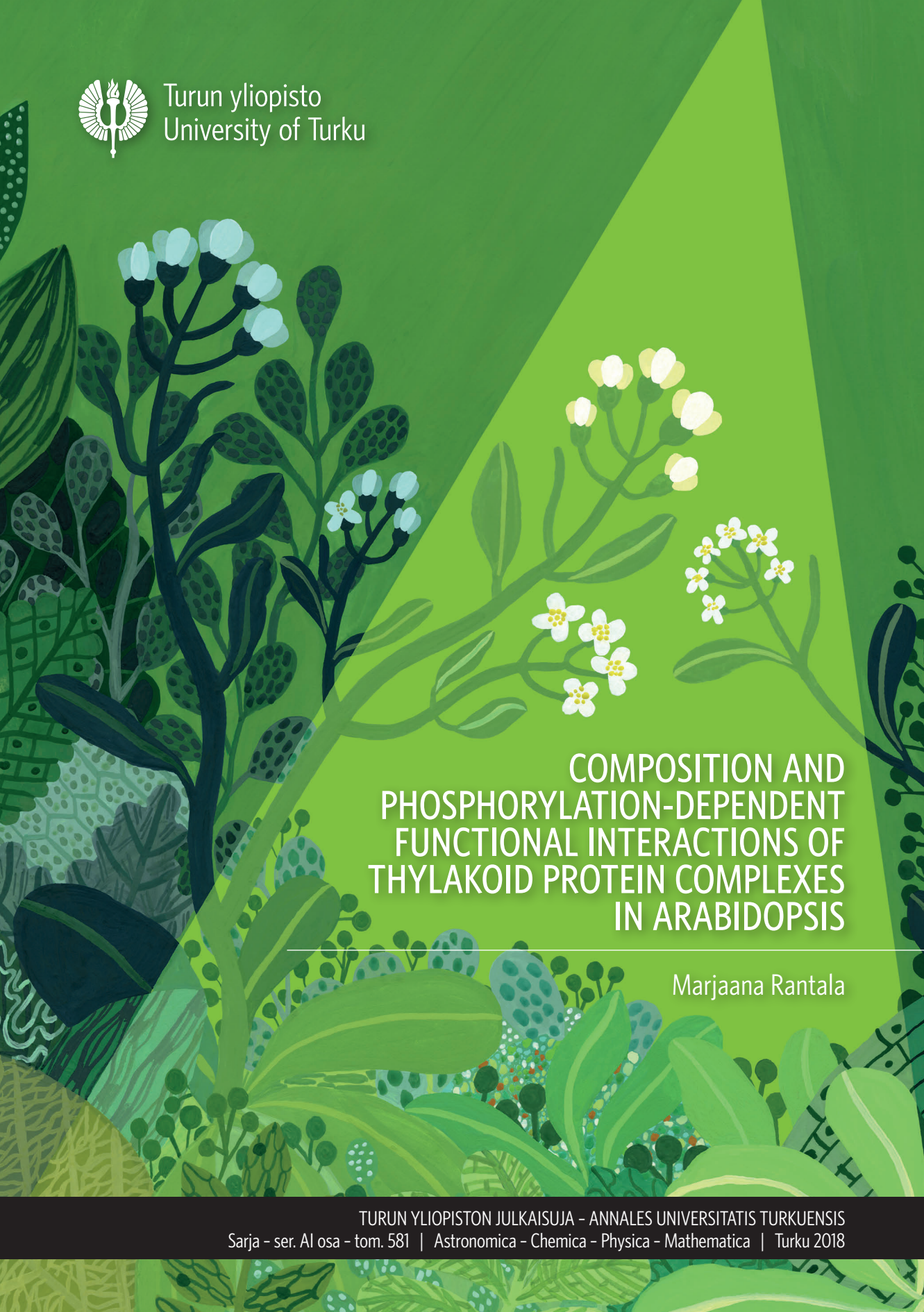

Turun yliopisto
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# LIST OF ORIGINAL PUBLICATIONS

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**II**. Nageswara RM, Suorsa M, Rantala M, Aro EM, Tikkanen M (2015) Plants Actively Avoid State Transitions upon Changes in Light Intensity: Role of Light-Harvesting Complex II Protein Dephosphorylation in High Light. *Plant Physiology* 168:721-34

**III**. Rantala M, Lehtimäki N, Aro EM, Suorsa M (2016) Downregulation of TAP38/PPH1 enables LHCII hyperphosphorylation in Arabidopsis mutant lacking LHCII docking site in PSI. *FEBS Letters* 590:787-94

**IV**. Rantala M, Tikkanen M, Aro EM (2017) Proteomic characterization of hierarchical megacomplex formation in Arabidopsis thylakoid membrane. *The Plant Journal 5:951*-962



Other recent publications related to the topic

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Plöchinger M, Torabi S, Rantala M, Tikkanen M, Suorsa M, Jensen PE, Aro EM, and Meurer J (2016) Impact of PsaI on the Stable Association of the LHCII Docking Site of PSI, State Transition, and Dark Reduction of the Plastoquinone Pool in *Nicotiana Tabacum. Plant Physiology* 172:450-63

Trotta A, Suorsa M, Rantala M, Lundin B, Aro EM (2016) Serine and threonine residues of plant STN7 kinase are differentially phosphorylated upon changing light conditions and specifically influence the activity and stability of the kinase. Plant Journal 87:484-94

Suorsa M, Rantala M, Danielsson R, Järvi S, Paakkarinen V, Schröder WP, Styring S, Mamedov F, Aro EM (2014) Dark-adapted spinach thylakoid protein heterogeneity offers insights into the Photosystem II repair cycle. Biochimica et Biophysica Acta 1837:1463-71

# ABSTRACT

Marjaana Rantala

**Composition and phosphorylation-dependent functional interactions of thylakoid protein complexes in Arabidopsis**

University of Turku, Department of Biochemistry, Molecular Plant Biology
Annales Universitatis Turkuensis, Turku 2018

In higher plants the thylakoid embedded pigment-protein complexes, photosystem (PS) I and PSII together with their respective light harvesting complex (LHC) antennas work in series to harvest solar energy and convert it into chemical energy. Profound understanding of the composition and interactions of the photosynthetic machinery is a prerequisite for understanding the function and regulation of the energy conversion reactions. The structure of individual components of the photosynthetic machinery has been resolved in high resolution and currently the interest is targeted at the supramolecular organization and light-dependent regulation of the photosynthetic protein complexes.

This thesis elucidates the overall organization of the thylakoid protein complex network providing structural insights into the hierarchical protein complex interactions in *Arabidopsis thaliana*. LHCII trimers are shown to mediate the supramolecular assemblies of the two photosystems and the strength of the interactions between individual subcomplexes is demonstrated using an optimized protein complex solubilization system together with two-dimensional native gel electrophoresis.

This thesis further addresses the dynamic nature of the protein super- and megacomplexes upon short-term light acclimation. It is shown that light intensity dependent LHCII phosphorylation causes re-arrangements of the protein complexes particularly in the non-appressed regions of the thylakoid membrane. The differential phosphorylation of distinct LHCII proteins, Lhcb1 and Lhcb2, is shown to occur in different pools of LHCII antenna trimers. Lhcb1 was found phosphorylated mainly in PSII-LHCII supercomplexes, whereas phosphorylated Lhcb2 is located exclusively in loosely bound LHCII, which is known to have a role in mediating the interaction of the PSI and PSII complexes upon dynamic changes in light intensity. The results of my thesis provide valuable information regarding the toolbox needed for the enhancement of photosynthetic productivity in the global attempts to meet the future challenges in food and bioenergy production.

# TIIVISTELMÄ

Marjaana Rantala

**Composition and phosphorylation-dependent functional interactions of thylakoid protein complexes in Arabidopsis**

Turun yliopisto, Biokemia, Molekulaarinen kasvibiologia
Annales Universitatis Turkuensis, Turku 2018

Kasvien viherhiukkasten tylakoidikalvostolla sijaitsevat suuret pigmentti-proteiinikompleksit, fotosysteemi (PS) I ja PSII yhdessä niitä ympäröivien valohaavien kanssa (LHC), keräävät auringon valoenergiaa ja muuttavat sen kemialliseksi energiaksi fotosynteesin valoreaktioissa. Fotosynteesiä katalysoivien yksittäisten proteiinikompleksien rakenteet on onnistuttu selvittämään lähes atomitarkkuudella, mutta fotosynteesikoneiston toiminnan ja säätelyn ymmärtäminen edellyttää lisäksi tylakoidikalvostolla sijaitsevien proteiinikompleksien välisten vuorovaikutusten ja järjestäytymisen tuntemista. Fotosynteesikoneiston säätelyn kannalta tärkeää on myös ymmärtää, kuinka muuttuvat ympäristöolosuhteet muokkaavat proteiinikompleksien välisiä vuorovaikutuksia.

Fotosynteettisten proteiinikompleksien analyysiä varten kompleksit on ensin eristettävä tylakoidikalvostolta miedoilla detergenteillä. Optimoimallani suurten ja labiilien proteiinikompleksien eristysmenetelmän avulla selvitin, kuinka yksittäiset fotosynteettiset proteiinikompleksit ovat järjestäytyneet lituruohon (*Arabidopsis thaliana*) viherhiukkasten tylakoidikalvostolla suuremmiksi komplekseiksi. Näiden kompleksien välisiä hierarkkisia vuorovaikutuksia tutkin optimoimallani 2D-natiivigeelielektroforessilla. Osoitin, että molemmat fotosysteemit, yhdessä hyvin löyhästi kiinnittyneen L-LHCII-valohaavin kanssa, muodostavat suuria super- ja megakomplekseja, jotka hajoavat alayksiköikseen L-LHCII-kompleksin irrotessa.

Fotosynteesikoneiston on sopeuduttava alati muuttuviin valo-olosuhteisiin, ja LHCII proteiinien fosforylaation tiedetään vaikuttavan ratkaisevasti kasvien valosopeutumiseen. Työssäni havainnollistin, kuinka LHCII-kompleksin Lhcb1- ja Lhcb2 -proteiinien palautuva fosforylaatio vaikuttaa koko fotosynteesikoneiston järjestäytymiseen, erityisesti tylakoidikalvoston pinoutumattomilla alueilla. Osoitin että Lhcb1-proteiinin fosforylaatio tapahtuu pääasiassa PSII-kompleksiin tiukasti kiinnittyneissä LHCII-komplekseissa, kun taas Lhcb2-proteiinin fosforylaatio, joka vaikuttaa erityisesti LHCII- ja PSI-kompleksin väliseen vuorovaikutukseen, tapahtuu lähes yksinomaan fotosysteemeihin löyhästi kiinnittyneessä L-LHCII-kompleksissa. Löytämäni tulokset auttavat ymmärtämään paremmin valoreaktioiden toimintaa, ja sen säätelyä muuttuvissa ympäristöolosuhteissa. Fotosynteesin säätelymekanismien tunteminen luo lisäksi perustan sovelluksille, joilla pyritään parantamaan kasvien tuottavuutta.

# ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine triphosphate |
| ACA | Aminocaproic acid |
| BN | Blue native |
| C | PSII core monomer |
| CET | Cyclic electron transfer |
| CBB | Coomassie Brilliant Blue |
| Chl | Chlorophyll |
| CURT1 | CURVATURE THYLAKOID1 |
| Cyt | Cytochrome |
| DIG | Digitonin |
| DM | Dodecyl-maltoside |
| ETC | Electron transfer chain |
| Fd | Ferredoxin |
| FNR | Fd-NADPH-oxidoreductase |
| GL | Growth light |
| HL | High light conditions |
| LET | Linear electron transfer |
| LHC | Light harvesting complex |
| LL | Low light |
| L-LHCII | Loosely bound LHCII |
| M-LHCII | Moderately bound LHCII |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced) |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate (oxidized) |
| NDH | NAD(P)H dehydrogenase-like complex |
| NPQ | Non-photochemical quenching |
| OEC | Oxygen evolving complex |
| $P680/P680^+$ | Red/ox primary electron donor of PSII |
| $P700/P700^+$ | Red/ox primary electron donor of PSI |
| PAGE | Polyacrylamide gel electrophoresis |
| PBCP | PSII CORE PHOSPHATASE |
| PC | Plastocyanin |
| pH | Negative logarithm of the proton concentration |
| *pmf* | Proton motive force |
| PS | Photosystem |
| P-Thr | Phosphothreonine |
| PQ | Plastoquinone |

| | |
|---|---|
| PQH2 | Plastoquinol |
| $Q_{A/B}$ | Primary electron-accepting plastoquinones of PSI |
| RC | Reaction center |
| RIQ | REDUCED INDUCTION OF NPQ |
| SDS | Sodium dodecyl sulphate |
| S-LHCII | Strongly bound LHCII |
| SMA | Styrene maleic acid |
| STN7 | STATE TRANSITION 7 kinase |
| STN8 | STATE TRANSITION 8 kinase |
| TAP38 | Thylakoid-associated phosphatase of 38 KDa |
| PBCP | PSII core phosphatase |
| VDE | Violaxanthin de-epoxidase |
| WT | Wild-type |
| Å | Ångström (= 0.1 nm) |

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# 1. INTRODUCTION

## 1.1 Photosynthesis

Almost all life on Earth relies on oxygenic photosynthesis, in which the energy from the sun is absorbed and converted into chemical energy by photoautotrophic organisms: plants, algae and cyanobacteria. Photosynthetic light reactions are catalyzed by membrane-embedded pigment-protein complexes that harvest solar energy, transfer it to reaction centers and ultimately store the energy in NADPH and ATP molecules. In the Calvin-Benson-Bassham cycle the energy of ATP and NADPH is used to assimilate the atmospheric carbon dioxide into organic compounds. The principal energy conversion reactions are similar in all oxygenic photosynthetic organisms, but the light harvesting, and regulation of the photosynthetic apparatus show distinct evolutionary diversity. During the past decades the knowledge of the function and structure of the photosynthetic apparatus has accumulated greatly, yet the dynamic organization and regulation of the energy conversion machinery still require extensive research. Since almost all chemical energy, most importantly food and fuel, is provided by photosynthesis, the thorough understanding of the photosynthetic process and its regulation is a prerequisite for developing applications that aim at improved productivity of photosynthetic organisms and work towards clean and renewable bioenergy production platforms.

## 1.2 Structure of photosynthetic pigment-protein complexes

In higher plants, photosynthesis takes place in specialized subcellular organelles, the chloroplasts, which originate from ancient endosymbiont cyanobacteria. Chloroplasts are separated from the cytoplasm by a double membrane envelope, which in turn, encloses an extensive internal membrane system known as the thylakoids. The thylakoid lipid bilayer segregates two aqueous compartments, the stroma and lumen, and provides the matrix for the photosynthetic protein complexes. Light absorption and the primary reactions of photosynthesis occur in the thylakoid membrane and are mediated by membrane embedded large multisubunit protein complexes: photosystem (PS) II and PSI, which are associated with peripheral light harvesting antennas. Cytochrome (Cyt) $b_6f$ interconnects the two photosystems whereas another complex, ATP synthase, functions in ATP formation.

### 1.2.1 Light harvesting antennas

In higher plants, solar energy is predominantly captured by the Lhc super-gene family-encoded light harvesting antennas that bind several chlorophylls and carotenoids (Jansson, 1999). Two distinct but structurally very similar chlorophylls, *a* and *b*, are typically present at a 3 to 1 ratio in higher plants. The principal photosynthetic pigment, Chl-*a*, is present in both photosystems and in the light harvesting antenna, while the majority of the accessory pigment, Chl-*b*, is present in the LHCII antenna trimers that bind half of all thylakoid chlorophyll molecules (Liu et al., 2004). The peripheral light harvesting antenna of PSI (LHCI) comprises four major light harvesting proteins (Lhca1-4) whereas three major (Lhcb1-3) and three minor lhcb proteins (Lhcb4-6, also known as CP29,CP26 and CP24) constitute the LHCII antenna of PSII (Jansson, 1999; Ballottari et al., 2004). LHCII proteins (Figure 1) consist of three transmembrane α-helices, labelled A, B and C, and of two luminal helices D and E (Kühlbrandt and Wang, 1991; Kühlbrandt et al., 1994; Liu et al., 2004).

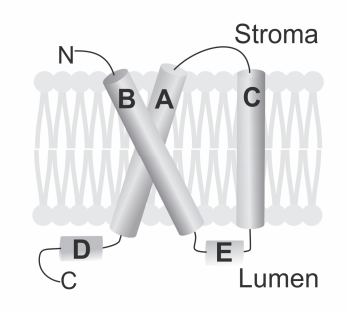


**Figure 1.** Schematic presentation of the LHCII monomer in parallel with a membrane plane. The α-helices (A-E), the loop regions between the helices and the N-and C-terminals are indicated.

The most abundant LHCII protein Lhcb1 together with the Lhcb2 and Lhcb3 proteins form hetero trimers, which along with the three minor antenna proteins Lhcb4, Lhcb5 and Lhcb6 account for light harvesting and excitation energy transfer to the PSII core. A fraction of the LHCII trimers also function as an antenna for PSI and the phosphorylation of the N-terminus of Lhcb1 and Lhcb2 proteins is known to play a key role in the regulation of excitation energy balance between PSII and PSI (Bennett, 1991; Allen, 1992; Tikkanen et al., 2012; Wientjes et al., 2013a). In addition, LHCII is involved in the dissipation of excess excitation energy in a photoprotective process called non-photochemical quenching (NPQ) (Müller et al., 2001). The activation of NPQ is dependent on low luminal pH, which induces protonation of acidic amino acids of the PsbS protein, and on the activation of the violaxanthin de-epoxidase entzyme (VDE). VDE catalyzes the conversion of LHCII bound xanthophyll, violaxanthin, into zeaxanthin, which is an effective excitation energy quencher (Niyogi et al., 1998). Further, the protonated PsbS induces conformational changes in LHCII proteins

resulting in safe dissipation of excess energy as heat (Li et al., 2000; Jahns and Holzwarth, 2012).

### 1.2.2 PSII, PSI and Cyt $b_6f$

The PSII reaction center complex (RC) is composed of the PsbA (D1), PsbD (D2) and PsbI proteins as well as of the α and β-subunits of the Cyt b559 complex. RC is the minimal unit required for primary photochemical reactions (Nanba and Satoh, 1987; Ikeuchi and Inoue, 1988). The PSII core monomer (C) consists the RC that is surrounded by integral, chl-*a* binding light harvesting antenna proteins PsbB (CP47) and PsbC (CP43) and several smaller intrinsic and extrinsic subunits (Wei et al., 2016; Bezouwen et al., 2017). The PSII core monomer coordinates a remarkable number of pigments including 37 chlorophylls, 10 carotenoids and other cofactors including quinones and non-heme iron (Wei et al., 2016). The extrinsic oxygen-evolving complex (OEC) stabilizes the $Mn_4CaO_5$ cluster, which is responsible for the water oxidation mechanism of PSII. The PSII core is assembled as a dimer (C2) in its active form. The core together with LHCII forms large C2S2M2 supercomplexes, where each of the core monomer is associated with one strongly bound (S)-LHCII and one moderately bound (M)-LHCII (Figure 2A). The structure of C2S2M2 supercomplex in *Arabidopsis thaliana* has been resolved at an overall resolution of 5.2 Å (Bezouwen et al., 2017) and from *Pisum sativum* at 2.7–3.2 Å resolution (Su et al., 2017). The S-LHCII is Lhcb1/Lhcb2-heterotrimer that is linked to the PSII core via the Lhcb5 protein (CP26), whereas M-LHCII consisting of Lhcb1 and Lhcb3 proteins, is associated to the core with Lhcb4 (CP29) and Lhcb6 (CP24) proteins (Boekema et al., 1999; Caffarri et al., 2009). Lhcb3 and Lhcb6 evolved after the divergence between algae and land plants, but interestingly, are missing from the gymnosperm family Pinaceae (Kouřil et al., 2016). The Lhcb3 protein is similar to Lhcb1/Lhcb2, but specific structural differences in the N-terminus and in AC and BC loops facilitate the interaction of the Lhcb3 protein with Lhcb6, and thereby determine the binding orientation of the trimer to the core complex (Su et al., 2017). Since the Lhcb1/3 trimer, together with Lhcb4 and Lhcb6, easily detaches from C2S2M2 supercomplexes during solubilization (Caffarri et al., 2009), M-LHCII may be considered a relatively stable pentamer. In addition to S- and M-LHCII, the C2S2M2 supercomplexes can bind several loosely bound (L)-LHCII trimers that occupy the spaces between individual PSII-LHCII supercomplexes in the grana core (Dekker and Boekema, 2005; Sznee et al., 2011; Ruban and Johnson, 2015). Occasionally, the PSII-LHCII supercomplexes laterally assemble into semi-crystalline arrays (Kirchhoff et al., 2007; Daum et al., 2010; Kouřil et al., 2012), and interact on their stromal surface with complexes on an adjacent membrane layer (Albanese et al., 2016, Su et al., 2017). The vertical interaction of PSII-LHCII supercomplexes probably plays a key role in the stabilization of grana-appressions (Daum et al., 2010).

The PSI reaction center is a PsaA/PsaB-heterodimer that is surrounded by several smaller subunits PsaC-PsaL, PsaN and PsaO. The PSI core is fused with the LHCI complex, which forms a half-moon shaped belt on the PsaF side of the core complex (Figure 2B) (Amunts et al., 2007). The LHCI antenna is composed of four Lhca proteins, of which the Lhca1/Lhca4 and Lhca2/Lhca3 form functional heterodimers (Wientjes and Croce, 2011). Similar to PSII, also the PSI-LHCI complex binds several prosthetic groups including chlorophylls, carotenoids and lipids. Located opposite to the LHCI binding site, the PSI subunits PsaH, PsaL, PsaO and PsaI provide a docking site for L-LHCII (Lunde et al., 2000; Galka et al., 2012; Mazor et al., 2015). Further, a pool of L-LHCII transfers excitation energy to PSI via LHCI antenna (Benson et al., 2015). Another two Lhca proteins of PSI, Lhca5 and Lhca6, have been shown to mediate the supercomplex formation between PSI and the NADPH dehydrogenase-like (NHD) complex (Peng et al., 2008; Peng et al., 2009; Kouřil et al., 2014).

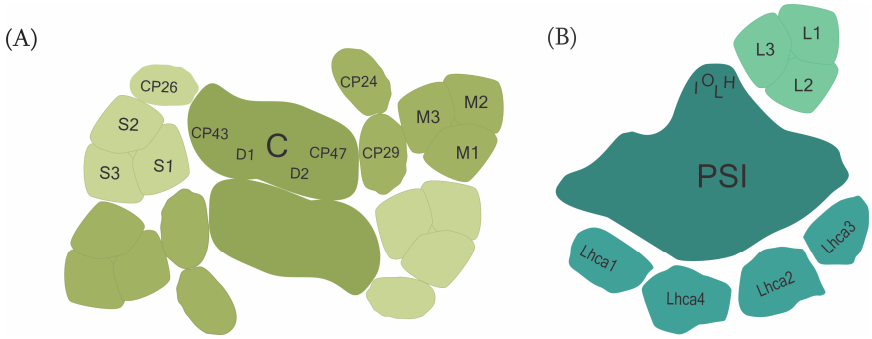


**Figure 2.** Overall structures of plant photosystem supercomplexes highlighting the light harvesting antennas (A) Top view of the C2S2M2 supercomplex demonstrates the binding sites of S (S1-S3)- and M-LHCII (M1-M3) to the PSII core (C) monomers. CP29, CP26 and CP24 denote the minor antenna proteins Lhcb4-Lhcb6, respectively. (B) Top view of PSI complex shows the location of the LHCI antenna and the L-LHCII (L1-L3) binding on a site composed of the PsaH, PsaL, PsaO and PsaI subunits.

The Cyt $b_6f$ dimer mediates the electron transfer between the two photosystems by oxidizing and reducing mobile electron carriers, plastoquinol ($PQH_2$) and plastocyanin (PC), respectively (Huang et al., 1994). Each Cyt $b_6f$ monomer is composed of Cyt f (PetA), Rieske (Pet C), Cyt $b_6$ (Pet B) and subunit IV (Pet D), which are all involved in the electron transfer reactions. Further, the complex contains at least four additional subunits and binds four hemes, one 2Fe-2S as well as a chlorophyll and a β-carotene with so far unknown functions (Kurisu et al., 2003).

## 1.3 Function of photosynthetic protein complexes

Solar energy is captured by the LHC-bound pigments, mainly by the chlorophylls that transfer the energy to the reaction centers. The antenna system does not perform any chemistry, the absorption of light and the transfer of the electronic excited states from one pigment to another is a purely physical process. The transformation of the physical energy of the chlorophyll excited state into chemical energy takes place in the reaction centers. The PSII RC contains a special Chl-*a* dimer P680, which is promoted to an excited state (P680*), either by direct photon absorption or by energy transfer from the antenna. P680* rapidly loses the excited electron to a first electron acceptor, Pheo, which immediately transfers the electron to plastoquinones. A mobile plastoquinone $Q_B$ is fully reduced to plastoquinol after receiving two electrons from plastoquinone Qa, and two protons from the stroma. $PQH_2$ is released from the $Q_B$ pocket of the D1 protein in PSII and oxidized twice by Cyt $b_6f$, which concomitantly expels the protons into the lumen. The electrons are further transferred from Cyt $b_6f$ to PC, which then re-reduces the PSI RC chlorophyll pair $P700^+$ that similarly to PSII has donated an excited electron to the primary electron acceptor of PSI. The electron from P700* moves via three Fe-S clusters to ferredoxin (Fd) and further to Fd-NADPH-oxidoreductase (FNR), which finally reduces $NADP^+$ to NADPH.

The primary electron donor ($P680^+$) of PSII is a highly oxidizing molecule that is re-reduced by electrons derived from water. The water splitting occurs at the OEC complex and is catalyzed by a manganese ($Mn_4CaO_5$) cluster that extracts four electrons from water, simultaneously expelling protons into the lumen, to release one molecule of oxygen. The protons expelled into the lumen during water oxidation, and those released from the stroma to the lumen simultaneously with the plastoquinol ($PQH_2$) oxidation at $Q_0$ site of the Cyt $b_6f$ complex, create a proton gradient ($\Delta pH$) and a membrane potential ($\Delta\Psi$) across the thylakoid membrane. This electrochemical gradient constitutes a proton motive force (*pmf*) utilized for ATP production by ATPase. The entire chain in which the two photosystems work in series to produce NADPH and ATP (Figure 3), is called linear electron transfer (LET) and the energy stored in these molecules is used in the fixation of $CO_2$ into carbohydrates, in photorespiration and in several other metabolic processes such as nitrogen and sulfur metabolism.

In addition to LET, alternative electron transfer routes take place in the thylakoid membrane. In cyclic electron transfer (CET) the electrons are cycled around PSI and only ATP is generated. Two CET-pathways, the so called PGR (proton gradient regulation) and NDH (NAD(P)H dehydrogenase-like complex) mediated CET are known (Munekage et al., 2002; DalCorso et al., 2008). Currently, both pathways are considered Fd-dependent, i.e. electrons from Fd are directed to the PQ pool and then transferred through Cyt $b_6f$ back to PSI (Yamori and Shikanai, 2016). The protons

pumped from the stroma to the lumen through Cyt $b_6f$ generate the *pmf,* which is utilized in ATP production. Since NADPH is not produced, CET functions in fine-tuning the ATP/NADPH ratio to meet the metabolic requirements. Further, the CET-induced acidification of the lumen and consequent downregulation of the Cyt $b_6f$, and the induction of the energy-dependent qE component of NPQ, prevent the over-reduction of the electron transfer chain (ETC) and thus protects both photosystems against photodamage (Suorsa et al., 2012).

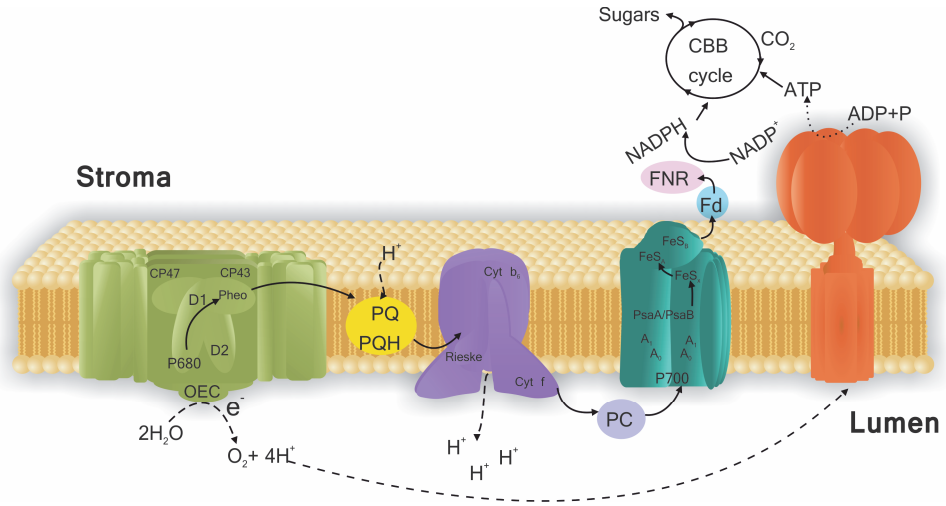


**Figure 3.** Simplified scheme of the linear electron transfer route in the plant thylakoid membrane. The electron and proton transfer reactions are indicated with solid and dashed line arrows, respectively. Only the elemental complexes involved in LET are illustrated in the membrane.

## 1.4 Organization of protein complexes in the thylakoid membrane

### *1.4.1 The Thylakoid membrane ultrastructure and lateral heterogeneity of protein complexes*

The thylakoid lipid bilayer is composed mainly of glycolipids, which constitute around 30% of the total membrane area (Kirchhoff et al., 2002). The remaining area is occupied by the photosynthetic protein complexes. In higher plants the ultrastructure of the thylakoid membrane is remarkably heterogenic consisting of appressed grana thylakoids and non-appressed stroma thylakoids (Figure 4A). The exact three-dimensional organization of the thylakoid network remains controversial. According to a helical fretwork model (Figure 4B), the stroma thylakoids wind around the grana stacks as right-handed helices that are interconnected to the stacks by fusion of narrow membrane protrusions (Paolillo, 1970; Brangeon and Mustardy, 1979; Mustárdy and Garab, 2003). The folded membrane model, on the contrary, postulates that the continuous thylakoid

system is folded into repeated units each containing three grana stacks (Arvidsson and Sundby, 1999). It has also been proposed that the grana is composed of paired layers that are bifurcations of fused stroma membrane sheets (Shimoni et al., 2005). According to this model, the neighboring grana units are further interconnected by membrane bridges.

The grana stacks have a diameter ranging from 300 to 600 nm, and contain 5-20 folded thylakoid layers depending on the plant species and the light condition (Mustárdy and Garab, 2003; Daum et al., 2010). The thylakoid membrane system encloses a continuous soluble space, the lumen (width 4.5-5 nm). The width of the partition gap on the stromal side of two appressed thylakoid membranes is 3.2-4 nm (Kirchhoff et al., 2007; Daum et al., 2010).

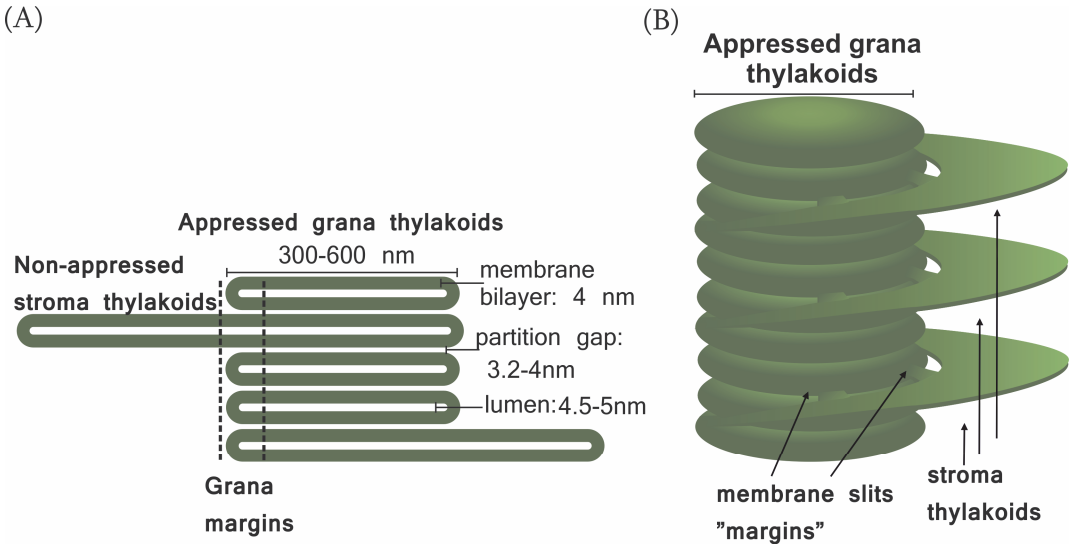


**Figure 4.** Models of the different compartments of the plant thylakoid network. (A) Simplified model of the plant thylakoid membrane shows the distinct thylakoid fractions: the appressed grana core, the surrounding grana margins and the non-appressed stroma thylakoids. The width of the lumen and the partition gap are indicated. (B) The different thylakoid fractions according to the helical model.

The grana stacks account for 80% of the total membrane area (Albertsson, 2001) and the individual stacks are interconnected by non-appressed stroma thylakoids. A distinct membrane region, grana margins, has been considered to represent an annular region in the interphase of the grana core and stroma thylakoids, near the curved regions of the grana (Wollenberger et al., 1994; Albertsson, 2001). However, electron microscopy data suggest that the space between the curved regions and the appressed grana core is too narrow to host protein complexes, and it was therefore suggested that the margins, in fact, comprise the junctional slits, which according to the helical fretwork model directly

connect the grana stacks to stroma thylakoids (Anderson et al., 2012; Ruban and Johnson, 2015).

The photosynthetic protein complexes are unevenly distributed in the heterogeneous thylakoid membrane (Andersson and Anderson, 1980). The majority of the PSII-LHCII supercomplexes are present in appressed thylakoids whereas PSI and ATP synthase are enriched in non-appressed thylakoids due to their large membrane-protruding structures that extend 5nm and 16 nm above the membrane on the stromal side, thereby preventing their access to the tightly packed grana appressions (Nevo et al., 2012). The location of Cyt $b_6f$ is controversial, but most studies report it is evenly distributed in all thylakoid fractions (Anderson, 1982; Albertsson et al., 1991). The distribution, however, is likely dependent on the light conditions (Kirchhoff et al., 2017). The grana margins, whose exact location remains enigmatic, host a different set of protein complexes than the pure grana core and the stroma thylakoids. Data obtained from mechanically fractionated thylakoid network samples demonstrate that this area accommodates both photosystems (Wollenberger et al., 1994; Albertsson, 2001), which have later been suggested to form large megacomplexes (Järvi et al., 2011).

### *1.4.2 Role of LHCII in thylakoid membrane stacking*

The whole complexity of the grana stacking is still elusive, but the role of cations in the stack formation was established already decades ago, when the removal of cations from isolated thylakoid suspension was shown to result in complete grana de-stacking and the randomization of the pigment protein complexes (Murata, 1969; Barber, 1980). It was discovered that the cations are screened between LHCII proteins on adjacent membrane layers and form strong salt bridges with the negatively charged amino acids of LHCII, thereby mediating the grana stacking (Barber, 1980). While the electrostatic screening of the negatively charged stromal loop of LHCII is considered to constitute the primary stabilizing force of the membrane appression, the interaction of the positively charged N-terminus with the negatively charged amino acids in the stromal loop of LHCII on neighboring membrane layers is also involved in grana formation (Standfuss et al., 2005). Further, the CURVATURE THYLAKOID1 (CURT1) and REDUCED INDUCTION OF NON-PHOTOCHEMICAL QUENCING (RIQ)1 and RIQ2 proteins were recently found to be involved in regulation of grana stacking (Armbruster et al., 2013; Yokoyama et al., 2016).

## 1.5 Phosphorylation dependent dynamic regulation of photosynthetic machinery

In natural environments the intensity and the spectral composition of light fluctuate rapidly, and the photosynthetic apparatus needs to respond to these changes to keep the

ETC balanced, to protect the machinery against damage by excess light energy and to allow the repair of damaged components. The thylakoid membrane is a remarkably flexible network that undergoes several structural changes such as swelling of the lumen, alterations in grana height and unstacking of the grana in response to fluctuations in light conditions (Anderson, 1986; Khatoon et al., 2009; Herbstová et al., 2012). These changes were initially thought to occur as long-term responses to environmental changes, but more recently they were shown to occur in time-scale of minutes (Rozak et al., 2002). Reversible phosphorylation of LHCII and PSII proteins appears to be among the most important regulators of these short-term changes. Post-translational protein phosphorylation, i.e. the covalent addition of a phosphate group to the side chains of serine (Ser), threonine (Thr) or tyrosine (Tyr) residues, serves as one of the most important regulatory mechanisms in plants. Almost 200 chloroplast phosphoproteins and 28 thylakoid-located phosphoproteins have been discovered from the model plant Arabidopsis (Reiland et al., 2009). Protein phosphorylation is catalyzed by protein kinases whereas the removal of the phosphate group is mediated by protein phosphatases. The reversible phosphorylation of several PSII core and LHCII proteins influences the interactions of the thylakoid protein complexes and affect to the attractive and repulsive forces of adjacent membrane layers in the grana. Thylakoid protein phosphorylation thus plays a key role in the regulation of photosynthetic light reactions upon changes in light conditions.

### *1.5.1 PSII core phosphorylation and repair cycle*

Light is essential for driving photosynthesis, but light energy beyond what is utilized by photosynthesis, can lead to oxidative damage of the photosynthetic apparatus, and consequently result in the reduction of photosynthetic capacity. PSII reaction center protein D1 is particularly prone to photodamage, which leads to the irreversible photoinactivation of the entire reaction center (Demmig-Adams and and Adams, 1992). Consequently, the PSII-LHCII complexes disassemble, and the frequency of PSII-semi-crystalline arrays in the grana decreases (Kouřil et al., 2013). Restoration of PSII function after photoinhibition occurs via a complex repair cycle in which the photoinhibited PSII complexes migrate from the grana core to the non-appressed membrane regions, where the damaged D1 protein is degraded by specific proteases and replaced with a new copy (Aro et al., 1993). The majority of the auxiliary proteins involved in the PSII repair cycle are located in the non-appressed thylakoids (Suorsa et al., 2014). The PSII core phosphorylation plays an important role in the PSII repair cycle as the negative charge introduced by the phosphate group creates electrostatic repulsion, which loosens the macroscopic folding of the grana and thereby facilitates the disassembly of the damaged PSII complexes thus allowing their migration to the non-appressed membrane regions (Tikkanen et al., 2008b; Fristedt et al., 2009; Järvi et al.,

2015). The reversible phosphorylation of D1, D2 , CP43 and PsbH on Thr residues upon exposure to high light is catalyzed by the STN8 kinase (Vainonen et al., 2005; Bonardi et al., 2005), which is associated with the PSII-LHCII supercomplexes (Wunder et al., 2013b). The dephosphorylation of the PSII proteins by PSII CORE PHOSPHATASE (PBCP) (Samol et al., 2012) is a prerequisite for proteolytic degradation of the D1 protein (Rintamäki et al., 1996).

### *1.5.2 LHCII phosphorylation in short-term light acclimation*

In addition to the PSII core, also the LHCII antenna undergo reversible phosphorylation (Bennett, 1977). The phosphorylation of the N-terminal Thr residue of Lhcb1 and Lhcb2 proteins is catalyzed by the STN7 kinase (Bellafiore et al., 2005). The LHCII phosphorylation serves as an important short-term acclimation mechanism that ensures excitation energy balance between PSII and PSI in changing environmental conditions (Bennett et al., 1980; Tikkanen et al., 2006; Pesaresi et al., 2009). The redox-dependent kinase is located in close proximity of Cyt $b_6f$ and is activated by the binding of plastoquinol to the $Q_0$ site of the complex (Vener et al., 1997). Further, STN7 possesses four phosphosites, three Thr and one Ser residue, and the activity and turn-over of the kinase was recently discovered to be linked to the phosphorylation of the Ser and two of the Thr residues (Trotta et al., 2016). The activity of the STN7 kinase is also dependent on the stromal redox state (Rintamäki et al., 2000).

The counteracting phosphatase required for LHCII dephosphorylation was discovered recently by two independent groups and was named THYLAKOID-ASSOCIATED PHOSPHATASE OF 38 KDa / PROTEIN PHOSPHATASE 1 (hereafter TAP38) (Pribil et al., 2010; Shapiguzov et al., 2010). The type 2C Ser/Thr protein phosphatase (PP2C) recognizes specifically two basic residues (arginine and lysine) on the N-terminus of the LHCII proteins (Wei et al., 2015). The regulation of the phosphatase has remained elusive but it is known to be constitutively expressed and redox-independent (Pribil et al., 2010).

The role of LHCII phosphorylation has been traditionally studied by exposing plants to different wavelengths of light that specifically excite one photosystem over another (Allen and Forsberg, 2001; Rochaix, 2007). ‘State 2’ light (650/470nm) treatment preferentially excites PSII and results in concomitant maximal phosphorylation of LHCII and the PSII core, whereas treatment with ‘state 1’ light (far-red light) favors the excitation of PSI and results in complete dephosphorylation of LHCII and the PSII core (Tikkanen et al., 2010). ‘State 2’ light induced phosphorylation of LHCII leads to a dramatic increase of PSI absorption cross-section, which according to a *state transition* model is explained by the migration of the LHCII trimer, associated to PSII in the grana, to PSI complexes in the stroma thylakoids. The LHCII phosphorylation, indeed, induces

a formation of an isolatable PSI-LHCII i.e. “state transition” complex (Pesaresi et al., 2009). The phosphorylated LHCII trimer interacts with the PSI complex at LHCII docking site composed of the PsaH, PsaL, PsaO and PsaI proteins (Lunde et al., 2000; Mazor et al., 2015; Plöchinger et al., 2016). The phosphorylated LHCII transfers excitation energy preferentially to PSI and the PSI absorption cross-section therefore increases.

Under more natural white light illumination, the LHCII proteins are always moderately phosphorylated throughout the thylakoid membrane (Tikkanen et al., 2006) and a fraction of LHCII has been shown to function as an innate antenna for PSI (Galka et al., 2012; Kouřil et al., 2013; Wientjes et al., 2013a). Importantly and differently from artificial ‘state light’ treatments, fluctuations in white light intensity affect phosphorylation of the PSII core and LHCII proteins in an opposite manner (Rintamäki et al., 1997). Upon exposure to low light, the LHCII phosphorylation reaches a maximum, whereas the PSII core proteins are only minimally phosphorylated. A study based on gentle mechanical fractionation of the thylakoid membrane revealed that although a fraction of phosphorylated (p)LHCII migrates to stroma thylakoids, the PSI absorption cross-section remains unchanged in this fraction, whereas in the grana margins the energy flow to PSI is enhanced although the amount of LHCII remains unchanged (Tikkanen et al., 2008a). It was therefore suggested that the grana margins, which host both photosystems, might play an important role in the regulation of light harvesting under light limiting conditions. Although it is clear that phosphorylation modulates the interaction of LHCII with the photosystems, it remains elusive, how it affects the organization of the entire protein complex network and the ultrastructure of the thylakoid membrane system.

Upon exposure of plants to high light, the STN8 kinase becomes fully activated and the PSII core phosphorylation extends to the maximum (Rintamäki et al., 1997). Concomitantly, the STN7 kinase become deactivated and LHCII becomes dephosphorylated. Despite the high-light-induced changes in PSII-LHCII phosphorylation, the relative excitation of PSII and PSI remains rather unchanged (Tikkanen et al., 2010) and the physiological role of the opposing phosphorylation of the PSII core and the LHCII antenna in high light remains poorly understood.

## 1.6 Current methods in the analysis of membrane protein complexes

Blue-native PAGE (BN-PAGE) is a gel-electrophoresis system that is well suited for the analysis of the composition and dynamics of the thylakoid pigment-protein complexes. The method was originally developed for the analysis of mitochondrial respiratory complexes (Schägger and von Jagow, 1991) and later adapted and further optimized for

the analysis of thylakoid membrane protein complexes (Kügler et al., 1997; Järvi et al., 2011). Prior to separation by native-PAGE, the protein complexes are isolated from the thylakoid membrane. The isolation mainly relies on the use of surface-active agents that destabilize the membrane and bind to the hydrophobic sectors of the isolated proteins. Mild nonionic detergents, such as α and β-dodecyl maltoside (β-DM) or digitonin (DIG), are typically used for isolation of native protein complexes from the thylakoid integrity (Aro et al., 2005; Caffarri et al., 2009; Järvi et al., 2011). β-DM solubilizes the protein complexes from the entire thylakoid membrane, but it destroys weak hydrophobic interactions between protein complexes (Wittig et al., 2006). DIG preserves labile interactions between protein complexes (Schägger and Pfeiffer, 2000), but the bulky structured DIG exclusively solubilizes protein complexes from the non-appressed regions of the membrane as the tight packing of the grana prevents the solubilization of the appressed regions (Järvi et al., 2011). Thus, the analysis of the entire thylakoid network is achieved only with slightly stronger detergents, such as β-DM, but this occurs at the cost of losing the labile hydrophobic interactions between individual protein complexes.

# 2. AIMS OF THE STUDY

Although high-resolution structures of photosynthetic protein complexes are available and important details on the molecular mechanisms behind the thylakoid protein phosphorylation have been reported during the past few decades, the dynamic organization of the photosynthetic protein complexes in the thylakoid membrane upon short-term light acclimation still remains elusive. The aim of my PhD work was to characterize the organization of the pigment-protein complexes in thylakoid membrane and elucidate the interactions between the protein complexes in response to short-term changes in light intensity. The specific aims were to:

(i) improve the protein isolation and separation methods for investigation of thylakoid pigment-protein complexes and their interactions,

(ii) determine the supramolecular organization and hierarchical formation of photosynthetic protein super- and megacomplexes

(iii) dissect the influence of thylakoid protein phosphorylation on the interactions of individual protein complexes and on the overall organization of the protein complex network.

# 3. METHODOLOGY

## 3.1 Plant material and standard growth conditions

A small flowering plant *Arabidopsis thaliana* was used in the experiments. Arabidopsis is one of the most important model organisms used in plant biology and the first plant to have its complete genome sequence available (Arabidopsis Genome Initiative, 2000). Importantly, several mutant lines are publicly available making the plant ideal for studying protein functions. *Arabidopsis thaliana* ecotype Columbia wildtype (WT) as well as several mutant lines (see table 1) were grown in a mixture of soil and vermiculite (1:1) under a photon flux density of 120 μmol photons $m^{-2}s^{-1}$ in an 8 h light/16 h dark regime (Growth light, GL) at 23°C. OSRAM PowerStar HQIT 400/D Metal Halide Lamps served as a light source. Mature rosette leaves from 5- to 6-week-old plants were used for the experiments.

**Table 1.** The Arabidopsis mutant lines used in this research. Description of the mutants can be found in the given references.

| Mutant | Defective protein | Publication | Reference |
|---|---|---|---|
| stn7 | STN7 kinase | I,II,III | Bellafiore et al., 2005 |
| stn8 | STN8 kinase | I,II,III | Bonardi et al., 2005; Vainonen et al., 2005 |
| tap38 | TAP38/PPH1 phosphatase | I.II,III | Pribil et al., 2010; Shapiguzov et al., 2010 |
| psal | PsaL | III | Lunde et al 2000 |

## 3.2 Light treatments, thylakoid isolation and chl determination

For specific experiments, the plants were treated with darkness, LL (20 μmol photons $m^{-2}s^{-1}$) or HL (800-1000 μmol photons $m^{-2}s^{-1}$) prior to membrane isolation (Publications I,II,III). The treatments with PSI and PSII specific lights (Publications I and II) were given as described in (Tikkanen et al., 2010). Thylakoid membranes from WT and the mutant lines were isolated from fresh leaf rosettes according to (Järvi et al., 2011). The chlorophyll content of the isolated thylakoids was determined according to (Porra et al., 1989).

## 3.3 Protein analysis with SDS-PAGE

Denaturating SDS-PAGE was used in all Publications for the analysis of thylakoid protein composition. Solubilized thylakoid proteins were separated on SDS-PAGE according to standard protocols described in Publications I-IV. Individual proteins were detected by Western blotting using protein or P-Thr specific antibodies (detailed

information of the used antibodies in the original Publications). The membranes were stained with Coomassie Brilliant Blue (CBB) to ensure equal (chlorophyll-based) loading.

## 3.4 Analysis of native protein complexes

For the analysis of native protein complexes, weak non-ionic detergents were used to gently isolate the complexes from the membrane without destroying their native structure. The protein complex solubilization was done essentially as described in (Järvi et al., 2011). β-D-dodecyl maltoside (β-DM) was used in all Publications, digitonin (DIG) was used in Publications I, III and IV. The detergents were diluted in bis-tris buffer (BTH) in all Publications, but in Publication IV aminocaproic acid (ACA) was used with DIG in the solubilization buffer.

The solubilized protein complexes were supplemented with Coomassie G-250 dye, which introduces negative charge to the protein complexes, which were separated according to their molecular mass with large pore blue native gel electrophoresis (lpBN-PAGE) (Schägger and von Jagow, 1991; Wittig et al., 2006; Järvi et al., 2011). The distinct subunits were resolved with two-dimensional (2D)-SDS-PAGE according to Järvi et al., (2011). In Publication I, the individual proteins separated by 2D-SDS-PAGE, were identified with mass spectrometry. In Publication IV, a new 3D-gel-electrophoresis system (lpBN-lpBN-SDS-PAGE) was optimized for the analysis of the subcomplex composition of larger protein complexes (detailed description in the original Publication IV). The proteins were visualized with silver staining (Blum et al., 1987) or SYPRO® Ruby staining according to Invitrogen Molecular Probes™ instructions.

## 3.5 77K chl fluorescence measurements

To characterize the state of the photosynthetic machinery, a low-temperature (77K, -196˚C) fluorescence emission spectrum was recorded. At this temperature, intact thylakoid membranes exhibit three major fluorescence peaks at F685, F695 (from PSII) and around F735 (from PSI). Disconnected LHCII antennas exhibit a strong fluorescence emission peak at 680 nm. The chlorophyll fluorescence spectra were recorded at 77K from intact thylakoids (Publications I, IV) and from supernatants after detergent-treatment (3 µg chl/100 µl storage buffer) (Publication IV) with an Ocean Optics S2000 spectrometer. Thylakoids were kept in liquid nitrogen and excited with blue light (480 nm wavelength). The fluorescence signal of intact thylakoids was normalized to 685 nm and that of the solubilized thylakoids to the PSI peak.

## 3.6 Transcript analysis

For the analysis of *TAP38* transcripts (Publication III) the cDNA was synthesized from purified RNA using iScript reverse transcriptase (Bio-Rad). RT-PCR reactions were performed with the BioRad IQ5 detection system using IQ SYBR Green Supermix (Bio-Rad) containing a fluorescent dye to detect the accumulation of amplicons. UBIQUITIN 3 and PP2AA3 were used as internal controls. Data were analyzed by using QBASE PLUS software

# 4. OVERVIEW OF THE RESULTS

## 4.1 Photosynthetic protein megacomplexes and regulatory proteins in the non-appressed regions of the thylakoid membrane

The composition of photosynthetic protein complexes in non-appressed thylakoid regions was analyzed by using a gentle detergent DIG for its ability to solubilize exclusively these membrane regions (Publication I). The chl a/b ratio of the DIG-soluble fraction, being around 4, was lower than what would be expected from the pure stroma thylakoids, indicating that the fraction also contains the interphase of the grana core and stroma thylakoids, i.e. the grana margins (Publication I). In line with an earlier report by (Järvi et al., 2011), the analysis of protein complexes with lpBN-PAGE confirmed the presence of several high molecular mass protein megacomplexes in this region of the thylakoid membrane. The megacomplexes were particularly abundant with PSI but the largest protein complex also contained PSII. Proteomic analysis of the non-appressed thylakoid fraction revealed that besides the structural subunits of the photosynthetic machinery, several regulatory proteins including PsbS, CURT1A and CURT1B were present (Publication I).

## 4.2 Reversible LHCII phosphorylation and protein complex reorganization upon changes in light intensity

### *4.2.1 STN7-dependent LHCII phosphorylation regulates protein complex interactions*

To elucidate how LHCII phosphorylation affects the interactions of the protein complexes in the non-appressed thylakoid regions, mutant lines defective in thylakoid protein (de)phosphorylation were analyzed from growth light acclimated plants. Protein complex analysis with lpBN-PAGE revealed that in the absence of functional STN7 kinase, the inability to phosphorylate LHCII proteins greatly affected the protein complex pattern of the *stn7* mutant (Publication I). The *stn7* mutant was not only unable to accumulate the PSI-LHCII complex (Pesaresi et al., 2009), but also the larger protein complexes were qualitatively different when compared to growth light acclimated WT plants, which exhibited highly phosphorylated LHCII (Publication I). In WT, a large fraction of PSI was allocated to the largest protein megacomplex, which also contained PSII, whereas in the *stn7* mutant the complex was less abundant and instead, a large amount of PSI was found in smaller supercomplexes (Publication I). To validate that the

peculiar organization of the thylakoid protein complexes in the *stn7* mutant was linked to dephosphorylated LHCII, and not to other features of the mutant, the effect of light-quality induced LHCII (de)phosphorylation in WT plants was analyzed. Illumination with 'state 1' light resulted in complete LHCII dephosphorylation and was followed by protein complex rearrangement, which resembled that of the *stn7* mutant. On the contrary, upon 'state 2' light-induced LHCII phosphorylation, the protein complex organization was similar to that observed in growth light acclimated WT (Publication I). It is therefore highly conceivable that protein complex rearrangements are linked to the STN7-mediated phosphorylation of the LHCII proteins.

In order to elucidate the role of LHCII phosphorylation in the modification of protein complex interactions in response to natural fluctuations of white light, LHCII phosphorylation dynamics and the consequent alterations in protein complex organization was analyzed from WT plants exposed to different light intensities (Publication I). The LHCII proteins were almost completely dephosphorylated after a 16 h period in darkness, but a subsequent short-term treatment in low light resulted in maximal LHCII phosphorylation and a following shift to high light resulted again in LHCII dephosphorylation (Publication I). Importantly, the protein complex pattern in dark acclimated and high light treated plants, both exhibiting dephosphorylated LHCII, was similar to that observed in *stn7*. The pattern of the low light treated WT plants resembled that of the growth light acclimated WT. This implies that the changes in protein complex interactions in non-appressed membrane regions is affected by LHCII phosphorylation also under natural changes in white light intensity (Publication I).

### *4.2.2 Role and regulation of TAP38 phosphatase in high light*

Upon exposure of plants to high light the PSII core proteins reach their maximal phosphorylation, whereas the LHCII proteins become concomitantly dephosphorylated (Rintamäki et al., 1997). To understand the functional significance of the opposite PSII-LHCII phosphorylation and the regulation of the LHCII phosphatase, WT plants and mutants lacking the LHCII phosphatase (*tap38*) or the LHCII docking site in PSI (*psal*) were analyzed (Publications II and III). The analysis of the *tap38* mutant demonstrated that in the absence of the TAP38 phosphatase, the LHCII proteins remained phosphorylated even upon exposure of plants to high light. Yet, the PSII core proteins were simultaneously maximally phosphorylated in the *tap38* mutant under high light. The resulting extensive phosphorylation of both the PSII core and the LHCII proteins is never observed in WT under high light. The 77K chl fluorescence spectra revealed that the coinciding PSII-LHCII phosphorylation in high light treated *tap38* resulted in a remarkable increase in the PSI absorption cross section, similar to that observed in WT upon illumination with an artificial 'state 2' light. In contrast, the shift of WT plants from low to high light induced only minimal changes in the relative excitation of the two

photosystems as a result of the LHCII dephosphorylation. These results provide compelling evidence that the phosphatase activity of TAP38 is needed in high light to maintain the excitation energy balance between the two photosystems (Publication II).

In Publication III it was demonstrated that the *psal* mutant, which completely lacks the LHCII docking site in PSI, is unable to accumulate TAP38 phosphatase. The marked down-regulation of the phosphatase did not take place at the transcriptional level, which implies that either the *TAP38* transcripts are poorly translated or the TAP38 protein is easily degraded (Publication III). Since the TAP38 phosphatase is responsible for the dephosphorylation of pLHCII, which interacts with PSI, the PSI-LHCII complex was assumed to be an association partner for the phosphatase. The absence of the PSI-LHCII complex in *psal* thus might induce instability and higher degradation rate for the phosphatase. To reveal the possible association of TAP38 with the PSI-LHCII complex or with other photosynthetic protein complexes, the localization of the TAP38 phosphatase was studied. Two-dimensional lpBN-SDS-PAGE analysis revealed that under low light conditions, the majority of TAP38 is present as a free protein unbound to any of the large membrane protein complexes. Nevertheless, upon subsequent exposure to high light, a fraction of TAP38 was found associated with the PSII-LHCII-PSI complex and concomitantly the pool of free phosphatases decreased (Publication III). Thus, although the TAP38 phosphatase in WT is constitutively expressed, its activity or stability might be dependent on its dynamic localization and interaction with the megacomplex.

## 4.3 Optimized detergent-treatment allows one-step solubilization of labile thylakoid protein complexes

As described above, DIG is a superior detergent when analyzing labile membrane protein super- and megacomplexes in the non-appressed regions of the thylakoid membrane. Generally DIG is excluded from grana core, which is thus left insolubilized and remains in the pellet (Publication I). To dissect the labile protein complex network from the entire thylakoid membrane system, specific attempts were taken towards improving the solubilization capacity of the thylakoid membrane by DIG. Excellent progress was achieved when DIG was applied in the presence of ACA to solubilize the thylakoid membrane. Intriguingly, based on the chlorophyll content and the chl-a/b ratio of the solubilized membrane fraction, it was demonstrated that DIG/ACA solubilizes the entire thylakoid membrane without discrimination between different membrane subdomains (Publication IV). DIG/BTH treatment typically solubilizes only 40% of the total membrane protein complexes, whereas the DIG/ACA treatment resulted in the solubilization of more than 90% of the thylakoid membrane complexes. The chl a/b ratio was similar to that of intact thylakoids, indicating non-selective solubilization

(Publication IV). It is suggested that ACA allows DIG to enter to the tightly appressed grana core. Importantly, recording the relative fluorescence of PSI and PSII by 77K chl fluorescence revealed that the addition of ACA does not lead to the randomization of the thylakoid protein complexes (Publication IV).

Several large protein super- and megacomplexes were obtained by lpBN-PAGE separation of the DIG/ACA solubilized thylakoids. The most abundant protein complexeses included the PSII-LHCII-PSI megacomplex, two C2S2M(2) supercomplexes, which co-migrated with PSI supercomplexes and finally, the PSI-LHCII complex. The protein complex patterns qualitatively resembled each other in DIG/ACA and DIG/BTH solubilized thylakoid samples. Nevertheless, the quantities of the individual complexes were different and provided an estimation of the sublocation of the protein complexes in the thylakoid membrane network. The two PSII-LHCII supercomplexes were considerably more abundant in the DIG/ACA treated thylakoid samples (Publication IV) than in the DIG/BTH samples that represents only the non-appressed membrane regions (Publication I). Thus, these supercomplexes likely reside in the grana core and represent large C2S2M(2) complexes (Publication IV). The PSII-LHCII-PSI megacomplex and the PSI-LHCII complex were abundant in the DIG/BTH solubilized thylakoid membranes thus providing evidence that these complexes are enriched in the non-appressed membrane regions (Publication I).

None of the isolated protein super- or megacomplexes contained the Cyt $b_6f$ complex or ATPase, which co-migrated in the gel with the PSII dimer and PSII monomer, respectively (Publication II and IV). Since Cyt $b_6f$ functionally interconnects the two photosystems and it is a crucial component of the electron transfer chain, it is probably located in the near proximity of the PSII-LHCII-PSI, although it does not appear to have physical interaction with the photosystems.

## 4.4 The role of LHCII in hierarchical formation of large protein complex assemblies

In Publication IV, I addressed the role of LHCII in the formation of large protein super- and megacomplexes. Although detergents always disconnect a fraction of LHCII from the thylakoid membrane integrity, DIG was shown to preserve a substantial fraction of LHCII still connected to the photosystems when compared to the commonly used detergent β-DM (Publication IV) or α-DM (unpublished). Importantly, the ability of DIG to preserve large protein super- and megacomplexes co-occurred with its ability to retain more LHCII bound to protein complexes as demonstrated by a relatively low fluorescence peak at 680 nm in the 77K chl fluorescence spectra, (Publication IV).

To elucidate the role of LHCII in preserving the supramolecular structures, I applied a two-step solubilization of the protein complexes from the entire thylakoid membrane. The protein complexes were first solubilized with DIG/ACA and separated on lpBN-PAGE. Subsequently, the gel lane containing the DIG/ACA separated protein complexes was treated with β-DM and the subcomplexes were separated on an orthogonal 2D-lpBN-PAGE (Figure 5A). During the second lpBN-PAGE, a fraction of M-LHCII and L-LHCII disconnected from the large DIG/ACA-derived protein complexes. Concomitantly with the release of LHCII, (i) the large PSII-LHCII-PSI megacomplex disassembled into smaller PSII and PSI subcomplexes, (ii) C2S2M(2) supercomplexes broke down into smaller PSII complexes and PSI monomers were released from the PSI supercomplexes. Finally, (iii) PSI-LHCII, i.e. the 'state transition' complex was fractioned into PSI monomer and L-LHCII-trimer (Publication IV). The PSI complex, PSII dimer, ATPase, Cyt $b_6f$ and PSII monomer retained their masses during the second solubilization.

Based on the results described above, it is concluded that DIG/ACA solubilization preserves LHCII interconnection with the photosystems and allows the analysis of supramolecular protein complex assemblies whereas the subsequent solubilization of the DIG/ACA derived complexes with β-DM allows the dissection of the hierarchical interactions of protein subcomplexes (Publication IV).

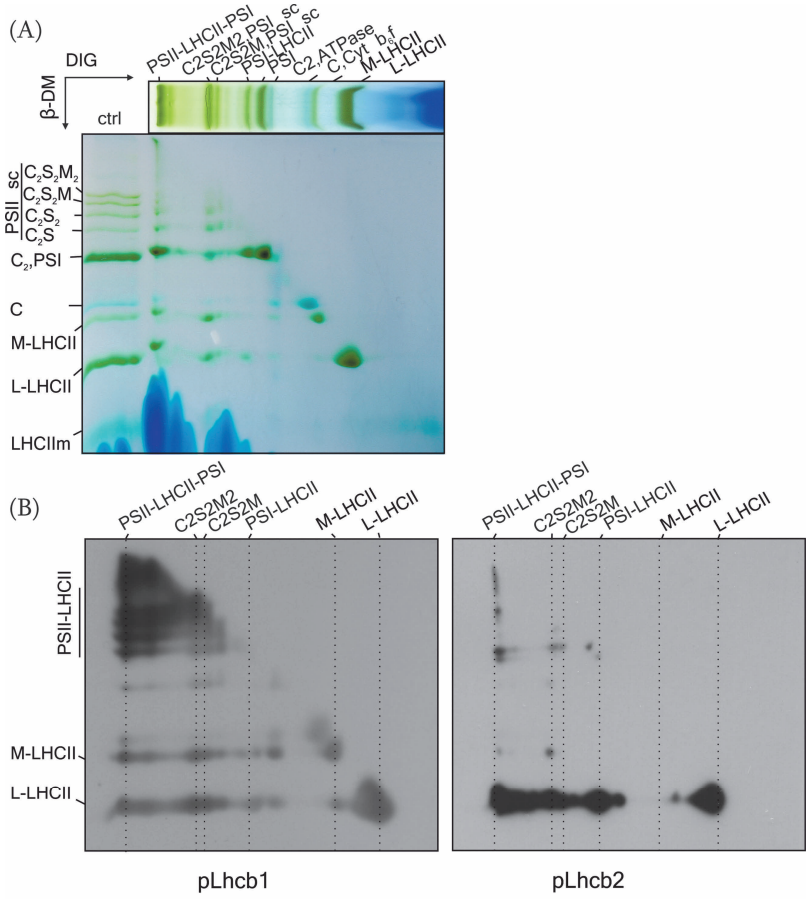


**Figure 5.** The subcomplex composition and LHCII phosphorylation of DIG-derived protein complexes. (A) Thylakoid protein complexes solubilized with DIG/ACA were separated on 1D-lpBN-PAGE and subsequently re-solubilized by β-DM and subjected to a second lpBN-PAGE to separate the disconnected subcomplexes. The control lane represents β-DM solubilized thylakoid membranes. (B) Western blot analysis of the 2D-lpBN gels demonstrates the localization of the phosphorylated Lhcb1 and Lhcb2 proteins.

## 4.5 Role of differential Lhcb1/Lhcb2 protein phosphorylation in protein super- and megacomplexes

To elucidate the role of distinct LHCII protein phosphorylation for the protein complex organization, the location of P-Lhcb1 and Lhcb2 in different protein super- and megacomplexes was analyzed from the thylakoids of growth light acclimated plants by immunoblotting of 2D-lpBN-gels (Publication IV, Figure 5B). Intriguingly, Lhcb2 was found phosphorylated almost exclusively in the L-LHCII trimers, which were disconnected from the megacomplexes during the second solubilization with β-DM. Lhcb1, on the contrary, was highly phosphorylated in the PSII-LHCII supercomplexes.

# 5. DISCUSSION

The photosynthetic machinery is composed of pigment-protein complexes that work in series to convert solar energy into chemical energy. Function of the system requires highly organized and regulated network of the protein complexes. There has been great progress in resolving the structures of individual complexes, but the overall organization of the photosynthetic protein complexes has remained largely elusive. Comprehensive examination of the labile thylakoid protein complex networks in this thesis confirmed that the majority of both photosystems and LHCII hierarchically form large protein super- and megacomplexes. The organization of the entire protein complex network is dictated by the reversible phosphorylation of the PSII and LHCII proteins. In the following two sections, I examine the obtained results in light of the recent literature and aim at integrating the thylakoid regulatory processes with the dynamic re-arrangements of the photosynthetic protein complexes.

## 5.1 Building blocks of thylakoid protein super-and megacomplexes

Structural analysis of thylakoid protein complexes mainly rely on the use of non-ionic detergents, which isolate the complexes from the membrane integrity by dispersing and replacing membrane lipids associated with the hydrophobic parts of the protein complexes. Detergents, most commonly α- and β-DM, have been successfully used to obtain the structural details of photosynthetic protein complexes, such as the PSII-LHCII complexes (Hankamer et al., 1997; Boekema et al., 1999; Eshaghi et al., 1999; Caffarri et al., 2009) and the PSI-NDH-supercomplex (Kouřil et al., 2014). DM, however, is not well suited for the characterization of the overall organization of the complexes in the thylakoid membrane, since it efficiently disconnects LHCII from the thylakoid integrity. For the analysis of labile protein super-and megacomplexes (Publications I, IV) a neutral detergent, DIG, was used for its ability to maintain weak interactions between protein complexes (Schägger and Pfeiffer, 2000). In Publication I, DIG was successfully used for the analysis of large protein complexes in the non-appressed thylakoid regions, yet supplementing DIG with ACA in the solubilization buffer (Publication IV) allowed, for the first time, one-step dissection of labile protein complexes from the entire thylakoid membrane.

Comparison of the protein complex composition of the non-appressed membrane regions (Publication I) with that obtained from the entire thylakoid membrane (Publication IV), allowed a general estimation of the localization of the protein complexes. In accordance with several other reports (Boekema et al., 1999; Caffarri et al., 2009), the majority of

PSII was found allocated to C2S2M(2) supercomplexes that predominate the grana core. The PSII-LHCII supercomplexes have been further shown to form larger megacomplexes, which may also contain additional LHCII trimers (Nosek et al., 2017). PSI complexes, as expected, were found enriched in the non-appressed membrane regions (Publication I). While a great quantity of PSI is present as a monomer, probably located in the stroma exposed membranes, a considerable fraction of PSI was discovered to be allocated to larger protein super- and megacomplexes (Publication I and IV). PSI was not only found in the PSI-LHCII complex (previously described by Pesaresi et al., 2009), but also in two larger PSI supercomplexes that are of similar size as the two C2S2M(2) supercomplexes (Publication IV). These complexes are postulated to represent PSI multimers (Galka et al., 2012; Yadav et al., 2017), but whether these are artefacts or physiologically relevant complexes, remains to be elucidated. The supercomplexes might also represent larger PSI-LHCII complexes in which more than one copy of L-LHCII trimers is associated to a single PSI monomer. Such association of several L-LHCII trimers with PSI has recently been suggested in several reports (Bell et al., 2015; Benson et al., 2015; Bos et al., 2017). Importantly, a large fraction of PSI and PSII-LHCII together with L-LHCII was discovered to be integrated in a mutual megacomplex (Publications I and IV), in line with earlier observation by (Järvi et al., 2011; Yokono et al., 2015). Such a megacomplex likely resides in the grana margins, which has been shown to host both photosystems (Svensson and Albertsson, 1989; Albertsson et al., 1990). The exact structure and stoichiometry of the complex remains to be resolved, but the size of the complex has been estimated to be >2.6 MDa, and the PSII to PSI ratio within the complex to be 1:1 (Yokono et al., 2015; Yokono and Akimoto, 2018). There is no consensus in the field whether the PSII-LHCII-PSI complex has a functional role or whether it is an artefact. Traditionally the two photosystems have been considered to be strictly spatially segregated in the thylakoid membrane. However, lately several reports have suggested that the photosystems become interconnected in grana margins via mutual LHCII antenna “lake” (Grieco et al., 2015; Benson et al., 2015; Rantala and Tikkanen, 2018) and I suggest that the isolated PSII-LHCII-PSI complex originates from such network.

To better understand the interactions between individual protein complexes within larger super- and megacomplexes, I optimized a two-step solubilization of the thylakoid membrane using two different detergents, DIG/ACA and β-DM (Publication IV). The first solubilization step was performed with DIG/ACA, which solubilizes the entire thylakoid network and preserves the supramolecular protein complexes. The second solubilization step with β-DM was shown to disconnect all L-LHCII and a large fraction of M-LHCII from the photosystems (Figure 6). The disconnection of M-LHCII, as expected, affects the stability of the C2S2M2 supercomplexes, whereas the L-LHCII disconnection detaches all PSI complexes from the super- and megacomplexes.

Importantly, the L-LHCII disconnection co-occurred with the disassembly of the two photosystems from the PSII-LHCII-PSI complex. Although it is not possible to confirm that the disconnection of LHCII precedes the disassembly of the two photosystems, I suggest that L-LHCII plays an essential role in mediating the interaction between PSII-LHCII and PSI. It is noteworthy that the amount of L-LHCII bound to the PSII-LHCII-PSI complex was significantly higher when compared to the amount of L-LHCII bound to the 'state transition' complex. It is therefore conceivable that the L-LHCII trimer bound to PSI in the 'state transition'-complex comprises only a minor fraction of the total L-LHCII associated with the photosystems.

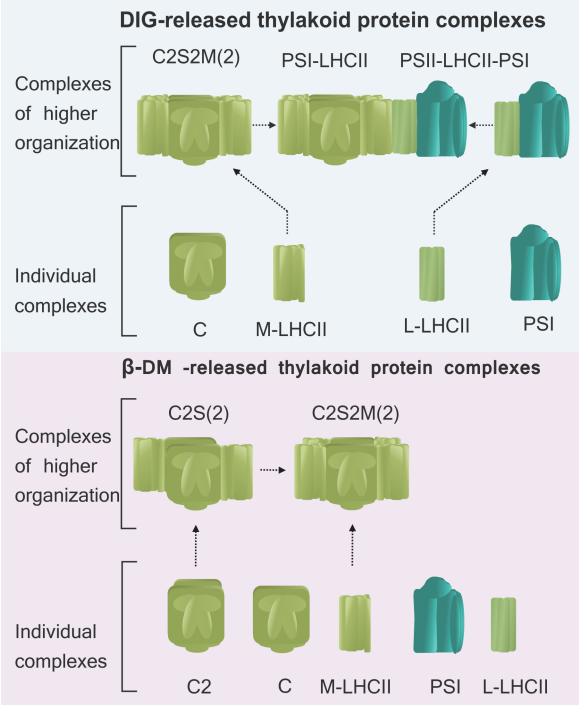


**Figure 6.** Schematic representation of the hierarchical formation of large thylakoid protein complexes via LHCII antenna. Detergents always remove a fraction of LHCII from the thylakoid integrity, yet DIG (upper panel) still preserves a large amount of LHCII interconnected with the photosystems. This is indicated by the preservation of the labile PSII–LHCII–PSI mc, C2S2M(2) sc. and PSI–LHCII sc. Unlike DIG, β-DM (lower panel), detaches all PS-bound L-LHCII, and the labile protein complexes dissociate during solubilization. β-DM further detaches some of the moderately bound M-LHCIIs from C2S2M2 complexes, thus producing smaller C2S2M/C2S2 supercomplexes and PSII dimer (C2). A fraction of PSII core monomer (C) and PSI are present as individual complexes after both detergent treatments.sc=supercomplex, mc=megacomplex.
Modified from Publication IV

It is important to note that L-LHCII is only loosely connected to the photosystems and majority of the loosely bound LHCII is inevitably lost when detergents are used (Grieco et al., 2015). Even DIG detaches large amount of LHCII from the thylakoid integrity and the PSII-LHCII-PSI megacomplex, which we obtained with DIG, therefore represents only a fraction of a large network in which the two photosystems are interconnected by a mutual L-LHCII lake. The whole complexity of the intact protein complex network still remains to be elucidated. An alternative approach, which recognizes the importance of the native lipid environment of the membrane protein complexes, has been introduced. Small organic polymer, styrene maleic acid (SMA), has been successfully used to isolate membrane nanodiscs in which protein complexes are embedded (Knowles et al., 2009;

Lee et al., 2016). SMA was recently shown to preserve LHCII energetically connected to the photosystems (Bell et al., 2015; Bos et al., 2017), implying that the native lipid environment is crucial for keeping LHCII interconnected within the thylakoid protein network. Further research is required to obtain more insights into the protein-lipid-interactions and their influence on the interactions between the thylakoid protein complexes.

Although the detergent-based protein complex analysis exploited in my thesis does not allow the analysis of the entirely intact protein complex network, it provides novel insights into the hierarchical interactions of the protein complexes, which reflect the overall organization of the photosynthetic apparatus in the thylakoid membrane.

## 5.2 Phosphorylation-dependent dynamic interactions of the protein complexes

Hierarchical interactions of the thylakoid protein complexes, as discussed above, are dynamic and dependent on environmental cues, most importantly on the changes in light conditions. The quantity and quality of light does not only fluctuate on seasonal and diurnal scale, but plants have to cope with rapid changes taking place in a scale of seconds and minutes in order to maintain fluent photosynthesis (Colombo et al., 2016). The light-dependent thylakoid protein phosphorylation is an important regulatory mechanism that allows plants to cope with ever-changing light conditions. The phosphorylation of the light harvesting proteins (Lhcb1 and Lhcb2) and the PSII core proteins (D1,D2, CP43) are catalysed by two different kinases, the STN7 (Bellafiore et al., 2005) and the STN8 kinase (Vainonen et al., 2005), respectively. The dephosphorylation of the LHCII proteins is catalysed by the TAP38 phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010) and the PSII core proteins by the PBCP phosphatase (Samol et al., 2012). The LHCII phosphorylation is known to modulate the interaction of LHCII with the two photosystems, whereas the PSII core phosphorylation facilitates the disassembly of the PSII-LHCII complexes during the PSII repair cycle (Rochaix, 2007; Pesaresi et al., 2011; Tikkanen et al., 2008b). In this thesis, the role of the protein phosphorylation-dependent regulatory processes in the thylakoid reorganization was assessed by studying both wild-type plants treated with different light intensities and mutant lines lacking the regulatory proteins. In the next paragraphs, I will consider the phosphorylation dynamics of LHCII and PSII proteins and the consequent reorganization of the thylakoid protein complexes, first upon shift from darkness to light, and then upon exposure to high light.

Upon shift from darkness to light, LHCII proteins become phosphorylated (Publication I) due to the activation of the STN7 kinase. Analysis of the localization of the pLhcb1 and pLhcb2 proteins from the entire thylakoid membrane revealed that Lhcb2

phosphorylation occurs almost exclusively in L-LHCII trimers, which are loosely bound to the protein super- and megacomplexes (Publication IV, Figure 5B). Recent reports have shown that the phosphorylation of the Lhcb2 protein is required for the formation of the PSI-LHCII complex and, more specifically, the phosphate group in the N-terminus of the Lhcb2 protein mediates the interaction of the L-LHCII trimer with the LHCII docking site in the PSI complex (Leoni et al., 2013; Pietrzykowska et al., 2014; Crepin and Caffarri, 2015; Longoni et al., 2015). In addition to the formation of an isolatable PSI-LHCII complex (Pesaresi et al., 2009), also the accumulation of the aforementioned PSII-LHCII-PSI megacomplex in the grana margins (section 5.1) is largely dependent on LHCII phosphorylation (Publication I). Upon complete LHCII dephosphorylation, as demonstrated by the *stn7* mutant, the amount of the PSII-LHCII-PSI megacomplex is clearly reduced, whereas the amount of large PSI supercomplexes concomitantly increased. A similar interdependence of the megacomplex formation was observed upon light induced LHCII phosphorylation (Publication I). Therefore it seems conceivable that the L-LHCII phosphorylation, occurring mainly at the Lhcb2 protein, is required for the stability of the PSII-LHCII-PSI complex.

Different from the location of pLhcb2, pLhcb1 was shown to be located predominantly in PSII-LHCII supercomplexes (Figure 5B). This is in line with recent report, which further showed that the Lhcb1 phosphorylation takes place mainly in the S-LHCII, and mostly in PSII-LHCII supercomplexes that are located near to the grana margins (Crepin and Caffarri, 2015). Lhcb1 phosphorylation also plays a role in the balancing of excitation energy between the two photosystems but importantly, the LHCII phosphorylation in PSII-LHCII supercomplexes does not induce their disassembly (Wientjes et al., 2013b). Thus, the phosphorylated S- or M-LHCII trimers are not directly involved in the formation of PSI-LHCII complexes. Instead, the Lhcb1 phosphorylation has been suggested to have a structural role in mediating the L-LHCII-PSI interaction (Pietrzykowska et al., 2014). Several alterations in the thylakoid ultrastructure take place when plants are shifted from darkness to light. The grana diameter decreases and the amount of appressed stacks per grana become reduced, but simultaneously the total proportion of grana per chloroplast increases (Rozak et al., 2002; Anderson et al., 2012) and the vertical stacking in the grana margins is loosened (Chuartzman et al., 2008). These changes have been proposed to arise from LHCII phosphorylation (Anderson et al., 2012), but also PSII core phosphorylation affects the grana ultrastructure.

Based on the phosphorylation dynamics (Publication I) and on the location of phosphorylated Lhcb1 and Lhcb2 proteins in protein super- and megacomplexes (Publication IV), as well as on the recent literature, I suggest the following model for the dynamic organization of thylakoid protein complexes. (i) Phosphorylation of Lhcb1 in PSII-LHCII complexes (Publication IV) upon exposure of plants to light, increases the flexibility of the grana membranes and brings a fraction of the PSII-LHCII

supercomplexes towards the edges of the grana. The proportion of this region, the grana margins, increases upon shift from darkness to light (Rozak et al., 2002) and further, LHCII phosphorylation in the grana margins disturbs the attractive forces between the adjacent grana membrane layers, causing the layers to retract from each other (Chuartzman et al., 2008). (ii) Subsequent opening of the edges of the grana allows a larger fraction of PSI complexes to become into contact with the PSII-LHCII supercomplexes via L-LHCII trimers (Publication I and IV, Figure 7A). The L-LHCII trimers are highly phosphorylated at Lhcb2 protein and therefore efficiently transfer energy to PSI (Galka et al., 2012), thus allowing fluent electron transfer between the photosystems. In line with previous suggestions (Tikkanen et al., 2008a; Anderson et al., 2012), the phosphorylation-dependent balancing of the excitation energy distribution between PSII and PSI likely takes place in grana margins. Importantly, the protein complex rearrangements upon LHCII phosphorylation are much more profound than previously anticipated and it is conceivable that the re-organization concerns the entire protein complex network rather than only a minor pool of mobile LHCII antenna. Mutant that lacks the STN7 kinase, fail to accumulate the PSII-LHCII-PSI megacomplex and consequently exhibit smaller PSI supercomplexes (Figure 7B), being unable to allocate PSI to the megacomplex (Publication II).

Contradicting to the above described model, it has been suggested that the formation of the PSII-PSI megacomplex is not dependent on LHCII phosphorylation, and the megacomplex was proposed to be a high light adaptation (Yokono et al., 2015). While this possibility cannot be ruled out, the data presented in this thesis, clearly demonstrate the essential role of L-LHCII to the stability of the PSII-LHCII-PSI complex and the dependence of the complex on LHCII phosphorylation. Since LHCII phosphorylation regulates the proportions of PSII-LHCII and PSI complexes in a mutual megacomplex, it is conceivable that balancing the excitation energy distribution relies on the dynamic organization of the PSII and PSI complexes (Publication I)

It has to be noted that the presented model of dynamic re-organizations of the protein complexes in the thylakoid membrane (Figure 7A) is a simplified 2D model that does not take into account the whole complexity of the thylakoid ultrastructure. Extensive research on thylakoid folding is still required to truly understand the relationship between the biochemical data obtained by analysis of the protein complex organization and the dynamics of thylakoid ultrastructure.

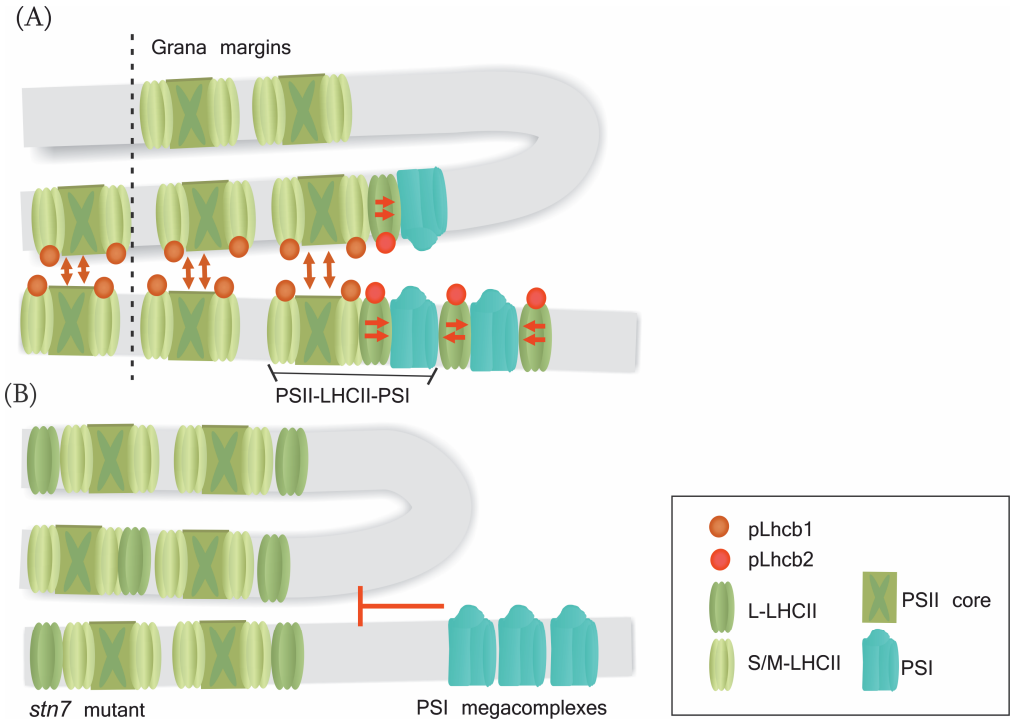


**Figure 7**. The influence of Lhcb1 and Lhcb2 phosphorylation on the organization of the two photosystems in grana margins. (A) The Lhcb1 phosphorylation in PSII-LHCII supercomplexes increases the membrane flexibility (membrane retraction indicated with red arrows) in the edges of grana. The Lhcb2 phosphorylation, which occurs exclusively in L-LHCII is followed by the accumulation of PSII-LHCII-PSI complex and facilitates the fluent electron transfer between the two photosystems. The blue arrows indicate the affinity of L-LHCII towards PSI. (B) The *stn7* mutant exhibits dephosphorylated Lhcb1 and Lhcb2 proteins, and is unable to preserve PSII-LHCII-PSI megacomplexes.

In Publications II and III, the role of TAP38 mediated LHCII dephosphorylation and the regulation of the phosphatase itself in high light, were addressed. Upon exposure to high light, plants exhibit a spectrum of responses in order to avoid damage caused by the excess of light. The PSII core proteins become maximally phosphorylated by the STN8 kinase and concomitantly, the LHCII proteins become dephosphorylated (Rintamäki et al., 1997; Tikkanen et al., 2010). The molecular mechanisms behind the inactivation and degradation of the STN7 kinase in high light have been studied extensively (Rintamäki et al., 2000; Willig et al., 2011; Wunder et al., 2013a; Trotta et al., 2016), whereas the role and regulation of the LHCII phosphatase, TAP38 in this process, have received less attention.

The analysis of the *tap38* mutant under high light revealed that TAP38 mediated LHCII dephosphorylation is required to avoid uncontrolled energy flow from LHCII to PSI. Indeed, the strong concomitant phosphorylation occurring simultaneously in the PSII

core and LHCII proteins, as demonstrated by the *tap38* mutant under high light, is shown to result in a drastic increase of PSI absorption cross section, whereas in WT the LHCII dephosphorylation by TAP38 phosphatase ensures that the relative excitation of the two photosystems remains rather unchanged despite the shift of plants to high light (Publication II). The exact regulation of the TAP38 phosphatase itself is enigmatic. Nevertheless, in Publication III, I discovered that a pool of free phosphatases associate to the PSII-LHCII-PSI megacomplex in high light and I suggest that the transient interaction might affect the stability or regulation of TAP38. The exact role of the dynamic localization of the phosphatase in its function remains to be elucidated, but it is clear that the phosphatase activity is important in high light. It is conceivable that the LHCII dephosphorylation in high light is required because (i) the PSII core phosphorylation reaches a maximum and consequently induces vertical loosening of the grana appressions to allow fluent PSII repair cycle (Tikkanen et al., 2008b; Fristedt et al., 2009; Kirchhoff, 2013). (ii) This results in the partial randomization of the thylakoid protein complexes, and enhanced energy spill-over to PSI. In this context the TAP38-mediated dephosphorylation of the LHCII proteins functions to prevent the over-reduction of PSI in high light (Publication II).

# CONCLUSIONS

In this thesis I have demonstrated the hierarchical formation of large protein super- and megacomplexes, and elucidated the role of LHCII in preserving these assemblies. I further discovered that the dynamic reorganization of the photosynthetic protein super- and megacomplexes upon shift from darkness to light is dependent on STN7 kinase-mediated LHCII phosphorylation and likely occur mainly in structurally dynamic grana margins, which accommodate both photosystems and a mutual L-LHCII antenna system. Lhcb1 phosphorylation in PSII-LHCII supercomplexes likely increase the structural flexibility of the grana margins whereas Lhcb2 phosphorylation in L-LHCII facilitates fluent electron flow and maintains the redox balance of ETC. Upon increasing light intensity the TAP38 phosphatase, which is shown to become associated with the grana margin-located PSII-LHCII-PSI megacomplex, prevents uncontrolled energy flow to PSI complex by dephosphorylating the LHCII proteins.

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