photosystem PSII and PSI is minimal in cyanobacteria due to huge and soluble phycobilisome antenna (PBS), the PSII and PSI in the higher plant thylakoid membrane are in connection in the grana margin regions *via* the Chl *a*/*b* binding light-harvesting (LHC)II antenna. For details, see the text.

Evolution of the membrane-embedded chlorophyll (Chl) *a*/*b* binding light-harvesting (LHCII) antenna in green algae and land plants led to a replacement of the PBS antenna and consequently allowed the development of the lateral heterogeneity of the thylakoid membrane. The thylakoid membrane network in

higher plant chloroplasts, and to a lesser extent also in green algae and mosses, is organized into appressed grana thylakoids and non-appressed stroma thylakoids. Specific thylakoid domains — the grana margins, grana end-membranes and stroma thylakoids — are exposed to the soluble chloroplast stroma where the carbon reduction reactions (CBB cycle) take place (Figure 1B). In 1980, Andersson and Anderson published their classical paper on the lateral heterogeneity of the thylakoid membrane in spinach chloroplasts and, by using mechanical thylakoid fractionation, demonstrated an extreme heterogeneity in the distribution of PSII and PSI in the thylakoid membrane [Andersson and Anderson, 1980]. Steric hindrance of the PSI complex, generated by a large domain protruding into the stroma, was demonstrated to prevent the location of the PSI complex in the tightly appressed grana thylakoids and PSI was shown to reside predominantly in the non-appressed thylakoid domains [Andersson and Anderson, 1980]. In turn, the most active form of PSII, the PSII-LHCII supercomplexes are localized in appressed thylakoid membranes [Andersson and Anderson, 1980; Danielsson *et al.*, 2006].

Since 1980, our knowledge of the lateral heterogeneity of the thylakoid membrane has further increased. Discovery of a plethora of photosynthetic regulatory mechanisms, which are generally induced by the exposure of the photosynthetic organism to light and relaxed in darkness, have stimulated researchers to investigate their role in thylakoid heterogeneity. In-depth characterization of specific thylakoid regulatory protein mutants, together with improved thylakoid fractionation and analysis methods, has changed the view of the strict lateral heterogeneity of the thylakoid membrane. Today, it is generally agreed that the light-induced dynamics in the lateral heterogeneity of higher plant thylakoid membrane allows fluent photosynthetic electron transfer and equal light harvesting capacity as well as efficient photoprotection of both photosystems in response to changes in the light environment.

3. Key Regulatory Mechanisms of Thylakoid Electron Transfer Reactions

3.1. *No clear model organism for regulation of light reactions*

The concept of the ~~dynamic~~, light intensity- and quality-dependent ~~flexibility~~ in the lateral heterogeneity of the thylakoid membrane developed concomitantly with accumulating evidence acquired from various mechanisms involved in regulation of photosynthetic light reactions, including the distribution of excitation energy to PSII and PSI and the various electron transfer routes in the thylakoid membrane.

In higher plants lacking the Flv proteins, a strict regulation of electron flow through the Cyt b_6f complex, which occupies a central position in the LET route (Figure 1), is of crucial importance [Joliot and Johnson, 2011; Suorsa *et al.*, 2012]. Likewise, the STN7 and STN8 kinase-dependent balancing of light energy distribution between PSII and PSI plays an important role in regulation of photosynthesis in response to changes in both the intensity and quality of light [Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005; Tikkanen *et al.*, 2008b]. On the other hand, *Chlamydomonas reinhardtii* (Chlamydomonas) has demonstrated distinct variance from both the cyanobacteria and higher plant regulatory mechanisms of thylakoid electron transfer reactions, making green algae unsuitable as a model system for plant chloroplasts. As far as it comes to mechanisms adjusting the photosynthetic light reactions according to environmental cues, the green algae systems represent an intermediate form providing evidence for gradual evolution of the higher plant-type photosynthesis regulation in the thylakoid membrane, first in the water environment and continuing upon the movement of organisms from the water to the land. Upon evolution of the higher plant thylakoid network, a wide variety of regulation mechanisms has developed and many of those typical for lower oxygenic photosynthetic organisms have vanished, thus ruling out the possibility that any specific organism or group of organisms could serve as a representative model.

3.2. *Evolution of key regulatory mechanisms of thylakoid electron transfer reactions*

Our knowledge on thylakoid regulation mechanisms, and particularly on the molecular basis of such regulation, is currently only emerging. Generally, there appears to be three key issues to be considered when evaluating how to maximize the functionality of both photosystems and how to avoid unwanted damage of the photosynthetic apparatus upon changes in environmental conditions. These include (i) the maintenance of the balance between forward electron transfer and thermal dissipation of excess excitation energy, (ii) the maintenance of redox balance in the electron transfer chain and (iii) ensuring equal distribution of excitation energy to the two photosystems, PSII and PSI. Different evolutionary groups of oxygenic photosynthetic organisms have solved these regulation needs in distinct ways, which are shortly discussed below (i)–(iii).

(i) Excess excitation energy absorbed by the antenna systems, *i.e.* the energy that cannot be utilized for photochemistry, has to be safely dissipated by non-photochemial quenching (NPQ) mechanisms. In cyanobacteria, the quenching of excess excitation energy absorbed by the PBS antenna at high light intensities is

mediated by a soluble carotenoid binding protein (orange carotenoid protein, OCP) that binds to the PBS antenna and triggers the dissipation of excess excitation energy [Wilson *et al.*, 2006]. In chloroplasts of plants and green algae, the thermal dissipation of excess excitation energy is to a large extent dependent on the protonation of the PsbS and LHCSR3 proteins, respectively, triggered by low lumenal pH upon illumination [Bonente *et al.*, 2011; Li *et al.*, 2000]. These thylakoid membrane proteins are likely responsible for conformational changes of the LHCII antenna proteins, which then alter the configuration of LHCII-bound pigments resulting in a safe energy dissipation as heat. In addition, violaxanthin deepoxidase, a key enzyme of xanthophyll cycle is activated by low lumenal pH, and is required for full activation of thermal energy dissipation in chloroplasts [Niyogi *et al.*, 1997]. Despite a plethora of literature published during the past decades on various NPQ mechanisms, including the PSII photoinhibition-related energy quenching, it is likely that still uncharacterized quenching mechanisms remain to be discovered. Moreover, the exact molecular mechanisms and the full evolutionary diversity of the quenching mechanisms remains to be elucidated.

(ii) Alternative electron transfer pathways play a crucial role in relieving the excitation pressure in the electron transfer chain. In cyanobacteria, the functional roles of the *flv4-2* operon and the *flv1* and *flv3* genes were already mentioned above. In addition to the Flv proteins, the respiratory terminal oxidases Cyd and Cox in the thylakoid membrane of cyanobacteria are important in protection of PSII during abrupt changes in the light intensity [Ermakova *et al.*, 2016; Lea-Smith *et al.*, 2013]. Moreover, both NAD(P)H:quinone oxidoreductases, NDH-1 and NDH-2, are functional in cyanobacteria. Compared to cyanobacteria, green algae have less complex electron transfer pathways. For example, Cyd, Cox, the *flv4-2* operon proteins and the NDH-1 complex are completely missing from Chlamydomonas, which in turn has a Proton Gradient Regulation-Like 1 (PGRL1)-dependent PSI cyclic electron transfer (CET) [Dang *et al.*, 2014]. The Chlamydomonas genome also contains *flv1* and *flv3* homologues, the *flvA* and *flvB* genes that are highly expressed in the *pgrl1* mutant cells, providing evidence for the presence of a FLV-dependent photoprotection mechanism of PSI [Dang *et al.*, 2014]. Mosses were recently demonstrated to possess a FLV-dependent safety valve [Gerotto *et al.*, 2016], and the related genes also exist in the genomes of lycophytes and gymnosperms [Yamamoto *et al.*, 2016; Zhang *et al.*, 2009]. Flowering plants, instead, seem to completely lack the FLV-dependent electron transfer pathways and instead rely on a proton motive force-dependent down-regulation of LET, being strictly dependent on the Proton Gradient Regulation5 (PGR5) protein [Suorsa *et al.*, 2012]. Such regulation of electron flow *via* the Cyt b_6f complex is crucial for protection of PSI against fluctuating light intensity-induced damage [Munekage

et al., 2002; Suorsa *et al.*, 2012; Tiwari *et al.*, 2016]. Further, higher plant chloroplasts contain a bacterial-like NDH-1 complex interacting with PSI and playing an important role in PSI-CET [Peltier *et al.*, 2016].

(iii) Mechanisms that allow the balanced distribution of excitation energy to PSII and PSI, including the "state transitions", play a central role in short-term light-acclimation processes of photosynthetic organisms. Since the demonstration of the concept of "state transitions" in 1969 [Bonaventura and Myers, 1969; Murata, 1969], our understanding on the complexity and physiological significance of these mechanisms has greatly advanced. The underlying molecular mechanism(s) of "state transitions" in cyanobacteria has remained fairly elusive. In Chlamydomonas and higher plants, reversible phosphorylation of the LHCII antenna plays an important role in "state transitions", yet several concomitant mechanisms are indispensable for balancing the distribution of excitation energy between PSII and PSI. The homologous serine/threonine-protein kinases responsible for phosphorylation of the LHCII proteins have been identified from Chlamydomonas (Stt7) [Depége *et al.*, 2003] and from *Arabidopsis thaliana* (Arabidopsis) (STN7) [Bellafiore *et al.*, 2005], the activity of the kinases being mainly regulated by changes in the redox state of the PQ pool. Binding of plastoquinol to the Qo site of the Cyt b_6f complex activates the STN7/Stt7 serine threonine kinases [Vener *et al.*, 1997]. Reduction of the lumenal Cys residues of STN7/Stt7 is likely to inhibit the kinase activity under high light intensity through the ferredoxin-thioredoxin system [Rintamäki *et al.*, 2000]. Dephosphorylation of LHCII proteins is dependent on the Thylakoid-Associated Phosphatase of 38 ~~KDA~~/Protein Phosphatase 1 (TAP38/PPH1) [Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010]. Importantly, not only the reversible phosphorylation of the LHCII proteins but also that of the PSII core proteins is essential for balancing the excitation energy between PSII and PSI [Mekala *et al.*, 2015; Rintamäki *et al.*, 1997]. A homologous kinase to STN7/Stt7, the STN8/Stl1, is responsible for phosphorylation of the PSII core proteins and the antagonist phosphatase, the Photosystem II Core Phosphatase (PBCP), has likewise been demonstrated [Bonardi *et al.*, 2005; Samol *et al.*, 2012].

Despite apparent similarity between the higher plant and green alga LHCII and PSII core protein phosphorylation, distinct differences also exist. It has been estimated that in Chlamydomonas up to 80% of the LHCII proteins are potentially phosphorylated giving rise not only to the control of energy balance between PSII and PSI, but also to a shift between the LET and PSI CET modes in order to balance the NADPH/ATP ratio according to environmental and cellular needs [Delosme *et al.*, 1996; Finazzi *et al.*, 1999, 2002]. PSI-CET specific megacomplex

PSI-LHCI-LHCII-Cytb_6f-PGRL1 has been isolated from Chlamydomonas cells grown under PSII-favoring light, which induces the phosphorylation of both the LHCII and PSII core proteins [Iwai *et al.*, 2010]. Yet, contradictory results exist indicating that the ~~PSI CET~~, in Chlamydomonas might be independent of LHCII phosphorylation and "state transitions" [Takahashi *et al.*, 2013]. In angiosperms like Arabidopsis, the STN7 kinase-mediated phosphorylation of LHCII leads to enhanced affinity of LHCII to PSI and consequently to an improved energy transfer from LHCII to PSI, which fully depends on the presence of the LHCII docking proteins PsaH and PsaL in PSI [Lunde *et al.*, 2000]. Intriguingly, phosphorylation of the LHCII proteins also regulates the formation of a labile PSI-PSII-LHCII megacomplex in non-appressed thylakoid membranes, boosting the energy distribution between PSII and PSI [Suorsa *et al.*, 2015], although direct interaction between PSI and PSII has also been documented [Yokono *et al.*, 2015]. Lack of LHCII phosphorylation or absence of LHCII docking proteins in PSI prevent the stable PSI-PSII-LHCII megacomplex formation in Arabidopsis [Leoni *et al.*, 2013; Suorsa *et al.*, 2015]. As mentioned above, in addition to LHCII phosphorylation, the balancing of excitation energy distribution between PSII and PSI in Arabidopsis has been demonstrated to also depend on the PSII core protein phosphorylation [Mekala *et al.*, 2015]. PSII core protein phosphorylation has a distinct role in regulation of thylakoid stacking (for details, see Section 4) and influences the physiological interactions between PSII, LHCII and PSI. Importantly, under natural fluctuations of the light intensity, the opposite phosphorylation of the LHCII antenna proteins and the PSII core proteins with respect to light intensity together ensure the optimal excitation energy distribution between PSII and PSI [Mekala *et al.*, 2015].

4. Factors Regulating the Thylakoid Architecture in Oxygenic Photosynthetic Organisms

~~The degree of thylakoid stacking is mainly determined by long-term light acclimation of plants, yet the reversible light-induced and short-term changes in thylakoid architecture are today well appreciated.~~ Indeed, high growth light intensity reduces thylakoid stacking, while low growth light intensity enhances thylakoid stacking [Anderson and Aro, 1994]. Intriguingly, independent of growth light intensity, the phosphorylation of the LHCII and PSII core proteins are maintained at a similar, moderate level [Mekala *et al.*, 2015]. This is because the chloroplast acquires a redox balance when environmental conditions remain constant. Such redox-balance within the chloroplast provides an advantage to re-adjust the phosphorylation of LHCII and PSII core proteins whenever the light conditions suddenly change [Mekala *et al.*, 2015]. Strong phosphorylation of the PSII core proteins

upon sudden increase in light intensity [Mekala *et al.*, 2015; Rintamäki *et al.*, 1997] has been suggested to increase the repulsion between thylakoid layers which, together with the osmotic swelling of thylakoid lumen caused by increased chloride and calcium influxes (counterbalanced by magnesium exflux), are likely to assist the destacking and lateral shrinkage of the grana stacks [Herbstová *et al.*, 2012; Kirchhoff, 2013]. In addition to the function of the proton and ion channels and the reversible thylakoid protein phosphorylation, also other post-translational protein modifications likely ~~to~~ regulate the dynamic light-intensity-dependent alterations in thylakoid architecture.

So far, only a few proteins specifically determining the thylakoid architecture in higher plant chloroplasts have been identified. These include the oxygen evolving complex protein PsbP and the trimeric LHCII antenna proteins, which likely regulate the lumen diameter and stabilize the appressed structures of the thylakoid membrane, respectively [Wan *et al.*, 2014; Yi *et al.*, 2009]. Further, the lack of grana margin localized Curvature Thylakoid1 (CURT1) and Reduced Induction of Non-Photochemical Quencing (RIQ) 1 and 2 proteins have been shown to decrease and enhance the granal stacking, respectively [Armbruster *et al.*, 2013; Yokoyama *et al.*, 2016]. Intriguingly, neither green algae nor cyanobacterial genomes contain orthologs of the *RIQ* genes [Yokoyama *et al.*, 2016]. RIQ proteins, which regulate the granal structures likely through reorganization of the LHCII antenna, have been localized exclusively to the appressed thylakoid domains, providing evidence for co-evolution of the LHCII antenna, thylakoid stacking and RIQ proteins. In turn, the role of CURT1 is evolutionarily conserved and knocking down of the CURT1 homolog in a cyanobacterium *Synechocystis* sp. PCC6803 affects the thylakoid architecture by disrupting the formation of thylakoid biogenesis centers *e.g.* the functional sites of the PSII biogenesis [Heinz *et al.*, 2016].

5. Revealing the Dynamics of Thylakoid Architecture in Higher Plant Chloroplasts

5.1. *Methods to study thylakoid heterogeneity — Dark acclimated plants*

For detailed characterization of thylakoid heterogeneity, the thylakoid membrane has traditionally been fractionated into different subdomains by mechanical or chemical fractionation. Whenever the thylakoid membrane architecture is investigated, specific attention should be put on the fact that the thylakoid architecture, for instance the degree of grana stacking, is highly dependent upon various factors such as the concentrations of ions and the pH, and thus the fractionation protocol

used should be carefully optimized. The strict lateral heterogeneity of the thylakoid membrane, published by Andersson and Anderson in 1980, was based on mechanical fractionation of thylakoids by Yeda press from dark-acclimated spinach leaves, followed by two-phase partitioning and differential centrifugation steps. Such a thylakoid fractionation protocol results in separation of the grana core, grana thylakoid membrane, margins, stroma thylakoid membrane and stroma core (known as Y100) fractions [Andersson and Anderson, 1980; Danielsson *et al.*, 2006]. The success of fractionation is generally estimated by the Chl *a*/*b* ratios of the obtained subfractions based on the fact that Chl *a* is bound to both photosystems, but Chl *b* is mostly bound to the LHCII antenna. In grana and stroma thylakoid membrane subfractions isolated by mechanical fractionation from spinach, the Chl *a*/*b* ratios are close to 2 and 6, respectively, indicating the enrichment of components of PSII-LHCII and PSI while in intact spinach thylakoids the Chl *a*/*b* ratio is around 3 [Tikkanen *et al.*, 2008b]. In the margin subfraction, the Chl *a*/*b* ratio is similar to that of the entire thylakoids, indicating the presence of both PSI and PSII complexes in the subdomain. Noteworthy, successful separation of the thylakoid membrane subdomains has been demonstrated only after acclimation of plants to darkness or low light [Tikkanen *et al.*, 2008b], while even short high light illumination leads to substantial loss of the yield of specific thylakoid membrane subfractions (for details, see Section 5.2).

Chemical approach for thylakoid membrane fractionation is based on the use of detergents. Mild, non-ionic detergents, such as dodecyl maltoside (DM), digitonin and Triton X-100 have uncharged, hydrophilic headgroups and have been used successfully for thylakoid fractionation, particularly to isolate native and enzymatically still active protein complexes from the lipid bilayer. Further, the design of a combination of detergents and differential centrifugation steps allows the separation and isolation of distinct thylakoid subfractions. β-DM solubilizes the entire thylakoid membrane network (both the appressed and non-appressed membrane domains) but concomitantly destroys the labile hydrophopic interactions between the protein complexes (for example, the interaction of LHCII antenna with PSI). Digitonin, on the contrary, preserves the weak protein–protein interactions, but has an access only to the non-appressed PSI-rich membrane domains, while the tightly appressed thylakoid domains are left insolubilized. Triton X-100 in high-salt buffer, in turn, can be used to isolate intact grana subfractions (BBY) by breaking-down the unstacked thylakoid regions.

It should be noted that, contrary to the mechanical fractionation of the thylakoid membrane, even mild detergents remove most of the lipids from the thylakoid membrane bilayer and cause a break-down of the labile protein–protein interactions thereby destroying the *in vivo* network of the photosynthetic protein complexes. The detergent treatments, especially β-DM and to a lesser extent digitonin,

always result in a detachment of a fraction of LHCII trimers from the thylakoid membrane integrity. This is clearly shown by the 77 K fluorescence emission spectra demonstrating the uncoupled LHCII trimer as a strong fluorescence emission peak at 680 nm [Grieco *et al.*, 2015]. In sharp contrast to thylakoid fractionation with detergents, the mechanical fractionation of the thylakoid membrane never reveals the 680 nm band in 77 K fluorescence emission spectra of any of the obtained fractions [Tikkanen *et al.*, 2008b] indicating that the release of LHCII from thylakoid integrity is an artefact due to the use of detergents.

For reasons mentioned above, the detergent-free methods for isolation of membrane protein complexes have recently drawn a lot of interest. A styrene-maleic acid (SMA) copolymer treatment of membranes has been shown to be capable of isolating membrane fractions in the form of nanodiscs that contain both the membrane lipids and the protein complexes. SMA has been successfully used to isolate the PSI complexes together with up to five LHCII trimers from the appressed regions of spinach thylakoid membrane [Bell *et al.*, 2015]. The PSI-LHCII complexes enriched within these membranes are highly susceptible to detergents, and addition of 1% β-DM leads to uncoupling of the LHCII antenna from PSI, indicated by an appearance of a large fluorescence emission peak at 680 nm at 77 K.

Despite partial release of LHCII antenna from the thylakoid integrity, the detergent-based thylakoid fractionation is still a valid method and the success of fractionation is, as in the case of mechanical fractionation, generally followed by measuring the Chl *a*/*b* ratios of obtained thylakoid fractions. Typically, three distinct fractions are isolated by detergent treatments and differential centrifugations *i.e.* the grana, margin and stroma thylakoid membrane subfractions with Chl *a*/*b* ratios for Arabidopsis typically being 2.5, 3.9 and 6.2 and for pumpkin 2.3, 3.3 and 9.3, respectively, when thylakoids are isolated from dark-acclimated leaves (Table 1).

Table 1. Typically obtained Chl *a*/*b* ratios of thylakoid subfractions isolated from differentially light-treated Arabidopsis and pumpkin leaves. Arabidopsis and pumpkin thylakoids were isolated from dark-acclimated leaves (dark) or from leaves exposed to low light (LL) or high light (HL) for 2 hours. Prior to Chl *a*/*b* determinations, thylakoids were fractionated into grana, margin and stroma thylakoid membranes using digitonin as described in Wunder *et al.* [2013] (Arabidopsis) or digitonin together with Triton X-100 as described in Rokka *et al.* [2000] (pumpkin). $n = 3$. Noteworthy, the yield of different subfractions is highly dependent on the pre-illumination light intensity of plants.

Arabidopsis	Dark	LL	HL	Pumpkin	Dark	LL	HL
Intact thylakoid	3.1	3.1	3.0	Intact thylakoid	3.7	3.9	3.7
Grana	2.5	2.6	2.4	Grana	2.3	2.5	2.3
Margin	3.9	3.7	3.3	Margin	3.3	3.5	3.5
Stroma thylakoid	6.2	5.0	5.5	Stroma thylakoid	9.3	6.7	6.8

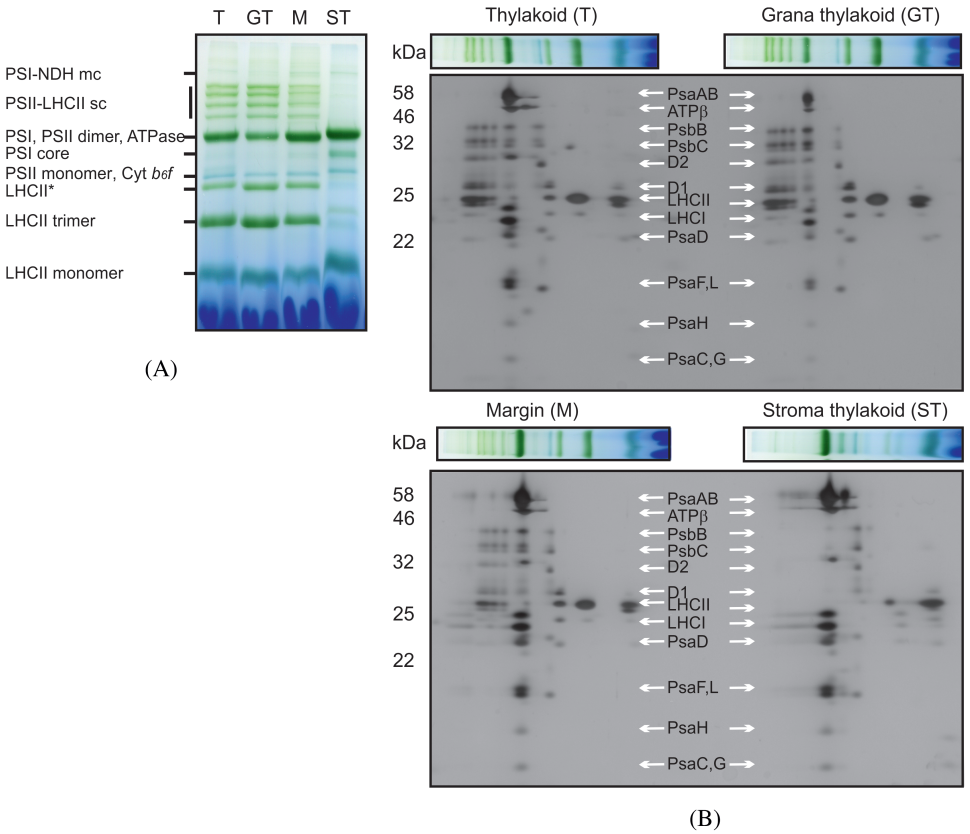

Figure 2. Large pore blue native (lpBN)-PAGE analysis of thylakoid subfractions solubilized with β-DM. Thylakoids were isolated from Arabidopsis leaves harvested in the end of the diurnal dark period. Intact thylakoid membrane (T) was fractionated into grana thylakoid (GT), margin (M) and stroma thylakoid (ST) subfractions with digitonin and differential centrifugation as described in Wunder *et al.* [2013]. Intact thylakoids and the thylakoid subfractions were solubilized with 1% β-DM before separation on lpBN-PAGE. (A) lpBN-PAGE. (B) Two-dimensional lpBN/SDS-PAGE stained with silver nitrate. Identification of the thylakoid membrane proteins is based on Aro *et al.* [2005].

A typical pattern of thylakoid protein complexes obtained from dark-acclimated Arabidopsis leaves (T) as well as from the digitonin-fractionated thylakoids yielding the appressed grana thylakoid (GT), margins (M) and non-appressed stroma thylakoid (ST) subfractions is demonstrated in Figure 2. Prior to the separation of the protein complexes by a large pore blue native (lpBN)-PAGE (Figure 2A), both the intact thylakoids and all thylakoid subfractions were solubilized with 1% β-DM. The subunits of the thylakoid protein complexes, separated by denaturing SDS-PAGE gel in the second dimension (Figure 2B), have been identified using protein specific antibodies and/or mass spectrometry [Aro *et al.*, 2005].

Intact thylakoid membrane isolated from dark-acclimated leaves and treated with 1% β-DM (Figure 2, lane T) reveals the following major photosynthetic protein complexes: the PSI-NDH-1 megacomplex, several PSII-LHCII supercomplexes with varying molecular mass, the co-migrating PSI complex, PSII dimer and ATP synthase, the PSI core complex, the co-migrating PSII monomer and Cyt b_6f complexes, the LHC*, the LHCII trimer and the LHCII monomer. The comparison of the protein complexes of intact thylakoids (T) and thylakoid subfractions (GT, M, ST) reveals thylakoid domain specific differences. In the grana thylakoid subfraction (Figure 2, lane GT) the amount of the PSII-LHCII supercomplexes is significantly higher while the amount of PSI and ATP synthase is significantly lower compared to the total thylakoids (T). The most prominent protein complexes in stroma thylakoid subfraction (Figure 2, lane ST), in turn, are the PSI-NDH-1 megacomplex, the PSI complex, ATP synthase, the PSI core complex (lacking the LHCI), PSII monomer, LHCII trimer and LHCII monomer, being in line with the classical thylakoid heterogeneity model of Andersson and Anderson [Andersson and Anderson, 1980]. The protein complex pattern of the margin subfraction (Figure 2, lane M) represents an intermediate of the grana and stroma thylakoids. Highly similar pattern of thylakoid protein complexes was obtained when thylakoids isolated from dark-acclimated leaves were fractionated mechanically prior to β-DM solubilization and BN/SDS-PAGE separation of the protein complexes [Danielsson *et al.*, 2006]

In order to gain information of more natural *in vivo* distribution of various protein complexes in distinct thylakoid subdomains, it is preferable to avoid extensive fractionation procedures and make use of the properties of mild detergents. 1% digitonin in 25BTH20G buffer solubilizes exclusively the stroma-exposed membrane domains during a five-minute incubation period and a centrifugation step to remove the detergent-inaccessible (insolubilized) thylakoid membrane fractions [Järvi *et al.*, 2011]. Figure 3 demonstrates the difference in the protein complexes solubilized from the thylakoid membrane by 1% β-DM and 1% digitonin. The lpBN/SDS-PAGE gel analysis reveals the presence of the subunits of all different protein complexes in 1% β-DM solubilized thylakoid membranes whereas the 1% digitonin solubilized complexes comprise mainly the subunits of PSI (*e.g.* PsaA/B), ATP synthase (*e.g.* ATPβ) and LHCII. Figure 3 also demonstrates that the tightly appressed thylakoid domain, which is left insolubilized by digitonin treatment, is mainly composed of PSII (*e.g.* PsbB) and LHCII, typical to the grana subdomain of the thylakoid membrane.

Taking together, Figures 2 and 3 clearly demonstrate that the thylakoid membrane solubilization with digitonin, which is capable of maintaining the weak protein–protein interactions, gives a unique pattern of protein complexes that differs from those obtained from the grana, margin or stroma thylakoid subfractions solubilized with β-DM prior to the native gel analysis. The key difference is that

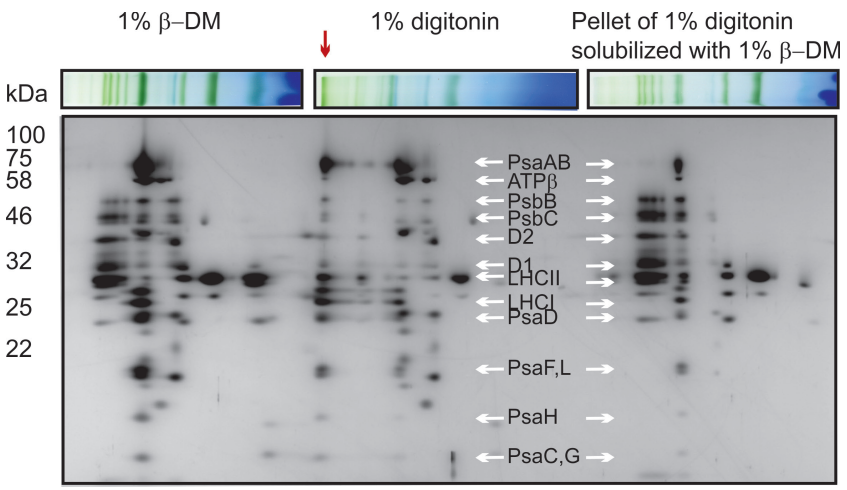

Figure 3. Large pore blue native (lpBN)/SDS-PAGE analysis of Arabidopsis thylakoid membrane protein complexes solubilized with β-DM and digitonin. Intact thylakoid membrane, isolated from dark-acclimated Arabidopsis leaves, was solubilized with 1% β-DM (solubilizing the entire thylakoid membrane) or 1% digitonin (solubilizing the non-appressed membranes) prior to lpBN-PAGE. The pellet from 1% digitonin sample was further solubilized with 1% β-DM. lpBN-PAGE lanes were subsequently submitted to separation of the individual subunits of the protein complexes in the second dimension by SDS-PAGE followed by silver staining. Identification of protein subunits is based on [Aro [*et al.*, 2005]. White arrows denote the major subunits of the photosynthetic protein complexes, red arrow indicates the PSI-PSII-LHCII-megacomplex.

the labile high molecular mass megacomplexes, of which the largest one represents the LHCII phosphorylation-dependent PSI-PSII-LHCII megacomplex (marked with red arrow in Figure 3), are enriched in the digitonin-solubilized thylakoid subfraction.

5.2. *Thylakoid fractionation from light-acclimated plants demonstrates light-dependent, reduced stringency of lateral heterogeneity*

It is important to improve the fractionation methods for light-acclimated plants in order to get better insights into the flexibility of the lateral heterogeneity and of the interactions between the thylakoid protein complexes upon changes in environmental conditions. Environmental changes, especially the light conditions, up- and down-regulate tens, even hundreds, of different regulatory mechanisms that, in concert, are responsible for fluent function of the photosynthetic light reactions (discussed in Section 4). It is particularly important to start understanding the

relationships between the thylakoid architecture, protein complex interactions and the thylakoid regulatory mechanisms, the latter being the driving force for all changes in the thylakoid structure.

As already mentioned above (Section 5.1), contrary to the relatively easy fractionation of the thylakoid membrane from dark-acclimated leaves, by both the mechanical and detergent-based fractionation mechanisms, the fractionation of the thylakoid membrane from light-acclimated leaves always produces more randomized thylakoid fractions, and results in different yields of the subfractions, as compared to the fractionation of dark-acclimated leaves. This is due to light-induced regulatory mechanisms and subsequent ~~reorganizations~~ occurring in the thylakoid architecture. Such regulation mechanisms induce the movement of thylakoid protein complexes laterally in the membrane, mixing the clear segregation of PSII, LHCII and PSI, typically present in dark-acclimated thylakoids. Enrichment of the "state transition"-related PSI-LHCII complex [Pesaresi *et al.*, 2009] is one example manifesting the low light-induced reorganization of the thylakoid membrane protein complexes (Figure 4A). Another low light-induced enhancement in protein-protein interactions is the accumulation of the PSI-PSII-LHCII megacomplex in grana margins as a result of differential thylakoid protein phosphorylation and accelerated need for maximal light harvesting to both photosystems [Suorsa *et al.*, 2015] (Figure 4A). Similarly, the light intensity-dependent PSII photodamage-repair cycle, evidenced by monomerization of the PSII-LHCII supercomplexes with increasing light intensity, is a typical example of the high light-induced consequences on thylakoid membrane architecture [Tikkanen *et al.*, 2008a] (Figure 4B).

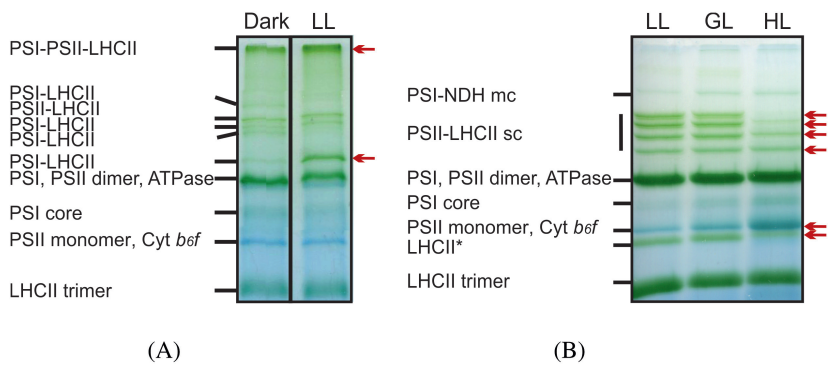

Figure 4. Large pore blue native (lpBN)-PAGE analysis of Arabidopsis thylakoid membrane protein complexes. Arabidopsis leaves were harvested in the end of the diurnal dark period and after 3 hours of exposure to low light (LL), growth light (GL) or high light (HL), and then immediately subjected to thylakoid isolation. Prior to gel electrophoresis, the thylakoid membrane was solubilized with (A) 1% digitonin and (B) 1% β-DM. Red arrows denote the light-induced dynamics at the level of thylakoid protein complexes.

Light-induced partial destacking of the grana and randomization of the protein complexes become evident also by monitoring the changes in the Chl *a*/*b* ratios of the margin and stroma thylakoid subfractions isolated from light-acclimated leaves and compared to those obtained from dark-acclimated leaves (Table 1). Spinach thylakoids isolated from dark-acclimated leaves and fractionated with the aqueous two-phase system typically give the Chl *a*/*b* ratios 2.2, 2.9 and 6.3 for the grana, margin and stroma thylakoid subfractions, respectively, while for low light-acclimated leaves the corresponding values were 2.3, 3.3 and 5.4 [Tikkanen *et al.*, 2008b]. Repeating lack of success in strict mechanical fractionation of the thylakoid membrane from either the growth- or high light-exposed spinach leaves, with respect to both the Chl *a*/*b* ratios and the yields of the subfractions, has to be interpreted as a result from light-induced modifications in the thylakoid membrane (unpublished results from Aro's lab). Similar problems are faced in detergent-based fractionation of the thylakoid membrane from light-acclimated leaves, *i.e*. the reorganizations of thylakoid protein complexes in light lead to a loss of strict thylakoid heterogeneity, as evidenced by light intensity-dependent changes in the yield of various subfractions and as a decrease in the Chl *a*/*b* ratio of the margin and stroma thylakoid subfractions isolated from Arabidopsis and pumpkin leaves (Table 1).

5.3. *Significance of thylakoid plasticity for photosynthetic light reactions and beyond*

Light-induced dynamic changes in thylakoid architecture are intimately linked to a high number of regulatory processes involved in the adjustment of the photosynthetic light reactions and carbon reduction reactions to rapid changes in environmental conditions, particularly to varying light intensities [Chow *et al.*, 2005]. Recently, there has been great advances in the high-resolution imaging techniques. However, as long as the imaging approaches of thylakoid membrane protein complexes, such as high-resolution cryo-electron tomography, do not provide sufficient resolution of the organization of the thylakoid pigment protein complexes, it is important to improve the fractionation methods for light-acclimated plants. It is highly conceivable that the development of the lateral heterogeneity of the thylakoid membrane is important not only for the performance of the photosynthetic processes in changing environments, but also reflects the wider signaling function of the photosynthetic apparatus in controlling the growth, development and stress acclimation of multicellular land plants [Gollan *et al.*, 2015; Kangasjärvi *et al.*, 2014; Piippo *et al.*, 2006]. To that end, it is highly likely that the mechanisms involved in regulation of thylakoid plasticity will remain in the focus of plant science also during the forthcoming years.

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